

# Fluorescence changes reveal kinetic steps of muscarinic receptor-mediated modulation of phosphoinositides and Kv7.2/7.3 K<sup>+</sup> channels

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G protein-coupled receptors initiate signaling cascades. M<sub>1</sub> muscarinic receptor (M<sub>1</sub>R) activation couples through G<sub>α<sub>q</sub></sub> to stimulate phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>). Depletion of PIP<sub>2</sub> closes PIP<sub>2</sub>-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 M<sub>1</sub>R activation, M<sub>1</sub>R/G<sub>β</sub> interaction, G<sub>α<sub>q</sub></sub>/G<sub>β</sub> separation, G<sub>α<sub>q</sub></sub>/PLC interaction, and PIP<sub>2</sub> hydrolysis. Electrophysiology was used to monitor channel closure. Time constants for M<sub>1</sub>R activation (<100 ms) and M<sub>1</sub>R/G<sub>β</sub> interaction (200 ms) are both fast, suggesting that neither of them is rate limiting during muscarinic suppression of M current. G<sub>α<sub>q</sub></sub>/G<sub>β</sub> separation and G<sub>α<sub>q</sub></sub>/PLC interaction have intermediate 1/e times (2.9 and 1.7 s, respectively), and PIP<sub>2</sub> 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 G<sub>α<sub>q</sub></sub>/PLC interaction. Evidently, channel release of PIP<sub>2</sub> 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<sup>+</sup> channel by muscarinic receptors.

Activation of G<sub>q/11</sub>-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 en-

coded by KCNQ2 and KCNQ3 (Kv7.2 and 7.3) channel subunits (Wang et al., 1998), requires phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) to be active (Suh and Hille, 2002; Zhang et al., 2003). Muscarinic modulation of M current acts through a chain of events: G<sub>α<sub>q</sub></sub> activates phospholipase C-β (PLCβ), which hydrolyzes PIP<sub>2</sub> to generate inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol. PIP<sub>2</sub> 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 M<sub>1</sub> muscarinic acetylcholine receptor (M<sub>1</sub>R) 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

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Abbreviations used in this paper: ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; FRET, fluorescence resonance energy transfer; GPCR, G protein-coupled receptor; GRK2, GPCR kinase 2; <sup>3</sup>H-NMS, N-methyl-<sup>3</sup>H-scopolamine; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; M<sub>1</sub>R, M<sub>1</sub> muscarinic receptor; oxo-M, oxotremorine-methiodide; PH, pleckstrin homology; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PLCβ, phospholipase C-β.

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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  $M_1R$ , 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  $M_1R$ -Cerulean. To generate the intramolecular fluorescent probe  $M_1R$ -EYFP-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  $G_{\alpha_q}$ -ECFP (Hughes et al., 2001; Scarlata and Dowal, 2004) from C. Berlot (Geisinger Clinic, Danville, PA); bovine EYFP- $G\beta_1$  and ECFP- $G\gamma_2$  (Ruiz-Velasco and Ikeda, 2001) from S. Ikeda (National Institutes of Health, Rockville, MD); rat EYFP-PLC $\beta_1$  (Scarlata and Dowal, 2004) from L. Runnels (University of Medicine and Dentistry, Piscataway, NJ); and human pleckstrin homology (PH) domain probes PH(PLC $\delta_1$ )-ECFP and PH(PLC $\delta_1$ )-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  $G_{\alpha_q}$ ,  $G\beta_1$ , and  $G\gamma_2$  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  $\mu$ l Lipofectamine-2000 and 0.2–0.8  $\mu$ g of each cDNA. For better membrane expression of any G protein subunit probe, we always transfected three G protein subunits ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) together. The next day, cells were plated onto poly-L-lysine-coated #0 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 $\times$ , 1.3 numerical aperture oil-immersion objective. Excitation light passed through a 0.2 neutral density filter and a cube containing a 440  $\pm$  10 nm band-pass 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  $\pm$  15 nm bandpass filter deflected light to one photomultiplier tube ("short-wavelength channel"), and a 570-nm dichroic mirror with a 535  $\pm$  12.5 nm bandpass filter deflected light to the other photomultiplier tube ("long-wavelength channel"). Cells were also epi-illuminated with red light, and a CCD camera with video monitor collected undeflected light above 570 nm to visualize the positioning 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 ( $YFP_C/CFP_C$ ) 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:

$$YFP_C = LW - 0.17 SW \quad (1)$$

$$CFP_C = SW \quad (2)$$

The ratio of these quantities,  $YFP_C/CFP_C$ , 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 FRET<sub>r</sub> to indicate that we use a common formula for FRET ratio but have not entirely proven that all the signals represent true FRET.

$$FRET_r = \frac{YFP_C}{CFP_C} = \frac{LW - 0.17 \cdot SW}{SW} \quad (3)$$

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 FRET<sub>r</sub> is in fact FRET. Nevertheless, in Results and Discussion we give lines of evidence that our calculated FRET<sub>r</sub> represents proper FRET. Slow photobleaching occurs during the measurements, but it had negligible effects on the FRET<sub>r</sub> 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

CFP<sub>C</sub> and YFP<sub>C</sub> values invariably changed in opposite directions with identical time courses. (2) The changes in the calculated FRET<sub>r</sub> ratio were nearly fully reversed by removing the agonist. (3) When strong illumination at 500 nm was used to bleach the YFP fluorophore, CFP<sub>C</sub> increased appreciably and the calculated baseline FRET<sub>r</sub> 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<sub>r</sub> measurements. Bleaching was accomplished by a 5-min 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 PLC-YFP, showed an average increase in CFP<sub>C</sub> 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 have all values reported 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 M<sub>1</sub>R-CFP construct coupled appropriately to modulate intracellular Ca<sup>2+</sup> and M current with standard kinetics and efficacy. However, the M<sub>1</sub>R-YFP-CFP 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 FRET<sub>r</sub> responses to muscarinic agonist oxotremorine-methiodide (oxo-M), we confirmed that incubation with 10 μM of muscarinic antagonist atropine blocked oxo-M-induced FRET<sub>r</sub> changes in all construct pairs studied. Atropine alone had no effect on FRET<sub>r</sub> for most pairs of constructs; when FRET<sub>r</sub> changes were observed, they were minimal and opposed the direction of oxo-M-induced changes.

#### Cell selection for photometry

After transfection of fluorescent proteins, the cell population is not uniform. Fewer than 10% of the cells are bright enough to use for photometry, and some of these are too bright. We selected cells for study under 440-nm illumination on the basis of several criteria. The short-wavelength counts had to be in the range of 500–12,000 counts (per 24 ms). The long-wavelength counts had to exceed the value expected from simple CFP bleed-through into the YFP channel. These criteria ensure adequate expression of CFP and YFP. The cell had to be firmly adherent to the substrate. For photometry, confocal microscopy, and patch clamp, we often chose cells that were slightly rounded rather than strongly flattened. They were easier to patch onto with a pipette, and in confocal optical section, they had a clearer vertical region of plasma membrane, permitting us to assess membrane localization of the probes. All such cells responded robustly in patch clamp (current measurement) and photometry to the muscarinic agonist oxo-M. Finally, we did not use cells that had bright fluorescent regions inside the cell.

#### Confocal fluorescence imaging

To verify membrane expression, cells were imaged using a Leica SP1 confocal microscope with a 63× water or 100× oil-immersion objective. The confocal images shown in several figures were used to determine subcellular localizations of probes, but not for any of the FRET<sub>r</sub> calculations. Cells pictured in confocal images are different from those on which FRET<sub>r</sub> calculations were per-

formed. For cyan images, the cells were illuminated with the 457-nm laser line (RSP465 beam splitter), and light from 462 to 551 nm was collected. For yellow images, the cells were illuminated with the 488-nm laser line (RSP500 beam splitter), and light from 523 to 593 nm was collected. Both because the 457-nm line excites CFP inefficiently (it is much weaker than the 488-nm line) and because CFP is intrinsically less bright, the confocal images for CFP required higher gain than those for YFP, in contrast to the epifluorescence photometry experiments using only 440-nm light, where the CFP<sub>C</sub> counts were larger than the YFP<sub>C</sub> counts. The confocal images shown are labeled cyan and yellow and represent the raw data with no corrections.

#### Current recording and analysis

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-ms 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 (*K<sub>d</sub>*) were determined by saturation binding assays with the M<sub>1</sub>R-specific antagonist *N*-methyl-<sup>3</sup>H-scopolamine (<sup>3</sup>H-NMS), and receptor inhibition constants (*K<sub>i</sub>*) were determined by competition binding experiments including 1 nM <sup>3</sup>H-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, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, and 8 glucose, adjusted to pH 7.4 with NaOH. The pipette solution contained (in mM): 175 KCl, 5 MgCl<sub>2</sub>, 5 HEPES, 0.1 BAPTA, 3 Na<sub>2</sub>ATP, and 0.1 Na<sub>3</sub>GTP, 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. <sup>3</sup>H-NMS was from PerkinElmer.

#### Data analysis

Data analysis was performed using IGOR Pro (Wavemetrics). Traces of FRET<sub>r</sub> 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:

$$R(t) = R_0 \quad \text{for } t < t_d \quad (4a)$$

$$R(t) = (R_0 - R_1) \exp(-(t - t_d) / \tau_{on}) + R_1 \quad \text{for } t > t_d, \quad (4b)$$

where *R* is the FRET<sub>r</sub>, *R*<sub>0</sub> and *R*<sub>1</sub> are the baseline and final values, and *t<sub>d</sub>* is the time delay. For receptor activation, *k<sub>on</sub>* was taken as



the slope of  $1/\tau_{\text{on}}$  versus oxo-M concentration, and  $k_{\text{off}}$  was  $1/\tau_{\text{off}}$ . Half-maximal effective concentrations ( $EC_{50}$ ) of agonist were obtained from fits of the Hill equation to graphs of normalized, steady-state amplitude change versus oxo-M concentration. Error for  $EC_{50}$  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>.

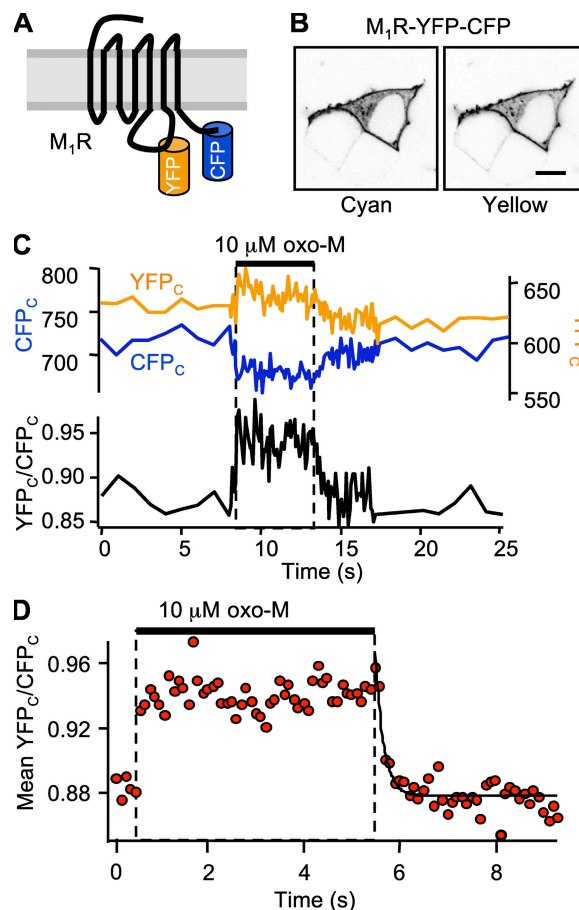
## RESULTS

### M<sub>1</sub>R activation

We examined receptor activation by measuring intramolecular FRET in the double-labeled receptor construct, M<sub>1</sub>R-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<sub>C</sub> (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<sub>r</sub> for the receptor construct (0.88) was much larger than the intermolecular FRET<sub>r</sub> for the other probe combinations we studied here. As evidence that this resting FRET<sub>r</sub> actually represents FRET between the fluorophores, we found that bleaching the YFP fluorophore with 5 min of 500 nm light increased CFP<sub>C</sub> counts by  $82 \pm 4\%$  and decreased the calculated baseline FRET<sub>r</sub> to 0.02 ( $n = 7$ ). Washing 10  $\mu\text{M}$  of the muscarinic agonist oxo-M onto cells expressing M<sub>1</sub>R-YFP-CFP resulted in a rapid increase of acceptor YFP<sub>C</sub> counts (Fig. 1 C, yellow line) and a decrease of donor CFP<sub>C</sub> counts (blue line) corresponding to an increase in FRET<sub>r</sub> (black line). Averaging five agonist exposures in a single cell, Fig. 1 D shows that the FRET<sub>r</sub> 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<sub>r</sub> 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.

### M<sub>1</sub>R affinity

Because coupling to G proteins was compromised in the M<sub>1</sub>R-YFP-CFP construct (see Materials and methods), we wanted to verify that its ligand binding was close to that for wild-type M<sub>1</sub>R. Using a radioactive ligand, we measured saturation (Fig. S1 A) and competition binding curves (Fig. S1 B) for membranes containing wild-type M<sub>1</sub>R, M<sub>1</sub>R-CFP, or M<sub>1</sub>R-YFP-CFP,



**Figure 1.** Kinetics of M<sub>1</sub>R activation. (A) Cartoon of the double-labeled M<sub>1</sub>R construct, M<sub>1</sub>R-YFP-CFP. (B) Confocal images of three resting cells, only one of which expresses the M<sub>1</sub>R. The transfected cell shows the distributions of cyan and yellow fluorescence. Bar, 10  $\mu\text{m}$ . (C) FRET<sub>r</sub> photometry time course for a single cell. The top panel shows corrected CFP<sub>C</sub> fluorescence (blue trace, left axis) and YFP<sub>C</sub> fluorescence (yellow trace, right axis), and the bottom panel shows the corrected ratio, YFP<sub>C</sub>/CFP<sub>C</sub> (black), for a 5-s exposure to 10  $\mu\text{M}$  oxo-M. Sampling frequency: 1 Hz during baseline and 10 Hz during agonist. (D) Normalized mean time course for five 5-s exposures to oxo-M in a single cell (same cell as C). Black line is a single-exponential fit with  $\tau_{\text{off}} = 180$  ms.

and for untransfected membranes. Dissociation constants ( $K_d$ ) for the radioactive M<sub>1</sub> receptor ligand <sup>3</sup>H-NMS were not significantly different among the three receptor constructs (mean  $\pm$  SEM): wild-type M<sub>1</sub>R,  $740 \pm 580$  pM; M<sub>1</sub>R-CFP,  $940 \pm 400$  pM; and M<sub>1</sub>R-YFP-CFP,  $760 \pm 510$  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 M<sub>1</sub>Rs, were also very similar: wild-type M<sub>1</sub>R,  $9.2 \pm 7.4$   $\mu\text{M}$ ; M<sub>1</sub>R-CFP,  $6.2 \pm 1.7$   $\mu\text{M}$ ; and M<sub>1</sub>R-YFP-CFP,  $4.2 \pm 1.0$   $\mu\text{M}$ . Thus, ligand binding remained normal in the compromised receptor.

### M<sub>1</sub>R/G protein interaction

Next, we measured coupling kinetics between receptor and G protein using M<sub>1</sub>R-CFP and G $\beta_1$ -YFP constructs

TABLE I  
Summary of kinetics

| Step                                           | Probes                                                                | Resting<br>FRET <sub>r</sub> % | ΔFRET <sub>r</sub><br>% | Delay <sub>on</sub><br>s | τ <sub>on</sub><br>s | Delay <sub>off</sub><br>s | τ <sub>off</sub><br>s | EC <sub>50</sub> (oxo-M)<br>nM |
|------------------------------------------------|-----------------------------------------------------------------------|--------------------------------|-------------------------|--------------------------|----------------------|---------------------------|-----------------------|--------------------------------|
| M <sub>1</sub> R activation                    | M <sub>1</sub> R-YFP-CFP                                              | 0.88                           | +6                      |                          | <0.1                 |                           | 0.18                  |                                |
| M <sub>1</sub> R/Gβ interaction                | M <sub>1</sub> R-CFP<br>Gβ <sub>1</sub> -YFP                          | 0.42 ± 0.07                    | +33 ± 6                 |                          | 0.20 ± 0.03          |                           | 3.7 ± 0.2             | 330 ± 150                      |
| Gα <sub>q</sub> /Gβ <sub>1</sub> separation    | Gα <sub>q</sub> -CFP<br>Gβ <sub>1</sub> -YFP<br>(with GRK2)           | 0.15 ± 0.01<br>0.26 ± 0.03     | −10<br>−17 ± 2          |                          | 2.0<br>2.8 ± 0.3     | 5.8<br>9.9 ± 2.9          | 35<br>28 ± 2          | 160 ± 100                      |
| Gα <sub>q</sub> /PLCβ <sub>1</sub> interaction | Gα <sub>q</sub> -CFP<br>PLCβ <sub>1</sub> -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                      |
| PIP <sub>2</sub> hydrolysis                    | PH(PLCδ <sub>1</sub> )-CFP<br>PH(PLCδ <sub>1</sub> )-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<br>with PLCβ                 | M current<br>PLCβ <sub>1</sub> -YFP                                   |                                |                         | 0.78 ± 0.07              | 1.2 ± 0.1            | 11 ± 7                    | 62 ± 22               |                                |
| Kv7.2/7.3 closure<br>with PH probes            | M current<br>PH(PLCδ <sub>1</sub> )-CFP<br>PH(PLCδ <sub>1</sub> )-YFP |                                |                         | 2.1 ± 0.1                | 5.7 ± 0.7            | 11 ± 5                    | 63 ± 9                |                                |

For each kinetic step, the probes used, average resting FRET<sub>r</sub> ratio, percent change, delays, and single-exponential time constants from onset and washout of 10 μM oxo-M are given. The reported EC<sub>50</sub> is based on a Hill fit to steady-state responses.

(Fig. 2 A). When coexpressed with unlabeled G protein subunits Gα<sub>q</sub> and Gγ<sub>2</sub>, these constructs localized primarily to the plasma membrane, with a small intracellular component (Fig. 2 B). Baseline FRET<sub>r</sub> averaged 0.42. Bleaching the YFP fluorophore with 5 min of 500 nm light increased F<sub>CFP</sub> by 10.2 ± 0.5% and decreased the baseline FRET<sub>r</sub> to 0.01 (*n* = 8). Application of 10 μM oxo-M consistently produced robust increases in YFP<sub>C</sub> and decreases in CFP<sub>C</sub>, and the FRET<sub>r</sub> rose 33% above baseline on average (Fig. 2 C). 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 FRET<sub>r</sub> as the oxo-M concentration was varied from 10 nM to 50 μM (Fig. 2 E). Normalizing responses like these to their maximal effect at 50 μM and averaging over several cells revealed a half-maximal effective concentration (EC<sub>50</sub>) of 330 nM oxo-M by Hill fit (Fig. 2 F). Apparently, half-maximal interaction between receptors and Gβ requires much less than half-maximal receptor occupancy (compare Fig. S1 B).

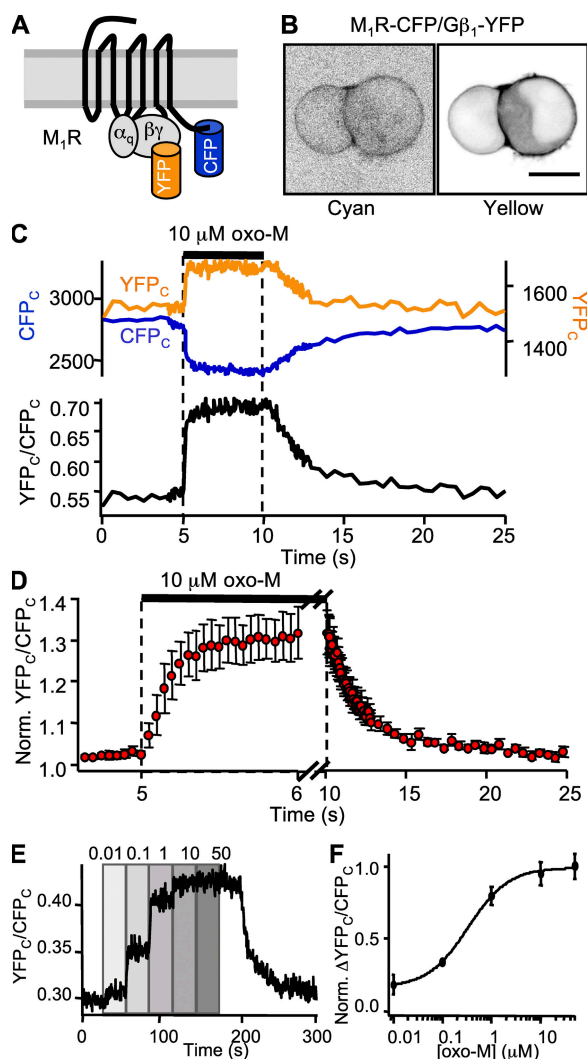
#### G protein separation

We looked for interactions within G protein heterotrimers by measuring FRET<sub>r</sub> changes between Gα<sub>q</sub>-CFP and Gβ<sub>1</sub>-YFP (Fig. 3 A). The resting FRET<sub>r</sub> ratio averaged 0.15 and always decreased after receptor activation with 10 μM oxo-M. However, on-kinetics varied widely across cells (τ<sub>on</sub> 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 FRET<sub>r</sub> with a mean τ<sub>on</sub> of 2.0 s and a τ<sub>off</sub> of 35 s after a 5.8-s delay (Fig. 3 D, open circles). The delay presumably reflects the time taken by preceding steps.

Experiments in the laboratory of Moritz Bünemann (Schliefenbaum, 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 FRET changes. In addition to binding GPCRs, GRK2 also binds both Gα<sub>q</sub> 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. 3 B), we found that GRK2 increased the resting FRET<sub>r</sub> and the agonist-induced loss of FRET<sub>r</sub> relative to the new baseline (Fig. 3 C). Resting FRET<sub>r</sub> averaged 0.26 and decreased 17% with 10 μM oxo-M (Fig. 3 D, closed circles). The kinetics were largely unchanged but more statistically robust compared with cells not transfected with GRK2. The average τ<sub>on</sub> was 2.8 s after a 140-ms delay, and the average τ<sub>off</sub> was 28 s after a 10-s delay. Serial concentration–response experiments (Fig. 3 E) gave an EC<sub>50</sub> of 160 nM oxo-M (Fig. 3 F), similar to that for receptor–Gβ interaction. Bleaching the YFP fluorophore with 5 min of 500 nm light increased CFP<sub>C</sub> by 18 ± 2% and decreased the baseline FRET<sub>r</sub> to 0.02 (*n* = 8).

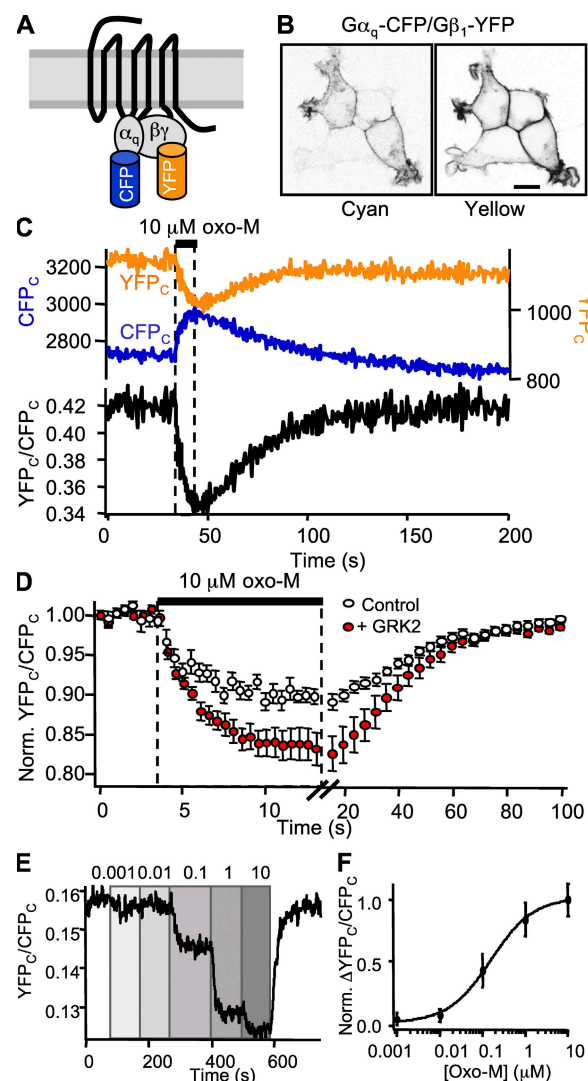
#### G protein/PLC interaction

To examine the kinetics of PLC activation, we measured FRET<sub>r</sub> between Gα<sub>q</sub>-CFP and PLCβ<sub>1</sub>-YFP (Fig. 4 A). These probes, when coexpressed with M<sub>1</sub>R and unlabeled G protein subunits Gβ<sub>1</sub> and Gγ<sub>2</sub>, localized primarily to the plasma membrane (Fig. 4 B). Some intracellular fluorescence could be seen in the cyan channel. Baseline FRET<sub>r</sub> averaged 0.14. Bleaching the YFP fluorophore with 5 min of 500 nm light increased CFP<sub>C</sub> by 12.1 ± 0.9% and decreased the baseline FRET<sub>r</sub> to 0.01 (*n* = 4).



**Figure 2.** Kinetics of M<sub>1</sub>R/Gβ<sub>1</sub> interaction. (A) Cartoon of M<sub>1</sub>R-CFP and Gβ<sub>1</sub>-YFP constructs and cognate G proteins. (B) Confocal images of a pair of cells expressing M<sub>1</sub>R-CFP and Gβ<sub>1</sub>-YFP. Bar, 10 μm. (C) FRET photometry time course for a single cell undergoing a 5-s exposure to 10 μM oxo-M. The top panel shows CFP<sub>C</sub> fluorescence (blue trace, left axis) and YFP<sub>C</sub> fluorescence (yellow trace, right axis), and the bottom panel shows the ratio, YFP<sub>C</sub>/CFP<sub>C</sub> (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) FRET time course for a single cell. Oxo-M was stepped to different concentrations ranging from 10 nM to 50 μM as labeled. (F) FRET concentration-response curve from steady-state values in E for six cells. Mean ± SEM.

Application of 10 μM oxo-M produced opposing changes in YFP and CFP fluorescence, and a reliable increase in FRET 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 FRET amplitude were concentration dependent (Fig. 4 E), with an EC<sub>50</sub> of 260 nM oxo-M (Fig. 4 F), similar to that for the two preceding steps.

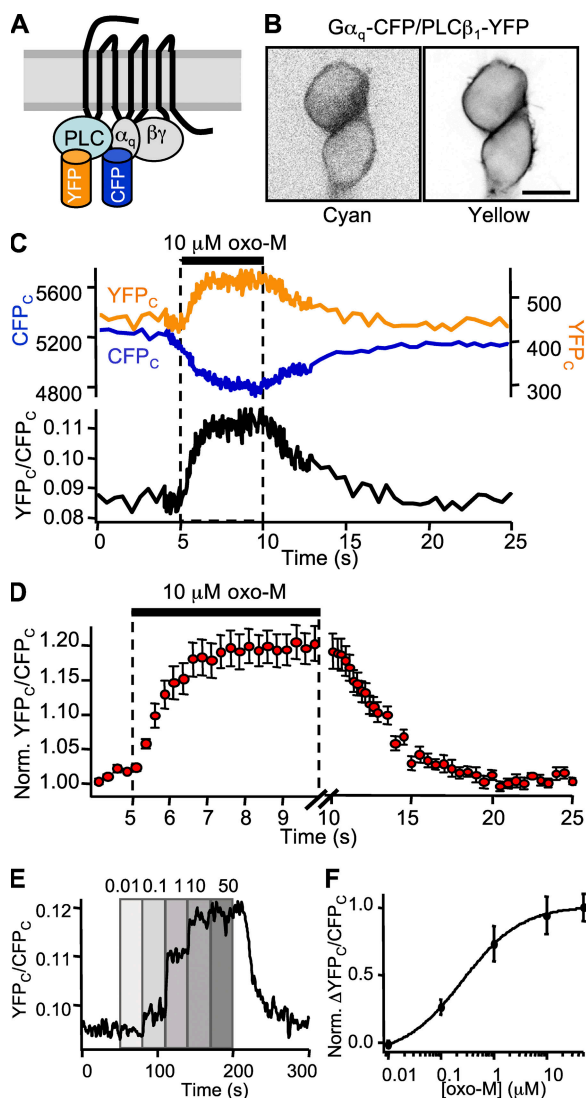


**Figure 3.** Kinetics of Gα<sub>q</sub>/Gβ<sub>1</sub> separation. All cells coexpress M<sub>1</sub>R, Gα<sub>q</sub>-CFP, Gβ<sub>1</sub>-YFP, Gγ<sub>2</sub>, and GRK2, except GRK2 is absent in one part of D. (A) Cartoon of Gα<sub>q</sub>-CFP and Gβ<sub>1</sub>-YFP constructs and cognate G proteins. (B) Confocal images of a group of cells expressing Gα<sub>q</sub>-CFP and Gβ<sub>1</sub>-YFP in the presence of GRK2. Bar, 10 μm. (C) FRET photometry time course for a single cell undergoing a 10-s exposure to 10 μM oxo-M. The top panel shows CFP<sub>C</sub> fluorescence (blue trace, left axis) and YFP<sub>C</sub> fluorescence (yellow trace, right axis), and the bottom panel shows the ratio, YFP<sub>C</sub>/CFP<sub>C</sub> (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) FRET time course for a single cell. Oxo-M was stepped to different concentrations ranging from 1 nM to 10 μM as labeled. For clarity in display, trace is smoothed. (F) FRET concentration-response curve from steady-state values in E for six cells. Mean ± SEM.

#### PIP<sub>2</sub> hydrolysis

To examine changes in PIP<sub>2</sub> concentration after PLC activation, we used the PIP<sub>2</sub>-binding PH(PLCδ<sub>1</sub>)





**Figure 4.** Kinetics of  $G\alpha_q/PLC\beta_1$  interaction. (A) Cartoon of  $G\alpha_q$ -CFP,  $PLC\beta_1$ -YFP, and cognate G proteins. (B) Confocal images of a pair of cells coexpressing  $G\alpha_q$ -CFP and  $PLC\beta_1$ -YFP. Bar, 10  $\mu$ m. (C) FRET photometry time course for a single cell undergoing a 5-s exposure to 10  $\mu$ M oxo-M. The top panel shows CFP<sub>c</sub> fluorescence (blue trace, left axis) and YFP<sub>c</sub> fluorescence (yellow trace, right axis), and the bottom panel shows the ratio, YFP<sub>c</sub>/CFP<sub>c</sub> (black). Sampling frequency: 2 Hz during baseline and 20 Hz during agonist. (D) Mean time course for 5-s exposures to oxo-M in 10 cells. Mean  $\pm$  SEM. Note the different time scales for onset and washout. For clarity in display, points from fast sampling were pooled in 200-ms bins. (E) FRET concentration-response time course for a single cell. Oxo-M from 10 nM to 50  $\mu$ M as labeled. For clarity in display, trace is smoothed. (F) FRET concentration-response curve from steady-state values in E for four cells. Mean  $\pm$  SEM.

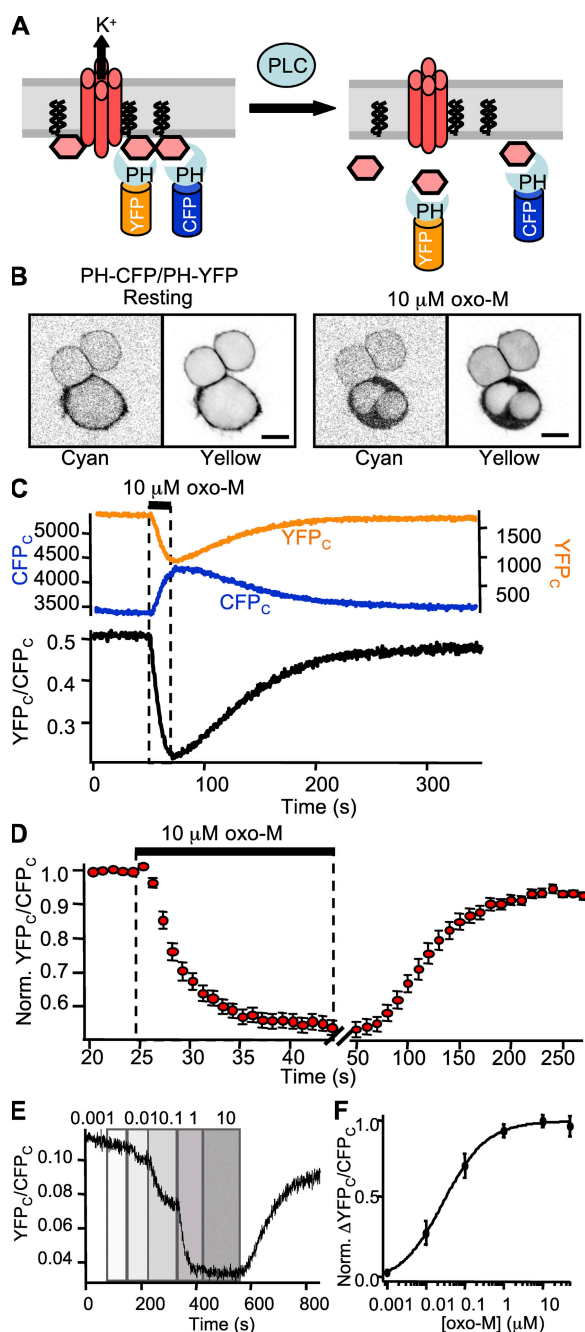
translocation probe (Fig. 5 A). This probe binds the phosphoinositol headgroup of  $PIP_2$  and  $IP_3$  within cells and translocates from the membrane to the cytosol when  $PIP_2$  is hydrolyzed to  $IP_3$  (Stauffer et al., 1998). We measured FRET between coexpressed PH-CFP and PH-YFP (van der Wal et al., 2001). At rest, the PH probes

were primarily localized to the plasma membrane where some of them were in sufficiently close proximity to allow optical interaction to occur (baseline FRET averaged 0.14). Bleaching the YFP fluorophore with 5 min of 500 nm light increased CFP<sub>c</sub> by  $24 \pm 4\%$  and decreased the baseline FRET to 0.02 ( $n = 9$ ). Upon application of 10  $\mu$ M oxo-M, translocation of fluorescence to the cytosol was evident in most cells. It was accompanied by opposing large changes in YFP<sub>c</sub> and CFP<sub>c</sub>, and a dramatic drop in the FRET as the probe molecules leave the membrane. The effect was reversible upon washout (Fig. 5 B). Fig. 5 C shows a robust decrease in FRET with 10  $\mu$ M oxo-M, averaging 44% (Fig. 5 D). The FRET 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 FRET amplitude were concentration dependent (Fig. 5 E) with an  $EC_{50}$  of 28 nM (Fig. 5 F), meaning that when compared with the  $EC_{50}$  of other steps (Table I), a very small receptor occupation and a small PLC activation suffice for extensive cleavage of  $PIP_2$ .

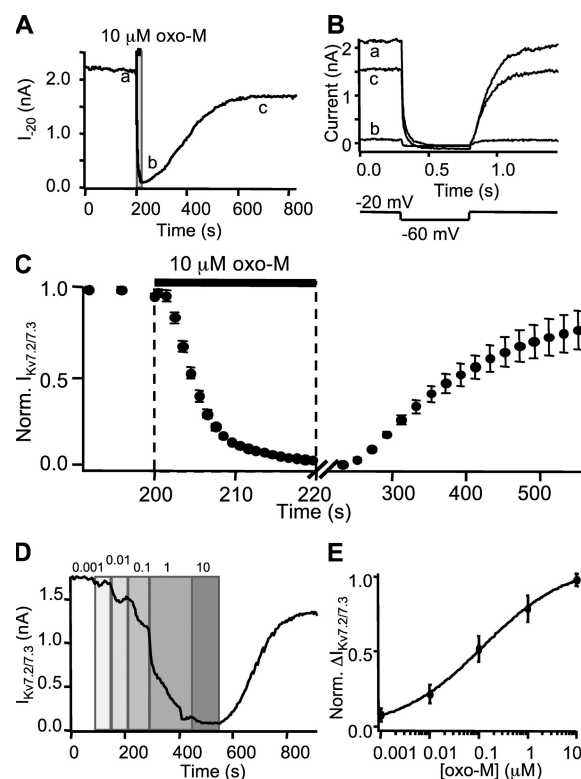
#### Channel closure

Whole cell voltage clamp was used to measure currents from cells expressing  $M_1R$  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  $-20$  mV was almost completely suppressed by 10  $\mu$ M oxo-M applied for 20 s (Fig. 6 A). On average, suppression of M current had a delay of 1.4 s and a  $\tau_{on}$  of 5.0 s. Washout was followed by a 34-s delay and recovery with a  $\tau_{off}$  of 123 s (Fig. 6 C). Current suppression was concentration dependent (Fig. 6 D) with an apparent  $EC_{50}$  of 120 nM oxo-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 ( $\alpha$ ,  $\beta$ , and  $\gamma$  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<sub>c</sub> < 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, oxo-M failed to suppress M current fully (not depicted).



**Figure 5.** Kinetics of  $\text{PIP}_2$  hydrolysis. (A) Cartoon of PH( $\text{PLC}\delta_1$ )-CFP, PH( $\text{PLC}\delta_1$ )-YFP,  $\text{Kv}7.2/7.3$  channels, and  $\text{PIP}_2$ . PLC hydrolyzes  $\text{PIP}_2$  to send PH probes to the cytosol. (B) Confocal images of three cells expressing PH-CFP and PH-YFP. Bar, 10  $\mu\text{m}$ . (C) FRET photometry time course for a single cell undergoing a 20-s exposure to 10  $\mu\text{M}$  oxo-M. The top panel shows  $\text{CFP}_c$  fluorescence (blue trace, left axis) and  $\text{YFP}_c$  fluorescence (yellow trace, right axis), and the bottom panel shows the ratio,  $\text{YFP}_c/\text{CFP}_c$  (black). Sampling frequency: 2 Hz throughout. (D) Mean time course for 20-s exposures to oxo-M in 22 cells. Mean  $\pm$  SEM. Note the different time scales for onset and washout. For clarity in display, points were pooled in 1-s bins for onset and 10-s bins for washout. (E) FRET concentration-response time course for a single cell. Oxo-M from 1 nM to 10  $\mu\text{M}$  as labeled. (F) FRET concentration-response curve from steady-state values in E for 12 cells. Mean  $\pm$  SEM.



**Figure 6.** Kinetics of  $\text{Kv}7.2/7.3$  channel closure. (A) Time course for current from a single voltage-clamped cell undergoing a 20-s exposure to 10  $\mu\text{M}$  oxo-M. Current is steady-state measured at  $-20$  mV. Sampling frequency: 0.25 Hz during baseline and 200 Hz during agonist. (B) Individual current traces corresponding to points in A. Voltage was stepped from  $-20$  to  $-60$  mV for 500 ms every 4 s. (C) Time course for normalized mean current in five cells. Note the different time scales for onset and washout. For clarity in display, points from fast sampling were pooled in 1-s bins and points from slow sampling were pooled in 20-s bins. (D) M current concentration-response time course for a single cell. Oxo-M from 1 nM to 10  $\mu\text{M}$  as labeled. (E) M current concentration-response curve from steady-state values for eight cells. Mean  $\pm$  SEM.

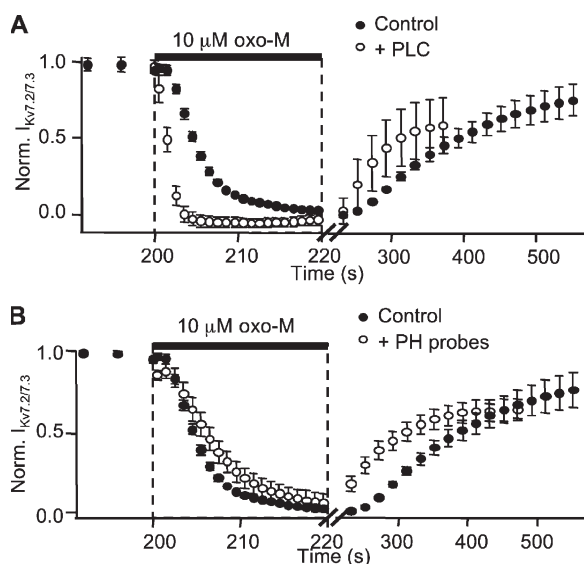
## DISCUSSION

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 within a couple of seconds with sub-second delays. Alterations of the  $\text{G}\alpha/\text{G}\beta\gamma$  complex and interactions with PLC occur within a couple of seconds with sub-second delays. And the depletion of  $\text{PIP}_2$  and closure of channels take  $\sim 5$  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 FRET values calculated with Eq. 3 are FRET due to resonance transfer of energy from CFP (donor) to nearby YFP (acceptor).

### Relation of FRET to FRET

With each pair of fluorophores that we studied, there were significant resting  $\text{YFP}_c$  counts (corrected for





**Figure 7.** PLC speeds and PH probes slow M current suppression. (A and B) Normalized mean M current at  $-20$  mV from six cells expressing either transfected PLC $\beta_1$ -YFP (open circles, A) or low levels of PH(PLC $\delta_1$ )-CFP and PH(PLC $\delta_1$ )-YFP (open circles, B). For comparison, control M current from five cells lacking exogenous PLC or PH probes (black circles). Note the different time scales for onset and washout.

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 increased resting CFP<sub>C</sub>, with the increase in CFP<sub>C</sub> 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<sub>C</sub> 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<sub>C</sub> and YFP<sub>C</sub> 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<sub>C</sub> dims, and, as for photobleaching of YFP, CFP<sub>C</sub> brightens. The time courses are exactly reciprocal and fully reversible. This is true of all five FRET pairs we studied. The G $\beta_1$ -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 M<sub>1</sub>R-CFP in Fig. 2 and with G $\alpha_q$ -CFP in Fig. 3.

The changes in YFP<sub>C</sub> take 0.2 s when partnered with M<sub>1</sub>R and 2.0–3.0 s and go in the opposite direction when partnered with G $\beta_1$ .

#### M<sub>1</sub>R activation is fast

The fast increase in intramolecular FRET within M<sub>1</sub>R-YFP-CFP upon the addition of 10  $\mu$ M oxo-M was finished by 100 ms and ought to reflect some receptor conformational change after agonist binding. We refer to this step as M<sub>1</sub>R 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  $\mu$ M oxo-M, and taking the slope of  $1/\tau_{on}$  versus [oxo-M], we estimate a  $k_{on}$  value of  $5.0 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ . 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  $5.6 \text{ s}^{-1}$ .

Our results fall within the range of FRET-based activation kinetics measured with other receptor types. Reported time constants for receptor activation are  $\sim 40$  ms for the G<sub>T</sub>-coupled  $\alpha_{2A}$ -adrenergic receptor with 10  $\mu$ M norepinephrine (Vilardaga et al., 2003),  $\sim 60$  ms for the G<sub>s</sub>-coupled  $\beta_1$ -adrenergic receptor with 10  $\mu$ M norepinephrine (Rochais et al., 2007), 66 ms for the G<sub>s</sub>-coupled adenosine A<sub>2A</sub> receptor with 1 mM adenosine (Hoffmann et al., 2005), and  $\sim 1$  s for the G<sub>s</sub>- and G<sub>q</sub>-coupled parathyroid receptor with 1  $\mu$ M parathyroid hormone (Vilardaga et al., 2003). The only deactivation time constant reported so far is  $\sim 2$  s for a FRET-labeled  $\alpha_{2A}$  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 M<sub>1</sub>R 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 M<sub>1</sub>R fluorescent constructs

To rule out altered ligand binding in our modified M<sub>1</sub> receptors, we measured dissociation constants for  $^3\text{H}$ -NMS binding and inhibition constants for oxo-M. Our results for the inhibition constant of oxo-M (4–9  $\mu$ M) are in the range of reported values: 8.1  $\mu$ M in Chinese hamster ovary cells in the presence of 0.5 mM GTP (Jakubik et al., 1997),  $2.2 \pm 0.2 \mu$ M for muscarinic receptors in rat cerebral cortex, and  $9.0 \pm 4.9 \mu$ M for M<sub>1</sub>-M<sub>4</sub> subtypes in a mixture of tissues (Sharif et al., 1995). If we take 4  $\mu$ M as the apparent dissociation constant for oxo-M and  $6 \text{ s}^{-1}$  as  $k_{off}$ , the predicted  $k_{on}$  ( $=k_{off}/K_d$ ) for the M<sub>1</sub>R would be  $1.5 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ . Dissociation constants

for  $^3\text{H-NMS}$  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 (Cortés 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  $M_1\text{R-CFP}$  and  $G\beta_1\text{-YFP}$  had a time constant of only 200 ms,  $\sim 30$ -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  $M_1\text{R-CFP}$  and  $G\beta_1\text{-YFP}$  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  $M_1\text{R-YFP-CFP}$  and faster than those of  $G\alpha_q\text{-CFP}/G\beta_1\text{-YFP}$ , 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  $G\beta$  constructs throughout the application of agonist. These results are consistent with the observation that  $M_1\text{R}$  activation increases receptor affinity for G proteins (Potter et al., 1988). Recovery of this signal ( $\tau_{\text{off}} = 3.7$  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  $G\beta_1$  or  $G\gamma_2$  increased with a  $t_{1/2}$  of  $\sim 300$  ms and recovered within a few seconds using 10  $\mu\text{M}$  isoproterenol (Galés 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  $\mu\text{M}$  norepinephrine in the presence of only endogenous  $G\alpha_i$  (Hein et al., 2005). In that study, coexpressing  $G\alpha_i$  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  $\tau_{\text{on}} = 50$  ms (1 mM adenosine) and recovered with  $\tau_{\text{off}} = 15$  s (100  $\mu\text{M}$  adenosine), and the  $\beta_1$  adrenergic receptor and  $G\gamma_2$  had  $\tau_{\text{on}} = 58$  ms (1 mM norepinephrine) and  $\tau_{\text{off}} = 8$  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  $G\alpha_q\text{-CFP}$  and  $G\beta_1\text{-YFP}$  would be consis-

tent 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  $G\alpha_q/G\beta_1$  FRET. It may have increased the resting value by recruiting more  $G\beta_1$  (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  $\tau_{\text{on}} = \sim 3$  s and delay plus  $\tau_{\text{off}} = \sim 40$  s. In our protocols, all of our measurements are on cells that coexpressed exogenous  $G\alpha$ ,  $\beta$ , and  $\gamma$  subunits. For comparison, Bünemann et al. (2003) found an increase in FRET between  $G\alpha_i$  and  $G\beta_1$  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  $\mu\text{M}$  norepinephrine. The same laboratory reported a decrease in FRET for  $G\alpha_s/G\gamma_2$  interaction with  $\tau_{\text{on}} = 500$  ms and  $\tau_{\text{off}} = 37$  s for  $A_{2A}$  adenosine receptor activation with 1 mM adenosine, and  $\tau_{\text{on}} = 440$  ms and  $\tau_{\text{off}} = 15$  s for  $\beta_1$  adrenergic receptor activation with 100  $\mu\text{M}$  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  $M_1\text{Rs}$  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  $G\alpha/G\beta$  or  $G\alpha/G\gamma$  FRET takes longer ( $\sim 15$ – $40$  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  $\tau_{\text{off}}$  for receptor–G protein interaction is only  $\sim 4$  s, we return to the idea of slow GTPase. Hydrolysis of  $G\alpha_q\text{-GTP}$  in vitro is supposed to be extremely slow without and accelerated almost 1,000-fold in the presence of  $\text{PLC}\beta_1$  ( $0.013\text{ s}^{-1}$  vs.  $9$ – $12\text{ s}^{-1}$ ) (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  $G\alpha$  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  $G\alpha_q$ -CFP and PLC-YFP (delay plus  $\tau_{on} = 1.7$  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 FRET change (unpublished data), indicating that activation of  $G\alpha_q$  by GTP is a prerequisite. Recovery from the FRET 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  $PLC\beta_1$  observed *in vitro* by Biddlecome et al. (1996). Using a vesicle preparation including  $M_1R$ ,  $G\alpha_q$ , and  $PLC\beta_1$  and measuring  $IP_3$  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/ $G\alpha_q$  interaction was rate limiting for PLC activation. Our data suggest that receptor/ $G\beta_1$  interaction is not rate limiting for PLC activation. Biddlecome et al. (1996) postulated that agonist exposure could induce the formation of receptor- $G\alpha_q$ -PLC complexes, which would exhibit accelerated activation over multiple GTPase cycles. In agreement, we see an increase of  $M_1R/G\beta$  FRET and an increase of  $G\alpha_q/PLC$  FRET. It is possible that overexpressing PLC promotes the formation of such complexes, permitting faster activation of PLC without agonist preincubation. Dowal et al. (2006) demonstrated baseline association between  $G\alpha_q$  and  $PLC\beta_1$  using FRET in PC12 and HEK293 cells, but did not observe an increase in  $G\alpha_q/PLC$  association upon the addition of cholinergic agonists. Two differences may explain this discrepancy between our studies: first, lower receptor expression levels in their cells may have failed to produce an observable response; second, the response may have been rapid enough to escape their lower sampling frequency (every 15 s).

#### PIP<sub>2</sub> hydrolysis is rate limiting

PIP<sub>2</sub> hydrolysis, as indicated by intermolecular FRET with PH domain probes, had similar on-kinetics (6–7 s combined delay and  $\tau_{on}$ ) to M current suppression. Because  $G\alpha_q$  interacts with PLC in <2 s, the rate-limiting step for channel closing must be the gradual depletion of PIP<sub>2</sub> after PLC activation. Thus, we found that over-

expression of PLC speeded M current suppression more than threefold, giving an on-rate nearly identical to that for interaction between  $G\alpha_q$ -CFP and PLC-YFP. With abundant PLC, the sum of the delay and the  $\tau_{on}$  for suppression of M current becomes only  $\sim 2$  s. In that short time PLC is activated, PIP<sub>2</sub> unbinds from channel subunits, PIP<sub>2</sub> is hydrolyzed, and channels close.

Comparison of steady-state concentration–response data from each step suggests that PIP<sub>2</sub> 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 PIP<sub>2</sub>. This suggests that PLC molecules undergo multiple activation cycles while receptors remain active, and that reduction of PIP<sub>2</sub> 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 PIP<sub>2</sub> 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 PIP<sub>2</sub> by the PH probes, which would reduce the availability of free PIP<sub>2</sub> and slow its access to PLC (Várnai 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 PIP<sub>2</sub> pool. If there normally is a metabolic set point for the level of free PIP<sub>2</sub> in the plasma membrane, sequestering of PIP<sub>2</sub> by PH domain probes for 24 h would induce a compensatory rise in the total membrane PIP<sub>2</sub> (free and bound). In agreement, cells with high PH probe expression had markedly slower declines in PH domain FRET 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 PIP<sub>2</sub> 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 PIP<sub>2</sub> (by enhanced hydrolysis or buffering, respectively) produces positive feedback on PIP<sub>2</sub> 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  $G\alpha_q$  (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  $IP_3$  production could increase the calcium signal and potentiate the calcium-dependent PI 4-kinase, accelerating PIP<sub>2</sub> resynthesis (Gamper and Shapiro, 2007).



### Pre-coupled or collision-coupled?

The mechanism of coupling among G protein-coupled signaling molecules is an important determinant of signaling kinetics and efficacy. Our baseline FRETr data and photobleaching results indicate some baseline proximity between M<sub>1</sub>R and Gβ<sub>1</sub>, Gα<sub>q</sub> and Gβ<sub>1</sub>, and Gα<sub>q</sub> and PLCβ<sub>1</sub>. In the case of both M<sub>1</sub>R/Gβ<sub>1</sub> and Gα<sub>q</sub>/PLCβ<sub>1</sub>, FRETr 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 Gα<sub>q</sub> 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 Gα<sub>q</sub> as well as to Gβγ and PLCβ<sub>1</sub> (Dowal et al., 2001), and may be involved in a signaling complex. Given the relatively low apparent affinity (640 nM) interaction between PLCβ<sub>1</sub> and Gα<sub>q</sub>(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 PLCβ<sub>1</sub> 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 Gα<sub>q</sub> and increase the frequency of collision between Gα<sub>q</sub> and PLC.

Although observing FRETr 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 PIP<sub>2</sub> 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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### REFERENCES

- Bal, M., O. Zaika, P. Martin, and M.S. Shapiro. 2008. Calmodulin binding to M-type K<sup>+</sup> channels assayed by TIRF/FRET in living cells. *J. Physiol.* 586:2307–2320.
- Biddlecome, G.H., G. Berstein, and E.M. Ross. 1996. Regulation of phospholipase C-β<sub>1</sub> by G<sub>q</sub> and m1 muscarinic cholinergic receptor. Steady-state balance of receptor-mediated activation and GTPase-activating protein-promoted deactivation. *J. Biol. Chem.* 271:7999–8007.
- Brown, D.A. 1983. Slow cholinergic excitation—a mechanism for increasing neuronal excitability. *Trends Neurosci.* 6:302–307.
- Brown, D.A., and P.R. Adams. 1980. Muscarinic suppression of a novel voltage-sensitive K<sup>+</sup> current in a vertebrate neurone. *Nature.* 283:673–676.
- Bünemann, M., M. Frank, and M.J. Lohse. 2003. Gi protein activation in intact cells involves subunit rearrangement rather than dissociation. *Proc. Natl. Acad. Sci. USA.* 100:16077–16082.
- Chen, Z., G. Rogge, C. Hague, D. Alewood, B. Colless, R.J. Lewis, and K.P. Minneman. 2004. Subtype-selective noncompetitive or competitive inhibition of human α<sub>1</sub>-adrenergic receptors by p-TIA. *J. Biol. Chem.* 279:35326–35333.
- Chisari, M., D.K. Saini, V. Kalyanaraman, and N. Gautam. 2007. Shuttling of G protein subunits between the plasma membrane and intracellular membranes. *J. Biol. Chem.* 282:24092–24098.
- Cortés, R., and J.M. Palacios. 1986. Muscarinic cholinergic receptor subtypes in the rat brain. I. Quantitative autoradiographic studies. *Brain Res.* 362:227–238.
- Digby, G.J., R.M. Lober, P.R. Sethi, and N.A. Lambert. 2006. Some G protein heterotrimers physically dissociate in living cells. *Proc. Natl. Acad. Sci. USA.* 103:17789–17794.
- Dowal, L., J. Elliott, S. Popov, T.M. Wilkie, and S. Scarlata. 2001. Determination of the contact energies between a regulator of G protein signaling and G protein subunits and phospholipase Cβ<sub>1</sub>. *Biochemistry.* 40:414–421.
- Dowal, L., P. Provitera, and S. Scarlata. 2006. Stable association between Gα<sub>q</sub> and phospholipase Cβ<sub>1</sub> in living cells. *J. Biol. Chem.* 281:23999–24014.
- Ehlert, F.J., and L.P. Tran. 1990. Regional distribution of M1, M2 and non-M1, non-M2 subtypes of muscarinic binding sites in rat brain. *J. Pharmacol. Exp. Ther.* 255:1148–1157.
- Evanko, D.S., M.M. Thiagarajan, S. Takida, and P.B. Wedegaertner. 2005. Loss of association between activated Gα<sub>q</sub> and Gβγ disrupts receptor-dependent and receptor-independent signaling. *Cell. Signal.* 17:1218–1228.
- Frank, M., L. Thumer, M.J. Lohse, and M. Bünemann. 2005. G Protein activation without subunit dissociation depends on a Gα<sub>i</sub>-specific region. *J. Biol. Chem.* 280:24584–24590.
- Galés, C., R.V. Rebois, M. Hogue, P. Trieu, A. Breit, T.E. Hébert, and M. Bouvier. 2005. Real-time monitoring of receptor and G-protein interactions in living cells. *Nat. Methods.* 2:177–184.

- Gamper, N., and M.S. Shapiro. 2007. Target-specific PIP<sub>2</sub> signalling: how might it work? *J. Physiol.* 582:967–975.
- Gamper, N., V. Reznikov, Y. Yamada, J. Yang, and M.S. Shapiro. 2004. Phosphatidylinositol [correction] 4,5-bisphosphate signals underlie receptor-specific G<sub>q/11</sub>-mediated modulation of N-type Ca<sup>2+</sup> channels. *J. Neurosci.* 24:10980–10992.
- Hein, P., M. Frank, C. Hoffmann, M.J. Lohse, and M. Bünemann. 2005. Dynamics of receptor/G protein coupling in living cells. *EMBO J.* 24:4106–4114.
- Hein, P., F. Rochais, C. Hoffmann, S. Dorsch, V.O. Nikolaev, S. Engelhardt, C.H. Berlot, M.J. Lohse, and M. Bünemann. 2006. Gs activation is time-limiting in initiating receptor-mediated signaling. *J. Biol. Chem.* 281:33345–33351.
- Hoffmann, C., G. Galetta, M. Bünemann, S.R. Adams, S. Oberdorff-Maass, B. Behr, J.P. Vilardaga, R.Y. Tsien, M.H. Ellisman, and M.J. Lohse. 2005. A FAsH-based FRET approach to determine G protein-coupled receptor activation in living cells. *Nat. Methods.* 2:171–176.
- Hughes, T.E., H. Zhang, D.E. Logothetis, and C.H. Berlot. 2001. Visualization of a functional Gα<sub>q</sub>-green fluorescent protein fusion in living cells. Association with the plasma membrane is disrupted by mutational activation and by elimination of palmitoylation sites, but not by activation mediated by receptors or AlF<sub>4</sub><sup>−</sup>. *J. Biol. Chem.* 276:4227–4235.
- Jakubik, J., L. Bacakova, E.E. el-Fakahany, and S. Tucek. 1995. Subtype selectivity of the positive allosteric action of alcuronium at cloned M<sub>1</sub>-M<sub>5</sub> muscarinic acetylcholine receptors. *J. Pharmacol. Exp. Ther.* 274:1077–1083.
- Jakubik, J., L. Bacakova, E.E. El-Fakahany, and S. Tucek. 1997. Positive cooperativity of acetylcholine and other agonists with allosteric ligands on muscarinic acetylcholine receptors. *Mol. Pharmacol.* 52:172–179.
- Kenakin, T. 1997. Agonist-specific receptor conformations. *Trends Pharmacol. Sci.* 18:416–417.
- Lodowski, D.T., J.A. Pitcher, W.D. Capel, R.J. Lefkowitz, and J.J. Tesmer. 2003. Keeping G proteins at bay: a complex between G protein-coupled receptor kinase 2 and Gβγ. *Science.* 300:1256–1262.
- Lohse, M.J., J.P. Vilardaga, and M. Bünemann. 2003. Direct optical recording of intrinsic efficacy at a G protein-coupled receptor. *Life Sci.* 74:397–404.
- Lohse, M.J., M. Bünemann, C. Hoffmann, J.P. Vilardaga, and V.O. Nikolaev. 2007a. Monitoring receptor signaling by intramolecular FRET. *Curr. Opin. Pharmacol.* 7:547–553.
- Lohse, M.J., C. Hoffmann, V.O. Nikolaev, J.P. Vilardaga, and M. Bünemann. 2007b. Kinetic analysis of G protein-coupled receptor signaling using fluorescence resonance energy transfer in living cells. *Adv. Protein Chem.* 74:167–188.
- Lohse, M.J., V.O. Nikolaev, P. Hein, C. Hoffmann, J.P. Vilardaga, and M. Bünemann. 2008. Optical techniques to analyze real-time activation and signaling of G-protein-coupled receptors. *Trends Pharmacol. Sci.* 29:159–165.
- Mukhopadhyay, S., and E.M. Ross. 1999. Rapid GTP binding and hydrolysis by G<sub>q</sub> promoted by receptor and GTPase-activating proteins. *Proc. Natl. Acad. Sci. USA.* 96:9539–9544.
- Nobles, M., A. Benians, and A. Tinker. 2005. Heterotrimeric G proteins precouple with G protein-coupled receptors in living cells. *Proc. Natl. Acad. Sci. USA.* 102:18706–18711.
- Potter, L.T., C.A. Ferrendelli, and H.E. Hanchett. 1988. Two affinity states of M1 muscarine receptors. *Cell. Mol. Neurobiol.* 8:181–191.
- Rochais, F., J.P. Vilardaga, V.O. Nikolaev, M. Bünemann, M.J. Lohse, and S. Engelhardt. 2007. Real-time optical recording of β<sub>1</sub>-adrenergic receptor activation reveals supersensitivity of the Arg389 variant to carvedilol. *J. Clin. Invest.* 117:229–235.
- Ruiz-Velasco, V., and S.R. Ikeda. 2001. Functional expression and FRET analysis of green fluorescent proteins fused to G-protein subunits in rat sympathetic neurons. *J. Physiol.* 537:679–692.
- Saini, D.K., V. Kalyanaraman, M. Chisari, and N. Gautam. 2007. A family of G protein βγ subunits translocate reversibly from the plasma membrane to endomembranes on receptor activation. *J. Biol. Chem.* 282:24099–24108.
- Scarlata, S., and L. Dowal. 2004. The use of green fluorescent proteins to view association between phospholipase Cβ and G protein subunits in cells. *Methods Mol. Biol.* 237:223–232.
- Sharif, N.A., G.W. Williams, and L.M. DeSantis. 1995. Affinities of muscarinic drugs for [<sup>3</sup>H]N-methylscopolamine (NMS) and [<sup>3</sup>H]oxotremorine (OXO) binding to a mixture of M<sub>1</sub>-M<sub>4</sub> muscarinic receptors: use of NMS/OXO-M ratios to group compounds into potential agonist, partial agonist, and antagonist classes. *Neurochem. Res.* 20:669–674.
- Stauffer, T.P., S. Ahn, and T. Meyer. 1998. Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P<sub>2</sub> concentration monitored in living cells. *Curr. Biol.* 8:343–346.
- Suh, B.C., and B. Hille. 2002. Recovery from muscarinic modulation of M current channels requires phosphatidylinositol 4,5-bisphosphate synthesis. *Neuron.* 35:507–520.
- Suh, B.C., L.F. Horowitz, W. Hirdes, K. Mackie, and B. Hille. 2004. Regulation of KCNQ2/KCNQ3 current by G protein cycling: the kinetics of receptor-mediated signaling by G<sub>q</sub>. *J. Gen. Physiol.* 123:663–683.
- Suh, B.C., T. Inoue, T. Meyer, and B. Hille. 2006. Rapid chemically induced changes of PtdIns(4,5)P<sub>2</sub> gate KCNQ ion channels. *Science.* 314:1454–1457.
- Turcotte, M., W. Tang, and E.M. Ross. 2008. Coordinate regulation of G protein signaling via dynamic interactions of receptor and GAP. *PLOS Comput. Biol.* 4:e1000148.
- van der Wal, J., R. Habets, P. Várnai, T. Balla, and K. Jalink. 2001. Monitoring agonist-induced phospholipase C activation in live cells by fluorescence resonance energy transfer. *J. Biol. Chem.* 276:15337–15344.
- Várnai, P., and T. Balla. 1998. Visualization of phosphoinositides that bind pleckstrin homology domains: calcium- and agonist-induced dynamic changes and relationship to myo-[<sup>3</sup>H]inositol-labeled phosphoinositide pools. *J. Cell Biol.* 143:501–510.
- Vilardaga, J.P., M. Bünemann, C. Krasel, M. Castro, and M.J. Lohse. 2003. Measurement of the millisecond activation switch of G protein-coupled receptors in living cells. *Nat. Biotechnol.* 21:807–812.
- Waelbroeck, M., M. Tastenoy, J. Camus, and J. Christophe. 1990. Binding of selective antagonists to four muscarinic receptors (M<sub>1</sub> to M<sub>4</sub>) in rat forebrain. *Mol. Pharmacol.* 38:267–273.
- Wang, H.S., Z. Pan, W. Shi, B.S. Brown, R.S. Wymore, I.S. Cohen, J.E. Dixon, and D. McKinnon. 1998. KCNQ2 and KCNQ3 potassium channel subunits: molecular correlates of the M-channel. *Science.* 282:1890–1893.
- Yuan, C., M. Sato, S.M. Lanier, and A.V. Smrcka. 2007. Signaling by a non-dissociated complex of G Protein βγ and α subunits stimulated by a receptor-independent activator of G protein signaling, AGS8. *J. Biol. Chem.* 282:19938–19947.
- Zhang, H., L.C. Craciun, T. Mirshahi, T. Rohács, C.M. Lopes, T. Jin, and D.E. Logothetis. 2003. PIP<sub>2</sub> activates KCNQ channels, and its hydrolysis underlies receptor-mediated inhibition of M currents. *Neuron.* 37:963–975.