Selective Interference of β-Arrestin 1 with κ and δ but Not μ Opioid Receptor/G Protein Coupling

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The role of β-arrestin 1 (β-ar1) in regulation of responsiveness of κ, δ, and μ opioid receptors has been investigated in human embryonic kidney 293 cells co-transfected with opioid receptor and β-ar1. Expression of human β-ar1 attenuated κ and δ opioid receptor subtype-mediated inhibition of cAMP production and resulted in a 100-fold increase of EC50 values for κ-agonist U69593 and δ-agonist [D-Pen2,D-Pen5]enkephalin and 30–40% reduction of their maximal responses. In contrast, coexpression of β-ar1 with μ opioid receptor did not affect the concentration-effect relationship of μ-agonist [d-Ala2,N-Me-Phe4,Gly5-ol]enkephalin. In parallel, κ and δ receptor-mediated G protein activation was also remarkably attenuated by overexpression of β-ar1, while the μ-agonist-stimulated response remained intact. These results indicate that β-ar1 interfere[es] receptor/G protein coupling and differentially regulates the responsiveness of opioid receptors. Truncation of κ and δ opioid receptors at carboxyl termini abolished inhibition of β-ar1 on the responsiveness of both receptors. Furthermore, μ opioid receptor became sensitive to β-ar1 regulation following replacement of its carboxyl terminus with the corresponding portion of the δ receptor. Removal of potential phosphorylation sites on the carboxyl terminus of κ opioid receptor led to reduced effect of β-ar1 on the receptor-mediated response. These results suggest that receptor carboxyl terminus and its phosphorylation play an important role in the interaction of β-ar1 and opioid receptors.

Opiates are effective analgesics. However, chronic use of opiates results in tolerance and dependence, which limit clinical application of opioid drugs. Opiates exert their effects in the central and peripheral nervous systems through interaction with μ, δ, and κ, three major types of opioid receptors, which are structurally homologues and belong to the G protein-coupled receptor (GPCR) family. It has been demonstrated clearly in opioid receptor gene knock-out experiments that analgesia, tolerance, and dependence induced by morphine are all mediated by the opioid receptor (1). However, the molecular mechanisms of opiate tolerance and dependence are not well understood. Desensitization of opioid receptors, the reduced responsiveness of opioid receptors upon agonist stimulation, has been implicated as one of the underlying mechanisms (2).

Accumulating evidence indicates that desensitization of opioid receptors involves receptor phosphorylation. Protein kinase C (3–7) and GPCR kinases (GRKs) (8–10) are known to be involved in desensitization of μ, δ, and κ opioid receptors. Agonist-stimulated phosphorylation of μ, δ, and κ opioid receptors has been demonstrated recently (7–11). Receptor desensitization is accepted as one of ubiquitous regulatory mechanisms of GPCRs (12). Homologous desensitization of GPCRs are well characterized using the β2-adrenergic receptor as a model, and GRKs and arrestins are involved (13). GRKs catalyze phosphorylation of agonist-occupied GPCRs, and their functional cofactor arrestins bind to the phosphorylated receptor, subsequently leading to quenching of G protein activation and reduction of GPCR-mediated responsiveness (12–18).

Four members of the arrestin family have been cloned and characterized to date (12, 14, 19). Visual arrestin is predominantly localized in retina where it regulates phototransduction (20). β-Arrestin 1 (β-ar1) and β-arrestin 2 (21) are widely expressed in many tissues, especially in the central nervous system, and both play important roles in the desensitization of several Gs- and Gi-coupled receptors (12, 14, 16, 21). Cone-specific arrestin, termed X-arrestin (22) or C-arrestin (23), a fourth member of the arrestin family, was cloned recently. Overexpression of β-ar1 and β-arrestin 2 attenuates cellular signaling events mediated by many GPCRs such as β2-adrenergic receptor (15), M4 muscarinic receptors (24), and α1B-adrenergic receptor (25).

Data suggesting the involvement of GRKs in phosphorylation and desensitization of opioid receptors have emerged recently. Overexpression of a dominant-negative mutant of β-adrenergic receptor kinase 1 (GRK2) blocks desensitization of κ opioid receptor (KOR) in COS-7 cells (10). Overexpression of GRK2 and GRK5 in human embryonic kidney (HEK) 293 cells enhances agonist-induced phosphorylation and desensitization of the δ opioid receptor (DOR), while the expression of a mutant form of GRK2 inhibits agonist-dependent DOR phosphorylation and desensitization (9). In addition, it was reported recently that agonist-induced receptor phosphorylation and internalization are greatly inhibited by truncation or substitution of serine or threonine residues at the carboxyl terminus of DOR (8, 26), and desensitization of μ opioid recep-

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The abbreviations used are: GPCR, G protein-coupled receptor; β-ar1, β-arrestin 1; HEK, human embryonic kidney; GRK, G protein-coupled receptor kinase; KOR, κ opioid receptor; DOR, δ opioid receptor; MOR, μ opioid receptor; GIRKs, G protein gated inwardly rectifying K+ channels; DOR-T, truncated DOR; KOR-T, truncated KOR; M/DOR, the MOR/DOR chimera; MEM, modified Eagle’s medium; DPDPE, [d-Pen2,d-Pen5]enkephalin; DAGO, [d-Ala2,N-Me-Phe4,Gly5-ol]enkephalin; GTP·S, guanosine 5′-3-O-(thiotriphosphate); PCR, polymerase chain reaction.
tor (MOR) is impaired after a potential GRK site on the carboxyl-terminal tail of the receptor was mutated (27). However, the contribution of arrestins in opioid receptor desensitization and the mechanisms of regulation of functional responses of opioid receptors by arrestins are to be investigated. It was observed that both GRK2 and β-arrestin levels increase in the rat locus coeruleus after chronic morphine administration (28).

Kovoor et al. (29) reported in a very recent study that overexpression of β-adrenergic receptor kinase 2 (GRK3) and β-arrestin 2 synergistically cause desensitization of DOR functionally coupled to the G protein gated inwardly rectifying K+ channels (GIRKs) when coexpressed in Xenopus oocytes with GIRK1 and GIRK4 (29). The same study also showed that the wild type MOR and the DOR mutants lacking serine and threonine residues on the carboxyl-terminal tail are insensitive to GRK3 and β-arrestin 2.

In the previous study, we have shown that DOR undergoes desensitization and phosphorylation upon agonist stimulation in HEK 293 cells overexpressing DOR and that endogenous GIRKs in HEK 293 cells contribute to desensitization and phosphorylation (9). More recently, we demonstrated that the agonist-dependent phosphorylation sites are located on the carboxyl terminus of DOR (8). In the current study, we further investigated the role of β-arrestin 1 in regulation of κ, δ, and μ opioid receptor subtype-mediated G protein activation in HEK 293 cells coexpressing opioid receptors and human β-arrestin 1. Our results show that overexpression of β-arrestin 1 in HEK 293 cells resulted in reduced responses of KOR and DOR while hardly affecting the signaling of MOR. The current study indicates that responsiveness of κ, δ, and μ opioid receptors is differentially regulated by β-arrestin 1, and the carboxyl terminus of opioid receptors is critical for the proposed interaction between opioid receptors and β-arrestin.

EXPERIMENTAL PROCEDURES

Constructs of Human β-arrestin and Wild-type and Mutant Opioid Receptors—Human β-arrestin 1 cDNA was amplified by reverse transcription-PCR using human brain mRNA as a template. The primer pair CGCATATGGGCGACAAAGGGACGCG and CACGAGAGTGGAGCCGG-AGC were designed according to sequence accession numbers L04685 and AA234534 in GenBankTM. The resultant PCR product was subcloned into pcDNA3 (Invitrogen). The mouse wild-type DOR cDNA and the truncated receptor lacking residue 342–372 (DOR-T) cDNA constructs were as described (8). The wild-type human KOR (GenBankTM accession number U11053) and MOR cDNA (7) constructs were generously provided by Drs. Brigitte L. Kieffer and Jia Bei Wang, respectively, and subcloned into pcDNA3. Human KOR-T lacking residues 354–381, human M/DOR chimera with residues 362–401 replaced by residues 342–372 of mouse DOR, KOR354T381 (T363A), KOR354T358 (S356A/T357G/S358G), and KOR354T358 (S356A/T357G/S358G/T363A) cDNA clones were constructed by PCR mutagenesis. All constructs used were in pcDNA3 (Invitrogen), and authenticity of sequences was confirmed by DNA sequencing.

Transfection and Expression of β-arrestin and Opioid Receptors—HEK 293 cells (American Type Culture Collection) were plated in 60-mm tissue culture dishes at 1 × 104 cells/dish in MEM (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum 20 h before transfection. Transfection was performed using indicated amounts of human β-arrestin 1 cDNA and 1.5–2.0 μg of opioid receptor cDNA/1 × 106 cells and the calcium-phosphate-DNA coprecipitation method as described (30). The total amount of DNA transfected was kept constant (6 μg of cDNA/1 × 106 cells) by addition of pcDNA3. The transiently transfected cells were harvested and used 48 h post-transfection. Levels of opioid receptor expression (1–3 pmol/mg of membrane proteins) were quantified by [3H]diprenorphine (Amerham) binding, and expression of μ opioid receptor was monitored with Western blotting and was kept at comparable levels.

Cyclic AMP Assay—Cells were challenged with agonist in the presence of 10 μM forskolin (Sigma) and 500 μM 1-methyl-3-isobutylxanthine (Sigma) at 37 °C for 15 min. The reactions were terminated with 1 N perchloric acid and neutralized with 2 N K2CO3. The cAMP level of each sample was determined using radioimmunoassay as described previously (31). Data were averaged from triplicate samples and calculated as 100 × (cAMP (for agonist) − cAMP (basal))/cAMP (for − cAMP (basal)) where cAMP (for + agonist) is cAMP accumulation in the presence of forskolin and agonist, cAMP (basal) is cAMP in the absence of forskolin and agonist, and cAMP (for) is cAMP in the presence of forskolin alone. U69593, [D-Pen2,D-Pen5]enkephalin (DPDPE), [D-Ala2,N-Me-Phe4,Gly](1-ol)enkephalin (DAGO), and other opioid ligands used in this study were obtained from Sigma.

[S]GTPγS Binding Assay—The experiments were performed as described previously (31). Cells were lysed in 5 mM Tris-HCl, pH 7.5, 5 mM EDTA, 50 mM EGTA at 4 °C, and the lysate was centrifuged at 30,000 × g for 10 min. The membrane pellet was resuspended, and an aliquot (containing 8 μg of protein) was incubated at 30 °C for 1 h in 50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 1 mM EGTA, 100 mM NaCl, 40 μM GDP, and 8 mM [35S]GTPγS (1200 Ci/nmol, NEN Life Science Products) in the presence or absence of agonist. The reaction was terminated by dilution in cold phosphate-buffered saline and filtered through GF/C filters under vacuum. Bound radioactivity was determined by liquid scintillation spectrophotometry.

Western Blot Analysis—Cells were lysed in 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 2% SDS, 1% 2-mercaptoethanol. Aliquots (50 μg of protein) of the whole cell extracts prepared were subjected to 10% SDS-polyacrylamide gel electrophoresis and then electroblotted onto nitrocellulose membranes. Immunoblotting was performed using anti-β-arrestin 1 monoclonal antibody (Transduction Laboratories) and ECL kit (Amersham) according to the manufacturer’s protocols.

Statistical Analysis—Data were analyzed with the Student’s t-test for comparison of independent means, with pooled estimates of common variances.

RESULTS

HEK 293 cells were cotransfected with β-arrestin 1 and κ, δ, or μ opioid receptors, and Western analysis shows that transfection of β-arrestin 1 cDNA resulted in a 5–10-fold increase of β-arrestin 1 over the endogenous basal level in HEK 293 cells, and expression of β-arrestin 1 in cells cotransfected with MOR was comparable with that in cells cotransfected with KOR or DOR (Fig. 1). The effects of overexpression of β-arrestin 1 on opioid agonist-induced inhibition of adenyl cyclase were assessed. As shown in Fig. 2, transfection of β-arrestin 1 attenuated agonist-induced, κ and δ opioid receptor subtype-mediated inhibition of cellular cyclic AMP production. The effect of β-arrestin 1 was dose-dependent, and transfection of over 1.2 μg of β-arrestin 1 cDNA resulted in strong inhibition on U69593- (κ-selective agonist) and DPDPE- (δ-selective agonist) induced responses (Fig. 2). Transfection of 2 μg of β-arrestin 1 cDNA construct caused 30–40% reduction in DOR- and KOR-mediated inhibition on cellular cAMP accumulation (Fig. 2). However, in strong contrast, overexpression of β-arrestin 1 did not affect the responsiveness of μ opioid receptor significantly under the same conditions (Fig. 2). Overexpression of...
β-arrest1 shifted the concentration-response curves of U69593 and DPDPE to the right, and the EC₅₀ values of U69593 and DPDPE increased over 100-fold (from 6 to 700 nM for U69593 and from 3 to 400 nM for DPDPE) in cells cotransfected with KOR and DOR, respectively (Fig. 3, panels A and B). Inhibition on cAMP production induced by 10 nM U69593 or DPDPE was almost completely abolished by transfection of β-arrest1 cDNA. However, coexpression of β-arrest1 with MOR did not significantly affect the concentration-effect relationship of DAGO, a μ-specific agonist (Fig. 3C). The above results clearly indicate that β, δ, and μ opioid receptor-mediated inhibition of adenylyl cyclase was differentially regulated by β-arrest1.

Opioid receptors are coupled to the inhibitory G proteins. Activation of β, δ, and μ opioid receptor subtypes stimulates Gi proteins and subsequently inhibits adenylyl cyclase. Therefore, the effects of overexpression of β-arrest1 on opioid receptor/G protein coupling were examined using [35S]GTPγS binding assay in cells coexpressing β-arrest1 and opioid receptors (32). As shown in Fig. 4, β, δ, and μ-selective opioid agonists U69593, DPDPE, and DAGO stimulated [35S]GTPγS binding to membranes from cells expressing exogenous β, δ, and μ opioid receptors, respectively. The G protein activation mediated by β and δ opioid receptors was strongly attenuated by overexpression of β-arrest1 in these cells (Fig. 4, panels A and B). In cells coexpressing β-arrest1 and KOR and DOR, 10⁻⁵–10⁻⁶ M U69593- or DPDPE-induced G protein activation was inhibited by over 50–70% (p < 0.01), while efficacy of DAGO to stimulate GTPγS binding was not affected significantly (Fig. 4C). These data imply that overexpression of β-arrest1 affects capabilities of β and δ opioid receptors to couple to Gi proteins and therefore results in attenuation of KOR- and DOR-mediated downstream inhibition of adenylyl cyclase. β-arrest1 exerts a less significant role in interference with coupling of MOR to Gi proteins in HEK 293 cells.

Arrestins interact with the third intracellular loop and the carboxyl terminus of the GPCRs. The structures of carboxyl termini of opioid receptors are believed to be essential for the regulation of the responsiveness of opioid receptors. It has been demonstrated recently that carboxyl-terminal structures in DOR and MOR are required for agonist-stimulated receptor phosphorylation, internalization, and desensitization (8, 26, 27, 33). To assess contribution of the carboxyl-terminal structures of opioid receptors to the differential regulation of responsiveness of β, δ, and μ opioid receptor subtypes by β-arrestin 1, DOR-T with the carboxyl-terminal 31 amino acid residues (including 7 serine/threonine) removed (8, 34) and KOR-T lacking the last 28 residues (containing 4 serine/threonine residues) corresponding to DOR at its carboxyl terminus were constructed (Fig. 5A). The effects of overexpression of β-arrest1 on opioid-stimulated inhibition of cAMP accumulation were investigated in cells coexpressing wild-type or carboxyl-terminal truncated opioid receptors. As shown in panels B and C of Fig. 5, truncation of β and δ opioid receptors at carboxyl terminus strongly attenuated inhibition of β-arrest1 on the responsiveness of both receptors. The responsiveness of the chimeric μ opioid receptor M/DOR with carboxyl terminus replaced by the corresponding portion of DOR was attenuated greatly following overexpression of β-arrest1, indicating that the essential structure required for the regulation of β-arrest1 is located on the carboxyl terminus of DOR (Fig. 5D) and suggesting that structures at the carboxyl termini of opioid receptors are critical in interaction with β-arrest1.

Arrestins bind to phosphorylated GPCRs at high affinity, and they are involved in the sequestration of GPCRs. It has been shown that agonist-dependent phosphorylation sites are located in the carboxyl terminus of DOR, and removal of six putative kinase phosphorylation sites on the carboxyl terminus of DOR inhibits agonist-stimulated receptor phosphorylation and internalization (8, 26). More recently, Kovoor et al. (29) found that coexpression of both GRK3 and β-arrestin 2 in Xenopus oocytes leads to a rapid desensitization of K⁺ conductance activated by DOR, and the desensitization is greatly reduced by substitution of all 5 serine or threonine residues on...
the carboxyl-terminal tail of DOR with alanine (28). These previous results and our current data demonstrate that phosphorylation of the carboxyl terminal of DOR is important for its interaction with arrestins. In the current study we have also shown that responsiveness of KOR, another type of opioid receptors, was modulated by β-arr1 and that the carboxyl-terminal structure of KOR was involved. Hence, additional mutant KOR constructs were made to assess the function of phosphorylation at the receptor carboxyl terminus in the modulation by β-arr1. The potential phosphorylation sites on KOR are listed in Table I. As shown in Fig. 6A, the response of KOR to U69593 stimulation was reduced greatly in the presence of overexpressed β-arr1 (the EC50 increased 60-fold), although to a somewhat less extent than the wild-type KOR does. However, mutation of the serine/threonine cluster between residues 356 and 358 impaired sensitivity of KOR to β-arr1 modulation remarkably (Fig. 6B). Moreover, removal of all four potential phosphorylation sites on the carboxyl terminus of KOR abolished the effect of β-arr1 on the receptor-mediated adenyl cyclase inactivation completely (Fig. 6C) and impaired its ability to uncouple KOR from G proteins (Fig. 7). These results confirm that the carboxyl terminus of KOR is essential for modulation of receptor response by β-arr1 and suggest that phosphorylation at the carboxyl termi-
modulation of GRK3 and β-arrestin 2 (29). In this study, we compared the impacts of overexpression of β-arrestin on the responsiveness of κ, δ, and μ three subtypes of opioid receptors in HEK 293 cells, which express GRK2 endogenously (35, 36). Our data revealed that expression of β-arrestin 1 significantly increased the EC_{50} and decreased the maximal responses of κ and δ opioids to stimulate [35S]GTPγS binding and to attenuate cAMP accumulation in HEK 293 cells transiently expressing κ, δ, or μ opioid receptors but left the concentration-effect relationship for DAGO unchanged. These results indicate that in addition to DOR, signaling of KOR, another opioid receptor subtype, is under regulation of arrestins. Thus, the opioid receptors break up into two groups according to their sensitivity to regulation of β-arrestins. Both KOR and DOR are regulated by β-arrestins while MOR, the most important opioid receptor in pain modulation, obviously belongs to the other group.

The results of Kovoor et al. (29) and the current study reveal a mechanism of differential desensitization of opioid responses mediated by both β-arrestins and the residues, phosphorylated by GRKs or other Kinases, on the carboxy terminal of the opioid receptors. Pak et al. (37) found that a splicing variant of rat MOR with a shorter C-tail lacking the potential GRK site is resistant to agonist-induced desensitization, and the distribution of the two isoforms of MOR is strikingly different in many regions of the brain. Differences in desensitization of opioid receptors have been observed previously in brain structures known to be involved in opiate drug actions following chronic morphine treatment (38). Both μ and δ opioid receptors undergo desensitization in the periaqueductal gray and the thalamus following chronic morphine treatment. However, in the nucleus accumbens and the caudate putamen, chronic morphine treatment results in desensitization of DOR but not MOR (38). These results suggest that changes in receptor responsiveness occurring during chronic morphine administration are not identical in all opiate-sensitive neural populations. Mechanisms of regulation of opioid receptor-mediated signals, especially the signal transduction through μ opioid receptor, the major opioid receptor involved in pain modulation, may include expression of receptor isoforms with different sensitivity to regulation or differential expression of protein kinases (such as GRKs) and arrestins (or arrestin-like proteins) interacting specifically with certain opioid receptors.

It is also demonstrated clearly in this study that the regulation of β-arrestin 1 on the responsiveness of KOR and DOR is through uncoupling of receptor and the inhibitory G proteins. Employing [35S]GTPγS binding and cyclase assays, we have determined the effects of β-arrestin 1 on opioid agonist-stimulated activation of G proteins and subsequent inhibition of cAMP production, and both signals were remarkably reduced by β-arrestin 1. The expression levels of opioid receptors have been carefully controlled and monitored by radioligand binding and flow cytometry analysis with fluorescent probe throughout this study. Therefore, the effect of β-arrestin 1 on the responsiveness of KOR and DOR appears to be a result of receptor/G protein uncoupling not receptor down-regulation.

For most members of GPCRs, the third intracellular loop and the carboxy-terminal tail of the receptor are key sites for signal initiation and termination. Agonist-stimulated phosphorylation of GPCRs occurs at serine and threonine residues in the intracellular domains, typically the third intracellular loop or carboxy terminus (39–41), and direct interaction of the third intracellular loop of the M_{3} subtypes of muscarinic, α_{2}-adrenergic receptors with β-arrestin 1 and β-arrestin 2 has been demonstrated (42). Our previous research showed that agonist-induced DOR internalization requires the cytoplasmic tail of DOR, and agonist-dependent (GRK2 and GRK5) phosphorylation sites are located within the 31 amino acid residues of the carboxy terminus of DOR (8). In this study, we demonstrated that the carboxy-terminal tails of KOR and DOR are required for the regulatory effect of β-arrestin 1 and that μ opioid receptor became sensitive to β-arrestin regulation following replacement of its carboxy terminal with the corresponding portion of δ receptor. These data agree with the result of Kovoor et al. (29) that 5 serine and threonine residues are required on the carboxy terminus of DOR for regulation of receptor desensitization (28) and indicate further that the 31 amino acid residues at the carboxy termin-
nus of DOR contain information essential and sufficient to induce "DOR/KOR"-type-specific response to β-arrestin regulation.

Recent x-ray crystal structure data of retinal arrestin revealed that arrestin interacts with the carboxyl-terminal phosphorylated sites of photoactivated rhodopsin through a dome-like binding pocket consisting of 11 positive charged amino acids (43). Mutating both threonine 363 and the 3 consecutive serine/threonine in the carboxyl-terminal tail of KOR abolished β-arr1-mediated inhibition on the agonist-induced inactivation of adenyl cyclase and partially inhibited the effect of β-arr1 on agonist-stimulated G protein activation, indicating that phosphorylation at the carboxyl terminus of KOR may play an important role in the interaction with β-arr1. In addition, our result from GTPγS binding experiments indicates that β-arr1 is able to interfere with coupling of KOR to G proteins to some extent, and that in the experiment conditions used, the overexpressed β-arr1 could bind nonphosphorylated κ opioid receptor. It suggests that opioid receptor phosphorylation is not an absolute requirement for β-arr1 binding. This is in agreement with previous studies on β2-adrenergic receptor that β-arrestin is capable of interacting with nonphosphorylated receptors (16, 17). Preliminary results of in vitro biochemical studies in our laboratory indicated that β-arr1 interacts with the nonphosphorylated carboxyl terminus of MOR and work is in progress to examine the characteristics of interaction of β-arr1 with carboxyl termini of opioid receptors.

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