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N-Terminal Residues Control Proteasomal Degradation of RGS2, RGS4, and RGS5 in Human Embryonic Kidney 293 Cells

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ABSTRACT

Regulator of G protein signaling (RGS) proteins modulate G protein-coupled receptor (GPCR) signaling. The N termini of some RGS4-family proteins provide receptor specificity and also contain an N-end rule determinant that results in ubiquitylation and decreased protein expression. The relevance of these mechanisms to other RGS proteins is not fully understood. Thus we examined function, receptor specificity, and expression of R4 subfamily RGS proteins (RGS2, -3, -4, -5, and -8). Although the N terminus plays a key role in protein stability in human embryonic kidney (HEK) 293 cells, we were unable to demonstrate specificity of RGS2, -3, -4, -5, or -8 for muscarinic receptors (M_1, M_3, and M_5). However, cellular RGS activity (8 = 3 > 2) was strongly correlated with expression; RGS4 and -5 had minimal expression and activity. Stabilizing mutations of RGS4 and -5 (C2S) enhanced expression and function with a greater influence on RGS4 than on RGS5. We were surprised to find that a predicted destabilizing mutation in RGS8 (A2C) did not markedly affect expression and had no effect on function. In contrast, a destabilizing mutation in RGS2 (RGS2-Q2L) recently identified as a rare N-terminal genetic variant in a Japanese hypertensive cohort (*J Hypertens* **23:**1497–1505, 2005) showed significantly reduced expression and inhibition of angiotensin II (AT_1) receptor-stimulated accumulation of inositol phosphates. We were surprised to find that RGS2-Q2R, also predicted to be destabilizing, showed nearly normal expression and function. Thus, proteasomal regulation of RGS expression in HEK293 cells strongly controls RGS function and a novel RGS2 mutation with decreased protein expression could be relevant to the pathophysiology of hypertension in humans.

G protein-coupled receptors (GPCRs) activate heterotrimeric G proteins, which mediate a wide array of signaling processes (Hepler and Gilman, 1992; Foord et al., 2005). A balance between activation and deactivation governs the amount of G protein activity. The regulator of G protein signaling (RGS) proteins accelerate deactivation and inhibit signaling by acting as GTPase-accelerating proteins at active Gα protein subunits (Ross and Wilkie, 2000). They strongly regulate signaling in cellular systems (Anger et al., 2004; Tovey and Willars, 2004) and have important in vivo functions such as the regulation of cardiac function (Fu et al., 2006; Zhang et al., 2006), blood pressure (Heximer et al., 2003), neurotransmission (Chen et al., 2004), and vision (Nishiguchi et al., 2004). There are more than 30 RGS domain-containing proteins divided into six subfamilies. Most RGS proteins act on members of the G_i or G_q families of G proteins (De Vries et al., 2000; Ross and Wilkie, 2000; Hollinger and Hepler, 2002). Within the RGS4 subfamily (R4), there is substantial promiscuity at Gα subunits in vitro (Watson et al., 1996; De Vries et al., 2000) and in cells (Hains et al., 2004) but less is known about the factors that regulate their cellular activity and specificity.

There is emerging evidence that RGS proteins can preferentially inhibit signaling through different GPCRs signaling through the same Gα subunit (Zeng et al., 1998; Xu et al., 1999; Saitoh et al., 2002; Wang et al., 2002). We recently

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ABBREVIATIONS: RGS, regulator of G protein signaling; mAChR, muscarinic acetylcholine receptor; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; PBS, phosphate-buffered saline; [^{3}H]NMS, *N*-methyl-[^{3}H]scopolamine; MG-132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; WT, wild type; AT_1, angiotensin II type 1; HA, hemagglutinin; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; $InsP_x$, inositol mono-, bis-, or trisphosphate; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; ANOVA, analysis of variance; fluo-4 AM, glycine *N*-[4-[6-[(acetyloxy)methoxy]-2,7-difluoro-3-oxo-3*H*-xanthen-9-yl]-2-[2-[2-[bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-5-methylphenoxy]ethoxy]phenyl]-*N*-[2-[(acetyloxy)methoxy]-2-oxoethyl], (acetyloxy)methyl ester.

demonstrated that endogenous RGS3 and RGS5 in vascular smooth muscle cells exhibit specificity for the M_3 muscarinic acetylcholine receptor (mAChR) and angiotensin AT_{1A} receptor, respectively (Wang et al., 2002). One potential mechanism for RGS-GPCR specificity is the formation of signaling complexes through direct binding between the GPCR and RGS proteins (Benians et al., 2005; Hague et al., 2005; Abramow-Newerly et al., 2006). Hepler and colleagues (Bernstein et al., 2004) recently demonstrated an interaction between RGS2 and RGS4 and the third intracellular loop (i3) of mAChRs, with tighter binding to the i3 loop of M_1 and M_5 than to that of M_3 mAChRs. In the present study, we assess the functional significance of this interaction in a cellular context.

Another potential mechanism controlling RGS action is ubiquitin-dependent proteasomal degradation (Varshavsky, 1997). Proteins bearing a degradation signal (*N*-degron) consisting of a destabilizing N-terminal residue (such as basic or bulky hydrophobic residues) are ubiquitylated on lysine, recognized by the proteasome, and degraded. Some proteins are not intrinsically unstable but are made unstable by *N*-arginylation through a nonribosomal arginine transferase (*ATE1* gene) that recognizes N-terminal acidic residues such as aspartate, glutamate, or oxidized cysteine (Lee et al., 2005). RGS4, -5 and -16, which have an N-terminal Cys2 (after removal of the initiator methionine), have a relatively short half-life in cells, with expression dramatically increased in the presence of proteasome inhibitors (Davydov and Varshavsky, 2000; Krumins et al., 2004; Hu et al., 2005; Lee et al., 2005). Although one would predict reduced function as a consequence of this reduced protein expression, there has been no direct correlation of expression and function, and the essential residues have not been fully defined.

A recent report on polymorphisms in RGS2 in a Japanese hypertensive cohort (Yang et al., 2005) suggests a possible role of proteasome-mediated degradation of mutant RGS2. Heximer et al. (2003) demonstrated that both homozygous and heterozygous Rgs2 knockout mice exhibit a strong hypertensive phenotype and prolonged responses to angiotensin II, so reduced RGS2 expression could be of considerable pathophysiological significance. Three studies of genetic variations in RGS2 in human hypertension have been reported recently (Yang et al., 2005; Riddle et al., 2006; Semplicini et al., 2006). Two defined coding sequence mutations (Yang et al., 2005; Riddle et al., 2006) and two of these mutations, RGS2-Q2L and RGS2-Q2R, are of particular significance because they would be predicted to increase proteasomal degradation by the N-end rule pathway (Leu and Arg are considered primary destabilizing residues) (Varshavsky, 1996).

Thus, in the present study, we investigate the function and expression of members of the R4 subfamily of RGS proteins. In particular, we asked:

1. Do RGS2, -3, -4, -5, and -8 proteins exhibit differential activities and specificity at the $G\alpha_{q/11}$-coupled M_1, M_3, and M_5 mAChRs in cells, and does the activity of RGS2 correlate with its recently reported in vitro binding specificity for the i3 loop of M_1 mAChRs?
2. To what degree does proteasomal regulation of RGS protein expression account for the differential effects of RGS transfection to inhibit GPCR signaling?
3. Which amino acids are involved in the N-end rule dependent destabilization of RGS4 and RGS5?
4. Are the N-terminal RGS2 genetic variants RGS2-Q2L or RGS2-Q2R destabilized by the N-end rule pathway of protein degradation?
5. Do RGS2-Q2L or RGS2-Q2R exhibit impaired functional activity to modulate angiotensin II signaling?

We were surprised to find that the differential binding of RGS2 and RGS4 to M_1 and M_5 versus M_3 mAChRs seems not to result in receptor selectivity in HEK293T cells. However, low expression of RGS4 and RGS5 does explain their minimal effects on mAChR signaling, although this regulation is more pronounced for RGS4 than RGS5 in HEK293T cells. The single Cys^2 in the RGS4 and RGS5 sequence is not sufficient for N-end rule-mediated destabilization, and even the four N-terminal residues of RGS4 and RGS5 do not confer substantial proteasome-dependent destabilization on RGS8. Furthermore, in HEK293T cells, only one of the two mutations of Gln^2 in RGS2 (RGS2-Q2L but not RGS2-Q2R) results in strong proteasomal degradation and lower expression in HEK293 cells, which reduces inhibition of angiotensin II signaling. This alteration may contribute to the pathogenesis of hypertension in patients with these rare mutations.

Materials and Methods

Materials. Cell culture media, pcDNA3.1(+) vector, and pcDNA3.1(−) vector, Lipofectamine 2000, natural mouse laminin, and phosphate-buffered saline (PBS) were from Invitrogen (Carlsbad, CA). High-molecular-weight poly(D-lysine) was from BD Biosciences (San Jose, CA). [*myo*-^{3}H]Inositol (82 Ci/mmol) and *N*-methyl-[^{3}H]scopolamine ([^{3}H]NMS; 84 Ci/mmol) were from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Human ^{125}I-Tyr^4-angiotensin II (2.2 kCi/mmol) was from PerkinElmer Life and Analytical Sciences (Boston, MA). Fluo-4 AM and Pluronic F-127 were from Invitrogen (Carlsbad, CA). Hank's basal saline solution was from Mediatech (Herndon, VA). Human angiotensin II acetate was from Sigma-Aldrich (St. Louis, MO). MG-132 was from Calbiochem (La Jolla, CA). Antisera were from Santa Cruz Biotechnology (Santa Cruz, CA). SuperSignal West Pico chemiluminescence substrate was from Pierce (Rockford, IL). Other reagents were from Sigma-Aldrich, Fisher Scientific (Fair Lawn, NJ), Calbiochem, and Merck (Darmstadt, Germany).

DNA Constructs. Mammalian expression vectors encoding the human, full-length, untagged wild-type (WT) angiotensin II type 1 (AT_1) receptor, M_1 mAChR, M_3 mAChR, M_5 mAChR, or RGS2, -3, -4, -5, or -8 in pcDNA3.1(+) were obtained from the University of Missouri-Rolla cDNA Resource Center (http://www.cdna.org). Constructs were generated in our laboratory and the primer sequences are available on request. RGS2-Q2L, RGS2-Q2R, and RGS4-C2S were generated by performing QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA). RGS5-C2S, RGS8-A2C, and RGS8-A2C/A3K/L4G were amplified from the polymerase chain reaction (PCR) as KpnI-XhoI fragments in which the primers introduced the mutations. RGS2-WT-HA, RGS2-Q2L-HA, RGS2-Q2R-HA, RGS4-WT-HA, RGS4-C2S-HA, RGS5-WT-HA, RGS5-C2S-HA, RGS8-WT-HA, RGS8-A2C-HA, and RGS8-A2C/A3K/L4G-HA were amplified from the PCR as KpnI-XhoI fragments in which the antisense primer encoded a C-terminal hemagglutinin (HA)-epitope tag followed by a stop codon. The fragments were isolated and subcloned into pcDNA3.1(+) vector. The open reading frame of all PCR generated constructs was verified by sequencing at the University of Michigan DNA Sequencing Core.

Cell Culture and Transfections. Human embryonic kidney (HEK) 293T cells were maintained in a humidified incubator at 37°C

with 5% CO_2 and grown to 95% confluence in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were transiently transfected by using Lipofectamine 2000 at 4 μl per microgram of plasmid DNA, and the manufacturer's recommended protocol was followed.

For the fluo-4 AM Ca^{2+} fluorescence assays, a black 96-well plate (Corning Life Sciences, Acton, MA) was coated with 50 μl laminin/well (80 μg/ml in PBS), incubated for 30 min at 37°C, and aspirated. Cells were trypsinized, suspended in DMEM without antibiotics, and seeded at 50,000 cells/well. Cells were transfected 24 h later with 0 or 10 ng of the appropriate mAChR, and 0 or 100 ng of RGS2, -3, -4, -5, or -8 plasmid DNA/well. The total amount of plasmid DNA was adjusted to 110 ng/well with pcDNA3.1(−). After transfection, the cells were incubated for 48 h before performing the fluo-4 AM Ca^{2+} fluorescence assays.

For the whole-cell radioligand binding and accumulation of [^{3}H]InsP_x assays, a 24-well plate was coated with 125 μl of poly(D-lysine)/well (80 μg/ml in water), incubated for 30 min at 37°C, and aspirated. The surface area per well of a 24-well plate is approximately 5-fold the surface area per well of a 96-well plate, so the number of cells seeded and plasmid DNA transfected was accordingly scaled up from the protocol for the fluo-4 AM Ca^{2+} fluorescence assays. Cells were trypsinized, suspended in DMEM without antibiotics, seeded at 250,000 cells/well, and, for the whole-cell accumulation of [^{3}H]InsP_x assays, labeled with 1 μCi/ml [*myo*-^{3}H]inositol. Cells were transfected 24 h later with 0 or 50 ng/well of the appropriate mAChR or 300 ng/well angiotensin AT_1 receptor, and the appropriate amount of pcDNA3.1(−) or RGS plasmid DNA. The cells were incubated for 48 h before performing the whole-cell radioligand binding and [^{3}H]InsP_x assays.

For Western blotting, a six-well plate was coated with 500 μl of poly(D-lysine)/well, incubated for 30 min at 37°C, and aspirated. Cells were trypsinized, suspended in DMEM without antibiotics, and seeded at 1.25×10^6 cells/well. Cells were transfected 24 h later with 2.5 μg of the appropriate RGS plasmid DNA/well. The cells were incubated for 48 h before pretreatment with MG-132 and preparation of the cell lysates.

Radioligand Binding Assays. Cells were rinsed once with PBS before incubation for 3 h with the appropriate concentration of [^{3}H]NMS or for 1 h with ^{125}I-Tyr^4-angiotensin II in Opti-MEM in saturation binding and competition binding assays. For mAChR binding assays, nonspecific binding was defined with 10 μM atropine. The K_D value of angiotensin II at angiotensin AT_1 receptors (1.1 nM) was measured with saturation binding analysis of specific binding of 10 pM to 10 nM ^{125}I-Tyr^4-angiotensin II and nonspecific binding was defined with 1 μM unlabeled angiotensin II. IC_{50} values were calculated by displacement of 50 pM ^{125}I-Tyr^4-angiotensin II by 100 pM to 1 μM unlabeled angiotensin II. The binding reaction was stopped by rinsing the cells with PBS once and replacing the labeling medium with ice-cold 5% (w/v) trichloroacetic acid. The plates were left at room temperature for 1 h to lyse the cells. Cell lysates were transferred to scintillation vials and the radioactivity counted.

Measurement of Whole-Cell Fluo-4 AM Ca^{2+} Fluorescence. The medium was aspirated and cells loaded with 5 μM fluo-4 AM and 0.01% (v/v) Pluronic F-127 (20% solution in dimethyl sulfoxide) in a loading buffer consisting of Hank's basal saline solution supplemented with 20 mM HEPES, pH 7.4, 1 mM Ca^{2+} (as calcium chloride), 1 mM Mg^{2+} (as magnesium chloride), and 2.5 mM probenecid. The cells were incubated for 45 min at 37°C, washed twice, and incubated for 30 min with loading buffer. A stock concentration of 10 μM carbachol was freshly prepared in loading buffer for automated injection into the wells by a Wallac Victor 1420 multilabel counter (PerkinElmer Life Sciences). Fluo-4 AM fluorescence (excitation at 485 nm; emission at 535 nm) was measured 10 times before and 100 times after the carbachol injection (final concentration, 1 μM).

Measurement of Whole-Cell Accumulation of [^{3}H]InsP_x. Previously described procedures were followed (Godfrey, 1992). The medium was aspirated, and the cells incubated for 1 h at 37°C with the appropriate concentration of carbachol or 2 h at 37°C with the appropriate concentration of angiotensin II in DMEM containing 25 mM HEPES, pH 7.4, and 20 mM lithium chloride. The medium was aspirated and replaced with chilled 10 mM formic acid, and the plates left for 1.5 h at 4°C to lyse the cells. The accumulated [^{3}H]InsP_x was separated by Dowex chromatography, the columns were eluted with 1 M ammonium formate and 100 mM formic acid, the eluates were transferred to scintillation vials, and the radioactivity was counted.

Preparation of HEK293T Cell Lysates. HEK293T cells were pretreated for 4 h with 0 or 20 μM MG-132 in DMEM. Cells were rinsed with PBS at room temperature, followed by the addition of 350 μl of immunoprecipitation buffer with protease inhibitors at 4°C. Immunoprecipitation buffer contained PBS plus 1% (v/v) Igepal CA-630, 0.5% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS, 0.01% (w/v) phenylmethylsulfonyl fluoride (PMSF), 0.03% (w/v) aprotinin, and 1 mM sodium orthovanadate. Cells were scraped from the well with a 1-ml pipette tip, and the lysate transferred to a microcentrifuge tube. The DNA was sheared by passing the cell lysate 20 times through a 21G, 1.5-inch needle, and 2 μl of 10 mg/ml PMSF added. The cell lysates were incubated for 1 h on ice, centrifuged at >10,000*g* (14,000 rpm, Eppendorf model 5415; Eppendorf-5 Prime, Inc., Boulder, CO) for 20 min at 4°C, and the supernatants were collected and frozen at −80°C. Protein concentrations were determined with the Bradford method (1976), using bovine serum albumin as standard.

Western Immunoblots. Protein samples (6 μg of lysate/lane) were resolved on a 12% SDS gel, transferred to an Immobilon-P membrane (Millipore, Billerica, MA) and subjected to Western immunoblot analysis. The membrane was blocked with 10 mM Tris, pH 8.0, 150 mM NaCl, and 5% (w/v) nonfat dry milk (blotto) for 15 min at room temperature on an orbital shaker. The membrane was probed overnight at 4°C with rabbit anti-actin and rabbit anti-HA primary antibodies diluted 1:300 and 1:800, respectively in the blotto. Thereafter, the membrane was washed with blocking buffer three times, and probed for 2 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody diluted 1:8000 in blotto. After three washes in blocking buffer, the RGS protein bands were visualized on a Kodak Digital Science Image Station (Eastman Kodak, Rochester, NY) using the SuperSignal West Pico chemiluminescent substrate, and images were quantified with Kodak 1D software.

Data Analysis. Data are reported as the mean ± S.E. of three to six independent experiments performed in duplicate or triplicate. Prism (version 4.03; GraphPad Software, San Diego, CA) was used to analyze the data. Saturation binding curves were fitted by nonlinear least-squares regression (one-site binding model) to determine the binding affinity (K_D) and maximal number of binding sites (B_{max}) of [^{3}H]NMS. One-site competition binding curves were analyzed by nonlinear least-squares regression with a homologous competition model to estimate both K_i and B_{max} values of ^{125}I-Tyr^4-angiotensin II. Semilogarithmic dose-response curves were fitted by nonlinear least-squares regression with a sigmoidal function with unity slope as least-squares nonlinear fits to determine the EC_{50} and maximal response (E_{max}) obtained with carbachol and the IC_{50} values of RGS proteins (potency as amount of transfected plasmid DNA per well). Corrected IC_{50} values were calculated using the Cheng-Prusoff correction by applying the carbachol concentration and EC_{50} value for carbachol obtained with the appropriate mAChR (see Table 2 legend). Statistical comparisons were done by using unpaired or paired two-tailed Student's t tests, or one-way ANOVA followed by Bonferroni's post-test to determine P values. A value of $P < 0.05$ was considered significant.

Results

Characterization of Function and Expression of mAChRs in Transiently Transfected HEK293T Cells. To characterize M_1, M_3, and M_5 mAChRs under the conditions of our transient transfections, receptor function was assessed by measuring accumulation of [^{3}H]InsP$_x$ with carbachol and the EC_{50}, and E_{max} values were calculated. Receptor expression levels were assessed from saturation binding of [^{3}H]NMS. Data obtained from the functional study and radioligand binding assays are shown in Table 1. M_1 and M_5 mAChRs expressed at equivalent levels, but M_3 mAChRs expressed at a somewhat higher level (2.8-fold, $P < 0.01$). In addition, the EC_{50} values for carbachol were slightly greater for M_1 versus M_3 ($P < 0.05$; EC_{50} ratio = 2.8) and M_1 versus M_5 ($P < 0.01$; EC_{50} ratio = 4.9), but the values for M_3 versus M_5 were not different ($P > 0.05$; EC_{50} ratio = 1.7). The E_{max} values for carbachol among the three mAChRs were not significantly different.

Differential Functional Activities of RGS Proteins to Inhibit Ca^{2+} Signaling. To assess the activities of different RGS proteins at the same mAChR and specificity of the same RGS at different mAChRs as an initial screen, HEK293T cells were transiently cotransfected with the M_1, M_3, or M_5 mAChR and RGS2, -3, -4, -5, or -8. In the absence of RGS proteins, stimulating the three receptors caused a rapid, transient increase in the intracellular Ca^{2+} concentration (4-fold over basal). For all three receptors, cotransfection of RGS2, -3, and -8 greatly reduced the Ca^{2+} signal compared with the control traces (Fig. 1). In contrast, RGS4 and RGS5 did not inhibit the Ca^{2+} response and cells with RGS4 even showed a small but not statistically significant increase in response. Radioligand binding assays confirmed that coexpression of RGS proteins did not significantly alter the level of [^{3}H]NMS binding (data not shown). Quantitation of peak Ca^{2+} responses (Fig. 1D) showed that RGS3 and RGS8 inhibited more than RGS2 did at all three receptors. RGS3 and RGS8 reduced signals 60 to 75% and RGS2 inhibited by 30 to 50%. For M_1 and M_5 but not M_3 mAChRs, the activities of RGS3 and RGS8 were significantly greater than that of RGS2 ($P < 0.05$ or $P < 0.01$). It is surprising that RGS2 inhibited the M_3 mAChR-stimulated Ca^{2+} signal at least as well as those of M_1 and M_5 mAChRs despite the previously reported stronger binding of RGS2 to the i3 loops of the M_1 and M_5 versus M_3 mAChRs (Bernstein et al., 2004).

RGS2 and RGS8 Inhibited Accumulation of [^{3}H]InsP$_x$. To more quantitatively assess the effects and specificity of RGS2 and RGS8, we investigated the DNA dependence of their effects on accumulation of inositol phosphates (Fig. 2). RGS3 was not included because it has an atypical, long N terminus with functions not well characterized (Hollinger and Hepler, 2002). Thus, we focused on RGS2 and RGS8, which are more structurally similar with short N termini. Whole-cell accumulation of [^{3}H]InsP$_x$ was measured after stimulating the cells with 200 nM carbachol for 1 h. As expected, RGS2 and RGS8 inhibited the [^{3}H]InsP$_x$ response in a DNA dose-dependent fashion (Table 2). Initial examination of the results showed a 4-fold greater IC_{50} value for both RGS2 and RGS8 at M_5 versus the M_1 mAChRs (Table 2); however, under the conditions of our transfections, there were differences in EC_{50} values of carbachol at the three receptors (see Table 1). Thus, we applied a Cheng-Prusoff correction (Cheng and Prusoff, 1973) to the RGS inhibition curves. This corrects the RGS plasmid DNA IC_{50} values for

TABLE 1

Radioligand binding and functional data for mAChRs

HEK293T cells were transiently transfected in 24-well plates with 50 ng/well of M_1, M_3, or M_5 mAChR plasmid DNA. Whole-cell saturation binding was measured with [^{3}H]NMS and the K_D and B_{max} values were calculated. Whole-cell accumulation of [^{3}H]InsP$_x$ was measured with increasing concentrations of carbachol, and the EC_{50} and E_{max} values were calculated. Results are the mean ± S.E. of three individual experiments performed in duplicate (functional assays) or triplicate (radioligand binding assays). Statistical comparisons were by one-way ANOVA followed by Bonferroni's post-test. The E_{max} values (counts per minute of radioactivity) obtained for the three receptors were not statistically different ($P > 0.05$).

mAChR	M_1	M_3	M_5
K_D (nM)	6 ± 0.4	17 ± 1[a]	6 ± 0.4
B_{max} (×10^6 receptors/cell)	1.3 ± 0.13	3.6 ± 0.14[b]	1.3 ± 0.16
EC_{50} (nM)	593 ± 55	209 ± 71[c]	114 ± 24[d]
E_{max} (cpm)	3845 ± 162	2734 ± 529	3004 ± 512

[a] $P < 0.001$ versus M_1 or M_5.
[b] $P < 0.001$ versus M_1 or M_5.
[c] $P < 0.05$ versus M_1.
[d] $P < 0.01$ versus M_1.

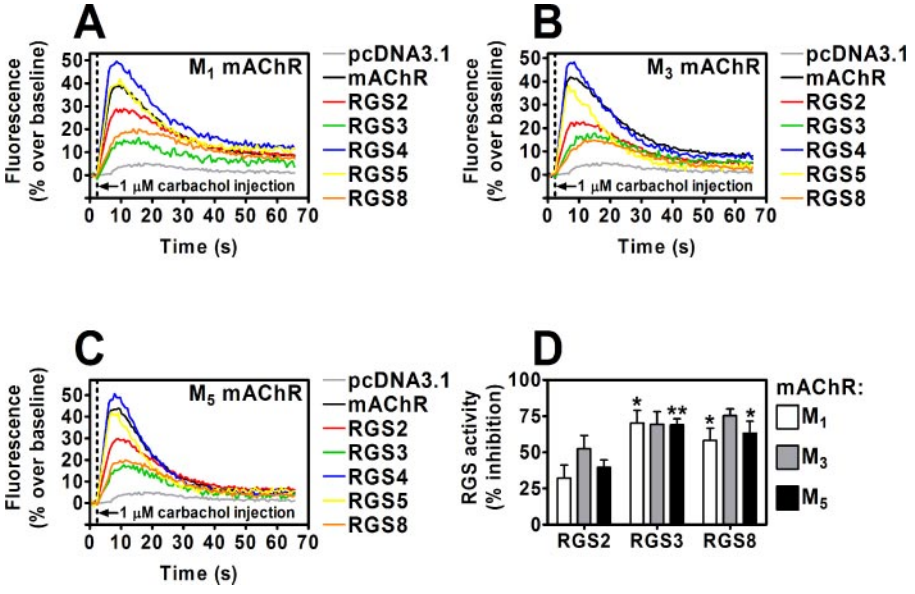

Fig. 1. RGS2, -3, and -8 inhibit Ca^{2+} signaling by $G\alpha_{q/11}$-coupled M_1, M_3, and M_5 mAChRs. HEK293T cells were loaded with fluo-4 AM, and intracellular Ca^{2+} changes were recorded after addition of 1 μM carbachol. The cells were transiently transfected in 96-well plates with 110 ng/well pcDNA3.1(−) vector, or 10 ng/well M_1 (A), M_3 (B), or M_5 (C) mAChR and with or without 100 ng/well RGS2, -3, -4, -5, or -8 plasmid DNA. The activities of RGS2, -3, and -8 to inhibit the peak mAChR responses are represented in D. Time course data are the averages of four independent experiments performed in duplicate or triplicate. The error bars in D represent S.E. Statistical comparisons were by one-way ANOVA followed by Bonferroni's post-test; *, $P < 0.05$; **, $P < 0.01$ versus RGS2.

the differences in carbachol EC_{50} values at the three receptors given the 200 nM dose of carbachol used (see Table 2 legend). After correction of the DNA plasmid IC_{50} values for RGS inhibition of receptor function, the potencies of RGS2 at the three receptors were all within a factor of 2, and differences were not statistically significant ($P > 0.05$). RGS8 seemed more active than did RGS2: 8-fold at M_1 mAChRs ($P < 0.05$) but not signficantly so at M_3 (2.7-fold) and M_5 mAChRs (1.4-fold). Thus, in contrast to our expectations from Bernstein et al. (2004) there was no significant difference in the activity of RGS2 among the three mAChRs, and it is certainly no more potent at M_1 than at M_3 receptors.

Transient Expression Levels of C-Terminal HA-Tagged RGS Proteins. One explanation for different effects of RGS transfection (e.g., lack of effect of RGS4 and RGS5) to inhibit muscarinic responses is differential expression levels of the RGS proteins. To compare expression of RGS2, -4, -5, and -8, a C-terminal HA-epitope tag was incorporated into the expression vector. The C terminus was chosen because the N terminus has been implicated in GPCR interactions and regulation of RGS stability (Zeng et al., 1998; Bernstein et al., 2000, 2004; Davydov and Varshavsky, 2000). Lysates from HEK293T cells transiently transfected with equal amounts of RGS2, -4, -5, or -8 plasmid DNA were probed with the anti-HA antibody, and proteins were detected at the expected molecular masses as shown in Fig. 3A. The lysates were also probed with an actin antibody to confirm equal loading among the lanes. Densitometric analysis of the protein bands (Fig. 3B) showed that the expression levels of RGS4 and RGS5 were equivalent to each other but markedly lower ($P < 0.001$) than those of either RGS2 or RGS8. RGS2 expressed at approximately twice the level of RGS8, although this was not statistically significant ($P > 0.05$). Thus, the slightly greater inhibition by RGS8 versus RGS2 could not be explained by a lower expression level of RGS2 in HEK293T cells. However, the lower expression levels of RGS4 and RGS5 probably account for their low activity in functional assays. Thus, we explored this question further with chemical and genetic manipulations to increase or decrease RGS protein expression.

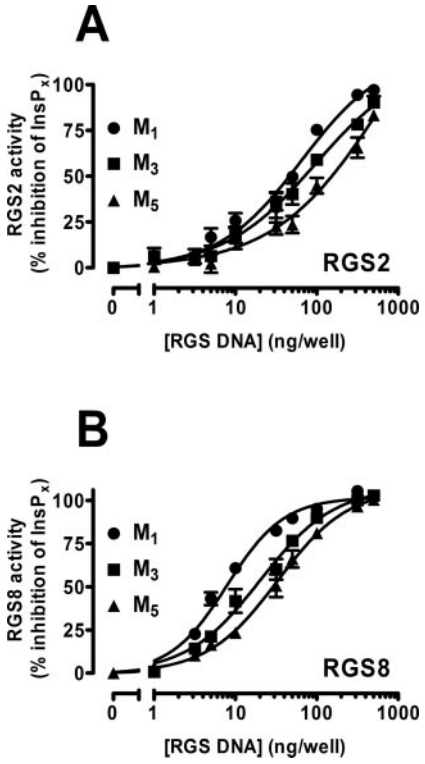

Fig. 2. Dose-dependent activities of RGS2 and RGS8 DNA to inhibit the accumulation of inositol phosphates. Whole-cell accumulation of [^{3}H]InsP_x was measured by stimulating the cells with 200 nM carbachol. The cells were transiently transfected in 24-well plates with 50 ng/well of M_1, M_3, or M_5 mAChR DNA and increasing amounts of RGS2 (A) or RGS8 (B) plasmid DNA. The total amount of DNA/well was balanced with pcDNA3.1(−) vector. Dose-response curves are averages of triplicate observations from three independent experiments and were fitted by nonlinear least-squares analysis. Error bars indicate S.E.

Role of N-Terminal Sequence and Proteasomal Degradation in Expression of RGS Proteins. Given the known role of proteasomal degradation of RGS4 and RGS5 (Davydov and Varshavsky, 2000; Krumins et al., 2004; Lee et al., 2005), we explored proteasomal degradation of the other RGS proteins by inhibiting the proteasome with MG-132 as described previously (Krumins et al., 2004). Inhibition of proteasome activity by MG-132 pretreatment (20 μM; 4 h) caused a 3-fold increase in expression of RGS2 (Fig. 4, A and B; $P < 0.001$), 20-fold increase for RGS4 (Fig. 4, C and D; $P < 0.001$), and 5-fold increase for RGS5 (Fig. 4, E and F; $P < 0.05$) and RGS8 (Fig. 4, G and H; $P < 0.001$). Thus, RGS4 is strongly modulated by proteasomal inhibition, whereas effects on RGS2, -5, and -8 are more modest.

To investigate the identity of N-terminal residues controlling the expression of RGS proteins, mutations of N-end rule determinants were made to attempt to stabilize RGS4 and RGS5 and to destabilize RGS8 (Fig. 5). Expression of RGS4-C2S was 50-fold ($P < 0.001$) greater than wild-type, an effect even larger than that of MG-132 (Fig. 4, C and D). Similar increases were seen for RGS4-C2G and RGS4-C2V mutations, as well as for a truncated RGS4 with the first N-terminal 18 amino acids deleted to initiate translation at the next methionine residue (data not shown). MG-132 pretreatment produced no additional increase in expression of the mutant RGS4-C2S ($P > 0.05$) and in the presence of MG-132

TABLE 2

RGS DNA plasmid dose dependency for inhibition of mAChR $InsP_x$ signals

HEK293T cells were transiently co-transfected in 24-well plates with 50 ng/well of the M_1, M_3, or M_5 mAChR and increasing amounts of RGS2 or RGS8 plasmid DNA. Whole-cell accumulation of [^{3}H]$InsP_x$ was measured with 200 nM carbachol, and the IC_{50} values were calculated. Cheng-Prusoff corrected IC_{50} values were calculated from the equation: Corrected $IC_{50} = IC_{50}/[1 + ([carbachol]/EC_{50})]$. Results are the mean ± S.E. of three individual experiments performed in triplicate.

mAChR	M_1	M_3	M_5
RGS2 DNA			
IC_{50} (ng of plasmid/well)	56 ± 16	65 ± 19	197 ± 86
Corrected IC_{50} (ng of plasmid/well)	43 ± 13	43 ± 13	84 ± 37
RGS8 DNA			
IC_{50} (ng of plasmid/well)	8 ± 2	20 ± 7	38 ± 11
Corrected IC_{50} (ng of plasmid/well)	7 ± 2	13 ± 5	16 ± 5

expression of the mutant was 3-fold higher than wild-type ($P < 0.01$).

For RGS5, the C2S mutation produced a 5-fold ($P < 0.05$) increase in expression compared with the wild-type (Fig. 4, E and F), which is similar to the increase seen with MG-132 ($P < 0.05$). There was no significant increase in expression of the mutant RGS5-C2S ($P > 0.05$) upon MG-132 pretreatment, and expression of the mutant in the presence of MG-132 was not significantly different from wild-type ($P > 0.05$).

To better understand the determinants of the N-end rule for RGS proteins, we attempted to reduce the stability of RGS8 by adding residues from RGS4 (Fig. 5). For RGS8 (Fig. 4, G and H), introduction of the single destabilizing Cys2 residue (RGS8-A2C) did not change its expression ($P > 0.05$). Introduction of the first three N-terminal residues from RGS4 or RGS5 (RGS8-A2C/A3K/L4G) decreased expression to 40 ± 10% of the control value ($P < 0.01$). MG-132 pretreatment caused RGS8-A2C to express at levels equivalent to those of wild-type ($P > 0.05$), but expression in the presence of MG-132 was still 5-fold lower ($P < 0.001$) for RGS8-A2C/A3K/L4G, suggesting effects independent of proteasomal degradation.

Thus, of the RGS proteins investigated here in HEK293T cells, RGS4 expression is most strongly regulated by both chemical and genetic manipulations designed to prevent proteasomal degradation, whereas all RGS proteins show some increase in expression with MG-132. Given the identical N-terminal sequences of RGS4 and RGS5 and literature on instability of RGS5, it is surprising that there is such a striking difference in our system. The identity of the single

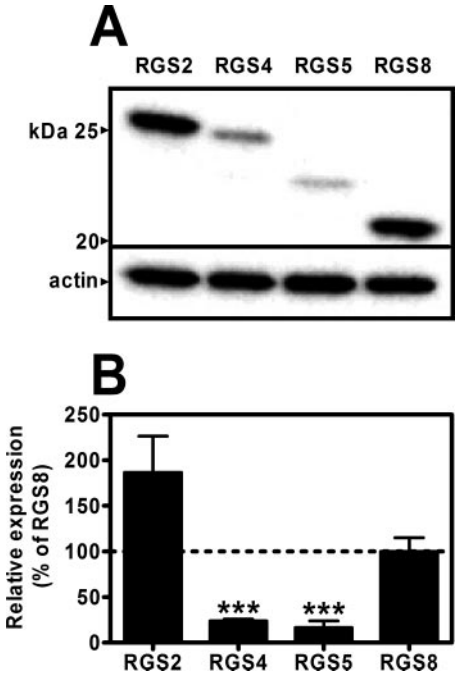

Fig. 3. RGS4 and RGS5 protein expression was much less than that of RGS2 or RGS8. HEK293T cells were transiently transfected in six-well plates with 2.5 μg/well of C-terminally HA-tagged RGS2, -4, -5, or -8 plasmid DNA. Cell lysates were analyzed by immunoblotting with antibodies specific for the HA tag and actin, and RGS protein levels were visualized in A. The blot migration of the molecular mass standards is indicated. Net band intensities were determined as described under *Materials and Methods*, and the RGS protein bands normalized to their corresponding actin bands. The quantified expression of RGS2, -4, and -5 relative to RGS8 is represented in B. The blot is representative of three different transient transfections with the summary from the three experiments in the bar graph. Error bars indicate S.E. and statistical comparisons of RGS expression versus RGS8 were by one-way ANOVA followed by Bonferroni's post-test; ***, $P < 0.001$.

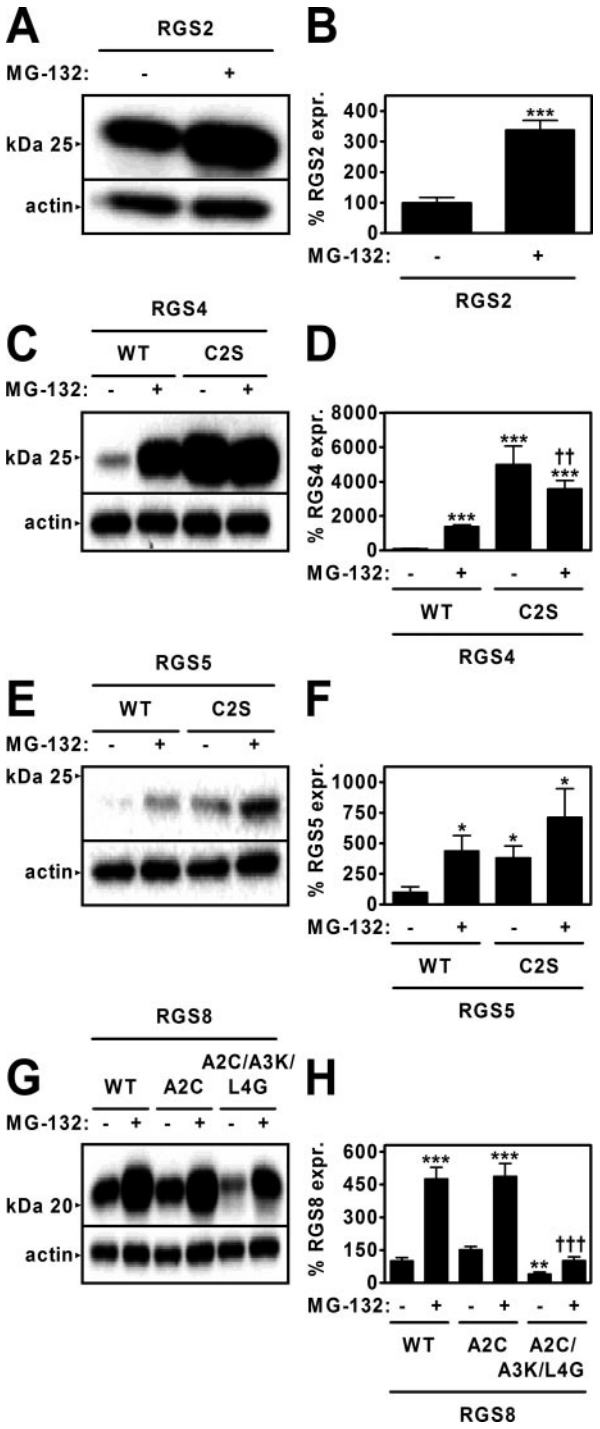

Fig. 4. The effects of MG-132 and mutations on the expression of RGS2, -4, -5, and -8. Expression of HA-tagged WT or mutant RGS proteins was measured in HEK293T cells as described for Fig. 4. Cells were also treated with or without the proteasome inhibitor MG-132 (20 μM; 4 h). Expression of each RGS protein relative to the wild-type without MG-132 pretreatment is represented for RGS2 (B), RGS4 (D), RGS5 (F), and RGS8 (H). The blots are representative of three different transient transfections and the error bars in graphs indicate S.E. Statistical comparisons were by Student's two-tailed unpaired t test (B), and one-way ANOVA followed by Bonferroni's post-test (D, F, and H): *, $P < 0.05$; ***, $P < 0.001$ versus WT without MG-132. ††, $P < 0.01$; †††, $P < 0.001$ versus WT with MG-132.

N-terminal residue at position 2 and even the first four residues are clearly insufficient to govern RGS protein stability. Furthermore, the N-end rule seems to apply only to a selective set of proteins because expression of the RGS8-A2C/A3K/L4G mutant was only increased modestly after pretreatment with MG-132.

Functional Activities of RGS4, RGS5, and RGS8 N-Terminal Mutants. We assessed the functional implications of higher expression of RGS4-C2S and RGS5-C2S and lower expression of RGS8-A2C/A3K/L4G to dose-dependently inhibit the accumulation of inositol phosphates. HEK293T cells were transiently cotransfected with the M_1 mAChR and increasing amounts of RGS4-WT and RGS4-C2S (Fig. 6A), RGS5-WT and RGS5-C2S (Fig. 6B), and RGS8-WT, RGS8-A2C, and RGS8-A2C/A3K/L4G (Fig. 6C) plasmid DNA. Whole-cell accumulation of [^{3}H]InsP_x was measured after stimulating the cells with 200 nM carbachol for 1 h. As shown previously for the Ca^{2+} signal (Fig. 1D), RGS4-WT and RGS5-WT at 300 ng of plasmid DNA/well produced minimal inhibition of the accumulation of [^{3}H]InsP_x (Fig. 6, A and B), although the highest plasmid DNA amounts of RGS5 did cause some effect. To prevent overloading the cells with plasmid DNA, which may cause significant cell death, even greater amounts were not used. The stable mutants (RGS4-C2S and RGS5-C2S) strongly inhibited the response with a maximal effect of ~75%. Although interpretation is complicated somewhat by the small dip in the curves for wild-type RGS4 and RGS5, the apparent leftward shifts in the predicted inhibition curves (50-fold for RGS4-C2S and 10-fold for RGS5-C2S) are largely consistent with the increases in expression observed with these mutants (Fig. 4, C & D and E & F, respectively).

For RGS8-WT, RGS8-A2C, and RGS8-A2C/A3K/L4G, all three constructs dose-dependently inhibited the response, and the curves are practically superimposed (Fig. 6C). Although it is somewhat surprising that the mutation RGS8-A2C/A3K/L4G did not decrease RGS8 activity, it should be noted that expression of the mutant is reduced by only 2.5-fold whereas that of RGS4-C2S and RGS5-C2S increased 50- and 5-fold, respectively, compared with the wild type. A 2.5-fold reduction in expression might be expected to produce only a modest rightward shift of the dose-response curve; however, it is possible that addition of the Cys^2 and Lys^3 residues enhances membrane localization as well as contributing to protein destabilization. Palmitoylation of N-terminal cysteine residues has been implicated in vitro (Tu et al.,

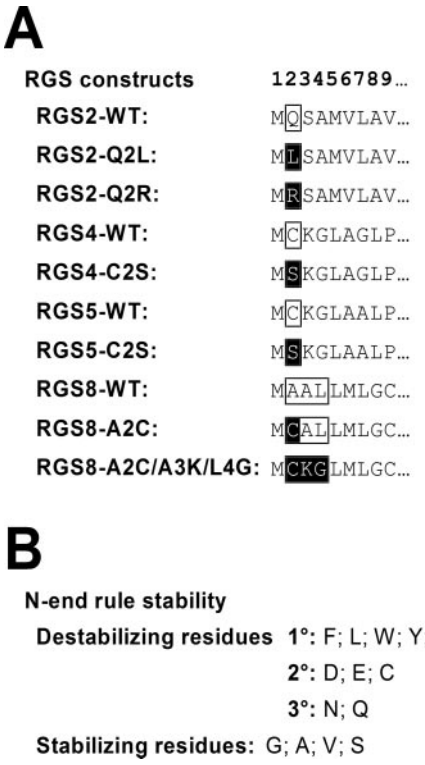

Fig. 5. The N-terminal sequences of wild-type RGS2, -4, -5, -8 and mutants (A) relative to the predictions of the *N*-end rule for protein stability (B) (Varshavsky, 1996). The Gln^2 of RGS2-WT is considered a tertiary destabilizing residue and was mutated to a primary destabilizing Leu^2 or Arg^2 and RGS2-Q2L or RGS2-Q2R, respectively, was generated. For RGS4-WT and RGS5-WT, the secondary destabilizing Cys^2 was mutated to a stabilizing Ser^2 and RGS4-C2S and RGS5-C2S were generated. For RGS8-WT, the stabilizing Ala2 was mutated to a destabilizing Cys^2 to generate RGS8-A2C, and the Ala2-Ala3-Leu4 sequence was mutated to Cys^2-Lys^3-Gly^4 to generate RGS8-A2C/A3K/L4G.

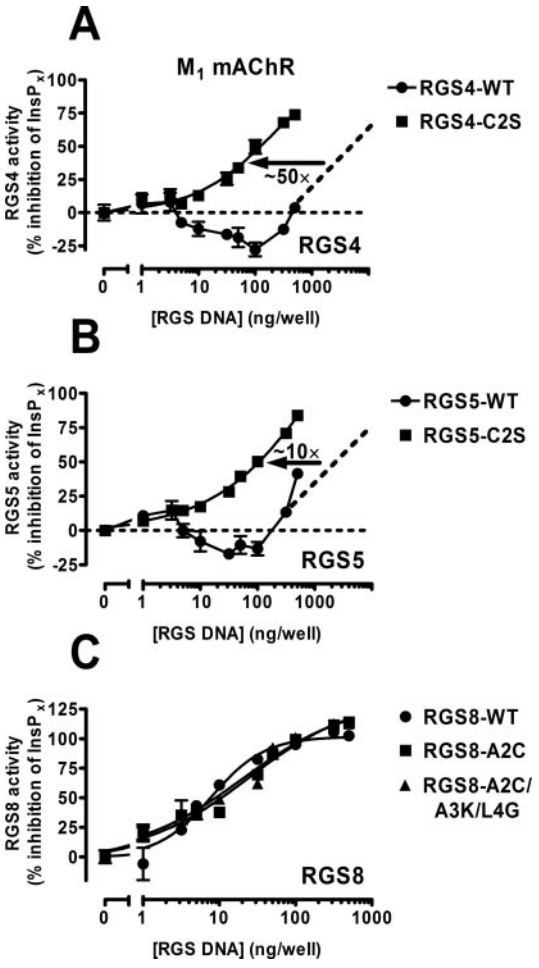

Fig. 6. Functional effects of RGS4, -5, and -8 mutants. Whole-cell accumulation of [^{3}H]InsP_x was measured by stimulating the cells with 200 nM carbachol. The cells were transiently transfected in 24-well plates with 50 ng/well of M_1 mAChR without RGS or with increasing amounts of RGS plasmid DNA: RGS4-WT or RGS4-C2S (A), RGS5-WT or RGS5-C2S (B), and RGS8-WT, RGS8-A2C, or RGS8-A2C/A3K/L4G (C). The total amount of DNA/well was balanced with pcDNA3.1(−) vector. Data are mean ± S.E. of three independent experiments, each performed in triplicate. Bold dashed lines indicate the predicted curves at higher amounts of WT RGS4 and RGS5 DNA; arrows indicate the leftward shift obtained with the mutants.

1999) and could aid in localizing and orienting the RGS to interact favorably with Gα (Jones, 2004), but determining the precise mechanism in this cellular context will require additional study.

Differential Expression and Stability of RGS2 Genetic Variants. Given the unpredictability of the second N-terminal residue in mammalian RGS protein degradation, it is unclear whether the RGS2-Q2L and RGS2-Q2R mutations found in the Japanese patients with hypertension would affect RGS2 expression. It is noteworthy that the RGS2-Q2L mutant was expressed at much lower levels than were the WT or Q2R mutant (Fig. 7A). In addition, its expression was markedly increased by MG-132. All RGS2 bands were detected at the expected molecular mass (~25 kDa), and equal loading among the lanes was confirmed by actin probing. To ensure that the effect was not due to a second mutation in the plasmid, two separate clones of RGS2-Q2L from the mutagenesis were tested and showed similar decreases in expression (data not shown). Densitometric analysis of the protein bands (Fig. 7B, left) shows that RGS2-Q2L expressed at a level 12-fold lower than that of RGS2-WT ($P < 0.001$), whereas the expression of RGS2-Q2R was not significantly different from WT. With MG-132 pretreatment (Fig. 7B, right), expression of RGS2-WT and RGS2-Q2R were increased 2.5- to 3-fold, which is no different from effects on RGS8 or the stabilized mutants of RGS4 and RGS5. This suggests that neither the RGS2 WT nor the Q2R mutant is unstable. In contrast, expression of RGS2-Q2L increased 17-fold with MG-132 ($P = 0.0009$), nearly as much as WT RGS4. Thus, of the two genetic variants, RGS2-Q2L was the most unstable (as confirmed by the lowest expression) and was most strongly regulated by the proteasome (as confirmed by greatest increase in expression with proteasomal inhibition).

RGS2-Q2L Function Correlated with Its Reduced Expression. The functional effects of RGS2 genetic variants to inhibit angiotensin AT_1 receptor-mediated signaling were also assessed. In Fig. 8A, the effect of angiotensin II on accumulation of $[^3H]InsP_x$ was measured in HEK293T cells transiently cotransfected with the human angiotensin AT_1 receptor and RGS2-WT, RGS2-Q2L, or RGS2-Q2R. The EC_{50} value for angiotensin II to increase the accumulation of $[^3H]InsP_x$ was 3 ± 0.6 nM, which is consistent with the results of other studies (Modrall et al., 2001). The EC_{50} value was not significantly changed ($P > 0.05$) by coexpression of the three RGS2 genetic variants. However, RGS2-WT reduced the angiotensin II E_{max} value by 82 ± 6% (Fig. 8, $P < 0.001$). There was no change in the B_{max} or K_d values for ^{125}I-Tyr^4-angiotensin II binding to receptor transfected alone compared with receptor transfected with RGS2 (data not shown). Therefore, as expected, the inhibition was distal to the receptor. In contrast, RGS2-Q2L decreased the E_{max} by only 46 ± 8%, and the magnitude of accumulation of inositol phosphates induced by angiotensin II with RGS2-Q2L transfection was significantly greater than that with WT RGS2 (Fig. 8B, $P = 0.03$). Furthermore, there were no differences in the E_{max} values for WT RGS2 and the RGS2-Q2R mutant. Thus, of the two Gln^2 mutants identified in the patients with hypertension, only RGS2-Q2L showed reduced functional activity in inhibiting angiotensin AT_1 receptor-mediated sig-

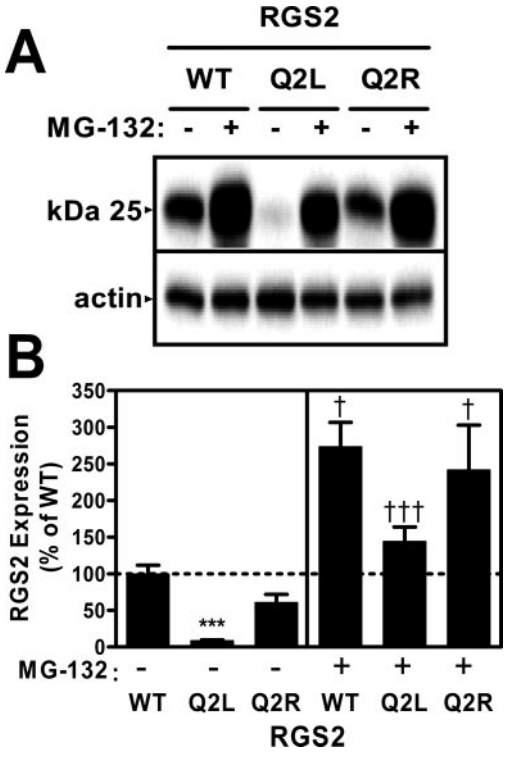

Fig. 7. Effects of RGS2 genetic variants and proteasomal inhibition on expression. HEK293T cells were transiently transfected in six-well plates with 2.5 μg/well of C-terminally HA-tagged RGS2-WT, RGS2-Q2L, or RGS2-Q2R plasmid DNA. At 48 h after transfection, cells were pretreated with the proteasome inhibitor MG-132 (0 or 20 μM; 4 h). Cell lysates were analyzed by immunoblotting with antibodies specific for the HA tag and actin, and RGS2 protein levels visualized. The migration of the molecular mass standard is indicated (A). Net band intensities were determined as described in *Materials and Methods*, and the RGS protein bands were normalized to their corresponding actin bands. The quantified expression of each RGS2 protein relative to WT without MG-132 pretreatment is shown (left, without MG-132; right, with MG-132) (B). The blot is representative of three different transient transfections with the summary from the three experiments in duplicate in the bar graph. Error bars indicate S.E. Statistical comparisons were by Student's two-tailed paired *t* test; ***, $P < 0.001$ versus RGS2-WT without MG-132; †, $P < 0.05$; †††, $P < 0.001$ versus RGS2 construct without MG-132.

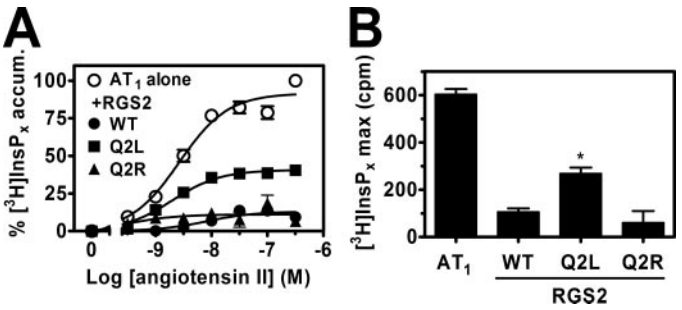

Fig. 8. Effects of RGS2 genetic variants on angiotensin AT_1 receptor-mediated function. HEK293T cells were transiently cotransfected in 24-well plates with 300 ng/well human angiotensin II type 1 (AT_1) receptor with 50 ng/well pcDNA3.1(−) vector or RGS2-WT, RGS2-Q2L, or RGS2-Q2R plasmid DNA. At 48 h after transfection, whole-cell accumulation of $[^3H]InsP_x$ was measured by stimulating the cells for 2 h with increasing concentrations of human angiotensin II (A). Data are expressed as mean peak $[^3H]InsP_x$ levels in counts per minute (i.e., radioactivity) for each condition after subtraction of basal levels without angiotensin II (B). Dose-response curves are averages of duplicate observations from three (AT_1 alone and AT_1 + RGS2-WT) to six experiments (AT_1 + RGS2-Q2L or RGS2-Q2R) normalized to the E_{max} values obtained with AT_1 alone and were fitted by nonlinear least-squares analysis. Error bars indicate S.E., and statistical comparison was by Student's two-tailed paired *t* test; *, $P < 0.05$ versus peak response obtained in the presence of RGS2-WT.

naling in HEK293 cells, a result consistent with its lower expression.

Discussion

This study investigated the specificity of several R4 subfamily RGS proteins at the $G\alpha_{q/11}$-coupled M_1, M_3, and M_5 mAChRs and determinants of their proteasomal degradation in HEK293T cells. Two major conclusions can be derived from this work: 1) Despite literature evidence for specific interactions of RGS2 and RGS4 with the M_1 mAChR third intracellular loop, we found little evidence for functional specificity of RGS2, -3, -4, -5, and -8 among mAChRs in HEK293T cells, and 2) differential proteasomal regulation of RGS protein expression plays a major role in the magnitude of their functional activities, and we identified a surprising difference between RGS4 and RGS5 in this system. Furthermore, the RGS2-Q2L mutation, identified as a rare nonsynonymous polymorphism in a group of Japanese patients with hypertension, dramatically reduced RGS2 protein stability and significantly impaired its modulation of angiotensin signaling.

RGS-GPCR Specificity in Overexpression Systems. RGS2, -3, -4, -5, and -8 have been identified to inhibit $G\alpha_q$, yet relatively little is known about determinants of their function. There is substantial published evidence for receptor-dependent specificity of RGS proteins (Zeng et al., 1998; Xu et al., 1999; Saitoh et al., 2002; Wang et al., 2002), and direct binding of RGS2 and -4 to the i3 loop of $G\alpha_{q/11}$-coupled mAChRs was recently demonstrated (Bernstein et al., 2004) with greater binding to the M_1 and M_5 i3 loops than for the M_3 i3 loop. However, association with full-length receptors and the functional significance of this interaction in cells was not investigated. In our study, we were unable to find evidence from two functional readouts that RGS2 exhibits any specificity at the full-length M_1, M_3, or M_5 mAChRs. This contrasts with a recent study on α_1-adrenoceptors (Hague et al., 2005) showing greater binding and functional specificity for the full-length α_{1A}- over the α_{1B}-adrenoceptor in transiently transfected HEK293 cells. Hence, direct association between RGS and receptor i3 loops may dictate signaling specificity but perhaps only for some GPCRs in a cellular context.

Our negative results for M_1/M_3 mAChR selectivity of RGS2 reported here do not invalidate literature on receptor/RGS specificity (Zeng et al., 1998; Xu et al., 1999; Saitoh et al., 2002; Wang et al., 2002); rather, they suggest that mechanisms may be more complex than just receptor/RGS binding. It is clear that cell-type specific processes, such as scaffold molecules [i.e., Gα-interacting protein C terminus (GIPC)] may play a role. Indeed, the specificity of endogenous RGS3 for the M_3 mAChR and RGS5 for the angiotensin AT_{1A} receptor in rat vascular smooth muscle cells (Wang et al., 2002) that we previously reported could also not be shown by transient overexpression in HEK293T cells. We found that RGS3 inhibited M_3 mAChRs and angiotensin AT_{1A} signals equally, whereas RGS5 inhibited neither (Q. Wang and R. Neubig, unpublished results). The latter effect is presumably due to the poor expression of RGS5, possibly suggesting that vascular smooth muscle-specific factors may be important for the specificity.

Role of Proteasome in Control of Signaling Pathways. A striking observation here and from others (Anger et al., 2004; Tovey and Willars, 2004) was the minimal functional activity that transfection with RGS4 and RGS5 exhibited compared with RGS2 and RGS8. Although it has been demonstrated that the expression of RGS4 (Krumins et al., 2004) and RGS5 (Lee et al., 2005) increased with proteasome inhibitors, surprisingly, there are no published functional data to assess the significance of proteasomal regulation. We provide evidence here that RGS2, -5, and -8 show markedly less regulation in HEK293 cells than RGS4. In addition, the degree of functional activity of the RGS proteins correlates fairly well with their expression levels (Figs. 1–3), but there were some discrepancies. RGS2 showed somewhat less activity than RGS8 despite its having approximately twice the level of expression (as detected by epitope immunoblotting). Furthermore, the mutants RGS4-C2S and RGS5-C2S showed relatively similar functional activity at 100 ng of plasmid, whereas the amount of immunoreactive HA-tagged RGS4 was substantially greater (Fig. 4, C and E). Thus, although proteasomal regulation plays a major role in controlling the function of each single RGS protein, there are obviously other determinants of activity comparing among RGS proteins.

It is surprising that our results show RGS5 to exhibit markedly less proteasomal regulation than RGS4. Although expression levels of RGS5 in HEK293T cells were low, the increase in its expression with the proteasome inhibitor MG-132 and upon mutation of the Cys^2 to Ser^2 was much less robust than the increases seen with RGS4. This was unexpected, because it was shown previously that both RGS4 and RGS5 were strongly degraded in reticulocyte lysates (Lee et al., 2005). Interestingly, in their report, the two splice variants of the ATE1 arginine transferase that led to destabilization of RGS4 and RGS5 seem to show differential activities (Lee et al., 2005). ATE1-1 strongly suppressed expression of RGS4 and RGS5 in *ATE1*$^{-/-}$ mouse embryonic fibroblasts, whereas the ATE1-2 splice variant seemed to more effectively suppress expression of RGS4 (Lee et al., 2005). Another difference between our study and their report (Lee et al., 2005) concerns their use of a very high MG-132 concentration of 2 mM, whereas we used 20 μM (4-h pretreatment), which has been reported previously in work on RGS proteins (Krumins et al., 2004). Thus, cell type-specific expression of ATE1 splice variants or differential sensitivity to MG-132 may account for the differences between RGS4 and RGS5 in the two studies. Regardless, both RGS4 and RGS5 showed substantial changes in functional activity when proteasomal regulation was reduced by N-terminal mutations. Thus, our results clearly support a role for regulation of RGS proteins by proteasomal degradation, but they raise new questions about cell type-dependent differences.

It is clear that an N-terminal Cys^2 is needed for the degradation of RGS4 by proteasomal mechanisms (Davydov and Varshavsky, 2000), but it has not been established in cell-based assays whether this is sufficient to increase degradation. We demonstrate with the RGS8-A2C mutant that the presence of an N-terminal Cys^2 was not sufficient for making a protein an N-end rule substrate. Furthermore, the mutant RGS8-A2C/A3K/L4G did have reduced expression, but there was not significantly enhanced proteasomal degradation based on the modest effect of MG-132. Thus, elements beyond just the N terminus probably affect this process. This conclusion is also supported by the different effects of the RGS2-

Q2L and Q2R mutations described below. This qualification of the N-end rule is important, given that the mammalian genome encodes ~350 proteins bearing an N-terminal Cys^2 (Lee et al., 2005), which is clearly not always intrinsically destabilizing but can become destabilizing depending on other factors. Compared with the short-lived RGS4 and RGS5, our results with the RGS4-C2S and RGS5-C2S mutants provide the first described gain of function in a classic GPCR-mediated readout. These stabilized analogs will be useful tools to more efficiently study potential therapeutic agents against RGS4 and RGS5 function (Zhong and Neubig, 2001; Roman et al., 2007).

In a Japanese cohort of 1724 patients with hypertension and 1102 patients without, Yang et al. (2005) identified two rare RGS2 mutations that would be predicted to affect protein stability, RGS2-Q2L and RGS2-Q2R. The former was seen only in patients with hypertension (two subjects, allele frequency = 0.12% but in no healthy subjects), and the latter was seen both in healthy patients (one subject, allele frequency = 0.09%) and in those with hypertension (three subjects, allele frequency = 0.17%). These mutations in RGS2 were of particular interest because the N-terminal residue is implicated in protein stability, and a decrease in RGS2 expression of even ~50% would be of clear pathophysiological significance given the hypertensive phenotype of both homozygous and heterozygous RGS2 knockout mice (Heximer et al., 2003). We show that RGS2-Q2L, but surprisingly not the Q2R genetic variant, is unstable in HEK293 cells, and its reduced expression results in impaired modulation of AT_1 receptor-mediated signaling. Because both Leu^2 and Arg^2 are considered primary destabilizing residues (Varshavsky, 1996), we had predicted that the Q2R mutant would also show reduced expression and ability to modulate signaling. It will be of interest to test expression in vascular smooth muscle cells as well. Such a substantial decrease in expression of RGS2-Q2L, even in heterozygous form, could well be sufficient to contribute to the clinical phenotype.

In conclusion, we were unable to provide functional evidence for RGS2 specificity at M_1 versus M_3 mAChRs despite published evidence for interactions with the M_1 receptor i3 loop. However, differential proteasomal regulation of RGS protein expression provides an important mechanism to control RGS activity and hence signaling specificity in cells. Further studies will be needed to fully define the determinants and control of this mechanism for RGS proteins in different cellular contexts, but our studies show that this control is quite complex. Furthermore, our results support emerging evidence (Semplicini et al., 2006) that decreased RGS2 protein levels may contribute to the pathogenesis of hypertension. In particular, we identify the nonsynonymous RGS2-Q2L polymorphism as having significant functional effects on RGS2 expression and regulation of angiotensin signaling.

Acknowledgments

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