Differential Effects of the Gβ5-RGS7 Complex on Muscarinic M3 Receptor–Induced Ca²⁺ Influx and Release

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Received January 22, 2014; accepted February 28, 2014

ABSTRACT

The G protein β subunit Gβ5 uniquely forms heterodimers with R7 family regulators of G protein signaling (RGS) proteins (RGS6, RGS7, RGS9, and RGS11) instead of Gγ. Although the Gβ5-RGS7 complex attenuates Ca²⁺ signaling mediated by the muscarinic M3 receptor (M3R), the route of Ca²⁺ entry (i.e., release from intracellular stores and/or influx across the plasma membrane) is unknown. Here, we show that, in addition to suppressing carbachol-stimulated Ca²⁺ release, Gβ5-RGS7 enhanced Ca²⁺ influx. This novel effect of Gβ5-RGS7 was blocked by nifedipine and 2-aminoethoxydiphenyl borate. Experiments with pertussis toxin, an RGS domain–deficient mutant of RGS7, and UBO-QIC [(l)-threonine, (3R)-N-acetyl-3-hydroxy-1-leucyl-(3R)-1-hydroxybenzene propanoyl-2,3-idehydro-N-methylalanyl-l-alanyl-N-methyl-l-alanyl-(3R)-3-[2S,3R)-3-hydroxy-4-methyl-1-oxo-2-[(1S)-oxopropyl]amino] pentyloxy]-1-leucyl-N.O-dimethyly-(7→1)-lactone (9Cl)], a novel inhibitor of Gq, showed that Gβ5-RGS7 modulated a Gq-mediated pathway. These studies indicate that Gβ5-RGS7, independent of RGS7 GTPase-accelerating protein activity, couples M3R to a nifedipine-sensitive Ca²⁺ channel. We also compared the action of Gβ5-RGS7 on M3R-induced Ca²⁺ influx and release elicited by different muscarinic agonists. Responses to Oxo-M [oxotremorine methiodide N,N,N-trimethyl-4-(2-oxo-1-pyrrolidinyl)]-2-butyn-1-ammonium iodide] were insensitive to Gβ5-RGS7. Pilocarpine responses consisted of a large release and modest influx components, of which the former was strongly inhibited whereas the latter was insensitive to Gβ5-RGS7. McN-A-343 [(4-hydroxy-2-butyryl)-1-trimethylammonium-3-chlorocarbanilate chloride] was the only compound whose total Ca²⁺ response was enhanced by Gβ5-RGS7, attributed to, in part, by the relatively small Ca²⁺ release this partial agonist stimulated. Together, these results show that distinct agonists not only have differential M3R functional selectivity, but also confer specific sensitivity to the Gβ5-RGS7 complex.

Introduction

In the canonical pathway, activated G protein–coupled receptors (GPCRs) promote the exchange of GDP for GTP on their cognate heterotrimeric (GαGβγ) G proteins. In the GTP-bound state, Gα-GTP and Gβγ dissociate and activate downstream effector enzymes and ion channels. Go subunits possess intrinsic GTPase activity, which inactivates the G protein and returns the cascade to the inactive state. In most G protein pathways, GTP hydrolysis is accelerated by a family of GTPase-accelerating proteins (GAPs) named regulators of protein signaling (RGS). The RGS homology superfamily consists of more than 30 mammalian RGS and RGS-like family members (Ross and Wilkie, 2000; Hollinger and Hepler, 2002). Some RGS proteins consist of little more than the RGS domain, whereas others have multidomain architecture and bind other signaling molecules. RGS proteins have other functions in addition to GAP activity, and may act as scaffolding proteins and effector modulators, and interact directly and selectively with GPCRs and ion channels (Schiff et al., 2000; Abramow-Newerly et al., 2006; Willars, 2006; McCoy and Hepler, 2009; Orlandi et al., 2012). RGS proteins have been proposed as pharmacological targets because they have a discrete expression profile, can specifically be recruited by activated GPCRs to modulate signaling, and selectively regulate Gi and/or Gq proteins (Roman and Traynor, 2011).

The R7 family of RGS proteins includes RGS6, 7, 9, and 11. In addition to the RGS domain, R7 family members have three other domains: Gγ-like, DEP (first identified in disheveled EGL-10 and pleckstrin), and DEP helical extension.

This work was supported by the National Institutes of Health National Institute of General Medical Sciences [Grant GM060019] and an American Heart Association Predoctoral fellowship [10PRE3600004].

dx.doi.org/10.1124/mol.114.091843.

ABBREVIATIONS: 2-APB, 2-aminoethoxydiphenyl borate; CCh, carbamylcholine chloride (2-hydroxyethyl)trimethylammonium chloride carbamate; DAG, diacylglycerol; DEP, disheveled-EGL10-pleckstrin homology; FLIPR, Fluorometric Imaging Plate Reader; GAP, GTPase-accelerating protein; GPCR, G protein–coupled receptor; HBSS, Hank’s balanced salt solution; HHBSS, Hank’s balanced salt solution containing 20 mM HEPES; M3R, muscarinic M3 receptor; McN-A-343, (4-hydroxy-2-butyryl)-1-trimethylammonium-3-chlorocarbanilate chloride; Oxo-M, oxotremorine methiodide N,N,N-trimethyl-4-(2-oxo-1-pyrrolidinyl)-2-butyn-1-ammonium iodide; PCR, polymerase chain reaction; PIP₂, phosphatidylinositol 4,5-bisphosphate; PLCβ1, phospholipase C β1 isoform; PMA, phorbol 12-myristate 13-acetate; PTX, pertussis toxin from Bordetella pertussis; RGS, regulators of G protein signaling; ROI, region of interest; TRP, transient receptor potential; UBO-QIC, (l)-threonine,(3R)-N-acetyl-3-hydroxy-1-leucyl-(3R)-1-hydroxybenzene propanoyl-2,3-idehydro-N-methylalanyl-l-alanyl-N-methyl-l-alanyl-(3R)-3-[2S,3R)-3-hydroxy-4-methyl-1-oxo-2-[(1S)-oxopropyl]amino] pentyloxy]-1-leucyl-N.O-dimethyly-(7→1)-lactone (9Cl); the plant depsipeptide Gq/11 inhibitor analogous to YM-254890; YM-254890, a novel Gq inhibitor purified from Chromobacterium sp.
The Gβ-like domain is responsible for the obligatory interaction with the unique G protein β subunit Gβ5 (Cabrera et al., 1998; Snow et al., 1998; Levay et al., 1999; Witherow et al., 2000). Dimerization protects the protein from rapid degradation, and the knockout of Gβ5 results in the loss of measurable amounts of all R7 RGS proteins (Witherow et al., 2000; Chen et al., 2003). The DEP domain is required for membrane localization of R7 RGS proteins, where it interacts with R7BP (Drenan et al., 2005; Martemyanov et al., 2005; Jayaraman et al., 2009). The DEP domain also interacts with Gβ5 (Narayanany et al., 2007; Cheever et al., 2008) and a select group of GPCRs (Kovoor et al., 2005; Cao et al., 2009; Sandiford and Slepak, 2009; Orlandi et al., 2012).

R7 proteins exhibit GAP activity toward Gt and Gi/o, but not Gq family G proteins (Hooks et al., 2003). In transfected CHO-K1 cells, Gβ5-RGS7 attenuates muscarinic M3 receptor (M3R)-induced Ca²⁺ signaling (Witherow et al., 2000; Narayanany et al., 2007). The action of Gβ5-RGS7 on Gq-mediated Ca²⁺ signaling is selective for the M3R, as the complex has no effect on several other Gq-coupled GPCRs (Sandiford and Slepak, 2009). This inhibition is not dependent on the GAP function (RGS domain) of RGS7, but instead is mediated by direct interaction via the DEP domain of RGS7 and the third intracellular loop of M3R (M3i3) (Sandiford and Slepak, 2009); the carboxy-terminal tail of the receptor and Gβ5 also appear to participate in the interaction (Sandiford et al., 2010). M3R is known to stimulate both Ca²⁺ release from intracellular stores and influx from the extracellular space (Parekh and Brading, 1992; Carroll and Peralta, 1998). In the present study, we sought to understand whether Gβ5-RGS7 modulates Ca²⁺ release from intracellular and extracellular sources. In addition, using available M3R agonists, we investigated the potential for functional pathway selectivity in Gβ5-RGS7-regulated M3R Ca²⁺ signaling.

Materials and Methods

Carbamylcholine chloride, McN-A-434 [4-hydroxy-2-butyln]-1-trimethylammonium-3-chlorocarbanilate chloride), Oxo-M (oxotremorine methiodide N,N,N',N'-trimethyl-4-(2-oxo-1-pyrrolidinyl)-2-buty1-1-ammonium iodide), pilocarpine hydrochloride, nifedipine, 2-APB (2-aminoethoxydiphenyl borate), and pertussis toxin were purchased from Sigma-Aldrich (St. Louis, MO). The selective Gq/11 inhibitor (2-aminoethoxydiphenyl borate), and pertussis toxin were purchased from Life Technologies (Carlsbad, CA). Glutathione and nickel-nitrilotriacetic acid resins were purchased from GE Healthcare (Piscataway, NJ). SuperSignal West Femto substrate and Fluorometric Imaging Plate Reader (FLIPR) membrane potential dye were acquired from Thermo Scientific (Waltham, MA) and Molecular Devices (Sunnyvale, CA), respectively.

Western Blot Analysis. Antibodies against the DEP domain of RGS7 were raised by immunizing rabbits (Cocalico Biologicals, Inc., Reamstown, PA) with recombinant DEP domain, amino acids 37–112. The DEP domain construct was generated by polymerase chain reaction (PCR) amplification of nucleotides 443–668 with the addition of a 6× His on the 5′ end. Then, the PCR product was inserted at BamHI and EcoRI sites of the pGEX-2T vector (GE Healthcare), which has a glutathione S-transferase tag and a thrombin cleavage site upstream of the ORF. The glutathione S-transferase–DEP–6× His protein was expressed in Escherichia coli and purified by glutathione chromatography as previously described (Narayanan et al., 2007). Next, fusion protein bound to glutathione beads was incubated with thrombin at room temperature for 4 hours with a thrombin to fusion protein ratio of 1:500 in thrombin cleavage buffer (50 mM Tris, 150 mM NaCl, 2.5 mM CaCl₂, and 0.1% 2-mercaptoethanol, pH 8.6). The supernatant was collected and DEP-6× His was purified by nickel-nitrilotriacetic acid resin chromatography (GE Healthcare) as described in Crowe et al. (1994), and the resulting eluate was used for immunization. The final bleed was used at a concentration of 1:5000 to detect recombinant RGS7 and RGS7ARG8 using SuperSignal West Femto substrate (Thermo Scientific) with secondary horseradish peroxidase–conjugated antirabbit antibody at a concentration of 1:75,000.

Constructs for Expression in Mammalian Cells. The cDNA for Gβ5 and RGS7 was cloned into the pcDNA3 vector at BamHI and NotI sites as described previously (Sandiford et al., 2010). The RGS7-ARG8 construct lacking the RGS domain was generated by PCR amplification of nucleotides 1–1310 and cloned into pcDNA3 vector linearized with BamHI and NotI. Construct encoding human M3R in pcDNA3.1 was purchased from clDNA.org.

Cell Culture and Transfection. As described earlier (Narayanany et al., 2007; Sandiford and Slepak, 2009; Sandiford et al., 2010), CHO-K1 cells were cultured in F-12K nutrient mixture with 10% fetal bovine serum and penicillin/streptomycin. Twenty-four hours prior to transfection, CHO-K1 cells were seeded on glass coverslips that were transiently transfected with M3R or M3R + Gβ5-RGS7 and loaded with fura-2 AM. Fluorescence images were recorded in real time using MetaFluor software as described in Materials and Methods. The entire visual field, containing 20–40 cells responding to muscarinic agonists, was used as a region of interest. The traces represent a mean of the 340/380 ratios recorded in at least two independent experiments, with three to six replicates per experiment. (A) CCh (1 μM) was applied for 30 seconds as indicated by the horizontal bar above the traces in the presence or absence of cotransfected Gβ5-RGS7 in complete HBSS buffer containing 1.26 mM CaCl₂. (B) Cells were transfected with Gβ5-RGS7 in Ca²⁺-free HBSS buffer. (C) The mean amplitude ± S.D. of the maximal responses (n = 9) with and without extracellular Ca²⁺. White bars represent cells transfected with M3R and pcDNA3 plasmid. Gray bars represent M3R cotransfected with Gβ5 and RGS7.

Fig. 1. Gβ5-RGS7 attenuation of M3R-stimulated Ca²⁺ release. CHO-K1 cells on glass coverslips were transiently transfected with M3R or M3R + Gβ5-RGS7 and loaded with fura-2 AM. Fluorescence images were recorded in real time using MetaFluor software as described in Materials and Methods. The entire visual field, containing 20–40 cells responding to muscarinic agonists, was used as a region of interest. The traces represent a mean of the 340/380 ratios recorded in at least two independent experiments, with three to six replicates per experiment. (A) CCh (1 μM) was applied for 30 seconds as indicated by the horizontal bar above the traces in the presence or absence of cotransfected Gβ5-RGS7 in complete HBSS buffer containing 1.26 mM CaCl₂. (B) Cells were transfected with Gβ5-RGS7 in Ca²⁺-free HBSS buffer. (C) The mean amplitude ± S.D. of the maximal responses (n = 9) with and without extracellular Ca²⁺. White bars represent cells transfected with M3R and pcDNA3 plasmid. Gray bars represent M3R cotransfected with Gβ5 and RGS7.
transfection, cells were seeded on 12-mm glass coverslips for Ca\textsuperscript{2+} imaging and T-75 flasks for membrane potential assay to achieve 50–75% confluency at the time of transfection. Lipofectamine 2000 transfection reagent was used according to manufacturer guidelines. For Ca\textsuperscript{2+} imaging, to identify transfected cells, we used a vector only expressing enhanced yellow fluorescent protein. The DNA ratio of yellow fluorescent protein to M3R to G\textsubscript{j5-RGS7} was 0.1:1:1.3 with a total of 0.51 \(\mu\)g of plasmid DNA per well. Empty pcDNA3.1 or LacZ vector DNA was used to ensure constant DNA loading in cotransfections. Forty-eight hours after transfection, cells were used for Ca\textsuperscript{2+} imaging or immunofluorescence studies. For membrane potential assay, cells were 80–90% confluent at the time of transfection. The ratio of DNA to Lipofectamine2000 was 1:2 with a total of 18.75 \(\mu\)g DNA per T-75 flask. The plasmid ratio of M3R to G\textsubscript{j5} to RGS7 was 1:1:3.

**Ca\textsuperscript{2+} Imaging.** Transiently transfected CHO-K1 cells grown on 12-mm glass coverslips were washed with culture media then incubated at 37°C in culture media containing 2 \(\mu\)M fura-2 AM for 25 minutes. After fura-2 AM loading, the cells were kept at ambient temperature for no longer than 1.5 hours before imaging. Coverslips were secured in a flow chamber and mounted on the stage of a Nikon TE2000 (Nikon, Tokyo, Japan) inverted fluorescence microscope. The cells were continuously superfused by gravity flow with HBSS either with or without CaCl\textsubscript{2}/MgCl\textsubscript{2}. As required by the experiment, flow was switched to agonist-containing HBSS for the specified time, then changed back to agonist-free buffer. Images were collected in real time every 3 seconds using a 20× UV objective lens and recorded using MetaFluor software (Molecular Devices). The excitation wavelengths were 340 nm (Ca\textsuperscript{2+} bound) and 380 nm (Ca\textsuperscript{2+} free), and the emission was set to 510 nm. The 340/380 ratio is representative of intracellular free [Ca\textsuperscript{2+}]\textsuperscript{i}. The entire field of view was selected as a region of interest (ROI). A typical ROI contained 50–70 cells, of which, 30–50 were yellow fluorescent protein-positive. The number of cells responding to muscarinic agents varied with agonist concentration, but was typically 30 cells in a particular ROI. Traces shown here are averages of two to four independent experiments with three replicate coverslips per experiment.

**FLIPR Membrane Potential Assay.** Twenty-four hours post-transfection, cells were detached with TrypLE Express (Life Technologies) and seeded in a 384-well, black-walled, clear-bottom plate (Corning, Inc., Corning, NY) at a density of 10,000 cells per well, and allowed to grow for 24 hours at 37°C, 5% CO\textsubscript{2}, and 95% relative humidity. As described previously (Baxter et al., 2002), cells were washed three times with assay buffer HBSS containing 20 mM HEPES, pH 7.4 (HHBSS). Then, 25 \(\mu\)l of assay buffer supplemented with 1.26 mM CaCl\textsubscript{2} was added to each well, and cells were allowed to equilibrate at room temperature for 10 minutes. Finally, 25 \(\mu\)l of blue FLIPR Membrane Potential Reagent (Molecular Devices), reconstituted per the manufacturer's instructions (final concentration of 20 \(\mu\)M), was added to the appropriate wells. The plate was incubated at 37°C for 30 minutes, and then placed into the FLIPR Tetra (Molecular Devices). A baseline read of plate fluorescence (510–545 nm excitation and 565–625 nm emission) was performed for 10 seconds. Next, HHBSS alone or CCh [carbamylcholine chloride (2-hydroxyethyl)trimethylammonium chloride carbamate] dissolved in HHBSS was dispensed using an integrated 384-well dispenser to the appropriate wells, during fluorescence measurements. Fluorescence was recorded every second for the remaining 590 seconds of the assay.

**Pertussis Toxin and UBO-QIC Treatment.** Pertussis toxin (PTX) was applied to the cells 4 hours prior to Ca\textsuperscript{2+} imaging at the final concentration of 200 ng/ml in culture media and incubated at 37°C. This concentration was maintained during fura-2 loading. UBO-QIC was dissolved in dimethylsulfoxide to a stock concentration of the maximal responses for total Ca\textsuperscript{2+} response (shown in Fig. 1A), Ca\textsuperscript{2+} release (Fig. 1B), and Ca\textsuperscript{2+} influx (Fig. 2C): *P ≤ 0.05.
of 2 mM and stored at −20°C, and applied to cells at the final concentration of 0.3 μM together with fura-2 AM.

Data Analysis. The raw data for the Ca²⁺ imaging experiments were exported from MetaFluor and into GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). The resulting mean traces were used to determine maximal values, which were compared by applying the t test equation for paired data. Statistically significant differences are indicated by one asterisk (*) for P < 0.05 and a double asterisk (**) for P < 0.01. The dose-response curves (Fig. 8) were fit with nonlinear regression using the GraphPad Prism sigmoidal (variable slope) equation.

Results

Our previous studies showed that Gβ5-RGS7 could attenuate M3R-mediated increases in free intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) in cotransfected CHO-K1 cells independent of its GAP activity (Narayanan et al., 2007; Sandiford and Slepak, 2009; Sandiford et al., 2010). To understand the mechanisms underlying Gβ5-RGS7–mediated inhibition of M3R, we examined the effects of Gβ5-RGS7 on two routes of Ca²⁺ entry into the cytoplasm: influx across plasma membrane and release from intracellular stores. We also compared the influence of Gβ5-RGS7 on signaling elicited by different muscarinic agonists.

Fig. 3. Effects of 2-APB, nifedipine, and PMA on M3R-stimulated Ca²⁺ signaling. Cells were transfected and treated as in Fig. 2. The panels show only the Ca²⁺ transients after depletion of intracellular stores. (A) Cells activated with 1 μM CCh in the presence of Ca²⁺. (B) Cells were treated with 100 μM 2-APB added prior to and during stimulation with 1 μM CCh. (C) Cells were treated with 10 μM nifedipine and stimulated with CCh as in B. (D) Cells expressing M3R or M3R + Gβ5-RGS7 were treated with 100 nM PMA. Each trace represents an average of three replicates in two (B and D) or three (C) independent experiments.

Effects of Gβ5-RGS7 on M3R-Induced Ca²⁺ Influx and Release. To investigate the effect of Gβ5-RGS7 on the different routes of Ca²⁺ entry, we first compared CCh-induced M3R activation in the presence and absence of extracellular Ca²⁺ (Fig. 1). We found that, in the presence of extracellular Ca²⁺, cotransfection with Gβ5-RGS7 resulted in a 10–30% reduction in the amplitude of the Ca²⁺ response (Fig. 1A). In the absence of extracellular Ca²⁺, Gβ5-RGS7–mediated inhibition was much greater, consistently reaching 50–70% (Fig. 1B). The stronger effect of Gβ5-RGS7 on the isolated release component (Fig. 1B) compared with the total (Fig. 1A) suggested that Gβ5-RGS7 might have a positive effect on Ca²⁺ influx.

To test this idea, we depleted intracellular Ca²⁺ stores by treating cells with 10 μM ATP, which activates endogenous ionotropic and Gq-coupled purinergic receptors in CHO cells (Iredale and Hill, 1993; Marcet et al., 2004) without affecting the transfected M3 receptor (Fig. 2). As expected, ATP-pretreated cells did not respond to CCh in Ca²⁺-free medium (Fig. 2A), demonstrating complete depletion of Ca²⁺ stores. After ATP-induced Ca²⁺ depletion, treatment of cells with Ca²⁺-containing buffer did not result in any significant signal (Fig. 2B), demonstrating inactivation of plasma membrane Ca²⁺ channels. However, CCh in Ca²⁺-containing HBSS buffer...
Fig. 4. Effect of Gß5-RGS7 on M3R-stimulated changes in membrane potential. CHO cells transfected with pcDNA, M3R, or M3R + Gß5-RGS7 were incubated with blue FLIPR Membrane Potential Reagent, as described in Materials and Methods. Fluorescence intensity was recorded using the FLIPR Tetra. An increase in fluorescence intensity indicates membrane depolarization. At the time of 10 seconds, CCh was added to final concentrations from 100 nM to 1 mM as indicated in A–E. The traces represent the mean changes in fluorescence intensity collected from quadruplicate wells on the 384-well plate. (F) Concentration dependencies of the maximal change in fluorescence at the last time point recorded in A–E.

Fig. 5. M3R Ca²⁺ signaling in transfected CHO cells only involves Gq. (A) Transfected cells were incubated with or without PTX, as described in Materials and Methods, and stimulated with 10 μM 5-hydroxytryptamine (5-HT). Traces show Ca²⁺ increase after store depletion as in Figs. 2 and 3. (B) Cells with or without PTX pretreatment were stimulated with CCh. (C) Cells were transfected with M3R or M3R together with Gß5 plus full-length RGS7 or the mutant lacking the RGS domain (RGS7DΔRGS). (D) Cells were treated with UBO-QIC, as described in Materials and Methods, and the total Ca²⁺ response to 1 μM CCh was recorded as in Fig. 1A. WT, wild-type.
induced a marked increase in [Ca^{2+}], showing that stimulation of M3R induces Ca^{2+} influx after ATP-induced Ca^{2+} store depletion (Fig. 2C). Gβ5-RGS7 augmented the CCh-mediated Ca^{2+} influx response, indicating that the complex has contrasting effects on the two routes of Ca^{2+} entry to the cytoplasm: it inhibits release from intracellular stores (Fig. 1) and stimulates influx across the plasma membrane (Fig. 2).

Evidently, when Ca^{2+} is present in both extracellular medium and intracellular stores, the net effect of Gβ5-RGS7 on CCh-induced increases in [Ca^{2+}], is slightly negative (Fig. 1A; Narayanan et al., 2007; Sandiford and Slepak, 2009; Sandiford et al., 2010). Since Ca^{2+} influx is essential in many cellular processes, including neurotransmitter release, the novel effect of Gβ5-RGS7 on M3R-stimulated Ca^{2+} influx was particularly interesting. Thus, we focused on delineating the mechanisms involved in this pathway.

**Gβ5-RGS7-Enhanced Ca^{2+} Influx Is Sensitive to Nifedipine, but Is Not Voltage-Dependent.** Multiple channels can mediate M3R-stimulated Ca^{2+} influx, including several transient receptor potential (TRP) (Montell, 2005) and voltage-dependent Ca^{2+} channels (Wuest et al., 2007). We tested the effect of the TRP channel inhibitor 2-APB and the L-type Ca^{2+} channel blocker nifedipine on M3R-stimulated Ca^{2+} influx (Fig. 3). 2-APB blocked Ca^{2+} influx by more than 90%, irrespective of the presence of Gβ5-RGS7 (Fig. 3, A and B). In contrast, nifedipine had little effect on M3R-stimulated influx in the absence of Gβ5-RGS7 (Fig. 3, A and C). However, nifedipine abolished the positive effect of Gβ5-RGS7 on Ca^{2+} influx (Fig. 3, A and C). In addition, we activated TRP channels by the diacylglycerol (DAG) analog PMA (phorbol 12-myristate 13-acetate) (Hardie, 2007). The Gβ5-RGS7 complex did not enhance PMA-stimulated Ca^{2+} influx. Taken together, these results indicate that M3R-elicited Ca^{2+} influx requires activation of a 2-APB–sensitive channel(s), and that Gβ5-RGS7 selectively enhances the activity of a nifedipine-sensitive Ca^{2+} channel(s).

Since nifedipine inhibits the positive effect of Gβ5-RGS7 (Fig. 3, A and C), we used a voltage-sensitive fluorescent dye assay (Baxter et al., 2002) (Fig. 4) to investigate whether Gβ5-RGS7 had an effect on M3R-stimulated changes in membrane potential. As is evident from the reduction of the fluorescence signal, at CCh concentrations below 10 μM, M3R activation caused hyperpolarization, and addition of Gβ5-RGS7 had no effect (Fig. 4, A–C). However, at CCh concentrations above 10 μM, M3R activity caused a notable membrane depolarization, consistent with earlier reports (Carroll and Peralta, 1998), and the Gβ5-RGS7 complex significantly attenuated this effect (Fig. 4, D and E). Considering that neurotransmitter in the synaptic cleft can reach millimolar concentrations (Scimemi and Beato, 2009) and the differential effect of micro- and

**Fig. 6.** Influence of Gβ5-RGS7 on acetylcholine-stimulated Ca^{2+} responses. We used 10 μM acetylcholine ACh [acetylcholine (2-acetyloxyethyl-trimethylazanium)] to elicit M3R-mediated Ca^{2+} release and influx as described in Figs. 1 and 2, respectively. (A) Total ACh response in complete HBSS buffer. (B) ACh-stimulated release was measured using Ca^{2+}-free HBSS buffer. (C) ACh in complete HBSS buffer stimulates Ca^{2+} influx response after ATP-induced Ca^{2+} depletion. (D) The bar graphs represent the mean amplitude ± S.D. of the Ca^{2+} responses from two independent experiments with three replicates in each. **P < 0.01.
millimolar [CCh] on M3R signal transduction (Carroll and Peralta, 1998), the effect of Gb5-RGS7 on depolarization at 0.01–1.0 mM CCh (Fig. 4F) is interesting and deserves further investigation.

The Action of Gb5-RGS7 Involves Gq, but Not Gi. It is well established that M3R couples to Gq; however, there are a few reports which suggest it can also couple to Gi (Offermanns et al., 1994). To rule out the possibility Gb5-RGS7–enhanced Ca2+ influx is mediated through GAP activity on Gi, we blocked Gi with PTX prior to M3R stimulation. Although PTX inhibited the endogenous 5-HT1B receptor (Fig. 5A), a Gi-coupled GPCR known to mobilize Ca2+ in CHO-K1 cells (Dickenson and Hill, 1995), it had no effect on M3R-stimulated Ca2+ influx in the presence or absence of Gb5-RGS7 (Fig. 5B). Consistent with this result, deletion of the RGS7 RGS domain (RGS7ΔRGS) had no significant effect (P = 0.08) on its ability to enhance M3R-stimulated Ca2+ influx (Fig. 5C). Next, we pharmacologically inhibited Gq with UBO-QIC, a cyclic depsipeptide also known as FR900359 (Zaima et al., 2013). This plant depsipeptide is closely related to the better described compound YM-254890 from Chromobacterium sp, which blocks Gq signaling by direct binding to Gq and inhibition of GDP release (Takasaki et al., 2004). UBO-QIC completely blocked the Ca2+ response to M3R stimulation (Fig. 5D). Together, these data confirm that the effect of Gb5-RGS7 on M3R-induced Ca2+ signaling is dependent on a Gq/11-mediated, but not Gi-mediated, mechanism.

Sensitivity of M3R to Gb5-RGS7 Regulation Is Determined by Distinct Agonists. Distinct muscarinic agonists are known to differentially activate Ca2+ influx and release (Hishinuma et al., 1997; Schaafsma et al., 2006). We investigated the effect of Gb5-RGS7 on M3R-mediated Ca2+ responses elicited by the full agonists acetylcholine, carbachol, and Oxo-M and the partial agonists pilocarpine and McN-A-343 (Figs. 6–10). We found that Ca2+ influx and release responses elicited by the tested compounds exhibited varying degrees of sensitivity to Gb5-RGS7. Whereas Gb5-RGS7 attenuated Ca2+ release induced by acetylcholine (Fig. 6B), it had no measurable effect on Ca2+ influx (Fig. 6C), resulting in an overall negative effect of Gb5-RGS7 on the total Ca2+ response (Fig. 6, A and D). When M3R was stimulated with Oxo-M, neither Ca2+ release (Fig. 7B) nor influx (Fig. 7C) was affected by Gb5-RGS7. As described earlier, Gb5-RGS7 affected both influx and release induced by CCh (Figs. 1 and 2).

Interestingly, pilocarpine-elicited responses were more sensitive to attenuation by Gb5-RGS7 compared with CCh (Fig. 8). Gb5-RGS7 reduced the Emax for CCh (Fig. 8A) and pilocarpine (Fig. 8B) responses to 56 and 37% of M3R alone, respectively (Table 1). The pilocarpine-elicited Ca2+ signals were composed of strong release and modest influx components (Fig. 9).
Together, these results (Table 2) indicate that the action of Gβ5-RGS7 on M3R-stimulated Ca\(^{2+}\) influx involves a specific receptor state that can only be induced by some ligands.

### Discussion

Earlier work in our laboratory (Narayanan et al., 2007; Sandiford and Slepak, 2009; Sandiford et al., 2010) demonstrated that the Gβ5-RGS7 complex attenuated M3R-dependent increases in intracellular [Ca\(^{2+}\)]. It is well documented that activated M3R initiates both release from intracellular stores and influx across the plasma membrane (Felder et al., 1992; Parekh and Brading, 1992; Carroll and Peralta, 1998). In this study, we experimentally isolated these constituents of Ca\(^{2+}\) entry and found that the Gβ5-RGS7 complex inhibits the release pathway (Fig. 1B), but augments Ca\(^{2+}\) influx (Fig. 2C). Apparently, this dual action produces a net negative effect on carbachol-stimulated increases in [Ca\(^{2+}\)], observed in Fig. 1A and in our earlier studies (Narayanan et al., 2007; Sandiford and Slepak, 2009; Sandiford et al., 2010).

Ca\(^{2+}\) release is mediated by the canonical pathway involving Gq, phospholipase C\(\beta\) (PLC\(\beta\)), and the second messenger inositol triphosphate, and the inhibitory action of Gβ5-RGS7 on M3R-stimulated Ca\(^{2+}\) release may be explained by its competition with Gq for the receptor (Blin et al., 1995; Singer-Lahat et al., 1996; Wuest et al., 2007). We began dissecting this pathway here by using G protein inhibitors and ion channel blockers (Figs. 3 and 5). Our assays utilizing PTX, the GAP-deficient RGS7 construct G5-RGS7 stimulated with CCh (A) and pilocarpine (B), respectively, this dual action produces a net negative effect on carbachol-stimulated increases in [Ca\(^{2+}\)], observed in Fig. 1A and in our earlier studies (Narayanan et al., 2007; Sandiford and Slepak, 2009; Sandiford et al., 2010).

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Receptor-induced Ca\(^{2+}\) influx involves two major pathways: Ca\(^{2+}\)-dependent, i.e., store-operated, and Ca\(^{2+}\)-independent (Putney, 2010). In our experiments, we eliminated potential contribution of store-operated channels by depleting intracellular Ca\(^{2+}\) stores prior to M3R stimulation. As Gβ5-RGS7 can potentiate Ca\(^{2+}\) influx under these conditions, it stands to reason that these channels are Ca\(^{2+}\)-independent. Our results with the L-type Ca\(^{2+}\) channel blocker nifedipine and the nonselective TRP channel blocker 2-APB show that both of these types of channels participate in this pathway. Although nifedipine had no significant effect on M3R-stimulated Ca\(^{2+}\) influx, it reduced the response in the presence of Gβ5-RGS7 (Fig. 3, A and C). This finding indicates that Gβ5-RGS7 facilitates coupling of M3R to a nifedipine-sensitive channel. Since Gβ5-RGS7 did not influence M3R-induced changes in membrane potential at CCh concentrations below 10 µM (Fig. 4), this channel does not appear to be voltage-dependent. Indeed, it has been shown that nifedipine can influence activity of channels other than the L-type family of voltage-dependent calcium channels (Curtis and Scholfield, 2001).

M3R-stimulated Ca\(^{2+}\) influx was nearly abolished by 2-APB regardless of the presence of Gβ5-RGS7 (Fig. 3D), suggesting TRP channel activation is necessary in this M3R-initiated pathway. Since some TRP channels can be activated by the membrane-delimited second messenger DAG (Montell, 2005), we tested if Gβ5-RGS7 could influence PMA-induced
Ca^{2+} influx, and found no such effect. These findings suggest the G\textsubscript{b}5-RGS7 complex does not directly influence DAG-activated TRP channels, and possibly acts upstream of the DAG-generating M3R effector enzyme PLC-\beta. In the past decade, it has become increasingly apparent that PIP\textsubscript{2} (phosphatidylinositol 4,5-bisphosphate) can either facilitate or inhibit pore opening of various ion channels (Suh and Hille, 2008). For example, ubiquitously expressed TRPM7 channels, which are Ca^{2+} permeant, are inhibited by activation of G\text{q} and depletion of PIP\textsubscript{2} (Runnels et al., 2002). Therefore, if G\textsubscript{b}5-RGS7 inhibits M3R–G\text{q} coupling, it should result in potentiation of Ca^{2+} conductance through TRPM7. This idea could explain an early observation by Carroll and Peralta (1998) that at low [CCh], the Ca^{2+} responses primarily consist of influx, and as M3R occupancy increases PLC activity, there is less influx because depletion of PIP2 reduces Ca^{2+} channel activity. It is also reasonable to speculate that G\textsubscript{b}5-RGS7 participates in a macromolecular complex that couples M3R to a nifedipine-sensitive channel, analogous to the complex containing G\textsubscript{b}5-RGS7 and some G protein–coupled inwardly rectifying potassium channels (Xie et al., 2010).

A single GPCR can control multiple signaling pathways within the same cell. The pleiotropic nature of GPCR signal transduction may be explained by compartmentalization (Ostrom and Insel, 2004; Halls, 2012), GPCR oligomerization (Terrillon and Bouvier, 2004), functional selectivity, and receptor conformation (Kobilka and Deupi, 2007; Jian et al., 2008; Kelly et al., 2008; Wacker et al., 2013). Previous reports showed that various muscarinic agonists can preferentially activate Ca^{2+} influx or release (Schaafsma et al., 2006). Consistent with this concept, we found that agonists used in our study were biased toward one of the two routes of the Ca^{2+} entry (Figs. 6–10; Table 2). Acetylcholine, oxotremorine-m, and CCh stimulated both influx and release. Pilocarpine strongly stimulated Ca^{2+} release, but had little impact on influx. Another partial agonist, McN-A-343, generated strong influx and rather weak Ca^{2+} release constituents. G\textsubscript{b}5-RGS7 inhibited Ca^{2+} release in the presence of all agonists except Oxo-M, suggesting that Oxo-M stabilizes an M3R conformation unfavorable to G\textsubscript{b}5-RGS7 binding. The positive effect on influx was only observed when M3R was stimulated with CCh and McN-A-343. Pilocarpine-evoked influx was not augmented by G\textsubscript{b}5-RGS7 (Fig. 6C). These results indicate that the action of G\textsubscript{b}5-RGS7 on influx involves a specific receptor state that can only be induced by some ligands.

When Ca^{2+} is present both intra- and extracellularly in CHO cells, the positive effect of G\textsubscript{b}5-RGS7 on influx may not be apparent because the negative effect on release is dominant. It is, however, plausible that G\textsubscript{b}5-RGS7 potentiation of influx may be particularly significant in nerve terminals or secretory cells where Ca^{2+} influx is crucial for vesicular fusion and content release. For example, in pancreatic \( \beta \) cells, M3R stimulation in the presence of high glucose leads to depolarization and insulin secretion that is independent of Ca^{2+} release and contingent upon Ca^{2+} influx (Henquin et al., 1988; Miura et al., 1996; Love et al., 1998; Yang and Berggren, 2006). It has recently been demonstrated that G\textsubscript{b}5 is expressed in pancreatic islet \( \beta \) cells.
in pancreatic islets (Wang et al., 2011; Nini et al., 2012). Therefore, the positive effect on Ca\(^{2+}\) influx identified in the CHO cell model could potentially explain the reduced plasma insulin and impaired ability to clear glucose in G\(\beta\)5-deficient mice (Wang et al., 2011).

In conclusion, we identified a dual effect of G\(\beta\)5-RGS7 on M3R-stimulated Ca\(^{2+}\) influx and release. The membrane-delimited pathway underlying influx requires Gq and involves activation of 2-APB–sensitive channel(s) and selective coupling of M3R to a nifedipine-sensitive Ca\(^{2+}\) channel by G\(\beta\)5-RGS7. Additionally, we showed that different agonists confer functional selectivity of M3R to G\(\beta\)5-RGS7, in accordance with the idea that ligands can modify GPCR interaction with protein binding partners.

### Acknowledgments
The authors thank Qiang Wang, Konstantin Levay, and Alexey Pronin for critical advice, and Peter Buchwald for critically reading the manuscript. The authors also thank Simone Sandiford, Junior Tayou, and Evangelos Liapis.

### Authorship Contributions
Participated in research design: Karpinsky-Semper, Volmar, Brothers, Slepak.

Conducted experiments: Karpinsky-Semper, Volmar.

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Wrote or contributed to the writing of the manuscript: Karpinsky-Semper, Volmar, Brothers, Slepak.

### References


![Fig. 10. Effects of G\(\beta\)5-RGS7 on McN-A-343–stimulated Ca\(^{2+}\) influx and release. (A) Total response of M3R to 100 μM McN-A-343 in complete HBSS buffer, in the absence and presence of G\(\beta\)5-RGS7. (B) McN-A-343–stimulated Ca\(^{2+}\) release. (C) Ca\(^{2+}\) response to McN-A-343 in complete HBSS following ATP depletion in Ca\(^{2+}\)-free buffer. (D) The mean amplitude ± S.D. of the Ca\(^{2+}\) responses from two independent experiments with three replicates in each. *P ≤ 0.05; **P ≤ 0.01.

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<th>Compound</th>
<th>Release</th>
<th>Influx</th>
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<tr>
<td>Acetylcholine</td>
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<td>Oxo-M</td>
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<td>Pilocarpine</td>
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<tr>
<td>McN-A-343</td>
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Release, measured in Ca\(^{2+}\)-free buffer; Influx, response elicited after Ca\(^{2+}\) store depletion; Net, measured in complete buffer.