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# Direct and Differential Interaction of β-Arrestins with the Intracellular Domains of Different Opioid Receptors

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## ABSTRACT

β-arrestins have been shown to play important roles in regulation of signaling and desensitization of opioid receptors in many in vivo studies. The current study was carried out to measure the direct interaction of β-arrestins with two functional intracellular domains, the third intracellular loop (I3L) and the carboxyl terminus (CT), of δ-, μ-, and κ-opioid receptors (DOR, MOR, and KOR, respectively). Results from the pull-down assay using glutathione *S*-transferase fusion proteins demonstrated that β-arrestins (1 and 2) were able to bind to the I3L of DOR and to the CT of DOR and KOR. Surface plasmon resonance measurement gave similar results with typical dissociation equilibrium constant ($K_D$) values in the micromolar range. The site-directed mutagenesis experiment further revealed that certain specific serine/threonine residues in these receptor domains play a critical role in their interaction with β-arrestins. Taken together, our data clearly indicated that β-arrestins interact differentially with the functional domains of different opioid receptors; this may provide a possible molecular basis for differential regulation of opioid receptors by β-arrestins.

Opiates display strong analgesic (Dickenson, 1991) and addictive (Koob, 1992) properties, and addiction to opiates such as morphine and heroin has been the subject of intense investigations. The analgesia, tolerance, and dependence induced by opiate drugs are mediated through stimulation of the membrane receptors known as δ- (DOR), μ- (MOR), and κ- (KOR) opioid receptors, as demonstrated by the lack of opiate actions observed in knockout mice deficient in the opioid receptors (Matthes et al., 1996; Simonin et al., 1998; Kieffer, 1999; Zhu et al., 1999). Desensitization of opioid receptors, the reduced responsiveness of opioid receptors upon agonist stimulation that involves receptor phosphorylation, uncoupling of receptor and G protein, and receptor internalization, has been implicated as one of the mechanisms underlying the onset and duration of tolerance and dependence (Nestler and Aghajanian, 1997). Interestingly, differences in desensitization of opioid receptors have been observed; in the rat nucleus accumbens and caudate putamen, chronic morphine treatment resulted in desensitization of DOR but not MOR (Noble and Cox, 1996).

Arrestins, which consist of four classes, visual arrestin, cone arrestin, β-arrestin 1 and β-arrestin 2, play a key role in G protein-coupled receptor (GPCR) regulation (for reviews, see Krupnick and Benovic, 1998; Lefkowitz, 1998). Visual arrestin and cone arrestin are expressed primarily in rod and cone cells in the visual system (Yamaki et al., 1987; Craft et al., 1994). β-arrestins 1 and 2 are widely expressed in many tissues (Lohse et al., 1990; Attramadal et al., 1992), with especially high-level expression in nervous and lymphatic tissues (Parruti et al., 1993), and have been shown to regulate various GPCRs (Krupnick and Benovic, 1998; Lefkowitz, 1998). As a subfamily of GPCRs, the opioid receptors are also functionally modulated by β-arrestins (Kovoor et al., 1997; Cheng et al., 1998; Zhang et al., 1998; Appleyard et al., 1999; Li et al., 1999). This concept is strongly supported by a recent study using the β-arrestin 2-deleted mice (Bohn et al., 1999). Furthermore, evidence from other laboratories and our own reveals that β-arrestins are able to differentially regulate three different members of opioid receptor family (Kovoor et al., 1997; Cheng et al., 1998). However, the underlying molecular mechanisms of this differential regulation of opioid receptors by β-arrestins, especially in terms of the direct interaction between opioid receptors and β-arrestins, are not reported yet.

It has been known that the third intracellular loop (I3L) and the carboxyl terminus (CT) of GPCRs are crucial domains for receptor function, and this is also the case for

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**ABBREVIATIONS:** DOR, δ-opioid receptor; MOR, μ-opioid receptor; KOR, κ-opioid receptor; GPCR, G protein-coupled receptor; I3L, third intracellular loop; CT, carboxyl terminus; GST, glutathione *S*-transferase; SPR, surface plasmon resonance; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; GSH, reduced glutathione; RU, resonance unit.

opioid receptors. The third intracellular loop of opioid receptors has been suggested as a regulation target by calmodulin-dependent protein kinase II (Koch et al., 1997), in addition to its established role in G protein activation (Merkouris et al., 1996; Georgoussi et al., 1997). In contrast, the carboxyl terminus of opioid receptors seems to be more significantly involved in the modulation of receptor function by protein kinases and β-arrestins (Kovoor et al., 1997; Cheng et al., 1998; Appleyard et al., 1999), as well as in the receptor coupling with G proteins (Merkouris et al., 1996; Georgoussi et al., 1997). The present work, with employment of glutathione *S*-transferase (GST) pull-down assay and surface plasmon resonance (SPR) technique, was thus designed to study in vitro the direct interaction of β-arrestins with those two functional domains, the third intracellular loop and the carboxyl terminus, of opioid receptors.

## Materials and Methods

**Construction of Expression Vectors.** GST fusion protein constructs for the third intracellular loops and the carboxyl termini of DOR, MOR, and KOR were generated from human DOR and MOR (generously provided by Dr. Jia Bei Wang, Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland at Baltimore) cDNA clones and KOR cDNA clone (generously provided by Dr. Brigitte L. Kieffer, University Louis Pasteur, Strasbourg, France) by amplification using the polymerase chain reaction (PCR). The PCR products were subcloned into pGEX-4T1 (Amersham Pharmacia Biotech, Piscataway, NJ) with *Bam*HI/*Xho*I sites for DOR and MOR and *Eco*RI/*Xho*I sites for KOR. The deletion and site-directed mutants of GST fusion proteins were subsequently obtained by PCR-based mutagenesis. Recombinant human β-arrestin 1 and 2 cDNA were amplified by reverse transcription-PCR using human brain mRNA as a template and subcloned into pET-30a (Novagen, Madison, WI). All constructs were confirmed by DNA sequencing.

**Expression and Purification of Recombinant Proteins.** Proteins were expressed in *Escherichia coli* BL21 (DE3) cells. GST fusion proteins were induced with 100 μM isopropyl-β-D-thiogalactoside for 3 h at 37°C. Cell lysate was applied to glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) and fusion proteins were purified according to the manufacture's instructions. Recombinant β-arrestin 1 and β-arrestin 2 were induced with 1 mM isopropyl-β-D-thiogalactoside for 7 h at 37°C. Cell lysate was sequentially applied to 50% saturated $(NH_4)_2SO_4$ solution and heparin- and Q-Sepharose. Each batch of protein was analyzed by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie Blue staining, showing a purity of more than 90%.

**Cell Culture.** Cells were obtained from American Type Culture Collection (Manassas, VA). THP-1 cells were cultured in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS and 2 mM glutamine. Neuroblastoma × glioma NG108–15 cells were cultured in Dulbecco's modifed Eagle's medium (Life Technologies) supplemented with 10% FBS with addition of 2 mM glutamine and 0.1 mM hypoxanthine, 10 μM aminopterin, and 16 μM thymidine. Cells were lysed by sonication in buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 0.1% Triton X-100), and centrifuged for 10 min at 12,000*g* at 4°C to obtain cytosol fraction.

**GST Pull-Down Assay.** Equimolar amounts of GST fusion proteins [0.15 nmol, equal to 5 μg of DCT (see under *Results*)] bound to glutathione-Sepharose 4B beads were incubated on a rotator with purified β-arrestin 1 (0.2 μg), β-arrestin 2 (0.2 μg), or cell cytosol fraction (150 μg of total protein) in 200 μl of buffer A at 4°C for 2 h. The beads were washed subsequently with 600 μl of buffer A and eluted with 10 mM reduced glutathione (GSH). Binding was quantified by immunoblotting of each fraction with anti-β-arrestin antibodies (Cheng et al., 2000) compared with a 10-fold range of known amounts of purified β-arrestin 1 resolved along with it.

**Western Blotting Analysis.** Protein samples were subjected to 10% SDS-PAGE and then electroblotted onto nitrocellulose membranes. Immunoblotting was performed using anti-β-arrestin antibodies as described previously (Cheng et al., 2000) and enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech) according to the manufacturer's protocols.

**Surface Plasmon Resonance Analysis.** Real-time analysis of interaction between β-arrestin 1 and GST fusion proteins was performed with a BIAcore-1000 instrument (Pharmacia Biosensor AB, Uppsala, Sweden). Assuming 1000 resonance units (RU) corresponds to a surface concentration of 1 ng/mm$^2$, β-arrestin 1 was immobilized to a CM5 biosensor chip (Pharmacia Biosensor AB) at a concentration of 2 ng/mm$^2$ (2000 RU) by amine coupling according to manufacturer's instructions. A blank surface was also prepared by applying the same treatment but without β-arrestin 1 to examine nonspecific protein interactions. The running buffer contained 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, and 0.005% Tween 20, and the flow rate was 30 μl/min. The sensor surface was regenerated between assays by treatment with 2 M NaCl. The kinetic analysis of the interaction between β-arrestin 1 and the GST fusion proteins was carried out using BIAevaluation software (version 3.0; Pharmacia Biosensor AB). A dissociation equilibrium constant ($K_D$) was determined by each measurement with a $\chi^2$ value $<$ 1, and the averages of $K_D$ values were then obtained by measurement of five different concentrations of GST fusion proteins over immobilized β-arrestin 1 surface.

## Results

**Interaction of Functional Domains of DOR, MOR, and KOR with β-Arrestins.** We first attempted to determine whether the intracellular domains of different opioid receptors could interact with β-arrestins in vitro, and we focused on the I3L and the CT, the known functional domains for signal initiation and termination for most GPCRs. The third intracellular loop of DOR (Leu$^{235}$-Ile$^{259}$), MOR (Leu$^{256}$-Ile$^{280}$), and KOR (Leu$^{248}$-Ile$^{272}$) were constructed as GST fusion proteins and termed DI3L, MI3L, and KI3L; DCT, MCT, and KCT stand for the GST fusion proteins of the carboxyl termini of DOR (Gln$^{331}$-Ala$^{372}$), MOR (Cys$^{348}$-Pro$^{400}$), and KOR (Cys$^{340}$-Val$^{380}$), respectively (Fig. 1A). All the GST fusion proteins were expressed in bacteria and purified to near homogeneity (Fig. 1B).

THP-1 and NG 108–15 cells have been shown to express high-level endogenous β-arrestins (Parruti et al., 1993), and our analysis with reverse-transcription PCR revealed that the majority was β-arrestin 1 (data not shown). Thus, the cytosolic β-arrestins were first tested for their interaction with GST fusion proteins generated above, and the results showed that β-arrestin was able to interact with DI3L, DCT, and KCT but not with MI3L, KI3L, MCT, or GST (Fig. 2, a and b). As another control for binding specificity, p42/44 mitogen-activated protein kinase in the cytosol fraction was not associated with those fusion proteins under such conditions (data not shown). Further experiments using purified β-arrestin 1 or β-arrestin 2 demonstrated that either β-arrestin can directly interact with these functional receptor domains (Fig. 2, c and d) and this interaction was not mediated by any other factors in the cytosol fraction.

**Real-Time Analysis of Direct Binding of the Receptor Functional Domains to Immobilized β-Arrestin 1.** The characteristics of association of β-arrestins with the

functional domains of opioid receptors were further investigated via a series of SPR measurements, using a BIAcore-1000 with the purified β-arrestin 1 immobilized on the sensor chip. There was a jump at the beginning and a drop at the end of each injection because of the slight difference in the refractive index between the running and sample buffers that did not significantly affect the measurement. As shown in Fig. 3, SPR measurements demonstrated that there were specific responses during the injections of DI3L, DCT, and KCT into the flow cell. In contrast, no such responses were detected during the injections of MI3L, KI3L, MCT, or controls of bovine serum albumin (BSA) and GST under the same conditions. The SPR data were in good agreement with those from the GST pull-down assay, regarding which receptor domain interacts with β-arrestin.

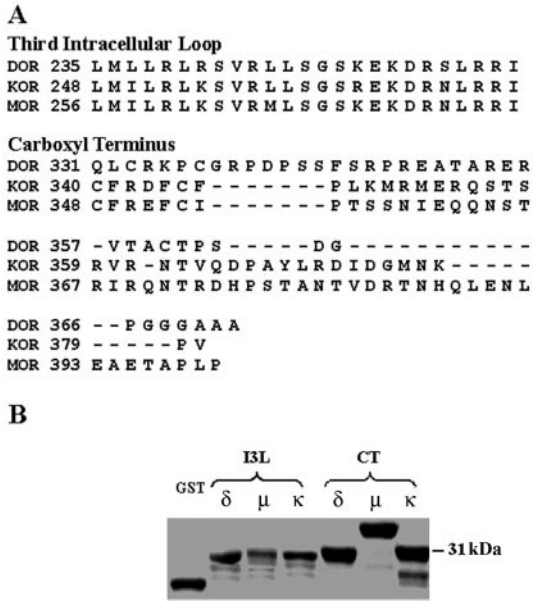


**Fig. 1.** Generation of GST fusion proteins of I3L and CT of opioid receptors. A, alignment of the amino acid sequences of the I3L and the CT of δ-, μ- and κ- opioid receptors fused with GST. Gaps served to generate the alignment are represented by dashes. B, GST fusion proteins were expressed in bacteria and purified as described under *Materials and Methods*. The purified GST and GST fusion proteins were subjected to 10% SDS-PAGE and visualized by Coomassie Blue staining. Molecular mass standard (kDa) is marked on the right.

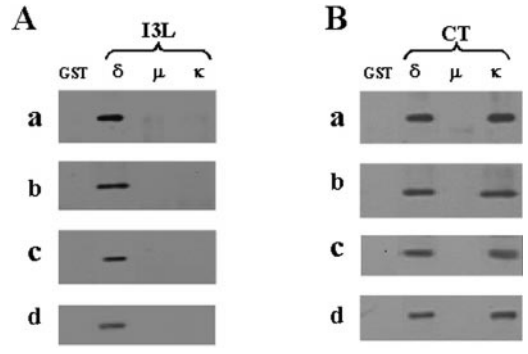


**Fig. 2.** Interaction of the I3L and the CT of opioid receptors with β-arrestins. Cytosol fraction of THP-1 (a) and NG108–15 (b) cells and purified β-arrestin 1 (c) and β-arrestin 2 (d) were incubated with GST, I3L (A), or CT (B) fusion proteins immobilized to glutathione-Sepharose 4B for 2 h at 4°C. The bound proteins were eluted with 10 mM GSH and analyzed with anti-β-arrestin antibodies after 10% SDS-PAGE. Results shown are representatives of at least four independent experiments.

Another advantage using SPR technique is to determine the disassociation equilibrium constant ($K_D$) of protein-protein interaction from the measured rates of the signal change. For example, the binding of KCT to immobilized β-arrestin 1 was found in a concentration-dependent manner by applying five different concentrations of KCT (Fig. 4A) and the $K_D$ value was calculated from these measurements. The averaged $K_D$ value between KCT and β-arrestin 1 was 2.3 ± 0.2 μM ($n$ = 2). The reciprocal of the slope of the fitted line (Fig. 4C) also gave a $K_D$ value (2.4 ± 0.4 μM). The values of $K_D$ obtained in two ways were comparable. This also agrees with the $K_D$ value between β-arrestin 1 and DI3L (7.6 ± 0.6 μM) and that between β-arrestin 1 and DCT (2.9 ± 0.3 μM) as described (Cen et al., 2001).

**Functional Role of Specific Ser/Thr Residues within I3L of Opioid Receptors in the Interaction between I3L and β-Arrestin 1.** The potential functional role of all four serine residues in DI3L in the interaction with β-arrestin 1 was examined by GST pull-down experiments. The results showed that substitution of Ser242, Ser247, or both with alanine had no significant effect on the β-arrestin binding to DI3L (Fig. 5A). However, substitution of Ser249 or Ser255 in DI3L to alanine resulted in ~50% reduction in β-arrestin binding and the double substitution of both Ser249 and Ser255 severely impaired the DI3L binding to β-arrestin (~80% reduction). These data indicated that Ser249 and

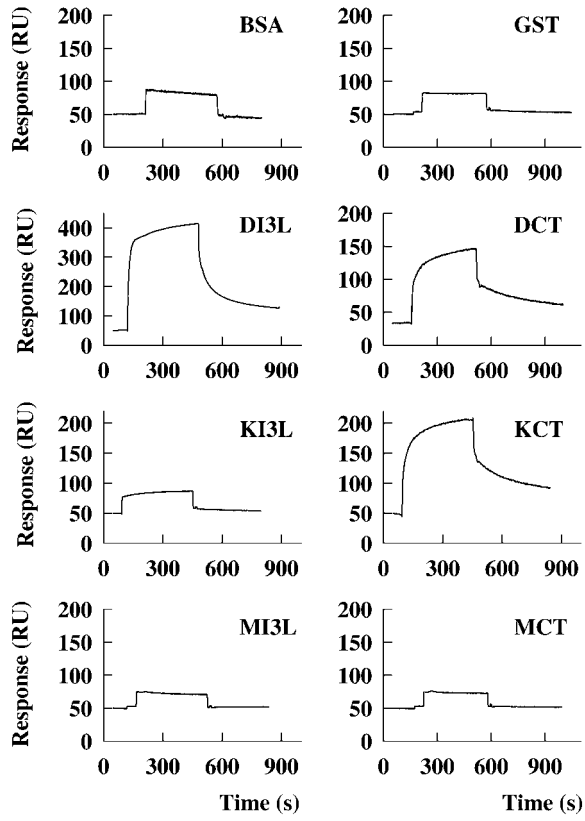


**Fig. 3.** Real-time SPR measurement of β-arrestin 1 interaction with I3L and CT of opioid receptors. About 2000 RU of β-arrestin 1 was immobilized on the surface of CM5 biosensor chip as described under *Materials and Methods*. 10 μM bovine serum albumin (BSA), GST, or GST fusion proteins of the third intracellular loops (DI3L, KI3L and MI3L) and the carboxyl termini (DCT, KCT and MCT) of three opioid receptors in running buffer were injected over the immobilized β-arrestin 1 surface at a flow rate of 30 μl/min. Representative data are shown from two independent experiments.

Ser255 but not Ser242 or Ser247 plays a critical role in the interaction between I3L of DOR and β-arrestin 1.

We further tried to determine why DI3L binds to β-arrestins, whereas MI3L and KI3L do not, even though they share homologous sequences with DI3L (~84%). After comparison of primary structures of the three I3Ls of opioid receptors, it was found that the corresponding residue to the critical Ser255 in DI3L is replaced by Asn268 in KI3L and by Asn276 in MI3L. So either Asn268 in KI3L or Asn276 in MI3L was thus purposely changed to serine by direct mutagenesis. The results showed that both mutants exhibited the comparable binding to β-arrestin 1 to that of DI3L (Fig. 5B). These data further confirmed the importance of specific serine residues in the interaction between functional domains of opioid receptors and β-arrestins.

**Functional Role of Specific Ser/Thr Residues within the CT of Opioid Receptors in the Interaction between the CT and β-Arrestins.** Earlier studies have reported that the mutation of some serine or threonine residues at the CT of opioid receptors greatly impairs the regulatory effect of β-arrestins in vivo (Kovoor et al., 1997; Cheng et al., 1998; Appleyard et al., 1999). DCT contains six Ser/Thr residues and KCT contains four Ser/Thr residues (Fig. 1A). To determine the role of these Ser/Thr residues in β-arrestins binding, we generated GST fusion proteins with DCT deletion mutation lacking the last 15 residues (Δ15) or various Ser/Thr substitution mutations. The results showed that the deletion of the last 15 residues at DCT, which includes Thr358, Thr361, and Ser363, led to complete abrogation of β-arrestin binding (Fig. 6), suggesting the critical role of these three Ser/Thr residues in the interaction. Further experiments with the mutation of all three Ser/Thr (T358A/T361A/S363A), which totally abolished binding of either β-arrestin to DCT (Fig. 6), strongly supported this notion.

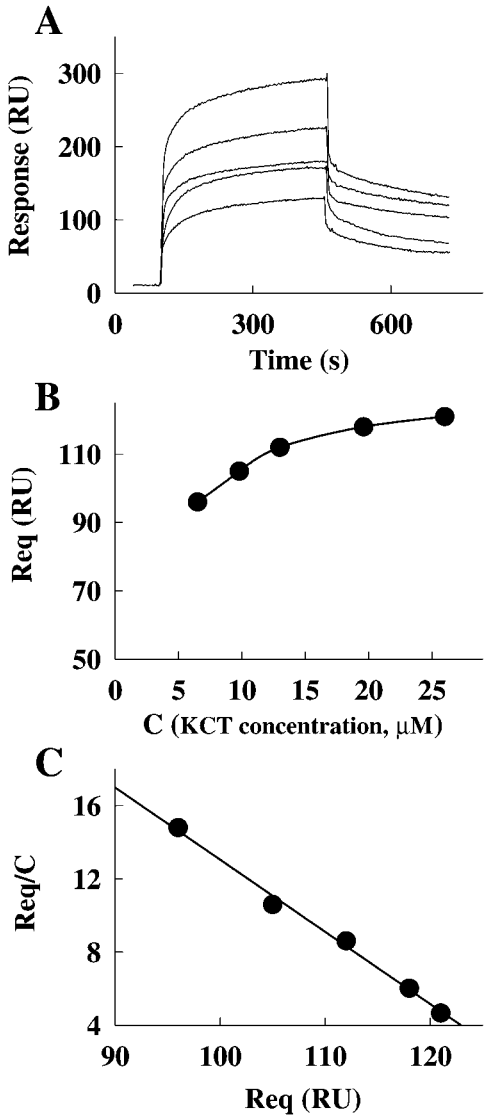


**Fig. 4.** Kinetics analysis of KCT binding to immobilized β-arrestin 1. A, increasing concentrations (6.5, 9.8, 13, 20, and 26 μM, lower to upper curves) of the GST fusion proteins of the CT of κ-opioid receptor in running buffer were injected over immobilized β-arrestin 1 surface at a flow rate of 30 μl/min. B, equilibrium binding data (Req versus C) obtained in A. Req, steady state binding value of KCT; C, concentrations of KCT injected. C, Scatchard plot analysis of data in B. The reciprocal of the slope of the fitted line gave a equilibrium dissociation constant ($K_D$) value of 2.4 ± 0.4 μM. The $K_D$ value ($K_D$ = 2.3 ± 0.2 μM; $n$ = 2) reported here was analyzed using the BIAevaluation software version 3.0 and comparable with the value derived from the Scatchard plot analysis.

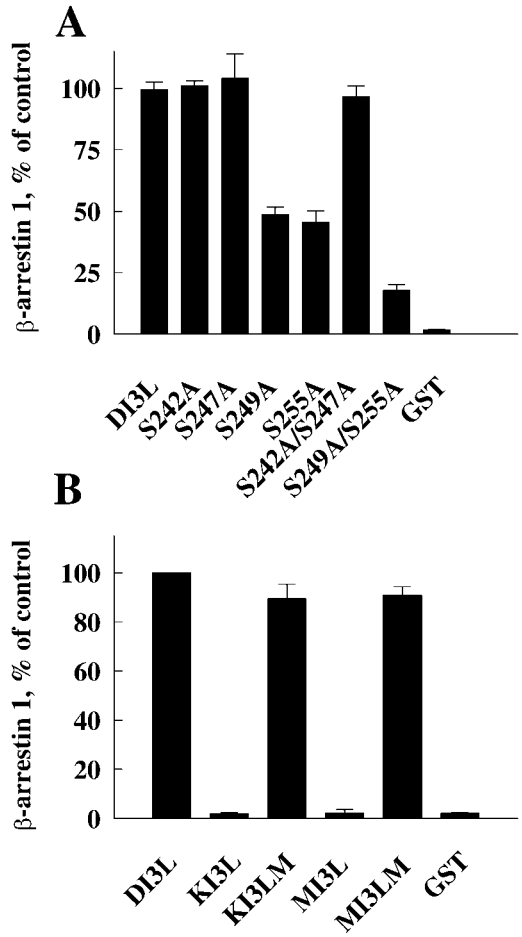


**Fig. 5.** Critical involvement of specific Ser/Thr residues within I3L of opioid receptors in the interaction with β-arrestin. The 750 nM GST fusion protein of DI3L and its site-directed mutants (A), or that of KI3L and MI3L as well as their site-directed mutants (N268S and N276S, respectively) (B) were incubated with 20 nM purified β-arrestin 1 for 2 h at 4°C in the presence of glutathione-Sepharose beads. The eluted proteins (with 10 mM GSH) were resolved on 10% SDS-PAGE and visualized by immunoblotting with β-arrestin antibodies. Data were normalized to the level of β-arrestin 1 bound to the wild-type DI3L. Means ± S.E. are shown from three independent experiments.

Our results also disclosed that any of the single mutation of these three Ser/Thr residues produced a loss of more than 50% and any of the double mutation caused reduction of more than 75% in β-arrestin 1 binding to DCT (Fig. 6A). Consistent with the above data, further SPR measurements revealed that the $K_D$ value for any single mutant increased about 3-fold compared with that of the wild-type DCT protein, and no binding was detected for the triple mutant, as in the case of DCT (Δ15) (Fig. 6A). In general, both β-arrestin 1 and β-arrestin 2 exhibited the same trends in binding to the DCT mutants, indicating that both β-arrestins interact similarly with residues in the carboxyl terminus of DOR. However, the inhibition of β-arrestin 2 binding was, in almost all cases, more complete than that of β-arrestin 1 (Fig. 6). The similar impairments in the binding of β-arrestin 1 were also observed when the four Ser/Thr residues at KCT were mutated to alanine or glycine (data not shown). Taken together, these data clearly indicated that the serine and threonine residues collectively serve as an essential element in the interaction of CT of opioid receptors with β-arrestins.

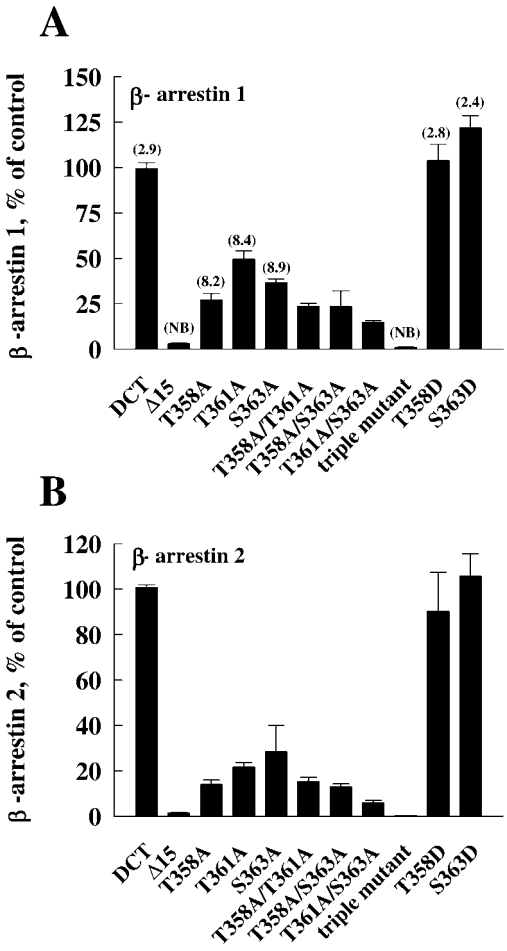


**Fig. 6.** Critical role of specific Ser/Thr residues within the CT of opioid receptors in the interaction with β-arrestins. The 750 nM GST fusion protein of the carboxyl terminus of DOR (DCT) and its various mutants were incubated with 20 nM purified β-arrestin 1 (A) and β-arrestin 2 (B) for 2 h at 4°C in the presence of glutathione-Sepharose beads. The eluted proteins (with 10 mM GSH) were resolved on 10% SDS-PAGE and visualized by immunoblotting with β-arrestin antibodies. The triple mutant refers to T358A/T361A/S363A mutation of DCT. Data were normalized to the level of β-arrestins bound to the wild-type DCT. Means ± S.E. are shown from three independent experiments. Dissociation equilibrium constants ($K_D$, in micromolar) for some of the interactions were obtained from SPR measurements and are indicated in parentheses on top of the corresponding columns. NB, no detectable binding.

Thr358 and Ser363 at the carboxyl terminal tail of DOR are the agonist-stimulated phosphorylation sites (Guo et al., 2000; Kouhen et al., 2000). The potential effect of the serine/threonine phosphorylation on the β-arrestin binding was assessed by using the DCT mutants with Thr358 and Ser363 residues replaced by negatively charged aspartic acid (Asp), which is thought to resemble phosphoserine and phosphothreonine. As shown in Fig. 6, in contrast to Ala substitutions, Asp substitutions of T358 and S363 retained a full capability to bind β-arrestins, although no statistical significant enhancement of β-arrestin binding was detected.

## Discussion

As recently demonstrated in the case of rhodopsin, a well studied model of GPCRs, the third intracellular loop and the carboxyl terminus of GPCRs are two independent intracellular domains with some structural characteristics, respectively (Palczewski et al., 2000). Although no structural data is available for other GPCRs so far, previous studies have revealed that the I3L and CT domains of opioid receptors can play independent roles in vivo with respect to G protein activation and regulation of opioid receptor signaling by some protein kinases and regulatory proteins. The current study, using GST pull-down assay and SPR technique, provided in vitro evidence that either I3L or CT functions as a sufficient structural domain capable of direct interaction with β-arrestins, as demonstrated in the case of DOR. Our data from KOR that showed differential ability of I3L and CT to interact with β-arrestins further support the above suggestion that each of these two structural domains can function independently and even distinctly, at least under such in vitro conditions. Our results agree with earlier reports that the I3L domain of muscarinic receptor (Wu et al., 1997) or 5-hydroxytryptamine2A receptor (Gelber et al., 1999) is able to bind to β-arrestins in vitro, although the CT domains of those receptors are not tested in these studies. The current data also suggest that the interaction of β-arrestins with two distinct domains of DOR may be involved in β-arrestin regulation of different receptor function as in the case of chemokine receptor CXCR4 (Cheng et al., 2000).

More than 1000 GPCRs, but only four arrestins, have been known so far. During last decade, extensive studies have revealed that β-arrestins can regulate various functions of many different GPCRs (Krupnick and Benovic, 1998; Lefkowitz, 1998), but much less is known for the specificity of or the difference in the β-arrestin regulation. Recent data from our and other laboratories exhibit that β-arrestins are able to differentially regulate the function of three different members of opioid receptor family in vivo (Kovoor et al., 1997; Cheng et al., 1998), but the underlying mechanism of β-arrestin regulation remains to be further investigated. The present study provided the in vitro evidence that two intracellular functional domains, the I3L and the CT, of opioid receptors interact with β-arrestins differentially. That is, both domains of DOR, one of KOR, and neither of MOR can directly bind to β-arrestins under similar in vitro conditions. This observation may thus offer a molecular explanation for the differential regulation of opioid receptor function by β-arrestins observed in vivo (Kovoor et al., 1997; Cheng et al.,

1998). These results, taken together, demonstrated the diversity and complexity of GPCR regulation by β-arrestins even in the same subfamily, such as the opioid receptor, which is probably caused by the differential physical interaction of β-arrestins with different GPCRs.

It has been demonstrated that arrestin binding to GPCRs is greatly enhanced after agonist stimulation and subsequent phosphorylation of receptors (Lohse et al., 1992; Gurevich et al., 1995, 1997), although there are reports that arrestins can effectively interact with GPCRs in the absence of receptor phosphorylation (Smith et al., 1994; Gurevich et al., 1995; Ferguson et al., 1996; Wu et al., 1997). However, it is not known whether the agonist-stimulated phosphorylation sites, the Ser/Thr residues, at GPCR, are directly involved in their interaction with β-arrestins. Very recently, we (Guo et al., 2000) and another group (Kouhen et al., 2000) have identified Thr358 and Ser363 at CT of DOR as agonist-stimulated phosphorylation sites. The current study further demonstrated that those Ser/Thr residues are also critically involved in the physical interaction of CT of DOR with β-arrestins in vitro. This may imply the existence of basal binding of β-arrestins to DOR even without Ser/Thr phosphorylation that serves as a mechanism to provide rapidly available β-arrestins. Our data from the mutation of the I3L of DOR also support this hypothesis. The arrangement of critical Ser/Thr residues involved in the interaction with β-arrestins seems to be in a typical pattern (-S/TX$_{4-5}$S/T-) that may serve as an essential motif for the GPCR interaction with β-arrestins (-**T**AC**T**P**S**- on DCT, -**S**KEKDR**S**- on DI3L, and–**STS**RVRN**T**- on KCT). Whether this is a general rule remains to be further investigated. Although it has been commonly accepted that GPCR kinase-catalyzed phosphorylation of receptors promotes their functional interaction with β-arrestins, this promotion was not observed in the current study under the in vitro conditions. The possible explanations are that an Asp (or Glu) substitution of Ser/Thr residues doesn't fully mimic the phosphorylated state of these Ser/Thr residues, or that the sensitivity of the assay used is not sufficient to detect the real enhancement. The third one could be that the phosphorylation of receptors can enhance the functional consequence of but not necessarily the apparent affinity of the receptor/β-arrestin interaction.

The published results from the knockout mice deficient in MOR (Matthes et al., 1996; Kieffer, 1999) and in β-arrestin 2 (Bohn et al., 1999) seem to suggest that β-arrestin 2 may regulate the function of MOR in vivo. However, it is unclear why β-arrestins failed to regulate MOR function in the transfected mammalian cells (Cheng et al., 1998) and to bind to either the CT or the I3L of MOR in the current study. Very recently, Celver et al., consistent with our observations, reported that mutation of serine or threonine residues to alanines in the putative third cytoplasmic loop and truncation of the carboxyl terminal tail did not block GRK3/β-arrestin 2-mediated desensitization of MOR in *Xenopus laevis* oocytes (Celver et al., 2000). They further showed that alanine substitution of a single threonine in the second cytoplasmic loop was sufficient to block homologous desensitization (Celver et al., 2000), which suggests an interaction between β-arrestin and the second cytoplasmic loop of MOR. The second reasonable interpretation could be that different splice variants of MOR (Pan et al., 1999) in vivo are subjected to differential regulation by β-arrestins. Several reports indirectly support this speculation that two alternatively spliced isoforms of rat MOR indeed differ in their agonist-induced desensitization (Zimprich et al., 1995), internalization and resensitization (Koch et al., 1998). The other possible explanation is that DOR is involved in the MOR regulation by β-arrestins in vivo because they can form functional oligomers (George et al., 2000).

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