Agonist-dependent Desensitization of the κ Opioid Receptor by G Protein Receptor Kinase and β-Arrestin*

We used the *Xenopus* oocyte expression system to examine the regulation of rat κ opioid receptor (rKOR) function by G protein receptor kinases (GRKs). κ agonists increased the conductance of G protein-activated inwardly rectifying potassium channels in oocytes coexpressing KOR with Kir3.1 and Kir3.4. In the absence of added GRK and β-arrestin 2, desensitization of the κ agonist-induced potassium current was modest. Coexpression of either GRK3 or GRK5 along with β-arrestin 2 significantly increased the rate of desensitization, whereas addition of either β-arrestin 2, GRK3, or GRK5 alone had no effect on the KOR desensitization rate. The desensitization was homologous as coexpressed δ opioid receptor-evoked responses were not affected by KOR desensitization. The rate of GRK3/β-arrestin 2-dependent desensitization was reduced by truncation of the C-terminal 26 amino acids, KOR(Q355A). In contrast, substitution of Ala for Ser within the third intracellular loop [KOR(S255A,S260A,S262A)] did not reduce the desensitization rate. Within the C-terminal region, KOR(S369A) substitution significantly attenuated desensitization, whereas the KOR(T363A) and KOR(S356A,T357A) point mutations did not. These results suggest that coexpression of GRK3 or GRK5 and β-arrestin 2 produced homologous, agonist-induced desensitization of the κ opioid receptor by a mechanism requiring the phosphorylation of the serine 369 of rKOR.

Prolonged exposure to opioid drugs often produces tolerance, dependence, and addiction. The molecular mechanisms underlying tolerance are complex and multifaceted (for review see Ref. 1). Because opioid receptors are members of the G protein-coupled receptor superfamily (for review see Ref. 2), one component of opioid tolerance is likely to be mediated by a phosphorylation-dependent receptor desensitization. Following agonist activation, other members of this receptor family are phosphorylated, then inactivated (for review see Ref. 3). Results of prior studies have suggested that the κ opioid receptor (KOR) undergoes a phosphorylation-dependent desensitization as well. Prolonged activation of KOR expressed in normal guinea pig brain slices resulted in receptor phosphorylation, and this increase in KOR phosphorylation correlated with the desensitization of the cellular response to κ agonists in the tissue (4).

The kinase responsible for regulation of KOR in vivo remains to be elucidated. Based on our understanding of other G protein-coupled receptors (for review see Ref. 5), one potential candidate is the family of G protein receptor kinases (for review see Ref. 6). In support of this hypothesis, agonist-induced desensitization of the κ opioid-evoked response was blocked by the expression of a dominant negative G protein-coupled receptor kinase in transfected cells (7). In addition, overexpression of β-arrestin 1 attenuated the κ opioid receptor-mediated response (8). However, the effects of specific G protein receptor kinases, the contribution of β-arrestins, and the regions of KOR required for agonist-induced desensitization remains to be elucidated. In this study, we used the *Xenopus* oocyte expression system to further characterize the potential mechanisms underlying agonist-induced desensitization of KOR. A better definition of the desensitization process is critical for a clearer understanding of the mechanisms underlying opioid tolerance.

**Experimental Procedures**

**Chemicals**—U69,593, U50,488H and naloxone were obtained from Research Biochemicals International. DPDPE was obtained from Peninsula Laboratories. All other chemicals were from Sigma.

**Mutagenesis of KOR**—The following mutations of the rat KOR cDNA in pGEM were made using an adaptation of the Quick Change protocol from Stratagene. Mutagenic oligonucleotides were as follows: GATGC-GAATGGAGCGCTAGAGCTCAAACAGAGTTAGAAACACAG (Q355A), CGCCTGAGGCGCTTGAAGGCTGTCCGGCTCCTCGCGGGAGCTCGAGAGAAGGAC (S255A, S260A, S262A), GCACAAACAGGTAGAAACCGGCGTGACAGATCTGCTTCATG(positions 363–370), and GACCAAGTGTGCGGTGCCTGCTTCATG (S356A, T357A). Mutant KOR (S356A,T357A) was made using the polymerase chain reaction overlap extension method (9) with the oligonucleotide GGAGCGCCAGGCCGAAAAGAAGGAC (S255A, S260A, S262A), GCACAAACAGGTAGAAACCGGCGTGACAGATCTGCTTCATG (position 363–370), and GACCAAGTGTGCGGTGCCTGCTTCATG (S356A, T357A). All mutations were confirmed by DNA sequencing.

**Complementary DNA Clones and cRNA Synthesis**—The rat KOR clone was in pGEM 3 such that the SP6 promoter directed sense transcripts. cDNA for the Kir3.1, Kir3.4, DOR, β-arrestin 2, and GRK3 were as described (10, 11). Plasmid templates for all constructs including KOR mutants were linearized before cRNA synthesis, and mMES-SAGE MACHINE kits (Ambion Corp.) were used to generate capped cRNA.

**Oocyte Culture and Injection**—Defolliculated, stage IV oocytes were prepared as described (12) and were incubated for 3–6 days after injection of the cRNA in normal oocyte saline buffer (96 mm NaCl, 2 mm KCl, 1 mm MgCl2, 1 mm CaCl2, and 5 mm HEPES, pH 7.5) solution supplemented with sodium pyruvate (2.5 mm) and gentamicin (50 μg/ml). cRNA were injected (50 nl/oocyte) with a Drummond microinjector.

**Electrophysiology**—Oocytes were voltage-clamped at −80 mV with two electrodes filled with 3 mm KCl having resistances of 0.5–2.0 ohms, penicillamine-2,5-enekephalin; U69,593, (+)-5(S,7α,8β)-N-methyl-N-(7- (1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl) benzeneacacetamide.
using a Geneclamp 500 amplifier and pCLAMP 6 software (Axon Instruments). Membrane current traces were recorded using a chart recorder. Data was also digitally recorded (Digidata, Axon Instruments, and Intel 386 PC) and filtered. To facilitate the recording of inward K\textsubscript{1} currents through the Kir3 channels, the normal oocyte saline buffer was modified to increase KCl concentration to 16 mM K\textsubscript{1}. The concentration of NaCl was correspondingly decreased to maintain osmolarity.

Data Analysis—

EC\textsubscript{50} values and curve fits were determined using Nfit (Island Products, Galestone, TX). Confidence intervals were used for comparison of the independent means. Statistical significance was determined using the Student’s t-test value for either 95 or 99% confidence levels.

FIG. 1. GRK3 and \(\beta\)-arr2 mediate agonist-induced desensitization. A, a representative trace from an oocyte injected with 1 ng of cRNA for the wtKOR and 0.05 ng each of the G protein-gated inwardly rectifying potassium channel subunits Kir3.1 and Kir3.4. The oocyte membrane potential was clamped at \(-80\) mV while bathed in normal saline buffer containing 2 mM KCl, as described under “Experimental Procedures.” Oocytes were then superfused with a saline buffer in which the KCl concentration was increased to 16 mM. This does not activate the channel, but allows the basal inward current to flow through the inwardly rectifying potassium channels at the \(-80\) mV holding potential (\(I_{basal}\)). After equilibration with the high potassium K\textsuperscript{+} buffer, application of the high potassium K\textsuperscript{+} buffer, application of \(\kappa\) opioid receptor agonist, 2 \(\mu M\) U69,593 increased the inward current (\(I_{peak}\)). Any change in basal inward current through Kir3 channels after agonist treatment was detected by superfusion of antagonist to reverse the receptor-activated response (\(I_{end}\)), and a baseline was plotted as shown by the dashed line. Current traces in subsequent figures show only the agonist-activated currents adjusted for changes in the baseline. B, representative traces depict baseline subtracted responses to 2 \(\mu M\) U69,593 or U50,488H that were recorded for at least 10 min in oocytes. The insets show the cRNA mixtures injected for the oocytes used to generate the trace above and the bar below the inset. C, comparison of the percent desensitization of the agonist response calculated as 1 - \(I_{at\ 10\ min}/I_{peak}\)\times100. Each bar represents the mean \(\pm\) S.E. calculated from 7 to 8 separate oocytes from 2 different donors (\(*\), denotes significance at 99% confidence compared with control oocytes).

FIG. 2. The GRK3/\(\beta\)-arr2-mediated agonist-induced desensitization is homologous. All oocytes were injected with the following cRNAs: 1 ng of the wtKOR and 0.05 ng each of the G protein-gated inwardly rectifying potassium channel subunits Kir3.1 and Kir3.4. The oocyte groups were also injected with 0.5 ng of GRK3 cRNA and/or 1–2 ng of \(\beta\)-arr2 cRNA as illustrated in the circled insets under the traces. All recordings were made 4–5 days after injection, and the responses measured in 16 mM K\textsuperscript{+} buffer. Responses were adjusted by baseline subtraction as described in the legend to Fig. 1. A, representative traces of the response to 1 \(\mu M\) DPDPE, 2 \(\mu M\) U69,593, and 1 \(\mu M\) naloxone applied as above the traces. B, the bar graph shows a comparison of the amount of both \(\kappa\) and \(\delta\) agonist response remaining after 10 min of treatment with U69,593 in the presence and absence of GRK3/\(\beta\)-arr2; the left pair was done using oocytes lacking GRK3/\(\beta\)-arr2, and the right pair was done with oocytes co-expressing GRK3 and \(\beta\)-arr2 as shown in the inset. For DPDPE activation of DOR, the percent desensitization equals 1 - \(I_{at\ 10\ min}/I_{peak}\)\times100. For U69,593 activation of KOR, the percent desensitization equals 1 - \(I_{at\ 10\ min}/I_{peak}\)\times100 (as in Fig. 1). Each bar represents the mean \(\pm\) S.E. calculated from 7 to 12 separate oocytes from at least 2 different donors (**, denotes significance at 99% confidence compared with control oocytes).
Homologous Desensitization of the \( \kappa \) Opioid Receptor

**First Cytoplasmic Loop**

| mDOR | R Y T K L K T A T N I Y |
|------|------------------------|
| rMOR | R Y T K M K T A T N I Y |
| rKOR | R Y T K M K T A T N I Y |

**Second Cytoplasmic Loop**

| mDOR | D R Y I A V C H P V K A L D F R T P A |
|------|-------------------------------------|
| rMOR | D R Y I A V C H P V K A L D F R T P R |
| rKOR | D R Y I A V C H P V K A L D F R T P L |

**Third Cytoplasmic Loop**

| mDOR | L M L R L R S V R L L S G S K E K D R S L R R T R M |
|------|--------------------------------------------------|
| rMOR | L M I L R K S V R M L S G S K E K D R N L R R T R M |
| rKOR | L M I L R K S V R L L S G S R E K D R N L R R T K L |

**Carboxy-terminal Tail**

| mDOR | Y A F L D E N F K R C F R Q L C R T P C G R Q E P G S L R R P |
|------|--------------------------------------------------------|
| rMOR | Y A F L D E N F K R C F R E F C I P T S S I E Q N S T R V |
| rKOR | Y A F L D E N F K R C F R D F C F F I K K R M E R Q S T N R V |

| mDOR | R Q A T T R E V T A C T P D G P G G G A A |
|------|-------------------------------------|
| rMOR | R Q N T R E H P S T A N T V D R T N H Q L E N L E A T A P L P |
| rKOR | R N T V Q D P A S M R D V G N N K P V |

Fig. 3. Comparison of the amino acid sequence of the predicted first, second, and third intracellular loops and the C-terminal tail of the KOR, DOR, and MOR. The amino acid sequences of the rat KOR (GenBank™ accession no. D16829), mouse DOR (accession no. S65335), and rat MOR (accession no. L13069) are shown. The single letter code for amino acids is used. Potential serine/threonine phosphorylation sites are shown in bold.

**Results**

Agonist-induced Desensitization of KOR Requires GRK3 and \( \beta\)-arr2—\( \kappa \) opioid receptors have been shown to couple to inwardly rectifying potassium channels in *Xenopus* oocytes co-injected with Kir3.1 (GRK1) and the \( \kappa \) opioid receptor (13, 14). As reported previously (11), co-expression of Kir3.1 and Kir3.4 and a reduction in the level of cRNA expression effectively minimized the heterologous desensitization of DOR- and MOR-evoked responses. The \( \kappa \) selective agonist U69,593 (2 \( \mu \)M) also activates an inwardly rectifying potassium conductance in *Xenopus* oocytes co-expressing the cRNA for the KOR, Kir3.1, and Kir3.4 (Fig. 1). The current was maximally activated by 2 \( \mu \)M U69,593, and this dose was used in subsequent experiments (EC\(_{50}\) = 240 nM).

For oocytes expressing KOR and Kir3 channel, the U69,593-induced response desensitized by about 20\% during a 10 min agonist application (Fig. 1, A–C). Additional co-injection of cRNAs for GRK3 and \( \beta\)-arrestin 2 caused a significant increase in the agonist-induced desensitization measured over 10 min (65\%, Fig. 1, B and C). In contrast, expression of either the cRNA for GRK3 or \( \beta\)-arrestin 2 alone did not increase the agonist-induced desensitization (Fig. 1, B and C). The finding that both GRK3 and \( \beta\)-arrestin 2 were required suggests that the desensitization observed was caused by receptor phosphorylation followed by arrestin binding rather than alternative kinase-independent mechanisms including G\( \beta\)\( \gamma\) sequestration by GRK3 (15).

**GRK3/\( \beta\)-arr2-dependent Desensitization Is Homologous—** The agonist-dependent desensitization of the KOR mediated by GRK3 and \( \beta\)-arr2 was found to be homologous (Fig. 2). The response to 1 \( \mu \)M DPDPE, a \( \delta \) selective opioid agonist, was significantly decreased in oocytes co-expressing GRK3 and \( \beta\)-arr2 (Fig. 2). The lack of change in the second DPDPE response following U69,593 in oocytes co-expressing GRK3 and \( \beta\)-arr2 indicates that the desensitization of the KOR-mediated response was homologous. The decrease in DPDPE response following \( \kappa \) agonist treatment was about 20–30\% in both the presence and absence of GRK3 and \( \beta\)-arr2. This is the same decrease as seen in the U69,593 response in the absence of GRK3 and \( \beta\)-arr2 (Figs. 1 and 2) suggesting that this GRK3 and \( \beta\)-arr2-independent change was homologous. Homologous desensitization is thought to occur by a change at the receptor (e.g. phosphorylation), whereas heterologous desensitization occurs at common downstream signaling steps (16).

Region of KOR Required for Desensitization—Studies with other G protein-coupled receptors have shown that specific serine and threonine residues in either the third intracellular loop or the C-terminal tail are required for regulation by GRKs (for review see Refs. 5 and 6). Fig. 3 shows a comparison of the amino acid sequences of the third intracellular loop and C-terminal tail of the rat \( \kappa \), rat \( \mu \), and mouse \( \delta \) opioid receptors. Potential phosphorylation sites for all three receptors are shown in bold. Previous studies with the \( \mu \) and \( \delta \) receptor showed that the serine and threonine residues in the C-terminal tail were important for GRK-mediated desensitization (11, 17, 18). We therefore made a mutation of KOR, which resulted in truncation of the C-terminal tail region containing the serine and threonine residues KOR(Q255A). In addition, we made point mutations of potential phosphorylation sites in the C-terminal tail. All the mutants expressed and coupled to the potassium channel with similar dose response curves for U69,593 (Figs. 4A and 5A). The EC\(_{50}\) values for both the wild type and mutant receptors are as follows (in \( nM \) with 95\% confidence intervals): wtKOR, 240 (190–291); KOR(Q355A), 173 (140–206); KOR(S255A,S260A,S262A), 110 (31–189); KOR(S356A,T357A), 208 (95–320); KOR(T363A), 165 (6–171); KOR(S369A), 131 (84–178). The EC\(_{50}\) values were not substantially different, suggesting that the mutations did not dramati-
The principal findings of this study are 4-fold. First, we found that agonist-induced desensitization of the k opioid receptor can be facilitated by co-expression of GRKs and β-arrestin 2 in the Xenopus oocyte. Second, that this desensitization is homologous. Third, that the C-terminal tail of rKOR, and in particular serine 369 was required for the agonist-induced desensitization observed. Last, that either GRK3 or GRK5 can produce agonist desensitization when co-expressed with β-arrestin 2. These results from oocyte expression studies demonstrate a potential mode of receptor regulation likely to be important in the intact nervous system.

G protein-coupled receptors have been shown to activate Kir3 channels through the release of Gβγ dimers (20). As has been discussed previously by Kovoor et al. (11), heterologous expression of GRK3 alone in the Xenopus oocyte may inhibit the ability of the opioid receptors to couple to Kir3 as both GRK2 and GRK3 have also been shown to bind Gβγ, and a fusion protein of a portion of GRK2 inhibited basal Kir3 activity (21). Indeed as can be seen in Fig. 1, co-expression of GRK3 appeared to decrease the peak response produced by κ agonists. However, expression of GRK3 alone did not produce any increase in the agonist-induced desensitization rate. The agonist-induced desensitization required co-expression of β-arrestin 2 suggesting the action of GRK3 was due to catalytic phosphorylation of the opioid receptor and not Gβγ sequestration. The fact that the GRK3 and β-arrestin 2-mediated desensitization was attenuated by a specific point mutation of serine 369 in the C-terminal tail of KOR further supports the conclusion that...
receptor phosphorylation was required. Last, co-expression of GRK5 and β-arrestin 2 also caused agonist-induced desensitization of KOR. As GRK5 does not bind to and is not recruited to the membrane by Gβγ; this again argues that the desensitization mediated by GRKs is not through Gβγ sequestration.

The mutagenesis approach provides indirect evidence that the receptor is phosphorylated at a critical serine residue. A direct test of this hypothesis requires a demonstration of phosphate incorporation at serine 369 following agonist stimulation. Although we tried that experiment, we were unable to get sufficient 32P incorporation into KOR expressed in oocytes to resolve the phosphopeptide fragments derived from immunoprecipitated receptor. Phosphospecific antibodies presently being developed in this laboratory may ultimately be useful in the detection of phosphoserine 369. Nevertheless, the hypothesis is supported by the present demonstration that the agonist-dependent desensitization required GRK and β-arrestin co-expression.

The finding that residues in the C-terminal tail are important for regulation of KOR by GRKs parallels the findings that the C-terminal tail of both the DOR and MOR receptors are important for their regulation by GRKs (11, 17, 18). These results suggest that opioid receptors differ from other Gi/Go-coupled receptors such as the m2 muscarinic and α2 adrenergic receptors whose regulation by GRKs requires phosphorylation sites in the third intracellular loop (19, 22, 23). The finding that serine 369 in KOR is essential for regulation by GRKs parallels the finding that threonine 394 appears to be the primary residue required for regulation of the μ opioid receptor (17, 24).

**FIG. 5. Serine 369 in KOR is required for GRK3/β-arrestin2-mediated agonist-induced desensitization.** Control oocytes were injected with the following cRNAs: either 1 ng of wtKOR, 1 ng of KOR(S369A), 3 ng of KOR(S356A,T357A), or 1–2 ng of KOR(T363A) with 0.05 ng of Kir3.1 and 0.05 ng of Kir3.4. Other oocytes were also injected with either 0.5 ng of GRK3 or 1 ng of GRK5 cRNA and 1–3 ng of β-arrestin 2 cRNA as shown under the graph. All recordings were made 4–5 days after injection, and the responses measured in 16 mM K+ buffer. Responses were adjusted by baseline subtraction as described in the legend to Fig. 1. The bar graph shows the percent desensitization for the wtKOR (black bars) or KOR(Q355Δ) (open bar), calculated as in Fig. 4 except that the experiments summarized by the middle bar lacked β-arrestin 2. Each bar represents the mean ± S.E. calculated from 6 to 8 separate oocytes from two donor frogs (** = significant to the 99% confidence interval; * = significant to the 95% confidence interval compared with control oocytes).

**FIG. 6. Effect of GRK5 and β-arrestin 2 on agonist-induced desensitization of the KOR.** Control oocytes were injected with the following cRNAs: 1 ng of KOR, 0.05 ng of Kir3.1, 0.05 ng of Kir3.4, 0.4 ng of DOR. Other oocytes were also injected with either 0.5 ng of GRK3 or 1 ng of GRK5 cRNA and 1–3 ng of β-arrestin 2 cRNA as shown under the graph. All recordings were made 4–5 days after injection, and the responses measured in 16 mM K+ buffer. Responses were adjusted by baseline subtraction as described in the legend to Fig. 1. The bar graph shows the percent desensitization for the wtKOR (black bars) or KOR(Q355Δ) (open bar), calculated as in Fig. 4 except that the experiments summarized by the middle bar lacked β-arrestin 2. Each bar represents the mean ± S.E. calculated from 7 to 12 separate oocytes from at least 2 different donors (**, denotes significance at 99% confidence compared with control oocytes).
Both serine 369 and threonine 394 are the most C-terminal Ser/Thr residues in the \( \kappa \) and \( \mu \) opioid receptors, respectively. Recently threonine 394 was found to be important for determining the rate of internalization and desensitization of the \( \mu \) opioid receptor, whether this is also true for KOR serine 369 remains to be determined (25). Similarly, the finding that the C-terminal threonine 353 is critical for \( \delta \) opioid receptor internalization and desensitization (26, 27) closely parallels the findings with KOR and MOR.

The data shown here suggests that GRK3 and GRK5 along with \( \beta \)-arrestin 2 are capable of regulating agonist-induced desensitization of the \( \kappa \) opioid receptor. This is the first demonstration of GRK3 and GRK5 regulating the rat KOR. Both the MOR and DOR have also been shown to be regulated by both GRK3 and GRK5 (11, 18). These results suggest that opioid receptors are again regulated differently than the \( \alpha_2 \) adrenergic receptor, which was shown to be phosphorylated and desensitized in an agonist-dependent manner by GRK3 but not by GRK5 (28). This provides further evidence that these kinases may differentially regulate G protein-coupled receptors. As was demonstrated by Rockman et al. (29), this specificity may also occur in vivo. It remains to be determined whether GRK3 and GRK5 are involved in the agonist-induced phosphorylation of opioid receptors seen in vivo. Localization studies show that GRK3 is expressed in many of the same regions of the brain as the \( \kappa \) opioid receptor supporting a role for this kinase in the in vivo regulation of KOR (30–32). However, this hypothesis remains to be directly tested. Characterization of the mechanism of desensitization in vitro and the determination of the critical residue for agonist desensitization of the \( \kappa \) receptor in vitro provides us with the knowledge needed to test whether this mechanism occurs in vivo.

Interestingly the removal of all the serine and threonine residues in the C-terminal tail of the KOR does not completely block the GRK/\( \beta \)-arrestin-mediated desensitization. The mechanism for this residual slow desensitization is not known. Potential mechanisms include phosphorylation of the remaining intracellular serine or threonine residues present in the putative cytoplasmic domains of the receptor or through the proposed adapter functions of \( \beta \)-arrestin that may bring in other proteins important for desensitization of the \( \kappa \) opioid receptor. In conclusion, the results show that GRKs and \( \beta \)-arrestin 2 are required for homologous agonist-induced desensitization of the \( \kappa \) opioid receptor expressed in the Xenopus oocyte and that the C-terminal tail of the rat \( \kappa \) receptor is required for this regulation. These results taken together with previously published findings support a role for GRKs and \( \beta \)-arrestin in the mechanism underlying the development of tolerance to opioids.

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