Opioid-induced Down-Regulation of RGS4

ROLE OF UBIQUITINATION AND IMPLICATIONS FOR RECEPTOR CROSS-TALK

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Regulator of G protein signaling protein 4 (RGS4) acts as a GTPase accelerating protein to modulate μ- and δ-opioid receptor (MOR and DOR, respectively) signaling. In turn, exposure to MOR agonists leads to changes in RGS4 at the mRNA and/or protein level. Here we have used human neuroblastoma SH-SY5Y cells that endogenously express MOR, DOR, and RGS4 to study opioid-mediated down-regulation of RGS4. Overnight treatment of SH-SY5Y cells with the MOR agonist DAMGO or the DOR agonist DPDPE decreased RGS4 protein by ~60% accompanied by a profound loss of opioid receptors but with no change in RGS4 mRNA. The decrease in RGS4 protein was prevented by the pretreatment with pertussis toxin or the opioid antagonist naloxone. The agonist-induced down-regulation of RGS4 proteins was completely blocked by treatment with the proteasome inhibitors MG132 or lactacystin or high concentrations of leupeptin, indicating involvement of ubiquitin-proteasome and lysosomal degradation. Polyubiquitinated RGS4 protein was observed in the presence of MG132 or the specific proteasome inhibitor lactacystin and promoted by opioid agonist. The loss of opioid receptors was not prevented by MG132, demonstrating a different degradation pathway. RGS4 is a GTPase accelerating protein for both Goi/o and Gqi proteins. After overnight treatment with DAMGO to reduce RGS4 protein, signaling at the Goi/o-coupled DOR and the Gqi-coupled M3 muscarinic receptor (M3R) was increased but not signaling of the α2 adrenergic receptor or bradykinin B2 receptor, suggesting the development of cross-talk between the DOR and M3R involving RGS4.

Regulator of G-protein signaling (RGS) proteins are a family of more than 30 molecules that control the duration of G protein-coupled receptor (GPCR)-mediated G protein signaling by acting as GTPase accelerating proteins (GAPs) (1–3). RGS4 is a small RGS protein (~28 kDa) and a member of the R4 subfamily of RGS proteins with a structure consisting of the GRS homology domain (RH domain) and a small N terminus that may be important for defining specificity of action at GPCRs (1). However, RGS4 is a promiscuous GAP and shares activity at both Gqi (4, 5) and Goi/o proteins (6, 7). The protein is unstable and subject to degradation by the N-end rule pathway (8–12). RGS4 is also regulated by transcription and RNA stabilization (13). This means that levels of this protein can be readily regulated. Indeed, stress and several drugs of abuse have been shown to alter RGS4 levels (13–15).

RGS4 has a wide distribution in brain, including regions important for the action of analgesic drugs acting at μ-opioid receptors (MOR) such as the cortex, thalamus, caudate putamen, nucleus accumbens, amygdala, locus ceruleus, periaqueductal gray, and dorsal horn of the spinal cord (16). Consequently, RGS4 has been linked to the regulation of MOR signaling in several in vitro systems (17–19), and a RGS4 knockout mouse shows phenotypic behaviors in response to MOR opioid agonist (20). In support of a role for RGS4 in the pharmacology of MOR agonists, several studies have shown that morphine can modulate RGS4 at both the mRNA and protein levels either in vitro (21) or in vivo (20, 22, 23). However, the results are not consistent, and there appears to be a disconnection between protein and mRNA changes (22).

SH-SY5Y human neuroblastoma cells endogenously express a variety of GPCRs (24–28), including MOR, δ-opioid (DOR), and α2 adrenergic receptors that couple to Gqi/o proteins and muscarinic M3 receptors (M3R) and bradykinin 2 receptors (B2R (29)) that couple to Gqi. They also express a high level of RGS4 (30). We have demonstrated (30) that RGS4 knockdown in SH-SY5Y cells enhances DOR but not MOR signaling. In contrast, in preliminary studies we found that chronic treatment of SH-SY5Y cells with either a MOR or a DOR agonist significantly reduced levels of RGS4 protein. Consequently, SH-SY5Y cells provide a model system to investigate opioid-induced changes in RGS4. In this study, we have tested the hypothesis that chronic treatment of SH-SY5Y cells with MOR or DOR agonists causes a reduction of RGS4 protein levels by promoting the turnover of RGS4 protein through the N-end rule pathway. Moreover, because RGS4 acts at Ga and Gqi (1, 2), we hypothesized that this reduction in RGS4 would modulate signaling of other GPCRs expressed in these cells.

The results show that chronic treatment with agonists at either MOR or DOR enhances the breakdown of RGS4 protein via the ubiquitin-proteasome pathway without changes in mRNA. This opioid-mediated reduction in RGS4 leads to selectively increased MOR and M3R signaling. The findings indicate the potential for RGS-mediated cross-talk between opioid...
receptors and other GPCRs that could have implications for cellular signaling in the face of chronic opioid exposure.

**EXPERIMENTAL PROCEDURES**

**Materials**—\[^{3}H\]DAMGO ([d-Ala,N-Me-Phe,Gly-ol\(^{-}\)]\)-enkephalin), \[^{3}H\]DPDPE ([d-Pen\(^{2}\),d-Pen\(^{5}\)]enkephalin), \[^{3}H\]quinuclidinyl benzilate, and \[^{3}H\]diprenorphine were from PerkinElmer Life Sciences. Morphine, SNC80, naloxone, and naltrindole were obtained through the Opioid Basic Research Center at the University of Michigan (Ann Arbor, MI); DAMGO, carbachol, UK14304, N-ethylmaleimide, leupeptin, retinoic acid, 3-isobutyl-1-methylxanthine (IBMX), forskolin, thapsigargin, and all other chemicals were from Sigma. Labradimil (Hyp\(^{3}\),\beta-(2-thienyl)-Ala\(^{5}\),Tyr(Me)\(^{8}\)-Arg\(^{9}\)bradykinin) was purchased from Bachem Americas, Inc. (Torrance, CA). Pertussis toxin (PTX) was purchased from List Biological Laboratories Inc. (Campbell, CA). Cyclic AMP radioimmunoassay kits were from GE Healthcare. Tissue culture medium, Lipofectamine 2000 reagent, Opti-MEM medium, fetal bovine serum, 100× penicillin-streptomycin, and trypsin were from Invitrogen. Antibodies were from the indicated sources: anti-phospho-p44/42 MAPK (ERK1/2) and anti-p44/42 MAPK (ERK1/2) (Cell Signaling Technology, Beverly, MA); anti-β-actin (Sigma); antimouse and anti-rabbit (Santa Cruz Biotechnology, Santa Cruz, CA); anti-ubiquitin (rabbit polyclonal antibody from Dako, Carpinteria, CA, and mouse monoclonal antibody from Zymed Laboratories Inc., S. San Francisco, CA); U1079 RGS4-antiserum was a kind gift from Dr. Stephen Gold (Merck). SuperSignal West Pico chemiluminescent substrate was from Pierce. Immobilon\(^{TM}\).P transfer membrane (0.45 μm pore size) was from Millipore Corp. (Bedford, MA). Protease inhibitor mixture tablets (Complete Mini, EDTA-free) were from Roche Diagnostics (Indianapolis, IN). MG132 and lactacystin were from Biomol International (Enzo Life Sciences, Plymouth Meeting, PA).

**Cell Culture**—SH-SY5Y cells were purchased from ATCC (Manassas, VA). SH-SY5Y cell lines stably expressing RGS4 shRNA or GFP shRNA were generated as previously reported (30). Cells were grown in DMEM medium containing 10% fetal bovine serum and penicillin (100 units/ml)-streptomycin (100 μg/ml). SH-SY5Y cells stably expressing RGS4 were obtained through the Opioid Basic Research Center at the University of Michigan (Ann Arbor, MI); DAMGO, carbachol, UK14304, N-ethylmaleimide, leupeptin, retinoic acid, 3-isobutyl-1-methylxanthine (IBMX), forskolin, thapsigargin, and all other chemicals were from Sigma. Labradimil (Hyp\(^{3}\),\β-(2-thienyl)-Ala\(^{5}\),Tyr(Me)\(^{8}\)-Arg\(^{9}\)bradykinin) was purchased from Bachem Americas, Inc. (Torrance, CA). Pertussis toxin (PTX) was purchased from List Biological Laboratories Inc. (Campbell, CA). Cyclic AMP radioimmunoassay kits were from GE Healthcare. Tissue culture medium, Lipofectamine 2000 reagent, Opti-MEM medium, fetal bovine serum, 100× penicillin-streptomycin, and trypsin were from Invitrogen. Antibodies were from the indicated sources: anti-phospho-p44/42 MAPK (ERK1/2) and anti-p44/42 MAPK (ERK1/2) (Cell Signaling Technology, Beverly, MA); anti-β-actin (Sigma); antimouse and anti-rabbit (Santa Cruz Biotechnology, Santa Cruz, CA); anti-ubiquitin (rabbit polyclonal antibody from Dako, Carpinteria, CA, and mouse monoclonal antibody from Zymed Laboratories Inc., S. San Francisco, CA); U1079 RGS4-antiserum was a kind gift from Dr. Stephen Gold (Merck). SuperSignal West Pico chemiluminescent substrate was from Pierce. Immobilon\(^{TM}\).P transfer membrane (0.45 μm pore size) was from Millipore Corp. (Bedford, MA). Protease inhibitor mixture tablets (Complete Mini, EDTA-free) were from Roche Diagnostics (Indianapolis, IN). MG132 and lactacystin were from Biomol International (Enzo Life Sciences, Plymouth Meeting, PA).

**Reverse Transcription–PCR (RT–PCR)**—Total RNA was prepared from SH-SY5Y cells using the VersaGENETM RNA purification system (Gentra Systems, Minneapolis, MN) and then subjected to the RT–PCR with SuperScript\(^{TM}\) One-Step\(^{TM}\) RT-PCR System according to the supplier’s manual (Invitrogen). Primers for detection of RGS4 were designed from the RGS4 coding region as follows: sense primer, 5’-GAAATGGGCTGAATC-3’; antisense primer, 5’-CATAGGTCTGTCG-3’. The primers were first checked by amplifying RGS4 plasmid DNA to make sure that the correct size of the PCR product (502 bp) was achieved. Total RNA (200 ng) was used with primers (0.3 μM each) and MgSO\(_4\) (1.2 mM) in a 25-μl volume. The reverse transcription was performed by incubating RNA at 45 °C for 30 min followed by PCR with 30 cycles at 95 °C for 30 s, 50 °C for 45 s, and 72 °C for 1 min. The RT-PCR products were separated by electrophoresis on a 1.8% agarose gel, stained with ethidium bromide, and photographed using a Kodak Image Station 440.

**Western Blot for RGS4 Protein**—Whole cell lysates were prepared from SH-SY5Y cells as described (30). Briefly, cells were suspended in ice-cold radioimmune precipitation lysis buffer containing protease inhibitors. Then 10 μg/ml phenylmethylsulfonyl fluoride was added, and the mixture was incubated for another hour on ice and finally centrifuged at 20,000 × g for 10 min. The supernatant was saved as total cell lysate, separated into aliquots, and stored at −20 °C. Protein (−30 μg) was subjected to SDS-polyacrylamide (SDS-PAGE) on a 12% mini-gel and transferred to an Immobilon\(^{TM}\) P transfer membrane. The membrane was blocked with 1% BSA in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 1 h and incubated with U1079 RGS4-antiserum at a 1:10,000 dilution for 1 h at room temperature or overnight in the cold room. After three consecutive washes with TBST (10 min for each), the membrane was incubated with a 1:20,000 dilution of secondary antibody (goat anti-rabbit IgG-HRP) for 45 min at room temperature. Prestained SDS-PAGE protein standards (Bio-Rad, Precision Plus Protein Standards, Kaleidoscope\(^{TM}\)) were used to determine the size of the detected proteins. The membranes were stripped and re-blotted with anti-β-actin antibody at a 1:1000 dilution as an internal control for protein loading. Proteins were visualized by chemiluminescence with SuperSignal West Pico (Pierce) and exposed to x-ray film.

**cAMP Accumulation**—Reticin acid-treated (10 μM for 6–7 days) SH-SY5Y cells at 80–90% confluency were washed once with fresh serum-free medium, and the medium was replaced with 1 mM IBMX in serum-free medium for 15 min at 37 °C and then replaced with medium containing 1 mM IBMX, 10–30 μM forskolin, and 1 μM SNC80 or UK14304 or carbachol at concentrations ranging from 10\(^{-3}\) to 10\(^{-5}\) M for 5 min at 37 °C. For thapsigargin treatment, 1 μM thapsigargin was preincubated together with 1 mM IBMX for 15 min, and then cells were treated with 100 μM carbachol for 5 min in medium containing 1 mM IBMX and 10 μM forskolin. Reactions were stopped by replacing the medium with ice-cold 3% perchloric acid, and samples were kept at 4 °C for at least 30 min. An aliquot (0.4 ml) from each sample was removed, neutralized with 0.08 ml of 2.5 M KHCO\(_3\), vortexed, and centrifuged at 15,000 × g for 1 min to pellet the precipitates. Accumulated cAMP was measured by radioimmunoassay in a 10–15-μl aliquot of the supernatant from each sample following the manufacturer’s instructions and calculated as pmol/μg of protein accumulation of cAMP or percent of control.

**MAPK Analysis**—Cells were plated in 24-well plates for 6–7 days, serum-starved for 24 or 48 h, and then washed once with fresh serum-free medium and stimulated with 0.1 or 1.0 μM SNC80 or 10 μM labradimil or vehicle, respectively, for 5 min at 37 °C. The reaction was stopped by adding 0.1 ml of ice-cold SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, and 0.01% bromphenol blue). Samples were removed from the wells, boiled for 5 min, and then subjected (10–15 μl each) to electrophoresis using a 12% SDS-PAGE mini gel followed by transfer to an Immobilon\(^{TM}\).P membrane for Western blotting. The blot was probed with a 1:6000 dilution of anti-phospho-p44/42 MAPK (Thr-202/Tyr-
antibody and visualized using horseradish peroxidase-conjugated anti-rabbit IgG followed by enhanced chemiluminescence detection to measure the activated phospho-ERK then exposed to Kodak x-ray film. To assure equal loading, the same membranes were stripped and re-blotted with a 1:4000 dilution of anti-p44/42 MAPK antibody to measure total ERK levels. MAPK activity was calculated as normalized arbitrary units of phosphorylated MAPK (ERK1/2) over total ERK1/2 by densitometry analysis of films in the linear range of exposure using a Kodak Image Station 440. Percent agonist-stimulated phospho-ERK over basal without agonist stimulation was calculated.

Immunoprecipitation of RGS4 Protein—After agonist treatment with or without proteasomal inhibitor MG132 or lactacystin, cells were lysed in buffer containing 50 mM Tris-HCl, pH 7.6, 5 mM MgCl$_2$, 0.5% Nonidet P-40, 0.1% Lubrol, 1 mM EDTA, 1 mM sodium orthovanadate, 1× protease inhibitor mixture, and 10 mM $N$-ethylmaleimide and centrifuged at 20,000 × g for 10 min. The supernatant was saved as total cell lysate. Equal amounts of protein lysates (500 µg each) were subjected to immunoprecipitation. The lysates were depleted of nuclei and cell debris by centrifugation for 10 min at 20,000 × g in an Eppendorf centrifuge and precleared once with 20 µl of protein A/G plus-agarose for 30 min at 4 °C on a rocking platform and incubated with or without (protein A/G plus-agarose only) anti-RGS4 antibody overnight at 4 °C with rocking. Subsequently, the immune complexes were washed 4 times in NET buffer (50 mM Tris-HCl, pH 7.6, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40). Finally, complexes were dissociated by heating for 5 min in SDS sample buffer, resolved on a 12% SDS-PAGE mini gel, transferred to an ImmobilonTM-P transfer membrane, and blotted with U1079 anti-RGS4 antibody at a 1:10,000 dilution. The secondary antibody (anti-rabbit) was used at 1:20,000 dilution. Proteins were visualized by enhanced chemiluminescence using Kodak x-ray film. The same membrane was stripped and re-blotted with anti-ubiquitin antibody either at concentrations of 1:1000 (rabbit) or at 1:500 (mouse). In some experiments the membrane was first blotted with anti-ubiquitin antibody, then stripped and re-blotted with U1079 anti-RGS4 antibody.

Receptor Binding Assay—Membranes from SH-SY5Y cells treated with or without agonists were prepared essentially as previously described (31), suspended in 50 mM Tris-HCl, pH 7.4, to 0.5–1.0 mg/ml protein and frozen in aliquots at −80 °C. Membrane protein (50 µg for MOR binding and 100 µg for DOR binding) was incubated in 50 mM Tris-HCl, pH 7.4, with 3.0 nM $[^{3}H]$DAMGO or 4.5 nM $[^{3}H]$DPDPE with or without 50 µM naloxone (to define nonspecific binding) in a total volume of
TABLE 1
Opioid receptor numbers in SH-SY5Y cells

| Treatment          | MOR fmol/mg of protein | DOR fmol/mg of protein |
|--------------------|------------------------|------------------------|
| Vehicle            | 150 ± 4                | 42 ± 3                 |
| Chronic agonist    | 4 ± 1                  | 14 ± 1                 |
| Chronic agonist + MG132 | 3 ± 2                  | 15 ± 2                 |

RESULTS

Down-regulation of RGS4 Protein by Opioid Receptor Agonists

To test the hypothesis that exposure to MOR or DOR agonists regulate RGS4 protein and/or mRNA levels, SH-SY5Y cells were chronically treated with either MOR (DAMGO) or DOR (DPDPE) agonists (10 μM) overnight. RGS4 expression was determined by Western blot using a validated RGS4-specific antibody U1079 (30) for mRNA. A strong protein band was detected at ~28 kDa in control SH-SY5Y cells comparable with the band seen in HEK293T cells overexpressing RGS4 (Fig. 1A, left panel). After DAMGO or DPDPE overnight treatment, the intensity of this band was decreased to 43 ± 11 and 45 ± 10% of that seen in control cells, respectively (Fig. 1A, right panel). The non-peptide agonists morphine and SNC80 (10 μM, overnight) caused a similar decrease in RGS4 protein expression (data not shown). Shorter (1 h and 4 h) treatment with either DAMGO or DPDPE (10 μM) did not change the level of RGS4 protein (data not shown). The agonist-induced loss of RGS4 was accompanied by a profound reduction in appropriate opioid receptor number. As shown in Table 1, the levels of MOR and DOR were reduced by 97 and 66%, respectively. When cells were treated overnight with DAMGO or DPDPE in the presence of naloxone (NLX, 10 μM) treatment (n = 3) after overnight DAMGO or DPDPE exposure (**, p < 0.001).

FIGURE 2. Effect of naloxone-precipitated withdrawal on RGS4 mRNA and protein. A, RGS4 mRNA level was not changed by overnight treatment with DAMGO (DA) or DPDPE (DA) or after naloxone (NLX, 10 μM, 6 h) precipitated withdrawal. Ctrl, control. Total RNA prepared from SH-SY5Y cells was subjected to RT-PCR using RGS4-specific primers, and PCR products were separated on a 1.8% agarose gel, stained with ethidium bromide, and photographed. The elongation factor EF-1α was used as a control. B and C, RGS4 protein levels (n = 9) recovered 2 h after naloxone (NLX, 10 μM) treatment (n = 3) after overnight DAMGO or DPDPE exposure (**, p < 0.001).
cally increased RGS4 protein levels by more than 3-fold. Moreover, in the presence of MG132, there was no evidence of DAMGO- or DPDPE-mediated down-regulation (Fig. 3A). In contrast, MG132 had no effect on agonist-induced receptor down-regulation (Table 1). Overnight treatment with the lysosome inhibitor leupeptin (25 μM) did not alter RGS4 protein levels or prevent the loss of RGS4 protein caused by DAMGO or DPDPE (Fig. 3B). However, increasing the leupeptin concentration to 100 μM for 24 h did increase RGS4 levels and block the opioid-induced RGS4 protein down-regulation (Fig. 3C).

Evidence for Ubiquitination of RGS4 in the Presence of Proteasome Inhibitor—Next, we asked whether ubiquitinated RGS4 could be identified. SH-SY5Y cells were treated with or without 20 μM MG132 in the absence or presence of 10 μM DAMGO or DPDPE for 18 h. In whole cell lysates a low level of RGS4 protein was seen after SDS-PAGE in the absence of MG132 treatment (Fig. 4A). In contrast, a heavy band of RGS4 protein was identified in samples incubated with MG132 either in the absence or presence of opioid agonists (Fig. 4A). SDS-PAGE of anti-RGS4-immunoprecipitated samples showed higher molecular weight bands (>60 kDa) recognized by the RGS4 antibody, indicating possible polyubiquitinated RGS4 protein. To confirm the presence of ubiquitinated RGS4, the membranes were stripped and re-blotted with anti-ubiquitin antibody (Fig. 4B). The presence of multiple high molecular weight bands was seen corresponding to polyubiquitinated RGS4 protein larger than 68 kDa (68 kDa equal to 5 ubiquitin molecules, ~110 kDa equal to 10 ubiquitin molecules, and ~200 kDa equal to 20 ubiquitin molecules). The same pattern was confirmed by first blotting with anti-ubiquitin antibody then stripping the same membrane and re-blotting with anti-RGS4 antibody (data not shown). Thus, in the presence of MG132, polyubiquitinated RGS4 protein was identified but at low levels, presumably due to rapid deubiquitination enzymatic activity (32).

After overnight treatment with MG132, we were unable to observe any additional agonist-induced polyubiquitination of RGS4 protein due to the already high level of RGS4 protein (Fig. 4B). However, as seen in the whole cell lysates, RGS4 accumulation (Fig. 5A) and accumulation of ubiquitinated protein (Fig. 5B) in the presence of MG132 was observed at earlier time points in the absence or presence of DAMGO (10 μM). To observe only ubiquitinated RGS4, we performed immunoprecipitation of RGS4 protein with anti-RGS4 antibody after 240 min of treatment with MG132 (20 μM) in the presence of
**Down-regulation of RGS4 Protein by Opioid Receptor Agonists**

**A. anti-RGS4**

|       | WL |       |       |
|-------|----|-------|-------|
|       | Ctr| DA    | DP    |
| MG132 | -  | +     |       |
| 250 kDa | 20% | 40%  | 60%  |
| 150 kDa | 70% |      |      |
| 100 kDa |     |      |      |
| 50 kDa  |     |      |      |
| 25 kDa  |     |      |      |
| 12 kDa  |     |      |      |
| 6 k kDa |     |      |      |

**B. anti-Ub**

|       |       |       |       |
|-------|-------|-------|-------|
|       | polyUb-RGS4 | IgG HC | IgG LC |
|       |             |        |       |
| MG132 | Ctr | DA    | DP    |
|       | -  | +     |       |
| 250 kDa | 20% | 40%  | 60%  |
| 150 kDa | 70% |      |      |
| 100 kDa |     |      |      |
| 50 kDa  |     |      |      |
| 25 kDa  |     |      |      |
| 12 kDa  |     |      |      |
| 6 k kDa |     |      |      |

DAMGO or DPDPE (10 μM). SDS-PAGE of the immunoprecipitated RGS4 protein compared with control (Fig. 5D, top panel). The same blot was stripped and re-blotted with anti-RGS4 antibody to confirm the presence of ubiquitinated RGS4 protein, although the signal was weak. To verify the presence of ubiquitinated RGS4, we used lactacystin as an alternative specific proteasomal inhibitor. This clearly showed (Fig. 5E) increased opioid-induced ubiquitinated RGS4. Both MG132 and lactacystin increase non-ubiquitated levels of RGS4 (data not shown).

**Modulation of DOR and M₂R Signaling by RGS4**—Previously, we showed that DOR signaling is increased by knockdown of endogenous RGS4 protein in SH-SY5Y cells (30). This is selective for DOR over MOR. Consequently, increases in RGS4 by MG132 should decrease DOR signaling. To test this, SH-SY5Y cells were treated with or without MG132 (20 μM) overnight, then the ability of the DOR agonist SNC80 (1 μM) to stimulate MAPK pathway and inhibit adenyl cyclase (AC) was examined. The SNC80-mediated increase in phosphorylation of MAPK was decreased from 129 ± 3 to 85 ± 2% (Fig. 6A and B). Similarly, SNC80-mediated inhibition of cAMP accumulation was decreased from 62 ± 1 to 47 ± 1% of control values (Fig. 6C). Basal ERK and cAMP levels with or without MG132 were not changed. There was no effect of MG132 on the ability of the α₂-AR agonist UK14304 to inhibit cAMP (Fig. 6D). In confirmation of this, UK14304 dose-dependently decreased cAMP accumulation equally in RGS4-deficient SH-SY5Y cells (30) prepared by infection with lentivirus-expressing shRNA against RGS4 and control GFP shRNA-infected cells (Fig. 6E).

SH-SY5Y cells also express Gαq-coupled M₂R. Carbachol-stimulated release of intracellular Ca²⁺ ([Ca²⁺]) in RGS4-deficient SH-SY5Y cells (30), so we tested whether the increase in [Ca²⁺] would cause an alteration in cAMP levels. In the RGS4-deficient SH-SY5Y cells, carbachol concentration-dependently increased forskolin-stimulated cAMP accumulation (Fig. 7D). This accumulation in cAMP in RGS4-deficient cells by carbachol (2.8 ± 0.1 pmol/µg protein) was abolished by pretreatment with thapsigargin (1 µM, 15 min) (Fig. 7E), indicating a mechanism involving release of [Ca²⁺], which leads to stimulation of AC activity.
Down-regulation of RGS4 Protein by Opioid Receptor Agonists

Because global increases or decreases in RGS4 in the cell modulate DOR and M₃R signaling, we wanted to examine whether the DAMGO-induced reduction in RGS4 would also alter signaling via these receptors. Overnight treatment with DAMGO (10 μM) caused an increase in SNC80-stimulated phosphorylation of MAPK from 81 ± 8 to 188 ± 31% (Fig. 8, A and B). Similarly, carbachol-stimulated cAMP accumulation was increased from 105 ± 4% in vehicle-treated cells to 137 ± 5% in cells treated overnight with 10 μM DAMGO (Fig. 8C). This effect of carbachol was again sensitive to thapsigargin pretreatment (1 μM, 15 min), confirming a role for increased [Ca²⁺],. There was no change in AC inhibition by UK14304 (1 μM) or DPDPE, then lysed in buffer containing a mixture of protease inhibitors as described under “Experimental Procedures.” A Western blot using anti-ubiquitin antibody (anti-Ub) of anti-RGS4-immunoprecipitated protein showed agonist-induced ubiquitination of RGS4 protein compared with the control without agonist (Ctrl). This same blot was stripped and re-blotted with anti-RGS4 antibody to confirm the presence of ubiquitinated RGS4 protein. This experiment was repeated three times with similar results.

**DISCUSSION**

Overnight treatment of human neuroblastoma SH-SY5Y cells with either a MOR or a DOR agonist caused a marked
reduction in levels of RGS4 protein. This decrease was prevented by the opioid antagonist naloxone and by pretreatment with PTX, confirming an action at G\(_{\alpha}\)-coupled opioid receptors. The proteasome inhibitor MG132 increased the basal level of RGS4 and blocked the agonist-induced down-regulation of RGS4 protein. Moreover, in the presence of MG132 or the more selective proteasomal inhibitor lactacystin, polyubiquitinated RGS4 was identified and promoted in the presence of agonist. The down-regulation of RGS4 was accompanied by a loss of opioid receptors, although the mechanism of degradation was different. The opioid agonist-induced reduction in RGS4 protein enhanced signaling at DOR and M\(_3\)R, but not \(\alpha_2\)-AR and BK\(_2\)R, suggesting receptor-specific cross-talk involving RGS4. The findings provide evidence that opioid agonist-induced down-regulation of RGS4 protein involves ubiquitination of the protein and demonstrate that agonist-induced loss of RGS4 leads to changes in signaling at co-expressed receptors that are normally regulated by RGS4.

There are several examples of MOR agonist-induced up- and/or down-regulation of RGS4 mRNA expression in both rodents (22, 23) and heterologous expression systems (21). A reduction in mRNA with normal turnover rate would explain the observed MOR- and DOR-induced loss of RGS4. However, we observed no change at the mRNA level. Consequently, our findings indicate that opioid agonists reduce levels of RGS4 protein by an action to promote degradation. RGS4 is an unstable protein known to be subjected to the N-end rule pathway after removal of the N-terminal methionine and enzymatic arginylation of the resulting N-terminal cysteine by arginine transferase to promote ubiquitination and proteasome degradation (8–12). Treatment of SH-SY5Y cells with MG132 or lactacystin markedly increased RGS4 protein in the presence or absence of MOR or DOR agonist and allowed direct identification of polyubiquitinated RGS4 protein, thereby confirming the route of degradation. There was also a protective effect of a high concentration of leupeptin, indicating a role for lysosomal breakdown. These results suggest that the agonist-induced down-regulation of RGS4 follows the same N-end rule pathway as the non-stimulated, endogenous turnover mechanism for this protein.

Although the present studies used a high (10 \(\mu\)M) concentration of the MOR agonist DAMGO, there is support that
changes in RGS4 levels do occur in vivo after administration of the MOR agonist morphine. Thus, MOR agonist treatment has been demonstrated to reduce RGS4 protein in mouse nucleus accumbens (20) on a shorter, 2-h, time scale, and in the rat locus ceruleus, chronic morphine treatment increased RGS4 protein (20, 22). It would appear that regulation of RGS4 protein level by MOR agonists is time- and tissue-dependent. We are currently investigating changes in brain expression of RGS4 protein after morphine administration in rat.

To act as a GAP, RGS4 protein binds to Gα protein and is in close proximity to the receptor. The agonist-induced loss of RGS4 is accompanied by a profound decrease in MOR or DOR number. Thus, a feasible explanation for the enhanced agonist-induced degradation of RGS4 protein is that RGS4 is co-internalized with MOR or DOR as may occur with other signaling proteins (35), and then this leads to degradation. On the other hand, the slow time course of down-regulation of RGS4 more closely matches that of MOR and DOR receptor degradation rather than receptor internalization (36, 37). Consequently, an alternative explanation is that RGS4 in complex with opioid receptors is protected from breakdown such that when receptor is degraded, RGS4 protein becomes a target for degradation. This would seem reasonable given the fact that the N terminus is the site for both metabolism and receptor association, at least for muscarinic and cholecystokinin receptors (8). Whatever the mechanism, it appears that down-regulation of receptors via the lysosome pathway (38), although the ubiquitin-proteasome pathway has been reported to be involved in the degradation of MOR and DOR exogenously expressed in HEK cells (39).

It is intriguing that both DOR and MOR agonists induced RGS4 breakdown, whereas only DOR signaling is sensitive to the GAP activity of RGS4 in SH-SY5Y cells (30). It is unlikely that MOR-mediated RGS4 knockdown is a result of a loss of DOR, either by a cross-tolerance mechanism (40) or internalization of MOR-DOR complexes (41) as there is no evidence of such cross down-regulation in SH-SY5Y cells (28) and we saw no change in DOR receptor numbers. On the other hand, there is evidence in vitro that RGS4 can act as a GAP for MOR signaling (18, 19) and conflicting evidence for RGS4-MOR association using immunoprecipitation (17, 30). Thus, it is possible that RGS4 and MOR are associated, although this association is weaker than between RGS4 and DOR. This would indicate that the lack of effect on MOR pharmacology seen by ourselves in SH-SY5Y cells (30) and others in RGS4 knock-out mice (42) can be explained by a redundancy of RGS action at MOR, such that after reduction of RGS4, other RGS proteins act as equally efficient GAPS for MOR signaling.

In SH-SY5Y cells, DOR agonists couple to Gαi/o proteins to inhibit Ca2+ -sensitive AC enzymes 1 and 8 (30) and muscarinic agonists such as carbachol act at M3R to activate Gαi and increase [Ca2+], with no observable effect on AC (34, 43, 44). Alteration of RGS4 levels in SH-SY5Y cells by shRNA knockdown or MG132 protection against degradation modulates sig-
naling of the DOR agonist SNC80 to AC and the MAPK pathway (Ref. 30 and present results). Under these conditions, there is also an alteration of the muscarinic agonist carbachol to release \([\text{Ca}^{2+}]_i\). Indeed, with knockdown of RGS4, the carbachol-evoked increase in \([\text{Ca}^{2+}]_i\) was sufficient to activate AC and, so, increase cAMP accumulation. We found no evidence that inhibition of AC by \(\alpha_2\)-AR agonist UK14304 or stimulation of MAPK by the BK2 agonist labradimil acting at BK2R was modulated by RGS4.

Treatment of SH-SY5Y cells overnight with the MOR agonist DAMGO mimicked the effects seen with RGS4 knockdown by shRNA; namely, SNC80 stimulated the MAPK pathway to a greater extent and carbachol afforded an increase in AC accumulation by a calcium-sensitive mechanism. This indicates a cross-talk mechanism whereby the cell responds to the MOR-mediated reduction in RGS4 by increasing signaling to DOR and \(M_3\)R normally modulated by RGS4 (Fig. 9). Presumably such cross-talk will apply only to other co-expressed GPCRs sensitive to RGS4 and, therefore, may be a compensatory mechanism for the cell to respond to the development of MOR tolerance. The increased signaling at DOR could be an alternative mechanism to the increase in DOR cell surface receptor numbers seen after chronic morphine exposure of cultured neurons or chronically morphine-treated rats (45). The ability of carbachol to increase activation of \(\text{Ca}^{2+}\)-sensitive AC enzymes in DAMGO-dependent cells might contribute to the known effects of chronic opioids to increase AC activity in SH-SY5Y cells and other systems expressing \(\text{Ca}^{2+}\)-dependent AC enzymes (46), including the locus ceruleus, which is an important site for opioid withdrawal (47). However, the loss of RGS4 was not persistent and was readily restored after naloxone-precipitated withdrawal, indicating the ease of regulation of this protein, allowing it respond to the signaling needs of the cell.

In conclusion, our findings suggest that opioid agonist-induced reduction in RGS4 occurs via the ubiquitin-proteasome pathway and may contribute to the maintenance of cell homeostasis in the morphine-dependent state. This would occur by increasing signaling via other co-expressed G\(_{a}\),\(\alpha\)-coupled receptors but also, given the promiscuity of RGS4 action, by increasing signaling of G\(_{a}\),-linked GPCRs, several of which, for example, CCK\(_{2}\) (48) and 5-HT\(_{1A}\) (49), are co-expressed with MOR in certain brain regions. Moreover, because activation of the heterologously expressed 5-HT\(_{1A}\) receptors has been shown to cause degradation of RGS20 via proteasome degradation in COS-7 cells and Neuro2A cells (50), agonist-induced alterations in RGS protein levels may provide a more general mechanism of cellular adaptation.

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