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β arrestin1-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 β arrestins are well known to regulate G protein-coupled receptors (GPCRs) signaling and trafficking, we therefore investigated the role of β arrestin1 (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 β arrestin1, its dominant negative mutant (β arrestin1^{319–418}), and shRNA directed against endogenous β arrestin1. Interestingly, both binding and confocal microscopy approaches demonstrated that β arrestin1 is required for hDOR endocytosis only when activated by etorphine. Conversely, functional experiments revealed that β arrestin1 is exclusively involved in hDOR desensitization promoted by the peptides. Taken together, these results provide substantial evidence for a β arrestin1-biased agonism at hDOR, where β arrestin1 is differentially involved during receptor desensitization and endocytosis depending on the ligand.

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Disclosure statement

All authors declare no conflict of interest.

Contributions to this work

BA, LC, AD, NM, AH, FL and SA: experimentations

GT and AB: synthesis of [³H]-TICP-[ψ]

VG: purification of β arrestin1 and generation of plasmid constructs for β arrestin1 mutants

BA, PJ and SA: writing and editing of the manuscript

Keywords

δ-opioid receptor; βarrestin; G protein-coupled receptor; Peptidic and alkaloid ligands

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 β2-adrenergic receptor. In this model, both βarrestins 1 and 2 (also named arrestin2 and 3, respectively) uncouple phosphorylated GPCRs from their cognate G proteins and promote internalization, which makes βarrestins the most important proteins playing a role in desensitization [1]. In case of opioid receptors, βarrestins have been shown to regulate μ (MOR), δ (DOR) and κ (KOR) uncoupling [2,3] and internalization [4,6]. Indeed, dominant-negative mutants of βarrestin (*i.e.* βarrestin1³¹⁹⁻⁴¹⁸ or βarrestin1-V53D) were shown to inhibit KOR [4], DOR [5] and MOR [6] internalization triggered by (*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidiny)-cyclohexyl]benzeneacetamide (U50488H), D-Penicillamine(2,5)-enkephalin (DPDPE) and etorphine, respectively. However, a recent report challenged this model of opioid receptor regulation by βarrestins. Indeed, while both receptor phosphorylation and βarrestin1 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 βarrestins 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, βarrestin recruitment, receptor phosphorylation and/or internalization) promoted by various ligands. For example, by studying a panel of β1 or β2-adrenergic receptors ligand-mediated responses of the adenylyl cyclase or the mitogen-activated protein kinase (MAPK) pathways, Galandrin and Bouvier (2006) [9] demonstrated the pluri-dimensionality 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, βarrestin 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 β-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.NH₂ (deltorphan 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 β arrestin1 (the only β arrestin 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 β arrestin1. We also knocked down endogenous β arrestin1 expression using shRNAs. We report that β arrestin1, 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 β arrestin1-biased mechanism depending on the ligand.

2. Material and methods

2.1. Plasmids

cDNAs for β arrestin1-GFP, β arrestin1³¹⁹⁻⁴¹⁸-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 β arrestin1 knockdown by shRNA

shRNAs used for specifically silencing β arrestin1 expression were originally described by Ahn et al. (2003) [16]. The double-stranded sequences targeting the endogenous β arrestin1 (5'-AAAGCCUUCUGCGCGGAGAAU-3') or a mismatch control sequence (5'-AAGGACCGCAAAGUGUUGUGU-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% CO₂.

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

SK-N-BE cells were transfected with GFP alone, β arrestin1-GFP, β arrestin1³¹⁹⁻⁴¹⁸-GFP and shRNAs (directed against β arrestin1 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 RNeasy total RNA isolation system (Qiagen), according to the manufacturer's instructions. 3 µg of total RNA were reverse transcribed and β -actin, β arrestins 1 and 2 expressions were studied by PCR using the following primers: 5' ATGGATGATGATATCGCCGCG3' (forward) and 5' TCCAGACGCAGGATGGCATGG3' (reverse) for β -actin, 5' TCA TGT CGG ACA

AGC CCT TGC3' (forward) and 5' CAC TTT GGG CTT GGG GTG CAT3' (reverse) for β arrestin 1, 5' CTT CAC CTT GAC CTT GTA GGA3' (forward) and 5' CAA GGA GCT GTA CTA CCA TGG3' (reverse) for β arrestin 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. β arrestins, GFP and tubulin were detected by immunoblotting with a monoclonal anti- β arrestin1 (BD Biosciences), the rabbit polyclonal anti- β arrestin1 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- β -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 MultiImager (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 *K_d* and *B_{max}* values.

2.8. Measurement of cAMP

Inhibition of adenylate cyclase was determined by measuring [³H] 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.

2.9. Quantification of hDOR endocytosis with [³H]TICP[]

[³H]TICP[ψ], a δ -selective antagonist [18], was used to measure cell surface opioid receptor internalization on attached cells, as previously described [17].

2.10. Confocal microscopy

SK-N-BE-hDOR cells, stably over-expressing the FLAG-tagged-hDOR (~2.6 pmol.mg⁻¹ of protein) were transfected with vectors encoding GFP (control), β arrestin 1-GFP, β arrestin 1³¹⁹⁻⁴¹⁸-GFP or shRNAs directed against the specific sequence of the endogenous β arrestin1, or with control mismatched sequence using the Amaxa's Nucleofector™. 48 h post-transfection, cells were treated with or without (control) opioid ligands. Cells were gently washed with PBS and fixed with a fresh solution of 4% (w/v) paraformaldehyde (PFA) for 15 min and then permeabilized with 0.1% (w/v) saponin for 10 min. Cells were rinsed thoroughly with PBS, blocked with PBS/1% (w/v) BSA for 30 min at room temperature, and incubated with the monoclonal anti-FLAG M2 (Sigma) antibody (5 μ g.ml⁻¹) in the blocking buffer for 2 h at room temperature. After washing with PBS, cells were incubated with TRITC-conjugated anti-mouse IgG (Sigma) in the blocking buffer for 2 h at room temperature. Coverslips were washed with PBS and mounted. Images were obtained using an Olympus confocal laser microscope (Fluo view), lens $\times 60$.

2.11. Quantification of endogenous β arrestin1 by western blotting

Purified β arrestin1 [19] was diluted in the lysis buffer to obtain a final concentration of 1 ng. μ l⁻¹. Protein concentration of cellular extracts obtained from the BE-WT was determined by the Bradford assay and 20, 30, 40, 50 and 60 μ g of proteins were loaded along with 7.5, 10, 15, 17.5, 20 and 25 ng of purified β arrestin 1 onto a 10% (w/v) acrylamide gel, separated by SDS-PAGE and transferred on to nitrocellulose membrane. β arrestin 1 was detected by immunoblotting with the mouse monoclonal anti- β arrestin 1 (BD Biosciences). Chemiluminescent detection and densitometric quantification were performed as described above.

2.12. β arrestin1-GFP translocation studies

SK-N-BE- β arrestin1-GFP clonal cells were transfected by the Nucleofector™ technology either with HA-V2R or FLAG-hDOR. 48 h post-transfection, cells were treated with vehicle (control), 1 μ M desmopressin (V2R agonist) or opioid agonists for 5 or 10 min. The medium was removed and cells were fixed with 4% (w/v) PFA. Coverslips were rinsed with PBS and mounted. Images were obtained on a Nikon Eclipse E-800 microscope (lens $\times$ 60).

2.13. Statistical analysis

All results are expressed as the mean $\pm$ standard error of the mean (S.E.M.) of n experiments. ANOVA (Graphpad Prism 4.0™) followed either by the Dunnet or the Bonferroni Test or Student t -test, where appropriate, were used to determine the statistical significance.

3. Results

3.1. Establishment and characterization of clonal lines from SK-N-BE cells

To characterize the regulation of hDOR by β arrestins, we first determined the expression of β arrestin isoforms in SK-N-BE cells. We amplified by RT-PCR mRNAs encoding both β arrestins 1 and 2 (Fig. 1A). However, Western blotting using the A1CT antibody (that recognizes both isoforms), revealed that SK-N-BE cells only express β arrestin1 (a band at $\sim$ 50 kDa), while in HEK293 cells (known to endogenously express both isoforms) a 45–50-kDa doublet was observed (Fig. 1B). This was also confirmed using a specific anti- β arrestin1 antibody (Fig. 6B).

Five clonal cell lines were generated from SK-N-BE cells (BE-WT, Fig. 1C, lane 1): three over-expressing GFP alone (lane 2), WT- β arrestin1-GFP (WT- β arr1-GFP, lane 3) or β arrestin1^{319–418}-GFP (DN- β arr1-GFP, dominant negative form of β arrestin1 corresponding to its carboxyl-tail domain, lane 4) and two expressing shRNAs, control (GFP-shRNA-MM, Fig. 1D, lane MM) or against the endogenous β arrestin1 (GFP-shRNA- β arr1, lane β arr1, Fig. 1D). We assessed the proper expression levels of β arrestin 1 (endogenous and GFP-tagged) by Western blots using the A1CT antibody. As shown in Fig. 1C, this antibody recognizes the endogenous β arrestin1 (a band at $\sim$ 50 kDa) in each cell line (lanes 1 to 4). GFP-tagged forms of β arrestin1 WT and DN were detected at $\sim$ 80 kDa (lane 3) and $\sim$ 40 kDa (lane 4), respectively. We confirmed that the two fusion proteins corresponded to the appropriate GFP-tagged forms by using an anti-GFP antibody, which also detected GFP alone (Fig. 1C, WB GFP, lanes 3 and 4 vs 2). In GFP-shRNA- β arr1 cells, a major and specific reduction ($\sim$ 70%) of endogenous β arrestin1 expression was observed, as compared to the GFP-shRNA-MM cells (Fig. 1D).

We ascertained that β arrestin1 overexpression or depletion did not change the pharmacological parameters ($B_{\max}$ and K_d values) of hDOR obtained in the different clonal cell lines compared to BE-WT (Table 1, first row).

3.2. Role of β arrestin1 in hDOR desensitization

To define the role of β arrestin1 in hDOR desensitization, we first used dose–response experiments to determine the agonist concentration ($[L]$) producing the maximal inhibition of adenylylate cyclase (AC) ($I_{\max}$) in each clonal cell line (Table 2).

We then determined the time course of hDOR-regulated AC activity in the clonal cells (where AC activity in naive cells was normalized to 100%, see Fig. 2). As previously reported [13], hDOR desensitization in BE-WT cells induced by peptides was both stronger and faster than with etorphine. Indeed, we observed a complete desensitization for the peptides after 15 min *vs* 50% desensitization after 30 min with etorphine (Fig. 2A). To address the involvement of β arrestin1 in this differential desensitization process, we first repeated the same experiments in the WT- β arr1-GFP cells. As shown in Fig. 2B, no potentiation of desensitization was evidenced upon peptides exposure, even at shorter time (5 min, data not shown), while consistently with our previous report [20], β arrestin1 overexpression significantly enhanced etorphine-promoted hDOR desensitization. Moreover, in this case the three ligands demonstrated the same desensitization profile (Fig. 2B).

To further delineate the role of β arrestin1 in hDOR desensitization, we then performed the same functional assays in β arrestin1-depleted cells (GFP-shRNA MM *vs* β arr1, Fig. 2C). Surprisingly, we observed a drastic reduction of hDOR desensitization, but only for the peptides (~55% and ~70% reduction for DPDPE and deltorphin I respectively, Fig. 2C). Indeed, we detected no change of etorphine-induced desensitization after 30 min exposure. Thus, the reduction of endogenous level of β arrestin1 by shRNA only affects peptide-induced hDOR desensitization, which strongly suggests a β arrestin1-biased agonism at the hDOR desensitization level.

3.3. Role of β arrestin1 in hDOR endocytosis

In order to determine the involvement of β arrestin1 in hDOR endocytosis quantitatively and qualitatively, we performed in parallel both binding and confocal microscopy experiments. Endocytosis was measured as a disappearance of cell surface receptors after ligand exposure, using the tritiated peptidic antagonist TICP- $[\psi]$, as previously described [17]. To increase the specific binding of $[^3H]$ TICP- $[\psi]$, clonal cell lines were transfected with a FLAG-tagged-hDOR. Binding experiments revealed a significant increase in receptor expression compared to clonal cell lines endogenously expressing hDOR, without any significant change in K_d values for $[^3H]$ diprenorphine (Table 1, second row).

In the BE-WT cells expressing FLAG-hDOR, a brief agonist peptidic exposure promoted a stronger hDOR endocytosis than with etorphine (~50–55% after 5 min *vs* ~40% after 10 min, respectively, Fig. 3A–C). Maximal endocytosis was reached after 30 min for etorphine and the peptides (~60% and ~80%, respectively, data not shown). Interestingly, overexpression of β arrestin1 significantly enhanced only etorphine-induced hDOR internalization (~60% *vs* ~40%, Fig. 3A–C). We then monitored FLAG-hDOR and β arrestin1-GFP intracellular trafficking by confocal microscopy (Fig. 3D). In untreated (naïve) WT- β arr1-GFP cells, β arrestin1-GFP exhibited an homogeneous distribution throughout the cytoplasm, whereas the FLAG-hDOR was localized at the plasma membrane. Upon agonist stimulation (30 min etorphine *vs* 15 min peptides at concentrations indicated in Table 2), we observed a strong FLAG-hDOR internalization in the presence of etorphine and peptides (as revealed by perinuclear punctuate staining, Fig. 3D, center column). Interestingly, internalized hDOR co-localized with β arrestin1-GFP only when activated by etorphine (Fig. 3D, merged panel), supporting our binding evidence for a β arrestin1-biased agonism at the hDOR endocytosis level.

In addition, in the DN- β arr1-GFP and shRNA- β arr1-GFP cells, hDOR endocytosis was strongly reduced in the presence of etorphine (~40–50% reduction after 30 min stimulation, Fig. 4A), while receptor endocytosis promoted by both peptides remained unaffected (Fig. 4B and C). These two observations strongly suggest that β arrestin 1 is only required for the endocytosis of hDOR activated by etorphine. Furthermore, upon overexpression of DN- β arr1-GFP and shRNA against β arrestin1 cells (containing GFP as a marker of transfected cells), we showed that hDOR endocytosis was dramatically affected only for etorphine (Fig. 4D and E). Indeed, after 30 min treatment with this ligand, immunostaining for the FLAG-hDOR was confined to the plasma membrane of transfected cells expressing GFP (*i.e.* Fig. 4D for DN- β arr1-GFP and Fig. 4E for shRNA- β arr1-GFP), while in non-transfected (GFP negative) cells, the endocytotic process was unaffected. In GFP-shRNA-MM cells, internalization promoted by the various agonists was similar to that obtained in BE-WT cells overexpressing the FLAG-hDOR (data not shown). Again, this β -arrestin-dependent endocytosis of hDOR appears etorphine-specific, since neither the dominant negative mutant of β arrestin 1 nor depletion of endogenous β arrestin 1 blocked internalization induced by peptides (Fig. 4D and E, peptides 15 min).

Thus, both radioligand binding and confocal microscopy studies demonstrate that hDOR endocytosis was regulated through a β arrestin-biased agonism process, since β -arrestin1 is only required in the presence of etorphine.

4. Discussion

The study presented here investigated the role of β arrestin 1 (the only isoform expressed in our cellular model) in the differential regulation of hDOR observed with alkaloid (etorphine) vs peptidic ligands (DPDPE and deltorphin I) [12–15].

4.1. Comparison with previous studies

1. When examining endocytosis, we found that β arrestin1 is only required upon etorphine activation. This was demonstrated by over-expression of WT- β arrestin1, which increased the alkaloid-induced hDOR internalization to the level produced by peptides. Furthermore, the expression of dominant negative β arrestin mutant or depletion of endogenous β arrestin1 by shRNA produced a major reduction of 50% of hDOR endocytosis, but only for etorphine. This was also confirmed by confocal microscopy experiments where we observed a blockade of endocytosis in GFP positive cells (only those expressing the DN- β arr1 or the shRNA directed against β arrestin 1). In contrast, neither potentiation nor reduction of hDOR endocytosis was detected upon exposure to peptides in cells where WT β arrestin1 was overexpressed or the expression of endogenous β arrestin1 was knocked down by shRNA. Our data are inconsistent with those obtained by Xiang and collaborators (2001) [5], who reported that DPDPE-induced DOR internalization could be increased by overexpression of WT β arrestin1. Such discrepancies could be explained by the different cellular models used (HEK cells in their study) and by the different DOR species (mouse in their study). Indeed, major differences are noted between carboxyl-tail domain of human vs mouse DOR, in the number and the localization of putative phosphorylation sites, known to be crucial for β arrestin interaction. Our data obtained in the neuroblastoma SK-N-BE strongly indicate that β arrestin1 is not required for internalization of hDOR activated by peptidic agonists. To the best of our knowledge, this is the first demonstration of β arrestin-independent opioid receptor internalization. While this protein was shown to be crucial for endocytosis of most GPCRs, such β arrestin-independent endocytosis has been previously reported for the protease-activated receptor-1 [21] or the N-formyl peptide receptor [22], and M2 muscarinic receptor [32].

2. Here we report two major findings. First, while in BE-WT cells hDOR desensitization was stronger with the peptidic ligands, over-expression of WT β arrestin1 abolished this difference by increasing the rate of etorphine-induced desensitization. This argues for a role of β arrestin1 in etorphine-promoted desensitization. Second, the reduction of endogenous β arrestin1 level by shRNA only inhibited desensitization induced by peptides, suggesting that etorphine-induced desensitization is β arrestin1-independent, which is unexpected in view of our first finding. To reconcile these two observations, we hypothesize that the affinity of etorphine-activated hDOR for β arrestin1 is not high enough to promote its recruitment that would lead to desensitization. However, this could be overcome by increasing the level of β arrestin1, as previously reported by our group for hDOR [20] as well as by others for morphine-activated MOR [24] and carbachol-activated M2 muscarinic receptor [32]. Regarding the peptidic ligands, it has been shown that DPDPE-induced mDOR desensitization was reduced in MEFs cells lacking β arrestins 1 and 2, as compared to WT-MEFs [23]. Thus, our data are in good agreement with those obtained by Law's group, showing that β arrestin is required for peptide-induced hDOR desensitization.

4.2. β arrestin1-biased agonism at hDOR

How to reconcile that β arrestin1 is involved in peptide-induced desensitization, but only appears to be recruited by etorphine-activated hDOR during endocytosis? Based on the observations of Chavkin and collaborators [25] showing that MOR contains one domain responsible for the GRK-dependent desensitization (Thr 180 in the second intracellular loop) and one for the GRK- and β arrestin-dependent endocytosis (in the carboxyl-tail domain), we hypothesize that hDOR may also contain two distinct interacting domains for β arrestin1: an “*uncoupling domain*” and an “*internalization domain*” responsible for β arrestin-dependent desensitization or internalization, respectively. Indeed, Cen et al. clearly demonstrated that DOR binds β arrestin via two distinct domains: its carboxyl-tail domain or its third intracellular loop [26]. Thus, we propose that those two regions would be differentially unmasked upon binding of peptidic or alkaloid ligands at the receptor (Fig. 5). Depending on the interacting domains, β arrestin 1 would be either involved in receptor uncoupling (in case of peptides) or in receptor endocytosis (in case of etorphine) by recruiting other partners such as clathrin or adaptin. This model is currently under investigation.

Another observation also requires an explanation: independently of the opioid ligand used in our cellular model, β arrestin1 is involved in hDOR regulation but does not translocate to the plasma membrane (Fig. 6A, +hDOR). First, we showed that activated V2R strongly promotes β arrestin1-GFP translocation in our cells (Fig. 6A, +V2R). Subsequently, we quantified β arrestin1 and hDOR endogenous levels in SK-N-BE cells and showed a 4:1 ratio (10.000 fmol.mg⁻¹ for β arrestin1 vs ~2.500 fmol.mg⁻¹ for hDOR, Fig. 6B). Since only β arrestin1 monomer is competent to bind GPCRs and β arrestin binds receptors at a 1:1 ratio [27], the most likely scenario is that endogenous β arrestin1 competes with β arrestin1-GFP for interacting with hDOR. Thus, the fraction of β arrestin1-GFP that translocates to the membrane upon hDOR activation is below detection threshold. However, this technical limitation is overcome by expressing higher levels of GPCRs, *e.g.*, V2R known to promote robust β arrestin-GFP translocation due to the presence of Ser/Thr cluster.

Traditional model of β arrestin-receptor interaction is based on the idea that a given β arrestin forms only one type of complex with a particular receptor, simultaneously producing all β arrestin-dependent effects on GPCR signaling and trafficking [28]. However, accumulating evidence suggests that β arrestin-receptor complexes are dynamic and highly regulated [29]. These complexes may come in different “flavors” depending on the ligand-promoted receptor conformation, the position and/or the number of receptor-attached phosphates

(reviewed in [30] and [31]). The first findings supporting this unorthodox notion were reported with the M2 muscarinic receptor, where β arrestin is involved in desensitization, but not internalization [32]. Subsequent discoveries that two phosphorylation domains of the N-formyl peptide receptor differentially regulate β arrestin and agonist affinity strongly supported this notion [33]. The phosphorylation of the same receptor by different GRKs promotes either sequestration or β arrestin-dependent ERK activation [34, 35], indicating that β arrestin binding to different phosphorylated regions of the TRH receptor has strikingly different functional consequences [36]. Rhodopsin, a prototypic GPCR, was also demonstrated to be differentially phosphorylated and form distinct complexes with rod arrestin [37]. Yet another traditional paradigm in GPCR field, that receptor activation by any agonist yields essentially the same active conformation, equally recognized by G proteins, GRKs, and β arrestins was challenged by recent findings that certain agonists differentially promote receptor interactions with G proteins [12] and β arrestins [38–40].

5. Conclusions

Our data not only bridge and further refine the emerging concept of β arrestin-biased agonism of GPCR signaling and trafficking, but also suggest that binding of alkaloid *vs* peptidic opioid ligands would unmask different intracellular regions of the hDOR that differentially interact with β arrestin1, mediating either uncoupling or internalization. Further work is required to clearly identify and delineate those two regions in the hDOR.

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Abbreviations

hDOR	human delta-opioid receptor
DPDPE	D-Penicillamine(2,5)-enkephalin
Deltorphin I	Tyr-D-Ala-Phe-Asp-Val-Val-Gly.NH ₂

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Fig. 1.

Characterization of β arrestin expression in the clonal cell lines derived from SK-N-BE cells. (A) β actin, β arrestins 1 and 2 expressions were studied in SK-N-BE cells (BE) by RT-PCR. A negative control (C-, without cDNA) was used to validate the specificity of amplification. A 100 bp ladder was used to determine the PCR products size. (B) Whole cell lysates were prepared both from wild type SK-N-BE (BE-WT) and HEK293 cells (used as a positive control for β arrestins 1 and 2 expression) and resolved by SDS/PAGE. Proteins were transferred onto a nitrocellulose sheet and probed for β arrestins 1 and 2 using the polyclonal antibody A1CT. (C) Whole cell lysates were prepared from wild type SK-N-BE cells (lane 1), GFP alone (lane 2), β arrestin 1-GFP (lane 3) or DN- β arr1-GFP (lane 4) clonal cell lines. Proteins were resolved by SDS/PAGE, transferred onto a nitrocellulose sheet and immunoblotted either with the anti- β arrestins A1CT antibody, a monoclonal anti-GFP antibody or an anti-tubulin antibody. (D) Proteins were extracted from the clonal cell lines expressing shRNA directed against a mismatch sequence (lane shRNA-MM) or the β arrestin 1 (lane shRNA- β arr1) and resolved by SDS/PAGE. The expression level of β arrestin 1 was

determined by using both the A1CT antibody and an anti-tubulin antibody used as an internal loading control. The ratio of β arrestin 1/tubulin was determined by densitometric analysis and revealed a profound and significant β arrestin 1 depletion in shRNA- β arr1-GFP vs shRNA-MM-GFP cells. Data are means $\pm$ S.E.M. of 3 different experiments (*, $p < 0.05$, Student t -test). Molecular weight standards are indicated in kDa.

Fig. 2.

Role of β arrestin1 in hDOR desensitization. BE-WT (A), β arrestin1-GFP (B), shRNA-MM-GFP and shRNA- β arr1-GFP (C) cells were pretreated in the presence or absence of etorphine for 30 min vs DPDPE or deltorphin I for 15 min to induce hDOR desensitization. Then the ability of those ligands to inhibit AC activity was measured. Ligand-induced inhibition in naive cells was referred as 100%. Data are means $\pm$ S.E.M. of 3–5 different experiments performed in triplicate. A and B, *, $p < 0.05$, one-way ANOVA followed by Dunnett's test compared to etorphine 30 min). C, *, $p < 0.05$ t -test compared to shRNA-MM. N.S., non significant.

Fig. 3. β arrestin1 is involved in etorphine-promoted hDOR endocytosis. BE-WT and β arrestin1-GFP cells, overexpressing the FLAG-hDOR, were stimulated 10 min with etorphine (A) or 5 min (B and C) with the peptides to induce endocytosis. Cell surface receptors were determined using [3 H]TICP-[ψ]. Values represent the % of internalized receptor in agonist-pretreated cells compared to untreated control cells. Means $\pm$ S.E.M. of 3–5 different experiments performed in triplicate are shown. (*, $p < 0.05$, Student t -test compared to BE-WT). N.S., not significant. (D) SK-N-BE cells stably overexpressing the FLAG-hDOR were transiently transfected with β arrestin1-GFP. Cells were incubated with or without (naive cells) etorphine, DPDPE or deltorphin I. After treatment, cells were fixed and immunostained with the anti-FLAG M2 antibody. Immunoreactivity was revealed with a TRITC-conjugated secondary antibody. Localization of the FLAG-hDOR (in red) and β arrestin1-GFP (green) was observed by confocal microscopy at a 60 $\times$ lens. Images are representative of 2 to 4 independent experiments. White arrows indicate the FLAG-hDOR.

Fig. 4.

βarrestin1-biased agonism at hDOR endocytosis level. BE-WT, DN-βarr1-GFP, shRNA-MM-GFP and shRNA-βarr1-GFP cells overexpressing FLAG-hDOR, were stimulated for 30 min with etorphine (A) or for 15 min with DPDPE (B) or deltorphin I (C) to induce endocytosis. Cell surface receptors were determined using [3 H]TICP- $[\psi]$. Values represent the % of internalized receptor in agonist-pretreated cells compared to untreated control cells. Means $\pm$ S.E.M. of 3–5 different experiments performed in triplicate are shown. (* and #, $p < 0.05$, t -test compared to BE-WT and shRNA MM, respectively). N.S., not significant. (D) SK-N-BE cells stably overexpressing FLAG-hDOR were transiently transfected with the DN-βarr1-GFP or shRNA-βarr1-GFP. Cells were incubated with or without (naive cells) etorphine, DPDPE or deltorphin I. After treatment, cells were fixed and immunostained with the anti-FLAG M2 antibody. Immunoreactivity was revealed with a TRITC-conjugated secondary antibody. Localization of the FLAG-hDOR (in red) and GFP (green) was observed by confocal microscopy at a 60 $\times$ lens. Images are representative of 2 to 4 separate experiments. White arrows indicate the FLAG-hDOR.

Fig. 5.

A model of βarrestin1-biased agonism at hDOR level. We propose that hDOR contains two distinct intracellular regions that could independently interact with βarrestin1. Upon etorphine activation, hDOR displays an R*1 conformation that unmaskes the "internalizing domain" (id). In contrast, receptor activation by peptide agonists preferentially stabilizes an R*2 conformation of the receptor exposing its "uncoupling domain" (ud).

Fig. 6.

Absence of plasma membrane β arrestin1-GFP translocation upon hDOR activation. (A) The β arr1-GFP cell line was transfected either with the V2R or the FLAG-hDOR. After addition of the ligand (*i.e.*, desmopressin for V2R vs Etorphine, DPDPE or deltorphin I for the FLAG-hDOR), a rapid (5 min) translocation of β arrestin1-GFP was observed only in the V2R transfected cells. (B) Quantification of the endogenous β arrestin1 in the BE-WT cells. Six samples containing 7.5, 10, 15, 17.5, 20 and 25 ng of purified β arrestin1 were analyzed by Western blot along with five samples containing 20, 30, 40, 50 and 60 μ g of cellular extract from the BE-WT cells. A calibration curve was constructed from quantification of the purified β arrestin1 bands by densitometry analysis (filled circles) to determine the amounts of β arrestin1 in the BE-WT cells (open circles). Data are a representative experiment repeated twice with similar results.

Binding parameters of the endogenous and the FLAG-hDOR in the different clonal cell lines. K_d (nm) and B_{max} (fmol.mg⁻¹) values were determined by Scatchard analysis from binding experiments using [³H]diprenorphine in the BE-WT and in the different clonal cell lines. FLAG-hDOR transfection of each clonal cell line effectively increased in opioid receptor expression compared to the BE-WT cells, endogenously expressing the opioid receptor. Means±S.E.M. of 3 different experiments performed in triplicate are shown.

Table 1

	BE-WT		WT-βarr1-GFP		DN-βarr1-GFP		shRNA MM		shRNA βarr1	
	Bmax	Kd	Bmax	Kd	Bmax	Kd	Bmax	Kd	Bmax	Kd
Endogenous hDOR	63.2±7.8	0.5±0.24	83.3±5.1	0.31±0.08	95.3±2.4	0.23±0.03	91.9±10.9	0.37±0.08	74.4±1.8	0.18±0.04
Transfected hDOR	121.1±2.1	0.34±0.01	535.5±73.3	0.56±0.06	187.9±8.2	0.53±0.01	776.9±3.9	0.94±0.01	1000.0±6.3	0.85±0.18

Table 2

Agonist-promoted inhibition of cAMP accumulation in the wild type SK-N-BE cells and in the different clonal cell lines. The ability of etorphine, DPDPE and deltorphin I to inhibit the FSK-stimulated cAMP accumulation was determined as described in Materials and methods. The values of maximum cAMP inhibition (I_{max}) in each cell line were determined from dose-response curves at 0.1X, 1X and 10X, where X is the concentration producing the maximum response in the BE-WT cells. [L] corresponds to the agonist concentration producing the maximal inhibition of AC. Means $\pm$ S.E.M. of 3–5 different experiments performed in triplicate are shown.

	BE-WT		WT- β arr1-GFP		DN- β arr1-GFP		GFP-shRNA β arr1		GFP-shRNA MM	
	[L] M	I_{max} (%)	[L] M	I_{max} (%)	[L] M	I_{max} (%)	[L] M	I_{max} (%)	[L] M	I_{max} (%)
Etorphine	10^{-7}	50.2 ± 2.6	10^{-7}	42.6 ± 3.7	10^{-7}	55.6 ± 2.4	10^{-7}	$31.3 \pm 5.6^{\#}$	10^{-7}	51.1 ± 2.8
DPDPE	10^{-7}	41.7 ± 3.3	10^{-6}	36.0 ± 2.3	10^{-6}	$27.7 \pm 1.7^{**}$	10^{-6}	36.7 ± 6.1	10^{-7}	42.5 ± 5.9
Deltorphin I	10^{-8}	38.4 ± 2.4	10^{-7}	32.3 ± 1.0	10^{-8}	$24.1 \pm 3.1^{**}$	10^{-7}	34.5 ± 1.4	10^{-8}	41.1 ± 3.4

* Significantly different compared to BE-WT (* and ** $p < 0.05$ and < 0.01 , ANOVA followed by Dunnett's, respectively).

[#] $p < 0.01$ t-test compared to shRNA MM.