Fluorescence changes reveal kinetic steps of muscarinic receptor–mediated modulation of phosphoinositides and Kv7.2/7.3 K⁺ channels

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G protein–coupled receptors initiate signaling cascades. M₁ muscarinic receptor (M₁R) activation couples through Goₐ to stimulate phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP₂). Depletion of PIP₂ closes PIP₂-requiring Kv7.2/7.3 potassium channels (M current), thereby increasing neuronal excitability. This modulation of M current is relatively slow (6.4 s to reach within 1/e of the steady-state value). To identify the rate-limiting steps, we investigated the kinetics of each step using pairwise optical interactions likely to represent fluorescence resonance energy transfer for M₁R activation, M₁R/Goₐ interaction, Goₐ/Goₐ/PLC interaction, and PIP₂ hydrolysis. Electrophysiology was used to monitor channel closure. Time constants for M₁R activation (<100 ms) and M₁R/Goₐ interaction (200 ms) are both fast, suggesting that neither of them is rate limiting during muscarinic suppression of M current. Goₐ/Goₐ/PLC interaction have intermediate 1/e times (2.9 and 1.7 s, respectively), and PIP₂ hydrolysis (6.7 s) occurs on the timescale of M current suppression. Overexpression of PLC accelerates the rate of M current suppression threefold (to 2.0 s) to become nearly contemporaneous with Goₐ/Goₐ/PLC interaction. Evidently, channel release of PIP₂ and closure are rapid, and the availability of active PLC limits the rate of M current suppression.

INTRODUCTION

G protein–coupled receptors (GPCRs) comprise the largest receptor family in the human genome, mediate a vast array of cellular processes, and constitute a large fraction of current pharmaceutical targets. GPCR signal transduction pathways use diverse signaling mechanisms and kinetics, and only a few G protein–coupled systems have received much quantitative attention. Recent studies reveal nuances in GPCR-G protein specificity (Kenakin, 1997), G protein heterotrimer stability (Evanko et al., 2005; Digby et al., 2006; Yuan et al., 2007), G protein trafficking among membranes (Chisari et al., 2007; Saini et al., 2007), and spatial organization of GPCRs with G proteins and effectors (Nobles et al., 2005; Dowal et al., 2006). We seek to deepen understanding of GPCR signaling by analyzing the underlying kinetics of the relatively slow modulation of a K⁺ channel by muscarinic receptors.

Activation of Goₐ₁₁–coupled muscarinic acetylcholine receptors in sympathetic neurons attenuates M-type potassium current and thus increases neuronal excitability (Brown and Adams, 1980; Brown, 1983). M current, an outwardly rectifying neuronal potassium current encoded by KCNQ2 and KCNQ3 (Kv7.2 and 7.3) channel subunits (Wang et al., 1998), requires phosphatidylinositol 4,5-bisphosphate (PIP₂) to be active (Suh and Hille, 2002; Zhang et al., 2003). Muscarinic modulation of M current acts through a chain of events: Goₐ activates phospholipase Cβ (PLCβ), which hydrolyzes PIP₂ to generate inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol. PIP₂ is a principal determinant of M current activity, and its depletion induces closure of Kv7.2/7.3 channels (Suh et al., 2006). Signal transduction through these steps from receptor to channel requires 10–15 s to come to completion.

Previously, we formulated a preliminary kinetic model for the steps from activation of the M₁ muscarinic acetylcholine receptor (M₁R) to closure of Kv7.2/7.3 channels (Suh et al., 2004). We found, however, that many intermediate rate constants were not constrained by empirical measurements. Here, we use optical signals likely to represent fluorescence resonance energy transfer (FRET) to tease apart these steps. We wish to resolve which steps contribute to the relative slowness of this signal. FRET is an optical technique that relies on the close proximity (<100 Å) of two fluorophores to monitor their relative molecular dynamics in intact cells in real time. Changes in FRET can reveal the kinetics of...
changes in protein conformation (intramolecular) or interaction (intermolecular). FRET has been used to determine the kinetics of signaling of several GPCRs, with a focus on G_{i/o} and G_s-coupled systems (Lohse et al., 2007a,b, 2008). Turning our attention to the G_q-coupled M_{1}R, we used FRET experiments to probe the kinetics of receptor activation, G protein activation and rearrangement, PLC activation, and PIP_2 hydrolysis. Electrophysiology was used to examine Kv7.2/7.3 channel closure. In this initial report, we emphasize the relative timing of the optical signals without close attention to their amplitude or to full kinetic modeling.

**MATERIALS AND METHODS**

**Constructs**

Cerulean, a variant of enhanced cyan fluorescent protein (ECFP), was appended to mouse M_{1} receptor cDNA (provided by N. Nathanson, University of Washington, Seattle, WA) after Cys460 at the C-terminus to generate M_{1}R-Cerulean. To generate the intramolecular fluorescent probe M_{1}R-ECFP-Cerulean, enhanced yellow fluorescent protein (EYFP) replaced a segment between Ala223 and Val358 in the third intracellular loop of the Cerulean-labeled receptor.

cDNAs for other fluorescent probes were obtained through the generosity of other laboratories: mouse G_{q}-ECFP (Hughes et al., 2001; Scarlata and Dowal, 2004) from C. Berlot (Geisinger Clinic, Danville, PA); bovine EYFP-G_{i0} and ECFP-G_{i0} (Ruiz-Velasco and Ikeda, 2001) from S. Ikeda (National Institutes of Health, Rockville, MD); rat EYFP-PLC_{β} (Scarlata and Dowal, 2004) from L. Runnels (University of Medicine and Dentistry, Piscataway, NJ); and human pleckstrin homology (PH) domain probes PH(PLC_{δ1})-ECFP and PH(PLC_{δ1})-EYFP (van der Wal et al., 2001) from K. Jalink (The Netherlands Cancer Institute, Amsterdam, Netherlands). For some controls we used ECFP-Mem, an ECFP that becomes palmitoylated and localizes mostly to the plasma membrane (Bal et al., 2008), from M. Shapiro (University of Texas Health Sciences, San Antonio, TX). Hereafter, we refer to fluorophores simply as CFP or YFP regardless of whether regular or enhanced fluorescent proteins were used.

Plasmids containing unlabeled human G_{α_1}, G_{β_3}, and G_{γ_2} were from the Missouri S&T cDNA Resource Center, human KCNQ2 and rat KCNQ3 were from D. McKinnon (State University of New York, Stony Brook, NY), and bovine GPCR kinase 2 (GRK2) was from M. Bünemann (University of Würzburg, Würzburg, Germany).

**Cell culture**

All experiments were performed on transiently transfected tsA-201 cells. The 2-ml transfection medium contained 10 μl Lipofectamine-2000 and 0.2–0.8 μg of each cDNA. For better membrane expression of any G protein subunit probe, we always transfected three G protein subunits (α, β, and γ) together. The next day, cells were plated onto poly-l-lysine–coated 80 glass coverslip chips, and fluorescent cells were studied 36–48 h after transfection.

**Epifluorescence photometry**

To measure fluorescence interactions between CFP and YFP, we made photometric measurements on single cells using an epifluorescence microscope equipped with two photomultipliers in photon-counting mode. The cells were excited by shutter-controlled light from a 75-W xenon arc lamp and measured on an inverted Nikon diaphot microscope using a 40×, 1.3 numerical aperture oil-immersion objective. Excitation light passed through a 0.2 neutral density filter and a cube containing a 440 ± 10 nm bandpass excitation filter and a 465-nm dichroic mirror. This cube excites CFP and not YFP, and transmits light from both CFP and YFP emissions. The entire cell was centered within a circular pinhole at the image plane of the side port of the microscope, and the total light in this circular field of view was pooled and counted. Emitted light was separated by two cubes in series: a 505-nm dichroic mirror with a 480 ± 15 nm bandpass filter deflected light to one photomultiplier tube (“short-wavelength channel”), and a 570-nm dichroic mirror with a 535 ± 12.5 nm bandpass filter deflected light to the other photomultiplier tube (“long-wavelength channel”). Cells were also epifluorescently illuminated with red light, and a CCD camera with video monitor collected undeflected light above 570 nm to visualize the position of the single cell within the pinhole.

For slow sampling, the shutter was opened for 24 ms every 100 or 500 ms. For fast sampling, the shutter remained open and the photon counters were activated for 24 ms every 50 ms. Shutter and counters were controlled by an in-house DOS-based program. Solution exchange was accomplished by a theta tube moved laterally by a step-driven motor (Warner Instruments) and was complete within 50 ms. Cells were simultaneously subjected to continuous slow bath flow of Ringer’s solution.

The fluorescence ratio was taken as the ratio of YFP to CFP emission (YFP/CFP) during 440-nm illumination after corrections for background fluorescence and bleed-through determined in separate experiments on cells transfected with single fluorophores. The subscript C is a reminder that the excitation light is exciting CFP in both cases. In single-fluorophore control experiments, the fraction of CFP emission that shows up in the long-wavelength channel is 0.17, and the fraction of YFP emission that shows up in the short-wavelength channel is 0.00. Direct excitation of YFP by 440 nm light was small and not corrected for. In principle, any correction would be proportional to YFP expression levels. If LW is the background-corrected number of counts in the long-wavelength channel, and SW is the number in the short-wavelength channel, the corrected fluorescence values are:

\[
YFP_C = LW - 0.17 \times SW \\
CFP_C = SW
\]

The ratio of these quantities, YFP/CFP, is often called the FRET ratio (Bünemann et al., 2003; Lohse et al., 2003; Vilardaga et al., 2003; Frank et al., 2005; Hein et al., 2005, 2006), but here we will call it FRETr to indicate that we use a common formula for FRET but have not entirely proven that all the signals represent true FRET.

\[
FRETr = \frac{YFP_C}{CFP_C} = \frac{LW - 0.17 \times SW}{SW}
\]

For questions of how long it takes for a certain step in the signaling cascade to be changed by agonist addition, it is not important whether FRETr is in fact FRET. Nevertheless, in Results and Discussion we give lines of evidence that our calculated FRETr represents proper FRET. Slow photobleaching occurs during the measurements, but it had negligible effects on the FRETr for the experiment durations and sampling frequencies we used.

For each pair of fluorescent constructs studied we provide three lines of evidence that the baseline ratios and agonist-induced signals calculated by Eq. 3 represent FRET rather than some other optical change. (1) During perfusion of agonist, the
CFPc and YFPc values invariably changed in opposite directions with identical time courses. (2) The changes in the calculated FRET ratio were nearly fully reversed by removing the agonist. (3) When strong illumination at 500 nm was used to bleach the YFP fluorophore, CFPc increased appreciably and the calculated baseline FRET ratio fell to near zero. This experiment, donor dequenching after acceptor photobleaching, was performed on separate populations of cells under the same transfection conditions used for kinetic FRET measurements. Bleaching was accomplished by a 5-nm illumination without the neutral density filter and using a YFP filter cube containing a 500 ± 10-nm bandpass excitation filter for YFP excitation, a 515-nm dichroic mirror, and a 535 ± 15-nm bandpass emission filter. Control experiments measuring YFP photon counts showed that YFP was bleached with an exponential time constant of ~60 s with this steady light, and YFP fluorescence was reduced by 94% after 5 min of illumination. In cells expressing membrane-directed CFP-Mem only, CFP was bleached 6.5 ± 1.5% (n = 6) in this time. Control experiments using a presumed non-interacting pair of fluorophores, CFP-Mem and PLCAFP, showed an average increase in CFPc of 9.0 ± 1.9% (n = 6) after acceptor photobleaching, confirming minimal energy transfer. This value has been corrected for 6.5% CFP bleaching, as all values expressed later for donor dequenching after acceptor photobleaching.

We performed control experiments to test the function of fluorescent constructs. Calcium photometry and electrophysiology confirmed that the M1R-CPF construct coupled appropriately to modulate intracellular Ca2+ and M current with standard kinetics and efficacy. However, the M1R-YFP-CPF construct failed to couple effectively to M current, likely because the YFP insert disrupts association with G proteins. Electrophysiology confirmed the coupling of other fluorescent constructs. To ensure the specificity of association with G proteins. Electrophysiology confirmed the coupling to M current, likely because the YFP insert disrupts association with G proteins. We recorded M currents from voltage-clamped cells in whole cell configuration at room temperature (23°C). Electrodes had resistances of 1–3 MΩ. The whole cell access resistance was 2–5 MΩ, and series-resistance errors were compensated 70%. Fast and slow capacitances were also compensated. M current was measured using a standard deactivation protocol: cells were held at −20 mV, and a 500-ns hyperpolarizing step to −60 mV was applied every 4 s. Data acquisition and analysis used PULSE software in combination with an EPC-9 amplifier (HEKA).

Radioligand binding

tsA cells were grown and transfected in 150-mm cell culture plates. Membranes were prepared using a cell harvester (Brandel) and radioligand binding was assayed as described previously (Chen et al., 2004). Receptor dissociation constants (Kd) were determined by saturation binding assays with the M1R-specific antagonist N-methyl-3H-scopolamine (3H-NMS), and receptor inhibition constants (Ki) were determined by competition binding experiments including 1 nM 3H-NMS and 0.1 nM to 300 μM oxo-M. Non-specific binding was determined in the presence of 10 μM atropine. Samples were counted with a Packard Tri-Carb 2200 CA liquid scintillation analyzer (PerkinElmer). Each result reflects two experiments performed in triplicate. Saturation and competition binding curves were fitted with rectangular hyperbolas for one-site binding. Inhibition constants were determined using the Cheng-Prusoff equation.

Solutions and materials

The external Ringer’s solution used for photometry and current recording contained (in mM): 160 NaCl, 2.5 KCl, 1.3 CaCl2, 1 MgCl2, 10 HEPES, and 8 glucose, adjusted to pH 7.4 with NaOH. The pipette solution contained (in mM): 175 KCl, 5 MgCl2, 5 HEPES, 0.1 BAFTa, 3 NaATP, and 0.1 Na2GTP, adjusted to pH 7.4 with KOH.

Atropine, oxo-M, and poly-L-lysine were from Sigma-Aldrich. DMEM, Lipofectamine-2000, and penicillin/streptomycin were from Invitrogen. Fetal bovine serum was from Gemini Bio-Products. 3H-NMS was from PerkinElmer.

Data analysis

Data analysis was performed using IGOR Pro (WaveMetrics). Traces of FRET or current versus time were fitted with a linear delay to accommodate the time required by preceding steps, followed by a single-exponential component. Fitting was performed with a least-squares criterion to determine delays and time constants (τ) of activation and deactivation. The fitted equations during agonist onset were:

\[
R(t) = R_b \quad \text{for } t < t_1 \quad (4a)
\]

\[
R(t) = (R_b - R_f) \exp(-(t - t_d)/\tau_m)) + R_f \quad \text{for } t > t_1, \quad (4b)
\]

where R is the FRET; Rb and Rf are the baseline and final values, and t1 is the time delay. For receptor activation, kact was taken as

Jensen et al. 349
the slope of $1/\tau_{on}$ versus oxo-M concentration, and $k_{off}$ was $1/\tau_{off}$. Half-maximal effective concentrations ($E_{C50}$) of agonist were obtained from fits of the Hill equation to graphs of normalized, steady-state amplitude change versus oxo-M concentration. Error for $E_{C50}$ is reported as the standard deviation of the fit parameter in IGOR, a measure analogous to the SEM. Elsewhere, reported errors are SEM.

Online supplemental material
Fig. S1 has two graphs showing radioligand saturation and competition binding data for receptor constructs expressed in tsA cells. It is available at http://www.jgp.org/cgi/content/full/jgp.200810075/DC1.

RESULTS

M₁R activation
We examined receptor activation by measuring intramolecular FRET in the double-labeled receptor construct, M₁R-YFP-CFP (Fig. 1 A). Imaging in a confocal microscope confirmed that the construct localized principally to the plasma membrane (Fig. 1 B). In our epifluorescence photometry apparatus, YFP C (acceptor) fluorescence was large, although the excitation light (440 nm) excited only CFP (donor), as would be expected for an intramolecular FRET interaction with fluorophores in close proximity. The calculated resting FRET for the receptor construct (0.88) was much larger than the intermolecular FRET for the other probe combinations we studied here. As evidence that this resting FRET actually represents FRET between the fluorophores, we found that bleaching the YFP fluorophore with 5 min of 500 nm light increased CFP C counts by 82 ± 4% and decreased the calculated baseline FRET to 0.02 ($n = 7$). Washing 10 μM of the muscarinic agonist oxo-M onto cells expressing M₁R-YFP-CFP resulted in a rapid increase of acceptor YFP C counts (Fig. 1 C, yellow line) and a decrease of donor CFP C counts (blue line) corresponding to an increase in FRET (black line). Averaging five agonist exposures in a single cell, Fig. 1 D shows that the FRET rose 6% above the already high baseline. The rising phase could not be resolved, as it exceeded the 10-Hz sampling frequency. Faster sampling required leaving the shutter open and resulted in excessive bleaching of the construct, which confounded kinetic measurements. The FRET change was readily reversed upon agonist washout; the falling phase was fitted with a single-exponential time constant of 180 ms. Table I summarizes these and subsequent kinetic measurements.

M₁R affinity
Because coupling to G proteins was compromised in the M₁R-YFP-CFP construct (see Materials and methods), we wanted to verify that its ligand binding was close to that for wild-type M₁R. Using a radioactive ligand, we measured saturation (Fig. S1 A) and competition binding curves (Fig. S1 B) for membranes containing wild-type M₁R, M₁R-CFP, or M₁R-YFP-CFP, and for untransfected membranes. Dissociation constants ($K_d$) for the radioactive M₁ receptor ligand [³H-NMS were not significantly different among the three receptor constructs (mean ± SEM): wild-type M₁R, 740 ± 580 pM; M₁R-CFP, 940 ± 400 pM; and M₁R-YFP-CFP, 760 ± 510 pM. The number of binding sites in untransfected membranes was negligible. Oxo-M inhibition constants, which should represent the apparent $K_d$ for oxo-M at M₁Rs, were also very similar: wild-type M₁R, 9.2 ± 7.4 μM; M₁R-CFP, 6.2 ± 1.7 μM; and M₁R-YFP-CFP, 4.2 ± 1.0 μM. Thus, ligand binding remained normal in the compromised receptor.

M₁R/G protein interaction
Next, we measured coupling kinetics between receptor and G protein using M₁R-CFP and Gβγ-YFP constructs.
Table 1
Summary of kinetics

| Step                                      | Probes                  | Resting FRETr % | ΔFRETr % | Delay_on s | τ_on s | Delay_off s | τ_off s | EC50 (nM) |
|-------------------------------------------|-------------------------|-----------------|----------|------------|--------|-------------|---------|-----------|
| M1R activation                            | M1R-YFP-CFP             | 0.88            | +6       | <0.1       | 0.18   |             |         |           |
| M1R/Gβ interaction                        | M1R-CFP, Gβ2-YFP       | 0.42 ± 0.07     | +33 ± 6  | 0.20 ± 0.03| 3.7 ± 0.2| 330 ± 150   |         |           |
| Gαq/Gβ separation                         | Gαq-CFP, Gβ2-YFP (with GRK2) | 0.15 ± 0.01   | -10      | 2.0        | 5.8    | 35          |         |           |
| Gαq/PLCβ1 interaction                     | Gαq-CFP, PLCβ1-YFP     | 0.14 ± 0.03     | +20 ± 2  | 0.38 ± 0.25| 1.3 ± 0.3| 0.34 ± 0.14| 3.6 ± 0.5| 260 ± 190 |
| PIP2 hydrolysis                            | PH(PLCβ1)-YFP          | 0.14 ± 0.03     | -44 ± 3  | 1.3 ± 0.2  | 5.4 ± 1.6| 29 ± 2      | 59 ± 7  | 28 ± 14   |
| Kv7.2/7.3 closure                          | M current               | 1.4 ± 0.3       | 5.0 ± 0.6| 34 ± 6     | 123 ± 20| 120 ± 100   |         |           |
| Kv7.2/7.3 closure with PLCβ                | PLCβ2-YFP              | 0.78 ± 0.07     | 1.2 ± 0.1| 11 ± 7     | 62 ± 22 |             |         |           |
| Kv7.2/7.3 closure with PH probes           | PH(PLCβ1)-CFP          | 2.1 ± 0.1       | 5.7 ± 0.7| 11 ± 5     | 63 ± 9  |             |         |           |

For each kinetic step, the probes used, average resting FRETr ratio, percent change, delays, and single-exponential time constants from onset and washout of 10 μM oxo-M are given. The reported EC50 is based on a Hill fit to steady-state responses.

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When coexpressed with unlabeled G protein subunits Gαq and Gγ2, these constructs localized primarily to the plasma membrane, with a small intracellular component (Fig. 2B). Baseline FRETr averaged 0.42. Bleaching the YFP fluorophore with 5 min of 500 nm light increased F_{CYP} by 10.2 ± 0.5% and decreased the baseline FRETr to 0.01 (n = 8). Application of 10 μM oxo-M consistently produced robust increases in YFP C and decreases in CFP C, and the FRETr rose 33% above baseline on average (Fig. 2C). The rising phase had an average time constant of 200 ms, and the falling phase had an average of 3.7 s. Changes in amplitude were concentration dependent, as shown in the time course of FRETr as the oxo-M concentration was varied from 1 nM to 50 μM (Fig. 2E). Normalizing responses like these to their maximal effect at 50 μM and averaging over several cells revealed a half-maximal effective concentration (EC50) of 330 nM oxo-M by Hill fit (Fig. 2F). Apparently, half-maximal interaction between receptors and Gβ requires much less than half-maximal receptor occupancy (compare Fig. S1B).

**G protein separation**

We looked for interactions within G protein heterotrimers by measuring FRETr changes between Gαq-CFP and Gβ2-YFP (Fig. 3A). The resting FRETr ratio averaged 0.15 and always decreased after receptor activation with 10 μM oxo-M. However, on-kinetics varied widely across cells (τ_{on} from 0.8 to 10 s) and were obscured by poor signal-to-noise ratios. Averaging records from 10 cells, we found a 10% reduction in FRETr with a mean τ_{on} of 2.0 s and a τ_{off} of 35 s after a 5.8-s delay (Fig. 3D, open circles). The delay presumably reflects the time taken by preceding steps.

Experiments in the laboratory of Moritz Bünemann (Schlieffenbaum, J., A.K. Kreile, M.J. Lohse, and M. Bünemann. 2008. Biophysical Society Meeting. Abstr. 1977) suggested that GRK2 could increase the amplitude of G protein FRETr changes. In addition to binding GPCRs, GRK2 also binds both Gαq and Gβγ. The binding sites for these subunits are separated by 80–100 Å, as deduced from the crystal structure (Lodowski et al., 2003). Selecting transfected cells with primarily plasma membrane fluorescence (Fig. 3B), we found that GRK2 increased the resting FRETr and the agonist-induced loss of FRETr relative to the new baseline (Fig. 3C). Resting FRETr averaged 0.26 and decreased 17% with 10 μM oxo-M (Fig. 3D, closed circles). The kinetics were largely unchanged but more statistically robust compared with cells not transfected with GRK2. The average τ_{on} was 2.8 s after a 140-ms delay, and the average τ_{off} was 28 s after a 10-s delay. Serial concentration-response experiments (Fig. 3E) gave an EC50 of 160 nM oxo-M (Fig. 3F), similar to that for receptor–Gβ interaction. Bleaching the YFP fluorophore with 5 min of 500 nm light increased CFP C by 18 ± 2% and decreased the baseline FRETr to 0.02 (n = 8).

**G protein/PLC interaction**

To examine the kinetics of PLC activation, we measured FRETr between Gαq-CFP and PLCβ2-YFP (Fig. 4A). These probes, when coexpressed with M1R and unlabeled G protein subunits Gβ2 and Gγ2, localized primarily to the plasma membrane (Fig. 4B). Some intracellular fluorescence could be seen in the cyan channel. Baseline FRETr averaged 0.14. Bleaching the YFP fluorophore with 5 min of 500 nm light increased CFP C by 12.1 ± 0.9% and decreased the baseline FRETr to 0.01 (n = 4).
To examine changes in PIP$_2$ concentration after PLC activation, we used the PIP$_2$-binding PH(PLC$_{H9254}$). Application of 10 μM oxo-M produced opposing changes in YFP and CFP fluorescence, and a reliable increase in FRETr averaging 20% above baseline (Fig. 4 C). Fitting with single exponentials yielded mean time constants of 1.3 s after a 380-ms delay for the rising phase and 3.6 s after a 340-ms delay for the falling phase. Changes in the FRETr amplitude were concentration dependent (Fig. 4 E), with an EC$_{50}$ of 260 nM oxo-M (Fig. 4 F), similar to that for the two preceding steps.

**Figure 2.** Kinetics of M$_1$R/G$_{H9251}$ interaction. (A) Cartoon of M$_1$R-CFP and G$_{H9252}$-YFP constructs and cognate G proteins. (B) Confocal images of a pair of cells expressing M$_1$R-CFP and G$_{H9252}$-YFP. Bar, 10 μm. (C) FRETr photometry time course for a single cell undergoing a 5-s exposure to 10 μM oxo-M. The top panel shows CFP$_C$ fluorescence (blue trace, left axis) and YFP$_C$ fluorescence (yellow trace, right axis), and the bottom panel shows the ratio, YFP$_C$/CFP$_C$ (black). Sampling frequency: 2 Hz during baseline and 20 Hz during agonist. (D) Mean time course for 5-s exposures to oxo-M in six cells. Note the different time scales for onset and washout. Mean ± SEM (E) FRETr concentration–response curve from steady-state values in E for six cells. Mean ± SEM.

**Figure 3.** Kinetics of G$_{H9251}$/G$_{H9252}$ separation. All cells coexpress M$_1$R, G$_{H9251}$-CFP, G$_{H9252}$-YFP, G$_{H9253}$, and GRK2, except GRK2 is absent in one part of D. (A) Cartoon of G$_{H9251}$-CFP and G$_{H9252}$-YFP constructs and cognate G proteins. (B) Confocal images of a group of cells expressing G$_{H9251}$-CFP and G$_{H9252}$-YFP in the presence of GRK2. Bar, 10 μm. (C) FRETr photometry time course for a single cell undergoing a 10-s exposure to 10 μM oxo-M. The top panel shows CFP$_C$ fluorescence (blue trace, left axis) and YFP$_C$ fluorescence (yellow trace, right axis), and the bottom panel shows the ratio, YFP$_C$/CFP$_C$ (black). Sampling frequency: 2 Hz during baseline and 10 Hz during agonist. (D) Mean time course for 10-s exposures to oxo-M in 10 cells in the absence (open circles) and 8 cells in the presence (closed circles) of GRK2. Note the different time scales for onset and washout. Mean ± SEM. For clarity in display, points were pooled in 500-ms bins for onset and 4-s bins for washout. (E) FRETr time course for a single cell. Oxo-M was stepped to different concentrations ranging from 1 nM to 50 μM as labeled. (F) FRETr concentration–response curve from steady-state values in E for six cells. Mean ± SEM.

**PIP$_2$ hydrolysis**

To examine changes in PIP$_2$ concentration after PLC activation, we used the PIP$_2$-binding PH(PLC$_{H9251}$).
were primarily localized to the plasma membrane where some of them were in sufficiently close proximity to allow optical interaction to occur (baseline FRETr averaged 0.14). Bleaching the YFP fluorophore with 5 min of 500 nm light increased CFP C by 24 ± 4% and decreased the baseline FRETr to 0.02 (n = 9). Upon application of 10 μM o xo-M, translocation of fluorescence to the cytosol was evident in most cells. It was accompanied by opposing large changes in YFP C and CFP C, and a dramatic drop in the FRETr as the probe molecules leave the membrane. The effect was reversible upon washout (Fig. 5 B). Fig. 5 C shows a robust decrease in FRETr with 10 μM o xo-M, averaging 44% (Fig. 5 D). The FRETr decayed after a 1.3-s delay with a time constant of 5.4 s. Recovery after washout had a 29-s latency and a time constant of 59 s. Decreases in the FRETr amplitude were concentration dependent (Fig. 5 E) with an EC50 of 28 nM (Fig. 5 F), meaning that when compared with the EC50 of other steps (Table I), a very small receptor occupation and a small PLC activation suffice for extensive cleavage of PIP2.

Channel closure
Whole cell voltage clamp was used to measure currents from cells expressing M1R and M channel subunits Kv7.2 and Kv7.3. We began with cells not transfected with additional G protein subunits or PLC. M current at V/H11002 = 20 mV was almost completely suppressed by 10 μM o xo-M applied for 20 s (Fig. 6 A). On average, suppression of M current had a delay of 1.4 s and a τoff of 5.0 s. Washout was followed by a 34-s delay and recovery with a τoff of 123 s (Fig. 6 C). Current suppression was concentration dependent (Fig. 6 D) with an apparent EC50 of 120 nM o xo-M (Fig. 6 E).

Because our optical measurements required the overexpression of additional fluorescent signaling components, we tested the effect of overexpression of these proteins on the kinetics of M current suppression. Whereas transfecting G proteins (α, β, and γ together) did not alter M current suppression (unpublished data), coexpressing PLC or PH probes with receptor and channel subunits did (Table I). Overexpression of PLC-YFP reduced the delay in current suppression from 1.4 to 0.78 s and shortened the time constant from 5.0 to 1.2 s. Recovery upon washout of agonist was also accelerated, reducing the delay from 34 to 11 s and the time constant from 123 to 62 s (Fig. 7 A). On the other hand, overexpression of PH probes slowed current suppression in a concentration-dependent fashion. Cells with low to moderate expression of PH probes (those with CFP C < 8,000 per 24-ms sampling period) had an average delay of 2.1 s and a time constant of 5.7 s for current suppression, and a delay of 11 s and a time constant of 63 s for recovery (Fig. 7 B). In cells with high expression of PH probes, o xo-M failed to suppress M current fully (not depicted).
The reaction times summarized in Table I fall into a satisfying sequence that agrees with our understanding of GPCR signaling pathways. Receptor binding and G protein interaction occur in <0.5 s and have minimal delays. Alterations of the Gα/Gβγ complex and interactions with PLC occur within a couple of seconds with sub-second delays. And the depletion of PIP2 and closure of channels take 5 s and start after a >1-s delay. We will consider the steps individually. It will be apparent that at present we do not know which of several biochemical steps each fluorescent protein pair reports, so we list major possibilities. First, however, we review the evidence that the FRETr values calculated with Eq. 3 are FRET due to resonance transfer of energy from CFP (donor) to nearby YFP (acceptor).

**DISCUSSION**

The reaction times summarized in Table I fall into a satisfying sequence that agrees with our understanding of GPCR signaling pathways. Receptor binding and G protein interaction occur in <0.5 s and have minimal delays. Alterations of the Gα/Gβγ complex and interactions with PLC occur within a couple of seconds with sub-second delays. And the depletion of PIP2 and closure of channels take ~5 s and start after a >1-s delay. We will consider the steps individually. It will be apparent that at present we do not know which of several biochemical steps each fluorescent protein pair reports, so we list major possibilities. First, however, we review the evidence that the FRETr values calculated with Eq. 3 are FRET due to resonance transfer of energy from CFP (donor) to nearby YFP (acceptor).

**Relation of FRETr to FRET**

With each pair of fluorophores that we studied, there were significant resting YFPc counts (corrected for...
background and CFP bleed-through), even though the excitation light excited only CFP. Energy is being transferred from CFP to YFP. The calculated mean resting FRET values were 0.14–0.88 (Table I). In addition, photobleaching the YFP fluorophore with 500 nm light always increases resting CFP, with the increase in CFP being largest for pairs that had the largest resting FRET. These criteria show that the resting FRET values reflect FRET. Less evident is whether the changes of FRET during stimulation also reflect FRET changes. It would be ideal to show that photobleaching of YFP increases CFP more (or less) during the oxo-M-activated state than at rest. However, the small size of the signals, the long time it takes to bleach, the irreversibility of bleaching, and the profound cellular changes that occur if agonist is applied for more than a few seconds do not facilitate doing this experiment. Instead, a clear indicator of FRET changes is the consistent reciprocal time course of CFP and YFP during agonist application. Consider Fig. 5, where we know there has to be a FRET decrease because the PH domain probes translocate away from the membrane during receptor activation. Because of their proximity decrease, YFP, CFP, and, as for photobleaching of YFP, CFP, brightens. The time courses are exactly reciprocal and fully reversible. This is true of all five FRET pairs we studied. The Gβ1-YFP fluorescence provides a nice demonstration that the intensity changes are not intrinsic to the single probe, but rather to the pair of molecules studied. This probe is paired with M1R-CFP in Fig. 2 and with Goα5-CFP in Fig. 3.

The changes in YFP take 0.2 s when partnered with M1R and 2.0–3.0 s and go in the opposite direction when partnered with Gβ1.

M1R activation is fast

The fast increase in intramolecular FRET within M1R-YFP-CFP upon the addition of 10 μM oxo-M was finished by 100 ms and ought to reflect some receptor conformational change after agonist binding. We refer to this step as M1R activation. Due to constraints from bleaching and perfusion speed, we were able to determine only a lower limit for the rate of receptor activation. Using kinetic data for 1 and 10 μM oxo-M, and taking the slope of 1/τ on versus [oxo-M], we estimate a k on value of 5.0 × 10^6 M^-1s^-1. Because this step was very rapid and the receptor construct possibly does not bind G proteins, it is unlikely to be affected by steps downstream in the signaling cascade. For receptor deactivation, we obtained a k off value of 5.6 s^-1.

Our results fall within the range of FRET-based activation kinetics measured with other receptor types. Reported time constants for receptor activation are ~40 ms for the Gβγ-coupled α2β-β3-adrenergic receptor with 10 μM norepinephrine (Vilardaga et al., 2003), ~60 ms for the Gβγ-coupled β1-adrenergic receptor with 1 μM norepinephrine (Rochais et al., 2007), 66 ms for the Gα1-coupled adenosine A2A receptor with 1 mM adenosine (Hoffmann et al., 2005), and ~1 s for the Gβγ and Gq-coupled parathyroid hormone receptor with 1 μM parathyroid hormone (Vilardaga et al., 2003). The only deactivation time constant reported so far is ~2 s for a FlAsH-labeled α2A receptor (Hein et al., 2005). Our estimate of deactivation is 10-fold faster. The four above-mentioned receptor constructs showed decreases in intra-receptor FRET with agonist, unlike ours, implying that the M1R C terminus might move closer to the insertion point in the third intracellular loop, whereas in the other receptors it might move away. However, because in our construct insertion of YFP into the third intracellular loop was compensated by removal of 134 residues of the normal receptor sequence (most of the loop), it may be unwise to try to infer the directions of relative movements of domains of unmodified receptors.

Ligand binding is normal in M1R fluorescent constructs

To rule out altered ligand binding in our modified M1 receptors, we measured dissociation constants for 3H-NMS binding and inhibition constants for oxo-M. Our results for the inhibition constant of oxo-M (4–9 μM) are in the range of reported values: 8.1 μM in Chinese hamster ovary cells in the presence of 0.5 mM GTP (Jakubik et al., 1997), 2.2 ± 0.2 μM for muscarinic receptors in rat cerebral cortex, and 9.0 ± 4.9 μM for M1-M4 subtypes in a mixture of tissues (Sharif et al., 1995). If we take 4 μM as the apparent dissociation constant for oxo-M and 6 s^-1 as k off, the predicted k on (=k off /K d ) for the M1R would be 1.5 × 10^8 M^-1s^-1. Dissociation constants
for $^3$H-NMS binding to three versions of M$_1$ receptors were internally consistent (580–670 pM) but were higher than those reported in the literature: 145 pM in Chinese hamster ovary cells (Jakubik et al., 1995), 120 pM in human neuroblastoma NB-OK1 cells (Waelbroeck et al., 1990), and 260 pM (Gortés and Palacios, 1986) or 300 pM in rat brain tissue (Ehlert and Tran, 1990).

**Signaling to G proteins is not rate limiting**

The change in FRET between M$_1$R-CFP and G$_{\beta\gamma}$-YFP had a time constant of only 200 ms, ~30-fold faster than that for M current suppression. Overexpressing G proteins did not accelerate M current suppression. Collectively, these data indicate that signaling to G proteins is not rate limiting for suppression of M current, and that the pool of endogenous G proteins suffices to keep up with the exogenously expressed M$_1$ receptors.

The FRET increase observed between M$_1$R-CFP and G$_{\beta\gamma}$-YFP likely represents either increased association between the two proteins or a conformational change within a preformed complex. Because the kinetics are slower than those of M$_1$R-YFP-CFP and faster than those of G$_{\alpha_q}$-CFP/G$_{\beta\gamma}$-YFP, the events represented probably occur between receptor activation and G protein activation. The large resting FRET (0.42) suggests that some significant fraction of receptors is pre-coupled to G proteins. There is no optical sign of dissociation of G$_{\beta\gamma}$ from receptors upon activation because we see a stable elevation in the FRET between receptor and G$_{\beta\gamma}$ constructs throughout the application of agonist. These results are consistent with the observation that M$_1$R activation increases receptor affinity for G proteins (Potter et al., 1988). Recovery of this signal ($\tau_{on} = 3.7$ s) may reflect partial receptor/G protein dissociation.

Our receptor/G protein kinetics are in the same range as those reported for other receptors and G proteins. Bioluminescence resonance energy transfer between the $G_s$-coupled $\beta_2$ adrenergic receptor and G$_{\beta\gamma}$ or G$_{\gamma}2$ increased with a $t_1/2$ of ~300 ms and recovered within a few seconds using 10 μM isoproterenol (Galès et al., 2005). FRET between the $\alpha_{2A}$ adrenergic receptor and G$_{\gamma}2$ subunits increased with a $t_1/2$ of 86 ms and recovered with a $t_1/2$ of 13 s using 100 μM norepinephrine in the presence of only endogenous G$_{\alpha_q}$ (Hein et al., 2005). In that study, coexpressing G$_{\alpha_q}$ accelerated the on-kinetics to 44 ms, so that they overlapped with receptor activation. FRET between the A$_2A$ adenosine receptor and G$_{\gamma}2$ increased with $\tau_{on} = 50$ ms (1 mM adenosine) and recovered with $\tau_{off} = 15$ s (100 μM adenosine), and the $\beta_1$ adrenergic receptor and G$_{\gamma}2$ had $\tau_{on} = 58$ ms (1 mM norepinephrine) and $\tau_{off} = 8$ s (Hein et al., 2006).

**G proteins rearrange or dissociate and slowly reset**

Traditionally the G$_{\alpha}/G_{\beta\gamma}$ complex is said to dissociate upon activation by GTP. Indeed, the decrease in FRET we see between G$_{\alpha_q}$-CFP and G$_{\beta\gamma}$-YFP would be consistent with such dissociation upon receptor activation or with some other rearrangement among the G proteins that increases the distance between the fluorophores. Recovery may reflect relaxation or reassociation of the G protein subunits. GRK2 increased the resting FRET and improved the signal-to-noise ratio for changes in G$_{\alpha_q}$/G$_{\beta\gamma}$ FRET. It may have increased the resting value by recruiting more G$_{\beta\gamma}$ (acceptors) to the cell surface. In addition, it may have bound one or both G protein subunits after separation, thus increasing the distance between the fluorophores considerably or increasing the fraction of subunits that are dissociated after activation (compare Schlieffenbaum, J., A.K. Kreile, M.J. Lohse, and M. Bünemann. 2008. Biophysical Society Meeting. Abstr. 1977).

Our kinetic measurements of G protein subunit rearrangement are similar to those reported for other GPCRs. We found a FRET decrease with $\tau_{on} = \sim3$ s and delay plus $\tau_{off} = \sim40$ s. In our protocols, all of our measurements are on cells that coexpressed exogenous G$_{\alpha_q}$, $\beta$, and $\gamma$ subunits. For comparison, Bünemann et al. (2003) found an increase in FRET between G$_{\alpha_q}$ and G$_{\beta\gamma}$ with $\alpha_{2A}$ adrenergic receptor activation, with a $t_1/2$ for onset of 1 s and a $t_1/2$ for washout of 38 s with 1 μM norepinephrine. The same laboratory reported a decrease in FRET for G$_{\alpha_q}/G_{\gamma}2$ interaction with $\tau_{on} = 500$ ms and $\tau_{off} = 37$ s for $A_{2A}$ adenosine receptor activation with 1 mM adenosine, and $\tau_{on} = 440$ ms and $\tau_{off} = 15$ s for $\beta_1$ adrenergic receptor activation with 100 μM norepinephrine (Hein et al., 2006). The off-kinetics we measured are consistent with these. Although the increase in FRET between G protein subunits seen for $\alpha_{2A}$ receptors does not suggest G protein dissociation, the decrease in FRET we see with M$_1$Rs could be explained either by subunit rearrangement or by dissociation.

For each example discussed above, recovery from G protein dissociation or rearrangement as measured by recovery of G$_{\alpha}/G_{\beta\gamma}$ or G$_{\alpha}/G_{\gamma}$ FRET takes longer (~15–40 s) than classically discussed G protein cycles. Are we overlooking some events? For example, some G$_{\beta\gamma}$ subunits (including $\beta_1$ but excluding $\gamma_2$) visit intracellular membranes after G protein activation and then would have to return to the plasma membrane to reassociate (Chisari et al., 2007; Saini et al., 2007). Additionally, in several published receptor–G protein FRET experiments already described, it seems that G protein takes as long as 8–15 s to dissociate from the receptor, suggesting a continued activation. Because in our work $\tau_{off}$ for receptor–G protein interaction is only ~4 s, we return to the idea of slow GTPase. Hydrolysis of G$_{\alpha_q}$-GTP in vitro is supposed to be extremely slow without and accelerated almost 1,000-fold in the presence of PLC$_{B_1}$ (0.013 s$^{-1}$ vs. 9.12 s$^{-1}$) (Mukhopadhyay and Ross, 1999). If we had expressed an excess of G proteins compared with PLC, the free G proteins would have an exceedingly slow GTPase rate and would have to wait to partner with a free
PLC to be able to complete GTP hydrolysis. This would slow overall deactivation of Go subunits and delay subsequent steps, such as rearrangement or reassembly of G protein subunits. We regard the widely observed slow recovery of G proteins as a puzzle that still needs further conceptual explanation.

**PLC activation is fast when PLC is abundant**

Interaction between Goq,CFP and PLC-YFP (delay plus τoq = 1.7 s) followed quickly after G protein activation. This step likely reflects G protein/PLC binding or conformational changes associated with PLC activation. Coexpression of RGS2 occludes this FRETr change (unpublished data), indicating that activation of Goq by GTP is a prerequisite. Recovery from the FRETr increase may reflect GTPase activity or G protein/PLC unbinding. The interpretation of this step is complicated by the fact that we must transfect PLC to measure its activation kinetics—this step might be slower in the presence of only endogenous PLC.

Our data are consistent with the “fast activation” of PLCβ1 observed in vitro by Biddlecome et al. (1996). Using a vesicle preparation including M1R, Goq, and PLCβ, and measuring IP3 production, they observed both fast (<2 s) and slow (12 s) activation of PLC. Fast activation occurred when GTP was added to vesicles preincubated with agonist, and slow activation occurred when agonist was added to vesicles preincubated with GTP, suggesting that guanine nucleotide exchange occurred rapidly and receptor/Goq interaction was rate limiting for PLC activation. In agreement, we see an increase of M current suppression in a calcium-dependent fashion. That is, enhanced IP3 production could increase the calcium signal and potentiate the calcium-dependent PI 4-kinase, accelerating PIP2 resynthesis (Gamper and Shapiro, 2007).

**Comparison of steady-state concentration–response data**

Comparison of steady-state concentration–response data from each step suggests that PIP2 hydrolysis comes to completion at agonist concentrations that activate receptors, G proteins, and PLC only partially. Evidently activating a fraction of G proteins and PLC can, given enough time, lead to hydrolysis of a large proportion of available PIP2. This suggests that PLC molecules undergo multiple activation cycles while receptors remain active, and that reduction of PIP2 levels is cumulative during agonist exposure. The normal excess of receptors, G proteins, and PLC permits much brisker physiological responses at higher agonist concentrations.

Consistent with PIP2 hydrolysis being rate limiting, expression of PH domain probes slowed M current suppression in a manner that depended on the PH probe expression level. This slowing probably reflects buffering of PIP2 by the PH probes, which would reduce the availability of free PIP2 and slow its access to PLC (Varnai and Balla, 1998; Gamper et al., 2004). This would imply that the amount of the PH probe expressed approaches or exceeds the size of the usual free PIP2 pool. If there normally is a metabolic set point for the level of free PIP2 in the plasma membrane, sequestering of PIP2 by PH domain probes for 24 h would induce a compensatory rise in the total membrane PIP2 (free and bound). In agreement, cells with high PH probe expression had markedly slower declines in PH domain FRETr with agonist. They were discarded from kinetic analysis. M current suppression was complete in the presence of PH probes, but was slowed by 1.4 s relative to cells not expressing PH probes. Accordingly, the reported time constant for PIP2 hydrolysis may be overestimated by up to 1.4 s.

Unexpectedly, recovery from suppression of M current was accelerated in cells transfected with PLC or PH probes. As a working hypothesis, we can suggest that chronic reduction in levels of free PIP2 (by enhanced hydrolysis or buffering, respectively) produces positive feedback on PIP2 synthesis via up-regulation of PI 4-kinase and/or PIP 5-kinase. For the case of PLC overexpression, we provide two additional concepts. Accelerated recovery may be partially explained by PLC’s function as a GTPase accelerating protein for Goq (Biddlecome et al., 1996)—when PLC is overexpressed, G protein activity (and downstream events) may be shut off more quickly. In addition, PLC overexpression may speed M current recovery in a calcium-dependent fashion. That is, enhanced IP3 production could increase the calcium signal and potentiate the calcium-dependent PI 4-kinase, accelerating PIP2 resynthesis (Gamper and Shapiro, 2007).
The mechanism of coupling among G protein–coupled signaling molecules is an important determinant of signaling kinetics and efficacy. Our baseline FRET data and photobleaching results indicate some baseline proximity between M1 R and Gαq and Gβγ and PLCβ1. In the case of both M1R/Gβγ and Gαq/PLCβ1, FRET increases substantially upon muscarinic activation, indicating that not every copy of these proteins is paired/active before stimulation.

The fast activation of PLC that we observe is consistent with the preformation of stable receptor/G protein/PLC complexes (Biddlecome et al., 1996), but may also be explained by an increase in the collisional frequency between Gαq and PLC or by potentiation of G protein activity when PLC is overexpressed. If complexes are formed, the fraction of one protein that enters the complex would be sensitive to the expression level of any other partner protein(s). Additionally, RGS4 binds to activated Gαq as well as to Gβγ and PLCβ1 (Dowal et al., 2001), and may be involved in a signaling complex. Given the relatively low apparent affinity (640 nM) interaction between PLCβ1 and Goq(GDP) (Dowal et al., 2006), formation of ternary complexes may require scaffold proteins or PLC in excess of endogenous levels. PLC overexpression might tilt the balance in favor of forming complexes. Results from a recent kinetic model for G protein–coupled signaling additionally suggest that PLCβ1 potentiates G protein activity by stabilizing receptor–G protein interaction and by increasing GDP/GTP exchange (Turcotte et al., 2008). Alternatively, PLC overexpression may simply provide a higher concentration of targets for diffusing Gαq and increase the frequency of collision between Gαq and PLC.

Although observing FRET in the resting condition is a positive indicator for proximity of the components, further experiments are needed to distinguish between these paradigms using multiple approaches. It would be instructive to constrain protein interaction dynamics, for instance by limiting diffusion in the membrane or linking proteins, and comparing activation kinetics and the mobility of possible partners with those in unconstrained systems. Additionally, developing quantitative models and comparing kinetic results with those collected in other systems will provide an important check on our data and potentially shed light on the underlying structure of the system.

These experiments show an orderly temporal progression of the receptor-mediated signaling cascade, and they demonstrate that the rate-limiting step for channel closure is the consumption of PIP2 by PLC. They supply the background material needed to develop a more quantitative model of the steps of the overall signaling pathway.
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