

**Conformational biosensors reveal allosteric interactions between heterodimeric AT1
angiotensin and prostaglandin F2 α receptors**

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Abstract

G protein-coupled receptors (GPCRs) are conformationally dynamic proteins transmitting ligand-encoded signals in multiple ways. This transmission is highly complex and achieved through induction of distinct GPCR conformations, which preferentially drive specific receptor-mediated signaling events. This

conformational capacity can be further enlarged via allosteric effects between dimers, warranting further study of these effects. Using GPCR conformation-sensitive biosensors, we investigated allosterically induced conformational changes in the recently reported F prostanoïd (FP)/angiotensin II type 1 receptor (AT1R) heterodimer. Ligand occupancy of the AT1R induced distinct

conformational changes in FP compared with those driven by PGF2 α binding to FP in bioluminescence resonance energy transfer (BRET)-based FP biosensors engineered with *Renilla* luciferase (RLuc) as an energy donor in the C-tail and fluorescein arsenical hairpin binder (FIAsh)-labeled acceptors at different positions in the intracellular loops. We also found that this allosteric communication is mediated through G α_q and may also involve proximal (phospholipase C) but not distal (protein kinase C) signaling partners. Interestingly, β -arrestin-biased AT1R agonists could also transmit a G α_q -dependent signal to FP without activation of downstream G α_q signaling. This transmission of information was specific to the AT1R/FP complex, as activation of G α_q by the oxytocin receptor (OTR) did not recapitulate the same phenomenon. Finally, information flow was asymmetric in the sense that FP activation had negligible effects on AT1R-based conformational biosensors. The identification of partner-induced GPCR conformations may help identify novel allosteric effects when investigating multiprotein receptor signaling complexes.

Introduction

G protein-coupled receptors (GPCRs) are conformationally dynamic proteins which transmit information following interaction with different ligands that can promote multiple, yet specific downstream outputs depending on cellular context. It is widely accepted that this complexity is achieved through induction of distinct conformations in the receptor, whereby distinct conformational states preferentially drive specific receptor-mediated signalling events. The available conformational capacity, and therefore possible receptor functions, can be further expanded when considering allosteric effects. Receptor signalling partners, such as

heterodimer partners, may provide novel conformational space generating new signalling modalities or protein life-cycle behaviours (1,2).

With the recent explosion in the number of G protein-coupled receptor (GPCR) structures, our understanding of the mechanics underlying GPCR function has evolved considerably. Structures of GPCRs bound to various ligands, nanobodies and co-crystals with G proteins demonstrate the conformation complexity of individual receptors (3-7). Although X-ray crystallography provides high-resolution snapshots of the receptor, it is limited with respect to reporting on dynamic events and to provide information on highly mobile protein domains or larger protein complexes. This is especially true when considering allosterically-mediated conformational change in the context of receptor heterodimers. Although there have been some reports of receptor oligomerization observed in crystal structures, this has not been a consistent observation and it is difficult to determine whether such arrangements represent artefacts of crystal packing, or reflect physiologically relevant interactions that occur in live cells (8-12). Some might argue that the paucity of GPCR oligomers in crystal structures refutes their existence, but many other biochemical and biophysical techniques reveal structural or functional profiles consistent with receptor oligomerization (13-18). At any rate, GPCR oligomerization must be interrogated from new perspectives that overcome some of these limitations.

Resonance energy transfer (RET) has been a key technology in characterizing GPCR oligomerization. High spatial and temporal resolution and applicability in live cell systems allows for highly robust assay development. RET experiments have been used extensively to support receptor

dimerization (19-24) but stringent controls are required to interpret the data (25-30). Newer fluorescence-based RET (FRET) assays have been developed that can capture intra-molecular rearrangements in GPCRs in response to agonist. These sensors make use of a small fluorescent molecule, fluorescein biarsenical hairpin binder (FAsH), as the acceptor and report on ligand binding-associated conformational rearrangements in multiple GPCRs (31-35). Such sensors have also been used to examine conformational dynamics of GPCRs in oligomeric states - specifically a class C homodimer of mGluR1 and a class A heterodimer of α_{2A} -adrenergic and μ -opioid receptors (36,37). Though valuable insight has been gained through the study of class C GPCRs, they are well accepted as obligate dimers. However, the latter article sheds some light on conformational crosstalk in a putative class A receptor heterodimer. These authors demonstrated that morphine, targeting the μ -opioid receptor, affected the conformation of the α_{2A} -adrenergic receptor in the presence of its ligand norepinephrine. This effect was shown to be G protein-independent and was still detected in isolated membranes, suggesting a simple mechanism of dimerization mediated through direct GPCR/GPCR contact.

We previously reported a functional interaction between FP and AT1R in vascular smooth muscle cells (38). Further, a physical interaction between the receptors was reported in HEK 293 cells heterologously expressing both receptors as well as with endogenous receptors in vascular smooth muscle cells. It was also shown that signalling pathways modulated by the putative heterodimer could be either symmetrically or asymmetrically regulated (i.e. respond or not to stimulation of either partner in a similar or dissimilar way) depending on the signalling output being measured. This differential regulation of

outputs lead to a functionally relevant bias attributed to AT1R/FP heterodimerization and may constitute a new druggable molecular target. To further understand the interplay between these two receptors, we generated a panel of FAsH/BRET-based conformation-sensitive biosensors (34,35,39-41) to explore the effect each of these receptors had on the conformational landscape of its dimer partner. We observed an asymmetrical transmission of conformational information from AT1R to FP whereby angiotensin II (Ang II) stimulation lead to a rearrangement in FP when tagged with biosensors engineered into the third intracellular loop with respect to its C-terminal RLuc moiety. This Ang II-induced conformational change was distinct from that driven by PGF2 α binding to FP. The effect of Ang II on the conformation of FP was dependent on the presence of active G α_q or G α_{11} and appeared to be mostly independent of downstream signalling and conformational information flow between the two receptors seemed to be unidirectional or asymmetric with respect to ICL3 and C-terminus. Finally, this transmission from AT1R to FP appeared to be specific as another G α_q -coupled receptor was unable to induce similar conformational rearrangements in FP.

Results

AT1R ligand binding induces a conformational change in FP

To investigate the interplay between the protomers in the FP/AT1R heterodimer, we began by co-expressing each of our previously published FP-ICL3-RLucII conformational biosensors (FAsH “walked” through the third intracellular loop (ICL3) at 5 different positions (39)) with wildtype AT1R (AT1R-WT) in HEK 293 cells. In this configuration, the assay strictly reports on

conformational rearrangements between different vantage points in ICL3 and the C-terminus of FP induced in response to ligand stimulation, i.e. only changes in FP conformation are reported. We noted a similar pattern across the different conformational biosensors in response to PGF2 α as previously observed when expressed alone (Fig. 1A). When comparing the position of the FAsH tags, the ICL3 P4 biosensor was again the FAsH tag position that showed the largest Δ BRET in response to PGF2 α in the panel (39). This response was dose-dependent (Supplemental Fig. 1A). Interestingly, when the cells were stimulated with Ang II, we not only observed a change in the BRET across all the FP conformational biosensors in the panel but opposite in direction - suggesting that the activation of the heterodimer partner caused a distinct conformational change in FP as compared to its cognate agonist (Fig. 1B). As with direct stimulation of the conformational biosensor with PGF2 α , the FP ICL3 P4 sensor also reported the largest Δ BRET in response to Ang II and the response was dose-dependent (Supplemental Fig. 1B). We therefore selected this biosensor as a focus for further study.

In order to assess the specificity of these effects, we next pre-treated cells with antagonists for either FP or AT1R (Fig. 1C). HEK 293 cells transfected with the FP-ICL3 P4-RLucII conformation-sensor and AT1R-WT were pre-treated with 10 μ M AS604872, a FP antagonist, or 10 μ M losartan, an AT1R antagonist, followed by stimulation with 1 μ M PGF2 α or 1 μ M Ang II. AS604872 was able to block the response to PGF2 α but interestingly had no effect on the response driven by Ang II, suggesting the Ang II-induced conformational rearrangements were uncoupled from the orthosteric binding pocket of FP. Pre-treatment with losartan had no effect on the PGF2 α -induced conformational

rearrangement in FP but was able to block the effect of Ang II. To ensure the dependence of AT1R to drive conformational change in FP across the heterodimer, HEK 293 cells were transfected with only the FP-ICL3 P4-RLucII conformation-sensor and in this case, were insensitive to either Ang II or losartan (Fig. 1D).

Though we represent the data as the ligand-induced change in BRET by averaging the readings pre-ligand injection subtracted from the average reading post ligand addition for simplicity of analysis, all our data sets were also temporally resolved. We noted a rapid response of the FP-ICL3 P4-RLucII sensor in response to 1 μ M PGF2 α when expressed alone (Fig 1E). This response reached a plateau and was sustained for the length of the recording, suggesting that such conformational changes are stable as long as agonist is present. When co-expressed with AT1R-WT, the response to 1 μ M PGF2 α was similar to FP alone, while the response to 1 μ M Ang II had a slower time constant before reaching a plateau (Fig. 1F). Additionally, co-expression of AT1R lead to an increase in the ligand-naïve BRET, suggesting expression of AT1R, independent of ligand binding, modulates the conformation of FP (compare Fig 1E vs. 1F). However, how this conformational information was transmitted was not clear from these experiments

AT1R-induced conformational change in FP is dependent on G α_q expression and activity

We next explored how the information was transmitted from AT1R to FP. Our initial focus was one step removed from the receptor, in the G proteins presumably shared by the heterodimeric receptor. Both FP and AT1R have been demonstrated to couple to G α_q , G α_{12} and G α_i (42-46). To investigate involvement of

different $G\alpha$ subunits, we used small molecule inhibitors, microbial toxins and cell lines with CRISPR-mediated knockout of G proteins and then re-assessed the ability of PGF2 α or Ang II to induce a conformational change in FP.

First, using the $G\alpha_q$ inhibitor FR900359 (47) on HEK 293 cells co-expressing the FP-ICL3 P4-RLucII conformational biosensor and AT1R-WT, we noted a slight effect on the PGF2 α -induced response where the magnitude of the response was slightly larger when $G\alpha_q$ activity was inhibited (Fig. 2A). In contrast, the Ang II response was completely abrogated upon $G\alpha_q$ inhibition. We next used a HEK 293 cell line made devoid of functional $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{12}$, and $G\alpha_{13}$ using CRISPR/Cas9 ($\Delta G\alpha_{q/11/12/13}$ line, Fig. 2B) (35). This cell line was especially valuable, as we could perform rescue experiments reintroducing individual $G\alpha$ subunits. Similar to our observations made using FR900359, there was minimal effect of the loss of these G proteins on the FP-ICL3 P4-RLucII response to PGF2 α . As with small molecule inhibition of $G_{q/11}$ however, the $\Delta G\alpha_{q/11/12/13}$ line also showed the dependence of Ang II-mediated conformational alterations in FP on re-expression of $G\alpha_q$ or $G\alpha_{11}$, highlighting the necessity of both expression and activity of $G\alpha_{q/11}$ for Ang II to affect FP conformation. However, co-expression of either $G\alpha_{12}$ or $G\alpha_{13}$ did not re-establish crosstalk between FP and the AT1R in the $\Delta G\alpha_{q/11/12/13}$ line.

We also examined the possible involvement of $G\alpha_i$ in the transmission of conformational information between the AT1R and FP using pertussis toxin (PTX, Fig 2C). Pre-treatment of the $\Delta G\alpha_{q/11/12/13}$ line expressing FP-ICL3 P4-RLucII, AT1R-WT and various co-expressed G proteins with PTX had no effect on the Ang II-induced response. Interestingly, the response

to PGF2 α was lost with PTX treatment only when $G\alpha_q$ was co-expressed suggesting a more complicated crosstalk we did not explore further here.

When examining the kinetic data, we noted that PGF2 α induced a sustained response regardless of the expression of $G\alpha_q$ while Ang II only induced a sustained response when $G\alpha_q$ was present (Fig. 2D and E). When compared to native HEK 293 cells, an increase in the time for the PGF2 α response to reach a plateau was observed (compare Fig. 2D vs. 1F). The effect was more clearly seen in the *inset* to Fig. 2D, where we have offset the baselines to match. This was also noted when $G\alpha_q$ was re-introduced for both PGF2 α and Ang II and therefore may be a property of the cell line as opposed to simple G protein expression *per se* (compare Fig. 2E vs. 1F). We also observed an increase in the ligand-naïve BRET when $G\alpha_q$ was re-introduced compared to native HEK 293 cells (compare Fig. 2E vs. 1F). This may again be due differences in gene expression profiles between the cells or the differences in G protein biosynthesis from endogenous and exogenous genetic templates. Taken together, our data showed that transmission of information from AT1R leading to a conformational change in FP was dependent on both the expression and activity of $G\alpha_{q/11}$ and independent of the other $G\alpha$ proteins tested.

Ang II-induced conformational change in FP required an intact cell membrane

Knowing that specific G proteins were required to mediate transmission of conformational information between the two protomers, we next focused on the involvement of other signalling proteins downstream of the receptor. In order to dissociate the receptor from more distal cytosolic components associated with

molecular crosstalk, we first prepared membranes from the $\Delta G\alpha_{q/11/12/13}$ line expressing the FP-ICL3 P4-RLucII conformation-sensor with and without AT1R-WT and $G\alpha_q$. We initially examined the effect of membrane preparation on basal BRET as reported from the FP conformational biosensor in the absence of ligand (Fig. 3A). We observed an increase in BRET in membranes that was reduced upon co-expression of $G\alpha_q$. Western blots on the membrane preparation samples confirmed co-expression of $G\alpha_q$ (Fig. 3B). We then compared intact cell and membrane preparation samples following stimulation with PGF2 α (Fig. 3C) or Ang II (Fig. 3D). In response to PGF2 α , we observed larger Δ BRET values in the membrane preparation samples compared to intact cells, but the effects were similar in direction. However, following stimulation with Ang II we noted a distinct Δ BRET (of opposite direction) when comparing intact cells with membranes regardless of whether or not $G\alpha_q$ was co-expressed.

As membrane preparation disrupted the ability of Ang II to induce Δ BRET and since $G\alpha_{q/11}$ was required to mediate transmission of information from AT1R to FP in intact cells, we assessed whether this was correlated with disruption of a physical interaction between the receptors *per se*. We demonstrated that neither membrane preparation itself, nor expression or absence of $G\alpha_q$, affected immunoprecipitation of FP with AT1R (Fig 3E). These observations suggest that cell integrity (and probably proper stoichiometric association of multiple signalling partners) is necessary to manifest allosteric interactions in the dimer even though a physical interaction between the two protomers was still maintained.

Proximal but not distal signalling partners affect Ang II-induced conformational rearrangement in FP

Having identified a requirement for both $G\alpha_q$ expression and activity as well as an intact cell membrane to allosterically connect FP and AT1R, we wanted to more explicitly examine whether conformational crosstalk from AT1R to FP was mediated through canonical molecular crosstalk. Since the membrane preparation procedure altered communication of conformational information from AT1R to FP, we investigated involvement of downstream signalling partners. We began by examining the distal effector protein kinase C (PKC) in the $G\alpha_q$ signalling pathway shared by both receptors. Using either an inhibitor (Gö6983, Fig. 4A) or an activator (phorbol-12-myristate-13-acetate, PMA, Fig. 4B), we detected no effect on the ability of either PGF2 α or Ang II to induce conformational changes in FP-ICL3 P4-RLucII when co-expressed with AT1R-WT in HEK 293 cells. We next examined a more proximal signalling protein in the $G\alpha_q$ signalling cascade, phospholipase C (PLC). When HEK 293 cells expressing the FP-ICL3 P4-RLucII conformation-sensor and AT1R-WT were pre-treated with the small molecule PLC inhibitor U73122, we noted no effect on the PGF2 α -mediated Δ BRET and a small but significant reduction in the response to Ang II suggesting that the receptor/ $G\alpha_{q/11}$ /PLC β complex may be shared by the heterodimer (Fig. 4C).

β -arrestin-biased ligands of AT1R are also capable of inducing a conformational change in FP

There is considerable interest in developing β -arrestin or G protein-biased ligands as therapeutics. However, given that receptors are constitutively associated with G proteins, there is still a lot we don't understand about how G proteins might be involved in how biased ligands signal. Thus, we next used AT1R biased ligands that selectively activate β -arrestin over $G\alpha_q$ (48).

Using all 5 of the FP-ICL3-RLucII conformational biosensors, we screened this group of ligands to see if they could induce conformational changes in FP (Fig. 5A). Our initial screen detected the ability of both SBpA and SI, β -arrestin biased AT1R agonists, to induce a small positive Δ BRET in the FP conformational biosensors at saturating concentrations. As with Ang II, the FP-ICL3 P4-RLucII conformational biosensor showed the largest magnitude Δ BRET across the panel of ligands and was therefore the focus of the subsequent experiment. To ensure that the heterodimerization between FP and AT1R did not alter the defined functional profile of these ligands as biased, we assessed their ability to activate $G\alpha_q$ using a BRET-based $G\alpha_q$ activation biosensor (Fig. 5A, *inset*). Neither SBpA or SI were able to elicit a response in this biosensor while a robust response was detected in response to Ang II. We tested SBpA and SI in the $\Delta G\alpha_{q11/12/13}$ line to assess whether there was any requirement for $G\alpha_q$ in mediating conformational rearrangement in FP. Interestingly, both SBpA- and SI-induced conformational changes were ablated in the $\Delta G\alpha_{q11/12/13}$ line, but could again be rescued when $G\alpha_q$ was reintroduced through transient co-expression (Fig. 5B and C comparing $G\alpha_q$ versus pcDNA3.1 vector control, respectively). These observations suggest that even biased ligands require $G\alpha_q$ to transmit information to FP even though they do not necessarily result in downstream $G\alpha_q$ signalling.

The $G\alpha_q$ -coupled OTR does not induce a similar conformational change in FP

Although one distal effector pathway was not involved in the conformational crosstalk between FP and AT1R, we did not systematically interrogate other pathways. However, to affirm receptor dimerization as a mechanism underlying the response of FP

conformational biosensors to Ang II, we explored whether another $G\alpha_q$ -coupled GPCR could lead to a similar response in FP (49,50) where similar patterns of molecular crosstalk might also be expected. We have preliminary data using BRET that FP and OTR do not dimerize (data not shown). We compared HEK 293 cells transfected with the FP-ICL3 P4-RLucII conformational biosensor and either AT1R-WT or OTR-WT (Fig. 6A and B). We noted FP conformational responses to Ang II and not OT in cells transfected with AT1R-WT or OTR-WT. To confirm that the absence of response to OT was not due to relative expression levels of the receptors, we titrated the expression of either AT1R-WT (Fig. 6C) or OTR-WT (Fig. 6D) and noted a saturating response AT1R-WT/Ang II conditions but no response across any of the OTR-WT/OT conditions. Finally, we confirmed that both AT1R-WT (Fig. 6E) and OT-WT (Fig. 6F) constructs were able to activate a BRET-based $G\alpha_q$ biosensor. This demonstrated that $G\alpha_q$ activation *per se* was not driving the conformational change in FP-ICL3 P4-RLucII, suggesting a shared G protein in the context of the AT1R/FP heterodimer.

Asymmetrical transmission of conformational information between protomers of the FP/AT1R heterodimer

Finally, we considered whether ligand binding to the FP protomer could induce a similar conformational change in its AT1R partner. Using a corresponding panel of conformational biosensors build into AT1R (35), we screened whether ligand binding to FP-WT induced changes in the relative orientation between the ICL3 and C-terminus of AT1R. Examining multiple vantage points in the ICL3 of AT1R demonstrated robust, position-dependent responses to orthosteric ligand (Ang II) in our AT1R conformational biosensors (Fig.

7A). Intriguingly, stimulation with PGF2 α was not able to induce a response in any of the AT1R conformational biosensors suggesting that the arrangement of the putative heterodimer was asymmetric (Fig. 7B). Examination of the kinetic data demonstrated a sustained response of the conformational biosensor to Ang II, similar to what we had observed with the FP sensor (Fig. 7C and D). The effect of co-expressing FP-WT was not as large on ligand-naïve BRET compared to AT1R-WT with the FP biosensor (compare Fig. 7C vs. D and Fig. 1E vs. F). As before, we noted no change in BRET across the entire recording period when in response to PGF2 α (Fig. 7D). When comparing traces from the $\Delta G\alpha_{q/11/12/13}$ with and without re-introducing $G\alpha_q$, we noted an increase in the sustained response to Ang II when $G\alpha_q$ was present (Fig. 7E vs. F). This is similar to observations we made when examining the sensor expressed alone without the co-expression of FP-WT (35).

Discussion

Here, we demonstrate asymmetric transmission of conformational information between protomers of the putative FP/AT1R heterodimer (summarized in Fig. 8). The AT1R-induced conformational rearrangement in FP was dependent on both expression and activation of $G\alpha_q$ and possible involvement of the proximal $G\alpha_q$ -effector PLC. This is consistent with reports showing that PLC β is stably associated with $G\alpha_q$ (51). Further, we demonstrate the AT1R-driven conformational change in FP was predominantly independent of a key distal downstream receptor signalling pathway. We propose that the transmission of information occurs at the level of the membrane and is most likely propagated via a shared G protein as part of a signalling complex. As we noted that even in the absence of $G\alpha_q$, the AT1R/FP heterodimer

remains intact, suggesting that $G\alpha_q$ subunits are not critical to the assembly of the receptor heterodimer, although other G protein heterotrimers might also serve this role in their absence. $G\beta\gamma$ subunits are also important in the formation of GPCR dimers and their associated signalling complexes, as suggested by our previous work (52,53). Our data here suggests that $G\alpha_q$ acts as a conduit, allosterically connecting the two receptors once assembled into a signalling complex. Surprisingly, β -arrestin biased AT1R ligands (48) also demonstrated a dependence on $G\alpha_q$ although they elicited no $G\alpha_q$ activation *per se*. This would further support the notion that $G\alpha_q$ plays a key structural role enabling conformational crosstalk between receptors, regardless of the nature of the bound ligand. Therefore, we demonstrate a novel mechanism in which allosteric interactions can transmit information between protomers of a GPCR heterodimer. Our observations are in contrast with a previous report demonstrating independence of the G proteins for conformational cross-talk between the receptors (37) suggesting that heterodimer-specific arrangements are possible.

We previously showed that within the AT1R/FP complex, each receptor was capable of modulating the functional output of the other through asymmetric allosteric interactions (38). Asymmetric structural arrangements have been noted in luteinizing hormone oligomers (54), rhodopsin (55), mGluR2/3 heterodimers (56) and leukotriene B4 receptor dimers (18). These studies support the notion that individual protomers in a receptor dimer may interact with a shared G protein through distinct interfaces (see also (2)), suggesting that structural asymmetries may translate into functional or conformational asymmetries. Our results here further strengthen the case for functional AT1R/FP heterodimeric complexes and provide insight into the

mechanism by which the two receptors communicate. Though the precise functional consequences of AT1R-induced change in FP conformation are yet to be determined, it also appears to be asymmetric in nature. We observed asymmetry in allosteric communication between receptors, with AT1R modulating FP but not the converse. Further, the AT1R to FP conformational crosstalk in the heterodimer may be biased toward $G\alpha_{q/11}$, as no effect was observed when we altered $G\alpha_{12/13}$ or $G\alpha_i$ function or levels. This could represent a coupling preference of the heterodimer or it may be possible that our biosensors are sensitive to conformations driven by particular G proteins coupled to the heterodimer. Capitalizing on such signal bias and asymmetric conformational crosstalk may provide novel venues for targeting heterodimers, ignored in most current drug discovery programs (57-59). As we have demonstrated previously, ligand binding to AT1R can modulate the functional output of FP (38). Since both AT1R and FP couple to $G\alpha_q$, it is difficult to explore the functional effect of the AT1R-induced conformational effects on FP, as they share a number of common signalling outputs. It is also important to acknowledge that there is also the possibility that the induced conformation may be silent with respect to signalling (60). A larger understanding of unique and shared receptor signalling outputs may help settle this question.

FP and AT1R are important targets at the core of many biological functions. AT1R is a primary target in the treatment of hypertension with AT1R antagonists of the sartan family being widely prescribed (61). A role for FP has also been demonstrated in regulating blood pressure where its blockade has been suggested to reduced blood pressure (62). FP is involved in parturition

with enhanced $PGF2\alpha$ signalling initiating labour by causing smooth muscle contraction of the myometrium (63,64). The AT1R is also expressed in the myometrium with increased levels coinciding with pregnancy (65-67). Examining the receptor complex may yield novel drug targets in these tissues. An understanding as to how these two receptors communicate at a structural level could facilitate rational drug design as our data indicates that at least as regards receptor conformation, Ang II is a biased ligand for FP, while the converse is not true for $PGF2\alpha$ and the AT1R. Regardless of the mechanisms, our conformational biosensors could be used to identify new conformational and allosteric connections between known and orphan GPCRs, without the requirement for knowledge about how downstream signalling or receptor trafficking is altered. This may help identify new targets for drug discovery as ligands for one receptor may act as allosteric modulators of heterodimer partners, provide a new vantage point from which to understand receptor dynamics, and foster the development of receptor screens that are portable from cell type to cell type regardless of *a priori* knowledge about downstream signalling.

Experimental procedures

Reagents

Unless noted otherwise, all chemicals were reagent-grade and purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. FR900395 was purchased from the Institute of Pharmaceutical Biology (University of Bonn, Germany). $PGF2\alpha$ was from Cayman Chemical while AS was a kind gift from Dr. Stéphane Laporte. SBpA, SI, SII, DVG were synthesized by Lifetein.

Western Lightning Plus-ECL was purchased from Perkin Elmer

Constructs

pIRES-SP-FLAG-AT1R-WT, pIRES-SP-FLAG-AT1R-ICL3 WT, P2, P3, P4, P5-RLucII were described in (35). pIRESpuro3-HA-FP-WT, pcDNA3.1 – SP-HA-FP ICL3 WT, P1, P2, P3, P4, P5-RLucII were previously described (39). pcDNA3.1-Gαq-EE and pcDNA3.1-Gα12-EE were obtained from the University of Missouri-Rolla cDNA Resource Center. pcDNA3.1-Gα11 and pcDNA3.1-Gα13 were obtained from Dr. Michel Bouvier. Generation of pcDNA3.1-myc-OTR-WT was previously described (68). Generation of pcDNA3.1-SF-DOR was previously described (40). The polycistronic Gα_q activation sensor was used as previously described (69).

Cell culture

HEK 293 SL or T cells and ΔGα_{q11/12/13} line (35) were cultured in Dulbecco's Modified Eagle's medium (DMEM, Wisent) supplemented with 10% vol/vol fetal bovine serum (FBS, Wisent). All cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cells were grown to ~80% confluency in T75 flasks (Corning) at which point they were plated for transfection. Gα_{q11/12/13} gene-deleted HEK 293 cells were generated as previously described (35).

Membrane preparation

FLAsH-labelled cells were resuspended in 1mL homogenizing buffer (10mM Tris-HCl pH 7.4, 5mM EDTA). Cells were homogenized with a glass Teflon homogenizer with 20 up-down strokes on ice. The homogenate was transferred to 1.5 mL microtubes and membrane was pelleted by centrifugation at 13,000 rpm at 4°C for 30 min in a Microlite RF from Thermo IEC.

Supernatant was collected for the reconstituted cell conditions and pelleted membrane was resuspended in TME buffer (50 mM Tris-HCl pH 7.4, 4.8 mM MgCl₂, 2 mM EDTA, 100 mM NaCl, 1 μM GDP, 30 nM GTP, 250x protease inhibitor cocktail (Sigma)). Total protein was quantified using a Bradford reagent (Bio-Rad) and 10 μg of protein was loaded/well in a white 96-well plate. For the reconstituted cell conditions, 1μM GDP and 30nM GTP were added to the collected supernatant after pelleting the membrane in which the membrane was resuspended. Total protein was quantified and 10 μg of protein was loaded/well in a white 96-well plate (Corning).

Conformational biosensor assays

Data collection with the FLAsH/BRET-based conformation biosensors was previously described (39). Briefly, labelled cells in white 96 well plates were treated with 2 μM coelenterazine H (Nanolight) for 5 minutes. Plates were loaded into a Victor X Light Luminescence plate reader (Perkin Elmer) and luminescence was captured through F460 and F535 filters for BRET 1. Filtered signals were collected every 0.6 s with an integration time of 0.2 s for 50 reads. Drug was then injected into the well using the attached injector module and signals through each filter were collected for another 100 reads. To calculate BRET, the signal through the F535 filter was divided by that through the F460 filter. The basal BRET refers to the average BRET across the first 50 reads prior to injection and the change in BRET due to drug binding (ΔBRET) is calculated by subtracting the basal BRET from the average BRET across the last 50 reads post-injection. Kinetic traces are the raw BRET calculated from the F535/F460 at the sampling frequency.

Gα_q-activation assay

As mentioned above, we used an established polycistronic $G\alpha_q$ activation sensor which is based on physical separation of $G\alpha$ and $G\gamma$ subunits following receptor activation (69). Briefly, cells in white 96-well plates (Corning), transfected with the biosensor, and GPCR of interest were washed once and then left with 80 μ L Krebs's Buffer in a white 96-well plate. Cells were left to rest for 2 hr. at RT before starting the assay. Data was collected on a Victor X Light luminescence plate reader (Perkin Elmer) using filters 410/80 and 515/30 for BRET 2 in well mode. Prior to data collection, 5 μ M coelenterazine 400a (Goldbio) was added to wells and incubated at RT for 5 minutes. Signal was collected through each filter with an integration time of 0.2 s every 0.6 s for 30 reads. The injector module then injected the drug of interest and the data was collected for another 60 reads. BRET was calculated by dividing the signal through the 515/30 filter by that captured through the 410/80 filter. The Δ BRET was then calculated by subtracting the average BRET pre-injection from the last 30 reads post-injection.

Co-immunoprecipitation

Transfected cells were lysed in solubilization buffer (25 mM HEPES pH 7.4, 5 mM EDTA, 50 mM NaCl, 10% glycerol, 1% Triton X-100, 1/250 protease inhibitor cocktail (Sigma)) for 1 hr at 4°C. Lysates were cleared by centrifugation at 13,500 rpm in a Microlite RF (Thermo IEC) microcentrifuge for 10 min. The supernatant was collected and protein was quantified using a Bradford assay (Bio-Rad). FLAG M2 beads (Sigma) were loaded with 500 μ g of protein and incubated at 4°C rocking overnight. The next day, beads were washed 3 times with solubilization buffer pelleting the beads at 1600 rpm for 2 min at 4°C between each wash. Protein was eluted from the beads in elution buffer (50 mM Tris pH 7.4, 150 mM NaCl and FLAG peptide (3

μ L/100 μ L TBS, Sigma) for 20 min at 4°C. Beads were spun down at 1600 rpm for 2 min and the supernatant was collected. 20 μ L sample buffer (62.5 mM Tris pH 6.8, 16.3% glycerol, 2% SDS, 5% β -mercaptoethanol, 2 mg bromophenol blue) was added and samples were heated to 65°C for 15 min.

SDS-PAGE and western blotting

Samples were loaded on a 10% polyacrylamide gel and ran for 1 hr at 120V. Resolved protein was transferred to PVDF membrane under 100V for 1 hr. Membranes were blocked for 1 hr in 5% milk in TBST. Anti-Rluc (Millipore) or anti-FLAG (Sigma) primary antibodies were prepared in TBST + 5% milk at a 1:2000 dilution. The membrane was incubated with primary antibodies at 4°C with gentle rocking overnight. The next day, the membrane was washed 3 times with TBST. Secondary antibodies conjugated to horseradish peroxidase (Sigma) were diluted at 1:20000 in TBST + 5% milk and incubated with the membrane for 1 hr at RT. The membrane was washed 3 times with TBST and then treated with enhanced chemiluminescence reagent (Perkin Elmer).

In-cell westerns

Cells plated in black 96-well plates (Corning), transfected with the proteins of interest were washed once with Krebs's buffer. Cells were then fixed with 2% paraformaldehyde which had been warmed to 37°C for 10 min at RT. Cells were then washed 3 times with Krebs's + 1% BSA. Primary antibodies for HA (Covance) FLAG (Sigma) were diluted in Krebs's + 1% BSA at 1:200. Cells were incubated with primary antibodies for 1 hr at RT. Cells were washed 3 times with Krebs's buffers and then incubated with anti-mouse or anti-rabbit secondary antibodies, respectively, conjugated to Alexa 488. Secondary

antibodies were diluted at 1:500 in Kreb's + 1% BSA and incubated with the cells for 1hr at RT. Hoechst DNA stain was also added during the incubation with secondary antibodies at a concentration of 1 μ g/mL. Fluorescent signals were measured using a Synergy 2 multimode plate reader (BioTek) using 360/40 excitation and 460/40 emission filters for Hoechst and 485/20 excitation and 528/20 emission filters for Alexa 488. The Alexa 488 signal was normalized to the Hoechst signal to quantify the average surface expression of the GPCR of interest/cell.

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Author contributions: T.E.H, R.S. and D.D. designed the study and R.S., D.D., D.P., A.Z., K.B. and Y.S. performed experiments. R.S. and D.D. analyzed the data. R.S., D.D., Y.S., A.I. and D.P. generated figures. R.S., D.D, A.I. and T.E.H. wrote and edited the paper.

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Figure Legends

Figure 1. Ang II binding to AT1R induces a conformational rearrangement in FP. A) + B) HEK 293 cells were transfected with FP conformational biosensors with a FAsH tag inserted at the indicated position (Px) along with AT1R-WT. Cells were stimulated with A) 1 μ M PGF2 α or B) Ang II and the change in BRET (Δ BRET) due to ligand stimulation is reported. C) + D) HEK 293 cells were transfected with FP-ICL3 P4-RlucII and C) AT1R-WT or D) FP-WT. Cells were pre-treated with assay buffer, 10 μ M AS604872 or 10 μ M losartan for 30 minutes prior to BRET recording. Cells were stimulated with 1 μ M PGF2 α or Ang II and the change in BRET (Δ BRET) due to ligand stimulation is reported. Bars represent the mean of 3 independent biological replicates and error bars represent SEM. A) + B) Tukey's test was performed where *** = $p < 0.001$, ** = $p < 0.01$ and * = $p < 0.05$, $n = 3$. C) + D) Dunnett's test was performed to compare treatments to buffer. No comparisons were statistically significant. Increasing n was not possible as availability of AS604872 was limited. E) + F) Kinetic traces of conformation biosensors in response to ligand stimulation. HEK 293 cells were transfected with E) FP-ICL3 P4-RlucII alone or F) co-transfected with AT1R-WT. The specified ligand was injected onto the cells at the 50th repeat measure as denoted by an arrow. Traces represent the mean of 3 independent experiments.

Figure 2. Functional $G_{\alpha_{11}}$ is required for Ang II-induced conformational crosstalk between AT1R and FP. A) HEK 293 cells were transfected with the FP-ICL3 P4-RLucII biosensor and AT1R-WT. Cells were pre-treated with 100 nM of the G_{α_q} inhibitor FR900359 for 1 hour followed by buffer, 10 μ M AS604872 or 10 μ M losartan for 30 minutes prior to BRET recording. Cells were stimulated with 1 μ M PGF2 α or Ang II and the change in BRET (Δ BRET) due to ligand stimulation is reported. B) $\Delta G_{\alpha_{11/12/13}}$ HEK 293 cells were transfected with FP-ICL3 P4-RlucII, AT1R-WT and pcDNA3.1 or the indicated G_{α} subunit. Cells were stimulated with 1 μ M PGF2 α or Ang II and the change in BRET (Δ BRET) due to ligand stimulation is reported. C) $\Delta G_{\alpha_{11/12/13}}$ HEK 293 cells were transfected with FP-ICL3 P4-RlucII, AT1R-WT and pcDNA3.1 or the indicated G_{α} subunit. Cells were pre-treated with buffer or 100 ng/mL pertussis toxin for 16 hours before FAsH labelling. Cells were stimulated with 1 μ M PGF2 α or Ang II and the change in BRET (Δ BRET) due to ligand stimulation is reported. Bars represent the mean of 3 independent biological replicates and error bars represent SEM. A) + B) Dunnett's test was performed to compare conditions with A) buffer or B) pcDNA3.1 where ** = $p < 0.01$. C) A two-way ANOVA was used to analyze the 2 graphs. For both graphs, the G protein factor and interaction were non-significant while the PTX factor was significant (PGF2 α = $p < 0.01$, Ang II = $p < 0.001$). Bonferroni corrected tests were used to make post-hoc comparisons where * = $p < 0.05$ and ** = $p < 0.01$. D) + E) Kinetic traces of conformational biosensors responding to ligand in the presence and absence of G proteins. $\Delta G_{\alpha_{11/12/13}}$ cells were transfected with D) FP-ICL3 P4-RlucII and AT1R-WT or E) FP-ICL3 P4-RlucII, AT1R-WT and G_{α_q} . The specified

ligand was injected onto the cells at the 50th repeat measure as denoted by an arrow. *Inset* to D): Offset basal traces for PGF2 α and Ang II to better demonstrate differences in biosensor responses. Traces represent the mean of 3 independent experiments.

Figure 3. Membrane preparation results in a loss of Ang II-induced AT1R to FP conformational crosstalk though the receptors still form a complex. A) $\Delta G\alpha_{q/11/12/13}$ HEK 293 cells were transfected with FP-ICL3 P4-RLucII, the indicated wildtype receptor and pcDNA3.1 or the indicated $G\alpha$ subunit. Cells were split into intact cell or membrane preparation groups. Each group was treated as described in *Experimental Procedures*. Basal BRET was recorded in the absence of receptor ligands. B) Western blot demonstrating that $G\alpha_q$ is still present in the sample after membrane preparation. C) + D) The same preparations used in A) and B) were stimulated with 1 μ M PGF2 α (C) or Ang II (D) and the change in BRET (Δ BRET) due to ligand stimulation is reported. E) Western blot demonstrating that AT1R (FLAG) can co-immunoprecipitate FP-ICL3 P4-RLucII in lysates from prepared membrane or intact $\Delta G\alpha_{q/11/12/13}$ HEK 293 cell samples, independent of the expression of $G\alpha_q$. A) + C) + D) Bars represent the mean of 3 independent biological replicates and error bars represent SEM. Tukey's test was used to compare all groups in graphs from where * = $p < 0.5$ and ** = $p < 0.01$. B) + E) are representative blots of $n=2$. E) Expression of FP-ICL3 P4-RLucII in all conditions has been demonstrated based on quantification of luminescence from RLucII (data not shown).

Figure 4. Proximal but not distal $G\alpha_q$ signalling crosstalk is involved in transmitting Ang II-induced conformational information to FP. A) + B) HEK 293 cells were transfected with FP-ICL3 P4-RLucII and AT1R-WT. Cells were pre-treated with A) 1 μ M of the PKC inhibitor Gö6983 for 1 hour or B) co-stimulated with 100 nM PMA, a direct PKC activator. Cells were stimulated with 1 μ M PGF2 α or Ang II and the change in BRET (Δ BRET) due to ligand stimulation is reported. D) HEK 293 cells were transfected with FP-ICL3 P4-RLucII and AT1R-WT. Cells were pre-treated with 1 μ M U73122 for 1 hour. Cells were stimulated with 1 μ M PGF2 α or Ang II and the change in BRET (Δ BRET) due to ligand stimulation is reported. Bars represent the mean of 3 independent biological replicates and error bars represent SEM. A t-test was performed to compare buffer vs. treatment where ** = $p < 0.01$.

Figure 5. β -arrestin-biased AT1R agonists induce a $G\alpha_q$ -dependent conformational change in FP. A) HEK 293 cells were transfected with the different FP ICL3 conformational biosensors and AT1R-WT. Cells were stimulated with the indicated ligand and concentration and the change in BRET (Δ BRET) due to ligand stimulation is reported. *Inset*: $\Delta G\alpha_{q/11/12/13}$ HEK 293 cells were transfected with FP-WT, AT1R-WT and a BRET-based $G\alpha_q$ activation sensor and stimulated with the indicated ligand and concentration and the change in BRET (Δ BRET) due to

ligand stimulation is reported, $n=2$. B) and C) $\Delta G\alpha_{q/11/12/13}$ HEK 293 cells were transfected with FP-ICL3 P4-RLucII, AT1R-WT and B) $G\alpha_q$ or C) pcDNA3.1. Cells were stimulated with the indicated ligand and concentration and the change in BRET ($\Delta BRET$) due to ligand stimulation is reported. Bars represent the mean of 3 independent biological replicates and error bars represent SEM. Dunnett's test was performed comparing to A) WT, or B) + C) buffer where ** = $p<0.01$.

Figure 6. Activation of $G\alpha_q$ by the oxytocin receptor does not induce a similar conformational change in FP. A) + B) HEK 293 cells were transfected with FP-ICL3 P4-RLucII and A) AT1R-WT or B) OTR-WT. Cells were stimulated with the indicated ligand/concentration and the change in BRET ($\Delta BRET$) due to ligand stimulation is reported. C) + D) HEK 293 cells were transfected with a constant amount of the cDNA for FP-ICL3 P4-RLucII and increasing amounts of C) AT1R-WT or D) OTR-WT cDNA. Cells were stimulated with 1 μM C) Ang II or D) oxytocin (OT) and the change in BRET ($\Delta BRET$) due to ligand stimulation is reported. Ligand-induced changes in BRET plotted against the relative C) AT1R or D) OTR surface expression as assessed by in-cell western normalized to the highest expression level. E) + F) HEK 293 cells were transfected with a BRET-based $G\alpha_q$ activation sensor, FP-WT and E) AT1R-WT or F) OTR. Cells were stimulated with the indicated ligand/concentration and the change in BRET ($\Delta BRET$) due to ligand stimulation is reported. A) + B) + E) + F) Bars represent the mean of 3 independent biological replicates and error bars represent SEM. Dunnett's test was performed comparing to buffer where ** = $p<0.01$. C) + D) A two-way ANOVA was performed, $n=3$. For C) both factors of relative expression and stimulation as well as the interaction were significant. For D), both factors of relative expression and stimulation were significant but the interaction was not. Bonferroni corrected t-tests were performed to compare the buffer vs. Ang II or OT at each relative expression level where ** = $p<0.01$ and *** = $p<0.001$.

Figure 7. Conformational communication between protomers of the FP/AT1R heterodimer is asymmetric. HEK 293 cells were transfected with AT1R conformation sensors with a FLAsH tag inserted at the indicated position along with FP-WT. Cells were stimulated with 1 μM PGF2 α A) or Ang II B) and the change in BRET ($\Delta BRET$) due to ligand stimulation is reported. Bars represent the mean of 3 independent biological replicates and error bars represent SEM. Tukey's test was performed where ** = $p<0.01$ and *** = $p<0.001$. C) + D) Kinetic traces of AT1R conformational biosensors responding to ligand. HEK 293 cells were transfected with C) AT1R-ICL3 P3-RLucII alone or D) co-transfected with FP-WT. The specified ligand was injected onto the cells at the 50th repeat measure as denoted by an arrow. Traces represent the mean of 3 independent experiments. E) + F) Kinetic traces of AT1R conformational biosensors responding to ligand in the presence and absence of G proteins. $\Delta G\alpha_{q/11/12/13}$ cells were transfected with E)

AT1R-ICL3 P3-RlucII and FP-WT or F) AT1R-ICL3 P3-RlucI, FP-WT and $G\alpha_q$. The specified ligand was injected onto the cells at the 50th repeat measure as denoted by an arrow. Traces represent the mean of 3 independent experiments.

Figure 8. Conformational information is transmitted asymmetrically between protomers of the FP/AT1R heterodimer and are dependent on $G\alpha$. In the absence (A) of $G\alpha_{q/11/12/13}$, PGF2 α binding to the sensor receptor can elicit a conformational (black arrow), but this response was blunted for the partner AT1R (red arrow). However, when $G\alpha_{q/11}$ is present (B), full responses to in the FP biosensor are observed in response to either receptor. When the AT1R is tagged with the conformational biosensor, responses are detected in response to Ang II but not PGF2 α (C and D, respectively), indicating that such conformational responses might be asymmetric depending on the relative positions of the sensor tags and the organization of the dimer/G protein complex.

Figure 1

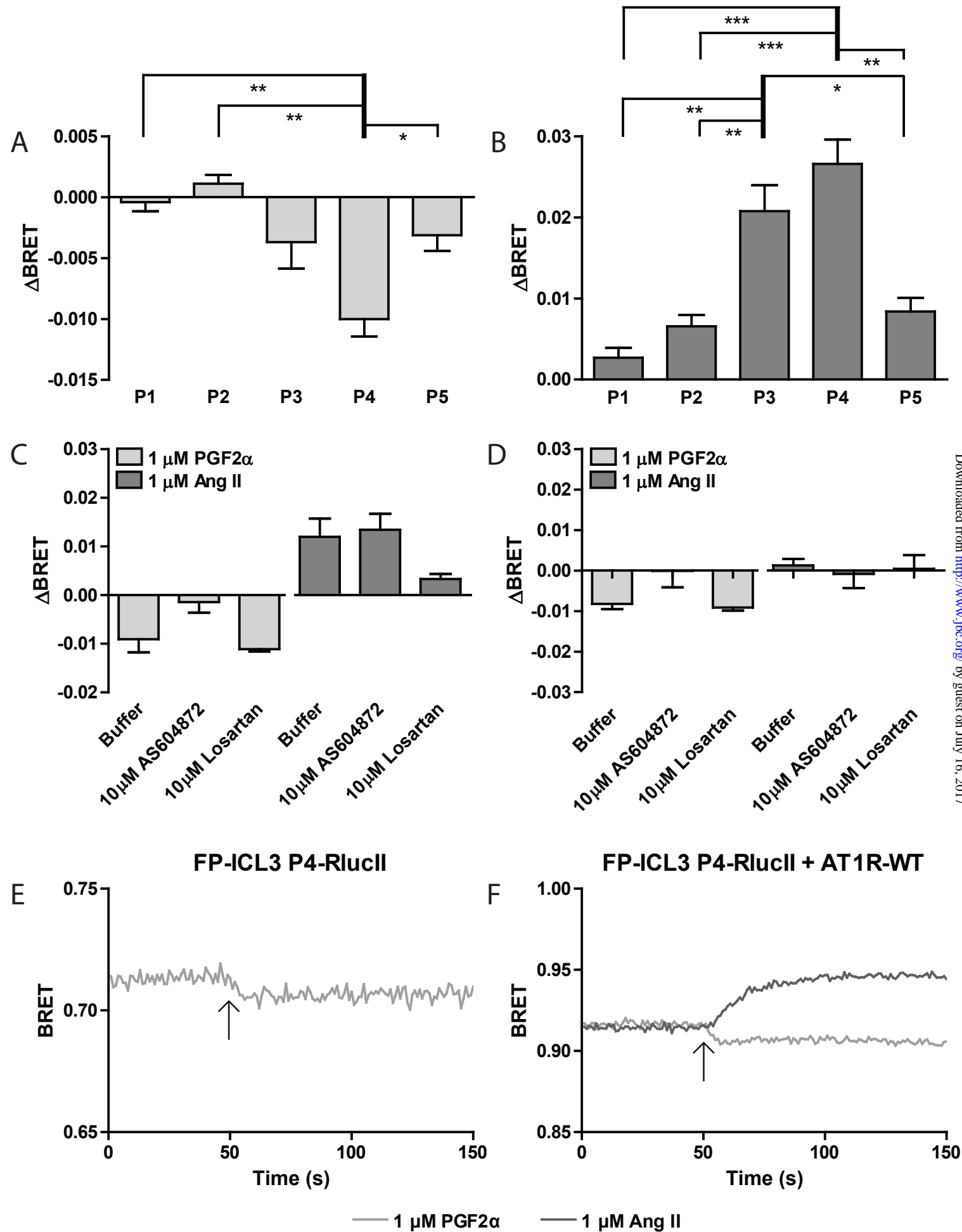


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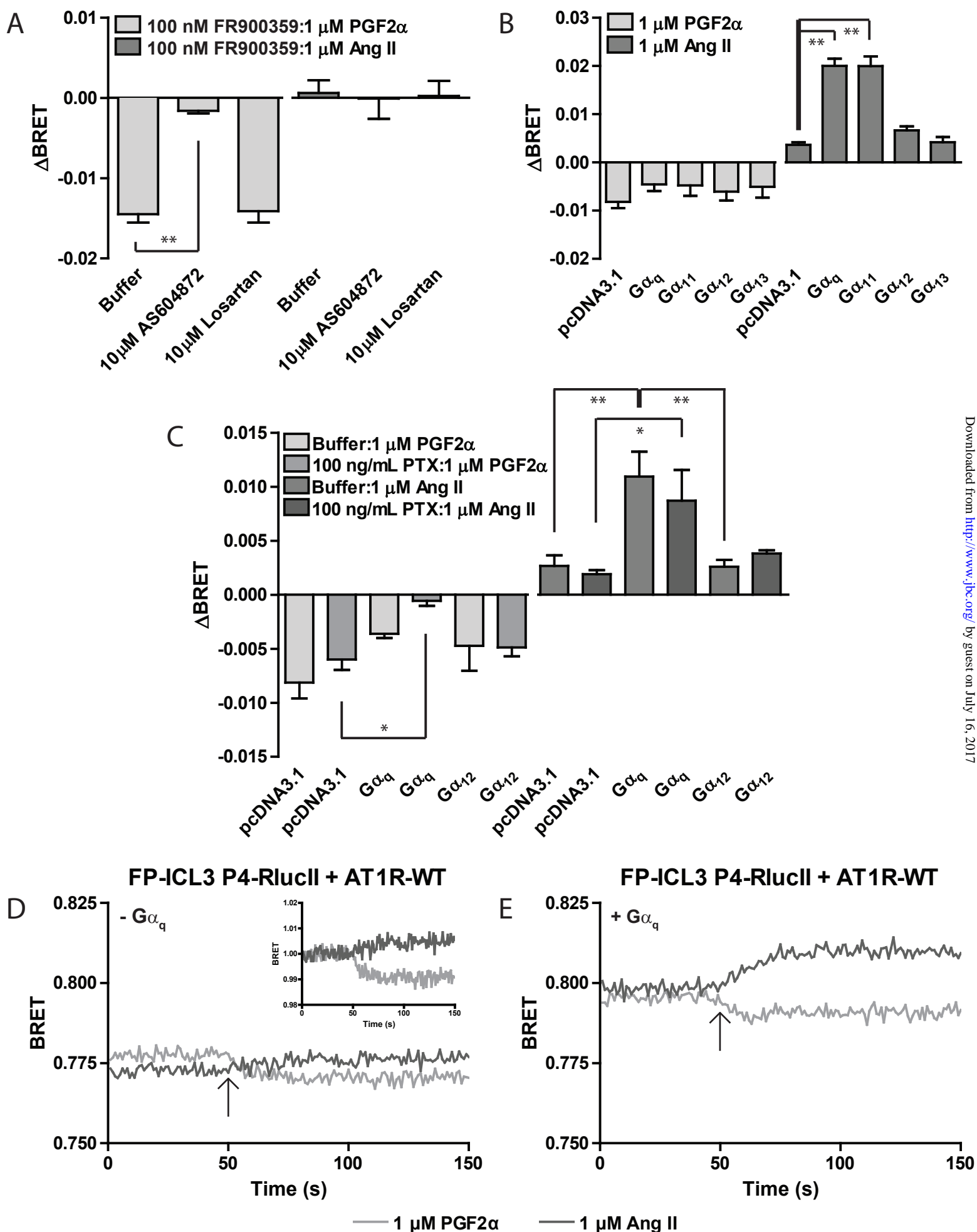


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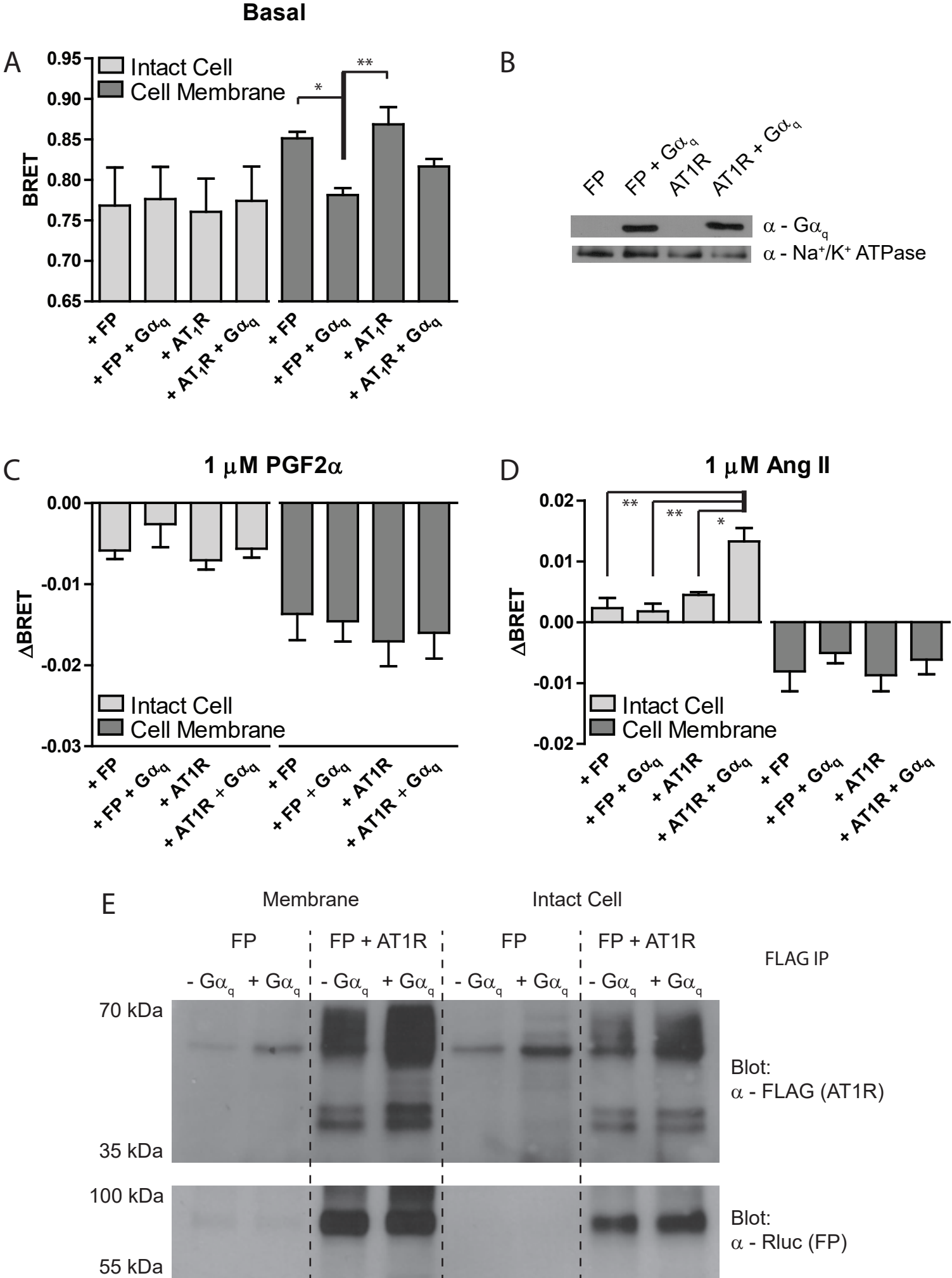


Figure 4

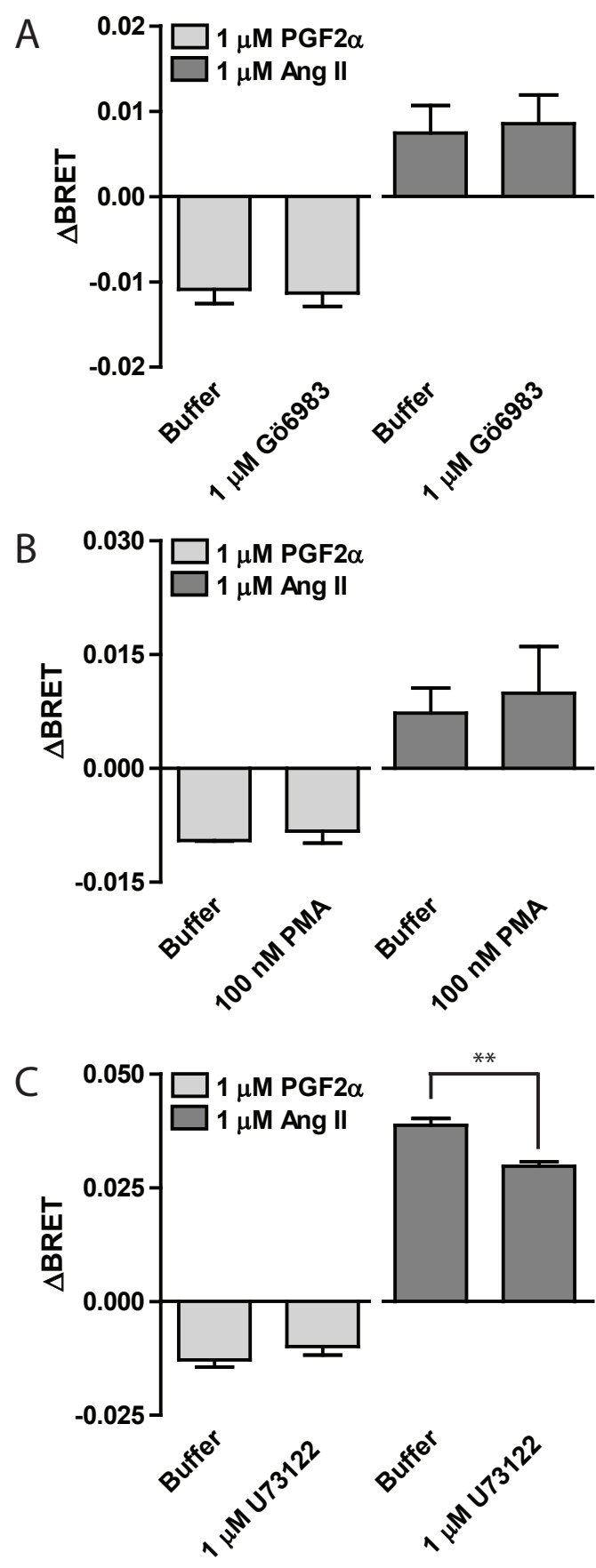


Figure 5

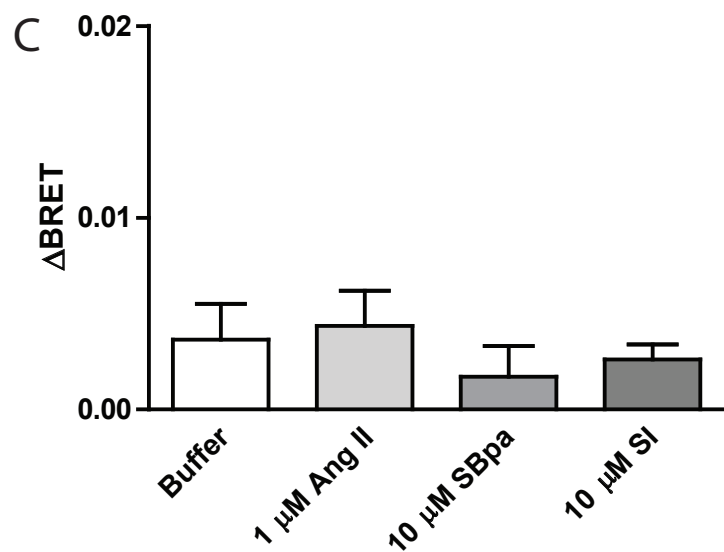
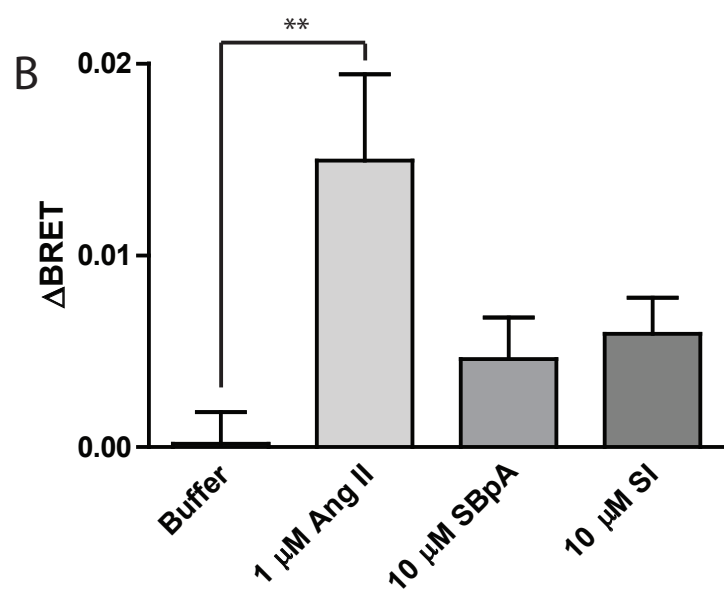
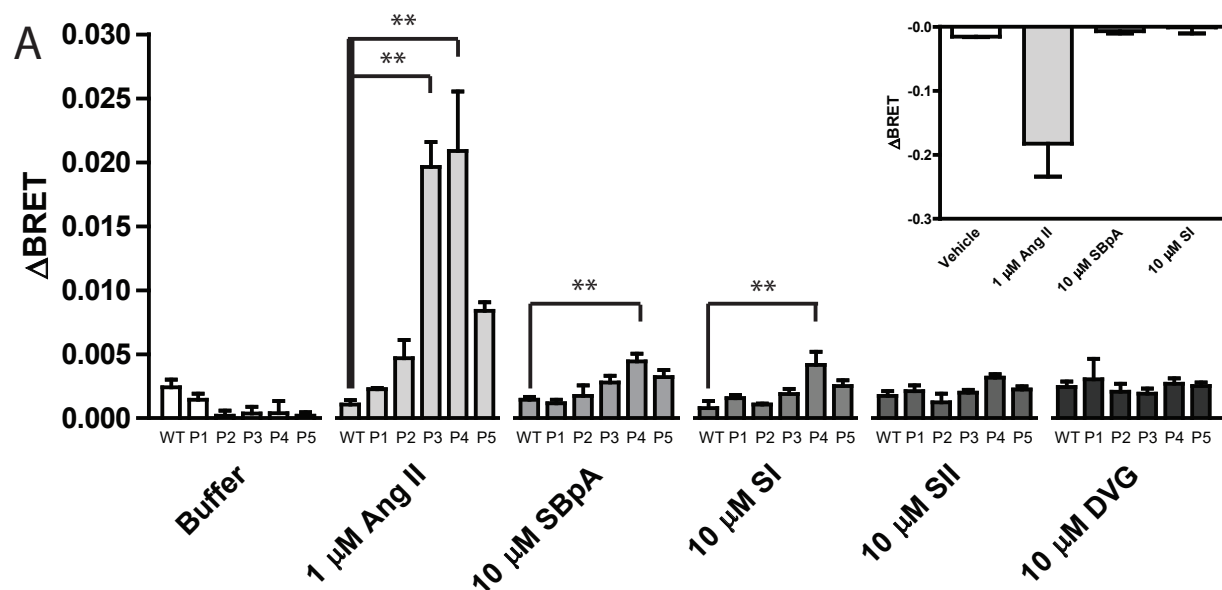
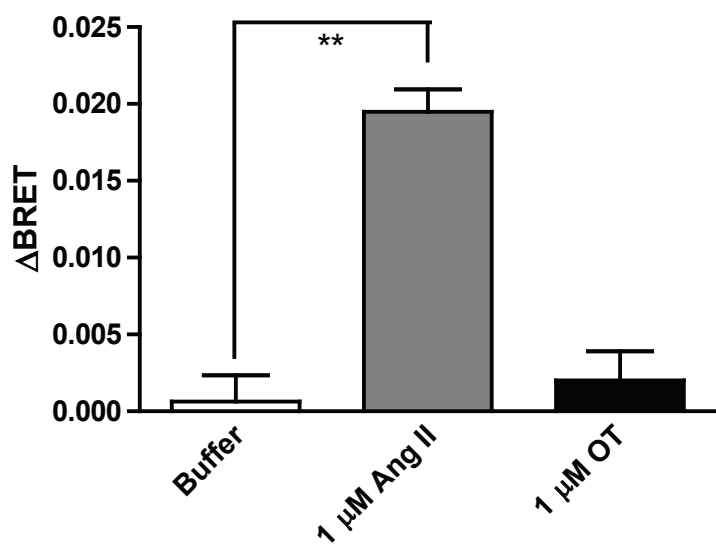
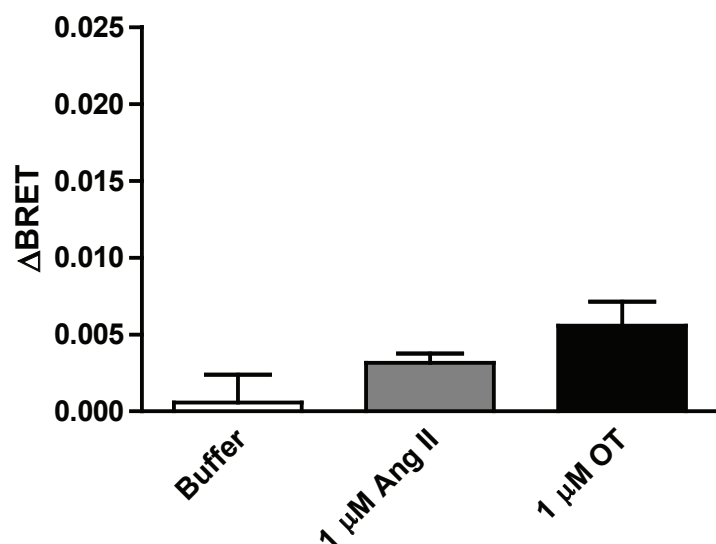


Figure 6

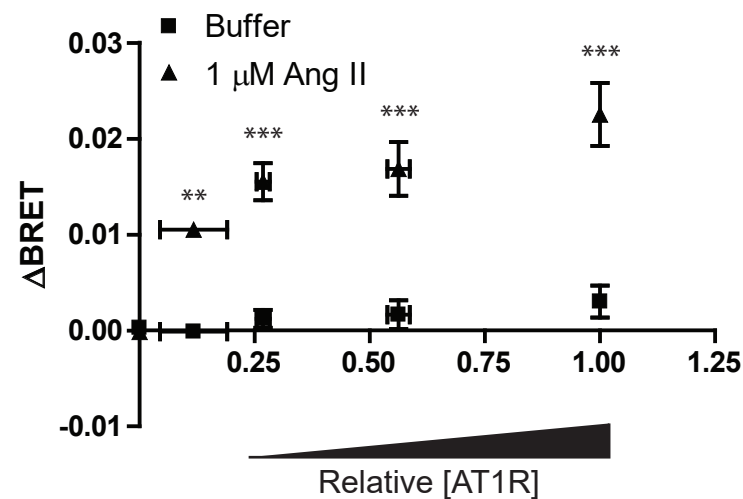
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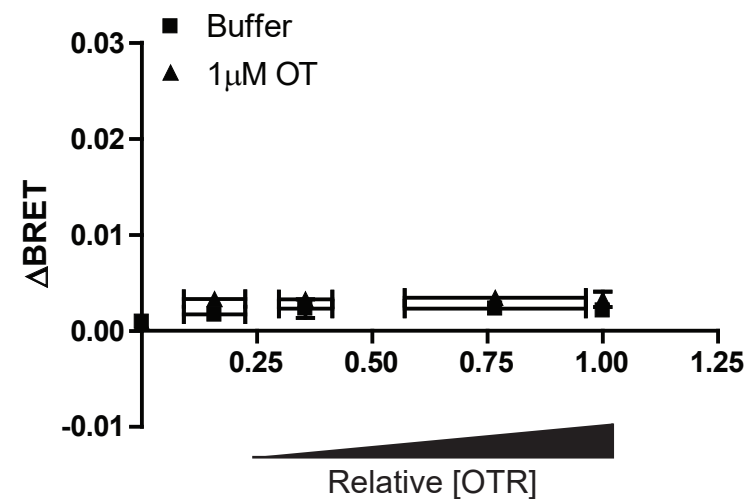
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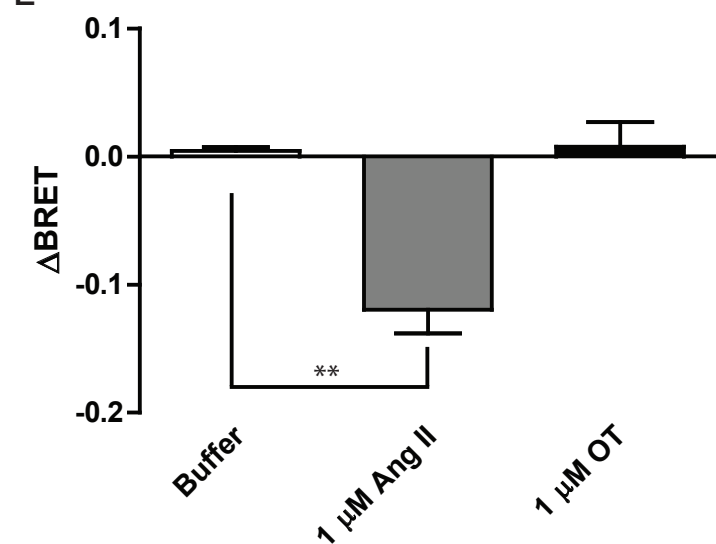
C FP-ICL3 P4-RlucII + FLAG-AT1R-WT



D FP-ICL3 P4-RlucII + myc-OT-WT



E HA-FP-WT + FLAG-AT1R-WT



F HA-FP-WT + myc-OT-WT

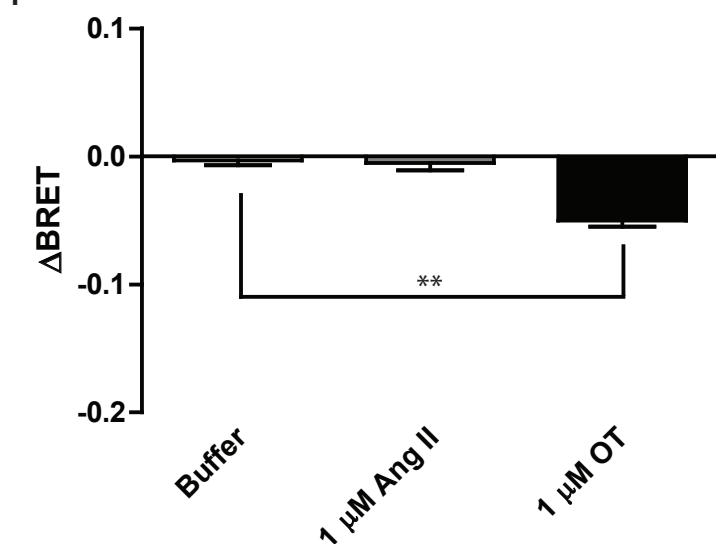


Figure 7

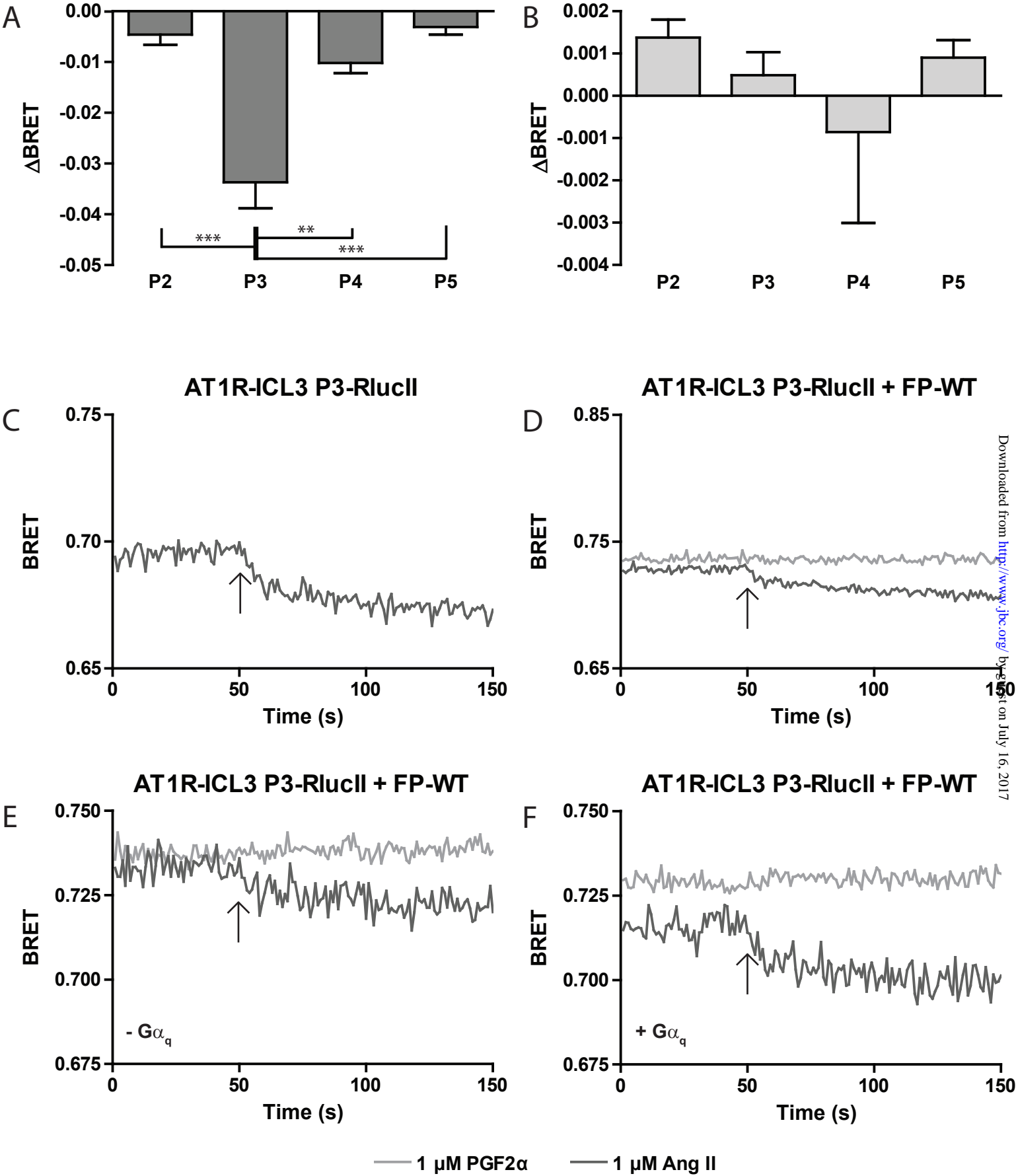
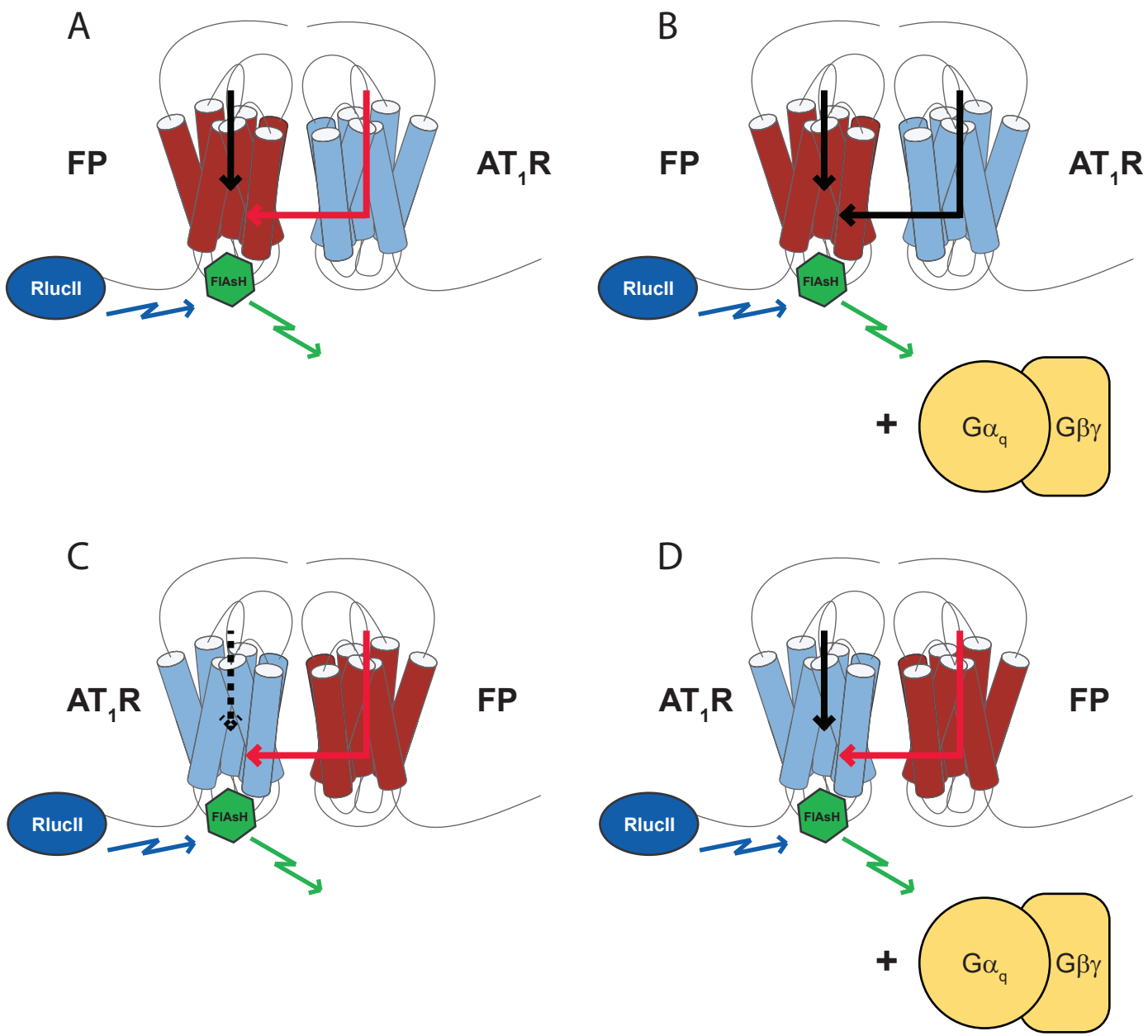


Figure 8



**Conformational biosensors reveal allosteric interactions between heterodimeric AT1
angiotensin and prostaglandin F2 α receptors**

Rory Sleno, Dominic Devost, Darlaine Pétrin, Alice Zhang, Kyla Bourque, Yuji Shinjo,
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