

Text S1 Materials and Methods.

Constructs and cell culture. All subunits ($\gamma 2$, $\gamma 3$, $\gamma 3$ C-terminal translocating mutant, $\gamma 9$ and $\gamma 11$) tagged with yellow fluorescent protein (YFP) or cyan FP (CFP) constructs have been described previously [1,2]. The chimera $\gamma 9$ -3 was made by substitution of the last 15 amino acids of the C terminus from $\gamma 3$ in $\gamma 9$ subunit. PH domain of PLC was obtained by PH-EGFP (from T. Balla [3]) and then the GFP was substituted with mCherry fluorescent protein (from R. Tsien) [4]. YFP-DBD was obtained from A. Newton [5]. The α -q-GFP in pcDNA1 (from C. Berlot, [6]) was used as a template in a Quik Change Multi Site Directed Mutagenesis reaction (Stratagene) and the GFP within the alpha-q was mutated to CFP. The entire α -q-CFP cDNA was then transferred to the HindII and NotI sites of pcDNA3.1.

CHO cells stably expressing the M3 muscarinic receptor (M3-CHO) have been described previously [7]. CHO cells were grown in CHO IIIa medium (Invitrogen) containing dialyzed fetal bovine serum (Atlanta Biologicals), methotrexate, penicillin, streptomycin, and glutamine. A549 cells were grown in Ham's F12 (Mediatech) with dialyzed fetal bovine serum, penicillin and streptomycin. All the transfections were performed using Lipofectamine 2000 (Invitrogen) as described previously [8].

Live Cell Imaging. Live cell imaging experiments were performed as follows. Cells were cultured on glass coverslips and transiently transfected with different cDNAs (details are in figure legend and text) using Lipofectamine 2000. After 16-24 h post-transfection, cells were washed with Hanks' buffered saline solution supplemented with 10 mM HEPES, pH 7.4, and mounted on an imaging chamber with an internal volume of 25 μ l (RC-30 chamber, Warner Instruments). A fluid delivery system including a

programmable valve controller and Teflon valves (Automate Scientific) was used to deliver buffer and agonist through the chamber at a rate of 0.5 - 0.6 ml/min with a regulated flow controller. The cells were visualized with a Zeiss Axioskop fluorescent microscope using a 63x oil immersion objective (1.4 NA) and 100-watt mercury arc lamp with a Hamamatsu CCD Orca-ER camera. The shutter and emission and excitation filter wheels were controlled by a Sutter Lambda 10-2 optical filter changer (Sutter Instrument Co.) run by MetaMorph 6.3.7 (Molecular Devices) software. The filter and beam splitter combinations (Chroma Technology) were as follows; for CFP, D436/10 excitation, D470/30 emission; for YFP, D500/20 excitation, D535/30 emission; for mCherry or DsRed D580/20 excitation, D630/60 emission, a polychroic beam splitter (Chroma 86002BS) and 10% neutral density filters. In FRET experiments, the FRET signal was determined by monitoring gain in CFP emission intensity in plasma membrane by photobleaching of YFP (acceptor photobleaching) [7]. Cells with equal expression levels of CFP and YFP were selected and experiments were performed as previously described [7]. Images for CFP were acquired before and after YFP photobleaching and intensity levels were compared and normalized to the prebleaching intensity. The emission intensity was corrected for CFP bleaching by determining it in cells expressing α q-CFP alone. In agonist treated cells, the cells were stimulated with 100 M carbachol before YFP photobleaching.

Although the citrine version of YFP which is less prone to bleaching was used, strong bleaching was observed in experiments where the images were captured in the streaming mode with 1 sec exposure time. In this mode images of cells were captured continuously with no interval time during acquisition. This bleaching does not affect the interpretation

of results shown. In other experiments, the interval time for acquisition was increased to 10 sec to minimize bleaching.

References

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