RXFP1 Recruits beta-arrestin 2 in a Relaxin-Dose-Dependent Fashion

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ABSTRACT

Human relaxin-2 (hRLX2) signaling is antifibrotic and anti-inflammatory, with several pathologies showing benefit from hRLX2 treatment in preclinical models. hRLX2 has passed safety requirements in multiple clinical trials. Relaxin signaling at Relaxin Family Peptide Receptor 1 (RXFP1) can also promote angiogenesis *via* cyclic adenosine monophosphate (cAMP), signaling that would be counter indicated in patients with increased risk or history of cancer. This suggests potential therapeutic benefit from the development of biased agonists of RXFP1 that signal more through β-arrestin 2 (ARRB2) than through G proteins (Fig. 1). However, the current view in the literature is that RXFP1, unlike the majority of other G-protein-coupled receptors (GPCRs), does not inducibly interact with ARRB2. We present data that both suggest that ARRB2 and RXFP1 interact and offer a methodology for screening candidate agonists of RXFP1.

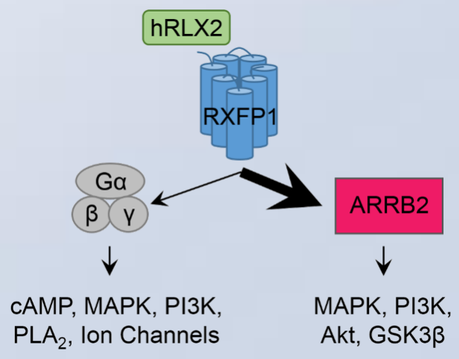

Figure 1. hRLX2-RXFP1 Signaling Overview. Signaling from most GPCRs can pass through G proteins and through complexes scaffolded by ARRB2.

METHODS

To determine protein subcellular localization, plasmids encoding fusion proteins were designed and cloned. ARRB2-mCerulean3-N1 and RXFP1-mCherry2-N1 were transfected into HEK-293T cells either singly or in combination. HEK-293T cells were seeded onto collagen coated glass coverslips at a density of 26,000 cells per square centimeter and then transfected with a total of 7.4*10^-13 g DNA per cell using Lipofectamine 3000 (Thermo Fisher Scientific) according to manufacturer instructions. Cells were fixed in 4% paraformaldehyde and mounted in SlowFade Diamond Antifade Mountant (Thermo Fisher Scientific).

To evaluate activation dependent ARRB2 recruitment to RXFP1 and RXFP2, we performed the TANGO assay, which generates signal based on reporter gene expression downstream of ARRB2 recruitment to a target GPCR transiently transfected into the HTLA cell line. TANGO plasmids for human RXFP1 and RXFP2 were obtained from Addgene (plasmids 66492 and 66493). HTLA cells were a generous gift from Bryan Roth and Wesley K Kroeze. HTLA cells were maintained in in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, 2 µg/ml puromycin and 100 µg/ml hygromycin B in a humidified atmosphere at 37 °C in 5% CO_2. Cells were seeded into 96-well plates at 114,000 cells per well. Cells were transfected with TANGO-RXFP1 or TANGO-RXFP2 at 42 ng DNA per well using Lipofectamine 3000 (Thermo Fisher Scientific) according to manufacturer instructions, with five replicate wells per condition. Luminescence was measured on a SpectraFluor plate reader (Tecan) using the Bright-Glo Luciferase Assay System (Promega) according to manufacturer instructions.

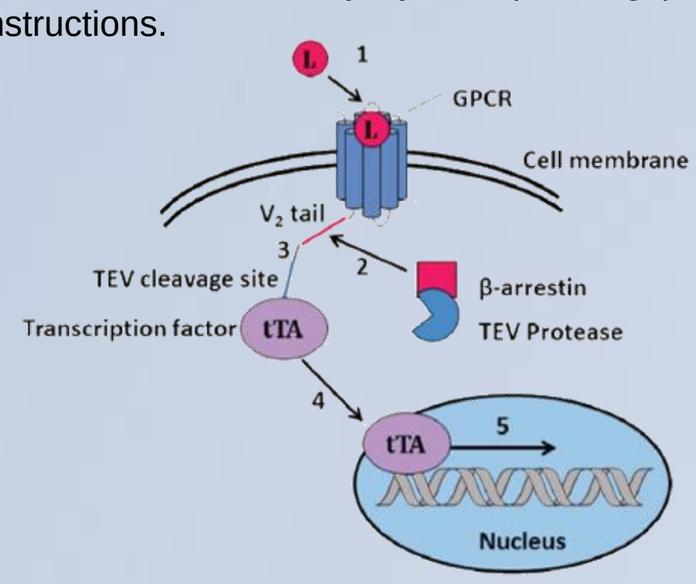

Figure 2. TANGO Assay. HTLA cells are a 293 derivative cell line with two modifications (1) an integrated expression cassette for luciferase that is controlled by the tetracycline transactivator (tTA) and (2) stable expression of a fusion protein of β-arrestin 2 and TEV protease. HTLA cells are transiently transfected with a target GPCR modified to include a C-terminal TEV cleavage site connecting to the tTA such that upon GPCR activation and recruitment of β-arrestin 2, the tTA moiety is cleaved and translocates to the nucleus to drive expression of luciferase.

RESULTS

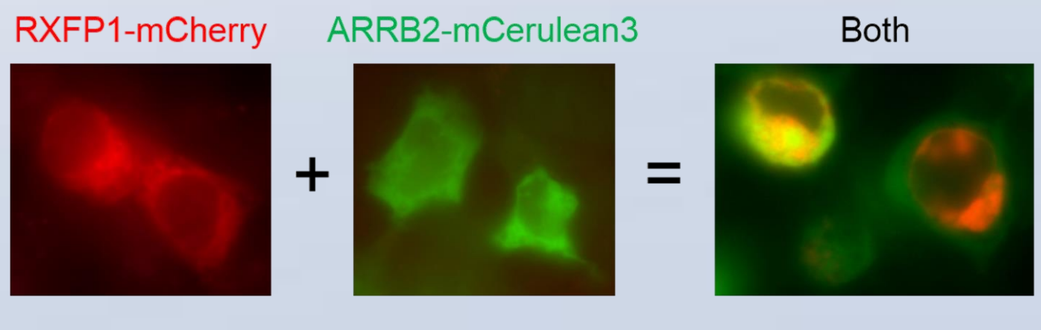

Figure 3. Assay. ARRB2 co-transfection perturbs subcellular localization of RXFP1 in HEK-293T cells. HEK-293T cells were transfected with either RXFP1-mCherry, ARRB2-mCerulean3, or both plasmids together (same total DNA/cell).

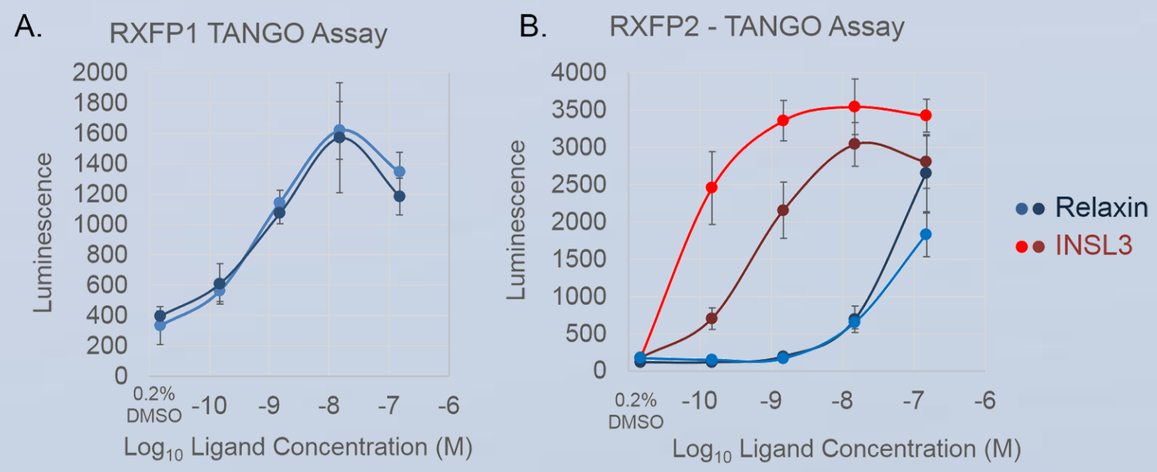

Figure 4. TANGO assay shows that hRLX2/INSL3 induce recruitment of ARRB2 to RXFP1 and RXFP2. HTLA cells were transfected with TANGO-RXFP1 (A) or TANGO-RXFP2 (B). 24 hours after transfection, cells were treated with the indicated ligands and incubated for 24 hours before reading luminescence using BrightGlo reagent and a plate reader. Two independent experiments plotted.

CONCLUSIONS

In cells derived from HEK 293, ARRB2 regulates RXFP1 protein subcellular localization. Transfection of both RXFP1 and ARRB2 led to the formation of large perinuclear deposits of RXFP1, yet when the same amount of RXFP1 DNA was transfected into cells without exogenous ARRB2, RXFP1 did not form these deposits and was instead distributed in a fashion consistent with localization on the cell membrane. This also provides a mechanistic explanation for the lack of observed ARRB2 relocalization after hRLX2 treatment seen in [1]. Working in HEK-293 cells, their work fluorescently labeled only ARRB2 and not RXFP1, so the loss of membrane expression of RXFP1 would not have been detected. In experiments adding hRLX2 to cells lacking membrane expression of RXFP1, no response would be predicted.

The TANGO assay provides evidence for the hRLX2 driven association of RXFP1 and ARRB2. The TANGO assay is a three part system. HTLA cells are stably modified to express ARRB2 fused to the Tobacco Etch Virus (TEV) protease and for tTA-driven expression of luciferase. The TANGO plasmids encode a GPCR of interest C-terminally fused to the tTA transcription factor, so that when ARRB2 binds the TANGO receptor, cleavage of the transcription factor ensues, promoting expression of luciferase. The assay is specific to the target receptor and independent of the G protein the receptor signals through.

RXFP1 TANGO assay showed a dose dependent response to hRLX2 with the bell-shaped curve reported previously [2]. The RXFP1 TANGO assay also showed higher baseline signal than the RXFP2 assay, which is consistent with the constitutive interaction between ARRB2 and RXFP1 deemed the “signalosome” [3]. RXFP2 TANGO assay results show receptor activation and ARRB2 recruitment in response to INSL3 and to a lesser extent, hRLX2, as well as a bell-shaped response curve for INSL3. All of these data are consistent with previous reports in the literature, serving to validate the TANGO assay and these results.

These data show that in at least two contexts, ARRB2 interacts with RXFP1/2. It is to be determined whether RXFP1 signals through ARRB2 to effect its biological responses. The implementation of a selective and sensitive assay for activation of RXFP1 and RXFP2 opens the door to the development of biased small molecule agonists of these receptors.

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Ethics approval: Univ. of Pgh. Institutional Animal Care and Use Committee.

REFERENCES

[1] Callander, G. E.; Thomas, W. G.; Bathgate, R. A. D. Prolonged RXFP1 and RXFP2 Signaling Can Be Explained by Poor Internalization and a Lack of β-Arrestin Recruitment. *Am. J. Physiol. - Cell Physiol.* **2009**, *296* (5), C1058–C1066.

[2] Sarwar M, Samuel C S, Bathgate R A, Stewart D R, and Summers R J. “Serelaxin-mediated Signal Transduction in Human Vascular Cells: Bell-shaped Concentration–Response Curves Reflect Differential Coupling to G Proteins.” *British Journal of Pharmacology* 172, no. 4 (October 8, 2014): 1005–19.

[3] Halls, M. L.; Cooper, D. M. F. Sub-Picomolar Relaxin Signalling by a Pre-Assembled RXFP1, AKAP79, AC2, β-Arrestin 2, PDE4D3 Complex. *EMBO J.* **2010**, *29* (16), 2772–2787.