Demonstration of a Direct Interaction between β₂-
Adrenergic Receptor and Insulin Receptor by BRET and
Bioinformatics

Maja Mandić¹, Luka Drinovec², Sanja Glisic³, Nevena Veljkovic³, Jane Nøhr⁴, Milka Vrecl¹*

¹ Institute of Anatomy, Histology & Embryology, Veterinary Faculty, University of Ljubljana, Ljubljana, Slovenia, ² Development Department, Aerosol d.o.o., Ljubljana, Slovenia, ³ Center for Multidisciplinary Research, Institute of Nuclear Sciences VINCA, University of Belgrade, Belgrade, Serbia, ⁴Department of Incretin & Islet Biology, Novo Nordisk A/S, Målev, Denmark

Abstract

Glucose metabolism is under the cooperative regulation of both insulin receptor (IR) and β₂-adrenergic receptor (β₂AR), which represent the receptor tyrosine kinases (RTKs) and seven transmembrane receptors (7TMRs), respectively. Studies demonstrating cross-talk between these two receptors and their endogenous coexpression have suggested their possible interactions. To evaluate the effect of IR and prospective heteromerization on β₂AR properties, we showed that IR coexpression had no effect on the ligand binding properties of β₂AR; however, IR reduced β₂AR surface expression and accelerated its internalization. Additionally, both receptors displayed a similar distribution pattern with a high degree of colocalization. To test the possible direct interaction between β₂AR and IR, we employed quantitative BRET² saturation and competition assays. Saturation assay data suggested constitutive β₂AR and IR homo- and heteromerization. Calculated acceptor/donor (AD) values as a measure of the relative affinity for homo- and heteromer formation differed among the heteromers that could not be explained by a simple dimer model. In heterologous competition assays, a transient increase in the BRET² signal with a subsequent hyperbolic decrease was observed, suggesting higher-order heteromer formation. To complement the BRET² data, we employed the informational spectrum method (ISM), a virtual spectroscopy method to investigate protein-protein interactions. Computational peptide scanning of β₂AR and IR identified intracellular domains encompassing residues at the end of the 7th TM domain and C-terminal tail of β₂AR and a cytoplasmic part of the IR β chain as prospective interaction domains. ISM further suggested a high probability of heteromer formation and homodimers as basic units engaged in heteromerization. In summary, our data suggest direct interaction and higher-order β₂AR:IR oligomer formation, likely comprising heteromers of homodimers.

Citation: Mandić M, Drinovec L, Glisic S, Veljkovic N, Nøhr J, et al. (2014) Demonstration of a Direct Interaction between β₂-Adrenergic Receptor and Insulin Receptor by BRET and Bioinformatics. PLoS ONE 9(11): e112664. doi:10.1371/journal.pone.0112664

Editor: Laszlo Buday, Hungarian Academy of Sciences, Hungary

Received June 7, 2014; Accepted October 6, 2014; Published November 17, 2014

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: The authors acknowledge funding from the Basileus S Program to M. Mandić, the Slovenian Research Agency program (P4-0053) to M. Vrecl, and the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant No. 173001) to S. Glisic and N Veljkovic. M. Vrecl, S. Glisic and N. Veljkovic participate in the European COST Action CM1207 (GLISTEN). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: It is true that two coauthors are employed by commercial companies; however this does not alter the authors’ adherence to PLOS ONE policies on sharing data and materials. The competing interests and financial disclosures of both co-authors employed by commercial companies are as follows: Luka Drinovec is employed by the company Aerosol d.o.o. This company produces instruments for the measurement of aerosolized soot (black carbon) and some accessories for aerosol measurement. Aerosol d.o.o. does not have any connection with molecular biology, development of medicines, etc. Thus, the authors declare that there are no competing interests for Luka Drinovec coauthoring the article “Demonstration of a direct interaction between β₂-adrenergic receptor and insulin receptor by BRET and bioinformatics”. Response to Reviewers: Jane Nørh is employed by Novo Nordisk A/S and owns Novo Nordisk A/S company stocks. The work on this paper was done as a part of collaboration with academia, i.e., co-supervision of Maja Mandic at the Veterinary faculty, University of Ljubljana. Therefore, the authors declare that there are no competing interests for Jane Nøhr coauthoring the article, “Demonstration of a direct interaction between β₂-adrenergic receptor and insulin receptor by BRET and bioinformatics”.

* Email: milka.vrecl@vf.uni-lj.si

Introduction

The functional interplay between different classes of receptors represents a means of fine-tuning the control of cellular functions that could be fundamental for understanding pathologic conditions and responses to therapeutic agents that interact with cell-surface receptors. Originally, 7 transmembrane receptors (7TMRs) and receptor tyrosine kinases (RTKs), together with their respective downstream effectors, were thought to represent distinct and linear signaling units; however, recent studies have provided evidence for functional crosstalk between these different types of receptors [1].

Glucose metabolism is under the cooperative regulation of the RTK insulin receptor (IR) and β₂-adrenergic receptor (β₂AR), a representative 7TMR. The anabolic action of insulin promotes glycogen synthesis, glucose uptake in skeletal muscle and lipid storage. By contrast, catecholamines act in opposition to insulin, stimulating glycogen breakdown, gluconeogenesis, and lipolysis [2]. They play an important role in counter-regulation of insulin-induced hypoglycemia. The ability of insulin to counter regulate
Interaction between β2AR and IR

catecholamine action at the tissue/cellular level is crucial for the “tight” regulation of serum glucose levels, and interaction between IR and β2AR could be a required action in this process.

At the molecular level, the ability of insulin to counter regulate β2ARs can be exerted through insulin-stimulated phosphorylation of β2AR and its subsequent internalization. An early in vitro study demonstrated that, in the presence of insulin, IR catalyzes the phosphorylation of β2AR predominantly at residues located in the cytoplasmic tail of β2AR-IR, Tyr350/Tyr354, and Tyr364 [3]—a finding that was consistent with the hypothesis that IR directly interacts with and phosphorylates β2AR. Phosphorylated β2AR binds to tyrosine phosphorylated PI3K, resulting in the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K) or the GTPase dynamin; all of the latter molecules are involved in 7TMR trafficking [4,5,6]. In addition, insulin-induced activation of MAPKs, ERK 1 and ERK 2, is potentiated by β2AR via a mechanism that requires the integrity of Tyr350 [4]. Thus, the functional cross-talk between β2AR and IR may be to fine tune the signals from multiple receptor signaling pathways. Recently, Fu et al. [7] described an IR-β2AR complex in the mouse heart, indicating that cross-talk between these two receptors could provide a molecular basis for the pathophysiology of metabolic and cardiovascular dysfunction under insulin-resistant states. β2AR and IR are also endogenously coexpressed in other cell types—i.e., pancreatic beta cells, adipocytes, liver cells, skeletal muscle cells and astrocytes [8,9,10]. Therefore, the formation of heteromeric complexes consisting of these two receptors may be plausible.

In the past decade, the concept that 7TMRs can exist as dimers or higher-order oligomers has advanced rapidly [11,12], and biophysical techniques based on resonance energy transfer (RET) such as BRET have become methods of choice in receptor oligomerization studies (recently reviewed in [13,14,15,16,17,18]). Utilizing BRET, β2AR homo- and heteromerization with different 7TMR family members have been reported [19,20,21,22,23,24,25].

In contrast to 7TMR-based BRET studies, BRET has been less exhaustively employed for RTK-based studies, and an overview of BRET studies associated with RTKs was recently reviewed by Issad et al. [26] and Siddiqui et al. [27]. RTKs often employ dimerization as a key factor in their activation; in fact, members of the IR subfamily are pre-dimerized by disulfide bonds (reviewed by De Meys [28]). In IR studies, BRET was used to monitor the activation state of IR [29,30,31] and its interactions with different intracellular binding partners, including protein tyrosine phosphatase-1B [32], Grb14 [33] and IRS1, IRS4 and She [34]. IR can also form hybrid heterotrimers with the insulin-like growth factor-1 (IGF-1) receptor, exemplifying that IR can comprise heteromers that are products of independent genes [35].

In addition to interfamily receptor heteromerization—i.e., 7TMR/7TMR and RTK:RTK pairs—a physiologically relevant, direct physical interaction between the 7TMR adenosine A2A receptor (A2A-AR) and RTK fibroblast growth factor receptor (FGFR) was demonstrated [36]. Evidence for other 7TMR:RTK heteromeric complexes consisting of these two receptors may be plausible.

Materials and Methods

Materials

Molecular biology reagents, as well as the tissue culture media and reagents, were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Gibco Invitrogen Corporation (Breda, the Netherlands), unless otherwise specified. [125I]-Iodopindolol was obtained from PerkinElmer (Boston, MA, USA). The ligands pindolol and isoproterenol were purchased from Sigma-Aldrich, and recombinant human insulin S100 was from Novo Nordisk A/S (Bagsværd, Denmark). Coelenterazine 400a was purchased from Biotrend Chemikalien GmbH (Köln, Germany). Anti-hemagglutinin (HA) high-affinity rat monoclonal antibodies were from Roche (Basel, Switzerland). Anti-rat horseradish peroxidase (HRP)-conjugated antibodies and anti-rat TRITC-conjugated antibodies were from Sigma-Aldrich.

Receptor fusion constructs

Human HA-tagged β2AR (HA-β2AR) cDNA in the vector pcDNA3.1 was purchased from Missouri S&T cDNA Resource Center (University of Missouri-Rolla, USA). HA-β2AR C-terminally tagged with Renilla luciferase 8 (RLuc8) (β2AR-RLuc8) was made using standard molecular biology techniques and was verified by sequencing. WT human IR isoform A lacks exon 11 and IR C-terminally tagged with the green fluorescent protein variant 2 (IR-GFP2) were generated and verified at Novo Nordisk A/S. C-terminally RLuc8-tagged IR (IR-RLuc8), C-terminally GFP2-tagged β2AR (β2AR-GFP2) and the membrane-inserted GFP2-tagged construct (GFP2-17aa) were the same as described previously [24,34,45]. All of the generated cDNA clones were inserted into the expression vector pcDNA3.1(+) .

Cell culture and transfection

HEK-293 cells (European Collection of Animal Cell Cultures, Salisbury, UK) were routinely maintained and passaged in Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM Glutamax-I, penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere of 5% (v/v) CO2. For transient transfection, HEK-293 cells were seeded at a density of ~1×106 cells per 60-mm tissue culture dish or at a density of 4×106 cells per 75-cm2 flask and transfections were performed the following day using Lipofectamine 2000 according to the manufacturer’s instructions. Cells were harvested 48 h after transfection, the cell number was determined using a cover-slipped hemocytometer, and the cells were resuspended in Dulbecco’s PBS supplemented with 0.2% (v/v) fetal calf serum, 1 g/L glucose and 36 mg/L sodium pyruvate to a density of 1×106 cells/mL, unless otherwise stated.
Luminescence and fluorescence measurements

The expression levels of RLuc8- and GFP2-tagged receptor constructs were monitored by total luminescence and fluorescence measurements as previously described [46]. For luminescence measurements, ~2×10^6 of resuspended cells were distributed in 96-well microplates (white Optiplate; Packard Bioscience, Meriden, CT, USA). After the addition of coelenterazine 400a to a final concentration of 5 μM, total luminescence was measured using a TriStar LB 942 microplate reader (Berthold Technologies, Bad Wildbad, Germany). For fluorescence measurements, ~2×10^6 of resuspended cells from the same transfections were plated in black 96-well FLA plates (Greiner Bio-One, Frickenhausen, Germany). Total fluorescence was measured using the TriStar LB 942 microplate reader with an excitation filter at 380 nm and an emission filter at 515 nm. Background values obtained with mock-transfected HEK-293 cells were subtracted in both measurements, and the mean values of triplicate wells/sample were then calculated.

Receptor Binding Assay

To establish a relationship between the luminescence/fluorescence signals generated by RLuc8 and GFP2-tagged β2ARs and cell-surface receptor number, radioligand binding assays were carried out on whole cells as previously described [47]. After transfections with increasing amounts of cDNA (0.01 to 2 μg) for either β2AR-RLuc8 or β2AR-GFP2, cells were plated onto 24-well plates at a density of ~1×10^5 cells per well. An aliquot of cells (~5×10^5) from each transfection was also transferred into a 60-mm dish for total luminescence/fluorescence measurements as described above. For the whole-cell radioligand binding assay, cells were washed once with assay buffer (HEPES-modified DMEM with 0.1% bovine serum albumin (BSA)) before being incubated with the β2AR antagonist [125I]-iodopindolol (30,000 cpm/well) and increasing concentrations of unlabeled pindolol (10–12 to 10–5 M final concentration) in assay buffer for 2 h at 4°C. Cells were then washed 5 times with ice-cold PBS and solubilized with 0.2 M NaOH and 1% sodium dodecyl sulfate (SDS) solution, and then the radioactivity levels were determined using a γ counter (LKB Wallac, Turku, Finland). Determination of the radioactivity levels was performed in triplicate. Binding parameters were determined from displacement curves generated by a sigmoidal dose-response curve fit (GraphPad Prism 5.0). Receptor density (Bmax), expressed as the receptor number per cell, was calculated as previously described by Ramsay et al. [48]. Whole-cell radioligand binding assays were also performed with cells expressing β2AR alone or in combination with IR. Displacement curves were generated using [125I]-iodopindolol (30,000 cpm/well) and increasing concentrations of isoproterenol or isoproterenol and insulin, and IC50 values were then determined using GraphPad Prism 5.0.

ELISA

ELISA assays for the measurement of surface-expressed HA-β2AR and quantification of receptor internalization were performed as described previously [49]. Briefly, HEK-293 cells were transiently transfected with either β2AR-RLuc8 alone or β2AR-RLuc8 and IR-GFP2. One microgram of each receptor was used, and the total amount of cDNA used for transfection was kept uniform by adding empty pcDNA3.1 vector. After transfection, cells were seeded at a density of ~1×10^5 cells per well in a 24-well plate. An aliquot of cells (~5×10^5) from each transfection was also transferred into a 60-mm dish for total luminescence/fluorescence

Table 1. Pharmacological properties of β2AR fusion constructs expressed in HEK-293 cells.

| Receptor          | IC50 (nM)    |
|-------------------|-------------|
| HA-β2AR           | 1.58±0.26   |
| HA-β2AR-RLuc8     | 1.16±0.11   |
| β2AR-GFP2         | 1.88±0.58   |
| β2AR-RLuc         | 0.94±0.09a  |

aData from Vrecl et al. [47].

Table 2. Effect of IR coexpression on the pharmacological properties of β2AR in HEK-293 cells.

| Ligands          | IC50 (nM) |
|------------------|-----------|
|                  | HA-β2AR   | HA-β2AR+IR |
| Pindolol         | 1.58±0.26 | 1.15±0.15  |
| Isoproterenol    | 576.7±134.2 | 406.5±36.5 |
| Insulin          | ND        | ND         |
| Pindolol+Insulin | 2.73±0.58 | 1.13±0.23  |

ND – not detected.

HEK-293 cells expressing HA-β2AR alone or HA-β2AR together with IR at a 1:1 cDNA ratio were incubated with [125I]-iodopindolol and increasing concentrations of the indicated ligands (10–12 to 10–5 M final concentration). When the concomitant effect of pindolol and insulin was tested, insulin was added to a 0.1 μM final concentration. IC50 values were generated using a sigmoidal dose-response curve fit (GraphPad Prism 5.0). Data are expressed as the means ± S.E. of three independent experiments performed in triplicate.

doi:10.1371/journal.pone.0112664.t002

doi:10.1371/journal.pone.0112664.t001

doi:10.1371/journal.pone.0112664.t002
compared with HA-independent experiments performed in triplicate. *, p,
fluorescence. Data are expressed as the means and total luminescence, and relative to hatched bars for total
at 515 nm. Fold change is relative to open bars for surface expression
was measured with an excitation filter at 380 nm and an emission filter
addition of the RLuc substrate coelenterazine 400a. Total fluorescence
measurement, respectively. Total luminescence was measured after the
relative expression was determined by luminescence and fluorescence
expression was measured in HEPES-modified DMEM for 10 min at 37 °C before fixing with 4% paraformaldehyde for 20 min at
4 °C. Cells were then washed 3 times in PBS and blocked (PBS
containing 1% BSA) for 60 min at room temperature. Cells were
kept at room temperature for all of the subsequent steps. First, cells
were incubated for 2 h with anti-HA antibody at a 1:600 dilution.
After 3 washes, cells were incubated with anti-rat horseradish
peroxidase-conjugated antibody at a 1:1000 dilution. After
extensive washing, the reaction was developed using the 3, 3’, 5,
5’-tetra-methylbenzidine (TMB) liquid substrate system. The
enzymatic reaction was stopped after 30 min at 37 °C by adding
0.2 N sulfuric acid. The absorbances were measured at 450 nm
using the microplate reader Rosys Anthos 2010 (Anthos Labtec
Instruments, Wals, Austria). Determinations were made in
triplicate.

Receptor internalization assay

The β2AR internalization assay was performed as described
previously [47]. Briefly, HEK-293 cells were transiently transfect-
ed with either β2AR alone or β2AR and IR. One microgram of
each receptor was used, and the total amount of cDNA used for
transfection was kept uniform by adding empty pcDNA3.1 vector.
After 2 days, cells were first subjected to a 2-h starvation period in
assay medium [HEPES-modified DMEM with 0.01% BSA] before
being incubated with 10 μM isoproterenol or a combination of
10 μM isoproterenol and 0.1 μM insulin S100 in assay medium
for time intervals ranging from 5 min to 60 min at 37 °C. Cells
were then placed on ice, washed 3 times with ice-cold PBS and
incubated for 2 h with [125I]-iodopindolol in the presence or
absence of 10 μM pindolol at 4 °C. Specific binding in each
fraction was determined as the difference between radiolabeled
ligand detected in the presence and absence of 10 μM pindolol.
Receptor sequestration was then defined as the decrease in specific
[125I]-iodopindolol binding compared with the total binding
obtained in untreated cells. The amount of internalized receptors
as a function of time was fitted using a one-site binding (hyperbola)
curve fit (GraphPad Prism 5.0) to estimate the half-time of
internalization (t1/2). All of the time points were performed in
triplicate.

Confocal microscopy

HEK-293 cells were transiently transfect ed with constructs
encoding HA-tagged and/or GFP2-tagged receptor constructs,
trypsinized, and plated on poly-L-lysine-coated glass coverslips
in complete DMEM. After 48 h, cells were treated as required with
either 10 μM isoproterenol, 0.1 μM insulin S100 or the combi-
nation of both in HEPES-modified DMEM for 10 min at 37 °C.
Upon treatment, cells were washed with ice-cold PBS and fixed
with 4% paraformaldehyde for 20 min at 4 °C. Following washing
(3 times in PBS), cells were permeabilized with PBS containing
0.01% Triton X-100 for 20 min. To reduce the nonspecific
binding, cells transfected with the HA-tagged receptors were
incubated in blocking solution (PBS containing 1% BSA) for
30 min. Subsequently, cells were incubated with a 1:100 dilution
of primary rat anti-HA antibodies in PBS overnight at 4 °C.
Following washing (3 times in PBS), cells were incubated with a
1:50 dilution of secondary rabbit anti-rat TRITC-conjugated
antibodies for 60 min at room temperature in the dark. Cells were

Figure 1. Effect of IR coexpression on β2AR surface expression and
internalization in HEK-293 cells. (A) ELISA was performed on intact HEK-
293 cells transiently transfected with HA-β2AR-RLuc8 alone (open bars)
or HA-β2AR-RLuc8 together with IR-GFP2 (hatched bars) at a 1:1 cDNA
ratio using an antibody directed against the HA epitope. Antibody
binding, as an index of receptor surface expression, was determined by
measuring the absorbance at 450 nm. HA-β2AR-RLuc8 and IR-GFP2 total
relative expression was determined by luminescence and fluorescence
measurement, respectively. Total luminescence was measured after the
addition of the RLuc substrate coelenterazine 400a. Total fluorescence
was measured with an excitation filter at 380 nm and an emission filter
at 515 nm. Fold change is relative to open bars for surface expression
and total luminescence, and relative to hatched bars for total
fluorescence. Data are expressed as the means ± S.E. of three
independent experiments performed in triplicate. *; p<0.05 as
compared with HA-β2AR-RLuc8 transfected cells. (B, C) Effect of IR
coexpression on the time-course of β2AR internalization. HEK-293 cells
were transiently transfected with either β2AR (dotted line) or β2AR
(n dotted line) at a 1:1 cDNA ratio. β2AR internalization was first induced by (B)
isoproterenol (10 μM) or (C) the combination of isoproterenol (10 μM) and insulin (0.1 μM) for the indicated time
intervals. Receptor sequestration was then defined as the decrease in specific
[125I]-iodopindolol binding compared with the total binding
obtained in untreated cells. The amount of internalized receptors
as a function of time was fitted using a one-site binding (hyperbola) curve fit
(GraphPad Prism 5.0). Data are expressed as the means ± S.E. from
three independent experiments performed in triplicate.
doi:10.1371/journal.pone.0112664.g001
then mounted using an anti-fading ProLong Gold reagent (Molecular Probes, the Netherlands), sealed and examined under an oil immersion objective (Planapo 40×6, N.A. = 1.25) using a Leica multispectral confocal laser microscope (Leica TCS NT, Heidelberg, Germany). The sequential detection of GFP2- and TRITC-stained receptors was achieved using excitation laser lines at 488 nm (argon) and 543 nm (helium-neon), respectively. The fluorescence from the channels was collected sequentially, and images were produced using an 8-fold frame averaging a resolution of 1024×1024 pixels. Optical sections (1.0 μm) were acquired, and representative sections corresponding to the middle of the cells were presented using Adobe Photoshop 7.0 computer software.

BRET2 saturation and competition assays
To derive BRET2 saturation curves, HEK-293 cells were transiently cotransfected with constant amounts of the constructs encoding RLuc8-tagged receptors together with increasing amounts of the constructs encoding GFP2-tagged receptors. For BRET2 competition assays, HEK-293 cells were cotransfected with constant amounts of RLuc8- and GFP2-tagged receptor constructs and with increasing amounts of untagged receptors. BRET2 assays were performed as described previously [24,46,47]. Briefly, 180 μl of resuspended cells containing ~2×10^5 cells was distributed in 96-well microplates (white Optiplate; Packard BioScience, Meriden, CT, USA). After the addition of coelenterazine 400a to a final concentration of 5 μM using an injector, readings were collected (TriStar LB 942 microplate reader, Berthold Technologies, Bad Wildbad, Germany). Signals at 410 nm (RLuc8 luminescence signal) and 515 nm (emission of light from excited GFP2) were measured sequentially, and 515/410 ratios (BRET2 signal) were calculated. The results were expressed in milliBRET units (mBU); BRET ratio × 1000. The expression levels of RLuc8- and GFP2-tagged constructs for each experiment were assessed by total luminescence and fluorescence measurements as described above. Determinations were made in triplicates.

Figure 2. Visualization of β2AR and IR cellular localization by confocal microscopy. IR-GFP2 and HA-β2AR-RLuc8 cellular localization is shown in untreated (control) cells (upper panels) and cells concomitantly treated with isoproterenol (10 μM) and insulin (0.1 μM) for 10 min at 37°C (lower panels). The green color indicates IR-GFP2; red indicates HA-β2AR-RLuc8; yellow/orange is the overlapping region indicating colocalization of IR-GFP2 and HA-β2AR-RLuc8. Note that both receptors have comparable localization in untreated and agonist-stimulated cells and that they exhibit a high degree of colocalization. However, in agonist-stimulated cells a proportion of intracellular receptors did not colocalize. Objective 40× and zoom factor 4 apply for all images.

doi:10.1371/journal.pone.0112664.g002
BRET\textsuperscript{2} assay data evaluation

The BRET\textsuperscript{2} values were fitted using the following equation for dimers: 

\[ BRET = BRET_{\text{max}} \left( \frac{1}{1 + \frac{A/D_{50}}{X}} \right) \]

where X is the ratio of acceptor (A; Receptor-GFP\textsuperscript{2}) to donor (D; Receptor-RLuc\textsuperscript{8}) molecules. BRET\textsubscript{max} is the maximum BRET\textsuperscript{2} signal when all of the donor molecules are interacting with acceptor molecules. A/D\textsubscript{50} value corresponds to the acceptor/donor ratio providing 50% of the BRET\textsubscript{max} and reflects the relative affinity of the acceptor (GFP\textsuperscript{2}-tagged receptor) for the donor molecules (RLuc\textsuperscript{8}-tagged receptor). Fitting parameters were compared using Welch’s t-test.

Simplistic BRET model for trimers with different association affinities

The BRET\textsuperscript{2} model for trimers is based on the Veacht and Steyer (1977) concept [50]. The theoretical saturation curve for trimers with the same association affinity is:

\[ BRET \sim \frac{I_{\text{acceptor}}}{I_{\text{donor}}} \sim \frac{2EAAD + 2EADD}{(1 - 2E)AAD + 2(1 - E)ADD + DDD} \]

BRET is defined as a ratio of light emission from acceptor divided by that of the donor. E is resonance energy transfer ratio:

\[ BRET = \frac{I_{\text{acceptor}}}{I_{\text{donor}}} = \frac{2EXAAD + 2EADD}{(1 - 2E)XAAD + 2(1 - E)ADD + DDD} \]  

BRET competition assay simulation for trimers with different energy transfer ratio

The BRET model for trimer competition is based on the Veacht and Steyer (1977) concept [50]. For receptors with equal affinities to form trimers we determine the frequency of each trimer type (A = acceptor, D = donor and W = wild type receptor):

\[ (A + D + W)^3 = AAA + 3AAD + 3AW + 3ADD + 6ADW + 3AWW + 3DDW + 3DWW + 3DDD + WWW \]

If the energy transfer ratio E is different for ADW trimers the following equation is obtained:
\[
BRET = \frac{I_{\text{acceptor}} - I_{\text{donor}}}{I_{\text{donor}}} = \frac{2E_1 AAD + 2E_1 ADD + 2E_2 ADW}{(1 - 2E_1 AAD + 2(1 - E_1) ADD + 2(1 - E_2) ADW + 2DDW + DWW + DDD}
\] (5)

Informational spectrum method (ISM)

The ISM is based on a model that assigns to each amino acid a defined parameter describing a physico-chemical property involved in the biological activity of the protein and corresponding to electron-ion interaction potential (EIIP). These values determine the electronic properties of the amino acids responsible for their intermolecular interactions [51].

The obtained numerical sequence, representing the primary structure of a protein, is then subjected to a discrete Fourier transformation defined as follows:

\[
X(n) = \sum x(m)e^{-j(2\pi/n)mn}, \quad n = 1, 2, \ldots, N/2
\] (6)

where \(x(m)\) is the \(m\)-th member of a given numerical series, \(N\) is the total number of points in this series, and \(X(n)\) are discrete Fourier transformation coefficients. These coefficients describe the amplitude, phase and frequency of sinusoids, which comprised the Fourier transformation coefficients. These coefficients describe the absolute value of the complex discrete Fourier amplitude, phase and frequency of sinusoids, which comprised the Fourier transformation coefficients. These coefficients describe the

The complete information concerning the original signal. The absolute value of the complex discrete Fourier transformation defines the amplitude spectrum and phase spectrum. The complete information concerning the original sequence is contained in both spectral functions. However, in the case of protein analysis, relevant information is presented in an energy density spectrum (for a review, see [51]) defined as follows:

\[
S(n) = X(n)X^*(n) = \frac{1}{2}X(n)^2, \quad n = 1, 2, \ldots, N/2.
\] (7)

Thus, the initial information defined by the sequence of amino acids is now presented in the form of the informational spectrum (IS), representing the series of frequencies and their amplitudes.

The IS frequencies correspond to the distribution of structural motifs with defined physico-chemical characteristics responsible for the biological function of a protein. When comparing proteins that share the same biological or biochemical function, ISM allows the detection of code/frequency pairs that are specific for their common biological properties or correlate with their specific interaction. This common informational characteristic of sequences is determined by a cross-spectrum (CS) for two proteins or consensus informational spectrum (CIS) for two or more proteins—i.e., the Fourier transformation of the correlation function for the spectrum. In this way, any spectral component (frequency) not present in all of the compared ISs is eliminated. Peak frequencies in CIS are common frequency components for the analyzed sequences. A measure of similarity for each peak is the signal-to-noise ratio (S/N), representing the ratio between the signal intensity at one particular IS frequency and the main value of the whole spectrum. If a CIS is calculated for a group of proteins with different primary structures, and strictly defined peak frequencies are found, the analyzed proteins likely participate in a mutual interaction or have a common biological function. The ISM was, thus far, successfully applied in the structure-function analysis of different protein sequences [51], prediction of new protein interactors [43] and identification of protein domains responsible for long-range interactions [52,53].

Computational peptide scanning

Computational peptide scanning was used to define linear protein regions that contribute the most to the amplitude and signal to noise ratio at the characteristic frequency and, therefore, are responsible for the interaction(s) described by the particular spectral characteristic. To identify the regions with the highest amplitudes at predefined Fourier frequencies, the entire sequences of \(\beta_2\)AR and IR were scanned by the ISM algorithm with overlapping windows of different lengths, leading to the identification of regions with the highest amplitudes at predefined Fourier frequencies.

Datasets

The sequences used for bioinformatics analysis were retrieved from the Uniprot database with the following accession numbers: P07550 (human \(\beta_2\)AR) and P06213 (human IR isoform A that lacks exon 11).

Statistical analysis

Statistical significance was determined using Student’s t-test and Welch’s t-test. Differences were considered statistically significant at a \(p\) value less than 0.05.

Ethics Statement

N/A.

Results

Characteristics of the \(\beta_2\)AR fusion constructs

Pharmacological characterization of the human HA-tagged \(\beta_2\)AR (HA-\(\beta_2\)AR) fused at the C-terminus with either the energy donor RLuc8 or energy acceptor GFP2 was performed using

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**Table 3. BRET\(^2\) saturation assay fitting results.**

| Receptor pair | BRET\(_{\text{max}}\) (mBU) | AD\(_{50}\) |
|---------------|-----------------|-------|
| \(\beta_2\)AR-RLuc8:HA-\(\beta_2\)AR-GFP2 | 437±35 | 1.7±0.5 |
| IR-RLuc8:IR-GFP2 | 143±10 | 1.3±0.4 |
| \(\beta_2\)AR-RLuc8:IR-GFP2 | 78±4 | 0.5±0.1 |
| IR-RLuc8:HA-\(\beta_2\)AR-GFP2 | 131±11 | 2.9±0.7 |

BRET\(^2\) data from saturation assays were fitted using the following equation for dimers: BRET = BRET\(_{\text{max}}\) (1/(1+AD\(_{50}\)/X)) where X is the ratio of acceptor (A: Receptor-GFP2) to donor (D: Receptor-RLuc8) molecules. The BRET\(_{\text{max}}\) is the maximal BRET obtained for a given pair and AD\(_{50}\) value corresponds to the A/D ratio providing 50% of the BRET\(_{\text{max}}\). The best-fit parameters and standard errors were derived from the data presented in Fig. 3. Fitting parameters were compared using Welch’s t-test. Statistical analysis shows that AD\(_{50}\) and BRET\(_{\text{max}}\) values differ significantly (p<0.05) between all of the tests.

[doi:10.1371/journal.pone.0112664.t003](https://doi.org/10.1371/journal.pone.0112664.t003)
radioligand binding assays (Table 1). The \( IC_{50} \) values of pindolol for HA-\( \beta_2 \)-AR-RLuc8 and \( \beta_2 \)-AR-GFP2 were in agreement with those obtained for HA-\( \beta_2 \)-AR and were also in the range previously reported for \( \beta_2 \)-AR-RLuc fusion construct [47]. IR fusion constructs were previously characterized [34]. Radioligand binding assays were also performed with HEK-293 cells cotransfected

\begin{figure}
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\caption{Homologous and heterologous BRET2 competition assay. (A, B) HEK-293 cells were cotransfected with a constant amount of RLuc8- and GFP2-tagged receptor while increasing the amount of untagged receptor. In the homologous competition assay (dotted line), BRET2 signals decreased with an increasing amount of WT receptor confirming the competition effect. In the heterologous BRET2 assay (solid line), where different WT receptors were used to compete with the tagged homomer receptor pair, a transient increase in the BRET2 signal with a subsequent hyperbolical decrease was observed. BRET0 is the BRET2 signal obtained in the absence of competitor. Data are expressed as the means \pm S.E. from three independent experiments performed in triplicate. (C, D) Receptor-GFP2/Receptor-RLuc8 expression ratio (GFP2/RLuc8 ratio) in each sample was evaluated for total luminescence and total fluorescence. Total luminescence and total fluorescence was measured as described under Material and Methods. Note that GFP2/RLuc8 ratio was roughly constant in the absence or presence of increasing concentrations of competitor (untagged \( \beta_2 \)-AR or IR). Data are expressed as the means \pm S.E. from three independent experiments performed in triplicate. doi:10.1371/journal.pone.0112664.g004}
\end{figure}

\begin{figure}
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\includegraphics[width=\textwidth]{figure5.png}
\caption{Informational spectrum (IS) of (A) \( \beta_2 \)-AR, (B) IR and (C) cross-spectrum (CS) of \( \beta_2 \)-AR and IR. CS of \( \beta_2 \)-AR and IR revealed common information corresponding to the IS frequency \( F(0.216) \) (panel C). doi:10.1371/journal.pone.0112664.g005}
\end{figure}
with the HA-β2AR and IR at a 1:1 cDNA ratio. The obtained IC50 values for the tested β2AR ligands—i.e., pindolol and isoproterenol—were comparable in β2AR- and in β2AR- and IR-expressing cells. Similarly, heteromerization with β3AR did not affect the binding properties of β2AR [20]. Insulin neither competed for binding against [125I]-iodopindolol nor affected the IC50 values of pindolol (Table 2).

β2AR surface expression and the internalization effect of IR coexpression

The effect of IR coexpression on β2AR surface expression and internalization properties was evaluated by ELISA in intact cells and by radioligand binding assays. As shown in Fig. 1A, a significant approximately 35% decrease in β2AR surface expression was observed in HEK-293 cells cotransfected with IR compared with β2AR-expressing cells (p<0.05). However, total β2AR expression was not affected by IR coexpression as shown by luminescence measurements, whereas IR expression was validated by fluorescence measurements. Analogous observations in β2AR surface expression were detected using radioligand binding assays, where β2AR surface expression decreased by around 30% (27.89±2.11) in HEK-293 cells cotransfected with IR compared with β2AR-expressing cells (p<0.05).

Additionally, ELISA measurements suggested that IR coexpression moderately increased isoproterenol-induced β2AR internalization, whereas concomitant treatment with isoproterenol and insulin evoked a comparable internalization rate. Treatment with insulin did not induce marked β2AR internalization (Figure S1).

To further examine the internalization kinetics of β2AR, HEK-293 cells expressing either β2AR or β2AR and IR were treated with either isoproterenol (10 μM) or a combination of both isoproterenol (10 μM) and insulin (0.1 μM) for varying periods of

Figure 6. Mapping of the domains with maximal contribution to the frequency component F(0.216) in the informational spectrum of (A) β2AR and (B) IR. Peptide scanning of the β2AR and IR identified regions encompassing residues at the end of the 7th TM domain and C-terminal tail of β2AR and a cytoplasmic part of the IR β chain as prospective interaction domains. The regions encompassing residues 325–364 in β2AR (A) and 1269–1314 IR (B) are essential for the information represented by the frequency F(0.216). The position of the first amino acid (aa) in the domain is shown. Panel B; the amino acid position denote the positions in IR β subunit starting from amino acid 763.

doi:10.1371/journal.pone.0112664.g006

Figure 7. Consensus informational spectrum (CIS) of β2AR:IR tetramers with the characteristic peak at F(0.216). (A) β2AR:β2AR:β2AR:IR, (B) β2AR:β2AR:IR:IR and (C) β2AR:IR:IR:IR tetramers. Note that the interaction affinities among the receptors decreased in the order β2AR:β2AR:β2AR:IR ≻ β2AR:β2AR:IR:IR ≫ β2AR:IR:IR:IR.

doi:10.1371/journal.pone.0112664.g007
Interaction between the β2AR and IR-BRET evidence

Considering the observed effects of IR coexpression on β2AR surface expression and internalization, as well as colocalization of both receptors in HEK-293 cells, we next investigated whether proximity indicative of direct interaction occurs between β2AR and IR by performing BRET dilution, saturation and competition assays. To obtain an absolute value of donor [D] and acceptor [A] molecules–i.e., Receptor-RLuc8 and Receptor-GFP2–we first generated correlation curves between total luminescence and fluorescence generated correlation curves between total luminescence and fluorescence versus the number of receptor-binding sites determined by radioligand binding. The linear correlation was obtained between the number of HA-β2AR-RLuc8 and β2AR-GFP2 binding sites and the total luminescence and total fluorescence, respectively (Figure S2).

The derived correlation factors to convert total luminescence and total fluorescence into receptor number were 0.00031 and 0.0089, respectively. Thereafter, BRET2 dilution assays with a constant [A]/[D] ratio were performed to set the concentration range for the saturation assays and distinguish monomers from dimers as previously described [54]. Due to the increasing noise in calculated BRET at low luminescence intensities, the lowest amount of HA-β2AR-RLuc8 and IR-RLuc8 cDNA used for transfection in saturation assays was determined at 0.05 and 0.015 μg, respectively.

BRET2 saturation assays were performed to investigate homodimerization of β2AR and IR. The BRET2 signal was measured in live HEK-293 cells that were transiently transfected with constant amounts of RLuc8-tagged (HA-β2AR-RLuc8, IR-RLuc8) and with increasing amounts of GFP2-tagged (β2AR-GFP2, IR-GFP2) receptor encoding constructs. In dimers and higher oligomers, the probability of BRET interaction increases with increasing acceptor/donor ratio until all of the acceptors have pairs, and the maximum BRET2 level (BRETmax) or plateau is reached. The AD50 value (also designated as BRET50; see methods) was also included to compare the affinity for homo- and heteromer formation. BRET2 saturation experiments data are shown in Figure 3 and the expression levels of RLuc8- and GFP2-tagged receptor constructs are shown in Figure S3. For all of the receptor combinations, the BRET2 signal was plotted as a function of the A/D ratio increased as a hyperbolic function reaching a saturation level (Fig. 3). Control experiments for the specificity of the interaction were performed by cotransfecting RLuc8-tagged receptors with increasing amounts of membrane-inserted GFP2-tagged construct (GFP2-17aa). Plasma membrane localization of the GFP2-17aa construct was previously demonstrated [45]. The BRET2 signal increased linearly with the increase in fluorescence/luminescence (GFP2/RLuc8) ratio, most likely reflecting random collisions between the RLuc8-tagged receptors and control unrelated GFP2-tagged construct (GFP2-17aa) (Figure S4). Saturation assay data were fitted using a dimer model in the approximation of small energy transfer [54] (Table 3). We observed the highest BRETmax–i.e., 437 mBU in the β2AR homologous saturation assay and considerably lower BRETmax values for other receptor combinations. It should be stressed that the BRETmax value depends on the distance between BRET pairs and is not a measure of the strength of interaction between the acceptor- and donor-tagged receptors. In homologous saturation

Table 4. The affinity of interaction between the β2AR and IR homo- and heteromers characterized by the signal-to-noise ratio (S/N) at the characteristic frequency (F) in the consensus informational spectrum (CIS).

| Dimer                      | S/N ratio |
|----------------------------|-----------|
| β2AR:β2AR:β2AR:β2AR       | 24.082    |
| β2AR:IR:IR                | 15.951    |
| IR:IR:IR:IR               | 22.407    |
| β2AR:IR:IR:IR:IR:IR:IR:IR | 52.867    |
| β2AR:IR:IR:IR:IR:IR:IR:IR | 43.476    |
| β2AR:IR:IR:IR:IR:IR:IR:IR | 25.605    |
| IR:IR:IR:IR:IR:IR:IR:IR:IR | 51.146    |
| IR:IR:IR:IR:IR:IR:IR:IR:IR | 89.617    |

doi:10.1371/journal.pone.0112664.t004
Assays, the value of AD_{50} for the IR homomer was significantly lower (p ≤ 0.05) than that for the β_2AR homomer (Table 3). For the receptor complex consisting of β_2AR-RLuc8:IR-GFP, the AD_{50} value was significantly lower (p ≤ 0.05) than the AD_{50} value for the IR-RLuc8:β_2AR-GFP. For heterodimers, there should be no difference in the relative affinity between AD and DA dimers. To interpret our data, we developed a simplistic trimer model for interpretation of AD_{50} values in a BRET saturation assay. According to this model, different AD_{50} values are derived from theoretical BRET saturation curves with different affinities for various types of trimer formation (Figure S5). This model should be used with caution because it does not take into account the simultaneous formation of dimers, trimers and higher-order oligomers. Stimulation with the agonists insulin or isoproterenol did not promote any detectable change in the BRET signal (data not shown), indicating that the receptor dimers/oligomers form constitutively and that addition of agonists does not induce a detectable change in the conformational or oligomerization state of the receptor complexes. However, it needs to be stressed that BRET only provides information concerning a steady-state population of dimer/higher-order oligomers and is not suited to monitor the rapid, “real-time” dynamic type of dimer/oligomerization [55].

To further support the findings from the saturation assays, we performed homologous and heterologous BRET competition assays where HEK-293 cells were co-transfected with a constant amount of RLuc8- and GFP-tagged receptor while increasing the amount of untagged receptor (Fig. 4A and B). Competition experiments were carried out at the constant Receptor-GFP/Receptor-RLuc8 expression ratio to avoid possible variations in the BRET signal due to fluctuation in the relative expression levels of the energy donor and acceptor (Fig. 4C and D). It is expected that the BRET signal would decrease if untagged receptors compete with the tagged receptors for the binding in complexes. In homologous BRET competition assays BRET signal decreased with increasing amount of competitor (un-tagged receptor). In the IR homologous competition assay, we observed a typical competition curve for dimers, where introduction of the same amount of untagged receptor produced an approximately 50% reduction in the observed BRET signal. In the β_2AR homologous competition assay, the reduction of the BRET signal was smaller. This observation cannot be explained by a simple dimer or trimer model; it could be attributed to clustering of the β_2AR where several acceptors can interact with each donor. In heterologous competition assays, we observed a transient increase in the BRET signal with a later hyperbolical decrease (see Fig. 4A and B). Untagged β_2AR caused approximately 1.4-fold increase in the IR BRET signal, while the effect of untagged IR on the β_2AR BRET signal was less obvious; maximal observed increase was less than 1.2-fold. The transient increase in the BRET signal with a subsequent hyperbolical decrease is theoretically predicted for trimers or higher-order oligomers, where the donor, acceptor and competitor are all present in the same complex (Figure S6).

Interaction between β_2AR and IR characterized by ISM

To support our experimental evidence with the bioinformatics data, we next applied the informational spectrum method (ISM), a virtual spectroscopy method to investigate protein-protein interactions and to analyze the structure/function relationship of proteins. ISM was utilized to identify important informational characteristic of the interaction between β_2AR and IR and identify the structural determinants potentially involved in receptor heteromerization. The primary structure of proteins encodes the information represented by the informational spectrum (IS) frequencies that correspond to the protein biological function. Mutually interacting proteins share common information that is represented by peaks in their cross-spectrum [51]. The informational spectrum of β_2AR is presented in Fig. 5A. It contains two characteristic peaks at the frequency F(0.216) and F(0.355). Fig. 5B represents the IS of the IR. By performing cross-spectral analysis of β_2AR and IR, we have identified that these two molecules share common information corresponding to the IS frequency F(0.216) (Fig. 5C). To further evaluate the importance of the peak at F(0.216) we performed CS analysis of β_2AR and IR with scrambled IR and β_2AR proteins as negative controls. The scrambled proteins with the identical amino acid composition to that of β_2AR and IR were created by random permutation of original proteins. CS analysis between wild type receptors and randomly selected scrambled β_2AR and IR are presented in Figure S7. The intensity of whole spectrum and the value of amplitudes at the characteristic peak F(0.216) is higher in CS of two wild type proteins compared to CS of original and scrambled proteins confirming the importance of the characteristic peak at the F(0.216) for interaction between β_2AR and IR.

Identification of the key protein domains responsible for the interaction between β_2AR and IR

Computational peptide scanning of β_2AR and IR was performed to identify the regions of proteins essential for information corresponding to the frequency F(0.216). The computer-assisted peptide scanning survey of the primary structure of β_2AR with overlapping windows of different lengths revealed that the region encompassing residues 325-364 is essential for the information represented by the frequency F(0.216) (Fig. 6A). Further peptide scanning of IR identified three principal regions as important for the information represented by the frequency F(0.216), however region encompassing residues 1269-1314 represents the most probable domain involved in this interaction (Fig. 6B).

Affinity of interaction between protomers

Peak frequencies in CIS represent common information encoded by the primary structures of analyzed proteins. Significance of information is determined by the signal-to-noise ratio (S/N), representing the ratio between the signal intensity at one particular IS frequency and main value of the whole spectrum. A higher S/N value at the characteristic frequency (F) in CS/CIS of two or more proteins suggests a higher propensity for their interaction.

The current analysis showed that the interaction affinities between the homomers of β_2AR and IR are similar and that at the level of dimer formation, both β_2AR and IR displayed a considerably lower affinity toward homodimerization than heterodimerization (Table 4). Considering that in the CS of the β_2AR and IR, the two receptors share common information corresponding to the IS frequency F(0.216), it can be assumed that this frequency is equally important for heterodimerization and for higher-order hetero-oligomer formation. The obtained S/N values at F(0.216) in the CS of β_2AR:β_2AR:β_2AR:IR and β_2AR:β_2AR:IR:IR tetrators were comparable, whereas that for the IR trimer displayed a considerably lower affinity for interaction with the β_2AR monomer (Fig. 7 and Table 4).

Discussion

7TMRs form the largest and most important pharmacotherapeutic target in drug discovery. Continual discovery of receptor heteromers expands the repertoire of functional 7TMR units and
The RLuc8/GFP2 BRET2 donor/acceptor pair used in our study showed a slightly lower propensity for heteromerization. However, simplistic model for interpretation of AD_{50} values and heterologous competition assays data provided evidence for higher-order oligomeric complex formation. A transient increase in the BRET signal suggests that the energy transfer efficiency E is increased in hetero-oligomeric complexes due to the smaller distance between the BRET donor/acceptor pair in the complex (see Figure S6). A similar effect—i.e., an increased homo-dimer BRET signal induced by an unrelated, untagged receptor—was previously observed with the gastric inhibitory polypeptide receptor [24]. Therefore, both the change in affinities and transient increase in the BRET signal could be a reflection of higher-order oligomeric complexes with affinities for distinct associations such as with trimers and tetrarsers with 2:1 and 2:2 stoichiometry, respectively, as proposed by Bréitwieser et al. [68]. At the level of dimer formation, both β2AR and IR displayed a higher propensity toward homodimerization than heterodimerization, suggesting homodimers as the basic units engaged in heteromerization. Affinity calculation for trimers and tetrarsers highlighted differences between β2AR and IR. Apparently, neither IR dimers nor trimers could form high affinity interactions with the β2AR monomer. However, IR monomers/homodimers can form high affinity heteromers with β2AR di-/trimers. Considering that both receptors displayed a higher propensity toward homodimerization and that IR is present in the plasma membrane as a disulfide-linked dimer [29], it is plausible to suggest that high-order 7TMR:RTK oligomers most likely comprise heterodimers of homomers (2:2 stoichiometry).

The computer-assisted peptide scanning survey of the primary structure of β2AR and IR revealed the domain encompassing residues 325–364 and 1269–1314 as prospective interaction domains. The identified region is located at the end of the 7th TM domain and C-terminal tail of β2AR and almost completely overlaps with helix 0 (helix adjacent to TM7 running along the internal membrane surface) of β2AR (residues L324–N357) [69]. A recent study also identified β2AR helix 0 as an important dimerization surface region [70]. In addition, this region contains major sites (Ty350/Tyr354 and Tyr356) for IR-mediated phosphorylation of β2AR [3,71] and consensus sites of Akt-catalyzed phosphorylation (Ser345/Ser 346) [72]. These results are in accordance with previous findings showing that β2AR is a substrate for IR and proposing direct interaction between these proteins [3,71]. The C-terminal cytoplasmic tail—more specifically, the 13-amino acid motif (residues 342–356)–was sufficient to confer the β2AR-β2AR tail chimera the ability to be regulated by insulin [62].

The prospective interaction domain identified in IR (residues 1269–1314) is positioned in the cytoplasmic part of the IR β chain. This region encompasses the terminal end of the tyrosine kinase catalytic domain and is a part of the C-terminal tail. The C-terminal domain of the IR β subunit has been found to play a key role in the regulation of tyrosine kinase activity [73,74]. The construct based on proteolytic cleavage used to solve the crystal structure of the IR tyrosine kinase domain of the human IR B-isoform [75] ends at residue 1293, which corresponds to the amino acid at position 1271 in the human IR isoform A used in our study. Therefore, it could be hypothesized that the terminal end of the IR tyrosine kinase domain and a part of the C-terminal tail are involved in the interaction with β2AR.

The involvement of intracellular domains (C-terminal tail and ICL3) was found to be fundamental for heterodimerization between the cannabinoid CB1 receptor, adenosine A2A, and dopamine D2 receptors, thus favoring the idea that electrostatic interactions between intracellular domains are more predominant.
in receptor heteromers and constitute a general mechanism for receptor heteromerization [76].

In summary, BRET data and ISM bioinformatics provided evidence for direct interaction and higher-order β2AR:IR oligomer formation that we hypothesize comprise heteromers of homodimers and identified prospective intracellular interaction domains engaged in heteromerization. In this regard, 7TMR:RTK heteromers could potentially generate a basis for the design of new therapeutics that can compete with today's epidemics, such as type-2 diabetes, obesity and cardiovascular diseases.

Supporting Information

Figure S1 HA-β2AR internalization as quantified by ELISA. Cells transiently transfected with HA-β2AR (open bars) or HA-β2AR together with IR (hatched bars) at a 1:1 cDNA ratio were incubated at 37°C with either isoproterenol (10 μM), insulin (0.1 μM) or combination of both ligands for 30 min. The amount of internalized receptor was then calculated from the decrease in the level of surface-expressed receptor after ligand treatment compared with untreated, control cells. Data are expressed as the means ± S.E. of three independent experiments performed in triplicate.

Figure S2 Correlation between total luminescence and fluorescence and the corresponding number of β2AR binding sites. HEK-293 cells were transfected with increasing amounts of HA-β2AR-RLuc8 (A) or β2AR-GFP2 (B) encoding constructs. The β2AR receptor density (Bmax) was determined by radioligand binding assays using [125I]-iodopindolol as a tracer as described in the Material and methods section. Total luminescence was measured after the addition of the RLuc8 substrate coelenterazine 400a. Total fluorescence was measured with an excitation filter at 380 nm and an emission filter at 515 nm. The linear regression curves were generated using GraphPad Prism 5.0. R2 fit values of 0.9705 and 0.9861 were obtained for HA-β2AR-IR-Luc8 (A) and β2AR-GFP2 (B), respectively.

Figure S3 Relationship between receptor-RLuc8 and receptor-GFP2 constructs expression. Expression levels of RLuc8- and GFP2-tagged constructs used in BRET2 saturation assays were also monitored by luminescence and fluorescence measurements. Total luminescence was measured after the addition of the RLuc8 substrate coelenterazine 400a. Total fluorescence was measured with an excitation filter at 380 nm and an emission filter at 515 nm. Data are expressed as the means±S.E. of 3–5 independent saturation experiments.

Figure S4 Random collisions between the RLuc8-tagged receptors and membrane-inserted GFP2-tagged construct (GFP2-17aa). HEK-293 cells were transiently cotransfected with a constant amount of RLuc8-tagged receptors and increasing amounts of GFP2-17aa encoding construct. BRET2 values were plotted as a function of the ratio between the total fluorescence/total luminescence (GFP2/RLuc8 ratio). Total luminescence was measured after the addition of the RLuc8 substrate coelenterazine 400a. Total fluorescence was measured with an excitation filter at 380 nm and an emission filter at 515 nm. Increasing the concentration of GFP2-17aa in cells expressing either the IR-RLuc8 or β2AR-RLuc8 resulted in high, but nonspecific linear increase of the BRET2 signal. Data are expressed as the means ± S.E. from three independent experiments performed in triplicate. Representative BRET2 saturation curves of β2AR and IR homomers are shown for comparison.

Figure S5 Comparison of theoretical BRET saturation curves with different affinities for trimer formation. Shown are simulated BRET saturation curves for case with the same affinity for AAD and ADD formation (solid line) and two special cases with different affinities for formation of AAD compared to ADD (hatched and dotted lines). Note that in all three cases the AD50 values are different. A: acceptor; D: donor.

Figure S6 Numerical simulation of heterologous BRET competition assay for trimers. Comparison of simulated BRET competition curves for trimers with the same (E1 = E2 = 0.1) and different (E1 = 0.1, E2 = 0.3) energy transfer ratios for ADD and AAD, where A, D and W are concentrations of acceptor (A = 1), donor (D = 1) and (W) wild type receptors i.e. competitor. Transient increase in BRET signal is observed in the case of different (E1 = 0.1, E2 = 0.3) energy transfer ratios (dotted line). BRET3 is the BRET signal obtained in the absence of competitor.

Figure S7 Cross-spectrum (CS) of (A) wild type β2AR and IR, (B) scrambled β2AR and wild type IR and (C) scrambled IR and wild type β2AR. Note that the value of amplitudes at the characteristic peak F(0.216) is higher in CS of two wild type proteins (panel A) compared to the CS of wild type and scrambled proteins (panels B and C).

Acknowledgments

The authors would like to thank Professor Pierre De Meyts for critical evaluation of the manuscript and American Journal Experts for proofreading the English usage in the manuscript.

Author Contributions

Conceived and designed the experiments: JN MV. Performed the experiments: MM SG MV. Analyzed the data: LD SG NV. Contributed reagents/materials/analysis tools: JN SG MV. Performed the experiments: MM. Analyzed the data: LD SG MV. Contributed reagents/materials/analysis tools: JN SG MV. Contributed to the writing of the manuscript: MM SG MV.

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