Muscarinic acetylcholine receptors belong to the large family of seven transmembrane G protein-coupled receptors (GPCRs). All five subtypes of this receptor type (M1–M5) are activated by the endogenous ligand acetylcholine and have been reported to be susceptible to allosteric receptor modulation by various ligands (1). In particular, the M2 receptor subtype has been studied intensely during the last years with regard to its allosteric modulation (1, 2). Several different allosteric ligands for muscarinic receptors have been identified, some of which, such as gallamine and W84 (hexamethylene-1,6-bis-[dimethyl-(3-phthalimidopropyl)-ammonium dibromide]), show some subtype selectivity for the M2 receptor (3–5), whereas others, such as tacrine, are rather unselective (6).

Allosteric ligands are defined by the fact that in the absence of an orthosteric ligand, they do not affect the activity of a receptor (7) but that they alter the affinities of orthosteric ligands, presumably because of conformational changes in the receptor (8–10). Recent experimental evidence supports the hypothesis that allosteric ligands can induce specific receptor conformations by direct binding to an allosteric ligand site and that this may result in changes of the agonist-induced activity of the receptor (11).

Most studies in the field of allosteric modulation have been made with radioligand binding assays (10, 12–14). These studies have shown alterations of antagonist [3H]N-methylscopolamine ([3H]NMS) radioligand binding, characterized by incomplete inhibition and a decreased speed of dissociation induced by allosteric ligands (15). At the same time, these compounds impaired agonist-induced receptor signals (13, 15). However, these effects appear to depend on the system and ligand studied and may reveal positive or negative effects on agonist or antagonist binding (15), leaving the exact mechanism of action of these compounds open.

Previous kinetic studies using radioligand binding suggest that the effects of some allosteric ligands might be quite rapid (5, 15). However, the precise analysis of such effects requires methods that can record effects on receptors in the subsecond time range. Recently, we have developed an approach that allows monitoring of conformational changes in GPCRs in living cells based on fluorescence (or Foerster) resonance energy transfer (FRET) (16–19). FRET is the transfer of energy from an excited donor to an acceptor molecule via long range dipole-dipole coupling mechanisms. To monitor conformational changes in the receptor, we initially developed fusions of GPCRs with cyan fluorescent protein (CFP) as the donor and yellow fluorescent protein (YFP) as the acceptor fluorophore (16, 17). However, the insertion of the relatively large fluores-
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cent proteins into an intracellular receptor loop may reduce their ability to couple to G proteins. Therefore, we have more recently adopted an approach replacing YFP by the small fluo-

rescein arsenical hairpin binder (FlAsH) as the acceptor fluoro-

phore; FlAsH can be used to covalently label a specific motif (CCPGGCC) inserted into the 3rd intracellular loop (ICL) of receptors. Compared with the CFP/ YFP approach, CFP/FlAsH con-

structs revealed larger agonist induced FRET changes and unaltered signaling properties of the receptors \((20, 21)\).

Here, we developed an $M_2$ muscarinic receptor sensor and used it for the FRET-based real-time mon-

itoring of receptor modulation by orthosteric and allosteric ligands. Analysis of receptor kinetics in-

duced by allosteric ligands in living cells supports the view that alloster-

ic modulation results in very rapid, actively induced conformational changes in the receptor.

EXPERIMENTAL PROCEDURES

Materials—The ligands acetyl-

choline, atropine, gallamine, and methoctramine were all obtained from Sigma-Aldrich. Carbachol was purchased from Alfa Aesar (Karlsruhe, Germany), and dimeth-

yl-W84 was purchased from Alexis Biochemicals/Enzo (Lörrach, Ger-

many). FlAsH is commercially avail-

able from Invitrogen as TC-FlAsH. All other chemicals were from standard sources and of the highest purity available.

Molecular Biology—To create a FRET-based sensor for the human muscarinic $M_2$ receptor (GenBank™ accession number AF498916), the sequence of the enhanced CFP was fused to the C terminus of the FLAG-tagged human muscarinic $M_2$ receptor via a 5-amino acid linker GSGEG, and the FlAsH motif CCPGCC was introduced into the shortened ICL3 with the indicated linkers. $B$, transfected HEK-TsA201 cells were labeled with FlAsH and analyzed by laser scanning microscopy. Confocal pictures show a distinct membrane staining in both the CFP and the FlAsH channels. $C$, radioligand binding studies with CHO cell membranes expressing wild-type $M_2$ receptor or $M_2$ receptor sensor are shown. Competitive [$^3H$]NMS displacement curves for carbachol and gallamine are presented (data are shown as means ± S.E., $n = 3$). The $IC_{50}$ values were $7.3 \pm 2.2$ and $10.0 \pm 1.7$ $\mu M$ for carbachol, $11.5 \pm 1.4$ and $11.5 \pm 3.9$ $\mu M$ for gallamine, for wild-type receptor, and $M_2$ receptor sensor, respectively. $D$, GIRK current measurements in HEK-TsA201 cells transfected with the $M_2$ receptor sensor and GIRK1/4 and activated with different concen-

trations of acetylcholine show the signaling of the receptor sensor to the GIRK channels with $EC_{50}$ values of $100 \pm 7$ $nM$ (mean ± S.E., $n = 5$). Cells without transfected receptor constructs showed no effects of acetylcho-

line on GIRK currents (data not shown). $E$, inhibition of forskolin-induced cAMP signals by the wild-type $M_2$ receptor and the $M_2$ receptor sensor was measured as described under "Experimental Procedures." Concentration-response curves for carbachol in absence or presence of $100 \mu M$ gallamine are presented. The $EC_{50}$ values were $1.6 \pm 0.3$ and $2.8 \pm 0.5$ $\mu M$ for carbachol, $31.4 \pm 0.3$ and $36.4 \pm 0.4$ $\mu M$ for carbachol in presence of gallamine, for wild-type receptor, and $M_2$ receptor sensor, respectively ($n = 4 – 5$). Error bars, S.E.

FIGURE 1. Generation and functionality of the muscarinic $M_2$ receptor sensor. $A$, a schematic structure of the sensor. CFP was fused to the C terminus of the FLAG-tagged human muscarinic $M_2$ receptor via a 5-amino acid linker GSGEG, and the FlAsH motif CCPGCC was introduced into the shortened ICL3 with the indicated linkers. $B$, transfected HEK-TsA201 cells were labeled with FlAsH and analyzed by laser scanning microscopy. Confocal pictures show a distinct membrane staining in both the CFP and the FlAsH channels. $C$, radioligand binding studies with CHO cell membranes expressing wild-type $M_2$ receptor or $M_2$ receptor sensor are shown. Competitive [$^3H$]NMS displacement curves for carbachol and gallamine are presented (data are shown as means ± S.E., $n = 3$). The $IC_{50}$ values were $7.3 \pm 2.2$ and $10.0 \pm 1.7$ $\mu M$ for carbachol, $11.5 \pm 1.4$ and $11.5 \pm 3.9$ $\mu M$ for gallamine, for wild-type receptor, and $M_2$ receptor sensor, respectively. $D$, GIRK current measurements in HEK-TsA201 cells transfected with the $M_2$ receptor sensor and GIRK1/4 and activated with different concen-

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culture media were supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were transfected using the Effectene reagent (Qiagen, Hilden, Germany). For fluorescence measurements, cells were seeded on poly-D-lysine-coated round glass coverslips, and the microscopy experiments were performed 48 h after transfection. CHO cells were maintained at 37 °C, 5% CO₂ in Dulbecco’s modified Eagle’s medium/F12 medium supplemented with 10% fetal calf serum. Transfections were performed using the Lipofectamine 2000 reagent (Invitrogen).

Radioligand Binding Studies—The binding of [³H]NMS (Amersham Biosciences) to the wild-type M₂ receptor and the M₂ receptor sensor was analyzed in membranes prepared from CHO cell lines expressing these receptors. Membranes containing 10 µg of membrane protein were incubated for 2 h at room temperature in the assay buffer (25 mM phosphate-buffered saline, 5 mM MgCl₂, pH 7.4) with 1–10 nM [³H]NMS. Nonspecific binding was determined in the presence of 10 µM atropine. The reactions were terminated by vacuum filtration through GF/B glass fiber filters (Millipore).

FlAsH Labeling—The labeling was done as described (20, 22). In brief, transfected cells were washed twice on the coverslips with phenol red-free Hanks’ balanced salt solution containing 1 g/liter glucose (HBSS; Invitrogen) and then incubated at 37 °C for 1 h with HBSS to which 500 nM FlAsH and 12.5 µM 1,2-ethanediol had been added. Subsequently, to reduce nonspecific labeling, the cells were washed twice with HBSS, incubated for 10 min with HBSS/250 µM 1,2-ethanediol, and again washed twice with HBSS before being used for fluorescence measurements.

Confocal Imaging and FRET Measurements—Confocal microscopy was performed using a Leica TCS SP2 system. FlAsH and CFP were excited with the 514 nm line of an argon ion laser or a 430-nm frequency-doubled diode laser, respectively.

FRET microscopy was performed essentially as described (20, 21). Live cells were maintained in FRET buffer: 144 mM NaCl, 5.4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, pH 7.3. Measurements were performed at room temperature using a Zeiss Axiosvert 200 inverted microscope equipped with an oil immersion ×100 objective, a dual emission photometric system and a Polychrome IV light source (Till Photonics, Gräfelfing, Germany). Excitation was done at 436 ± 10 nm (beam splitter DCLP 460 nm), applied with a frequency between 10 and 50 Hz depending on the nature of experiment. Fluorescence was recorded from entire single cells in FlAsH and CFP emission channels using 535 ± 15 nm and 480 ± 20 nm emission filters and a DCLP 505-nm beam splitter. FRET was monitored as the emission ratio FlAsH/CFP, which was corrected offline for the spillover of CFP into the FlAsH channel, direct FlAsH excitation, and photobleaching. To study ligand-induced changes in FRET, cells were continuously superfused with FRET buffer supplemented with various ligands as indicated, applied with the help of a computer-assisted solenoid valve-controlled rapid superfusion device ALA-VM8 (solution exchange in 5–10 ms; ALA Scientific Instruments).

To measure M₂ receptor-induced inhibition of cAMP signaling, CHO cells expressing the wild-type receptor or the M₂ receptor sensor were transfected with the FRET-based cAMP sensor Epac1-camps, and the cAMP levels were measured by FRET imaging as described previously (23). After stimulation of cAMP production by 2 µM forskolin, increasing concentrations of carbachol were added to the cells in presence or absence of 100 µM gallamine.

Measurement of Membrane Currents—For the measurement of potassium currents, an extracellular solution of the following composition was used: 120 mM NaCl, 20 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES-NaOH, pH 7.3. The internal (pipette) solution contained 100 mM potassium aspartate, 40 mM KCl, 5 mM MgATP, 10 mM HEPES-KOH, 5 mM NaCl, 2 mM EGTA, 1 mM MgCl₂, 0.01 mM GTP, pH 7.3. Membrane currents were recorded under voltage-clamp conditions, using a conventional whole cell patch-clamp technique (24). Patch-pipettes were fabricated from borosilicate glass capillaries (GF-150–10, Warner Instrument Corp.) using a horizontal puller (P-95, Fleming & Poulsen), with a DC resistance of 3–6 MΩ. Membrane currents were recorded using either a patch-clamp amplifier (Axopatch 200; Axon Instruments) or an EPC 9 (HEKA Instruments) as described previously (24). Signals were analog-filtered using a low pass Bessel filter (1–3-kHz corner frequency). Iₖ,ACH was measured as an inward current using a holding potential of −90 mV as described (24). Voltage ramps (from −120 mV to +60 mV in 500 ms, every 10 s) were used to determine current-voltage (I-V) relationships.

Data Processing—Fluorescence intensities were acquired using Clampex (Axon Instruments). Values are given as mean ± S.E. of n independent experiments. Statistical analyses and curve fitting were performed using Prism 4.0 (GraphPad), Origin (OriginLab), or Clampfit (Axon Instruments).

RESULTS

Design and Functionality of a FRET-based M₂ Receptor Sensor—First, we created constructs in which the whole M₂ receptor sequence was directly fused to the N terminus of CFP. Because such receptor constructs showed low expression in cells and poor membrane localization (data not shown), we inserted the 5-amino acid linker GSGEG between the receptor C terminus and CFP (Arg⁴⁶⁷) and introduced a FLAG tag on the N terminus (position 2) of the receptor, which resulted in significantly improved receptor expression and membrane localization (Fig. 1, A and B). The human M₂ muscarinic receptor has a long ICL3. To improve the FRET efficiency between the fluorophores, we deleted a part of ICL3 (Pro²³⁰ to Thr³⁶⁹) that is not involved in G protein coupling (25). Similar to the previously described α₂A-adrenergic receptor sensor (17, 21, 22), we inserted the CCGGCC FlAsH binding motif into the shortened loop. We chose FlAsH as the acceptor fluorophore because earlier studies have shown that FlAsH, compared with YFP, provided higher signal amplitudes and preserved the receptor functionality in terms of activation of downstream signaling (20, 21). Using radioligand binding assays, we confirmed that the M₂ receptor sensor and the wild-type M₂ receptor have similar affinities for carbachol and a negative allosteric ligand gallamine, as shown by the competitive displacement of [³H]NMS (Fig. 1C).
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FIGURE 2. Effects of agonists on the FRET response of the M₂ receptor sensor. Fluorescence emission was measured at 480 nm (F₄₈₀) and at 535 nm (F₅₃₅) from cells expressing the M₂ receptor sensor and superfused for the indicated period of time with agonist. A, individual recording from a single cell shows that acetylcholine induced a rapid increase in F₄₈₀ and a corresponding decrease in F₅₃₅ resulting in a reduced ratio F₅₃₅/F₄₈₀ representing FRET (bottom trace). Upon washout of acetylcholine, all traces reverted to their original values. B, concentration-response curves for the FRET changes evoked by acetylcholine and carbachol revealed EC₅₀ values of 1.5 ± 0.3 µM and 5.1 ± 0.4 µM, respectively (means ± S.E. (error bars), n = 6–8).

FIGURE 3. Reversal of agonist-induced FRET signals of the M₂ receptor sensor by antagonists. Shown are responses to the agonists acetylcholine (A) or carbachol (B). Top panels; F₄₈₀/F₅₃₅ traces (normalized to the starting values) as in Fig. 2, representative of at least six independent experiments. Washout of the agonist as well as addition of atropine in the continued presence of the respective agonist resulted in a fast reversal of the FRET signal. Bottom panels, time constants for the respective experiments, in which statistical significance was assessed using a t test. Error bars, S.E. **, p < 0.01.

To confirm that the resultant M₂ receptor sensor can effectively couple to G proteins and induce downstream signaling, we first measured the G protein-mediated activation of GIRK channels and observed a concentration-dependent activation of the GIRK current (Fig. 1D). Next, we compared the efficacy of the wild-type M₂ receptor and of the M₂ receptor sensor to inhibit forskolin-stimulated cAMP production in CHO cells. Both receptors showed similar efficacies and potencies of cAMP inhibition by carbachol in the absence and presence of the negative allosteric ligand gallamine (Fig. 1E), suggesting that the receptor sensor maintained the full functionality of the native M₂ receptor.

The M₂ Receptor Sensor Reports Conformational Changes in Response to Orthosteric Ligands—For FRET experiments, we used single HEK-TsA201 cells, which showed clear membrane localization of the receptor sensor (Fig. 1B). Stimulation of fluorescent cells with the full receptor agonist acetylcholine resulted in a fast, reversible decrease in FlAsH and a concomitant increase in CFP fluorescence, indicative of a decrease in FRET (Fig. 2A). The changes in CFP and FlAsH fluorescence were clearly agonist-dependent and had maximal amplitudes of ~5–6%, which can be easily measured without visible desensitization of the FRET signals after multiple stimulations (supplemental Fig. 1). Next, we used various concentrations of two M₂ receptor agonists, acetylcholine and carbachol, to establish concentration-response curves with EC₅₀ values of 1.5 or 5.1 µM, respectively (Fig. 2B). These values are high compared with potencies determined in other types of experiments, but it must be kept in mind that in the FRET experiments there is no receptor reserve, and they are measured under nonequilibrium conditions.

We then investigated whether the M₂ receptor sensor also reports effects of receptor antagonists. Atropine given alone did not cause any change in FRET (data not shown). However, when we applied atropine to cells that had been first stimulated with the agonists acetylcholine (Fig. 3A) or carbachol (Fig. 3B), this led to a rapid and complete reversal of the agonist-induced signals. The reversal of the FRET signals induced by atropine at saturating concentrations occurred faster than that induced by mere washout of the agonists (Fig. 3, bottom panels). This may indicate that in the latter case rebinding of agonists occurred and that the rates measured in the presence of atropine reflected the true dissociation rates of acetylcholine (τ ≈ 0.8 s) and carbachol (τ ≈ 0.5 s).

Modulation of Receptor Activity by Allosteric Ligands Can Be Observed by FRET—Next, we investigated whether the M₂ receptor sensor can be utilized to observe effects of allosteric receptor modulators. In this study, we used gallamine and dimethyl-W84, which have been characterized as negative allosteric modulators of muscarinic receptors; both dimethyl-W84 and gallamine have been described as relatively selective for the M₂ receptor (26–28).

We initially used a 10 µM concentration of each ligand to determine whether the allosteric ligands produce a receptor signal in the absence of an orthosteric ligand (Figs. 4 and 5). Cells were first stimulated with 100 µM acetylcholine or carbachol to show that the receptor was functional. After washout of the agonists, cells were superfused with an allosteric ligand. Gallamine (Fig. 4A) did not change the FRET signal when given alone; W84 (Fig. 5A) induced only a very small change in the F₅₃₅/F₄₈₀ ratio, caused by an isolated increase in F₅₃₅ presumably due to its intrinsic fluorescent properties. These data indicate that the allosteric ligands alone do not affect the FRET signal of the M₂ receptor sensor.

In contrast, when given in the presence of an orthosteric agonist, both allosteric ligands partially reversed the FRET
signals induced by acetylcholine (Figs. 4B and 5B) and carbachol (Figs. 4C and 5C). The extent of this reversal varied as a function of the nature and concentration of the allosteric ligand.

At high concentrations, both allosteric ligands were able to reverse the agonist-induced FRET signals fully (Figs. 4, B and C, and 5, B and C, right panels). Dimethyl-W84 was more than 1 order of magnitude more potent than gallamine, with half-maximal reversal at less than 10 μM (Fig. 5, B and C). Interestingly, following W84 application and washout, neither acetylcholine nor carbachol evoked full FRET responses (Fig. 5, B and C), suggesting a high affinity of dimethyl-W84 for the M₂ receptor or incomplete washout.

Kinetics of Allosteric Ligand Effects—Because our FRET-based approach represents a unique tool to monitor changes in receptor conformation in real time, we analyzed the kinetics of the different orthosteric and allosteric ligands used in this study. As in the preceding experiments, single cells were first superfused with an orthosteric ligand, acetylcholine or carbachol (both at a saturating concentration of 100 μM), and then the allosteric ligands were added at saturating concentrations (1 mM) on top of either acetylcholine (Fig. 6A) or carbachol (Fig. 6B) to determine the maximal speeds of the receptor conformational change induced by allosteric ligands. The reversal of FRET signals induced by saturating concentrations of the orthosteric antagonists atropine and methoctramine served as controls; the resultant off-rates were τ_{ACh,Atr} = 677 ms ± 68 ms and τ_{ACh,Metho} = 609 ms ± 87 ms for acetylcholine compared with τ_{Carb,Atr} = 494 ms ± 20 ms and τ_{Carb,Metho} = 432 ms ± 66 ms for carbachol. As shown above (Fig. 3), these rates are faster than those obtained by simple washout of the agonists, which were ∼0.1 s (acetylcholine) and ∼0.9 s (methoctramine), respectively.

In contrast, gallamine and dimethyl-W84 reverted the agonist-induced FRET signals considerably faster. We were able to estimate the kinetic values accurately by measuring at 50 Hz (i.e. 20 data points/s) and fitting the FRET traces with the monoexponential function as shown in Fig. 6, C–E. The values obtained for gallamine were τ_{ACh,Gall} = 87.2 ms ± 6.6 ms and τ_{Carb,Gall} = 105 ms ± 17 ms; the values for W84 were τ_{ACh,W84} = 212 ms ± 46 ms and τ_{Carb,W84} = 175 ms ± 30 ms. A direct overlay of the traces caused by saturating concentrations of atropine and gallamine is shown in Fig. 6F and illustrates the much higher speed of the effect of the allosteric ligand.

Because some allosteric ligands such as dimethyl-W84 may be hydrolyzed by cellular esterases, we tested whether the kinetics of dimethyl-W84 responses are affected by these
enzymes. Inhibition of esterases with physostigmine did not result in any change of kinetic constants (supplemental Fig. 1B), suggesting that in our measuring system the esterases do not show any significant effects.

Analysis of the kinetics at various concentrations of the ligands (Fig. 7A) clearly shows that the used concentrations were indeed saturating for the kinetic effects, and in particular that even very high concentrations of atropine (up to 1 mM) did not produce faster signals. Fig. 7B shows the respective rate constants (k_{obs} values) for saturating concentrations of atropine, methoctramine, and gallamine and illustrates that these are the same for the two orthosteric ligands, but that the k_{obs} value of gallamine is much higher.

DISCUSSION

Allosteric ligands for GPCRs have gained increasing interest in recent years, and numerous compounds with presumed allosteric properties are currently in development as potential drugs (29). It appears that such compounds can affect either the affinity or the efficacy (or both) of conventional orthosteric ligands, but their exact mode of action remains to be established.

In this report, we have used rapid kinetic techniques to investigate the effects of presumed allosteric ligands on the M₂ muscarinic receptor. Using an approach developed previously to monitor the activation of parathyroid hormone-1 receptor, we designed an M₂ receptor carrying a CFP and a FlAsH binding motif to permit the direct monitoring of activation-dependent conformational changes of the receptor. This permitted us to study effects of the various compounds on the receptor protein itself rather than monitoring effects mediated via downstream processes.

Our results indicate that despite the addition of the two different fluorophores, this receptor construct retained its downstream signaling properties as evidenced by GIRK channel activation and inhibition of cAMP signaling. The EC_{50} value of the functional GIRK channel response (≈100 nM), cAMP responses, and the direct measures of affinity reported by the FRET signals (1.5 µM for acetylcholine and 5.1 µM for carbachol) and also the high sensitivity toward atropine are all in agreement with data from native receptors (30–32) and indicate the functional integrity of the receptor construct. This is in line with observations made with the A_{2A}-adenosine receptor, where the insertion of a FlAsH-labeling motif into ICL3 was functionally tolerated whereas the insertion of the much larger YFP as a label disrupted receptor/G protein signaling (21).

Exposure of this M₂ receptor construct to agonists resulted in a rapid decrease of FRET, as seen from a simultaneous increase in F_{480} and decrease in F_{535}. This is in line with similar data obtained with FRET constructs of other GPCRs (16–21).

FIGURE 6. Kinetics of the reversal of agonist-induced FRET signals. Data are derived from experiments as in Figs. 4 and 5. A and B, average time constants (τ) of the effects of atropine (10 µM), methoctramine (100 µM), dimethyl-W84 (1 mM), and gallamine (1 mM) in reversing the FRET signals induced by 100 µM acetylcholine (A) or 100 µM carbachol (B); (means ± S.E. (error bars), n = 6 – 8). Statistical significance of the differences between the kinetics of atropine and allosteric ligands was assessed by analysis of variance. ***, p < 0.001. C–E, representative individual FRET traces for atropine, dimethyl-W84, and gallamine applied after 100 µM acetylcholine and fitted with a monoexponential function to estimate the τ values shown in A. F, reversal of the FRET signal in the continued presence of 100 µM acetylcholine induced by addition of gallamine (1 mM) or atropine (10 µM) to the superfusion buffer; shown is the percent reversal of the acetylcholine-induced FRET signal. Traces are representative of six to eight independent experiments.
This signal was rapidly lost upon washout of the agonists or even slightly faster by addition of the antagonist atropine, presumably because the latter prevents rebinding of the agonists.

Gallamine and dimethyl-W84 have been described as negative allosteric compounds at M₂ muscarinic receptors (29, 33–35). They have been reported to reduce the equilibrium affinity of antagonists, but even more so of agonists at these receptors and to slow down both the association and, less so, the dissociation of antagonist radioligands (10). In line with these earlier data, our M₂ receptor FRET sensor did not respond to gallamine or dimethyl-W84 alone. However, in the presence of agonists, both compounds blocked agonist-induced FRET signals in a concentration-dependent manner, compatible with a reversal of the receptors into an inactive state.

Interestingly, the kinetics of this reversal were very rapid. At saturating concentrations, these kinetics occurred with time constants of ≈80–200 ms. This is up to 8-fold faster than the off-rates seen with orthosteric antagonists. This indicates that the allosteric ligands actively induce an inactivation process that is faster than the “normal” dissociation of the agonist. As mentioned above, gallamine and atropine decrease both the speed of association and dissociation of radioligand binding to muscarinic receptors (10, 35). Thus, it appears unlikely that the very rapid effects of the two compounds on the M₂ receptor FRET sensor are mediated by forced dissociation of the agonists from their binding site. In radioligand binding experiments, such rapid forced dissociation would have been apparent in those radioligand binding studies as an almost instantaneous dissociation. Thus, it seems more plausible that the allosteric ligands caused the transition of the receptors into an inactive conformation.

Although GPCRs have long been regarded as monomeric entities, it has become increasingly clear that some GPCRs exist and function as dimers or even occur in larger receptor assemblies (36–38). This raises the question of whether allosteric ligands might act across a dimer. Early studies on the effects of gallamine on [3H]NMS binding noted that the allosteric effects persisted in solubilized receptors (10); these had an apparent molecular weight of ≈80,000, a value that is between the actual values of a mono- and a dimer.

Our kinetic data appear to argue for an action of allosteric and orthosteric ligands on the same protein. We observed switch times of allosteric ligands down to <100 ms. This is in the same range as the activation times for most GPCRs, including the M₂ receptor studied here, by their orthosteric agonists (16–21). In contrast, the fastest switch times that we observed across a receptor dimer, the inactivation of an α₂-adrenergic receptor by a μ-opiate receptor in an α₂/μ-receptor dimer, was 450 ms (17). Similar switch times on the order of 500 ms were also observed from receptors to G proteins (39–41). Interactions of GPCRs with receptor kinases and β-arrestins are even slower (42–43). Thus, the switch times observed here for the allosteric ligands gallamine and dimethyl-W84 are well below the switch times that have so far been observed between a GPCR and any other protein, in particular a neighboring receptor in a dimer and a G protein. In fact, these switch times for allosteric ligands are more compatible with intramolecular switch times in GPCRs. This would argue for the binding of allosteric and orthosteric ligands to the same receptor moiety. Thus, although dimers or larger assemblies of M₂ receptors appear to be of importance in receptor function, this does not seem to be the case for allosteric ligands. Instead, our data indicate that a single GPCR molecule may be switched from different sites to integrate different signals into a receptor output.

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