Influence of Goαq on the Dynamics of M₃-Acetylcholine Receptor–G-Protein–Coupled Receptor Kinase 2 Interaction

Valerie Wolters, Cornelius Krasel, Jörg Brockmann, and Moritz Bünemann

Institute for Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Philippus-University Marburg, Marburg, Germany (V.W., C.K., M.B.); and Department of Pharmacology and Toxicology, University of Würzburg, Würzburg, Germany (J.B.)

Received July 10, 2014; accepted October 10, 2014

ABSTRACT

G-protein–coupled receptor kinase 2 (GRK2) is a serine/threonine kinase with an important function in the desensitization of G-protein–coupled receptors. By phosphorylating cytoplasmic regions of the receptor, they induce binding of arrestin proteins to activated receptors, resulting in an uncoupling of receptor signaling and initiation of receptor internalization (Krupnick and Benovic, 1998; Wu et al., 1998). Most GRKs are ubiquitously expressed (Pitcher et al., 1998), and alterations in their expression levels have been shown to be associated with several diseases, such as heart failure (Ungerer et al., 1993; Hata and Koch, 2003). The importance of GRKs in the cardiovascular system and other fields demonstrates that a comprehensive knowledge about the various functions of GRKs is of great interest.

Structurally, GRKs are multidomain proteins, containing an N-terminal RGS (regulator of G-protein signaling) homology domain, followed by a central kinase domain and a C-terminal domain (Pitcher et al., 1998). The C-terminal domain is variable

Introduction

G-protein–coupled receptor kinases (GRKs) are a family of serine/threonine kinases that initiate the desensitization of G-protein–coupled receptors. Based on its ability to bind G-protein βγ subunits as well as activated Goα subunits, it can be considered as an effector for G-proteins. The recruitment of GRK2 with activated Gq-protein subunits as well as negatively charged membrane phospholipids (Pitcher et al., 1992; Touhara et al., 1995). Because of overlapping binding sites, these GRKs compete with inactive Goα subunits for Gβγ binding. Therefore, they only interact with Gβγ and translocate to the membrane after G-protein activation (Ford et al., 1998).

The RGS homology domain of GRK2 and GRK3 is known to interact with activated Goα-proteins, but not with Goα– or Goγ–proteins (Carman et al., 1999; Sallese et al., 2000). It has been shown by a crystal structure that even a simultaneous binding of Gβγ and Goα is possible (Tesmer et al., 2005). The GRK binding site on Goα includes the Goα switching region, which enables the discrimination between the active and inactive state of Goα (Carman et al., 1999). The GRK surface that binds Goα (C-site) differs from the binding site RGS proteins use (A-site) (Zhong and Neubig, 2001; Sterne-Marr et al., 2003), which is a possible explanation for the weak or complete lack of GAP (GTPase-activating protein) function of GRK2 (Carman et al., 1999). The sequestration of activated G-protein subunits by GRK2 has been shown to desensitize the signaling of G-protein–coupled receptors independent between the different GRKs, but in all cases is essential for their membrane targeting. GRK2 and GRK3 contain the largest C-terminal domain, including a pleckstrin homology domain that interacts with Gβγ subunits as well as negatively charged membrane phospholipids (Pitcher et al., 1992; Touhara et al., 1995). Because of overlapping binding sites, these GRKs compete with inactive Goα subunits for Gβγ binding. Therefore, they only interact with Gβγ and translocate to the membrane after G-protein activation (Ford et al., 1998).

This study was funded by the Collaborative Research Centre grant SFB 593 TPA 13 to M.B.

dx.doi.org/10.1124/mol.114.094722

This article has supplemental material available at molpharm.aspetjournals.org.
of phosphorylation by preventing their interaction with downstream effectors (Raveh et al., 2010; Fernandez et al., 2011). However, whether binding of activated Goq affects the function of GRK2 is still unknown and needs further investigation.

With the present study, we aimed to clarify the effects of Goq on the interaction of GRK2 with activated Gq-protein–coupled receptors. Of special importance for our study were previously published GRK2 mutants with reduced binding affinity toward Goq (GRK2(D110A)) (Sterne-Marr et al., 2003) and Gqγ [GRK2(R587Q)] (Carman et al., 2000). These mutants allowed determination of the individual contribution of the Gq-protein subunits for the recruitment and interaction of GRK2 with G-protein–coupled receptors. We established new Förster resonance energy transfer (FRET)–based assays to study the interaction of GRK2 with the M2-acetylcholine (M2-ACh) receptor (M2-AChR) as well as Gqα-protein subunits with high spatiotemporal resolution in single living human embryonic kidney 293T (HEK293T) cells. Combining advantages of FRET imaging with the GRK2 mutants provided new insight into the influence of Goq on extent and stability of GRK2 interaction with the M3-AChR.

Materials and Methods

Chemicals. Agar, ampicillin, enhanced chemiluminescence solution, and lysogeny broth were purchased from AppliChem (Darmstadt, Germany); acrylamide, glycine, MgCl2, milk powder, SDS, and tetramethylethylenediamine were from Carl Roth (Karlsruhe, Germany); agarose was from Biozym (Hessisch Oldendorf, Germany); CaCl2 was from MERCK (Darmstadt, Germany); and Dulbecco’s modified Eagle’s medium, fetal calf serum, phosphate-buffered saline, penicillin/streptomycin, L-glutamine, and trypsin-EDTA were from Biochrom (Berlin, Germany). All other substances were purchased from Sigma-Aldrich (Steinheim, Germany).

Plasmids. The M3-AChR was obtained from the Missouri S&T cDNA Resource Center (Rolla, MO). cDNAs for Goq, Goαγ–yellow fluorescent protein (YFP) (Hughes et al., 2001), Gβ1, Gγ2, Gq2, Goα2-adrenergic receptor (Goα2-AR), Goq1–YFP (C351I) (Bünemann et al., 2003), Goq1 (C351I) (Wise et al., 1997), Goqα, Gβ1-Cer (Frank et al., 2005), M2-AChR–YFP (Hoffmann et al., 2012), arrestin3-YFP (Krasel et al., 2009), and GRK2 (Winstel et al., 1996) were described previously. GRK2–mTurquoise fluorescent protein (mTurq) was cloned analogously to M2-AChR–YFP. M–AChR–cyan fluorescent protein (CFP) was generated by cloning CFP with a Ser-Arg linker to the C-terminus of human M2-AChR. GRK2-YFP and GRK2-mTurq were cloned by fusing the open reading frames of YFP and mTurquoise, respectively, to the C-terminus of human GRK2 using polymerase chain reaction. In these constructs, the last amino acid of GRK2 is connected to the second amino acid of the fluorescent protein by a Ser-Arg linker. The GRK2 mutants were generated from these plasmids by site-directed mutagenesis using the following primers: 5′ CAAGATGTACGCGCATGAGGCTCGACAAAGA 3′ (K220R), 5′ CCGGGAGATTCTGCCATCACACATG 3′ (D110A), 5′ CCTGTTCCACCAACTCACGTGGGTTGGC 3′ (R587Q).

Cell Culture and Transfection. Experiments were performed in HEK293T cells cultured at 37°C in a humidified atmosphere with 5% CO2 in Dulbecco’s modified Eagle’s medium (4.5 g/l glucose) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Transient transfection was conducted with Qiagen Effectene Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The general transfection protocol contained 0.5 μg M2-AChR, 1.6 μg Goqα, 0.5 μg Gβ1, 0.2 μg Gγ2, and 0.5 μg GRK2. In the experiments shown in Figs. 1, 4, and 6, the protocol was adapted to 1 μg M2-AChR and 0.1 μg GRK2 to adjust the expression levels. Experiments were performed 40–48 hours after transfection at room temperature.

Translocation Experiments. Translocation experiments were performed with an inverted microscope (IX71; Olympus, Hamburg, Germany) with a 100× immersion objective (UPlanSApo 100/1.40 Oil; Olympus) equipped with a confocal fluorescence recovery after photobleaching imaging system (VT-HAWK; VisiTech International, Sunderland, UK) and the beam splitter 405/491/642 DC (VisiTech International). The samples were illuminated with 405 nm and 491 nm lasers (VisiTech International). An Optosplit II with T495plxr, ET 470/40x and ET 535/30m (Chroma, Bellows Falls, USA) was used to split YFP and CFP emission on a CCD (charge-coupled device) camera (EM-CCD Digital Camera, Hamamatsu, Herrsching am Ammersee, Germany). Fluorescence recordings were processed using the software VoxxCell Scan (VisiTech International). A pressurized perfusion system (Ala-VC1–SSP; ALA Scientific Instruments, Farmingdale, NY) was used to continuously superfuse the cells with buffer (137 mM NaCl, 5.4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM Heps, pH 7.3) or agonist-containing buffer. For translocation experiments, GRK2–mTurq–transfected cells were excited at 405 nm, and mTurq emission was recorded at 2 Hz. Subsequently, YFP was directly excited at 491 nm, and YFP fluorescence recorded at 2 Hz to determine the relative expression level of M2-AChR and GRK2. To analyze membrane translocation of GRK2, two ROIs (region of interest) were defined: a polygonal ROI including the cell membrane and a rectangular ROI in the cytosol. Then the quotient of the fluorescence intensities (Imembrane/Icytosol) was calculated. ΔImembrane/Icytosol of the individual recordings was averaged to compare translocation of the different GRK2 mutants.

Single-Cell FRET Imaging. FRET experiments were performed on an inverted fluorescence microscope (Eclipse Ti; Nikon, Düsseldorf, Germany) as described in Milde et al. (2013). In some experiments, cells were not excited with the Lambda DG-4 (Sutter Instrument, Novato, CA) but with a light-emitting diode (LED) excitation system (PE-2; CoolLED, Andover, UK) containing LEDs emitting light at 425 and 500 nm, respectively. The intensity of both LEDs was set to 2%. All filters were unchanged. The recording interval is indicated in the figure legend of the respective experiment. Fluorescence data were corrected for background fluorescence, bleed-through, and false excitation, and the FRET ratio was determined as the quotient of the fluorescence intensities of YFP and CFP (Fyfp/Fcfp). The data shown in Fig. 6A and Supplemental Figs. 3 and 4A were additionally corrected for bleaching effects. Individual FRET recordings were averaged and displayed either as absolute alterations in FRET ratio or as data normalized to the individual maximal agonist-induced response to compare onset and offset kinetics of the agonist-induced effect. Absolute alterations in FRET were determined as the difference between the averaged FRET ratio of the last 10 time points before stimulation with and withdrawal of the agonist for each individual recording. Monoeponential onset and offset kinetics were fitted with an exponential function for each individual recording. The obtained k values were used for further statistics. Bixonponential kinetics was analyzed with the mean traces of the different conditions.

Quantification of Relative Expression Levels. Because of an influence on the extent of the FRET signal, the relative expression level of CFP and YFP was controlled. For calibration of the stoichiometry of the relative expression level, the construct YFP–β2–AR–CFP (Dorsch et al., 2009) and analogously cloned reference constructs with different fluorophores were used. Both fluorophores were excited individually, and fluorescence intensities were recorded and corrected for background fluorescence. The calibration factor was calculated as Fcfp/Fyfp. For each individual FRET recording, the factor Fcfp/Fyfp was calculated the same way. This factor was divided by the respective calibration factor to calculate the individual expression ratio.

For a reliable comparison of FRET signals, the relative expression ratio should not significantly differ between different conditions. An
excess of the FRET acceptor YFP is advantageous, as an influence on the extent of the FRET signal is unlikely in that case.

**Evaluation and Statistics.** Data evaluation was conducted with Excel 2010 (Microsoft, Redmond, WA) and Origin Pro 9.1 (OriginLab, Northampton, MA). Arithmetic mean and standard error were calculated where applicable. Statistics were analyzed by analysis of variance (ANOVA) with Bonferroni post-hoc test. *P* <0.05 were considered to represent significant differences between tested conditions.

**Results**

**Interaction of GRK2 with M3-AChR.** To study dynamics of GRK2 interaction with M3-AChR, we set out to image FRET between mTurq-labeled M3-AChR and YFP-labeled GRK2. Functionality of a similarly C-terminally labeled M3-AChR was described previously (Ziegler et al., 2011). The C-terminally YFP-labeled GRK2 was functional, as it phosphorylated rhodopsin similarly to wild-type GRK2 (Supplemental Fig. 1). The fluorescently labeled constructs were transiently transfected in HEK293T cells and subjected to single-cell dual-emission FRET recording. mTurq was excited at 425 nm, and fluorescence of mTurq and YFP was recorded simultaneously, while cells were continuously superfused with buffer with or without agonist, allowing for determination of onset and offset kinetics as a function of agonist addition or withdrawal. Receptor stimulation with a saturating agonist concentration of ACh (10 μM) resulted in a reversible increase in YFP fluorescence (yellow trace) and a corresponding decrease in mTurq fluorescence (blue trace), reflecting the development of FRET due to the interaction between GRK2 and the M3-AChR (Fig. 1A). The emission ratio of both recordings was determined as Δ(FYFP/FCFP) and is referred to as FRET in the following paragraphs.

The kinase-deficient GRK2(K220R) (Kong et al., 1994), which was used to control for effects due to receptor phosphorylation by GRK2, was comparable to wild-type GRK2 in FRET change (Fig. 1B) and agonist-mediated membrane translocation (Fig. 1C). Therefore, a major influence of receptor phosphorylation on kinetics of GRK2–M3-AChR interactions can be excluded. To verify specificity of the agonist-dependent alteration of FRET, HEK293T cells were transfected with the same constructs, but additionally with unlabeled α2A-AR and Goβγ. GRK2 was recruited to the membrane after stimulation of α2A-AR with 100 μM norepinephrine (Fig. 1C). However, this membrane recruitment resulted in only a very minor development of “bystander” FRET between GRK2 and M3-AChR (Fig. 1B), thereby confirming high specificity of the GRK2–M3-AChR interaction.

**Interaction of GRK2 with Goα and Gβγ.** The Goα-protein binding–attenuated GRK2 variant GRK2(D110A) and the Gβγ binding–attenuated GRK2(K220R) were used to investigate the effect of Goα and Gβγ binding by GRK2 on the GRK2–M3-AChR interaction. To determine the effectiveness of the inserted point mutations, FRET-based interaction assays between GRK2 and Gβγ were established in HEK293T cells. M3-AChR stimulation with 10 μM ACh resulted in a reversible development of FRET that reflects Gβγ–GRK2 and Goα–GRK2 interaction (Fig. 2, A and B). Between GRK2 and Goα only a minor rise in FRET was detectable, verifying specificity of the Goα–GRK2 interaction (Supplemental Fig. 2A). GRK2(K220R) showed a small FRET signal with Gγ and GRK2(D110A) showed a small FRET signal with Goα.

**Fig. 1.** FRET imaging enables investigations of GRK2 recruitment to M3-AChR. (A) HEK293T cells transiently transfected with YFP-labeled GRK2, M3-AChR–mTurq, and unlabeled Goα, Gβ, and Gγ were subjected to single-cell FRET imaging recorded at 2 Hz. mTurq was excited at 425 nm, and mTurq and YFP fluorescence were recorded simultaneously. M3-AChR stimulation with 10 μM ACh, as indicated by the black bar, resulted in a reversible increase in YFP fluorescence (yellow trace) and a corresponding decrease in CFP fluorescence (blue trace), reflecting the interaction between GRK2 and M3-AChR (lower panel). The FRET ratio was determined as Δ(FYFP/FCFP) (upper panel). (B) Single-cell FRET recordings were averaged (mean ± S.E.M.; n = 12) and displayed as absolute alterations in FRET. Experiments with α2A-AR [stimulated with 100 μM norepinephrine (NE) and Goα] were used to control signal specificity. The absolute FRET amplitude of the underlying individual experiments was determined as described in the Materials and Methods section, and statistical analysis was performed by ANOVA with Bonferroni post-hoc test (*P* <0.05). (C) Images of the different conditions in the absence and presence of agonist (average of five sequential images recorded by live-cell confocal microscopy).
confirming that these mutants exhibit a substantially reduced affinity with respect to the binding of either Gbg or Gag, or both (Fig. 2, C and D; Supplemental Fig. 2, B and C). However, Gag binding deficiency appeared not as potent as Gbg binding deficiency.

Furthermore, the FRET signal between Gag and GRK2 (R587Q) was significantly diminished compared with wild-type GRK2, which confirms the importance of Gbg for GRK2 translocation. However, Gag alone recruited a substantial amount of this mutant to the membrane, which argues in favor of a contribution of Gag to GRK2 recruitment.

Onset kinetics of the changes in FRET was evaluated by monoexponential fitting. The FRET signal between GRK2 and Gag (half time \( t_{1/2} = 53.34 \) seconds) developed about 3 times slower than between GRK2 and Gbg (\( t_{1/2} = 0.95 \) seconds) (Fig. 2E). This suggests that membrane recruitment of GRK2 by Gag is delayed compared with Gbg.

Monoexponential fitting did not reveal significant differences in dissociation kinetics of Gbg (\( t_{1/2} = 25.1 \) seconds) and Gag (\( t_{1/2} = 27.1 \) seconds) from GRK2. However, the onset of Gag dissociation was clearly delayed (Fig. 2F), which argues in favor of a higher binding affinity of GRK2 to Gag than to Gbg.

Dissociation of Gbg was significantly accelerated with GRK2(D110A) (\( t_{1/2} = 16.3 \) seconds), which suggests that Gag binding affects interaction stability of GRK2 with Gbg.

Both Gag and Gbg Recruit GRK2 to the Membrane. GRK2 membrane translocation was investigated by live-cell confocal microscopy with high temporal resolution. Representative examples demonstrated a clear agonist-dependent membrane targeting of GRK2, GRK2(D110A), and GRK2(R587Q) (Fig. 3A). In contrast, the double mutant was barely targeted to the membrane. Statistical evaluation confirmed these observations (Fig. 3B; Supplemental Fig. 2D), suggesting that Gag as well as Gbg can recruit GRK2 to the membrane.

Effects of Gag Binding on GRK2–M3-AChR Interaction. Next we tested the contribution of Gbg, Gag, and both together in the interaction of GRK2 with M3-AChR by means of FRET. Only a minor rise in FRET between M3-AChR and GRK2 was observed with the Gbg binding–attenuated GRK2 (R587Q) and GRK2(D110A + R587Q), confirming the importance of Gbg for GRK2 recruitment (Fig. 4A). However, the FRET development was significantly diminished with the Gag binding–attenuated GRK2(D110A) as well, which supports our hypothesis that Gag is significantly involved in membrane
targeting of GRK2. In this context, it was very important to exclude that the reduction in the amplitude of the FRET signal was due to unfavorable relative expression levels of the fluorescent proteins. Therefore, relative expression levels of GRK2 and M3-AChR were determined for each individual recording as described in the Materials and Methods section (Fig. 4B). The results clearly showed that the observed differences in the amplitude of the FRET signal were not due to alterations in the relative expression levels of the fluorescent proteins.

We also compared onset kinetics and found no significant difference between wild-type GRK2 ($t_{1/2} = 1.44$ seconds) and GRK2(D110A), but significantly slowed onset kinetics with GRK2(R587Q) ($t_{1/2} = 2.39$ seconds) (Fig. 4C). As GRK2(R587Q), unlike the others, is recruited to the receptor primarily by $G_q$, these slowed kinetics correspond with the results of the GRK2–G-protein interaction assay that already hint at a delayed membrane targeting of GRK2 compared with $G_b$ (Fig. 2E). $G_a$ binding by GRK2(D110A) ($t_{1/2} = 1.21$ seconds) occurred shortly before M3-AChR interaction ($t_{1/2} = 1.76$ seconds). $G_a$ binding by GRK2(R587Q) ($t_{1/2} = 2.79$ seconds) was not significantly different from its $G_b$-AChR interaction ($t_{1/2} = 2.39$ seconds). These data suggest that GRK2 membrane translocation is the rate-limiting step for its receptor interaction.

Dissociation after withdrawal of the agonist proceeded with biphasic kinetics. An initial fast decline was followed by a slower phase that formed a major part (Fig. 4D). This indicates that the majority of GRK2 receptor complexes exhibited a prolonged interaction. GRK2(R587Q) showed a delayed dissociation, which was comparable to the delayed dissociation of $G_a$ from GRK2. This suggests that, in the absence of $G_b$ binding, the dissociation of GRK2 from the receptor is mostly dependent on the dissociation from $G_a$.

The role of $G_a$ in GRK2–M3-AChR interaction was further investigated in the same FRET assay without cotransfection of $G_b$, $G_q$, or the whole heterotrimer. The reduced FRET signal without overexpressed G-proteins suggests that the endogenous G-proteins are not sufficient to effectively recruit the exogenous GRK2 to the exogenous receptors (Fig. 4E; Supplemental Fig. 2E). Also, with overexpressed $G_a$ or $G_b$, the development of FRET was diminished, which could argue that less functional G-proteins were formed. Dissociation of GRK2 was significantly accelerated without overexpressed $G_a$, suggesting that $G_a$ binding to GRK2 prolongs interaction of GRK2 with M3-AChR (Fig. 4F).

Concentration-Response Curves. To determine the sensitivity of the GRK2 interactions, we measured concentration-response curves for $G_a$-GRK2, $G_b$-GRK2, and M3-AChR–GRK2 interactions under very similar conditions by utilizing the corresponding FRET assays (Fig. 5). Interestingly, the highest sensitivity was observed for the interaction between $G_q$ and GRK2, which exhibited a concentration-response curve that was significantly left-shifted compared with the $G_b$-GRK2 interaction. This argues in favor of a higher binding affinity between GRK2 and $G_a$ compared with GRK2 and $G_b$, which was already suggested by the GRK2–G-protein interaction assay (Fig. 2F). The concentration-response curve of the
M3-AChR–GRK2 interaction was further right-shifted, which can be attributed to the requirement of an active conformation of the receptor. This is known as the spare receptor phenomenon and corresponds well with the concentration-response curve of M3-AChR activation (Ziegler et al., 2011).

**Functional Effects of Gαq Binding by GRK2.** To investigate, whether the effects of Gαq and Gβγ on GRK2 recruitment influence GRK2 function, we analyzed the interaction of arrestin3 with the M3-ACh receptor by measuring FRET between arrestin3-YFP and M3-AChR–mTurq. Arrestin binding to G-protein–coupled receptors requires both GRK-induced receptor phosphorylation (Lohse et al., 1990) and agonist binding to the receptor (Krasel et al., 2005). The latter study further describes that, upon agonist washout, the GRK2-induced receptor phosphorylation is more sustained than the arrestin binding to the receptor. For this reason, arrestin binds very rapidly to prephosphorylated receptors upon repeated stimulation. We confirmed this result with the M3-AChR and observed an accelerated arrestin recruitment during the second stimulation compared with the first stimulation (Supplemental Fig. 3). Accordingly, arrestin binding to the receptor reflects the GRK2-induced receptor phosphorylation, which demonstrates that real-time measurement of arrestin recruitment in response to agonist is a sensitive assay to study functionality of the different GRK2 mutants. Indeed, in the absence of overexpressed GRK2 or in the presence of the kinase-dead GRK2(K220R), the interaction of arrestin3 with the receptor was significantly reduced compared with wild-type GRK2 (Fig. 6A; Supplemental Fig. 4A). In these experiments, arrestin3 was always in excess of receptor (Supplemental Fig. 4B), and equal expression of the GRK2 mutants was verified by western blotting (Supplemental Fig. 4C). Importantly, the interaction between receptor and arrestin was significantly diminished in the presence of Gαq binding–attenuated GRK2 mutant as well, suggesting that binding of Gβγ and Gαq to GRK2 is required for full kinase function. 

**Fig. 4.** Gαq improves the extent and stability of agonist-dependent interaction of GRK2 with M3-AChR. Individual single-cell FRET recordings were averaged (mean ± S.E.M.; n ≥ 12) and displayed either as absolute alterations in FRET (A) or as data normalized to the individual maximal agonist-induced response to compare onset kinetics (C) and offset kinetics (D) of the agonist-induced effect. The trace of wild-type (WT) GRK2 was already shown in Fig. 1B. Statistics are given for analysis of absolute amplitudes or kinetics by ANOVA with Bonferroni post-hoc test (*P < 0.05). (B) Relative expression levels of GRK2 mutants and M3-AChR in the assay shown in (A) were not significantly different between the conditions. (F) HEK293T cells transiently transfected analogously to (A), but without Gαq, Gβγ, or the whole heterotrimer were subjected to single-cell FRET imaging recorded at 2 Hz. Individual single-cell FRET recordings were averaged (mean ± S.E.M.; n ≥ 12) and displayed either as absolute alterations in FRET (E) or as data normalized to the individual maximal agonist-induced response to compare offset kinetics (F) of the agonist-induced effect.
arrestin binding to the M3-AChR appeared faster in the presence of wild-type GRK2 compared with the GRK2 mutants, although a detailed analysis was precluded by the increased noise due to the much-reduced amplitude in the context of the mutants. In all conditions, we observed a fast initial rise in the FRET signal, which presumably can be attributed to arrestin binding to prephosphorylated receptors. However, in cases where no functional GRK2 was expressed, the signal decreased subsequently to various degrees, probably due to competition with either G-proteins or even GRKs. Offset kinetics after withdrawal of the agonist was not significantly different between the conditions ($t_{1/2} = 2.82$ seconds for GRK2), and was markedly faster than dissociation of GRK2 from the receptor.

Having demonstrated that $\alpha_q$ binding to GRK2 contributes to functional GRK2–M3-AChr receptor interactions, we wondered whether it also affects interactions of GRK2 with G-protein–coupled receptors. We therefore studied the interaction of GRK2 with the G-protein–coupled M3-ACh receptor in the presence of the M3-ACh receptor and overexpressed $\alpha_q$-proteins. We found no significant difference between the receptor interaction of wild-type GRK2 and the $\alpha_q$ binding–attenuated GRK2 mutant, which suggests that $\alpha_q$ does not contribute to an increased GRK2 binding to G-protein–coupled receptors (Fig. 6B; Supplemental Fig. 4D).

**Discussion**

In the present study, we investigated the contributions of $\alpha_q$ and $\beta_\gamma$ to the agonist-dependent interaction of GRK2 with G-protein–coupled receptors and its function. For the first time, we were able to observe interactions between GRK2 and receptors or G-protein subunits in real time. $\alpha_q$ contributed to GRK2 targeting to the membrane, enhanced the extent and stability of the interaction between GRK2 and the M3-AChR, and was important for maximal GRK2 function. These results were obtained from newly established FRET assays using GRK2 and GRK2 point mutants with impaired $\alpha_q$ or $\beta_\gamma$ interaction. Single-cell FRET imaging allowed resolving association and dissociation kinetics in response to fast application or withdrawal of agonist. The loss of function of the $\alpha_q$ or $\beta_\gamma$ binding–deficient GRK2 mutants used in this study was verified by FRET experiments (Fig. 2, C and D). Each of these mutants contains a point mutation which we consider unlikely to have a major effect on GRK2 interaction with the receptor, the respective unaffected G-protein subunit, or overall GRK2 folding. In addition, we controlled expression levels for each recording to ensure comparability of the FRET signals. Confocal imaging demonstrated that interaction of GRK2 with either $\alpha_q$ or $\beta_\gamma$ was sufficient for membrane targeting upon receptor stimulation (Fig. 3). The role of $\beta_\gamma$ is more prominent, which was reflected in faster

---

**Fig. 5.** Concentration-response curves of the different interaction assays. HEK293T cells transiently transfected as indicated in Figs. 1 and 2 were subjected to single-cell FRET imaging recorded at 2 Hz. The M3-AChR was stimulated with increasing ACh concentrations, and the agonist-mediated responses were normalized to the saturating ACh concentration (mean ± S.E.M.; $n \geq 9$). Log(EC$_{50}$) of the underlying individual experiments was determined, and statistical analysis was performed by ANOVA with Bonferroni post-hoc test (*$P < 0.05$). Compared with $\alpha_q$–GRK2 interaction [log(EC$_{50}$) = −8.60 ± 0.07, EC$_{50}$ = 2.52 nM ACh], the concentration-response curves of the $\beta_\gamma$–GRK2 interaction [log(EC$_{50}$) = −8.24 ± 0.08, EC$_{50}$ = 5.82 nM ACh] and M3-AChR–GRK2 interaction [log(EC$_{50}$) = −7.14 ± 0.09, EC$_{50}$ = 71.9 nM ACh] were significantly right-shifted.

**Fig. 6.** Functional effects of G-protein binding to GRK2. (A) HEK293T cells transiently transfected with M3-AChR–mTurq, arrestin3–YFP, unlabeled $\alpha_q$, $\beta_6$, $\gamma_7$, and the different GRK2 mutants were subjected to single-cell FRET imaging recorded at 5 Hz. Individual single-cell FRET recordings were corrected for bleaching, averaged (mean ± S.E.M.; $n \geq 13$), and displayed as absolute agonist-induced alterations in FRET. Statistics are given for analysis of absolute amplitudes by ANOVA with Bonferroni post-hoc test (*$P < 0.05$). (B) HEK293T cells transiently transfected with M3-AChR–CFP, GRK2–YFP, or GRK2(D110A), unlabeled M3-AChR, $\alpha_q$, $\beta_6$, and $\gamma_7$ were subjected to single-cell FRET imaging recorded at 2 Hz. Individual single-cell FRET recordings were averaged (mean ± S.E.M.; $n \geq 14$) and displayed as absolute alterations in FRET. Statistics are given for analysis of absolute amplitudes or onset kinetics by ANOVA with Bonferroni post-hoc test (*$P < 0.05$).
interaction of GRK2 with Gβγ than with Goq (Fig. 2E), and in a higher extent of FRET between GRK2 and the receptor for the Goq binding–attenuated GRK2 mutant than the Gβγ binding–attenuated mutant (Fig. 4A). However, Goq binding to GRK2 significantly enhanced the extent and stability of the GRK2-M3-AChR interaction. This was shown by the reduced receptor interaction of the Goq binding–attenuated GRK2 compared with wild-type GRK2 (Fig. 4A), and by the accelerated dissociation of GRK2 from M3-AChR after withdrawal of the agonist in the absence of Goq (Fig. 4F). However, the Goq binding–attenuated GRK2(D110A) did not dissociate faster from M3-AChR than wild-type GRK2 (Fig. 4D), which may be explained by the fact that this mutation only reduces the interaction between GRK2 and Goq by about 70%. Furthermore, the affinity between GRK2 and Goq seemed higher than between GRK2 and Gβγ. This was suggested by delayed dissociation of Goq from GRK2 compared with Gβγ (Fig. 2F) and a significantly higher sensitivity of the GRK2-Goq interaction to increasing agonist concentrations compared to the GRK2-Gβγ interaction (Fig. 5). This may suggest that the contribution of Goq to GRK2 recruitment is higher at lower agonist concentrations. It has been previously shown that negatively charged phospholipids also play a role in the membrane targeting of GRK2 (Touhara, 1997). Most likely, these charged phospholipids will work in concert with G-protein subunits to recruit GRK2 to membranes and receptors. However, we did not address this issue in the present study. The phospholipid binding sites on GRK2 have been mapped and do not overlap with either the Goq or the Gβγ binding site (Carman et al., 2000).

It has been shown previously that both agonist occupancy and receptor phosphorylation are necessary for arrestin binding to G-protein–coupled receptors (Krasel et al., 2005). Therefore, we used arrestin binding to the M3-AChR as a readout for receptor phosphorylation. Gβγ binding was absolutely required for GRK2 function (Fig. 6A), probably by placing GRK2 and the receptor in a favorable orientation as suggested by previous studies (Wu et al., 1998). In the absence of Goq binding, some GRK2 activity was retained. However, efficient arrestin recruitment to the M3-AChR was observed only in the context of interactions of GRK2 with both Goq and Gβγ. We therefore propose that simultaneous binding of GRK2 to Gβγ and Goq (Tesmer et al., 2005) brings GRK2 to a position where it can optimally phosphorylate the sites on the M3-AChR that are required for arrestin binding. Accordingly, we hypothesize that, dependent on binding of Goq or Gβγ or both, differential orientations or conformations of GRK2 in the receptor complex exist. In agreement with this hypothesis, we observed a delayed dissociation of the Gβγ binding–attenuated GRK2 mutant from the receptor upon agonist washout (Fig. 4D), which most likely was now dependent on the dissociation of Goq from GRK2, which was similarly delayed (Fig. 2F).

Comparing the dynamics of GRK2 and arrestin3 interactions with M3-AChRs, we found that surprisingly, the desorption of arrestin from the receptor was much faster than the dissociation of GRK2 (Figs. 4A and 6A). It is generally assumed that receptor phosphorylation does not affect receptor–G-protein coupling, and that receptor desensitization occurs only after binding of arrestins to the receptor (Loehse et al., 1992). However, the prolonged interaction of GRK2 with the M3-AChR that we observed suggests that, at least for this receptor, G-protein–mediated signaling could already be inhibited by GRK2 binding. Hence, GRK2 presumably contributes to the phosphorylation-independent desensitization of receptor signaling that was previously attributed to the sequestration of Goq and Gβγ by GRK2 (Luo et al., 2008).

Taken together, we could visualize interactions of GRK2 with Gγ-protein subunits and receptors in single cells and resolve detailed kinetics. Our results uncovered that Goq binding to GRK2 enhances the recruitment of GRK2 to M3-ACh receptors. Goq significantly prolongs the interaction of GRK2 with M3-AChR, leading to longer occupancy times of receptors, and improves GRK2 function, which likely contributes to altered signaling and desensitization of receptors.

Authorship Contributions

Participated in research design: Wolters, Krasel, Brockmann, Bünemann.

Conducted experiments: Wolters, Brockmann.

Performed data analysis: Wolters, Brockmann, Bünemann.

Wrote or contributed to the writing of the manuscript: Wolters, Krasel, Bünemann.

References


Address correspondence to: Moritz Büemann, Institute for Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Philipps-University Marburg, Karl-von-Frisch-Str. 1, 35043 Marburg, Germany. E-mail: moritz.bueemann@staff.uni-marburg.de