Biochemical and Biophysical Research Communications 445 (2014) 486–490

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Human orexin/hypocretin receptors form constitutive homo- and heteromeric complexes with each other and with human CB_1 cannabinoid receptors

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ARTICLE INFO

Article history:
Received 29 January 2014
Available online 13 February 2014

Keywords:
Orexin
Hypocretin
Cannabinoid
G-protein-coupled receptor
dimerization
Bioluminescence resonance energy transfer

ABSTRACT

Human OX_1 orexin receptors have been shown to homodimerize and they have also been suggested to heterodimerize with CB_1 cannabinoid receptors. The latter has been suggested to be important for orexin receptor responses and trafficking. In this study, we wanted to assess the ability of the other combinations of receptors to also form similar complexes. Vectors for expression of human OX_1, OX_2 and CB_1 receptors, C-terminally fused with either *Renilla* luciferase or GFP^2 green fluorescent protein variant, were generated. The constructs were transiently expressed in Chinese hamster ovary cells, and constitutive dimerization between the receptors was assessed by bioluminescence energy transfer (BRET). Orexin receptor subtypes readily formed homo- and hetero(di)mers, as suggested by significant BRET signals. CB_1 receptors formed homodimers, and they also heterodimerized with both orexin receptors. Interestingly, BRET efficiency was higher for homodimers than for almost all heterodimers. This is likely to be due to the geometry of the interaction; the putatively symmetric dimers may place the C-termini in a more suitable orientation in homomers. Fusion of luciferase to an orexin receptor and GFP^2 to CB_1 produced more effective BRET than the opposite fusions, also suggesting differences in geometry. Similar was seen for the OX_1–OX_2 interaction. In conclusion, orexin receptors have a significant propensity to make homo- and heterodi-/oligomeric complexes. However, it is unclear whether this affects their signaling. As orexin receptors efficiently signal via endocannabinoid production to CB_1 receptors, dimerization could be an effective way of forming signal complexes with optimal cannabinoid concentrations available for cannabinoid receptors.

1. Introduction

Early on, G-protein-coupled receptors (GPCRs) were assumed to exists as monomers, but the view has widened during the last 15–20 years. Family C GPCRs exists as obligate homo- or heterodimers (or some probably as dimers of dimers) [1,2]. For family A GPCRs, homo- and heteromerization seems to show different degrees of dynamic regulation [3–5]. Heteromerization with another GPCR enhances cell surface trafficking of some GPCRs, such as α_{1D} and α_{2C} adrenoceptors [6,7]. For other GPCRs, such as opiate receptors, properties like internalization and pharmacology are affected by complex partners [8,9]. The methods for investigation of di-/oligomerization include optical methods such as BRET and FRET (bioluminescence and fluorescence resonance energy transfer, respectively) and fluorescence colocalization spectroscopy, and biochemical methods such as solubilization and immunoprecipitation followed by denaturing or native gel electrophoresis. So-called bi- or multivalent ligands, which specifically act on receptor di-/oligomers, or antibodies, which detect receptor di-/oligomers, have been developed, allowing assessment of native receptor complexes with some selectivity [10,11]. On the other hand, there are no means for effectively blocking complex formation between GPCRs. Thus, it is difficult to fully estimate the importance of di-/oligomerization for GPCR function. Nevertheless, there are notable dimer-specific pharmacological findings for especially opi-

Abbreviations: BRET, bioluminescence energy transfer; FRET, fluorescence resonance energy transfer; GFP^2, a generated mutant of green fluorescent protein; Rluc, *Renilla reniformis* luciferase; GPCR, G-protein-coupled receptor.

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http://dx.doi.org/10.1016/j.bbrc.2014.02.026
0006-291X/

ate receptors (above) and development of bi-/multivalent ligands could expand the therapeutic potential of this.

Human orexin receptors OX_1 and OX_2 are class A GPCRs, which bind neuropeptides orexin-A and -B. Orexin system is involved, for example, in the regulation of wakefulness and sleep pattern, appetite, pain gating and reward [12,13]. Orexin system has been actively investigated with respect to these physiological functions but also in the perspective of the complex signaling of orexin receptors [14,15]. The role of the orexin system in maintaining wakefulness is dramatically expressed upon disruption of the orexin system in narcolepsy [13]. On the other hand, blocking of orexin receptors allows sleep induction in the therapy of insomnia [16]. CB_1 cannabinoid receptors also belong to family A GPCRs. Their native ligands are lipid metabolites called endocannabinoids among which 2-arachidonoylglycerol (2-AG) and anandamide are the most well-known [17]. The endocannabinoid system is the most widespread GPCR system in the brain. Endocannabinoids engage in the regulation of many physiological functions including analgesia, appetite, learning and memory [17]. On the physiological level, orexins and endocannabinoids seem to have overlapping functions. On some brain *loci* as well as in recombinant systems, orexin receptor signaling has been suggested to trigger production of 2-AG, leading to the activation of CB_1 receptors [18–21]. In recombinant cells, human OX_1 receptors have been shown to form heteromeric complexes with CB_1 receptors, suggesting an additional way of interaction of these systems [22]. OX_1 receptors also seem to form homomeric complexes [23]. The role of human OX_2 receptors in homo- or heteromeric complexes has not been investigated, and therefore we set out in this study to explore all possible two-partner complexes among human OX_1, OX_2 and CB_1 receptors under similar conditions in recombinant CHO cells.

2. Materials and methods

2.1. Cell culture

Chinese hamster ovary (CHO) cells were propagated on circular plastic culture dishes (56-cm^2 bottom area; Greiner Bio-One GmbH, Frickenhausen, Germany) in nutrient mixture (Ham's F-12) medium (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 100 U/ml penicillin G (Sigma–Aldrich, St. Louis, MO), 80 U/ml streptomycin (Sigma) and 10% (v/v) fetal calf serum (Life Technologies, Inc.) at 37 °C in 5% CO_2 in an air ventilated humidified incubator. For BRET experiments, the cells were cultured on six-well plates (9.6-cm^2 well bottom area, Greiner Bio-One GmbH).

2.2. Construction of the fusion protein cDNA constructs for $BRET^2$

The cDNAs encoding human OX_1 and OX_2 in the pEGFP-N3 vector [24], and the human CB_{1a} receptor in the pcDNA3.1-CB_1-GFP [hCB_{1a} receptor with C-terminal (enhanced) green fluorescent protein] [25] from Dr. Debra Kendall (University of Connecticut, Storrs, CT, USA) were used as templates for PCR amplification. Expression vectors for Rluc (*Renilla reniformis* luciferase) fusion receptors were generated by subcloning the receptor PCR products without the stop codon in frame into pCIneo-Rluc vector (5′ from Rluc) ([26] using NheI site (OX_2 and CB_1) or XbaI site (OX_1). A 6-amino acid linker (GSGTGS) was included between the receptor and Rluc. For the construction of GFP^2 (a generated mutant of green fluorescent protein) fusion receptors, the PCR products without the stop codon were inserted into HindIII and BamHI sites of pGFP^2-N2 vector (PerkinElmer) (5′ of GFP^2). The resulting constructs were verified by DNA sequencing. As a positive control for the $BRET^2$ studies, a vector coding for a Rluc–GFP^2 fusion protein was used [26].

2.3. Transfection

For transfection the cells were grown on six-well plates to 40–60% confluence and transfected in Ham's F-12 with 2 μg/well DNA and 5 μl/well FugeneHD (Roche, Mannheim, Germany). Cells were transfected with different combinations of Rluc and GFP^2 constructs. The amount of Rluc construct DNA was fixed and the amount of GFP construct DNA varied. The total amount of DNA in each well was kept constant with the empty pcDNA3.1 vector. Rluc–GFP^2 fusion and each receptor-Rluc alone were used as positive and negative controls, respectively. All GFP^2 and Rluc fusion constructs lead to expression of proteins in CHO cells, as verified from the fluorescence (GFP^2) or luminescence (Rluc) (see also Figs. 1 and 2).

2.4. $BRET^2$ assay

The cells were transfected as described above and after 24 h they were washed with PBS, detached with PBS + 0.2 g/l EDTA solution and collected to Eppendorf tubes. The cells were then quickly spun down, resuspended in Hepes-buffered medium (HBM; composition in mM: NaCl, 137; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1.2; KH_2PO_4, 0.44; $NaHCO_3$, 4.2; glucose, 10; and Hepes, 20; adjusted to pH 7.4 with NaOH) and pipetted onto a white 384-well microplate (CulturPlate-384, PerkinElmer, Waltham, MA, USA). Luciferase substrate coelenterazine 400a [2,8-dibenzyl-6-phenyl-imidazol(1,2a)pyrazin-3-(7H)-one; Gold biotechnology, St. Louis, MO, USA] was added to the wells in 5 μM final concentration with automated reagent injector. The plate was measured with Pherastar FS (BMG, Labtech GmbH, Ortenberg, Germany) using $BRET^2$ plus optic module. For some experiments (Fig. 2D), the cells were stimulated for 10 min with 100 nM orexin-A (or HBM as the control) at 37 °C before addition of the substrate. BRET ratio was calculated by dividing the measured fluorescence with luminescence after subtracting the reader background (fluorescence counts before the addition of the substrate).

The leak of each Rluc construct in the GFP^2 channel was 0.0366 ± 0.0005 (n = 14; negative control) and the response of the Rluc–GFP^2 0.1837 ± 0.0024 [n = 11 (leak not subtracted); positive control].

2.5. Data analysis

All the data are presented as mean ± SE; n refers to the number of batches of cells. Each experiment was performed in 4–6 replicates at least three times. Microsoft Excel was used for all data analyses including t test and nonlinear curve-fitting to a simple hyperbolic equation.

3. Results and discussion

Significant BRET signal was observed upon co-expression of OX_1–Rluc and OX_1–GFP^2 (Fig. 1A). The response showed saturation when plotted against the relative amount of cDNA for the GFP fusion construct (Fig. 1A). The same was seen for the combination OX_2–Rluc–OX_2–GFP^2 (Fig. 1A). The saturating curves confirm that complex formation is specific, and thus that both orexin receptor subtypes are capable of forming constitutive homodi-/oligomeric structures. We next investigated possible heteromerization of orexin receptor subtypes. The pair OX_2–Rluc–OX_1GFP^2 showed a strong response and a nicely saturating BRET curve (Fig. 1B), while the response of the pair OX_1–Rluc–OX_2GFP^2 was clearly weaker and did not show good saturation (Fig. 1B).

CB_1 receptor has been shown to heterodimerize with other class A GPCRs, namely D_2 dopamine, μ opioid, AT_1 angiotensin, A_1

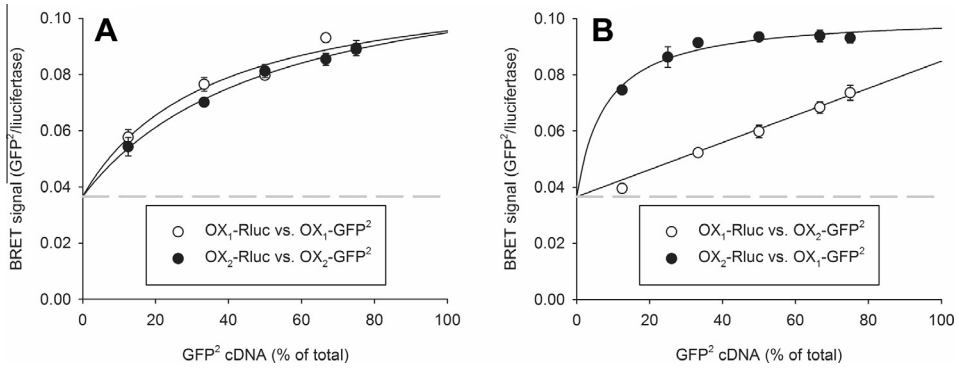

Fig. 1. Orexin receptor subtype homomerization (A) and heteromerization (B). The level of the leak of Rluc light in the GFP^2 channel in the absence of BRET is indicated by the gray dashed line.

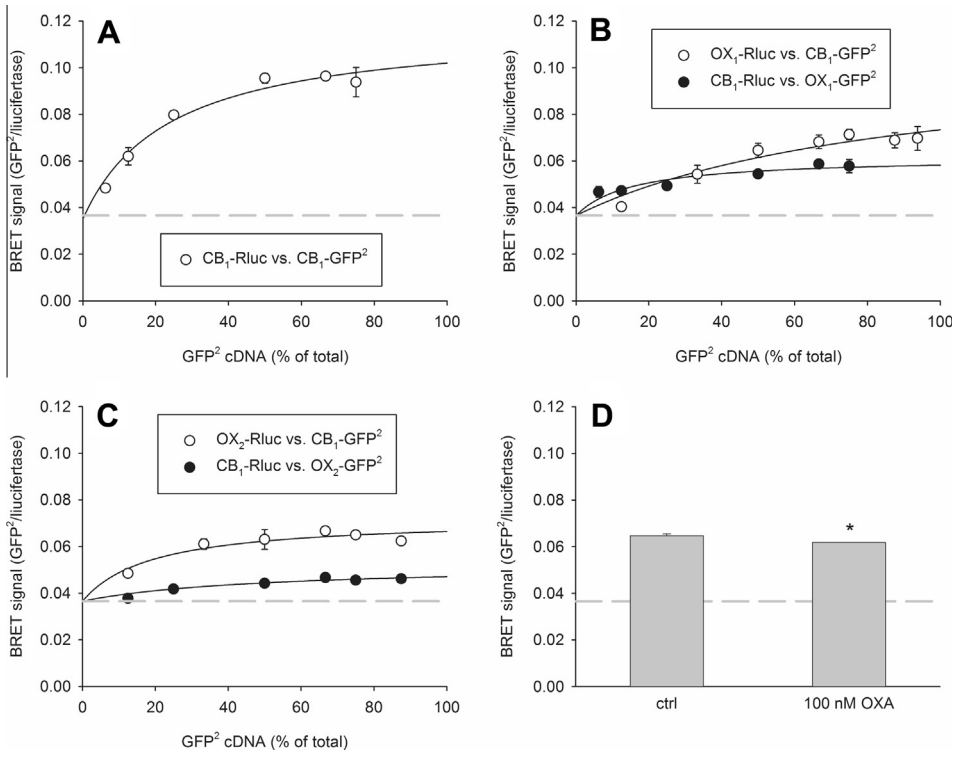

Fig. 2. Orexin and cannabinoid receptor interactions. (A) CB_1 cannabinoid receptor homomerization. (B, C) Heteromerization between CB_1 cannabinoid receptors and OX_1 (B) and OX_2 (C) orexin receptors. (D) The effect of orexin-A stimulation (100 nM, 10 min), on BRET between OX_1–Rluc and CB_1–GFP^2. The significance is the same ($^*p < 0.05$) for both paired and non-paired *t* tests. The level of the leak of Rluc light in the GFP^2 channel in the absence of BRET is indicated by the gray dashed line.

adenosine and OX_1 orexin receptors [4,27–29,22]. It is generally believed that CB_1 receptors also form homomeric complexes, but there is little evidence for this, except for studies utilizing a reputedly specific antibody towards the dimer [3]. In the BRET assay, CB_1 receptors were seen to readily homodimerize (Fig. 2A) and heterodimerize with OX_1 and OX_2 receptors (Fig. 2B and C). Results with OX_1 and CB_1 receptor BRET studies resembled those between OX_1 and OX_2 receptors in so far as the two combination variants showed rather different results. CB_1–Rluc–OX_1–GFP^2 combination showed good saturation but very weak maximum BRET response (Fig. 2B). In contrast, the response was larger for the OX_1–Rluc–CB_1–GFP^2 combination, but it did not saturate equally well (Fig. 2B). Both combinations of CB_1 and OX_2 receptors showed saturating BRET responses, but the BRET efficiency was very weak for the CB_1–Rluc–OX_2–GFP^2 (Fig. 2C).

The results thus strongly suggest that orexin receptor subtypes readily form constitutive homo- and heteromeric complexes with one-another. Both orexin receptor subtypes also formed complexes with CB_1 cannabinoid receptors. However, BRET efficiency vastly varied between different complexes. This was most pronounced for the principally same receptors with the fusion proteins added in different partners, i.e. OX_1–Rluc–OX_2–GFP^2 vs. OX_2–Rluc–OX_1–GFP^2, OX_1–Rluc–CB_1–GFP^2 vs. CB_1–Rluc–OX_1–GFP^2, and OX_2–Rluc–CB_1–GFP^2 vs. CB_1–Rluc–OX_2–GFP^2. Receptor expression levels – which are not solely defined by the transfection cDNA amounts – should determine the apparent DNA amount at which saturation is obtained, but not the saturating BRET efficiency as such. Instead, this is likely to reflect the usual FRET and BRET problem, i.e. that the geometry (distance, orientation, freedom of movement, etc.) of the interacting proteins determines the energy transfer efficiency. The homomeric complexes of OX_1, OX_2 and CB_1 showed clearly best BRET efficacy. It is imaginable that the likely symmetric complexes place the C-terminal Rluc and GFP^2 in a symmetrical orientation in these homomeric complexes. Nevertheless,

due to the geometry issues, the FRET or BRET efficiency cannot be used to determine the affinity for complex formation, and the different receptor combinations cannot be compared quantitatively. The results therefore only establish that such complexes can be formed.

The findings of the present study are in agreement with previous FRET studies suggesting OX_1–CB_1 heteromerization [30,22]. OX_1 receptors have also been shown to form larger molecular aggregates on native gel [23]; while this suggests homomerization of the receptors, the results, in fact, do not specifically identify OX_1 receptors to be the sole complex members. However, also FRET and BRET studies support OX_1 homomerization [23]. Orexin-A was, further, suggested to enhance di/-oligomerization, and antagonists to reduce that below the basal level; however, the behavior of different receptor constructs was different [23]. Due to alternative splicing, mouse OX_2 receptor is expressed in two isoforms, named $OX_{2\alpha}$ and $OX_{2\beta}$ [31]. Upon expression in HEK-293 cells, these two isoforms form heteromeric complexes based on BRET measurements [32]. In contrast, the homomeric complexes showed barely any BRET [32]. This is interesting since the human OX_2 receptors, which show good homodimerization, are very similar to mouse $OX_{2\alpha}$. Altogether, it is beyond doubt that human (and other mammalian) orexin receptors can form homo- and heteromeric complexes, possibly even so that these dominate over monomeric structures in native cells. However, we do not know whether these serve any physiological function, except for mouse receptors, for which heterodimerization of OX_2 receptors may enhance expression (and thus signaling) [32].

We finally tested whether OX_1–CB_1 dimerization would be affected by orexin receptor activation, which should also induce CB_1 receptor activation via 2-AG production [20,21]. Under the experimental conditions, we saw only a minor reduction in the BRET efficiency (Fig. 2D). This may be due to slightly reduced dimerization but also equally well to some other conformational reorientation induced by, e.g. ligand binding or phosphorylation. This is one of the weaknesses of FRET and BRET methods. Oligomerization in response to receptor stimulation has previously been studied in detail with homomers of OX_1 receptors in recombinant HEK-293 cells [23]. Under conditions of low receptor expression levels, OX_1 receptor activation enhanced the FRET signal while under high receptor expression levels this was not seen. Similar was suggested by studies utilizing native gel electrophoresis. This indeed indicates that homodi-/oligomerization can be dynamically regulated in orexin receptor signaling. Di-/oligomerization of GPCRs may be a very dynamic process, as shown in studies with M_1 muscarinic receptors [5]. Orexin stimulation of mouse $OX_{2\alpha}$–$OX_{2\beta}$ heterodimers also induces and increases the BRET response [32]. For some GPCRs, the dimers may be rather stable. As concerns CB_1 receptors, the data are in some contradiction: rapid formation of CB_1–D_2 receptor complexes is seen upon receptor co-stimulation but, on the other hand, the CB_1–D_2 as well as homomeric CB_1 receptor complexes appear to mostly resist even strongly denaturing conditions [3,4]. We only observed a minor (10%) reduction in BRET between OX_1 and CB_1 receptors upon orexin stimulation (Fig. 2D). However, it is possible that there is not much of a chance of seeing any dynamic regulation of OX_1–CB_1 complexes if the complexes formed are stable. However, the methodology used in the present study cannot resolve this, and further studies would be required.

In summary, we show that both orexin receptor subtypes and CB_1 cannabinoid receptors are capable of forming constitutive homo- and heteromeric complexes. Whether these have physiological function or whether they would be dynamically regulated, remains to be shown. An interesting way of determining the physiological significance is presented by the so-called bivalent ligands. Such ligands would show some selectivity for the receptor homo- or heterodimers, as opposed to monomeric receptors. Such ligands have also been designed for some CB_1 receptor dimers [33,34]. It would be interesting to see if such bivalent ligand could be made for OX_1–OX_2 heteromers or homomeric OX_1 or OX_2 complexes. OX_1 and OX_2 receptor mRNA is coexpressed in a number of tissues and it would be altogether interesting to see, whether heteromeric complexes with a pharmacology or signaling properties different from the homomeric receptors and receptor complexes may be formed.

Acknowledgments

This study was supported by the Academy of Finland, the Magnus Ehrnrooth Foundation and the Liv & Hälsa Foundation. Maria Jäntti is affiliated with the FinPharma Doctoral program-Drug Discovery Section (FPDP-D). We would also like to thank Dr. Jani Saarela from Institute for Molecular Medicine Finland (FIMM) for technical assistance and access to PherastarFS.

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