ßarrestin1-biased agonism at human δ-opioid receptor by peptidic and alkaloid ligands

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ABSTRACT

We have previously reported on the differential regulation of the human δ-opioid receptor (hDOR) by alkaloid (etorphine) and peptidic (DPDPE and deltorphin I) ligands, in terms of both receptor desensitization and post-endocytic sorting. Since ßarrestins are well known to regulate G protein-coupled receptors (GPCRs) signaling and trafficking, we therefore investigated the role of ßarrestin1 (the only isoform expressed in our cellular model) in the context of the hDOR. We established clonal cell lines of SK-N-BE cells over-expressing ßarrestin1, its dominant negative mutant (ßarrestin1$^{319–418}$), and shRNA directed against endogenous ßarrestin1. Interestingly, both binding and confocal microscopy approaches demonstrated that ßarrestin1 is required for hDOR endocytosis only when activated by etorphine. Conversely, functional experiments revealed that ßarrestin1 is exclusively involved in hDOR desensitization promoted by the peptides. Taken together, these results provide substantial evidence for a ßarrestin1-biased agonism at hDOR, where ßarrestin1 is differentially involved during receptor desensitization and endocytosis depending on the ligand.

1. Introduction

One third of drugs used in clinical practice, including opioids, target G protein-coupled receptors (GPCRs) to produce their therapeutic effects. However, when prolonged treatment is required, a reduction of their potency, called *tachyphylaxis*, is often observed. A lot of effort has been dedicated to elucidate the molecular basis of such phenomenon referred to as *desensitization*. Most of these data were obtained from Lefkowitz's group using the ß2-adrenergic receptor. In this model, both ßarrestins 1 and 2 (also named arrestin2 and 3, respectively) uncouple phosphorylated GPCRs from their cognate G proteins and promote internalization, which makes ßarrestins the most important proteins playing a role in

desensitization [1]. In case of opioid receptors, ßarrestins have been shown to regulate μ (MOR), δ (DOR) and κ (KOR) uncoupling [2,3] and internalization [4,6]. Indeed, dominant-negative mutants of ßarrestin (i.e. ßarrestin1$^{319–418}$ or ßarrestin1-V53D) were shown to inhibit KOR [4], DOR [5] and MOR [6] internalization triggered by (trans)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide (U50488H), D-Penicillamine(2,5)-enkephalin (DPDPE) and etorphine, respectively. However, a recent report challenged this model of opioid receptor regulation by ßarrestins. Indeed, while both receptor phosphorylation and ßarrestin1 recruitment are required for the selective μ-agonist [D-Ala(2),N-Me-Phe(4),Gly(5)-ol]-enkephalin (DAMGO)-induced MOR desensitization, neither the MOR-S375A mutation (eliminating the key phosphorylation site) nor the absence of ßarrestins expression (in the mouse embryonic fibroblasts; MEFs) reduced morphine-promoted desensitization [7].

An emerging concept termed "biased agonism", "functional selectivity" or "agonist-directed trafficking" adds yet another level of complexity to the classical view of GPCR regulation. According to this concept, a ligand-occupied receptor can preferentially activate different signaling pathways (and consequently, promotes different responses *in vivo*), depending upon which ligand is bound (see for review [8]). Such observations were reported when examining

different readouts (G protein coupling, second messenger production, ßarrestin recruitment, receptor phosphorylation and/or internalization) promoted by various ligands. For example, by studying a panel of ß1 or ß2-adrenergic receptors ligand-mediated responses of the adenylyl cyclase or the mitogen-activated protein kinase (MAPK) pathways, Galandrin and Bouvier (2006) [9] demonstrated the pluridimensionality of ligand efficacy. In fact, a given ligand can act as an agonist on the MAPK pathway while simultaneously acting as an antagonist on the cAMP pathway. This ligand-specific response of a GPCR is likely a result of selective stabilization of a limited set of receptor conformations that preferentially interact with specific partners. Recently, McPherson and collaborators (2010) [10] used the same approach with MOR (examining G protein coupling, ßarrestin interaction, receptor phosphorylation and internalization) by using a panel of 21 opioid ligands. In this study, the authors clearly demonstrated that opioid ligands, similar to ß-adrenergic ligands, also display a biased agonism. Therefore, we decided to test this biased agonism phenomenon in our cellular model: the human neuroblastoma SK-N-BE, endogenously expressing the hDOR [11].

We have previously shown that the hDOR is subject to complex differential regulation upon alkaloid (etorphine) or peptidic agonist exposure (i.e. DPDPE and Tyr-D-Ala-Phe-Asp-Val-Val-Gly.NH$_2$ (deltorphin I)). This differential regulation was observed in terms of G-protein coupling [12], receptor desensitization [13], post-endocytic trafficking [14] and GRK requirement [15]. Here, we investigated the role of ßarrestin1 (the only ßarrestin isoform expressed in our cellular model) in this differential regulation, focusing on hDOR desensitization and endocytosis induced by these three ligands. We generated clonal lines of SK-N-BE cells over-expressing either wild type or mutant form of ßarrestin1. We also knocked down endogenous ßarrestin1 expression using shRNAs. We report that ßarrestin1, is essential for peptide-induced hDOR desensitization of the cAMP pathway, and also exclusively involved in hDOR endocytosis promoted by etorphine. These results demonstrate for the first time that the human DOR is differentially regulated via ßarrestin1-biased mechanism depending on the ligand.

2. Material and methods

2.1. Plasmids

cDNAs for ßarrestin1-GFP, ßarrestin1$^{319-418}$-GFP, FLAG-hDOR and hemagglutinin tagged-Vasopressin type 2 receptor (HA-V2R) were kindly provided by Prof. S. Cotecchia (Université de Lausanne, Switzerland), Prof. N.W. Bunnett (University of California, San Francisco, USA) and Prof. M. Bouvier (Université de Montréal, QC, Canada), respectively. The expression plasmid for pcDNA3.1-hygro (+)-FLAG-hDOR was purchased from TOP Gene Technologies (Montreal, Quebec, Canada).

2.2. Specific ßarrestin1 knockdown by shRNA

shRNAs used for specifically silencing ßarrestin1 expression were originally described by Ahn et al. (2003) [16]. The double-stranded sequences targeting the endogenous ßarrestin1 (5′-AAAGCCUU-CUGCGCGGAGAAU-3′) or a mismatch control sequence (5′-AAGGACCG-CAAAGUGUUGUGU-3′) were cloned into the pRetroSuper-NeoGFP vector (Oligoengine).

2.3. Cell culture

SK-N-BE and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma), supplemented with 10% fetal calf serum (FCS) (Biowest), 1% antibiotic–antimycotic mixture (Sigma), and 2 mM L-glutamine at 37 °C in a water-saturated atmosphere containing 5% CO$_2$.

2.4. Generation of the SK-N-BE clonal cell lines

SK-N-BE cells were transfected with GFP alone, ßarrestin1-GFP, ßarrestin1$^{319-418}$-GFP and shRNAs (directed against ßarrestin1 or a mismatch sequence) by the Amaxa's Nucleofector™ technology (Kit L, program A-20), according to the manufacturer's instructions. Stably transfected cells were generated after selection with 1 mg.ml^{-1} geneticin (G418, Sigma Aldrich), and clonal cell lines were obtained from G418-resistant cells by standard techniques, and further checked by Western blotting. For the binding experiments, these clonal cell lines were transfected by the Nucleofector™ technology with a pcDNA3.1-hygro(+)-FLAG-tagged-hDOR construct. Stably transfected cells were obtained after selection with 0.5 mg.ml^{-1} hygromycin B (Sigma), and the whole pool of resistant cells was used without further clonal selection.

2.5. RNA isolation and RT-PCR experiments

Total RNA was extracted from SK-N-BE cells using RNAgent^R total RNA isolation system (Promega), according to the manufacturer's instructions. 3 μg of total RNA were reverse transcribed and ß-actin, ßarrestins 1 and 2 expressions were studied by PCR using the following primers: 5′ATGGATGATGATATCGCCGCG3′ (forward) and 5′TCCA-GACGCAGGATGGCATGG3′ (reverse) for ß-actin, 5′TCA TGT CGG ACA AGC CCT TGC3′ (forward) and 5′CAC TTT GGG CTT GGG GTG CAT3′ (reverse) for ßarrestin 1, 5′CTT CAC CTT GAC CTT GTA GGA3′ (forward) and 5′CAA GGA GCT GTA CTA CCA TGG3′ (reverse) for ßarrestin 2.

2.6. Western blotting

SK-N-BE and HEK293 cells were harvested by centrifugation (100 g, 5 min) and the resulting pellet was dissolved in lysis buffer (10 mM Tris–HCl, 1 mM EDTA, 0.1% (v/v) Triton-X100, pH 7.4), sonicated and then clarified by centrifugation for 15 min at 20,000 g and 4 °C. Total protein concentration was determined by the Bradford assay and equal amounts of supernatant protein were separated on 10% (w/v) acrylamide gels by SDS-PAGE and transferred onto nitrocellulose membranes. ßarrestins, GFP and tubulin were detected by immunoblotting with a monoclonal anti-ßarrestin1 (BD Biosciences), the rabbit polyclonal anti-ßarrestin1 and 2 (A1CT, kindly provided by Profs. R.J. Lefkowitz, Duke University Medical Center, North Carolina, USA and S.A. Laporte, McGill University, QC, Canada), the monoclonal anti-GFP antibody (BD Biosciences) and the rabbit polyclonal anti-ß-tubulin antibody (Santa Cruz), respectively, followed by peroxidase-coupled secondary antibodies. Proteins were visualized by enhanced chemiluminescence system (Super-Signal West Pico, Pierce); densitometric quantification was performed with a Fluor-S Multilmager (Bio-Rad).

2.7. Binding experiments

Radioligand binding studies were performed on attached cells as described previously [17]. Each determination was carried out in triplicate. Scatchard analysis was performed using SigmaPlot software to calculate Kd and Bmax values.

2.8. Measurement of cAMP

Inhibition of adenylate cyclase was determined by measuring [^{3}H] cAMP accumulation as previously described [17]. Maximal inhibitory levels of opioid agonists were determined for each clonal cell line at 0.1X, 1X and 10X of the concentration producing the maximum response in the non-transfected SK-N-BE (BE-WT) cells [13]. All experiments were carried out in triplicate and repeated at least three times with similar results.