Supporting Information

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SI Text

Conventional expression of the M_2–GIRK1/GIRK4 system with both RGS4 and $G\alpha_{oA}$ produced consistent and accurate wild-type dose–response relationships. We next proceeded to evaluate the nonsense suppression protocols by using the wild-type recovery experiment, in which a stop codon was placed at a specific site within the M2 receptor gene, and THG73 tRNA ligated with the wild-type amino acid was injected into the cell to reestablish the wild-type protein. In these nonsense suppression experiments, we injected $G\alpha_{oA}$ and RGS4 mRNA, along with the GIRK1/GIRK4 and mutant M2 receptor mRNA. Analysis of the nonsense suppression data revealed a significant increase in EC_{50} for W7.40Trp (440 nM) compared with the conventional wild-type experiment (240 nM). In addition, there was considerable variability among different batches of *Xenopus* oocytes.

To remedy the failed wild-type recovery experiment and, hopefully, the batch-to-batch data variability, we began to search for trends between individual cell data and other properties of our M2/GIRK1/GIRK4 signaling system. In our normal EC_{50} measurements, the responses to each drug dose are averaged across all cells, and these averaged responses are fit to the Hill equation. For individual cell analyses, we used the measurement cEC_{50} to refer to the EC_{50} obtained when each cell's dose–response relationship data were individually fit to the Hill.

When we analyzed the relationship between $I_{K,Agonist}$ and cEC_{50}, we found that the nonsense suppression and conventional expression cEC_{50} datasets diverged only when comparing cells with low $I_{K,Agonist}$ (Fig. S1). By separating cells from both conventional and W7.40Trp experiments into 2-μA bins, we showed that in the lowest-current bin (0–2 μA), cells from our nonsense suppression experiments had significantly higher cEC_{50} values on average than cells in the conventional expression experiments (conventional, 380 nM; nonsense suppression, 680 nM; t test $P < 0.001$). If cells with $I_{K,Agonist}$ less than 2 μA were removed from both datasets, the difference between the 2 EC_{50} values narrowed (230 nM and 300 nM for the conventional and nonsense suppression experiments, respectively). We concluded that low levels of M2 receptor expression in the nonsense suppression experiments—the source of low $I_{K,Agonist}$ levels—produced abnormally shifted ACh dose–response relationships.

One possible explanation for this connection between expression levels and dose–response relationships is the injection of $G\alpha_{oA}$ mRNA. Nonsense suppression produces lower levels of receptor expression than conventional expression methods, and this would produce a lower flux of free $G\beta\gamma$ subunits in response to ACh application. Injection of $G\alpha_{oA}$ mRNA increases the level of free $G\alpha$ inside the cell, which could bind not only to the endogenous *Xenopus* oocyte $G\beta\gamma$ subunits (the desired outcome), but also to $G\beta\gamma$ subunits liberated by activation of the GPCR. This would prematurely terminate M2–GIRK signaling. Under such conditions, a higher dose of ACh would be needed in low-expressing cells to produce the equivalent degree of signal saturation compared with normally expressing cells. Such a phenomenon would shift the dose–response relationship to higher cEC_{50} values.

To test this hypothesis, we performed a series of wild-type recovery experiments in which we reduced the amount of $G\alpha_{oA}$ mRNA injected and monitored the change in mean cEC_{50}. Cells with 0 ng of injected $G\alpha_{oA}$ mRNA had cEC_{50}s that were not significantly different from the conventional expression experiments (suppressed, 260 ± 30 nM; conventional, 380 ± 40 nM; t test $P = 0.1$) and were significantly lower than cells with 2 ng (680 ± 60 nM; t test $P = 0.002$) or 1 ng (530 ± 120 nM; t test $P = 0.02$) of $G\alpha_{oA}$. When all of the cells from each condition were pooled together, the EC_{50} for W7.40Trp without $G\alpha_{oA}$ was 290 nM, similar to the conventional wild-type EC_{50} of 240 nM. As further confirmation of our hypothesis, we observed that $G\alpha_{oA}$ injection does not change the dose–response relationship of the conventionally expressed M2 receptor: the EC_{50} measured in the conventional expression experiment with $G\alpha_{oA}$ was 250 nm, whereas the EC_{50} recorded without the G protein was 240 nM.

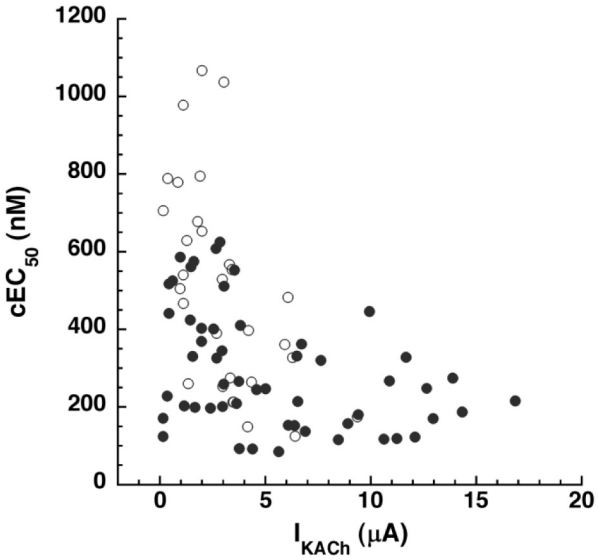

Fig. S1. Suppression M2AChR experiments exhibit higher cEC_{50} values in cells with low $I_{K,Agonist}$. When $I_{K,Agonist}$ is plotted with cEC_{50}, conventional and suppressed wild-type data diverge most for cells with $I_{K,Agonist} < 2$ μA.

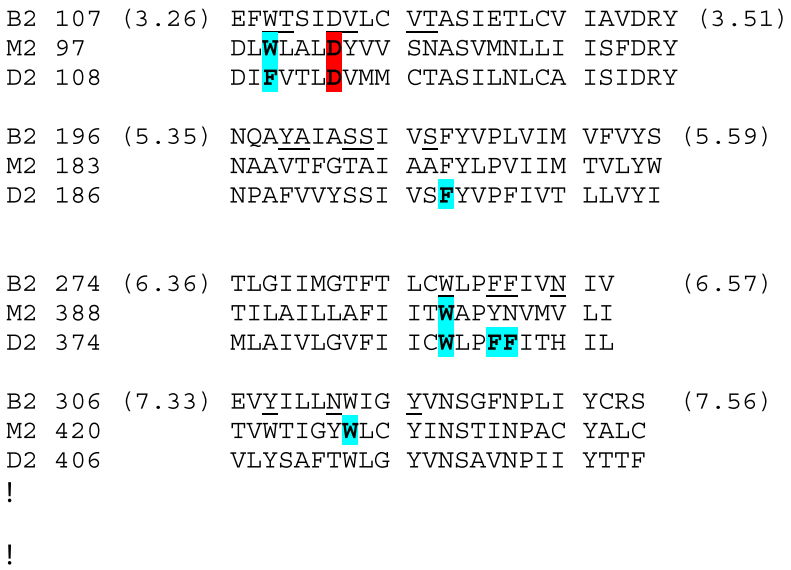

Fig. S2. Alignment of β_2AR, M2AChR, and D2DR binding-site sequences. Residues within 5 Å of the ligand in the β2AR crystal structure are underlined. Aromatic residues examined in this study are highlighted. We have adopted the numbering system, in which the most highly conserved residue of transmembrane helix X is designated X.50. A residue 5 to the N terminus would be X.45; 5 to the C terminus would be X.55. Note that the X.50 residue does not necessarily lie in the middle of the transmembrane helix; it is simply the most conserved residue [see: Ballesteros JA, Shi L, Javitch JA (2001) *Mol Pharmacol* 60:1–19].

Table S1. Data for M2 AChR at W7.40, W6.48, and W3.28 and for the D2 receptor at F3.28, F5.47, W6.48, F6.51, and F6.52*

	EC_{50}	n_H†	n
M2 AChR			
WT			
W3.28			
dCA	1,900 ± 80	0.8 ± 0.02	12
W6.48			
Trp	310 ± 6	0.8 ± 0.01	17
F_2Trp	1,100 ± 70	0.8 ± 0.04	12
F_3Trp	420 ± 30	1.1 ± 0.06	14
W7.40			
Trp	190 ± 20	0.9 ± 0.1	41
F_1Trp	240 ± 9	0.9 ± 0.03	26
F_2Trp	1,000 ± 80	0.8 ± 0.04	20
F_3Trp	170 ± 10	0.9 ± 0.05	12
D2 receptor			
WT	59 ± 3	1.06 ± 0.02	44
F3.28			
Phe (WT)	55 ± 1	1.13 ± 0.04	5
F_1Phe	140 ± 10	0.84 ± 0.03	4
F_2Phe	36 ± 1	1.0 ± 0.1	3
F_3Phe	140 ± 10	0.89 ± 0.14	3
Cha	97 ± 2	1.03 ± 0.04	5
F5.47			
Cha	78 ± 1	1.30 ± 0.14	7
W6.48			
Trp (WT)	42 ± 4	0.96 ± 0.05	15
F_1Trp	120 ± 10	0.98 ± 0.05	14
F_2Trp	290 ± 30	0.95 ± 0.06	11
F_3Trp	840 ± 60	0.85 ± 0.05	13
F_4Trp	1,800 ± 300	0.84 ± 0.06	16
Nap	190 ± 20	1.12 ± 0.06	8
F6.51			
Phe (WT)	65 ± 4	1.03 ± 0.04	6
F_1Phe	76 ± 6	0.98 ± 0.03	12
F_2Phe	4,200 ± 350	0.97 ± 0.04	7
F_3Phe	6,200 ± 400	0.095 ± 0.03	18
Cha	55,000 ± 4,000	0.87 ± 0.06	4
4-CNPhe	1340 ± 160	0.94 ± 0.06	4
4-MePhe	690 ± 40	1.01 ± 0.02	6
3,5-Me_2Phe	75,000 ± 5000	0.89 ± 0.05	6
F6.52			
Phe (WT)	45 ± 3	1.04 ± 0.11	6
F_1Phe	41 ± 2	1.01 ± 0.11	7
F_2Phe	1,700 ± 100	1.14 ± 0.03	8
F_3Phe	5,500 ± 400	1.09 ± 0.03	7
4-CNPhe	240 ± 30	1.06 ± 0.05	5
4-BrPhe	1,500 ± 100	1.02 ± 0.04	4
4-MePhe	91 ± 6	1.00 ± 0.05	5
3,5-Me_2Phe	33,000 ± 3,000	1.11 ± 0.10	7
Conventional			
T7.39V	100 ± 20	0.89 ± 0.12	4
D3.32E	50,000 ± 4000	1.07 ± 0.08	9
D3.32N	140,000 ± 10,000	1.12 ± 0.03	6
D3.32S	730,000 ± 60,000	1.13 ± 0.09	5
D3.32A	2,000,000 ± 300,000	0.83 ± 0.07	2

*EC_{50} (nM) and n_H values are ± SEM.
†Hill coefficient.