Science Signaling
AAAS

www.sciencesignaling.org/cgi/content/full/11/542/eaar4309/DC1

# Supplementary Materials for

## G protein signaling–biased agonism at the κ-opioid receptor is maintained in striatal neurons

Jo-Hao Ho, Edward L. Stahl, Cullen L. Schmid, Sarah M. Scarry, Jeffrey Aubé, Laura M. Bohn*

*Corresponding author. Email: lbohn@scripps.edu

Published 7 August 2018, *Sci. Signal.* **11**, eaar4309 (2018)
DOI: 10.1126/scisignal.aar4309

**This PDF file includes:**

Fig. S1. KOR agonists do not stimulate cAMP accumulation.
Fig. S2. KOR agonist potency for inhibiting forskolin-stimulated cAMP accumulation is not affected by changing the incubation time.
Fig. S3. RGS protein effects on KOR-regulated adenylyl cyclase activity.
Fig. S4. Triazole 1.1 and iso 2.1 display similar signaling profiles in CHO and U2OS cells stably expressing mouse KOR as they do expressing human KOR.
Fig. S5. β-arrestins are not required for KOR-regulated adenylyl cyclase activity.
Fig. S6. β-arrestins are required for KOR internalization.
Table S1. Signaling parameters for the time course inhibition of forskolin-stimulated cAMP accumulation in CHO-hKOR cells.
Table S2. Signaling parameters for the KOR agonists in the functional assays in CHO-mKOR cells.
Table S3. Signaling parameters for the KOR agonists in the inhibition of forskolin-stimulated cAMP accumulation in WT and β-arrestin1/2-KO MEF-mKOR cells.
Table S4. Signaling parameters for the inhibition of cAMP accumulation in CHO cells stably expressing hKOR alone or with hRGS4, hRGS9.2, or hRGS12.3.
Table S5. Primer sequences.

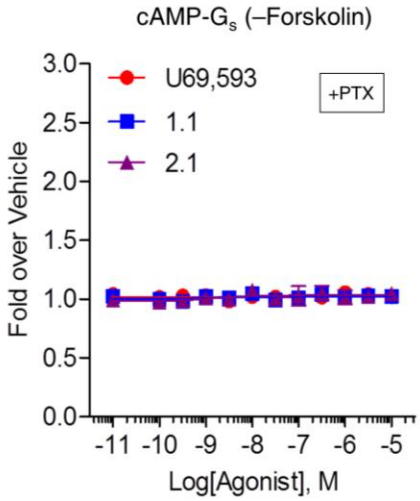


**Fig. S1. KOR agonists do not stimulate cAMP accumulation.** The cAMP accumulation assay was performed using CHO-hKOR cells with pertussis toxin pretreatment and without forskolin stimulation. Data are presented as means ± S.E.M. of N = 3 independent experiments.

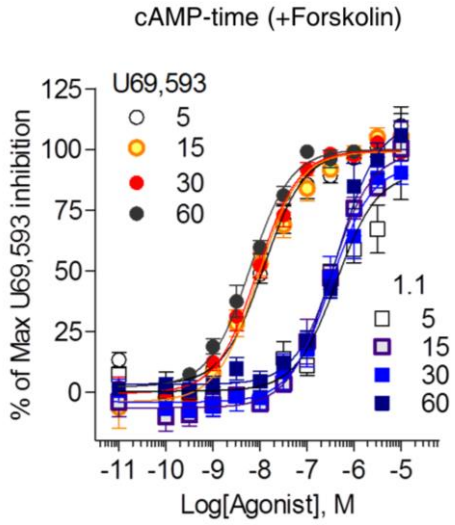


**Fig. S2. KOR agonist potency for inhibiting forskolin-stimulated cAMP accumulation is not affected by changing the incubation time**. CHO-hKOR cells were cotreated with KOR agonist, U69,593 or triazole 1.1, and 20 μM forskolin and 25 μM PDEIV inhibitor for 5, 15, 30 and 60 minutes. The cAMP levels were determined by using the Cisbio cAMP HTRF kit as described in the Methods. Data are shown as mean ± S.E.M. from N ≥ 3 independent experiments.

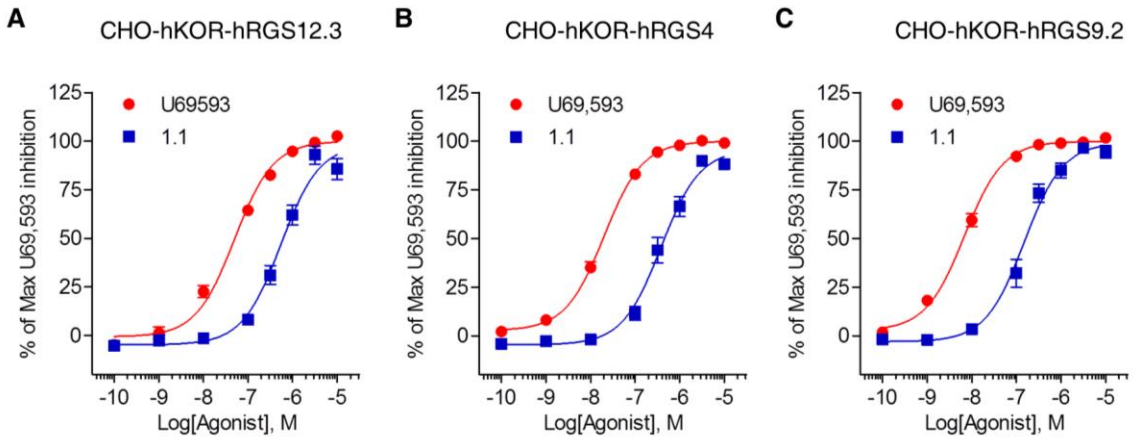


**Fig. S3. RGS protein effects on KOR-regulated adenylyl cyclase activity.** (**A to C**) Overexpression of RGS12.3, RGS4 or RGS9.2 differentially impact the potency for the KOR agonists in inhibiting forskolin-stimulated cAMP accumulation in CHO cells stably expressing hKOR with hRGS12.3 (A), hRGS4 (B), or hRGS9.2 (C). Data are presented as mean ± S.E.M. of $N \geq 4$ independent experiments.

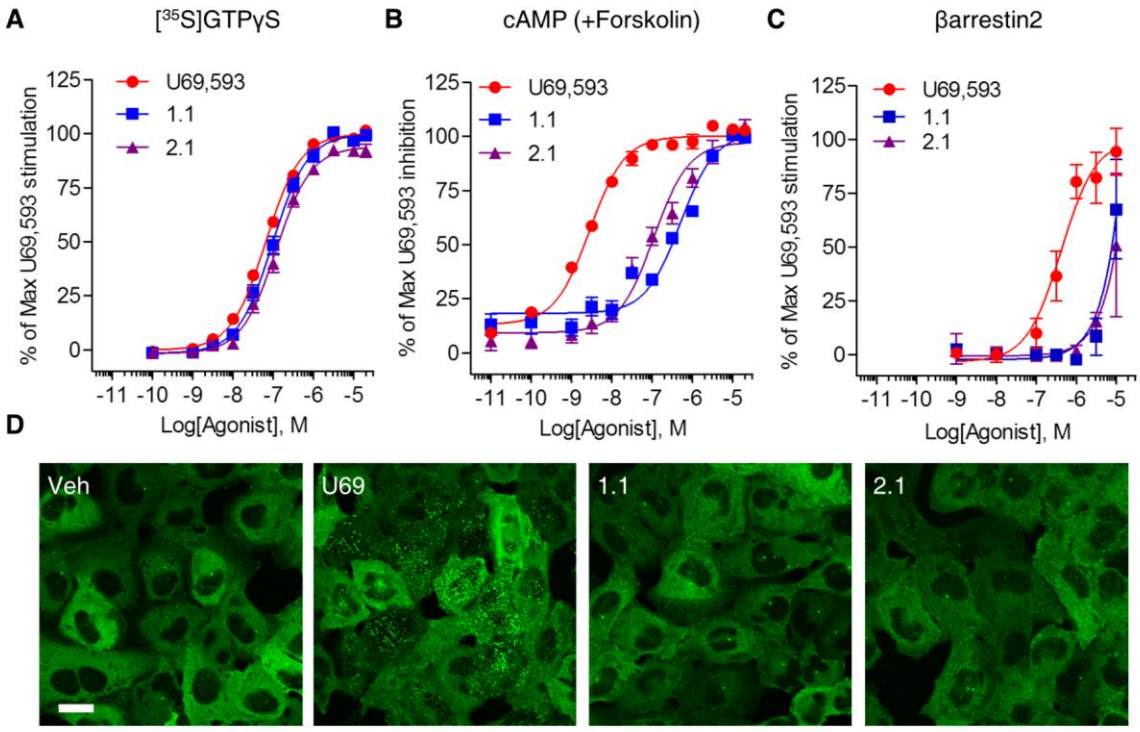


**Fig. S4. Triazole 1.1 and iso 2.1 display similar signaling profiles in CHO and U2OS cells stably expressing mouse KOR as they do expressing human KOR.** (**A**) [$^{35}$S]GTPγS binding assay using membranes prepared from CHO-mKOR cells. (**B**) Forskolin-stimulated cAMP accumulation assay using CHO-mKOR cells. (**C**) β-Arrestin2 recruitment assay using confocal microscopy in U2OS-hKOR-β-arrestin2-EGFP cells. (**D**) Representative confocal images of β-arrestin2 recruitment in U2OS-hKOR-β-arrestin2-EGFP cells. Scale bar: 20 μm. Graphs are presented as mean ± S.E.M. of N ≥ 3 independent experiments.

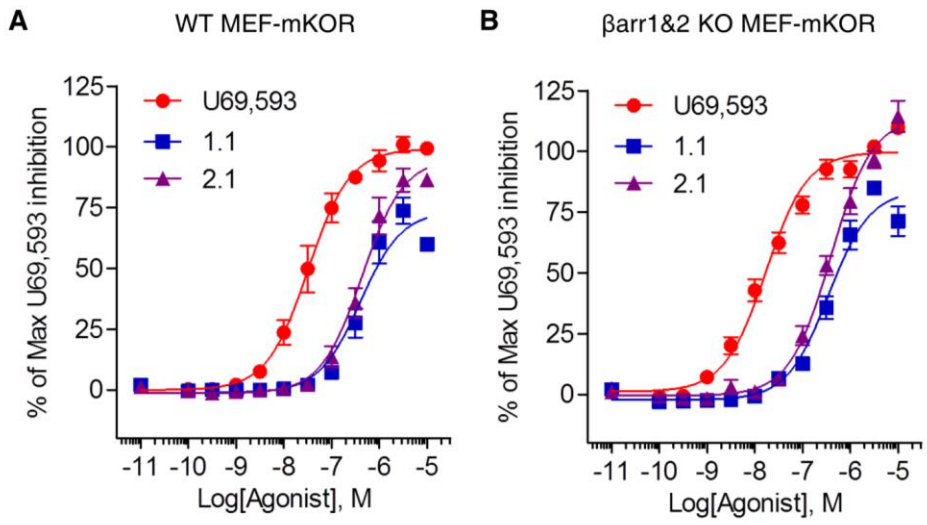


**Fig. S5. β-arrestins are not required for KOR-regulated adenylyl cyclase activity.** (**A and B**) The weak potency of triazole 1.1 and isoquinolinone 2.1 in inhibiting forskolin-stimulated cAMP accumulation is β-arrestin-independent as shown in WT MEF-mKOR cells (A) compared to β-arrestin1/2-KO MEF-mKOR cells (B). Data are presented as mean ± S.E.M. of N = 3 independent experiments.

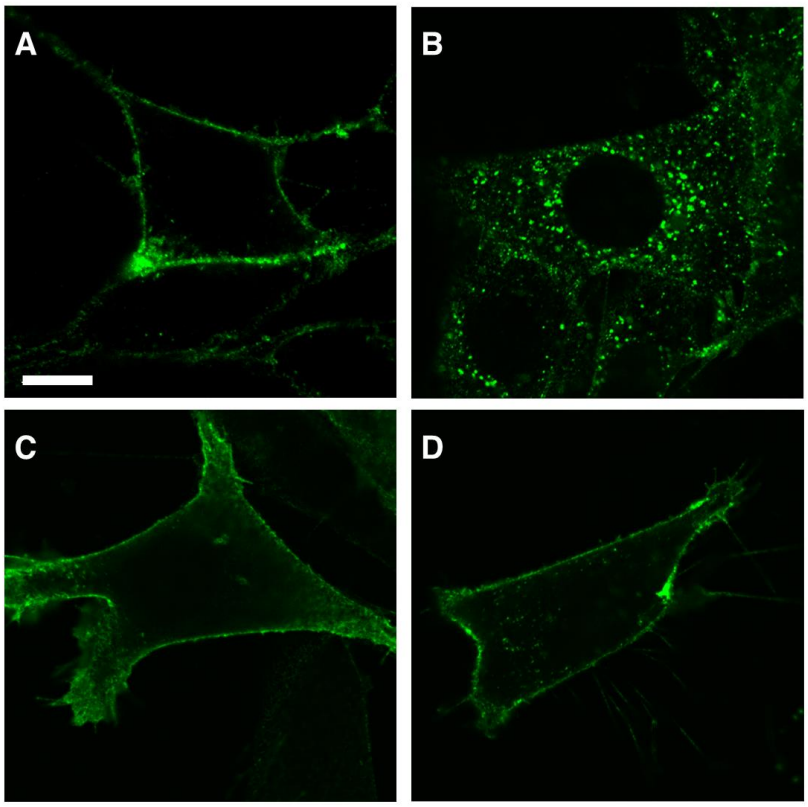


**Fig. S6. β-arrestins are required for KOR internalization.** (**A to D**) Representative confocal images of KOR internalization in WT MEF-hKOR cells **(**A: vehicle, and B: 10 μM U69,593) or in β-arrestin1/2-KO MEF-hKOR cells (C: vehicle, and D: 10 μM U69,593**)**. N = 3 independent experiments, 21 to 24 images for vehicle or U69,593 treatment in WT MEF hKOR or β-arrestin1/2-KO hKOR cells. Scale bar: 10 μm.

| Time point | | U69,593 | 1.1 |
|---|---|---|---|
| 5 min | $EC_{50}$ (nM) | 13 ± 3 | 481 ± 166 |
| | $E_{MAX}$ (%) | 100 | 93 ± 19 |
| 15 min | $EC_{50}$ (nM) | 11 ± 3 | 333 ± 97 |
| | $E_{MAX}$ (%) | 100 | 100 ± 6 |
| 30 min | $EC_{50}$ (nM) | 10 ± 2 | 448 ± 194 |
| | $E_{MAX}$ (%) | 100 | 96 ± 3 |
| 60 min | $EC_{50}$ (nM) | 6 ± 0.9 | 483 ± 62 |
| | $E_{MAX}$ (%) | 100 | 112 ± 6 |

**Table S1. Signaling parameters for the time course forskolin-stimulated cAMP accumulation in CHO-hKOR cells.** Data are presented as mean ± S.E.M. from N ≥ 3 independent experiments performed in duplicate to quadruplicate. Potency for both KOR agonists at 5, 15, and 60 minutes are not significantly different for their potency at 30 minutes. ($P > 0.05$, one-way ANOVA.)

| Compound | [$^{35}$S]GTPγS binding | | cAMP | | βarr2 (Image) | |
|---|---|---|---|---|---|---|
| | $EC_{50}$ (nM) | $E_{MAX}$ (%) | $EC_{50}$ (nM) | $E_{MAX}$ (%) | $EC_{50}$ (nM) | $E_{MAX}$ (%) |
| U69,593 | 66 ± 5 | 100 | 0.8 ± 0.04 | 100 | 134 ± 23 | 100 |
| 1.1 | 98 ± 16 | 100 ± 2 | 157 ± 11 | 101 ± 1 | > 10,000 | (62 ± 7) |
| 2.1 | 123 ± 27 | 94 ± 2 | 41 ± 12 | 98 ± 3 | > 10,000 | (70 ± 14) |

**Table S2. Signaling parameters for the KOR agonists in the functional assays in CHO-mKOR cells.** Data are presented as mean ± S.E.M. from N ≥ 3 independent experiments performed in duplicate to quadruplicate. cAMP: Forskolin-stimulated cAMP accumulation assay, βarr2 (Image): β-arrestin2 recruitment assay using confocal microscopy. Where $EC_{50}$ values did not converge, the % maximum stimulation at 10 μM are shown in parentheses.

| Cell type | | U69,593 | 1.1 | 2.1 |
|---|---|---|---|---|
| WT MEF-mKOR | $EC_{50}$ (nM) | 26 ± 5 | 333 ± 28 | 386 ± 64 |
| | $E_{MAX}$ (%) | 100 | 76 ± 3 | 98 ± 2 |
| βarr1&2 KO MEF-mKOR | $EC_{50}$ (nM) | 15 ± 2 | 279 ± 26 | 321 ± 35 |
| | $E_{MAX}$ (%) | 100 | 82 ± 1 | 111 ± 1 |

**Table S3. Signaling parameters for the KOR agonists in the forskolin-stimulated cAMP accumulation in WT and β-arrestin1/2-KO MEF-mKOR cells.** Data are presented as mean ± S.E.M. of N = 3 independent experiments performed in duplicate to quadruplicate.

| Cellular system | | U69,593 | 1.1 |
|---|---|---|---|
| hKOR[a] | $EC_{50}$ (nM) | 12 ± 2 | 309 ± 65 |
| | $E_{MAX}$ (%) | 100 | 94 ± 5 |
| hKOR, hRGS12.3 | $EC_{50}$ (nM) | 51 ± 7**** | 603 ±124 |
| | $E_{MAX}$ (%) | 100 | 100 ± 5 |
| hKOR, hRGS4 | $EC_{50}$ (nM) | 21 ± 2 | 419 ± 105 |
| | $E_{MAX}$ (%) | 100 | 96 ± 2 |
| hKOR, hRGS9.2 | $EC_{50}$ (nM) | 7 ± 1 | 160 ± 34 |
| | $E_{MAX}$ (%) | 100 | 99 ± 2 |

**Table S4. Signaling parameters for the forskolin-stimulated cAMP accumulation in CHO cells stably expressing hKOR alone or with hRGS4, hRGS9.2, or hRGS12.3.** Data are presented as mean ± S.E.M. of N ≥ 4 independent experiments performed in duplicate to quadruplicate. (****p < 0.0001 for U69,593 potency in CHO-hKOR cells versus U69,593 potency in CHO-hKOR-hRGS12.3 cells, one-way ANOVA, Bonferroni's post-hoc test). [a]: Forskolin-stimulated cAMP accumulation assay in CHO-hKOR cells was shown in Table1 for comparison with the RGS protein effect.

| Primers for cloning genes to MSCV vector | |
|---|---|
| 3xHA-hKOR_F | CGATACCTCGAGCACCATGTACCCATACGATG |
| 3xHA-hKOR_R | AACTCATACTGGTTTATTCATCCCATCGATGTCCC |
| 3HA-hKOR_noTGA_F | AGATCTCTCGAGGTTGCCAGATATACGCGTTGACA |
| 3HA-hKOR_noTGA_R | CCGGTAGAATTCGTTAACTACTGGTTTATTCATCCCATCGA |
| P2A_F | AATAAACCAGTAGTTgccactaacttctccctgttgaaacaagcaggggatgtcgaagagaatcccgggccaGTTAACGAATTCTACCGG |
| P2A_R | CCGGTAGAATTCGTTAACtggcccgggattctcttcgacatcccctgcttgtttcaacagggagaagttagtggcAACTACTGGTTTATT |
| KORP2AMyc_F | GTTAACGAATTCTACCGGGT |
| KORP2AMyc_R | CAGGTCCTCCTCTGAGATCAGCTTCTGCTCCATtggcccgggattctctt |
| hGIRK1_F | aatcccgggccaGTTatgtctgcactccgaaggaa |
| hGIRK1 _R | CCGGTAGAATTCGTTAACtgtgaagcgatcagagttcat |
| hGIRK1P2A_F | gatcgcttcacaGTTgccactaacttctccctgttgaaacaagcaggggatgtcgaagagaatcccgggccaGTTAACGAATTCTACCGG |
| hGIRK1P2A_R | CCGGTAGAATTCGTTAACtggcccgggattctcttcgacatcccctgcttgtttcaacagggagaagttagtggcAACtgtgaagcgatc |
| hGIRK2_F | aatcccgggccaGTTatggccaagctgacagaatc |
| hGIRK2_R | ccggtagaattcGTTcagctagggcactaaactttgg |
| RGS4_F | TCAGAGGAGGACCTGGATTGCAAAGGGCTTGCAGG |
| RGS4_R | GTAGAATTCGTTAACTTAGGCACACTGAGGGACCA |
| RGS9.2_F | TCAGAGGAGGACCTGACAATCCGACACCAAGGC |
| RGS9.2_R | GTAGAATTCGTTAACTTACAGGCTCTCCCAGGG |
| RGS12.3_F | TCAGAGGAGGACCTGAATTTGGGGAAAGAGTTGTCAAACG |
| RGS12.3_R | GTAGAATTCGTTAACTCAGACGAAGGTGGCGT |
| HA-mKOR_F | AGATCTCTCGAGGTTCGTTACATAACTTACGGTAAATGGC |
| HA-mKOR_R | CCGGTAGAATTCGTTccacgaCTAGTCATACTGGCT |
| Primers for cloning genes to pCMV HA vector | |
| mKOR_F | attacgctcttatggctGAGTCCCCCATTCAGATCT |
| mKOR_R | ttcgggcctccatggccacgaCTAGTCATACTGGCT |

**Table S5.** Primer sequences. F: forward, R: reverse.