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Identification of two C-terminal amino acids, Ser^{355} and Thr^{357}, required for short-term homologous desensitization of μ-opioid receptors

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Abstract

Our recent study suggests that a cluster of Ser/Thr residues ($T^{354}S^{355}S^{356}T^{357}$) at the intracellular carboxyl tail of rat μ-opioid receptor (MOR1) is required for the development of short-term homologous desensitization. To investigate the functional role played by individual serine or threonine residue of this (TSST) cluster in the agonist-induced μ-opioid receptor desensitization, point mutant (T354A), (S355A), (S356A) and (T357A) μ-opioid receptors were prepared and stably expressed in human embryonic kidney 293 cells (HEK 293 cells). Similar to wild-type μ-opioid receptors, mutant (T354A) and (S356A) μ-opioid receptors stably expressed in HEK 293 cells developed homologous desensitization after 30 min pretreatment of DAMGO ([D-Ala^2,*N*-methyl-Phe^4,Gly-ol^5]enkephalin), a specific μ-opioid receptor agonist. Substituting Ser^{355}or Thr^{357} with alanine resulted in a significant attenuation of agonist-induced μ-opioid receptor desensitization. In HEK 293 cells stably expressing double mutant (S355A/T357A) μ-opioid receptors, DAMGO pretreatment failed to significantly affect the efficacy and potency by which DAMGO inhibits forskolin-stimulated adenylyl cyclase activity. Consistent with the general belief that agonist-induced phosphorylation of guanine nucleotide binding protein (G protein)-coupled receptors is involved in homologous desensitization. Treating HEK 293 cells expressing wild-type μ-opioid receptors with 5 μM DAMGO for 30 min induced the receptor phosphorylation. Mutation of Ser^{355} and Thr^{357} also greatly impaired DAMGO-induced μ-opioid receptor phosphorylation. These results suggest that two C-terminal amino acids, Ser^{355} and Thr^{357}, are required for short-term homologous desensitization and agonist-induced phosphorylation of μ-opioid receptors expressed in HEK 293 cells.

Keywords: μ-Opioid receptor; Homologous desensitization; HEK 293 cells; DAMGO; Adenylyl cyclase; Agonist-induced phosphorylation

1. Introduction

Morphine and endogenous opioid peptides produce various biological effects including the analgesia by activating μ-opioid receptors [1–3]. Continuous administration of morphine-like drugs results in the development of tolerance and dependence [3–5]. Following the sustained or repeated exposure to agonists, μ-opioid receptor activity is also rapidly attenuated by a process referred to as the desensitization. Therefore, homologous desensitization of μ-opioid receptors *in vivo* is likely to be involved in the development of morphine-induced tolerance [4,5]. Molecular cloning studies indicated that μ-opioid receptor is a member of G protein-coupled receptor family [1,3]. Recently, multiple lines of evidence indicated that G protein-coupled receptor kinase (GRK)-mediated phosphorylation of G protein-coupled receptors in their active or stimulated conformations results in homologous desensitization [6–8]. According to this proposal, agonist-bound G protein-coupled receptor causes the dissociation of G_α and $G_{\beta\gamma}$ subunits. In addition to the subsequent G_α- or $G_{\beta\gamma}$-mediated activation of effector systems, $G_{\beta\gamma}$ subunits also bind to GRK and translocate GRK from the cytoplasm to the cell membrane. After being phosphorylated by GRK,

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Abbreviations: G protein, guanine nucleotide binding protein; DAMGO, [D-Ala^2,*N*-methyl-Phe^4,Gly-ol^5]enkephalin; cAMP, cyclic adenosine 3′,5′-monophosphate; HEK 293 cells, human embryonic kidney 293 cells; GRK, G protein-coupled receptor kinase.

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the receptors bind to inhibitory proteins, β-arrestins, and are uncoupled from G proteins, which results in the homologous desensitization. The physiological importance of GRK and β-arrestin regulation of μ-opioid receptor activity *in vivo* is clearly demonstrated by a recent study showing that in mice lacking β-arrestin-2 gene, chronic morphine treatment fails to induce μ-opioid receptor desensitization and opioid tolerance [9].

Up to now, six subtypes of GRKs have been cloned and characterized [7,8]. Among these GRKs, GRK2 is highly expressed in the nervous system and likely to mediate homologous desensitization of μ-opioid receptors *in vivo* [10]. Consistent with this hypothesis, it has been reported that agonist-induced desensitization of μ-opioid receptors was accompanied by the phosphorylation of receptor [11,12]. Overexpression of GRK2 in cell lines expressing μ-opioid receptors has been shown to promote agonist-induced receptor phosphorylation and attenuate the agonist inhibition of adenylate cyclase activity [13]. Our recent investigation also demonstrated that short-term homologous desensitization of rat μ-opioid receptors (MOR1) expressed in HEK 293 cells was greatly decreased following the transfection of cDNA fragment encoding GRK2(495-689) polypeptide [14], which acts as a specific $G_{\beta\gamma}$ antagonist and blocks various $G_{\beta\gamma}$-mediated transduction events including the activation of GRK2 [15]. These findings suggest that GRK2-mediated phosphorylation is involved in the development of agonist-induced μ-opioid receptor desensitization.

Similar to other G protein-coupled receptors, GRK2 is likely to induce μ-opioid receptor desensitization by phosphorylating serine and threonine residues within the third intracellular loop or cytoplasmic carboxyl tail [6–8]. Our recent study using various C-terminal deletion mutants of rat μ-opioid receptor indicated that a cluster of Ser/Thr residues ($T^{354}S^{355}S^{356}T^{357}$) at the C terminus plays an essential role in GRK2-mediated μ-opioid receptor desensitization [14]. In the present study, the functional role played by the individual serine or threonine residue of C-terminal TSST cluster in mediating μ-opioid receptor desensitization was investigated by preparing point mutant receptors. The results presented here indicate that two C-terminal amino acids, Ser^{355} and Thr^{357}, are required for short-term homologous desensitization of μ-opioid receptors expressed in HEK 293 cells. Further phosphorylation study also demonstrates that Ser^{355} and Thr^{357} play a critical role in agonist-induced μ-opioid receptor phosphorylation.

2. Materials and methods

2.1. Materials

[^{3}H][D-Ala^2, *N*-methyl-Phe^4, Gly-ol^5]enkephalin (DAMGO), [^{3}H]naloxone, [^{32}P]orthophosphate, Biotrak cAMP-binding protein assay kit and enhanced chemiluminescence kit were purchased from Amersham. Mammalian expression vectors, pBKCMV and pCMV-Tag3, were obtained from Stratagene. Human embryonic kidney 293 cells (HEK 293 cells) were purchased from American Type Culture Collection. G418 (geneticin sulfate) and cell culture reagents were obtained from GIBCO BRL. Restriction enzymes and DNA ligase were purchased from New England Biolabs. DOTAP was obtained from Boehringer Mannheim. Forskolin and isobutylmethylxanthine were purchased from RBI, and DAMGO was from Peninsula. Protease inhibitors and protein G-agarose were obtained from Sigma, and monoclonal anti-myc antibody was from Calbiochem. Sequenase 2.0 DNA sequencing kit and protein assay kit were purchased from United States Biochemical and Bio-Rad, respectively.

2.2. Construction of mutant μ-opioid receptors

A full-length cDNA encoding the rat μ-opioid receptor (MOR1) was obtained by performing PCR amplification using cDNA synthesized from rat brainstem mRNA [14,16]. pBKCMV vector containing the cDNA of wild-type rat μ-opioid receptor was used as DNA template for the oligonucleotide-directed mutagenesis using PCR amplification [16,17]. The following oligonucleotide primers were used for preparing mutant μ-opioid receptors: 5′TTCGATCGTGGACGAGGCTGGGATGCAGAACTC-TC3′ for mutant (T354A) μ receptor; 5′CTGTTCGATC-GTGGACGCGGTTGGGATGCAGAACT3′ for (S355A) μ-opioid receptor; 5′TTGCTGTTCGATCGTGGCCGAG-GTTGGGATCAGA3′ for mutant (S356A) μ receptor; 5′GTTTTGCTGTTCGATCGCGGACGAGGTTG GGAT-GC3′ for (T357A) μ-opioid receptor; 5′GTTTTGCTGT-TCGATCGCGGACGCGGTTGGGATGC AGAA3′ for mutant (S355A/T357A) μ receptor. Amplified DNA fragments were purified and subcloned into the pBKCMV vector. The mutations were confirmed by performing the dideoxy DNA sequencing.

2.3. Stable expression of μ-opioid receptors in HEK 293 cells

HEK 293 cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum, 50 U/mL penicillin and 50 ug/mL streptomycin. Cell cultures were maintained at 37° in a humidified 5% CO_2 incubator.

The cDNA clone (3 μg per well) of wild-type or mutant μ-opioid receptor was transfected into HEK 293 cells in six-well dishes by performing the lipofection using *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl-sulfate (DOTAP). Two days after the transfection, HEK 293 cells expressing μ-opioid receptors were selected by adding 0.8 mg/mL G418 (geneticin sulfate) to the culture medium. Positive clones were maintained in the culture

medium containing 0.2 mg/mL G418. After selecting stable cell lines expressing wild-type or mutant μ-opioid receptors, cells with the passage number of 2–20 were used for the present investigation.

2.4. Intracellular cAMP assays

To study μ-opioid receptor-mediated inhibition of forskolin-stimulated cAMP production, HEK 293 cells were seeded onto six-well dishes. On the experimental day, culture medium was removed, and cells were washed with serum-free medium. Subsequently, cells were incubated for 20 min at 37° with 0.2 mM isobutylmethylxanthine and 20 mM forskolin in the absence or presence of DAMGO, a specific μ-opioid receptor agonist. The reaction was terminated by removing the medium, and cellular cAMP was extracted by adding 80% ethanol. Cells were then pelleted, and the supernatant was lyophilized. Subsequently, cAMP level was determined by using the Biotrak cAMP-binding protein assay kit (TRK 432; Amersham). Prism program (GraphPad Software) was used to analyze the dose-response curve for DAMGO-mediated inhibition of cAMP formation.

2.5. Radioligand binding assays

HEK 293 cells expressing μ-opioid receptors were harvested by using the binding buffer containing 50 mM Tris–HCl (pH = 7.4), 1 mM EDTA, 5 mM $MgCl_2$ and 0.2 mg/mL bacitracin. Then, HEK 293 cells were pelleted by a centrifugation at 24,000 *g* for 15 min at 4°, and the pellet was homogenized in the binding buffer using a Polytron homogenizer. The homogenate was then recentrifugated at 48,000 *g* for 25 min at 4°. The pellet was resuspended in the binding buffer and used as the membrane preparation for the radioligand binding assay. Protein concentration in prepared membrane was measured by using the Bradford method (Bio-Rad protein assay kit).

For the saturation binding assay of μ-opioid receptors, membrane preparations (30–40 μg of protein/assay tube) were incubated for 1 hr at 25° with various concentrations of [^{3}H]DAMGO (65 Ci/mmol) or [^{3}H]naloxone (60 Ci/mmol). Specific μ-opioid receptor binding was defined as that displaced by 10 μM DAMGO. The binding reaction was terminated by vacuum filtration through GF/C filters. Filters were washed with ice-cold binding buffer (without bacitracin), and bound radioactivity was measured using a liquid scintillation counter.

For displacement studies, membrane preparations (30–40 μg of protein/assay tube) were incubated for 1 hr at 25° with 3 nM [^{3}H]naloxone and varying concentrations of DAMGO. Non-specific binding was determined by adding 10 uM DAMGO to the reaction mixture. The binding assay was terminated by the rapid filtration through GF/C filters. Filters were washed with ice-cold binding buffer (without bacitracin), and bound radioactivity was counted. Prism program (GraphPad Software) was used to analyze the data derived from the saturation and competition binding assays.

2.6. Immunoblotting analysis of epitope-tagged μ-opioid receptors

The cDNA of wild-type or mutant μ-opioid receptor was subcloned into a mammalian expression vector pCMV-Tag3 (Stratagene), which provides myc peptide tag (EQKLISEEDL) to the N terminus of receptor protein. Following the transfection, HEK 293 cells expressing myc-tagged μ-opioid receptors were selected by adding 0.8 mg/mL G418 (geneticin sulfate) to the culture medium.

HEK 293 cells stably expressing myc-tagged μ-opioid receptors were washed with ice-cold phosphate buffered saline (PBS). Cells were then solubilized with ice-cold lysis buffer A containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris–HCl (pH = 8), 5 mM EDTA, 10 μg/mL leupetin, 10 μg/mL benzamidine, 10 μg/mL aprotinin, 2 μg/mL pepstatin and 0.1 mM phenylmethylsulfonyl fluoride. Following the centrifugation at 15,000 *g* for 20 min, solubilized cell supernatants were fractionated on the 10% SDS-polyacrylamide gel. Proteins on the gel were transferred to polyvinylidene difluoride (PVDF) membrane, and the membrane was blocked with PBS containing 5% non-fat dried milk and 0.1% Tween-20. PVDF membrane was then incubated with diluted monoclonal anti-myc antibody (Calbiochem) for 2 hr at room temperature. After being washed, the membrane was incubated with sheep anti-mouse horseradish peroxidase-linked secondary antibody (Amersham). Subsequently, immunoreactive proteins on the membrane were visualized by using the enhanced chemiluminescence protocol (ECL Kit, Amersham).

2.7. Agonist-induced phosphorylation of μ-opioid receptors

On the experimental day, HEK 293 cells stably expressing myc-tagged wild-type or mutant μ-opioid receptors were washed phosphate-free minimal essential medium and labeled with [^{32}P]orthophosphate (0.2 mCi/mL) for 3 hr at 37°. To induce agonist-dependent receptor phosphorylation, labeled cells were then stimulated with 5 μM DAMGO for 30 min at 37°. After being washed with ice-cold PBS, cells were solubilized with ice-cold lysis buffer A (please see the previous section) supplemented with 10 mM NaF and 10 mM disodium pyrophosphate. Following the centrifugation at 15,000 *g* for 20 min, aliquots of solubilized cell supernatants were used for protein determination. Subsequently, cell lysates were precleared with 50 μL of protein G-agarose beads for 2 hr at 4°. Myc-tagged μ-opioid receptors were then immunoprecipitated with 5 μg of monoclonal anti-myc antibody (Calbiochem) and 50 μL of protein G-agarose suspension overnight at 4°.

Immune complexes were collected by the centrifugation and washed three times with lysis buffer A. Immunoprecipitates resuspended in SDS-sample buffer were dissociated from the agarose beads and resolved by SDS-polyacrylmide gel electrophoresis (10% gel). Each lane of gel was loaded with the equal amount of wild-type or mutant μ receptor protein. Following the electrophoresis, the gel was dried and exposed to the X-ray film. Phosphorylation of μ-opioid receptor was quantitatively determined by using a BAS 1500 PhosphoImager (Fuji).

2.8. Statistics

All results are expressed as the mean ± SE value of N experiments. Mann–Whitney test (two-tailed) was used to determine whether the difference was statistically significant ($P < 0.05$).

3. Results

3.1. Impairment of short-term μ-opioid receptor desensitization by substituting Ser^{355} and Thr^{357} with alanines

Saturable and specific binding sites for [^{3}H]DAMGO, a selective μ-opioid receptor agonist, were detected in HEK 293 cells stably transfected with the cDNA encoding the rat μ-opioid receptor. Scatchard analysis of [^{3}H]DAMGO binding revealed the expression of single population of high-affinity binding sites ($B_{max} = 475 \pm 52$ fmol/mg protein; $K_d = 2.8 \pm 0.3$ nM; Table 1). No specific [^{3}H]DAMGO binding was observed in non-transfected HEK 293 cells. DAMGO inhibited forskolin (20 μM)-stimulated cAMP formation with a concentration-dependent manner. The maximal DAMGO (5 μM)-mediated inhibition was 41 ± 4%, and EC_{50} value was 32 ± 3 nM (Table 2).

As described previously by us and other groups of workers [12,14,18], μ-opioid receptors expressed in HEK 293 cells or *Xenopus* oocytes exhibited the phenomenon of short-term homologous desensitization. Pretreating HEK 293 cells with a saturating dose of DAMGO (5 μM) for 15–30 min decreased both potency and efficacy of DAMGO-mediated inhibition of forskolin-stimulated adenylyl cyclase activity. Following 30 min pretreatment of 5 μM DAMGO, the maximal DAMGO inhibition of forskolin-stimulated cAMP production was reduced by 52 ± 3%, and EC_{50} value increased from 32 ± 3 to 62 ± 5 nM (N = 10 experiments). Saturation binding assays indicated that pretreating HEK 293 cells expressing μ-opioid receptors with DAMGO did not affect binding properties of [^{3}H]naloxone, a specific opioid receptor antagonist. For control cell membranes, B_{max} and K_d values of [^{3}H]naloxone binding were 465 ± 35 fmol/mg protein and 2.1 ± 0.2 nM (N = 4 experiments), respectively. For membranes prepared from HEK 293 cells pretreated with 5 μM DAMGO for 30 min, B_{max} and K_d values of [^{3}H]naloxone binding were 456 ± 40 fmol/mg protein and 2.2 ± 0.3 nM (N = 4 experiments), respectively. Competition binding assay using [^{3}H]naloxone showed that DAMGO pretreatment significantly reduced the affinity of DAMGO for μ-opioid receptors. Control K_i value for DAMGO inhibition of [^{3}H]naloxone binding was 1.4 ± 0.1 nM (N = 4). Following DAMGO (5 μM) pretreatment for 30 min, DAMGO displaced [^{3}H]naloxone binding with a K_i value of 5.6 ± 0.4 nM (N = 4). These results suggest that short-term homologous desensitization of μ-opioid receptors expressed in HEK 293 cells results from a decrease in the agonist affinity.

Our previous investigation indicated that a cluster of Ser/Thr residues ($T^{354}S^{355}S^{356}T^{357}$) located in the intracellular carboxyl tail of the μ-opioid receptor plays an essential role in GRK2-mediated homologous desensitization [14]. To investigate functional role played by the individual Ser/Thr residue of C-terminal ($T^{354}S^{355}S^{356}T^{357}$) cluster in the development of μ-opioid receptor desensitization, point mutant (T354A), (S355A), (S356A) and (T357A) μ-opioid receptors were prepared. The cDNA encoding each mutant μ-opioid receptor was stably transfected into HEK 293 cells, and a stable cell line with a B_{max} value similar to that of wild-type μ-opioid receptor was selected for further studies (Table 1). Radioligand binding studies demonstrated that mutant (T354A), (S355A), (S356A) or (T357A) μ-opioid receptor binds to [^{3}H]DAMGO with a similar affinity (K_d) as the wild-type μ-opioid receptor (Table 1). In HEK 293 cells stably expressing point mutant

Table 1
Ligand binding characteristics of wild-type and mutant μ-opioid receptors expressed in HEK 293 cells

Receptor type	B_{max} (fmol/mg protein)	K_d (nM)
Wild-type	475 ± 52	2.8 ± 0.3
(T354A)	430 ± 25	3.1 ± 0.3
(S355A)	460 ± 43	2.6 ± 0.2
(S356A)	483 ± 50	2.8 ± 0.2
(T357A)	450 ± 48	2.5 ± 0.3
(S355A/T357A)	527 ± 35	2.9 ± 0.2

Saturation binding assays were performed using [^{3}H]DAMGO. Each value represents the mean ± SE of four experiments performed in triplicate.

Table 2
Inhibition of forskolin-stimulated cAMP formation by wild-type and mutant μ-opioid receptors expressed in HEK 293 cells

Receptor type	Maximal inhibition (%)	EC_{50} (nM)
Wild-type	41 ± 4	32 ± 3
(T354A)	38 ± 3	29 ± 3
(S355A)	39 ± 2	30 ± 4
(S356A)	45 ± 2	31 ± 2
(T357A)	46 ± 2	27 ± 2
(S355A/T357A)	46 ± 3	28 ± 3

Each value represents the mean ± SE of 8–10 experiments performed in duplicate.

μ-opioid receptors, the maximal inhibition of forskolin-stimulated cAMP accumulation by DAMGO and EC_{50} value were similar to those obtained from HEK 293 cells expressing wild-type μ-opioid receptors (Table 2).

Similar to wild-type μ-opioid receptors, mutant (T354A) and (S356A) μ-opioid receptors stably expressed in HEK 293 cells developed homologous desensitization after 30 min exposure to 5 μM DAMGO. In HEK 293 cells stably transfected with the cDNA of (T354A) μ-opioid receptor (Fig. 1A), 30 min pretreatment of 5 μM DAMGO greatly reduced both the efficacy (control percentage of inhibition = 38 ± 3; with DAMGO preincubation, inhibition = 18 ± 3%); N = 8) and potency (control EC_{50} value = 29 ± 3 nM; with DAMGO pretreatment, EC_{50} value = 55 ± 4 nM; N = 8) by which DAMGO inhibits forskolin-stimulated cAMP formation. Pretreating HEK 293 cells expressing (S356A) μ-opioid receptors with 5 μM DAMGO for 30 min (Fig. 1B) also resulted in a reduced ability of DAMGO to inhibit forskolin-stimulated adenylyl cyclase activity (control maximal inhibition = 45 ± 2%, EC_{50} value = 31 ± 2 nM; with DAMGO pretreatment, inhibition = 24 ± 2%, EC_{50} value = 57 ± 3 nM; N = 8).

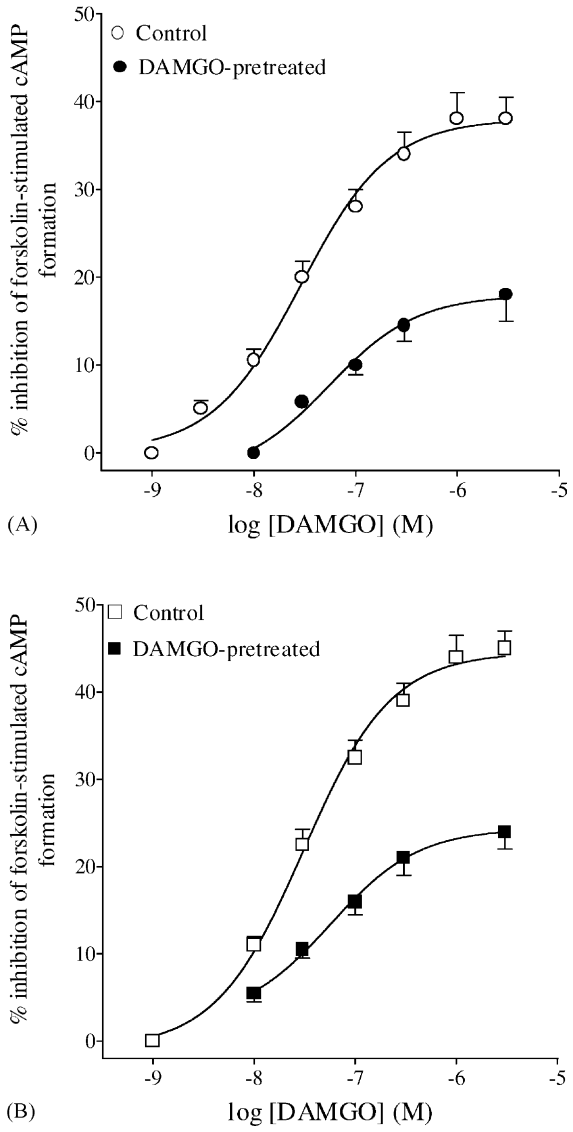

Fig. 1. Agonist-induced short-term desensitization of (T354A) or (S356A) μ-opioid receptors. (A) In HEK 293 cells stably expressing mutant (T354A) μ receptors, 30 min preincubation of 5 μM DAMGO greatly reduced both the efficacy and potency by which DAMGO inhibits forskolin-stimulated adenylyl cyclase activity. (B) Pretreating HEK 293 cells expressing (S356A) μ-opioid receptors with 5 μM DAMGO for 30 min also resulted in a reduced ability of DAMGO to inhibit forskolin-stimulated cAMP formation. Each point shows the mean ± SE value of eight experiments performed in duplicate.

Substituting Ser^{355}or Thr^{357} with alanine significantly attenuated the agonist-induced μ-opioid receptor desensitization. Compared to HEK 293 cells expressing wild-type μ-opioid receptors (desensitization = 52 ± 3%; N = 10), pretreating HEK 293 cells expressing (S355A) or (T357A) μ-opioid receptors with 5 μM DAMGO for 30 min led to a significant reduction in the desensitization of DAMGO (5 μM) inhibition of forskolin-stimulated cAMP formation. Percentages of desensitization for (S355A) and (T357A) μ-opioid receptors were 32 ± 3 and 20 ± 2%, respectively (Fig. 2). The functional significance of Ser^{355} and Thr^{357} in the development of μ-opioid receptor desensitization was further confirmed by using another HEK 293 cell line stably expressing (S355A) μ-opioid receptors (B_{max} = 355 ± 38 fmol/mg protein, K_d = 3.1 ± 0.2 nM, N = 4) or mutant (T357A) μ receptors (B_{max} = 343 ± 35 fmol/mg protein, K_d = 2.7 ± 0.3 nM, N = 4). In these cell lines, short-term desensitization induced by 30 min pretreatment of 5 μM DAMGO was also

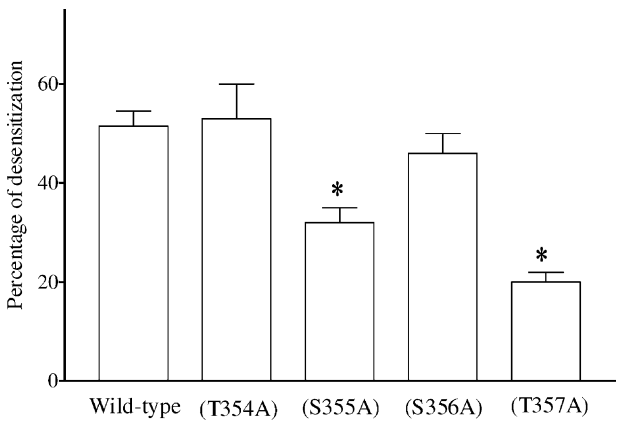

Fig. 2. Identification of C-terminal Ser/Thr residues involved in homologous desensitization of the μ-opioid receptor. Similar to wild-type μ-opioid receptors (T354A) and (S356A) mutant μ-opioid receptors developed the short-term desensitization after 30 min pretreatment of 5 mM DAMGO. Point mutation of S^{355} or T^{357} partially impaired the development of the μ-opioid receptor desensitization. The maximal DAMGO (5 μM)-mediated inhibition of forskolin-stimulated cAMP production was measured in control and DAMGO-pretreated cells. Percentage of desensitization was calculated as [1-(DAMGO inhibition of cAMP formation in agonist-pretreated cells/ control DAMGO inhibition of cAMP formation)] × 100. Each bar shows the mean ± SE value of 8–10 experiments performed in duplicate. The (*) indicates the significant difference ($P < 0.05$) between wild-type μ-opioid receptor and (S355A) or (T357A) mutant μ receptor.

greatly reduced. Percentages of desensitization for (S355A) and (T357A) μ-opioid receptors were 29 ± 2% (N = 4) and 18 ± 2% (N = 4), respectively (control percentage of desensitization = 52 ± 3). These results indicate that $Ser^{355}$and Thr^{357} play an important role in agonist-induced short-term desensitization of μ-opioid receptors.

To test the possibility that both $Ser^{355}$and Thr^{357} are required for homologous desensitization of μ-opioid receptors, mutant (S355A/T357A) μ-opioid receptor was prepared and stably expressed in HEK 293 cells. Radioligand binding studies indicated that both B_{max} and K_d values of [^{3}H]DAMGO bindings in HEK 293 cells stably transfected with the cDNA of (S355A/T357A) μ-opioid receptor were not significantly different from those measured in HEK 293 cells expressing wild-type μ-opioid receptors (Table 1). In HEK 293 cells stably expressing mutant (S355A/T357A) μ-opioid receptors, the maximal inhibition of forskolin-stimulated cAMP accumulation by DAMGO (46 ± 3%, N = 10) and EC_{50} value (28 ± 3 nM, N = 10) were similar to those obtained from HEK 293 cells expressing wild-type μ-opioid receptors (Table 2). However, in contrast to wild-type μ-opioid receptors, pretreating HEK 293 cells expressing (S355A/T357A) μ-opioid receptors with 5 μM DAMGO for 30 min did not significantly affect the efficacy and potency by which DAMGO inhibits forskolin-stimulated adenylyl cyclase activity (the maximal inhibition = 42 ± 3%; EC_{50} value = 31 ± 2 nM; N = 9 experiments; Fig. 3). In another HEK 293 cell line stably expressing (S355A/T357A) μ-opioid receptors (B_{max} value of [^{3}H]DAMGO binding = 357 ± 31 fmol/mg protein; K_d = 3.2 ± 0.4 nM; N = 4), 30 min pretreatment of 5 μM DAMGO also failed to affect the ability of DAMGO to inhibit forskolin-stimulated cAMP formation (control maximal inhibition = 33 ± 4%, EC_{50} value = 29 ± 2 nM; with DAMGO preincubation, maximal inhibition = 31 ± 3%, EC_{50} value = 32 ± 2 nM; N = 4). These results suggest that two C-terminal amino acids, Ser^{355} and Thr^{357}, are required for short-term homologous desensitization of μ-opioid receptors expressed in HEK 293 cells.

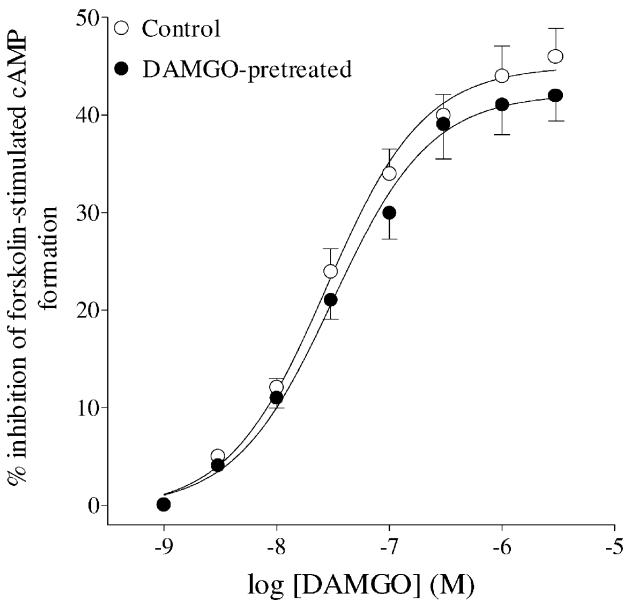

Fig. 3. Attenuation of short-term μ-opioid receptor desensitization by the mutation of S^{355} and T^{357} residues. In contrast to wild-type μ-opioid receptors, pretreating HEK 293 cells expressing (S355A/T357A) μ-opioid receptors with 5 μM DAMGO for 30 min did not significantly affect the maximal DAMGO-mediated inhibition of forskolin-stimulated cAMP formation and EC_{50} value by which DAMGO inhibits the adenylyl cyclase activity. Each point represents the mean ± SE of nine experiments performed in duplicate.

3.2. Impairment of agonist-induced μ-opioid receptor phosphorylation by substituting Ser^{355} and Thr^{357} with alanines

To investigate the functional role of Ser^{355} and Thr^{357} in agonist-induced μ-opioid receptor phosphorylation, we prepared HEK 293 cell line stably expressing myc-tagged wild-type μ-opioid receptors (B_{max} = 465 ± 42 fmol/mg protein, K_d for [^{3}H]DAMGO = 2.9 ± 0.2 nM, N = 3) or myc-tagged (S355A/T357A) μ receptors (B_{max} = 486 ± 35 fmol/mg protein, K_d for [^{3}H]DAMGO = 2.7 ± 0.3 nM, N = 3). Similar to non-tagged μ-opioid receptors (Table 2), DAMGO dose-dependently inhibited forskolin-stimulated cAMP formation in HEK 293 cells expressing myc-tagged wild-type μ-opioid receptors (inhibition = 42 ± 3%, EC_{50} value = 28 ± 3 nM, N = 4) or myc-tagged (S355A/T357A) μ receptors (inhibition = 44 ± 5%, EC_{50} value = 27 ± 2 nM, N = 4). Myc-tagged wild-type μ-opioid receptors stably expressed in HEK 293 cells developed homologous desensitization after 30 min exposure to 5 mM DAMGO (desensitization = 49 ± 4%; N = 4). However, pretreating HEK 293 cells expressing myc-tagged (S355A/T357A) μ receptors with 5 μM DAMGO for 30 min failed to affect the ability of DAMGO to inhibit forskolin-stimulated adenylyl cyclase activity (control maximal inhibition = 44 ± 5%, EC_{50} value = 27 ± 2 nM; with DAMGO preincubation, maximal inhibition = 40 ± 3%, EC_{50} value = 29 ± 3 nM; N = 4). These results demonstrate that addition of myc tag does not affect the agonist activation and homologous desensitization of μ-opioid receptors.

In accordance with a previous study [19], Western blotting analysis using monoclonal anti-myc antibody showed that HEK 293 cell line stably transfected with cDNA encoding myc-tagged wild-type or (S355A/T357A) μ receptors expressed a specific ~85 kDa-immunoreactive protein, which represents glycosylated monomer of μ-opioid receptor (Fig. 4A). This protein band was not detected in 293 cells transfected with empty pCMV-Tag3 vector. To study whether short-term μ-opioid receptor desensitization reported in the present study is accompanied by agonist-induced receptor phosphorylation, HEK 293 cells expressing myc-tagged μ-opioid receptors were labeled with $^{32}P_i$ and then stimulated with 5 μM DAMGO for 30 min. Subsequently, monoclonal anti-myc antibody was used to immunoprecipitate phosphorylated μ receptors.

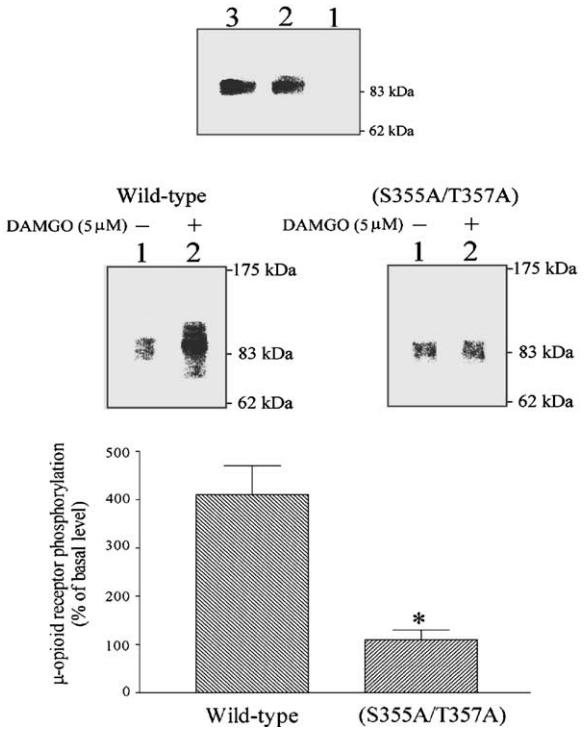

Fig. 4. Attenuation of DAMGO-induced μ-opioid receptor phosphorylation by the mutation of S^{355} and T^{357} residues. (A) Immunoblotting analysis using monoclonal anti-myc antibody indicated that a ~85 kDa-immunoreactive protein, corresponding to glycosylated monomer of μ-opioid receptor, was observed from HEK 293 cells expressing myc-tagged wild-type (lane 2) or (S355A/T357A) (lane 3) μ-opioid receptors. This protein band was absent in HEK 293 cells transfected with empty pCMV-Tag3 vector (lane 1). (B) ^{32}P-labeled HEK 293 cells stably expressing wild-type or (S355A/T357A) μ-opioid receptors were incubated with or without 5 μM DAMGO for 30 min as indicated. Myc-tagged μ-opioid receptors were then immunoprecipitated, resolved by SDS–PAGE, and receptor phosphorylation was quantitatively determined by PhosphoImager analysis. In HEK 293 cells expressing wild-type μ receptors, DAMGO (5 μM) induced the phosphorylation of a ~85 kDa protein, which represents glycosylated μ-opioid receptor observed in immunoblotting analysis. In contrast, DAMGO-induced phosphorylation of (S355A/T357A) μ-opioid receptors was greatly impaired. (C) DAMGO-stimulated phosphorylation of wild-type or (S355A/T357A) μ receptors was quantitatively analyzed and shown as the percentage of basal phosphorylation level seen in the absence of agonist stimulation. Each bar represents the mean ± SE value of four experiments. The (*) indicates the significant difference ($P < 0.01$) between wild-type μ-opioid receptor and mutant (S355A/T357A) μ receptor.

Compared to unstimulated cells, DAMGO stimulation of HEK 293 cells expressing wild-type μ-opioid receptors caused a 4-fold increase in phosphorylation of ~85 kDa protein, which corresponds to myc-tagged μ receptors observed in Western blotting study (Fig. 4B and C). DAMGO-induced phosphorylation of ~85 kDa protein was not observed in non-transfected cells. Furthermore, DAMGO-stimulated phosphorylation of myc-tagged wild-type μ receptors was blocked by 5 μM naloxone (data not shown). In contrast to wild-type μ-opioid receptors, (S355A/T357A) μ receptor phosphorylation stimulated by 30 min incubation of 5 μM DAMGO was dramatically inhibited (Fig. 4B and C). This finding suggests that Ser^{355} and Thr^{357} play a critical role in DAMGO-induced phosphorylation of μ-opioid receptors expressed in HEK 293 cells.

4. Discussion

Several lines of evidence propose that GRK-mediated phosphorylation of G protein-coupled receptors including μ-opioid receptors in their agonist-stimulated conformations leads to the development of homologous desensitization [6–8]. In accordance with this hypothesis, our previous study demonstrated that short-term homologous desensitization of μ-opioid receptors expressed in HEK 293 cells is greatly impaired following the transient transfection of cDNA fragment encoding GRK2(495-689) polypeptide, which blocks $G_{\beta\gamma}$-mediated translocation and activation of GRK2 [14,15]. Further studies using the C-terminal deletion mutants showed that a cluster of Ser/Thr residues ($T^{354}S^{355}S^{356}T^{357}$) at the carboxyl tail is required for GRK2-mediated μ-opioid receptor desensitization [14]. To determine which of these amino acids plays an essential role in the development of short-term agonist-induced desensitization, we constructed point mutant μ-opioid receptors and stably expressed them in HEK 293 cells. The results presented here suggest that two amino acids in the C-terminal loop, Ser^{355} and Thr^{357}, play a critical role in short-term homologous desensitization of μ-opioid receptors. Up to now, six alternatively spliced isoforms of μ-opioid receptors with different intracellular carboxyl tails have been identified [20]. Interestingly, Ser^{355} and Thr^{357} are conserved among the six variants of μ-opioid receptors, indicating the functional importance of these two C-terminal amino acids in mediating short-term μ receptor desensitization.

Previous studies demonstrated that GRK2 prefers to phosphorylate serine and threonine residues flanked by the upstream acidic amino acids [6–8]. Ser^{355} and Thr^{357}, which are believed to play an important role in GRK2-mediated μ-opioid receptor desensitization in HEK 293 cells, are located downstream of an acidic amino acid (Glu349). It has been reported that in HEK 293 cells expressing μ-opioid receptors, overexpression of GRK2 enhances the μ-opioid receptor phosphorylation and attenuates agonist inhibition of adenylate cyclase activity [13]. Therefore, it is reasonable to hypothesize that GRK2 mediates short-term homologous desensitization of μ-opioid receptors by phosphorylating Ser^{355} and Thr^{357} located in the intracellular carboxyl tail. In accordance with this hypothesis, the present investigation demonstrates that DAMGO-induced μ-opioid receptor phosphorylation is greatly impaired in HEK 293 cells expressing (S355A/T357A) μ-opioid receptors. However, further study is required to provide the direct evidence that Ser^{355} and Thr^{357} of μ-opioid receptor are phosphorylated by GRK2 following agonist activation.

Our recent investigation using acutely isolated neurons of the nucleus raphe magnus (NRM) also strongly supports the hypothesis that GRK2 mediates short-term μ-opioid receptor desensitization in the brain. DAMGO hyperpolarizes and inhibits NRM GABAergic neurons by enhancing the inwardly rectifying K^+ conductance [21]. Our single-cell RT-PCR assays showed that NRM GABAergic neurons, which exhibit short-term homologous desensitization of DAMGO enhancement of the inward rectifier K^+ conductance, contained GRK2 mRNA without expressing GRK3 mRNA [22]. Furthermore, following the internal perfusion of a synthetic peptide, which corresponds to $G_{\beta\gamma}$-binding domain of GRK2 and has been shown to inhibit $G_{\beta\gamma}$ activation of GRK2, short-term homologous desensitization of DAMGO-evoked K^+ currents in NRM GABAergic neurons was greatly impaired [22].

Recent mutational analysis of μ-opioid receptor demonstrated that cytoplasmic C-terminal loop contains Ser/Thr residues that are phosphorylated following the agonist stimulation [23,24]. Consistent with this finding, our results suggest that DAMGO stimulation results in phosphorylation of C-terminal Ser^{355} and Thr^{357} residues. However, it has been shown that mutation of Thr^{394} greatly reduced agonist-induced phosphorylation of μ-opioid receptors expressed in CHO cells [23]. Identification of different phosphorylation sites in HEK 293 and CHO cells suggests that agonist activation of μ-opioid receptor phosphorylates C-terminal Ser/Thr residues in a cell type-specific manner. It is possible that HEK 293 and CHO cells express different subtypes and levels of intrinsic kinases that are activated by DAMGO. As a result, Thr^{394} serves as the critical residue for agonist-induced phosphorylation of μ-opioid receptors expressed in CHO cells [23].

A recent study by El-Kouhen *et al.* [24] demonstrated that Thr^{370} and Ser^{375} residues at the C terminus play an essential role in DAMGO-induced phosphorylation of μ-opioid receptors expressed in HEK 293 cells. Despite of using the same expression system, different C-terminal Ser/Thr residues required for DAMGO-induced μ receptor phosphorylation were identified by El-Kouhen *et al.* [24] and us. This discrepancy is likely to result from a significant difference in the expression level of μ-opioid receptors in HEK 293 cells. El-Kouhen *et al.* [24] studied agonist-induced μ receptor phosphorylation by using HEK 293 cell lines expressing a high density (B_{max} = 1300–6900 fmol/mg protein) of μ-opioid receptors. Due to a large receptor reserve and rapid recycling and resensitization of μ-opioid receptors [25], short-term μ receptor desensitization observed in the present study is absent in cell lines used by El-Kouhen *et al.* [24]. Previous binding assays showed that B_{max} values of μ-opioid receptors in the brain lie in the range of 150–170 fmol/mg protein [26]. Therefore, we investigated DAMGO-induced desensitization and phosphorylation using HEK 293 cells with a lower expression level of μ-opioid receptors (B_{max} = 340–500 fmol/mg protein). The activity and effector coupling efficiency of opioid receptors expressed in mammalian cells are believed to depend on the receptor density [27,28]. Therefore, it is possible that depending on the expression level in HEK 293 cells, agonist stimulation of μ-opioid receptors activates intrinsic kinases, β-arrestins and other regulatory proteins with a different manner, which results in the phosphorylation of different C-terminal Ser/Thr residues and differential regulation of μ receptor activity. Consistent with this hypothesis, the data presented here suggests that DAMGO stimulation of μ-opioid receptors results in phosphorylation of Ser^{355} and Thr^{357} residues at the C terminus, which subsequently leads to receptor uncoupling and short-term homologous desensitization. On the other hand, El-Kouhen *et al.* [24] reported that in HEK 293 cells expressing a high density of μ-opioid receptors, C-terminal Thr^{370} and Ser^{375} residues function as phosphorylation sites following the DAMGO stimulation and play a critical role in regulating agonist-induced μ receptor internalization.

In agreement with the present study, recent studies suggest that serine and threonine residues located in the cytoplasmic carboxyl loops of δ- and κ-opioid receptors also play an essential role in GRK-mediated homologous desensitization. It has been reported that GRK2 phosphorylates δ-opioid receptors expressed in mammalian cell lines and mediates the short-term desensitization of δ-opioid receptors [29]. Pretreating hippocampal slices with U50,488H, a specific κ-opioid receptor agonist, results in the phosphorylation of κ-opioid receptor protein [30]. GRK2 and GRK3 have been shown to mediate agonist-induced desensitization of κ-opioid receptors expressed in mammalian cell lines or *Xenopus* oocytes [31,32]. Site-directed mutagenesis studies further demonstrated that serine or threonine residues located in the intracellular C-terminal loop play an important role in homologous desensitization of δ- or κ-opioid receptors [32–34].

It has been shown that in HEK 293 cells expressing μ-opioid receptors, overexpression of β-arrestin facilitated short-term desensitization of agonist inhibition of adenylate cyclase activity by causing μ receptor-G_i protein uncoupling [18]. Consistent with this finding, our membrane binding assays indicate that 30 min pretreatment of DAMGO fails to affect B_{max} value of [^{3}H]naloxone binding and that short-term μ-opioid receptor desensitization is accompanied by a reduced DAMGO affinity for μ receptor. Thus, together with our previous finding that GRK2 mediates μ-opioid receptor desensitization [14,22], the results presented here suggest that β-arrestin uncouples μ-opioid receptor from G_i protein and causes short-term homologous desensitization by binding to phosphorylated μ receptors.

In addition to uncoupling receptors from G proteins, β-arrestin is also believed to facilitate the receptor internalization by targeting phosphorylated G protein-coupled receptors to the clathrin-mediated endocytotic machinery [7,35]. Following the internalization, receptors

are dephosphorylated in early endosomes and recycled back to the cell membrane in a reactivated state. This recycling pathway could contribute to the functional resensitization of G protein-coupled receptors [7,35]. Alternatively, endocytozed receptors can be targeted to lysosomes for proteolytic down-regulation, which results in a reduction in the number of functional receptors [36]. The functional significance of endocytosis in regulating μ receptor activity is indicated by recent studies showing that C-terminal splice variants of μ-opioid receptor differ in agonist-induced internalization and receptor resensitization, which leads to varied desensitization kinetics of these μ receptor isoforms [37–40]. Compared to the major isoform of μ-opioid receptor (MOR1) studied in the present study, MOR1B isoform of μ receptor lacks one phosphorylation site (Thr^{394}) at the carboxyl tail and desensitizes with a slower rate than MOR1 [37]. Mutating Thr394 of MOR1 results in a slower homologous desensitization and a reduction in agonist-induced receptor phosphorylation [23,40]. Interestingly, further studies indicated that Thr^{394} at the C terminus functions as a negative regulator of μ-opioid receptor internalization and recycling process [37,38]. Therefore, slower rate of agonist-induced desensitization exhibited by MOR1B isoform, which lacks the C-terminal Thr^{394}, results from the facilitated μ receptor endocytosis and enhanced resensitization [38]. A recent study also indicated that three C-terminal splice variants of the mouse μ-opioid receptor, MOR1C, MOR1D and MOR1E, differ in their DAMGO- and morphine-induced endocytosis, receptor reactivation and down-regulation [39].

Homologous desensitization of μ-opioid receptor-mediated inhibition of adenylyl cyclase activity could result from a decrease in the agonist affinity or down-regulation of μ-opioid receptors [3]. In contrast to short-term pretreatment, a prolonged exposure (over a period of several hours) to agonist induces the functional desensitization of μ-opioid receptors by decreasing the density of μ receptors in the cell membrane *via* the degradation pathway [41,42]. It has been reported that two serine residues (Ser^{356} and Ser^{363}) at the C terminus play an essential role in agonist-induced down-regulation of μ-opioid receptors [42]. These findings suggest that different C-terminal Ser/Thr residues are involved in mediating two distinct molecular mechanisms underlying homologous desensitization of μ-opioid receptors, a reduced agonist affinity and receptor down-regulation. A better understanding of molecular mechanisms by which μ-opioid receptors desensitize *in vivo* should be useful in designing therapeutic agents that prevent opioid-induced tolerance.

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References

[1] Reisine T, Bell GI. Molecular biology of the opiate receptors. Trends Neurosci 1993;16:384–9.
[2] Matthes HWD, Maldonado R, Simonin F, Valverde O, Slowe S, Kitchen I, Befort K, Dierich A, Le Meur M, Dolle P, Tzavara E, Hanoune J, Roques BP, Kieffer BL. Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the μ-opioid receptor gene. Nature 1996;383:819–23.
[3] Law PY, Loh HH. Regulation of opioid receptor activities. J Pharmacol Exp Ther 1999;289:607–24.
[4] Nestler EJ. Under siege: the brain on opiates. Neuron 1996;16:897–900.
[5] Chavkin C, McLaughlin JP, Celver JP. Regulation of opioid receptor function by chronic agonist exposure: constitutive activity and desensitization. Mol Pharmacol 2001;60:20–5.
[6] Haga T, Haga K, Kameyama K. G protein-coupled receptor kinases. J Neurochem 1994;63:400–12.
[7] Krupnick JG, Benovic JL. The role of receptor kinases and arrestins in G protein-coupled receptor regulation. Annu Rev Pharmacol Toxicol 1998;38:289–319.
[8] Pitcher JA, Freedman NJ, Lefkowitz RJ. G protein-coupled receptor kinases. Annu Rev Biochem 1998;67:653–92.
[9] Bohn LM, Gainetdinov RR, Lin FT, Lefkowtiz RJ, Caron MG. μ-Opioid receptor desensitization by β-arrestin-2 determines morphine tolerance but not dependence. Nature 2000;408:720–3.
[10] Arriza JL, Dawson TM, Simerly RB, Martin LJ, Caron MG, Snyder SH, Lefkowitz RJ. The G protein-coupled receptor kinases βARK1 and βARK2 are widely distributed at synapses in rat brain. J Neurosci 1992;12:4045–55.
[11] Zhang L, Yu Y, Seamus M, Weight FF, Uhl GR, Wang JB. Differential μ-opiate receptor phosphorylation and desensitization induced by agonists and phorbol esters. J Biol Chem 1996;271:11449–54.
[12] Yu Y, Zhang L, Yin X, Sun H, Uhl GR, Wang JB. μ-Opioid receptor phosphorylation, desensitization, and ligand efficacy. J Biol Chem 1997;272:28869–74.
[13] Zhang J, Ferguson SSG, Barak LS, Bodduluri SR, Laporte SA, Law PY, Caron MG. Role for G protein-coupled receptor kinase in agonist-specific regulation of μ-opioid receptor responsiveness. Proc Natl Acad Sci USA 1998;95:7157–62.
[14] Wang HL. A cluster of Ser/Thr residues at the C terminus of μ-opioid receptor is required for G protein-coupled receptor kinase 2-mediated desensitization. Neuropharmacology 2000;39:353–63.
[15] Koch WJ, Hawes BE, Inglese J, Luttrell LM, Lefkowitz RJ. Cellular expression of the carboxyl terminus of a G protein-coupled receptor kinase attenuate $G_{\beta\gamma}$-mediated signaling. J Biol Chem 1994;269:6193–7.
[16] Wang HL. A conserved arginine in the distal third intracellular loop of the μ-opioid receptor is required for G protein activation. J Neurochem 1999;72:1307–14.
[17] Wang HL. Basic amino acids at the C terminus of the third intracellular loop are required for the activation of phospholipase C by cholecystokinin-B receptors. J Neurochem 1997;68:1728–35.
[18] Whistler JL, von Zastrow M. Morphine-activated opioid receptor elude desensitization by β-arrestin. Proc Natl Acad Sci USA 1998;95:9914–9.
[19] George SR, Fan T, Xie Z, Tse R, Tam V, Varghese G, O'Dowd BF. Oligomerization of μ- and δ-opioid receptors: generation of novel functional properties. J Biol Chem 2000;275:26128–35.
[20] Pan YX, Xu J, Bolan E, Abbadie C, Chang A, Zuckerman A, Rossi G, Pasternak GW. Identification and characterization of three new alternatively spliced μ-opioid receptor isoforms. Mol Pharmacol 1999;56:396–403.
[21] Pan ZZ, Tershner SA, Fields HL. Cellular mechanism for anti-analgesic action of agonists of the κ-opioid receptor. Nature 1997;389:382–5.

[22] Li AH, Wang HL. G protein-coupled receptor kinase 2 mediates μ-opioid receptor desensitization in GABAergic neurons of the nucleus raphe magnus. J Neurochem 2001;77:435–44.
[23] Deng HB, Yu YK, Pak YS, O'Dowd BF, George S, Surratt CK, Uhl GR, Wang JB. Role for the C terminus in agonist-induced μ opioid receptor phosphorylation and desensitization. Biochemistry 20 00;39:5492–9.
[24] El-Kouhen R, Burd AL, Erickson-Herbrandson LJ, Chang CY, Law PY, Loh HH. Phosphorylation of Ser^{363}, Thr^{370}, and Ser^{375}, residues within the carboxyl tail differentially regulates μ-opioid receptor internalization. J Biol Chem 2001;276:12774–80.
[25] Law PY, Erickson LJ, El- Kouhen R, Dicker L, Solberg J, Wang W, Miller E, Burd AL, Loh HH. Receptor density and recycling affect the agonist-induced desensitization of μ-opioid receptor. Mol Pharmacol 2000;58:388–98.
[26] Zukin RS, Pellegrini-Giampietro DE, Knapp CM, Tempel A. Opioid receptor regulation. In: Herz A, editor. Opioids I, handbook of experimental pharmacology, vol. 104/I. Berlin: Springer-Verlag, 1993. p. 107–24.
[27] Pak YS, Kouvelas A, Scheideler MA, Ramussen J, O'Dowd BF, George SR. Agonist-induced functional desensitization of the μ-opioid receptor is mediated by loss of membrane receptors rather than uncoupling from G protein. Mol Pharmacol 1996;50:1214–22.
[28] Hirst RA, Hirota K, Grandy DK, Lambert DG. Coupling of the cloned κ-opioid receptor to adenylyl cyclase is dependent on receptor expression. Neurosci Lett 1997;232:119–22.
[29] Pei G, Kieffer BL, Lefkowitz RJ, Freedman NJ. Agonist-dependent phosphorylation of the mouse δ-opioid receptor: involvement of G protein-coupled receptor kinases but not protein kinase C. Mol Pharmacol 1995;48:173–7.
[30] Appleyard SM, Patterson TA, Jin WZ, Chavkin C. Agonist-induced phosphorylation of the κ-opioid receptor. J Neurochem 1997;69: 2405–12.
[31] Raynor K, Kong H, Hines J, Kong G, Benovic J, Yasuda K, Bell GI, Reisine T. Molecular mechanisms of agonist-induced desensitization of the cloned mouse kappa-opioid receptor. J Pharmacol Exp Ther 1994;270:1381–6.
[32] Appleyard SM, Celver J, Pineda V, Kovoor A, Wayman GA, Chavkin C. Agonist-dependent desensitization of the κ-opioid receptor by G protein receptor kinase and β-arrestin. J Biol Chem 1999;274: 23802–7.
[33] Kovoor A, Nappey V, Kieffer BL, Chavkin C. μ and δ receptors are differentially desensitized by the coexpression of β-adrenergic receptor kinase 2 and β-arrestin-2 in *Xenopus* oocytes. J Biol Chem 1997;272:27605–11.
[34] Law PY, Maestri-El Kouhen O, Solberg J, Wang W, Erickson LJ, Loh HH. Deltorphin II-induced rapid desensitization of δ-opioid receptor requires both phosphorylation and internalization of the receptor. J Biol Chem 2000;275:32057–65.
[35] Oakley RH, Laporte SA, Holt JA, Barak LK, Caron MG. Association of β-arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitization. J Biol Chem 1999;271:11449–54.
[36] Tsao P, von Zastrow M. Down-regulation of G protein-coupled receptors. Curr Opin Neurobiol 2000;10:365–9.
[37] Koch T, Schulz S, Schroder H, Wolf R, Raulf E, Hollt V. Carboxyl-terminal splicing of the rat μ-opioid receptor modulates agonist-induced internalization and receptor resensitization. J Biol Chem 1998;273:13652–7.
[38] Wolf R, Koch T, Schulz S, Klutzny M, Schroder H, Raulf E, Bling F, Hollt V. Replacement of threonine 394 by alanine facilitates internalization and resensitization of the rat μ-opioid receptor. Mol Pharmacol 1999;55:263–8.
[39] Koch T, Schulz S, Pfeiffer M, Klutzny M, Schroder H, Kahl E, Hollt V. C-terminal splice variants of the mouse μ-opioid receptor differ in morphine-induced internalization and receptor resensitization. J Biol Chem 2001;276:31408–14.
[40] Pak YS, O'Dowd BF, George S. Agonist-induced desensitization of the μ opioid receptor is determined by threonine 394 preceded by acidic amino acids of the COOH-terminal tail. J Biol Chem 1997;272: 24961–5.
[41] Chakrabarti S, Yang W, Law PY, Loh HH. The μ-opioid receptor down-regulates differently from the δ-opioid receptor: requirement of a high-affinity receptor-G protein complex. Mol Pharmacol 1997;52:105–13.
[42] Burd AL, El-Kouhen R, Loh HH, Law PY. Identification of serine 356 and serine 363 as the amino acids involved in etorphine-induced down-regulation of the μ-opioid receptor. J Biol Chem 1998;273:34488–95.