Identification of Protein Kinase C Activation as a Novel Mechanism for RGS2 Protein Upregulation through Phenotypic Screening of Natural Product Extracts

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ABSTRACT

Biochemical high-throughput screening is widely used in drug discovery, using a variety of small molecule libraries. However, broader screening strategies may be more beneficial to identify novel biologic mechanisms. In the current study we used a β-galactosidase complementation method to screen a selection of microbial-derived pre-fractionated natural product extracts for those that increase regulator of G protein signaling 2 (RGS2) protein levels. RGS2 is a member of a large family of proteins that all regulate signaling through coupled receptors (GPCRs) by accelerating GTPase activity on active Gα as well as through other mechanisms. RGS2-/- mice are hypertensive, show increased anxiety, and are prone to heart failure. RGS2 has a very short protein half-life due to rapid proteasomal degradation, and we propose that enhancement of RGS2 protein levels could be a beneficial therapeutic strategy. Bioassay-guided fractionation of one of the hit strains yielded a pure compound, Indolactam V, a known protein kinase C (PKC) activator, which selectively increased RGS2 protein levels in a time- and concentration-dependent manner. Similar results were obtained with phorbol 12-myristate 13-acetate as well as activation of the Gq-coupled muscarinic M3 receptor. The effect on RGS2 protein levels was blocked by the nonselective PKC inhibitor Gö6983 (3-[1-[3-(dimethylamino)propyl]-5-methoxy-1H-indol-3-yl]-1H-pyrrole-2,5-dione), the PKCβ-selective inhibitor Ruboxastaurin, as well as small interfering RNA-mediated knockdown of PKCβ. Indolactam V-mediated increases in RGS2 protein levels also had functional effects on GPCR signaling. This study provides important proof-of-concept for our screening strategy and could define a negative feedback mechanism in Gq/Phospholipase C signaling through RGS2 protein upregulation.

Introduction

Much progress has been made in the rational design of drug-like compounds against specific proteins, with biochemical high-throughput screening strategies emerging as the primary driver in drug discovery. However, the expected effects in a cell or a whole animal are difficult to predict from a single assay. Broader, phenotypic screening strategies may therefore be required to develop a more complete picture of potential drug targets. We were thus motivated to apply a broad phenotypic screening approach to identify novel compounds that increase protein expression of regulator of G protein signaling (RGS) 2 in a selection of prefractionated natural product extracts.

Over the centuries, natural products have been an excellent source of medicines with antibacterial, antifungal, antitumor, anhelemic, enzyme inhibition, and other activities for the treatment of a wide spectrum of diseases. Plants, in particular,
have formed the basis of the traditional medicine systems; however, marine and terrestrial microorganisms have also proven to be a good source of novel bioactive compounds (Molinski et al., 2009; Cragg and Newman, 2013). Marine actinomycetes are one of the most prolific groups of bioactive secondary metabolite producers (Fiedler et al., 2005). These microbes produce a variety of chemical structures including terpenoids, peptides, macrolides, polycyclic compounds, and quinones (Zotchev, 2012). The structural diversity and wide range of bioactivity have established marine natural products as a promising frontier in future drug discovery.

A significant percentage of clinically approved drugs target G protein–coupled receptors (GPCRs) or related processes. These receptors all couple to heterotrimeric G proteins consisting of an α- and a βγ-subunit. Upon agonist binding to the receptor, conformational changes are induced in the G protein enabling exchange of GDP for GTP on the α-subunit, thereby activating the G protein. The α- and βγ-subunits then dissociate, and both can initiate signal transduction cascades within the cell. The signal is turned off by hydrolysis of the GTP back to GDP. Although Gα has intrinsic GTPase activity, this process is very slow. RGS proteins act as GTPase activating proteins (GAPs), greatly accelerating GTP hydrolysis by direct binding to Gα (>1,000 fold) (Berman et al., 1996; Zhong and Neubig, 2001; Sjogren et al., 2010). They thereby serve as important negative regulators of G protein–mediated signal transduction.

Of the more than 20 known classic RGS proteins, RGS2 has been proposed as a viable drug target in several pathophysiological conditions, such as hypertension (Tsang et al., 2010), heart failure (Zhang et al., 2006; Takimoto et al., 2009), anxiety disorders (Otowa et al., 2011), and prostate cancer (Cao et al., 2006). RGS2−/− mice are hypertensive, show increased anxiety, and are prone to heart failure (Heximer et al., 2003; Zhang et al., 2006). RGS2 has a very short protein half-life due to rapid proteasomal degradation (Bodenstein et al., 2007; Sjogren et al., 2012). Several rare human mutations that speed RGS2 degradation and/or reduce function have been identified in hypertensive patients in Japanese, Chinese, and African-American cohorts (Yang et al., 2005; Zhao et al., 2008; Watanabe et al., 2010). Furthermore, human mutations leading to decreased RGS2 expression have been associated with a decreased response to antihypertensive treatment (Semplicini et al., 2010; Sugimoto et al., 2010). Consequently, pharmacological upregulation of RGS2 protein levels could be a novel beneficial approach in drug discovery.

In our previous work we identified digoxin and other cardiotonic steroids in a small molecule screen as selective stabilizers of RGS2 protein (Sjogren et al., 2012). Digoxin increases RGS2 protein levels two- to threefold, and this has functional effects on GPCR signaling in cells. From those studies we identified a novel mechanism by which digoxin could have beneficial effects in heart failure. However, novel drug candidates are still needed, because the toxic properties of digoxin are well known. Therefore in the current study we undertook a similar screen using a prefractionated natural product extract library from Costa Rica marine biodiversity resources. This was motivated by the hypothesis that natural products might provide greater structural diversity and bioactivity for identification of novel mechanisms and effective small molecule mediators of RGS2 upregulation compared with commercially available drug libraries.

Materials and Methods

**MG-132** [N-(benzoyloxycarbonyl)leucinylleucinylleucinalin] was from Calbiochem (Quincy, MA). Indolactam V, phorbol 12-myristate 13-acetate (PMA), Ruboxastaurin were from Sigma-Aldrich (St. Louis, MO) and G06983 (3-[1-[3-(dimethylamino)propyl]-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione) was from Cell Signaling (Danvers, MA). If not otherwise indicated all chemicals were from Sigma-Aldrich, and all tissue culture supplies were from Invitrogen/ Gibco (Grand Island, NY).

**Preparation of Natural Product Extracts Library for High-Throughput Screening.** The Sherman laboratory natural products extracts (NPEs) collection of actinomyces samples were collected from various locations around the world including Costa Rica, Papua New Guinea, and Panama as marine sediments. The procedure for isolation of actinomyces from marine source materials was previously described by Hirsch and Christensen (1983) by sprinkling sediment onto a mixed cellulose filter (Millipore, Billerica, MA) on top of a Bennett’s agar (glucose 1%, NZ amine 0.2%, beef extract 0.1%, yeast extract 0.1%, and agar 1.5%) plate containing 25 μg/ml of cyclohexamide and 25 μg/ml of nalidixic acid. The plate was incubated at 28°C for 3 days before the filter was carefully removed. The plate was then incubated another 7–10 days until colonies were visible. The colony was picked off the plate and streaked onto ISP2 agar until pure. Seed cultures were grown in 17-ml dual position cap tubes containing 2 ml of ISP2 and grown for 4 days on a rotary shaker at 200 rpm. The seed culture was then poured into a 250-ml baffled flask containing 100 ml of ISP2 and grown for 18 days on a rotary shaker at 200 rpm. The culture was centrifuged at 4000 rpm for 10 minutes to remove the cells, and 2 g of XAD16 resin (Sigma-Aldrich) contained within a polypropylene mesh bag was added to the broth and incubated overnight on the rotary shaker. The resin bag was removed and placed into 10 ml of MeOH followed by 10 ml of acetone and 10 ml of ethyl acetate. Each of the three fractions was evaporated and reconstituted to a final concentration of 15 mg/ml in dimethylsulfoxide (DMSO) and stored at the Center for Chemical Genomics, Life Sciences Institute, University of Michigan, Ann Arbor.

**Cell Culture and Transfections.** Human embryonic kidney (HEK)-293 cells were maintained in a humidified incubator at 37°C with 5% CO₂ and grown to 95% confluence in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO), containing 4.5 g/l glucose, 2 mM L-glutamine, 25 mM HEPES with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected using Lipofectamine 2000 (Invitrogen) at 5 μl/μg of plasmid DNA, according to the manufacturer’s recommended protocol. All transfections were performed under serum-free conditions in Opti-MEM (GIBCO). Transfections were allowed to proceed for 4–5 hours before the media were changed to DMEM with 10% FBS. For transient transfections, experiments were run 24-hours after transfection. Stable HEK-293 cell lines expressing human RGS2 or RGS4 tagged with the 4-kDa ProLabel tag were described previously (Sjogren et al., 2012). Primary rat vascular smooth muscle cells (VSMC)
were prepared as previously described (Atkins et al., 2009). Cells were used from passages 3 to 8.

**Celltiter Fluor Viability Assay and PathHunter ProLabel Assay.** The 4-kDa ProLabel (PL) tag on the C terminus of RGS2 and RGS4 permitted rapid and quantitative assessment of protein expression. HEK-293 cells expressing RGS2-PL or RGS4-PL were trypsinized and resuspended in DMEM without phenol red (GIBCO) containing 4.5 g/l glucose, 2 mM L-glutamine, 25 mM HEPES supplemented with 0.1% bovine serum albumin (BSA), and counted using the Countess automatic cell counter (Invitrogen). Cell concentration was adjusted to 500,000 cells/ml and plated in a white 384-well plate (Corning, Corning, NY) in 30 µl (15,000 cells/well) DMEM without phenol red plus 0.1% BSA. Cells were allowed to attach for at least 3 hours before treatment with compounds. At the end of treatment, the medium was removed using an ELx406 plate washer (BioTek, Winooski, VT) and 5 µl/well of Celltiter Fluor viability reagent was added (Promega, Madison, WI). Celltiter Fluor reagent is a peptide substrate (glycylphenylalanyl-aminofluorocoumarin) that enters intact cells where it is cleaved by the live-cell protease activity to generate a fluorescent signal proportional to the number of living cells. The plate was shaken at 400 rpm for 2 minutes and incubated at 37°C for 30 minutes before reading fluorescence (Ex 390 nm/Em 505 nm) on a Pherastar plate reader (BMG, Cary, NC).

The PathHunter ProLabel protein expression assay (DiscoveRx, Fremont, CA) was performed immediately after the viability assay following the manufacturer’s general protocol. Briefly, CL/lysis reagent was prepared by combining 2 parts CL lysis buffer, 1 part ProLabel lysis buffer and 25 parts lysis reagent. Ten microliters per well CL/lysis reagent was added to each well and the plate was shaken at 400 rpm for 2 minutes. Plates were incubated at room temperature for an additional 5 minutes to allow complete cell lysis. Five microliters enzyme acceptor (incomplete β-galactosidase) was then added, and the plate was shaken at 400 rpm for 2 minutes and then incubated in the dark at room temperature for 3 hours. Chemiluminescence corresponding to relative RGS2 or RGS4 protein expression was detected on a Pherastar plate reader.

**High-Throughput Screening for NPEs that Increase RGS2 Protein Levels.** NPEs (3840) representing 1280 pure culture bacterial strains (as a result of 3 extraction methods for each strain, see above) were used in a high-throughput screen against both RGS2 and RGS4, using the in-house-developed HEK-293 cell lines and the PathHunter ProLabel assay multiplexed with the Celltiter Fluor viability assay as described above. The screen was run in white 384-well plates (Corning) using 15,000 cells/well (30 µl at 500,000 cells/well in DMEM without phenol red, 0.1% BSA). Cells were allowed to attach for 3 hours before compound treatment overnight at 37°C. NPE (200 nM/well at 15 mg/ml in DMSO as described above) was added to the cells using a Biomek FX liquid handling system (Beckman Coulter, Indianapolis, IN). MG-132 (10 µM) was used as a positive control (columns 2 and 24) and Passive Cell lysis buffer (Promega) was added to all corner wells as a cell viability negative control. Columns 1 and 23 were Passive Cell lysis buffer (Promega) was added to all corner wells as a cell viability negative control. Columns 1 and 23 were

**The quality of the high-throughput screen was determined by calculating the Z’ for the PathHunter ProLabel using the following formula:**

\[
Z' = 1 - \frac{3 \sigma_p + 3 \sigma_n}{\mu_p - \mu_n}
\]

The signal from wells treated with 10 µM MG-132 was used as the positive control (p) and the signal from wells treated with DMSO only was used as the negative control (n). An assay with a Z’ value of >0.5 is considered a good quality assay.

**Collection and Purification of Streptomyces manzanensis.** Streptomyces manzanensis (Sherman laboratory Streptomyces strain 12610-H1) was isolated from marine sediments collected near Manzanillo on the Caribbean coast of Costa Rica during June 2007 (collection permit R-CM-INBio-30-2007-OT); the sample was purified and extracted as described above.

**General Analytical Chemistry Methodology.** NMR spectra were acquired on a Varian INOVA 700 MHz NMR Facility, Department of Chemistry, University of Michigan. A high-resolution atmospheric pressure chemical ionization–MS spectrum was measured using an Agilent Q-TOF high-performance liquid chromatography (HPLC)-APCI-MS at the mass spectrometry technical services, Department of Chemistry, University of Michigan. Low resolution liquid chromatography–MS analysis of HPLC was measured using a Shimadzu 2010 electrospray spectrometer. HPLC separations were performed using an Agilent 1100 HPLC system (Agilent, Santa Clara, CA) using Waters XBridge Prep (5 µm OBD 19 × 150 mm; Waters, Milford, MA) and Phenomenex Luna C8 (2) (250 × 21.2 mm, 5 µm) columns (Phenomenex, Torrance, CA).

**Streptomyces manzanensis Culture Maintenance and Fermentation.** An oatmeal plate (6% oatmeal, 1.25% agar, 3% NaCl) was streaked from a glycerol spore stock and incubated for 5 days. Seed cultures of 3 ml (×5) of ISP2 media (1% malt extract, 0.4% yeast extract, 0.4% dextrose, 3% sodium chloride) were inoculated with a loop full of vegetative cells from an oatmeal plate culture of Streptomyces manzanensis and incubated with shaking (200 rpm) at 28°C for 5 days and then transferred to 100-ml culture (×5) for the same procedure. A 25-ml portion of the seed cultures was transferred to a 2.8-l Fernbach flask containing 1.5 l of the ISP2 medium, and the fermentation was carried out on a rotary shaker (200 rpm) at 28°C for 24 days. This process was performed with 16 Fernbach flasks (total culture 24 l). After 24 days the cultures were harvested by centrifugation, and the resulting cell free broth was subjected to solid phase extraction using 20 g/l of culture of Amberlite XAD-16 resin (Dow Chemical Company, Midland, MI). The resin was then separated by filtration and subjected to organic extraction using different organic solvents, and the first culture was extracted three times: twice with MeOH and once with 1:1 MeOH: EtOAc.

protein levels as described above. Hits in the screen were defined as NPEs that 1) increased RGS2-PL chemiluminescence >3 S.D. above DMSO control; 2) showed <20% decrease in viability compared with DMSO control (viability assay window defined as: DMSO control = 100% versus passive cell lysis buffer = 0%); and 3) NPEs that showed effects according to criteria 1 and 2 using more than one extraction method.

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solvent mixture (500 ml each time) to yield 5.2 g of dried crude extract.

Isolation and Purification of Indolactam V. The organic extracts were evaporated to dryness yielding 5.2 g of the crude extract. The crude extract was dissolved in 200 ml of H2O and was subjected to a C18-silica gel column (30 x 2.6 cm, YMC Gel ODS-A, 12 nm, S-150 μm). The C18 column was eluted with a stepwise gradient of H2O/ACN (100:0 → 100) to give eight fractions (Fr.1–Fr.8) and then washed with EtOAc. All fractions were concentrated under vacuum and assayed using the RGS2 PathHunter ProLabel assay. Fraction 4 (4.6 H2O/ACN, ~600 mg) was the most active and was subjected to a Sephadex LH-20 column (GE Healthcare, Pittsburgh, PA) in 1:1 CHCl3/MeOH to obtain 5 fractions (A–E) and a column wash fraction (F) that were assayed again to give three potent fractions 4B–4D, whereas fraction 4C showed the highest potency. Fraction 4C (88.7 mg) was separated on a reversed-phase HPLC column (Phenomenex Luna C8 (2) 250 x 21.2 mm, 5 μm; DAD at 210, 238, and 254 nm; flow rate 5 ml/min) eluted with 45:55 ACN/H2O to give five 4 fractions (4C1–5–wash). Based on the RGS2 upregulation assay activity, fraction 4C5 (9.1 mg) was further separated using different reversed phase HPLC column (Waters XBridge, 250 x 20 mm, 5 μm; 65:35 MeOH/H2O; DAD at 210, 238, and 254 nm; flow rate 5 ml/min) to three fractions (4C5a–c). Fraction 4C5b (14.7 minutes, 5.2 mg (0.1% of the dried crude extract)) is the most potent molecule at the RGS2 upregulation assay. Two milligrams of the same compound was isolated from the size exclusion chromatography fraction 4B; the two samples were combined for structure elucidation experiments. The structure of this potent molecule was determined using an extensive array of 1D and 2D NMR techniques along with high-resolution–MS analysis as Indolactam V, a previously known compound. On the basis of the proton NMR of Indolactam V in DMSO-d6 integration, our compound is a mixture of the (−) and (+) conformers in 6:1 ratio, respectively, as described previously (Endo et al., 1985).

Small interfering RNA Transfections. For small interfering RNA (siRNA) transfections, cells were transfected with 25 nM siGENOME SMART-POOL siRNA (Dharmacon/Thermo Scientific, Pittsburgh, PA) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s recommended protocol. All transfections were performed under serum-free conditions in Opti-MEM, and experiments were performed for 3 hours in DMEM with 0.5% FBS. Indolactam V treatment was performed for 3 hours in DMEM with 10% FBS. Measurement of isoproterenol-induced cAMP levels was performed using the LANCE Ultra cAMP kit (PerkinElmer, Waltham, MA) according to the manufacturer’s instructions. Briefly, cells were dissociated using Versene (Gibco), harvested in phosphate-buffered saline, and pelleted at 300 g for 5 minutes. Cells were resuspended in Hanks’ balanced salt solution, counted, pelleted, and resuspended in assay buffer (Hanks’ balanced salt solution, 5 mM HEPES, 0.5 mM 3-isobutyl-1-methylxantine, 0.1% BSA; pH 7.4) at a concentration of 400,000 cells/mL. A 5-μl cell suspension (2,000 cells wells) and 5 μl 2 x isoproterenol (in assay buffer) was added to a white 384-well plate (Corning), and the reaction was incubated for 30 minutes at room temperature. Five microliters of each 4X Eu-cAMP tracer and 4X ULight-anti-cAMP working solutions were added to the wells, and the plate was incubated for 1 hour at room temperature on an orbital shaker. The membrane was probed overnight at 4°C with primary antibody diluted in TBS-T with 5% (w/v) nonfat dry milk. Rat anti-HA antibody was from Roche (1:1000), and rabbit RGS2 antibody was a gift from Dr. David Siderovski (1:2000).

The membrane was washed with TBS-T four times and probed for 1 hour at room temperature with horseradish peroxidase–conjugated secondary antibody diluted in TBS-T, 5% (w/v) nonfat dry milk. Rabbit anti-rat (1:10,000) and goat anti-rabbit (1:10,000) antibodies were from Sigma-Aldrich. Horseradish peroxidase–conjugated anti-actin (Santa Cruz Laboratories, Santa Cruz, CA; 1:10,000) antibody was used as a loading control. After four washes in TBS-T, the protein bands were visualized on autoradiography film using the Super Signal West Pico chemiluminescent substrate (Pierce), and images were scanned and quantified using the ImageJ software (NIH, Bethesda, MD).

CAMP Measurements. HEK-293T cells in 60-mm dishes were transiently transfected with the β2-adrenergic receptor and RGS2-HA and allowed to grow to 95% confluency in DMEM with 10% FBS. Indolactam V treatment was performed for 3 hours in DMEM with 0.5% FBS. Measurement of isoproterenol-induced cAMP levels was performed using the LANCE Ultra cAMP kit (PerkinElmer) according to the manufacturer’s instructions. Briefly, cells were dissociated using Versene (Gibco), harvested in phosphate-buffered saline, and pelleted at 300 g for 5 minutes. Cells were resuspended in Hanks’ balanced salt solution, counted, pelleted, and resuspended in assay buffer (Hanks’ balanced salt solution, 5 mM HEPES, 0.5 mM 3-isobutyl-1-methylxantine, 0.1% BSA; pH 7.4) at a concentration of 400,000 cells/mL. A 5-μl cell suspension (2,000 cells wells) and 5 μl 2 x isoproterenol (in assay buffer) was added to a white 384-well plate (Corning), and the reaction was incubated for 30 minutes at room temperature. Five microliters of each 4X Eu-cAMP tracer and 4X ULight-anti-cAMP working solutions were added to the wells, and the plate was incubated for 1 hour at room temperature in the dark. Time-resolved fluorescence resonance energy transfer signal was detected using a Tecan Infinite M1000 PRO plate reader (Tecan Group, Männedorf, Switzerland).
Data Analysis and Statistics. All data were analyzed using GraphPad Prism 6.0 (GraphPad; LaJolla, CA). Dose-response curves were fit using nonlinear regression. Datasets with three or more groups were analyzed with one-way analysis of variance with Bonferroni’s post hoc test for multiple comparisons. Data are presented as mean ± S.D., and a \( P \) value less than 0.05 was considered significant.

Results

High-Throughput Screen for RGS2 Modulators in a Collection of Natural Product Extracts. To identify novel, natural product modulators of RGS2 protein expression, we used a previously described (Sjogren et al., 2012) \( \beta \)-galactosidase complementation method (PathHunter ProLabel; DiscoveRx) to screen a selection of microbial-derived pre-fractionated for those that increase RGS2 protein levels. Detection of RGS protein levels took advantage of HEK-293 cell lines stably expressing RGS2 or RGS4 C-terminally tagged with a small (4 kDa) part of \( \beta \)-galactosidase (hereafter referred to as ProLabel). The development of these cell lines has been described previously (Sjogren et al., 2012). The overall \( Z^* \) score (DMSO versus 10 \( \mu \)M MG-132) in the screen was 0.58, demonstrating acceptable quality of the results obtained.

Both RGS2- and RGS4-PL expressing cell lines were used in a high-throughput screen at the University of Michigan Center for Chemical Genomics using a collection of 1280 natural product extracts (see overview in Supplemental Fig. 1). Each extract in the collection is represented three times in the

![Fig. 1. Structure elucidation of a pure active compound from fractionation and purification of the natural product extract from Streptomyces manzanensis (12610-H1). (A) Isolated Indolactam V \( ^{1} \text{H} \) NMR in DMSO-\( d_{6} \), 700 MHz. (B) Isolated Indolactam V \( ^{13} \text{C} \) NMR in DMSO-\( d_{6} \), 700 MHz. (C) Indolactam V NMR chemical shifts in DMSO-\( d_{6} \) (700 MHz). d, doublet; m, multiplet; s, singlet; t, triplet. (D) Structure of \((-)\)-Indolactam V.](image-url)
screen because of the use of different extraction methods, rendering the total number of assay points to be 3840. The PathHunter ProLabel assay was multiplexed with a fluorescent viability assay (Celltiter Fluor, Promega) to detect and correct for cell toxicity as described in Materials and Methods. Hits were defined as extracts that increased RGS2 protein levels >3 S.D. over baseline (DMSO-treated cells), had no effect on RGS4 protein levels, and that were represented more than once (i.e., by more than one extraction solvent method). Also, NPEs that reduced cell viability >20% versus DMSO control were excluded as hits. Not surprisingly, the overall rate of toxicity among the NPEs was fairly high (~50% showed >20% reduction in cell viability). Interestingly, however, among the NPEs that lead to increased RGS2 protein levels, none of the hit strains had to be excluded based solely on toxicity.

With these criteria above we identified three strain extracts that increased RGS2 protein levels with no effect on the closely related RGS4 protein in the primary screen (see triage in Supplemental Fig. 2). One of these was chosen for follow up studies. The other two strains are the subject of an ongoing study and will not be discussed here. However, our efforts so far with those strains indicate that the active molecules in the strains are unrelated to the components of the current strain 12610-H1. To determine the identity of this strain, we performed 16S rRNA extraction and phylogenetic analyses using GENEIOUS pro (Biomatters, Auckland, New Zealand; Supplemental Methods). The resulting phylogenetic tree of the Streptomyces manzanensis strain (12610-H1) is presented in Supplemental Fig. 3.

**Isolation of a Pure Active Compound from Streptomyces manzanensis.** The methanol extract from Streptomyces manzanensis was subjected to bioassay-guided fractionation and purification using different chromatographic methods, including reverse-phase C18, size exclusion chromatography, and HPLC (Supplemental Fig. 4). After each fractionation step samples were tested for effects on RGS2 protein levels using the PathHunter ProLabel assay, and positive fractions were further purified until one single compound was obtained. The structure of this compound was elucidated using various spectroscopic methods such as MS, UV, infrared, and NMR (Fig. 1, A and B). Structure elucidation revealed the identity of the compound to be a known (PKC) activator, Indolactam V (Fig. 1, C and D). Further analysis also demonstrated that Indolactam V was present predominantly as the (−) stereoisomer (6:1 ratio versus the (+) stereoisomer). Hence, in subsequent follow-up studies we used the commercially available (−)-Indolactam V (Sigma-Aldrich) for comparative analysis.

**Indolactam V Increases RGS2 Protein Levels in a Time- and Dose-Dependent Manner.** To characterize Indolactam V-mediated increases in RGS2 protein levels, we initially performed time course experiments, with Indolactam V added at 1, 2, 3, 4 hours or overnight, using the PathHunter ProLabel assay. Indolactam V selectively increases RGS2 protein levels in a time- and concentration-dependent manner. HEK-293 cells stably transfected with RGS2-PL were treated at different time points with 5 μM Indolactam V (ILV; A) or 10 nM PMA (B) in a 384-well plate. RGS2-PL expression was detected using the PathHunter ProLabel assay. Both ILV and PMA cause a time-dependent increase in RGS2 protein levels. Both Indolactam V isolated from the natural product extract (Fraction 4C5b; C) and commercially available (−)-Indolactam V (Sigma-Aldrich; D) increased RGS2 protein levels 6-fold over basal in a concentration-dependent manner as measured with the PathHunter ProLabel assay with similar EC50 values as measured with the PathHunter ProLabel assay. PMA (E) also increased RGS2 protein levels to a similar level as Indolactam V. In contrast, RGS4 protein levels were not increased by PKC activation either through Indolactam V or PMA treatment. Data in each panel are presented as mean ± S.D. from three independent experiments run in triplicate. ***P < 0.001 using one-way analysis of variance with Bonferroni’s post hoc test for pairwise comparison with control.
ProLabel assay. Both commercially available (−)-Indolactam V (Sigma-Aldrich; Fig. 2A) and another PKC activator, PMA (Fig. 2B), increased RGS2 protein levels in a time-dependent manner. A significant increase in RGS2 protein levels could be observed after 1 hour of treatment with 5 μM Indolactam V and 2 hour of treatment with 10 nM of PMA, with both compounds reaching a maximum effect at 3 hours.

Next, we investigated concentration dependence as well as selectivity of Indolactam V for RGS2 versus RGS4 upregulation. Overnight treatment with our in-house purified and isolated fraction 4C5b from *S. manzanensis* (Fig. 2C) as well as commercially available (−)-Indolactam V (Fig. 2D) increased RGS2 protein levels 6-fold in a concentration-dependent manner with very similar EC₅₀ values [4C5b 777 nM; 95% CI (464 nM, 1.3 μM) versus Sigma-Aldrich 680 nM; 95% CI (473 nM, 972 nM)]. Neither of the compounds had any effect on RGS4 protein levels. PMA showed similar selectivity for RGS2 over RGS4 with an EC₅₀ value of 1.4 nM; 95% CI (0.69 nM, 2.7 nM; Fig. 2E), suggesting a general mechanism mediated by PKC rather than a nonspecific effect of Indolactam V specifically on RGS2 protein levels.

**PKC Activators Increase Both Exogenous and Endogenous RGS2 Protein Levels.** To confirm the results obtained with the PathHunter ProLabel assay we performed Western blot in HEK-293T cells transiently transfected with RGS2-HA. Indolactam V, as well as PMA, significantly increased RGS2-HA protein levels (Fig. 3A), confirming our results with the PathHunter ProLabel assay and ruling out assay artifacts.

We next investigated whether the effect of PKC activation could be seen also with endogenously expressed RGS2 protein. We used primary rat VSMC that have endogenous expression of RGS2 protein (Sjogren et al., 2012). As demonstrated in Fig. 3B, both Indolactam V and PMA treatment significantly increased RGS2 protein expression in VSMC, although with a more modest response than in the recombinant cell lines. This demonstrates that RGS2 protein levels are increased by PKC activators independent of the expression system.

**Effects of Indolactam V and PMA on RGS2 Protein Levels Are Due to PKC Activation.** To determine whether the increase in RGS2 protein levels by Indolactam V and PMA was caused by PKC activation we performed concentration-response experiments using the PathHunter ProLabel assay. HEK-293-RGS2-PL cells were treated with increasing concentrations of Indolactam V or PMA in the presence or absence of the nonselective PKC inhibitor Gö6983. The increase in RGS2 protein levels induced by both Indolactam V and PMA was completely blocked with 100 nM Gö6983, whereas 50 nM Gö6983 resulted in partial blockade of RGS2 protein upregulation (Fig. 4). In Western blot using HEK-293 cells transiently transfected with RGS2-HA (Fig. 4D). Thus, activation of PKC leads to increased RGS2 protein levels.

**PKCβ Mediates Increases in RGS2 Protein Levels by Indolactam V.** To determine which PKC isoform is responsible for increased RGS2 protein levels we transfected HEK-293-RGS2-PL cells with siRNA targeting five individual PKC subtypes (PKCα, β, γ, δ, and ε) and subjected them to treatment with Indolactam V followed by the PathHunter ProLabel assay. As a positive control for the efficiency of siRNA effects, siRNA targeting RGS2 resulted in a 50% reduction in RGS2 protein levels compared with cells transfected with nontargeting siRNA (Fig. 5A). However, even with such low transfection efficiency, siRNA-mediated knockdown of PKCβ resulted in a significant decrease in the maximum increase in RGS2 protein levels induced by Indolactam V (Fig. 5A). Knockdown of the other PKC subtypes had no effect on Indolactam V–induced RGS2 protein levels. We confirmed these results using Western blot and siRNA against PKCα and PKCβ (Fig. 5B). Both siRNAs lead to efficient knockdown of the PKC isoforms. The effect of Indolactam V on RGS2 protein levels was completely blocked by PKCβ knockdown, whereas knockdown of PKCα had no effect (Fig. 5B). Hence, PKCβ seems to be mediating the effects of Indolactam V on RGS2 protein levels.

To confirm this, we used a PKCβ-selective inhibitor, Ruboxastaurin, to block the effects of Indolactam V and PMA, respectively. The effects of both Indolactam V and PMA on RGS2 protein levels could be blocked by 100 nM Ruboxastaurin as demonstrated by both Western blot (Fig. 5C) and the PathHunter ProLabel assay (Fig. 5D–G). This further establishes PKCβ as the subtype responsible for PKC-induced increases in RGS2 protein levels.

**GPCR Activation Leads to a PKC-Mediated Increase in RGS2 Protein Levels.** Given that RGS2 modulates signaling through Gq-coupled receptors and activation of PKC is a major downstream effector of Gq, we next investigated whether activation of a Gq-coupled receptor would result in increased RGS2 protein levels. HEK-293T cells were transiently transfected with the M₃ muscarinic receptor and RGS2-HA. Cells were stimulated with 1 μM carbachol for 1 hour before analysis of RGS2 protein levels by Western blot. Carbachol treatment resulted in a significant increase in RGS2 protein levels that could be blocked by both Gö6983 and Ruboxastaurin (Fig. 6), suggesting that RGS2 protein levels are increased by GPCR activation through a PKC-dependent mechanism.
PKC-Mediated Increase in RGS2 Protein Levels Has Functional Effects on GPCR Signaling. Finally we investigated whether PKC-mediated increases in RGS2 protein levels have functional effects on GPCR signaling. Because Indolactam V treatment will result in activation of signaling outputs related to Gq activation, it proved difficult to measure effects of increased RGS2 protein levels on Gq signaling outputs. Several groups have demonstrated that RGS2 can inhibit Gs-mediated signaling through direct interaction with certain adenylate cyclase subtypes (Salim et al., 2003; Roy et al., 2006). We therefore investigated the effects of Indolactam V–mediated RGS2 protein increase on β2-adrenergic receptor–mediated cAMP signaling. HEK-293T cells were transiently transfected with the β2 receptor agonist isoproterenol caused a robust, concentration-dependent cAMP response that could be partially suppressed by RGS2 (Fig. 7, A and B). In the absence of RGS2, Indolactam V had no effect on the maximum cAMP response to isoproterenol. However, Indolactam V–mediated increased RGS2 protein levels (Fig. 7C) caused a further suppression the cAMP response, suggesting that PKC-mediated increases in RGS2 protein levels has functional effects on GPCR signaling at least in this system.

Discussion

In the present study we used a novel screening strategy to identify small molecule enhancers of RGS2 protein expression. We used a selection of NPEs to enable the identification of novel, naturally occurring molecules. Traditional small molecule high-throughput screening campaigns use a single, high concentration of compounds (typically 10 μM). The complex nature of the NPEs makes it impossible to determine molar concentrations and as such they are instead referred to as microgram per milliliter until a single compound with known molecular weight has been isolated. Several issues arise when screening NPE libraries. Compounds present at high concentration will dominate the response and toxic compounds included in a NPE might exclude it as a hit. Thus, both the false-negative and false-positive hit rate in these types of screens are usually fairly high. Nevertheless, several novel compounds have been discovered from marine natural product extracts that have proven clinical efficacy in various diseases (for review see Molinski et al., 2009; Cragg and Newman, 2013).

The primary aim of the study was to identify novel compounds that increase RGS2 protein levels as possible candidates for cardiovascular drug development. Although the identified compound, Indolactam V, has been described previously, these results demonstrate a new mechanism of RGS2 protein regulation and facilitate increased understanding of the role of protein kinase action for RGS2 function. The knowledge of such mechanisms will aid in the characterization of future novel RGS2 protein stabilizers.

We previously showed that RGS2 is rapidly degraded through the proteasome and that cardiotonic steroids such as ouabain and digoxin increase RGS2 protein levels (Bodenstein et al., 2007; Sjogren et al., 2012). Furthermore
low RGS2 protein levels are associated with hypertension and increased severity of heart failure in animal models. Also, human mutations that lead to decreased RGS2 protein expression have been identified in hypertensive patients in Japanese, Chinese, and African-American populations (Yang et al., 2005; Zhao et al., 2008; Watanabe et al., 2010).

Phosphorylation of RGS proteins was previously demonstrated. RGS5, which is closely related to RGS2, is phosphorylated by PKC at Ser166, with attenuating effects on GAP activity (Moroi et al., 2007). PKC is also known to phosphorylate RGS2 in vitro, leading to attenuated GTPase activating protein activity as well as attenuated ability for RGS2 to inhibit β-adrenergic receptor–mediated Phospholipase C activation in erythrocyte membranes (Cunningham et al., 2001). Although seemingly contradictory to the current results, this study was performed in isolated in vitro systems, where the machinery regulating RGS2 protein levels has been removed. Hence, the effects on GAP activity versus protein levels of RGS2 cannot be directly compared between these studies. Indeed, we found that 3-hour treatment with Indolactam V in HEK-293T cells leads to an increase in RGS2 protein levels and a subsequent augmentation of RGS2-mediated suppression of β2-adrenergic receptor–mediated cAMP production. The mechanism behind this may be very different to what has previously been observed. Indeed, in the case of RGS16, phosphorylation of either Ser53 or Ser194 inhibits GAP activity (Chen et al., 2001), whereas phosphorylation of Tyr168 by Src kinase promotes protein stability (Derrien et al., 2003). Further studies are required to determine the mechanism by which PKC acts to increase RGS2 protein levels.
levels. These studies are under way but are beyond the scope of the current manuscript.

Previous work showed that RGS2 mRNA is downregulated by lysophosphatidic acid stimulation in foam cells, and this could be prevented by inhibiting PKC (Lee et al., 2010). The discrepancies between those results and our current data could be due to cell type-specific effects. Also, RGS2 mRNA and protein levels are likely to be differentially regulated as we previously showed that RGS2 protein is rapidly degraded by the ubiquitin-proteasomal pathway. This was certainly true in the case of RGS4 in a study by the group of Yaping Tu (Xie et al., 2009). They found that RGS4 mRNA levels in metastatic breast cancer cells were 20,000 higher than in control cells. At the protein level, however, RGS4 was virtually undetectable, whereas the nonmetastatic cells demonstrated robust RGS4 protein expression. That study as well as our current data demonstrates that mRNA levels do not necessarily correlate to protein levels, especially in the case of short-lived proteasome targets like RGS2 and RGS4.

At this stage, the effect of Indolactam V (or PMA) does not appear to be due to alterations at the transcriptional level. In the PathHunter ProLabel HEK-293 cell lines both RGS2 and RGS4 are expressed under control of the cytomegalovirus promoter. The selectivity obtained in this system for RGS2 versus RGS4 suggests that the increased RGS2 protein expression by PKC activation occurs through a posttranscriptional mechanism. Future work is needed to determine the precise mechanism of PKC-mediated increases in RGS2 protein expression.

It is unclear whether Indolactam V- or PMA-mediated increases in RGS2 protein levels are a result of direct phosphorylation of RGS2 by PKC or an indirect effect through other mechanisms. RGS2 mRNA has been shown to be upregulated by activation of several Gq-coupled GPCRs, including endothelin-1 and angiotensin II receptors (Grant et al., 2000; Mittmann et al., 2002). In the current study we demonstrated increases in RGS2 also at the protein level after activation of the M3 muscarinic receptor, making it reasonable to suggest that increased RGS2 expression could be a result of downstream activation of PKC by Gq pathways, thus functioning as a negative feedback mechanism for GPCR signaling.

In conclusion, the current study is an important proof-of-concept for our chosen screening strategy. We successfully purified one active compound from a microbial-derived natural product extract through bioassay-guided fractionation. Furthermore, our work effectively facilitates increased understanding of how RGS2 protein levels are regulated.
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Authorship Contributions

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