Influence of $\alpha_q$ on the Dynamics of M$_3$-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, Philipps-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. Based on its ability to bind G-protein $\beta\gamma$ subunits as well as activated $\alpha_q$ subunits, it can be considered as an effector for G-proteins. The recruitment of GRK2 to activated receptors is well known to be mediated by $\beta\gamma$ together with negatively charged membrane phospholipids. In the current study, we address the role of $\alpha_q$ on the interaction of GRK2 with activated $\alpha_q$-protein–coupled receptors. Therefore, we established new Förster resonance energy transfer (FRET)–based assays to study the interaction of GRK2 with the $M_3$-acyethylcholine ($M_3$-ACH) receptor as well as $\alpha_q$-protein subunits with high spatiotemporal resolution in single living human embryonic kidney 293T cells. $M_3$-ACH receptor stimulation with 10 µM acetylcholine resulted in distinct changes in FRET, which reflects interaction of the respective proteins. GRK2 mutants with reduced binding affinity toward $\alpha_q$ [GRK2(D110A)] and $G_{\beta\gamma}$ [GRK2(R587Q)] were used to determine the specific role of $G_q$-protein–binding by GRK2. Comparison of absolute FRET amplitudes demonstrated that $\alpha_q$ enhances the extent and stability of the GRK2–$M_3$-ACH receptor interaction, and that not only $G_{\beta\gamma}$ but also $\alpha_q$ can target GRK2 to the membrane. This reveals an important role of $\alpha_q$ in efficient recruitment of GRK2 to $M_3$-ACH receptors. Furthermore, interactions between $\alpha_q$ and GRK2 were associated with a prolongation of the interaction between GRK2 and the $M_3$-ACH receptor and enhanced arrestin recruitment by these receptors, indicating that $\alpha_q$ influences signaling and desensitization.

Introduction

G-protein–coupled receptor kinases (GRKs) are a family of serine/threonine kinases that initiate 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., 1998; Hata and Koch, 2003). The important influence 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 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 $G_{\beta\gamma}$ 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 $\alpha_q$ subunits for $G_{\beta\gamma}$ binding. Therefore, they only interact with $G_{\beta\gamma}$ 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 $G_q$-proteins, but not with $G_{\alpha}$- or $G_{\alpha_q}$-proteins (Carman et al., 1999; Sallese et al., 2000). It has been shown by a crystal structure that even a simultaneous binding of $G_{\beta\gamma}$ and $G_q$ is possible (Tesmer et al., 2005). The GRK binding site on $G_q$ includes the $G_q$ switching region, which enables the discrimination between the active and inactive state of $G_q$ (Carman et al., 1999). The GRK surface that binds $G_q$ (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 of ABBREVIATIONS: ANOVA, analysis of variance; $\alpha_{2A}$-AR, $\alpha_{2A}$-adrenergic receptor; ACh, acetylcholine; AChR, acetylcholine receptor; CFP, cyan fluorescent protein; FRET, Förster resonance energy transfer; F$_{m\text{CFP}/m\text{YFP}}$, FRET ratio; GRK, G-protein–coupled receptor kinase; HEK, human embryonic kidney; LED, light-emitting diode; $M_3$-ACHR, $M_3$-ACH receptor; mTurq, mTurquoise fluorescent protein; RGS, regulator of G-protein signaling; ROI, region of interest; $t_{1/2}$, half time; YFP, yellow fluorescent protein.
of phosphorylation by preventing their interaction with downstream effectors (Raveh et al., 2010; Fernandez et al., 2011). However, whether binding of activated Gq affects the function of GRK2 is still unknown and needs further investigation.

With the present study, we aimed to clarify the effects of Gq 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 Gq (GRK2(D110A)) (Sterne-Marr et al., 2003) and Gγ [GRK2(R87Q)] (Carman et al., 2000). These mutants allowed determination of the individual contribution of the Gγ–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-α-cholinoceptor (M2-AChR) receptor (M2-AChR) as well as Gγ–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 Gq on extent and stability of GRK2 interaction with the M2-AChR.

Materials and Methods

Chemicals. Agar, ampicillin, enhanced chemiluminescence solution, and lysogenic broth were purchased from AppliChem (Darmstadt, Germany); acrylamide, glycine, MgCl2, milk powder, SDS, and tetramethylethylenediamine were from Carl Roth (Karlruhe, Germany); agarose was from Biozym (Hessisch Oldendorf, Germany); CaCl2 and tetramethylethylenediamine were from Carl Roth (Karlsruhe, Germany); CaCl2 was from MERCK (Darmstadt, Germany); and Dulbecco’s modified Eagle’s medium, fetal calf serum, phosphate-buffered saline, penicillin/streptomycin, 1-glutamate, and trypsin-EDTA were from Biochrom (Berlin, Germany). All other substances were purchased from Sigma-Aldrich (Steinheim, Germany).

Plasmids. The M2-AChR was obtained from the Missouri S&T cDNA Resource Center (Rolla, MO). cDNAs for Gαq, Gαq–yellow fluorescent protein (YFP) (Hughes et al., 2001), Gβ1, Gγ2, Gqα2–adrenergic receptor (α2-AR), Gq11–YFP (C351I) (Bünemann et al., 2003), Gα13 (C351I) (Wise et al., 1997), Gαqα13–YFP (Frank et al., 2005), M2-AChR–YFP (Hoffmann et al., 2012), arrestin3–YFP (Krasel et al., 2005), and GRK2 (Wistat et al., 1996) were described previously. M2-AChR–mTurquoise fluorescent protein (mTurq) was cloned analogously to M2-AChR–YFP. M2–AChR–cyano 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. The GRK2 interaction with the M2-AChR 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. The GRK2 interaction with the M2-AChR was generated by cloning CFP with a Ser-Arg linker to the C-terminus of human M2-AChR.
between GRK2 and the M3-AChR (Fig. 1A). The emission reflecting the development of FRET due to the interaction a corresponding decrease in mTurq fluorescence (blue trace), reversible increase in YFP fluorescence (yellow trace) and conditions.

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 its 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 saturation of onset and offset kinetics as a function of 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(F<sub>FRET</sub>/F<sub>CFP</sub>) 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 α<sub>2A</sub>-AR and G<sub>q11</sub>. GRK2 was recruited to the membrane after stimulation of α<sub>2A</sub>-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 G<sub>αq</sub> and G<sub>βγ</sub>. The G<sub>αq</sub>-protein binding–attenuated GRK2 variant GRK2(D110A) and the G<sub>βγ</sub> binding–attenuated GRK2(R587Q) were used to investigate the effect of G<sub>αq</sub> and G<sub>βγ</sub> 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 G<sub>βγ</sub> or G<sub>αq</sub> were established in HEK293T cells. M3-AChR stimulation with 10 μM ACh resulted in a reversible development of FRET that reflects G<sub>βγ</sub>-GRK2 and G<sub>αq</sub>-GRK2 interaction (Fig. 2, A and B). Between GRK2 and G<sub>βγ</sub> only a minor rise in FRET was detectable, verifying specificity of the G<sub>αq</sub>-GRK2 interaction (Supplemental Fig. 2A). GRK2(R587Q) showed a small FRET signal with G<sub>βγ</sub>, and GRK2(D110A) showed a small FRET signal with G<sub>αq</sub>.
confirming that these mutants exhibit a substantially reduced affinity with respect to the binding of either Gβγ or Gαq, or both (Fig. 2, C and D; Supplemental Fig. 2, B and C). However, Gαq binding deficiency appeared not as potent as Gβγ binding deficiency. Furthermore, the FRET signal between Gαq and GRK2(R587Q) was significantly diminished compared with wild-type GRK2, which confirms the importance of Gβγ for GRK2 translocation. However, Gαq alone recruited a substantial amount of this mutant to the membrane, which argues in favor of a contribution of Gαq to GRK2 recruitment.

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

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

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

Both Gαq and Gβγ 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 Gαq as well as Gβγ can recruit GRK2 to the membrane.

Effects of Gαq Binding on GRK2–M3-AChR Interaction. Next we tested the contribution of Gβγ, Gαq, 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 Gβγ binding–attenuated GRK2 (R587Q) and GRK2(D110A + R587Q), confirming the importance of Gβγ for GRK2 recruitment (Fig. 4A). However, the FRET development was significantly diminished with the Gαq binding–attenuated GRK2(D110A) as well, which supports our hypothesis that Gαq 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 $\alpha_q$, these slowed kinetics correspond with the results of the GRK2–G-protein interaction assay that already hint at a delayed membrane targeting of GRK2 by $\alpha_q$ (Fig. 2E). $\gamma$ binding by GRK2(R587Q), unlike the others, is delayed significantly slower than the interaction with GRK2. This suggests that, in the absence of $\gamma$ binding, the dissociation of GRK2 from the receptor is mostly dependent on the dissociation from $\alpha_q$.

Concentration-Response Curves. To determine the sensitivity of the GRK2 interactions, we measured concentration-response curves for $\alpha_q$-GRK2, $\beta\gamma$-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 $\gamma$ and GRK2, which exhibited a concentration-response curve that was significantly left-shifted compared with the $\beta\gamma$-GRK2 interaction. This argues in favor of a higher binding affinity between GRK2 and $\gamma$ compared with GRK2 and $\beta\gamma$, which was already suggested by the GRK2–$\gamma$-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 $G_{q}$ Binding by GRK2. To investigate, whether the effects of $G_{q}$ and $G_{bg}$ on GRK2 function influence GRK2 interaction, 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 $G_{q}$ binding–attenuated GRK2 mutant as well, suggesting that binding of $G_{bg}$ and $G_{q}$ to GRK2 is required for full kinase function.

**Fig. 4.** $G_{q}$ 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.; $n \geq 12$) 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 $G_{q}$, $G_{bg}$, 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.; $n \geq 12$) 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-ACh receptor interactions, we wondered whether it also affects interactions of GRK2 with Gq-protein–coupled receptors. We therefore studied the interaction of GRK2 with the Gq-protein–coupled M3-ACh receptor in the presence of the M3-ACh receptor and overexpressed Gq-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 Gq-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 Gq-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
interaction of GRK2 with Gbgγ than with Gαq (Fig. 2E), and in a higher extent of FRET between GRK2 and the receptor for the Gαq binding–attenuated GRK2 mutant than the Gbgγ binding–attenuated mutant (Fig. 4A). However, Gαq 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 Gαq 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 Gαq (Fig. 4F). However, the Gαq 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 Gαq by about 70%. Furthermore, the affinity between GRK2 and Gαq seemed higher than between GRK2 and Gbgγ. This was suggested by delayed dissociation of Gαq from GRK2 compared with Gbgγ (Fig. 2F) and a significantly higher sensitivity of the GRK2-Gαq interaction to increasing agonist concentrations compared to the GRK2-Gbgγ interaction (Fig. 5). This may suggest that the contribution of Gαq 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 Gαq or the Gbgγ 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. Gbgγ 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 Gαq 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 Gαq and Gbgγ. We therefore propose that simultaneous binding of GRK2 to Gbgγ and Gαq (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 Gαq or Gbgγ 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 Gbgγ binding–attenuated GRK2 mutant from the receptor upon agonist washout (Fig. 4D), which most likely was now dependent on the dissociation of Gαq from GRK2, which was similarly delayed (Fig. 2F).

Comparing the dynamics of GRK2 and arrestin3 interactions with M3-AChRs, we found that surprisingly, the dissociation 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 (Lohse 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 Gαq and Gbgγ by GRK2 (Luo et al., 2008).

Taken together, we could visualize interactions of GRK2 with Gbgγ-protein subunits and receptors in single cells and resolve detailed kinetics. Our results uncovered that Gαq binding to GRK2 enhances the recruitment of GRK2 to M3-AChR receptors. Gαq 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 Bünemann, Institute for Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Philipps-University Marburg, Karl-von-Frisch-Str. 1, 35043 Marburg, Germany. E-mail: moritz.buene-mann@staff.uni-marburg.de

Gαq Effects on M3-ACh Receptor–GRK2 Interaction