Supporting Information

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SI Materials and Methods

G Protein-Activated Inwardly Rectifying K^+ Current Measurements. Currents were recorded by using the standard two-electrode voltage-clamp technique (Axoclamp 2B amplifier; Axon Instruments). pCLAMP8 software (Axon Instruments) was used for data acquisition and analysis. G protein-activated inwardly rectifying K^+ (GIRK) currents were measured as in ref. 1. Briefly, oocytes were injected with 200 pg of the receptor (Cys416 or mutants), 200 pg of each of the two subunits of the GIRK channel (GIRK1 and GIRK2), and with 1 ng of the $G\alpha_{i3}$ subunit (the expression of the $G\alpha_{i3}$ subunit reduces basal GIRK currents and ensures robust activation of the channel by the receptor). The oocyte was first clamped randomly at either −80 mV or +40 mV in the ND96 solution (2 mM K^+). Basal K^+ currents were developed upon replacement of the ND96 by a 24 mM K^+ solution (72 mM NaCl, 24 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, pH adjusted to 7.4 with KOH). Then, increasing concentrations of ACh (Sigma-Aldrich) or Oxotremorine (Oxotremorine Sesquifumarate; Sigma-Aldrich) were applied, and K^+ current, induced by these agonists, appeared. This current was terminated upon washout of the agonists. After a 10-min wash, the protocol was repeated at the other holding potential.

[^{3}H]ACh Binding Experiments. Binding experiments were conducted as previously described (1, 2). Briefly, oocytes were injected with 10 ng of the receptor and 200 pg of each GIRK1 and GIRK2 per oocyte. Binding of [^{3}H]ACh (specific activity 80 Ci/mmol, American Radiolabeled Chemicals) was measured at two holding potentials that were induced by modifying the KCl concentration. Each oocyte was incubated either in ND96 [inducing membrane potential of −88 mV (1, 2)] or in high K^+ solution (2 mM NaCl, 96 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, pH adjusted to 7.4 with KOH) [inducing membrane potential of +5 mV (1, 2)] containing a given concentration of [^{3}H]ACh. After 30 s of incubation the oocyte was rapidly washed in ice-cold ND96 or high K^+ solution and counted in a scintillation counter (total binding). To determine the nonspecific binding, binding of the same [^{3}H]ACh concentrations to oocytes expressing only the GIRK channel was measured. Specific binding was calculated by subtracting the average nonspecific binding from the total binding of each oocyte. Experiments in which the difference between the total binding and the nonspecific binding was not significant ($P > 0.05$) were discarded.

Pertussis Toxin Treatment. The A-protomer of pertussis toxin (PTX) was purchased from List Biological Laboratories. Thirty nanoliters in a concentration of 20 ng/μL were injected into M_2R-expressing oocytes 10–20 h before measurements.

***Q − V* Curve.** To construct the $Q - V$ curve, the charge that moved (Q) was calculated as the time integral of the gating currents at each potential, and plotted as a function of membrane potential. The resulting values were normalized to the maximal charge that moved.

M_2R Model. The homology model seen in Figs. 1*A* and 3*A* was constructed by Gregory et al. (3) based on the structure of the β_2 adrenergic receptor (PDB 2RH1). The illustrations in these figures were produced using the Visual Molecular Dynamics program (4).

1. Ben-Chaim Y, et al. (2006) Movement of 'gating charge' is coupled to ligand binding in a G-protein-coupled receptor. *Nature* 444:106–109.
2. Ben-Chaim Y, Tour O, Dascal N, Parnas I, Parnas H (2003) The M2 muscarinic G-protein-coupled receptor is voltage-sensitive. *J Biol Chem* 278:22482–22491.
3. Gregory KJ, Hall NE, Tobin AB, Sexton PM, Christopoulos A (2010) Identification of orthosteric and allosteric site mutations in M2 muscarinic acetylcholine receptors that contribute to ligand-selective signaling bias. *J Biol Chem* 285: 7459–7474.
4. Humphrey W, Dalke A, Schulten K (1996) VMD: Visual molecular dynamics. *J Mol Graph* 14:33–38, 27–28.

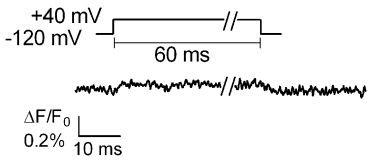

Fig. S1. Fluorescence signal in the mutant in which Cys413 and Cys416 were mutated to serine. This very small signal is representative of the tetramethylrhodamine maleimide (TMRM)-labeled mutant, elicited by depolarizing pulse from a holding potential of −120 mV to +40 mV. The time course of this signal suggests that it may be electrochromic in origin but its small magnitude prevented further study. The pulse protocol is shown above.

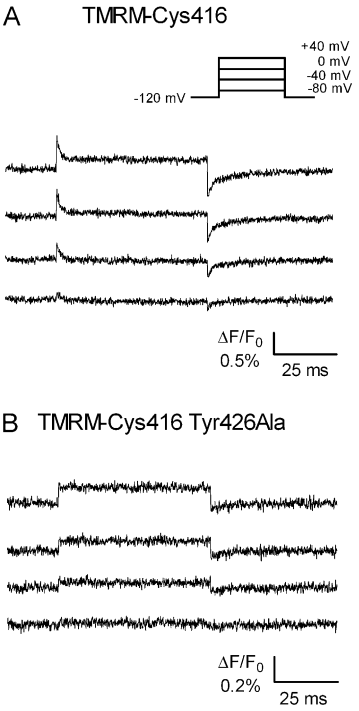

Fig. S2. Raw traces of TMRM-Cys416 and TMRM-Cys416 Tyr426Ala. (*A*) Representative fluorescence signals in the TMRM-Cys416, elicited by depolarizing pulses. The pulse protocol is shown above. (*B*) Same as in *A*, but for the mutant TMRM-Cys416 Tyr426Ala.

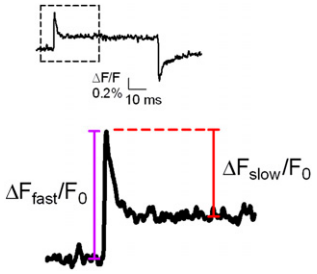

Fig. S3. Identification of $\Delta F_{fast} - V$ and $\Delta F_{slow} - V$. (*Inset*) The complete fluorescence signal. The "on" response is marked by the dashed square. In the main figure, the "on" response in a higher resolution. To construct $(\Delta F_{fast}/F_0) - V$, the amplitude of the fast component, indicated by a purple line, was measured and plotted against the applied voltage. To construct $(\Delta F_{slow}/F_0) - V$, fluorescence was calculated as the difference between the fluorescence level at the end of $\Delta F_{fast}/F_0$ and the fluorescence level after reaching a steady state, as indicated by the red line, and plotted against the applied voltage.

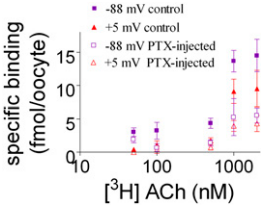

Fig. S4. [^{3}H]ACh binding to M_2R-expressing oocytes, control (filled symbols) or following PTX injection (empty symbols) at resting potential (purple squares) or depolarization (red triangles). Results are given as mean ± SEM. Data taken with permission from ref. 2.