Cardiac myocytes express mRNA for ten RGS proteins: changes in RGS mRNA expression in ventricular myocytes and cultured atria

Tarik Kardestuncer[1], Haiguo Wu[1], Angela L. Lim, Eva J. Neer*

Department of Medicine, Cardiovascular Division, Brigham and Women's Hospital and Harvard Medical School, 75 Francis Street, Boston, MA 02115, USA

Abstract **Regulators of G-protein signalling (RGS) are recently identified proteins that shorten the lifetime of the activated G protein. We now show that rat cardiac myocytes express mRNA for at least 10 RGS. The mRNA for RGS-r is barely detectable in rat ventricles, but increases more than 20-fold during the 60- to 90-min process of isolating ventricular myocytes, and after 90 min of culture of atrial pieces in medium with Ca^{2+}. Both in myocytes and in atria, the rise in RGS-r is transient. The mRNA for cardiac RGS5, but not RGS-r, is developmentally regulated. These studies suggest that rapid regulation of RGS levels may be a new mechanism that governs how signals are transmitted across the cardiac cell membrane.**
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Key words: RGS protein; G protein; Heart; Cardiocyte; mRNA expression

1. Introduction

Signals that control cellular responses are transmitted across the plasma membrane by an information relay system that involves specific receptors, a set of coupling proteins (called G proteins because they bind GTP), and intracellular effectors. Over the last few years, it has become clear that additional components can modulate the function of the transmembrane signalling system by acting on the G proteins [1,2]. The RGS proteins bind to the $G\alpha$ (GTP-binding) subunit of the G proteins and, among other actions, increase the rate of GTP hydrolysis. By this and other mechanisms, RGS proteins increase the rate at which G-protein-mediated signals are turned off [3].

Many studies have shown that G-protein levels change rather little with changing physiological conditions. For example, in various cardiac diseases, G-protein levels rise and fall usually by a factor of 2, but not by an order of magnitude [4]. Similarly, as PC12 cells develop, the levels of G proteins do change, but rather modestly [5]. When changes in G-protein levels do occur, they tend to occur slowly. In contrast, the first RGS protein discovered, the yeast Sst2 protein, is strongly induced by yeast pheromone within 60 min [6]. Similarly, treatment of B lymphocytes with mitogen or an activator of protein kinase C for 24 h induces expression of RGS1 mRNA [7]. The ability of RGS proteins to be rapidly regulated in-

troduces a potentially important new element in understanding the mechanisms by which cells control their responses to changing external and internal environments.

So far, over 20 RGS proteins have been described [8]. Since it is apparent that the RGS family of proteins is very large, the question of specificity becomes extremely important. RGS proteins can bind to and increase the GTPase activity of many G proteins in the $G\alpha_i$ and/or $G\alpha_q$ classes, but none in the $G\alpha_s$ class [9–11]. The potential for crosstalk among different RGS proteins would not be of any biological relevance if each RGS protein occurred in a different cell type. But if cells contain many different RGS, then what determines which G protein is regulated and when? The distribution of RGS proteins has been analyzed across many tissue types, but so far, there has been no report of the number of different RGS proteins that occur within a single cell type. The cell must control the function of each of its RGS proteins to allow any signal to move specifically and with the right duration through the G-protein network. In this manuscript, we report that cardiac myocytes express mRNA for at least 10 different RGS proteins. The mRNA for one of these, RGS-r, is rapidly and markedly elevated in the process of isolating myocytes from rat ventricles or in cultured atria. RGS mRNA levels also change in culture and in development. These studies reveal the complexity and dynamic regulation of the RGS regulatory system in cardiac myocytes.

2. Materials and methods

2.1. RT-PCR and generation of RGS probes
Reverse transcription (RT) was carried out using the Superscript preamplication system according to the manufacturer's instructions (Gibco-BRL). For PCR amplification, a pair of degenerate primers spanning the RGS core domain were used: Forward – G(G/A)IGA(G/A)AA(T/C)(A/T/C)TIGA(G/A)TT(T/C)TGG; Reverse – G(G/A)TAIGA(G/A)T(T/C)ITT(T/C)TT(T/C)CAT. PCR products of the expected size were cloned into a TA-cloning vector pCR2.1 (Invitrogen). By using this approach, we cloned RGS 3, 4, 5 and r. To clone other RGS members, specific PCR primers were designed, based on their published nucleotide sequences [12,13]. All positive clones were verified by sequencing. Plasmid DNA containing each RGS insert was digested with appropriate restriction enzyme(s) to release the insert for use as a probe in Northern blot.

2.2. Myocyte and atrial preparation
Myocytes were isolated from adult rat ventricles as described previously, and cultured in DMEM supplemented with albumin, carnitine, creatine, insulin, taurine and triiodothyronine (ACCITT) [14]. Rats (150–200 g) were sacrificed by CO_2 inhalation for 3 min by procedures that meet institutional guidelines. The left and right atria were cut into half and placed in 24-well plates containing Ca^{2+}-free DMEM (Gibco-BRL), plus 1 mM Ca^{2+} or 5 mM EGTA. Each half atrium was further chopped into small pieces and incubated in a 37°C incubator with 6% CO_2 for different times. At the end of each time point, atrial pieces, together with the medium, were transferred into

2.0-ml tubes, centrifuged for 2 min and liquid was removed. The atrial pieces were frozen in dry ice for RNA preparation.

2.3. RNA preparation and Northern blot analysis

Total RNA was isolated from rat atria, ventricles, brain and cardiomyocytes using RNAzol according to the manufacturer's instructions (Tel-Test, TX, USA). For myocytes, RNAzol was added directly to the 100-mm dishes after the culture medium was removed. Ten μg of total RNA were loaded on a formaldehyde-containing gel, blotted onto a nylon membrane and hybridized with ^{32}P-labelled RGS probe [15]. The membrane was re-probed with ^{32}P-end-labeled 24-mer oligonucleotide specific for rat 18S rRNA (5′-ACGGTATCT-GATCGTCTTCGAACC-3′) [16]. The bands were scanned by Desk-Scan II program and analyzed by NIH Image Program. The amount of RGS mRNA was normalized to 18S rRNA.

3. Results

3.1. Ventricular myocytes express mRNA for 10 different RGS proteins

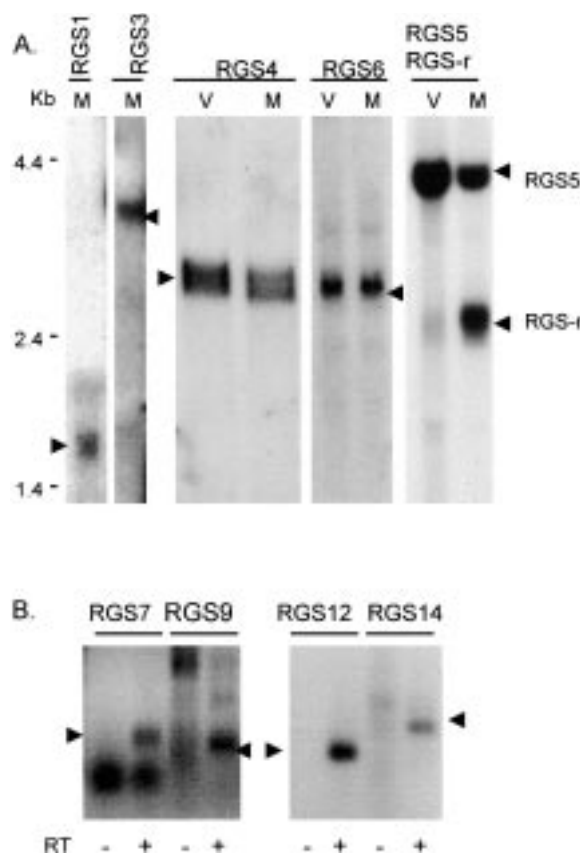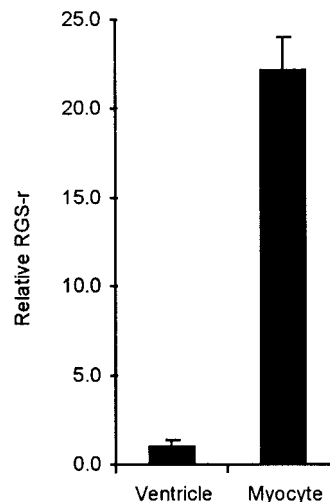We used two RT-PCR based approaches to determine the

Fig. 2. Comparison of RGS-r expression in rat ventricle and in freshly prepared myocytes. RNAs from adult rat ventricle and freshly-made ventricular myocytes were analyzed in parallel by Northern hybridization with the rat RGS-r probe (core domain, 241 bp) and reprobed with rat 18S rRNA (see Section 2). The films were scanned, analyzed by NIH Image Program, and each lane normalized to 18S rRNA. The normalized amount of RGS-r mRNA in freshly frozen ventricle was set as 1. Data shown represent means ± S.E.M. of 3 independent preparations of ventricular mRNA and 8 independent preparations of myocyte mRNA analyzed on one membrane. The same ratio of mRNA in ventricle to myocytes was seen in 8 other experiments each analyzed separately.

Fig. 1. RGS mRNA expression in rat ventricles and cardiomyocytes. Ten μg of total RNA isolated from rat ventricles and isolated cardiomyocytes was used for Northern blots as described in Section 2. Panel A shows autoradiograms of the Northern blots for the six RGS mRNAs we could detect by this method. Washing conditions for RGS 1, 3, 4 and 6 are: 2×SSC (1×SSC=0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1% SDS at room temperature for 20 min; 0.25×SSC, 0.1% SDS at 60°C for 15 min, twice. For RGS5 and RGS-r, an additional wash (0.1×SSC, 0.1% SDS at 60°C for 10 min) was included. The transcript sizes for RGS1, 3, 4, 5, 6 and r are 1.6 kb, 4.1 kb, 3.3 kb, 4.4 kb, 3.1 kb and 2.5 kb, respectively. Some RGS that were negative by Northern blots could be detected by RT-PCR (panel B). The expected product sizes for RGS7, RGS9, RGS12 and RGS14 (184, 159, 602 and 666 bp, respectively) are indicated by arrows.

expression of RGS proteins in the heart and to develop a set of probes for Northern blot analysis, as described in Section 2. The first was to use degenerate primers to identify known and, potentially, novel RGS proteins. The second was to use oligonucleotides specific for known RGS sequences. To prepare probes, RT-PCR was carried out using heart and brain mRNA. For analysis of the complement of mRNA for RGS proteins in the heart, we analyzed mRNA from whole ventricles and isolated cardiomyocytes. In cardiomyocytes, the transcripts for some RGS (RGS1, RGS3, RGS4, RGS5, RGS6 and RGS-r) were relatively abundant and could be detected by both RT-PCR and Northern blot (Fig. 1A; RT-PCR data not shown), others (RGS7, RGS9, RGS12 and RGS14) were less abundant and could only be detected by RT-PCR but not by Northern blot (Fig. 1B and Northern blot, data not shown). RGS8 was undetectable even by RT-PCR, while RGS10 and RGS11 gave faint bands that were not clearly interpretable (data not shown). Thus, the cardiomyocyte expresses mRNA for at least 10 different RGS proteins.

The levels of mRNA for RGS4, 5, 6 (Fig. 1A) and 3 (data not shown) were similar in whole ventricles and in cardiomyocytes. RGS1 mRNA was slightly higher in cardiomyocytes than in intact ventricle but the overall expression level was so low that we did not investigate it further. We conclude that these RGS mRNAs are found both in ventricular myocytes and in non-myocyte cells.

3.2. RGS-r mRNA increases rapidly in isolated ventricular myocytes and cultured atria

In contrast to RGS1, 3, 4, 5 and 6, the expression of RGS-r was dramatically different between the intact ventricle and the isolated cardiomyocyte (Figs. 1A and 2). Analysis of 8 inde-
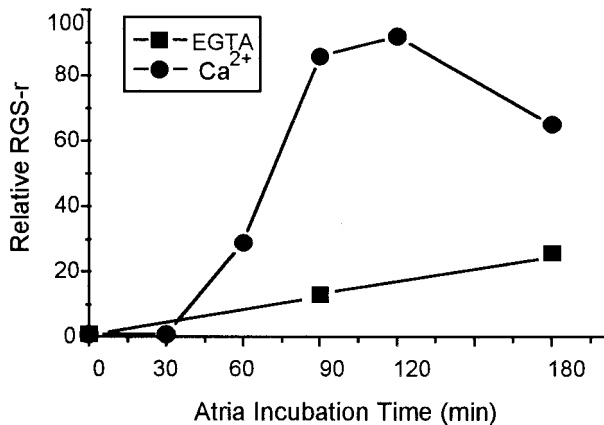
Fig. 3. RGS-r mRNA increases upon culture of rat atria. Rat atria were cut into half and incubated in DMEM with 1 mM Ca^{2+} or 5 mM EGTA at 37°C. At each time point, atrial pieces were collected and frozen on dry ice. Total RNAs were then isolated simultaneously and analyzed for RGS-r mRNA by Northern blot. The amount of RGS-r mRNA was normalized to 18S RNA. The amount of RGS-r mRNA in atria frozen immediately after removal from the animal was set as 1. Left atria and right atria were analyzed separately and gave similar results. The figure shows a representative experiment from right atria. Similar results were obtained in 3 independent experiments analyzing both right and left atria.

pendent preparations of cardiomyocytes showed that the mRNA for RGS-r was 22 ± 2-fold higher in freshly prepared myocytes than in whole ventricle (Fig. 2). The increase cannot be due simply to purification of myocytes because myocytes make up about 20–30% of cells in the ventricle so purification could only account for a 3- to 5-fold increase. The 60- to 90-min procedure for making myocytes from ventricles involves hanging the hearts and perfusing them with a mixture of enzymes to dissociate the cells, followed by centrifugation and differential plating, as described in Section 2. We measured the mRNA for RGS-r at each step of myocyte preparation and found that the rise in RGS-r mRNA occurs when the Ca^{2+} concentration is increased to 1.0 mM. In the absence of Ca^{2+}, collagenase alone or treatment with trypsin and DNase increase RGS-r slightly, but far less than the level seen in the full myocyte preparation (data not shown).

To define further the factors that regulate RGS-r mRNA in the heart, we tried to make myocytes without increasing RGS-r mRNA but were unsuccessful, as we were unable to obtain significant yields of dissociated myocytes in Ca^{2+}-free medium. Therefore, to define Ca^{2+} dependence of RGS-r mRNA expression, we used atria in organ culture. Atrial pieces can be kept in culture for several hours and are suitable for study of agonist stimulated enzyme activities such as PLCβ [17]. Left and right atrial pieces were always incubated separately, but the time course and magnitude of RGS-r expression was similar in both. As shown in Fig. 3, the RGS-r transcript was very low in rat atria frozen immediately after collection, but began to rise after 60 min of culture in DMEM with 1 mM Ca^{2+}. By 120 min, the mRNA for RGS-r increased approximately 90-fold over the initial level, then declined. In Ca^{2+}-free medium (DMEM with 5 mM EGTA), RGS-r mRNA also rose, but more slowly reaching a level only 20% of that with Ca^{2+}. Thus, there seem to be both Ca^{2+}-dependent and -independent steps regulating RGS-r expression.

We were unable to cause elevation of RGS-r mRNA by

isoproterenol (10 μM), carbachol (1 μM) or endothelin (0.1 μM) either in media containing or lacking Ca^{2+} (data not shown). Recently, Buckbinder et al. [18] showed that expression of RGS-r (that they called 'A28-RGS14') can be induced in a human carcinoma cell line by the p53 tumor suppressor or by doxorubicin. Doxorubicin is a potent anti-tumor drug whose clinical value is limited because it causes cardiomyopathy. However, 16 h of culture with 1 μM doxorubicin does not elevate RGS-r in the atria (data not shown), so it is unlikely that this mechanism contributes to doxorubicin cardiac toxicity.

3.3. RGS content changes on extended culture of cardiomyocytes

Although RGS-r mRNA increased sharply when cardiac myocytes are isolated, it decreased by about 70% within 8 h of culture in Ca^{2+}-containing DMEM supplemented with AC-CITT (Fig. 4A). However, even after 32 h, the level was higher than in the freshly frozen ventricle. Similarly, in cultured
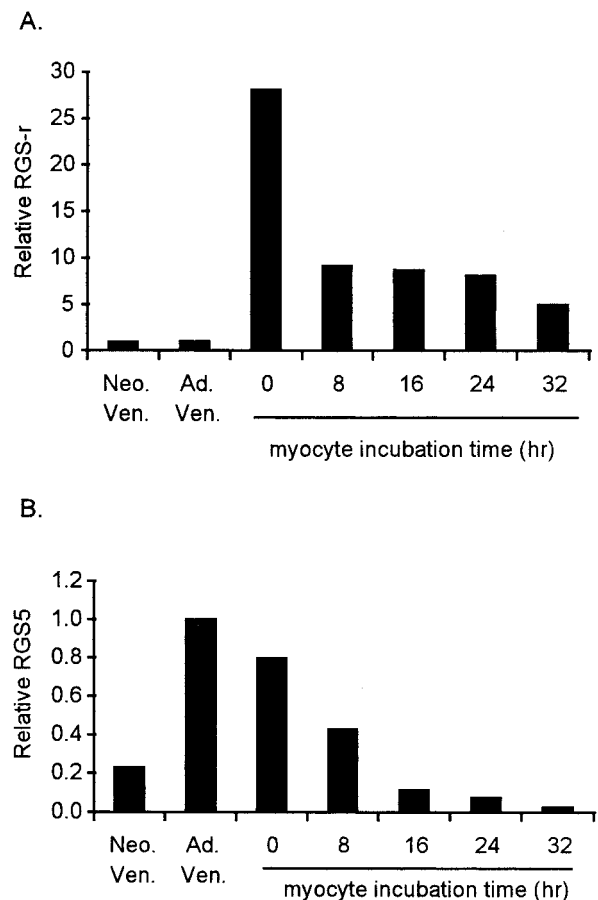
A.

B.

Fig. 4. RGS expression decreases upon culture of ventricular myocyte cells. A: Total mRNA was isolated from adult rat ventricle, neonatal rat ventricle (2-day old), and adult ventricular myocytes incubated in DMEM medium for the time period indicated. The RNAs were analyzed by Northern hybridization with RGS-r probe (core domain, 241 bp) and reprobed with 18S rRNA oligonucleotide. The band densities were quantitated by the NIH Image Program and normalized to 18S rRNA. The amount of RGS-r mRNA in adult ventricle was set as 1. The experiment shown is representative of two similar ones. B: The same membrane as was shown in A was re-analyzed using the RGS5 probe (core domain, 241 bp). The data were analyzed as described in A. Similar results were seen in 2 additional independent experiments.

atria, RGS-r expression began to decline after 2 h (Fig. 3). The decline in RGS-r mRNA is not unique because RGS5 also declined in culture, although with a different time course and disappeared almost completely within 32 h (Fig. 4B). Note that RGS5 mRNA is developmentally regulated since it is about 5-fold higher in adult ventricle than in neonatal ventricle. There is no striking difference in RGS-r mRNA between neonatal and adult ventricles, but the levels in both are extremely low.

4. Discussion

The cardiomyocyte contains at least 9 different $G\alpha$ subunits, with members representing each of the four families of $G\alpha$ subunits ($G\alpha_i$, $G\alpha_q$, $G\alpha_s$, $G\alpha_{12}$) [19,20]. $G\alpha_{12}$ is found in heart, but it is not known whether it occurs in cardiac myocytes. The RGS proteins characterized so far activate members of the $G\alpha_i$ and $G\alpha_q$ families. Cardiomyocytes have at least 6 isoforms of these RGS-sensitive G proteins ($G\alpha_o$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_q$, $G\alpha_{11}$, $G\alpha_z$). We now show that ventricular myocytes express mRNA for at least 10 different RGS proteins. Because antibodies for all these RGS proteins are not yet available, we cannot know for the whole set how mRNA abundance relates to protein abundance. Potentially, cardiac myocytes could contain as many (or more) different types of RGS proteins as they have target $G\alpha$ subunits. This observation suggests that different RGS proteins may be called into play under different physiological circumstances.

Our data show that mRNA for at least one RGS protein in the heart, RGS-r, is dramatically increased in the process of isolating myocytes from intact ventricle. This observation shows that it is very difficult to know exactly what the full complement of RGS proteins in any cell actually is. For example, some of the RGS proteins that we were not able to detect in the heart or detect only by RT-PCR may, in fact, be abundantly expressed under the correct physiological condition which we did not happen to replicate in the course of our experiments. Therefore, the example of RGS-r tells us that what we have found is a minimum number, but not necessarily the total complement of RGS mRNAs that cardiocytes are capable of expressing.

Isolated ventricular myocytes are used as a model of myocyte function in an intact ventricle. We now show that the very process of creating myocytes dramatically changes the mRNA for at least one RGS protein. While it is well known that changes in mRNA levels are not always accompanied by an equivalent rise in protein, it would be extremely unusual for such a dramatic increase in mRNA not to be accompanied by a substantial increase in cellular protein levels. Development of anti-RGS-r antibodies will eventually allow us to determine the extent to which the rise in RGS-r mRNA is reflected by a rise in protein. In the atrial pieces, we showed that there is both a Ca^{2+}-dependent and a Ca^{2+}-independent process that leads to the elevation of RGS-r mRNA.

These studies illustrate the complex regulation of cardiac signal transduction systems. Changing levels of RGS proteins in cultured cells are likely to be reflected in altered agonist sensitivity of some receptors. Our studies reemphasize the dynamic character of signal transduction systems in cardiac myocytes and suggest that rapid regulation of RGS levels may be a new regulatory mechanism that governs how signals are transmitted across the cardiac cell membrane.

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