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Dominant negative actions of human prostacyclin receptor variant through dimerization: implications for cardiovascular disease

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Abstract

Objective—Prostacyclin and thromboxane mediate opposing cardiovascular effects through their receptors, the IP and TP, respectively. Individuals heterozygous for an IP variant, IP^{R212C}, displayed exaggerated loss of platelet IP responsiveness and accelerated cardiovascular disease. We examined association of IP^{R212C} into homo- and hetero- dimeric receptor complexes and the impact on prostacyclin and thromboxane biology.

Methods and Results—Dimerization of the IP, IP^{R212C} and TPα and was examined by Bioluminescent Resonance Energy Transfer in transfected HEK293 cells. We observed an equal propensity for formation of IPIP homo- and IPTPα hetero- dimers. Compared to the IP alone, IP^{R212C} displayed reduced cAMP generation and increased ER localization, but underwent normal homo- and hetero- dimerization. When the IP^{R212C} and IP were co-expressed a dominant negative action of variant was evident with enhanced wild type IP localization to the ER and reduced agonist-dependent signaling. Further, the TPα activation response, which was shifted from inositol phosphate to cAMP generation following IPTPα heterodimerization, was normalized when the TPα instead dimerized with IP^{R212C}.

Conclusions—IP^{R212C} exerts a dominant action on the wild type IP and TPα through dimerization. This likely contributes to accelerated cardiovascular disease in individuals carrying one copy of the variant allele.

Introduction

The prostanoids are formed following conversion of arachidonic acid by cyclooxygenases (COX)-1 and −2[1]. Thromboxane A_2 (TxA_2), which is derived predominantly from platelet COX-1, is a well-established contributor to cardiovascular disease (CVD)[2]. Indeed, the cardioprotective effects of aspirin result from irreversible inhibition of platelet COX-1-derived TxA_2[3], reflecting the central role of TxA_2 as a platelet agonist in CVD. A second major vascular prostanoid, prostacyclin (PGI_2), which is derived predominantly from vascular endothelial COX-2[4], is an established anti-thrombotic, anti-proliferative

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vasodilator[2]. PGI_2's cardiovascular effects - platelet inhibition, vasodilation and decreased proliferation - directly oppose those of TxA_2[4]. Indeed, depression of PGI_2 generation, with unrestricted biosynthesis of TxA_2 via COX-1, provides a mechanistic explanation for the cardiovascular hazard associated with selective COX-2 inhibitors[4].

Distinct G-protein-coupled receptor (GPCR) signaling pathways drive the opposing effects of PGI_2 and TxA_2[1, 2]. PGI_2, via its receptor (the IP), elevates cellular cAMP while activation of the TxA_2 receptor (the TP) elevates inositol phosphates (InsP) and intracellular calcium. We[5, 6], and others[7, 8], report that, similar to other GPCRs[9], IP and TP associate to form homo- and hetero-dimers. Interestingly, hetero-dimerization with the IP facilitated coupling of the TPα to cAMP generation (an IP-like cellular response)[6] and rendered the TP sensitive to regulation by IP agonists[5]. Importantly, this occurred not only in transfected cells but also in vascular smooth muscle cells (SMC), which endogenously express IP and TP. Therefore, when IP and TP are present in the same cell, a common occurrence in cardiovascular tissues, cAMP can be generated via the PGI_2-IPIP and TxA_2-IPTP pathways. This signaling shift likely contributes to the limit placed by the IP on the deleterious cardiovascular effects of TP activation.

Genetic IP variants display altered expression, ligand binding and function[10]. One variant, IP^{R212C}, which occurs at low frequency (0.8% in white and Asian cohorts), displayed impaired signaling and was associated with accelerated CVD in two studies[11, 12]. Strikingly, all but one IP^{R212C} carrier identified to date are heterozygous for the mutant. Platelets from IP^{R212C} heterozygote individuals are functionally non-responsive to a PGI_2 analog[11]. This contrasts with the gene dosage effect in mice heterozygous for IP deletion in which the *ex vivo* platelet response to a PGI_2 analog, as well as accelerated thrombosis *in vivo*, fell midway between wild type and IP null animals[13]. Taken together, these data suggest a dominant negative action of IP^{R212C} on the wild type receptor in heterozygous individuals. Whether and how IP^{R212C} modifies wild type IP function has not been examined. However, according to the current information regarding structural requirements for Class A GPCRs dimerization[14, 15], IP^{R212C} should dimerize normally. Since IP is a dimeric partner for at least two GPCRs – itself[7], to form IPIP homodimers, and the TPα, to form IPTPα heterodimers[5, 6] - this raises the intriguing possibility that the mutant may modify the function of an associated receptor in a homo- or hetero- dimeric assembly. The aim of this study was to examine the effect of IP^{R212C} on its dimeric partners. We evaluated whether dimerization of IP^{R212C} with the wild type IP might account its dominant negative action and whether association of IP^{R212C} with IP and/or TP may contribute to accelerated CVD in IP^{R212C} carriers.

Methods

Detailed methods are provided as supplemental information. Receptors were fused to renilla luciferase (rLUC) or yellow fluorescent protein (YFP) or tagged with hemagglutinin (HA) or Myc epitope tags. Receptor dimerization (by Bioluminesence Resonance Energy Transfer), signaling (by quantifying cellular cAMP and InsP generation) and cellular localization (by immunofluorescent microscopy) were examined in HEK293 cells, 48 hours after transfection.

Results

Homodimerization of IP and TPα by BRET

IP homodimerization has been observed, previously, by Western blot and co-immunoprecipitation[6, 7]. Because these are qualitative methods for indirect analysis of dimerization, we first confirmed and refined these studies using a quantitative methodology,

Bioluminesence Resonance Energy Transfer (BRET)[15]. We chose the BRET[1] method, in which receptors are fused to either a donor (renilla luciferase; rLuc) or acceptor (yellow fluorescent protein; YFP)[16]. Transfer of energy, upon addition of rLuc substrate, reflects the physical interaction of donor and acceptor to within 10nm.

IP homodimerization was confirmed in HEK293 cells transfected with IPrLuc plus IPYFP. In BRET saturation experiments a constant amount of IPrLuc was combined with gradually increasing levels of IPYFP. Receptor expression was at low physiologically relevant levels (<50 fmol/mg protein by ^{3}H-iloprost binding to membranes prepared from transfected cells). The IPrLuc:IPYFP curve was saturable, indicating a specific interaction (Fig 1A and 1B). Total YFP emission was measured, in separate samples, to quantify IPYFP expression (Fig 1B). The $BRET_{50}$ (the level of YFP fusion protein, as fold over basal, that produced 50% of the maximal signal) was 1.27 ± 0.06 for IP homodimerization, consistent with high affinity association. Lower non-saturable BRET was observed when IPrLuc was combined with YFP-fused DP_1, a receptor for PGD_2 (Fig 1A and 1C), indicating no interaction. PGD_2 is vasoactive prostaglandin that, similar to PGI_2, mediates vasodilation and platelet inhibition via a Gs-coupled receptor[17], making it an excellent and biologically relevant control. The absence of a IP + DP_1 signal was not due to poor DP_1YFP expression; in unrelated studies that used DP_1YFP as an acceptor, a normal and saturable BRET signal was recorded (not shown). Low non-saturable BRET was also observed when IPrLuc was combined with empty YFP vector (not shown).

We next preformed competition analyses in which BRET was measured with or without unfused (i.e. neither rLuc- nor YFP- fused) HA-tagged IP. Inclusion of HA-IP significantly reduced the IPrLuc:IPYFP BRET signal (Fig 1D). The apparently small absolute reduction in BRET likely reflects the array of possible assemblies that arise from the combination of three IP species (IPrLUC, IPYFP and HA-IP). Thus, each monomer can interact with an identical or alternatively labeled monomer. However, it is only through interruption of the IPrLuc:IPYFP association that unfused HA-IP can alter the BRET signal, resulting in the apparently small reduction. The consistent and highly significant reduction in BRET by unfused IP supports strongly the saturation data and validates our BRET[1] system for measurement of receptor dimerization.

High affinity TPα homodimerization was also readily evident by BRET saturation analysis (Fig 1E and 1F; $BRET_{50}$ = 1.24 ± 0.1, n=6). Thus, similar to the IP, high affinity association of the TPα to form homodimers was observed.

Heterodimerization of IP with TPα by BRET

We reported previously heterodimerization of the TPα with the IP, with consequent changes in TPα signaling and regulation[5, 6]. Here, we confirmed IPTPα heterodimerization by BRET. IPrLuc:TPαYFP BRET was saturable (Fig 2A and 2B; $BRET_{50}$ = 1.49 ± 0.4; n=4). Interestingly, despite the clear indication of IPTPα heterodimerization by BRET (and by co-immunoprecipitation[6]), unfused HA-IP did not reduce the IPrLuc:TPαYFP BRET signal. This was evident with either an excess (20×) of HA-IP (Fig 2C), or with increasing HA-IP combined with a single mid-range level of IPrLuc + IPYFP (Fig 2D). Similar results were obtained when unfused TPα was the competitor (not shown). These data support the notion that IPTPα heterodimerization occurs in a manner that is distinct from the IPIP homodimers.

IP^{R212C} dimerization with IP or TPα

Structural studies suggest that the dimerization of Class A GPCRs is mediated through their transmembrane domains[18, 19], however the specific site(s) that direct IP dimerization is unknown. It was unclear, therefore, whether the IP^{R212C} would display an altered propensity

to dimerize. We examined dimerization of IP^{R212C} with itself, with wild type IP or with TPα. A specific and saturable interaction was evident in IP^{R212C}rLucI:IP^{R212C}YFP saturations (Fig 3A and 3B, $BRET_{50}$ = 1.13±0.08, n=3). Similar results were obtained with IPrLUC + IP^{R212C}YFP or IP^{R212C}rLuc + IPYFP (Fig 3A). Apparent differences in the curves in Fig 3A most likely arise from differences in the efficiency of IP versus IP^{R212C} fusion protein expression. IP^{R212C} reflect also dimerized efficiently with the TPα (Fig 3C). Together, these data indicate no detectable impact of the mutation on IP^{R212C} homo- or hetero- dimerization (Supplemental Table 1).

Cellular localization of IP and IP^{R212C}

IP^{R212C} is retained in the ER, reducing its plasma membrane expression[11]. We considered whether, similar to other ER retained mutant GPCRs[20], IP^{R212C} could promote ER retention of the wild type IP. Myc-IP and HA-IP^{R212C} were expressed, alone or in combination. Because of the equivalent affinity for IP to interact with another IP or with IP^{R212C} (Supplemental Table 1), we used IP^{R212C} at a 2:1 excess, minimizing the amount of wild type IP not associated with the variant. When transfected alone, Myc-IP was distributed diffusely in cytoplasm, ER and plasma membrane (Fig 4A and supplemental Fig S1A and B). This pattern is typical for transiently transfected GPCRs and was observed also in cells transfected with HA-DP1 (Fig 4B). MycIP was clearly observed in regions that were negative for the ER marker calnexin (Supplemental Fig S1A and B; dotted lines) and was not modified by co-expression of HA-DP1, a non-interacting control (Fig 4B). As previously reported[11], HA-IP^{R212C} was concentrated in the ER (Fig 4C). When coexpressed with HA-IP^{R212C}, Myc-IP distribution was markedly altered with intense localization of the wild type receptor in focal calnexin-positive areas (Fig 4C and Supplemental Fig S1C). These data provide qualitative support for the physical interaction of IP and IP^{R212C} leading to ER retention of the wild type receptor.

Effect of IP^{R212C} on Wild Type IP function

Accelerated CVD in IP^{R212C} heterozygote individuals was associated with an exaggerated loss of IP-dependent platelet cAMP generation to non-functional levels[11]. We considered, therefore, the impact of IP^{R212C} on wild type IP function. First, HEK293 cells were transfected individually, with HA-IP^{R212C} or HA-IP, and the response to a PGI_2 analog (cicaprost) examined. Significantly lower cAMP was generated via IP^{R212C} compared with IP (Fig 5A), confirming the mutant's previously reported signaling deficiency[11]. The IP^{R212C} response remained depressed when the variant was increased to 2× (Fig 5B), 3× (Fig 5D, white bar) or 4× (not shown), in single transfectants. Thus, the maximal ability for IP^{R212C} to signal was significantly below that of the wild type receptor.

In cells cotransfected with HA-IP^{R212C} + HA-IP (2:1), cAMP generation was significantly lower that the expected additive value calculated from parallel single transfections (Fig 5C). This impaired response was also observed when IP^{R212C} was introduced at 2× or 3× the level of the co-expressed wild type IP (Fig 5D, hatched and striped columns). Indeed, across all single and co-transfection experiments that included IP^{R212C}, cAMP levels were depressed by 50–70% compared to the wild type IP alone (Fig 5D). Thus, IP^{R212C} acted in a dominant fashion to “impose” its signaling deficiency on the wild type receptor. We hypothesize that this occurs through interruption of IPIP homodimerization and/or formation of an IPIP^{R212C} dimer.

IP^{R212C} does not Facilitate TPα-cAMP Signaling

When associated with the IP, activation of the TPα led to a robust cAMP response[6]. We posited this as a mechanism through which the IP can limit TP function. Given that IP^{R212C} efficiently heterodimerizes with the TPα, we considered the impact of IP^{R212C} on TPα

signaling. Co-expression of the wild type IP (2×) with TPα (1×) facilitated a cAMP response to TPα activation (Fig. 6A), as reported[6]. In contrast, the cAMP response to a TxA_2 analog, IBOP, in IP^{R212C} + TPα co-transfectants, was returned similar to that observed in parallel singly TPα-transfected cells (Fig 6A). These data demonstrate that, although its ability to heterodimerize with the TPα was intact, IP^{R212C} did not recapitulate the impact of the wild type IP on TPα-cAMP signaling.

IP^{R212C} was less effective that IP in reducing TPα-InsP signaling

Finally, we examined how IP and IP^{R212C} modified the ability of TPα to activate phospholipase C. When cotransfected with wild type IP (2×), the InsP response to TPα (1×) activation was significantly reduced (Fig 6B). Taken together with the data in Figure 6A, it appears that, when heterodimerized with IP, TPα coupling was shifted from phospholipase C to adenylyl cyclase. In contrast, and concomitant with the absence of a TPα-cAMP signal, IBOP-induced InsP generation was normalized in HA-IP^{R212C} (2×) + HA-TPα (1×) cotransfectants (Fig 6B). This is consistent with the concept that the mutant was unable to shift TP signaling and instead allowed the InsP signal to dominate. Thus, the deficiency associated with the IP^{R212C} extended beyond its own signaling pathway to impact its heterodimeric partner.

Discussion

Studies in mice and humans indicate the critical contribution made by PGI_2-IP and TxA_2-TP to cardiovascular function and disease[1, 2]. Similar to other GPCRs, the IP and TP form homo- and hetero- dimers, that may be integral to their function and regulation[5–8]. Of particular relevance to CVD, we reported how heterodimerization with the TP may contribute to the limit placed by the IP on TxA_2 function[5, 6]. Recent studies reveal a significant level of genetic variability in the human IP[21] with at least one variant, IP^{R212C}, associated with accelerated CVD[11]. Since expression of IP^{R212C} is almost exclusively heterozygous[11], we considered it important to examine this variant in the context of co-incident expression of wild type IP, as well as TP. We reasoned that IP^{R212C} could alter, through dimerization, the function of wild type IP in heterozygote individuals. Furthermore, IP^{R212C} may compete with the IP for heterodimerization with the TP. Either or both of these events may modify the cardiovascular response to PGI_2 and/or TxA_2.

We confirmed the IP^{R212C} signaling deficiency and report its normal capacity to homo- and hetero- dimerize. We describe a dominant negative action of IP^{R212C} on wild type IP function, which enhanced ER retention of the IP and reduced signaling in response to a PGI_2 analog. These observations provide a mechanistic basis for the exaggerated impact of IP^{R212C} on the cAMP response to a PGI_2 analog in platelets from heterozygous individuals[11]. Further, the response to a TxA_2 analog, which was shifted from InsP to cAMP generation following IPTPα heterodimerization, was normalized when the TPα instead dimerized with IP^{R212C}. Thus, a cellular mechanism through which the IP can limit TP function was minimized likely contributing to accelerated CVD in IP^{R212C} carriers.

Our study is the first to use BRET to examine prostanoid receptor dimerization. In BRET studies it is critical to establish that energy transfer results from specific, non-random interactions between the rLUC-fused donor and YFP-fused acceptor. Protein conformation and 3-D structure can alter acceptor/donor orientation. Hence the magnitude of the BRET signal does not fully reflect the amount of dimer or the affinity of the interactors. Consequently, it is insufficient to conclude protein interaction based on measurable BRET above background and it is critical to demonstrate saturable BRET[16, 22]. We performed BRET saturations combining a fixed level of donor with gradually increasing acceptor. BRET was plotted against the molar ratio of transfected receptor-YFP to receptor-rLuc to

assess saturability and reproducibility. In this analysis, saturation of the BRET signal indicates a specific interaction but the slope of the curve, which is dependent on the actual cellular expression of receptor-YFP achieved, does not reflect affinity. Therefore, BRET was also plotted against total YFP emission (excited at 485nm), expressed as fold over basal, as a measure of actual YFP-receptor expression. Saturation again indicates specificity, while the slope reflects affinity and may be quantified, in each data set, as the $BRET_{50}$.

A highly reproducible and saturable BRET signal was recorded in cells transfected with IPrLUC + IPYFP, consistent with a high affinity homodimerization. Similar high affinity interactions were evident for TP homo- and IPTP hetero- dimerization, with no significant difference in $BRET_{50}$ for these three interactions (Supplemental Table 1). This is important when considering the likelihood of dimerization of native receptors. Indeed, although saturable BRET was observed in αA1-adrenoreceptor + ∂-opioid receptor co-transfection studies, the markedly higher $BRET_{50}$ value for the heterodimer suggests that this interaction is unlikely to occur *in vivo*[23]. Our data supports equal propensity for IP and TP homo- and hetero- dimerization providing for at least three native dimeric species – IPIP, TPTP and IPTP. Concordantly, we observed cellular signaling consistent with formation of all three species in aortic SMC[6], supporting homo- and hetero- dimerization of the native receptors.

Fusion to energy donor or acceptor moieties is essential for BRET thus we relied on a transfection system for our studies. This is a major limitation of BRET and raises concerns of artifactual interactions. We are confident that this is not the case for several reasons. First, receptor expression in BRET studies was at low physiological levels. Second, we examined extensively saturation of BRET, and not simple measurement of a BRET signal, as evidence of true dimerization. Finally, the lack of saturable BRET between IP and DP1 strengthens considerably our confidence that BRET is not an artifact of receptor expression in non-native cells. We recognize, however, the importance of confirming our BRET studies in native cells.

Currently it is believed that the association of GPCRs occurs in the ER from where correctly folded and dimerized receptors are transported to the Golgi. Indeed, dimer/oligmerization may be a necessary quality control step for ER/Golgi export[9, 15]. The factors and molecular pathways that regulate this process are largely unknown but the concentration of individual receptors may be a contributing factor. As an example, augmented expression of the B2 receptor for bradykinin increased heterodimerization with the AT1 angiotensin II receptor with consequent elevation in angiotensin II responses[24]. We reported recently augmented TP expression following its activation[25], a process that may contribute to elevated TP levels in animal models of CVD and in human disease. Our studies suggest that IP dimerization with the TP, and the consequent shift in TP signaling to cAMP generation, may limit the negative impact of enhanced TP expression. Other factors, that may regulate the dimerization process, such as association with G proteins or chaperone proteins, are a focus of our current research.

We noted a distinct difference between the process of IP homodimerization compared with IPTP heterodimerization. While IPrLuc:IPYFP BRET was reduced by an excess of unfused IP, neither unfused IP nor TPα modified the IPrLuc:TPαYFP signal. The trivial explanation, that the unfused receptors simply did not interact with the IPrLuc:TPαYFP heterodimer, seems unlikely. Both BRET saturation (Fig 2) and co-immunoprecipitation[6] experiments clearly demonstrate IP-TP interaction. It is possible that the presence of rLuc and/or YFP sterically prevented competitor access to the site of heterodimer interaction. However, our competition studies with IPrLuc:IPYFP homodimers, together with reports using similarly labeled GPCRs[26], argue against such blockade. It is more likely, therefore, that within the IPTPα heterodimer the site(s) for IP and TPα homodimerization remain accessible, leaving

the IPrLuc-TPαYFP BRET signal unchanged by excess unfused receptors. Multiple dimerization motifs[18, 19] are present in the IP and TP, making it possible that distinct interactions direct their homo- and heterodimerization. Indeed, our data is consistent with oligomerization, a phenomenon reported for several Class A GPCRs[18, 27, 28], in which the IPTPα heterodimer can bind additional IP and/or TPα molecules. The design of the current BRET studies does not discriminate between formation of dimers and oligomers. Currently, we are using a modified BRET technique to investigate IP-TP oligomerization.

Impaired cAMP generation via IP^{R212C} was evident in HEK293 (Fig 5A) and COS-7[11] cells. Platelets from IP^{R212C} heterozygote individuals displayed an exaggerated loss of PGI_2 responsiveness[11] that exceeded the relative impairment observed in IP+/− heterozygous mice[13], consistent with a dominant negative action of the variant. We established IP^{R212C} dimerization with the wild type IP and observed a dominant impact of the mutant on wild type IP cellular distribution and function. We believe that, through formation of the IP:IP^{R212C} dimer, loss of PGI_2 responsiveness is augmented beyond the IP^{R212C}'s discrete signaling deficiency. In addition, co-expression of the TP with IP^{R212C} markedly modified TP signaling, compared to TP + wild type IP co-transfectants, such that “normal” TP signaling was restored. Thus, along with the depression of wild type IP function, IP^{R212C} fails to allow generation of a TP-dependent cAMP signal. Instead, InsP generation approached the level recorded in cells expressing the TP alone. Thus, the overall outcome in IP^{R212C}-expressing cells, stimulated with either IP or TP agonists, was less cAMP and more InsP. In functional terms this would facilitate deleterious cardiovascular events contributing to the accelerated CVD observed in IP^{R212C} carriers[11].

It is unclear how IP^{R212C} affected signaling changes in its dimeric partners. IP^{R212C}, which is itself concentrated in the ER (reference 11 and Fig 4C) promoted retention of the wild type receptor to the ER. This is consistent with IP homodimerization early in biogenesis and suggests that signaling changes reflect reduced surface receptor expression. In native cells it is difficult, and misleading, to express sufficiently high levels of IP^{R212C} to impact the wild type receptor. Thus, we have not confirmed IP^{R212C}-dependent changes in localization of the endogenous IP. It is also possible that the presence of IP^{R212C} interferes with G protein-coupling of the partner receptor modifying directly the signal. Indeed, GPCRs are proposed to function as a pentameric complex containing a receptor dimer and one heterotrimeric G protein[29, 30]. Recent elegant investigations of the dopamine D2 receptor demonstrated allosteric modulation of one receptor by the other, and transmission of an altered conformation across the dimer interface. Thus, the intrinsic changes associated with the IP^{R212C} mutation may be transmitted to its dimeric partners modifying the signaling outcome. Given that cAMP generation via both IP and TP were offset by IP^{R212C}, we speculate that a modified IP^{R212C}-Gs interaction controls the signaling outcome of the dimeric complex. In keeping with this model, when complexed to the IP^{R212C}, the TP can signal through a Gq-InsP pathway, perhaps because TP-Gq coupling is now permitted.

Numerous studies support the critical anti-thrombotic and cardiovascular protective role of PGI_2 as a natural restraint on vasoactive mediators including TxA_2[2, 4]. Our data suggests that dimerization of the IP and the TP is an integral part of this system. Our studies support the concept that considering the IP and TP as discrete signaling entities is an oversimplification of biological reality. Full understanding of these pathways will require definition of the cell- and context- specific parameters that direct homo- and hetero-dimerization in native cells. Importantly, however, our study provides the first evidence for allosteric influence of genetic receptor variants on the function of other receptors providing a mechanistic basis for dominant effects through dimerization.

Condensed abstract

Individuals heterozygous for a variant prostacyclin receptor, IP^{R212C}, display exaggerated loss in platelet IP responsiveness and accelerated cardiovascular disease. We report a dominant effect of IP^{R212C} on the wild type IP, and on the receptor for thromboxane, through dimerization as a mechanism underlying deleterious cardiovascular outcomes in IP^{R212C} carriers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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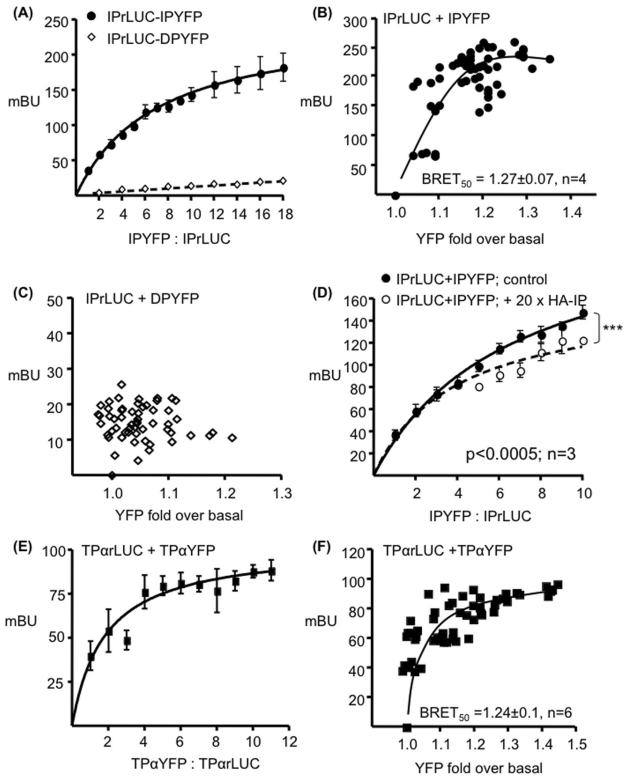

Figure 1. BRET analysis of IP and TP homodimerization

HEK293 cells were transfected with a fixed amount (0.1 μg) of (A-D) IPrLUC or (E, F) TPαrLUC together with increasing amounts of (A, B, D) IPYFP, (A, C) DP_1YFP or (D, E) TPαYFP. BRET saturation curves were constructed by plotting milli BRET units (mBu) against (A, C, D, E) the relative amount of receptor-YFP transfected or (B, F) direct YFP fluorescence (fold over basal). In (D) the BRET was examined in the presence or absence of HA-IP at 20 times IPrLUC. Data are mean ± sem of 6–8 experiments unless otherwise indicated; B, C and F are representative experiments, that were repeated several times with similar results. $BRET_{50}$ values are mean YFP fold over basal ± sem.

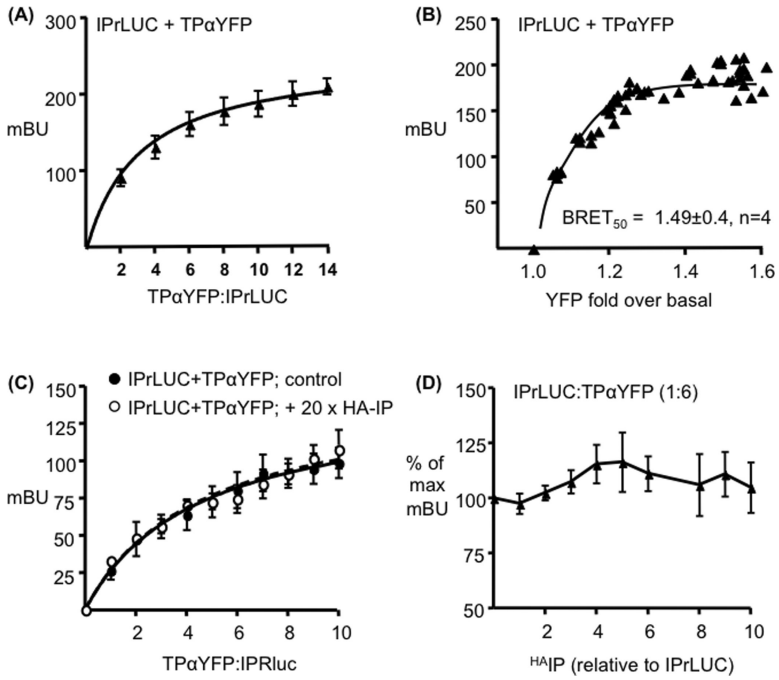

Figure 2. BRET analysis TPαIP heterodimerization

(A-C) HEK293 cells were transfected with a fixed amount (0.1 μg) of IPrLUC together with increasing amounts of TPαYFP. BRET saturation curves were constructed by plotting milli BRET units (mBu) against (A and C) the relative amount of receptor-YFP transfected or (B) direct YFP fluorescence (fold over basal). In (C) the BRET signal was examined in the presence or absence of HA-tagged IP at 20 times IPrLUC. In (D) cells were co-transfected with IPrLUC:TPαYFP (1:6) plus increasing amounts of HA-IP. Data are mean ± sem of 4–5 experiments; B is a representative experiment, that was repeated several times with similar results. The $BRET_{50}$ for IPTP heterodimerization is given as mean YFP fold over basal ± sem.

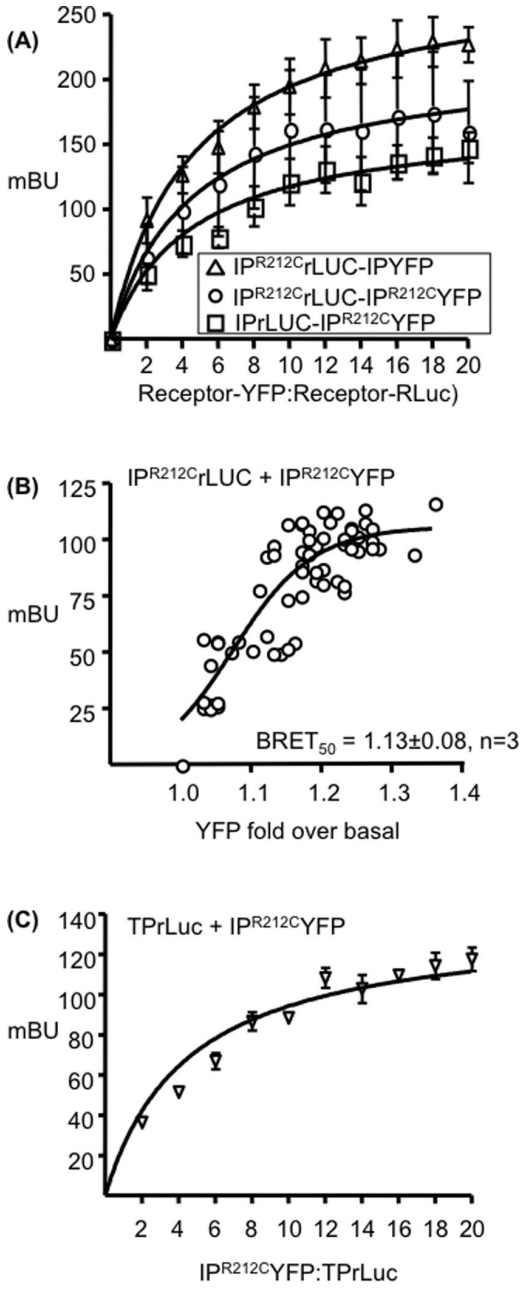

Figure 3. BRET analysis of IP^{R212C} homo- and heter- dimerization

HEK293 cells were transfected with a fixed amount (0.1 μg) of receptor-rLUC together with increasing amounts of receptor-YFP. BRET saturation curves were constructed by plotting milli BRET units (mBu) against (A and C) the relative amount of receptor-YFP transfected or (B) direct YFP fluorescence (fold over basal). Data are mean ± sem of 3–5 experiments (A and C); B is a representative experiment, that was repeated several times with similar results. The $BRET_{50}$ for $IP^{R212C}IP^{R212C}$ homodimerization is given as mean YFP fold over basal ± sem.

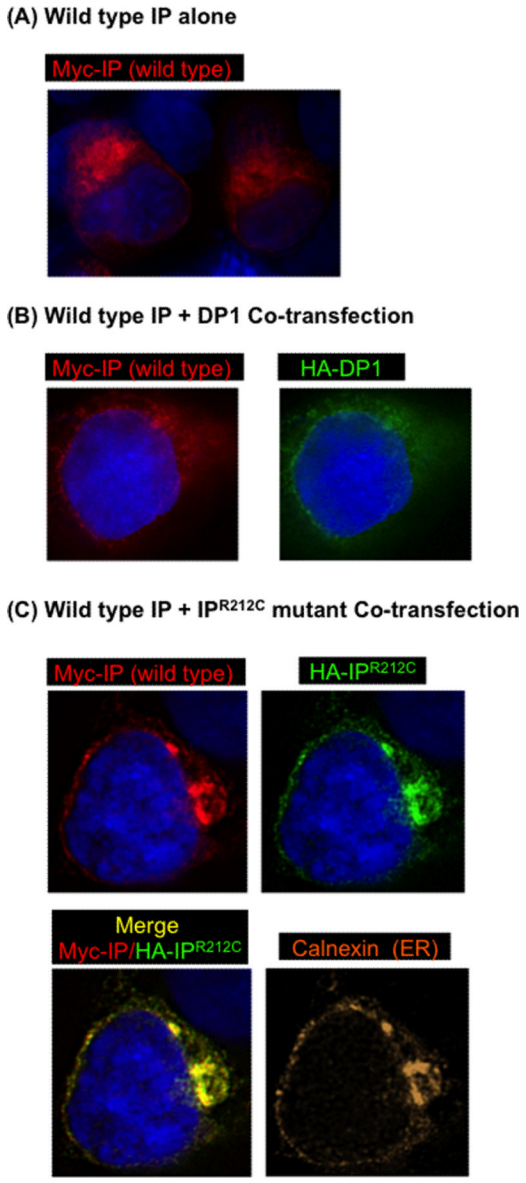

Figure 4. Cellular localization of wild type IP and IP^{R212C}

HEK293 cells were transfected with Myc-IP (A) alone or in combination with (B) 2 fold HA-DP1 or (C) 2 fold HA-IP^{R212C}. Receptor localization was examined by deconvolution immunofluorescence microscopy in cells co-stained for endoplasmic reticulum (ER; calnexin) and nuclei (DAPI, blue stain).

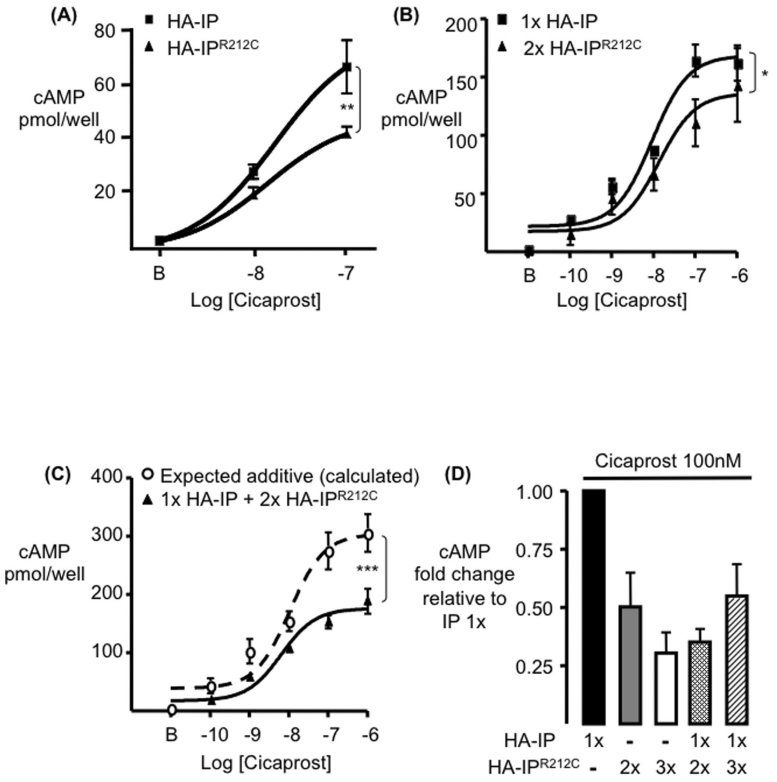

Figure 5. Impact of IP^{R212C} on wild type IP signaling

HEK293 cells were transfected with HA-IP, with or without HA-IP^{R212C}. HA-IP^{R212C} quantities are given as relative (2x, 3x) to the amount of HA-IP transfected (1x) except in (A) where equivalent levels were used. Cells were treated with an IP agonist, cicaprost, and cAMP quantified. (A) Comparison of equivalent levels of HA-IP versus HA-IP^{R212C}. (B) Comparison of 1x HA-IP versus 2x HA-IP^{R212C}. In (C) the expected additive was calculated from cells transfected with either 1x HA-IP or 2x HA-IP^{R212C}. In (D) cAMP levels were normalized to cells transfected with 1x HA-IP alone. Data are mean ± sem of 3 experiments. * $p<0.05$; ** $p<0.01$; ***$p<0.0005$

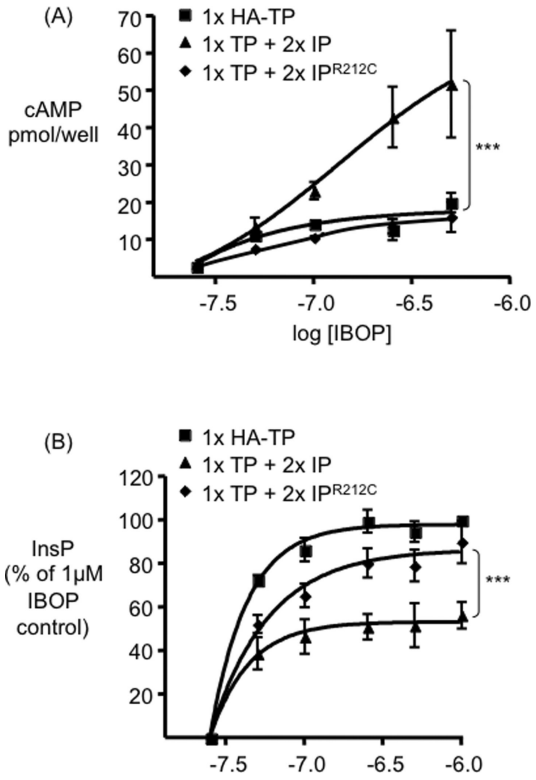

Figure 6. Impact of IP^{R212C} on TP and IPTP signaling

HEK293 cells were transfected with HA-TP, with or without HA-IP or HA-IP^{R212C}. IP constructs were present at twice (2x) the level of the TP. Cells were treated with a TP agonist, IBOP, for 10 min and (A) cAMP or (B) inositol phosphate (InsP) levels quantified. Data are mean ± sem or 3 experiments. *** $p<0.0005$.