μ and δ Opioid Receptors Are Differentially Desensitized by the Coexpression of β-Adrenergic Receptor Kinase 2 and β-Arrestin 2 in Xenopus Oocytes*

(Received for publication, May 15, 1997, and in revised form, July 8, 1997)

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The Xenopus oocyte expression system was used to test the hypothesis that homologous opioid receptor desensitization results from receptor phosphorylation by G protein-coupled receptor kinases. Activation of δ (DOR), μ (MOR), or β₂-adrenergic receptors increased K⁺ conductance in oocytes coexpressing the G protein-gated inwardly rectifying K⁺ channel subunits GIRK1 and GIRK4, and the intrinsic rate of desensitization was small. Coexpression of β-adrenergic receptor kinase 2 (β₂-ARK2) and β-arrestin 2 (β-arrestin2) synergistically produced a rapid desensitization of both DOR and β₂-adrenergic receptor signaling with a t½ < 4 min. β₂-ARK2 and β-arrestin2 more slowly desensitized MOR responses; a similar synergistic effect on MOR required 2–3 h of agonist treatment. DOR mutants lacking serine and threonine residues at the end of the cytoplasmic tail coupled effectively to GIRK channels but were insensitive to β₂-ARK2 and β-arrestin2. However, a DOR mutant having serine residues mutated to alanine in the third cytoplasmic loop was indistinguishable in coupling and desensitization from the wild type DOR. These studies establish that opioid receptors can be regulated by β₂-ARK2 and β-arrestin2 and that a portion of the COOH terminus of DOR enhances sensitivity to this modulation.

Opioid agonists are the drugs of choice in the management of severe and chronic pain; however, continuous use of opioids can cause undesirable side effects including drug tolerance and addiction. The processes underlying tolerance are complex and involve learning mechanisms, compensatory changes in neuronal circuits, and desensitization of signal transduction mechanisms (1). Three classes of opioid receptors: μ, δ, and κ, have been defined pharmacologically, shown to couple to G proteins of the Gᵡ, family, and cloned (2, 3). Previous studies have examined the molecular mechanisms underlying opiate tolerance. Chronic treatment of animals with opiates has not revealed a functionally significant change in opioid receptor number (4, 5) or mRNA levels (Ref. 6, but see also Refs. 7 and 8). However, opioid receptor desensitization has been consistently implicated as one of the molecular events accompanying tolerance (9). Continuous infusion of guinea pigs or rats with morphine results in an uncoupling of μ opioid receptors from associated G proteins as measured biochemically (10, 11). Recordings from single rat brain neurons of the locus coeruleus show a homologous desensitization to opioid-induced increases in potassium conductance after chronic morphine treatment (12). Prolonged morphine administration reduced the ability of DAMGO,1 a selective μ opioid agonist, to stimulate [(35)S]GTPγS binding in distinct rat brain areas (13). Chronic morphine treatment of rats also led to a homologous (e.g., receptor-specific) desensitization of either δ or μ opioid receptor activity as measured by the ability of specific agonists to inhibit adenyl cyclase in several brain structures (14).

Termination of signaling of other G protein-coupled receptors results from agonist-dependent phosphorylation by G protein-coupled receptor kinases and subsequent binding of arrestins (15–17). Data implicating similar mechanisms in opioid receptor desensitization have emerged. G protein-coupled receptor kinase levels are increased in rat locus coeruleus after morphine administration (18). A dominant negative mutant of β₂-adrenergic receptor kinase 1 (β₂-ARK1, a member of the G protein-coupled receptor kinase family, blocks κ opioid desensitization in COS-7 cells (19). In addition, agonist-dependent phosphorylation of opioid receptors accompanies desensitization (20–22). Agonist-dependent desensitization and phosphorylation of the δ opioid receptor was enhanced by overexpression of β₂-ARK1 (20); however, the effect of other G protein-coupled receptor kinases on δ opioid receptor desensitization, the contribution of arrestins, and the regions of the δ opioid receptor involved in receptor uncoupling remains to be elucidated.

To further characterize the molecular mechanisms underlying opioid receptor desensitization, we studied the regulation of the coupling between opioid receptors and potassium channels coexpressed in Xenopus oocytes. In many neuronal types, stimulation of opioid receptors activates G protein-gated inwardly rectifying potassium channel (GIRK channel) conductance (23). Previously, we showed that the δ, μ, and κ opioid receptors functionally couple to a GIRK conductance in Xenopus oocytes injected with cRNA for the receptor and the GIRK1 channel subunit (24–26). We found that sustained agonist exposure caused the channel response to desensitize. Attenuation of the response was heterologous, since it resulted from changes in coupling downstream of the receptor (25), which hindered our studies investigating homologous receptor desensitization.

*This work was supported by U.S. Public Health Service Grant DA04123 from the National Institute on Drug Abuse and by a grant from the Ministère de la Recherche et de la Technologie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: DAMGO, [d-Ala²,methyl-Phe⁴, glycyl⁷]enkephalin; DOR, δ opioid receptor; DOR TT, DOR tail truncation mutant; DOR TS, DOR tail substitution mutant; DOR LS, DOR loop substitution mutant; MOR, μ opioid receptor; β₂-AR, β₂-adrenergic receptor; β₁-AR1 and β₁-AR2, β₂-adrenergic receptor kinase 1 and 2, respectively; β₂-arr2, β-arrestin 2; DPDPE, [n-penicillamine-2,5]enkephalin; GIRK channel, G-protein gated inwardly rectifying potassium channel; GTPγS, guanosine 5‘-3-O-(thio)triphosphate.
mechanisms. We now report that in Xenopus oocytes coexpressing the GIRK4 subunit in addition to GIRK1, little desensitization of opioid receptor-activated GIRK conductance was observed.

Mammalian cell lines endogenously express many proteins involved in receptor desensitization and down-regulation including members of the G protein-coupled receptor kinase family, arrestins, clathrin, protein kinase A, and protein kinase C. Reconstitution experiments in cell lines performed while investigating opioid receptor desensitization are complicated by rapid receptor sequestration and down-regulation (26). In this study, we exploit the absence of endogenous opioid receptor desensitization in Xenopus oocytes coexpressing the GIRK1 and GIRK4 channel subunits to examine the effect of exogenously expressed β-adrenergic receptor kinase 2 (β-ARK2) and β-arrestin 2 (β-arrr2) on μ and δ opioid receptor activity.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—DPDPE and DAMGO were from Peninsula Laboratories. Naloxone was from Research Biochemicals International. All other chemicals were from Sigma.

**Oocyte Preparation**—The mouse DOR cDNA (28) was subcloned into HindIII/BamHI sites of pCDNAI/Amp (Invitrogen). Construction of DOR LS (S242A, S247A, S249A, S255A) and DOR TS (T352A, T353A, T358A, T361A, S363A) mutants was performed as described previously for the construction of a different mutant DOR (29), except that mixtures of oligonucleotides were annealed to single-stranded templates to generate multiple mutations. Mutagenic oligonucleotides were as follows: GCCTGGGGCGCTGCGTC (S242A), CTGCTGGGCCGTGACCA (S247A, S249A), AGGACCGCGCCCTGCCG (S255A), CCAGGGCCGGCCGGTGA (T352A, T353A), GAGGCTGCTGGCGCTGTC (S358A), and GCGGGGCGCCGGGACG (T361A, S363A). The 3′ tail truncation mutant DOR TT encodes the mouse DOR receptor with a 33-amino acid COOH-terminal deletion and was constructed by excision of an NotI fragment encompassing part of the 3′-coding sequence as well as the entire 3′ noncoding region.

**Complementary DNA Clones and cRNA Synthesis**—The rat μ opioid receptor clone obtained from Dr. Lei Yu (GenBankTM accession number L13069) was put into the pCS2+ vector such that the SP6 promoter directed sense transcripts. Mu opioid receptor cDNA template was also generated using polymerase chain reaction as described previously (25). Gαq and Gβδ were that described by Graziano et al. (30). GIRK4 (clone provided by Dr. John Adelman, GenBankTM accession number X83584) and β-arrr2 cDNA (clone provided by Dr. Robert Lefkowitz, GenBankTM accession number M191590) was first amplified by the utilization of Amplitaq DNA Polymerase (Perkin-Elmer) in a standard polymerase chain reaction using oligonucleotides designed to add a BamHI cloning site to the 5′ end and a 45-base poly(A) tail. The rat β-ARK2 cDNA was provided by Dr. Shaun Coughlin (31). Plasmid templates for all constructs including DOR and DOR mutants were linearized prior to cRNA synthesis, and mMESSAGE MACHINE kits (Ambion Corp.) were used to generate capped cRNA.

**Oocyte Culture and Injection**—Oocytes were prepared as described (25) and were incubated for 3–7 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). See below for the composition of the recording buffer. cRNA was injected (50 nl/oocyte) with a Drummond microinjector.

**Electrophysiology**—Oocytes were clamped at −80 mV with two electrodes filled with 3 mM KCl having resistances of 0.5–1.5 MΩ, using a Geneclamp 500 amplifier and pCLAMP 6 software (Axon Instruments). All data were digitally recorded (Digidata, Axon Instruments, and Intel 386PC) and filtered. Membrane current traces were also recorded using a chart recorder. To enable inward K⁺ currents to flow through the GIRK channels, the normal oocyte saline buffer was modified to increase KCl concentration to the K⁺ concentration values described under “Results.” The concentration of NaCl was correspondingly decreased to maintain isosmolarity.

**Statistical Analysis**—We used Student’s test for comparison of the independent means. Values given are two-tailed p values. Data from the dose response experiments were fitted to a simple Emax model using the nonlinear regression analysis package PCNONLIN version 4.2 (SCI Software) to determine the EC₅₀ and 95% confidence intervals.

**RESULTS**

Previously we reported that DAMGO, a selective μ opioid agonist, elicited a GIRK conductance in Xenopus oocytes injected with cRNA for the μ opioid receptor (MOR) and the GIRK channel subunit GIRK1 (25). In those studies, the expression of functional GIRK channels was dependent upon the formation of heteromultimers between the enzogenously expressed GIRK1 subunit and the endogenously expressed Xenopus GIRK4 homologue, GIRK5 (32). As shown previously, the total potassium current flowing through the GIRK channels in those oocytes was relatively small (Fig. 1A, Ref. 25) since the endogenously expressed levels of GIRK5 limited the expression of functional GIRK channels. The modest expression of GIRK channels required that the configuration of the channel and the electrochemical driving force be enhanced by perfusing the cell with a high K⁺ saline buffer containing 96 mM K⁺ (Fig. 1A, Ref. 25). Furthermore, to measure MOR-GIRK coupling reliably, it was necessary to inject oocytes with high levels of cRNA for MOR (1 ng) and the GIRK1 channel subunit (1–2 ng) (25). Sustained agonist exposure led to a slow reduction in the

![Fig. 1. Heterologous desensitization of MOR-GIRK coupling is abolished by the expression of GIRK1-GIRK4 heteromultimeric GIRK channels.](image-url)
evoked current (Fig. 1A) that was previously shown to be a result of changes occurring downstream of the receptor (25). This heterologous desensitization limited the utility of this system in investigating homologous receptor desensitization mechanisms.

The expression of functional GIRK channels was greatly enhanced by the coexpression of both GIRK1 and GIRK4 channel subunits that have been shown to assemble as heteromultimers (33). In *Xenopus* oocytes cojected with cRNA for both GIRK1 and GIRK4 channel subunits (0.02 ng each), large inward currents were observed even in lower K⁺-containing solutions (Fig. 1B). The μ-opioid agonist, DAMGO, elicited a substantial activation of GIRK conductance in oocytes injected with as little as 0.02 ng of MOR cRNA. Under these conditions, prolonged MOR activation by DAMGO produced no desensitization of the inward current (Fig. 1B). The amplitudes of the MOR-activated inward currents could be varied over a large range (50–1500 nA in 16 mM K⁺ recording buffer) by injecting oocytes with various amounts of MOR cRNA (0.005–0.2 ng). No significant acute desensitization of the receptor-elicited GIRK current was observed across this large range of MOR functional expression (data not shown). The absence of desensitization of the receptor-elicited GIRK responses allowed us to characterize the effect of exogenously expressed β-ARK2 and β-arr2 on receptor activity.

In our investigation of the modulation of opioid receptor signaling by β-ARK2 and β-arr2, we tested for the functional expression of the β-ARK2 and β-arr2 combination by coexpressing the β₂-adrenergic receptor (β₂-AR) in the same cells. In addition to activation by Gₛ/Gₛ- coupled receptors, GIRK channels in *Xenopus* oocytes can also be activated by the Gₛ-coupled β₂-AR but only in those cells cojected with cRNA for Gₛ (34). Therefore, control oocytes were injected with a mixture of cRNA for β₂-AR, Gₛ-δ opioid receptor (DOR), MOR, GIRK1, and GIRK4. In the control oocytes, application of 1 μM isoproterenol evoked a GIRK current that decayed at a rate of about 8% of the peak response/min (Fig. 2). Since phosphorylation of the β₂-AR by protein kinase A inhibits coupling to Gₛ, and causes desensitization of the receptor (35), the slow desensitization of the response to isoproterenol possibly reflects an effect of the activation of the endogenous oocyte cAMP system by the activated β₂-AR. The coexpression of either β-arr2 or β-ARK2 alone did not significantly increase the rate of response desensitization compared with control. As predicted (see Refs. 15–17), coexpression of both β-ARK2 and β-arr2 increased the rate of β₂-AR desensitization by more than 2-fold and suggested functional expression of β-ARK2 and β-arr2 following the injection of their cRNA transcripts. DOR signaling was desensitized in an even more dramatic manner by the combination of β-ARK2 and β-arr2 (Fig. 2). Injection of cRNA for either β-ARK2 or β-arr2 alone (along with receptor and channel) did not produce this rapid desensitization.

The increased rate of desensitization of β₂-AR and DOR signaling caused by the coexpression of β-ARK2 and β-arr2 was not produced by any changes in functional receptor expression. In the example shown (Fig. 2), the mean peak isoproterenol-induced inward current was 195 ± 13 nA (± S.E.) in the control oocytes and 225 ± 22 nA in cells injected with β-ARK2 and β-arr2 cRNA. The peak response of control oocytes to δ receptor activation could be varied by changing the amount of DOR cRNA injected and also differed from oocyte batch to batch (50–1500 nA). However, the normalized DOR desensitization rate of control oocytes never exceeded 8%/min. The normalized desensitization rate of DOR in oocytes containing functional β-ARK2 and β-arr2 was always significantly greater than 8%/min (p < 0.001).

**FIG. 2.** Effect of β-ARK2 and β-arr2 on the acute response desensitization of the β₂-AR, DOR, and MOR. Control oocytes were injected with a mixture of the following cRNAs: 0.05 ng of β₂-AR, 1 ng of Gₛ, 0.02 ng of MOR, 0.1 ng of DOR, 0.02 ng of GIRK1, and 0.02 ng of GIRK4. The inset (circle) summarizes the cRNA injected into the control oocytes; the GIRK1-GIRK4 complex is represented as GIRK. Some of the control oocytes were also injected with 0.5 ng of β-ARK2, 1 ng of β-arr2, or both. All recordings were performed 3–4 days postinjection, and responses were measured in 16 mM K⁺ buffer. Note that the short vertical lines through the traces indicate when agonist treatment was discontinued and antagonist perfusion began. Responses were adjusted by base-line subtraction as described in the legend to Fig. 1. A, representative traces depicting base line-subtracted responses to 1 μM isoproterenol (ISO), 40 nM DPDPE, or 40 nM DAMGO that were recorded for at least 4 min. To determine the amount of response desensitization, agonist treatment was followed by antagonist perfusion. Isoproterenol-elicited responses were reversed with 1 μM propranolol, whereas DPDPE and DAMGO treatments were followed with 1 μM naloxone. Calibration scales are the same for each trace: 100 nA, 2 min. B, comparison of the normalized desensitization rates of the drug responses in the different oocyte groups. The normalized desensitization rate is the change in response/min expressed as a percentage of the peak response. The data summarize the results of three separate experiments with oocytes obtained from three different donors; once a response was recorded, the oocyte was not used again. Each bar represents the mean ± S.E. calculated from 4–9 separate oocytes (†, p < 0.01; ††, p < 0.001 compared with the corresponding controls).

Strikingly, this combination of β-ARK2 and β-arr2 was much less effective in desensitizing MOR signaling acutely (Fig. 2). After 5 min of DAMGO application (40 nM, the EC₅₀ value), MOR desensitization in oocytes expressing β-ARK2 and β-arr2 was not significantly different from control (Fig. 2B). The differing sensitivities of MOR and DOR to β-ARK2 and β-arr2 modulation was not explained by differences in receptor expression. We examined the desensitization rate of the DAMGO response in β-ARK2- and β-arr2-expressing oocytes that had been injected with different amounts of MOR cRNA (0.02–0.64 ng/oocyte). Using the gene expression conditions described, the peak amplitude of the agonist-induced response was limited by the levels of receptor, not channel, expression. Over a wide
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A

First Cytoplasmic Loop
DOR: R Y T K K T A T N I Y 87
MOR: R Y T K K T A T N I Y 106

Second Cytoplasmic Loop
DOR: C H P V K A L D F R T P A 163
MOR: C H P V K A L D F R T P A 182

Third Cytoplasmic Loop
DOR: L M L R L R L S V R L S G S K E K D R S L L R T I R M 262
MOR: L M L R L R L S V R L S G S K E K D R S L L R T I R M 281

Carboxy-terminal Tail
DOR: Y A F L D E N F K R C F R O L C R T P C G R O B P G S L 345
MOR: Y A F L D E N F K R C F R E F C T P S S T I D Q O N S 363

B

Fig. 3. Comparison of the amino acid sequence of the predicted first, second, and third cytoplasmic loops and the carboxy-terminal tail of the δ and μ opioid receptors and diagram of the DOR mutants tested. A, the sequences of the mouse δ and the rat μ opioid receptors are shown using the single-letter amino acid abbreviations. Residues that are different between the two proteins are boxed. Gaps used to generate the alignment are represented by dashes. The arrow after Arg335 represents the point of truncation of the mutant DOR construct DOR TT. In the loop-substituted mutant DOR LS, the four serine residues in the third cytoplasmic loop that were mutated to alanine are outlined. In the tail-substituted mutant DOR TS, the outlined residues in the carboxy-terminal tail were substituted with alanine. B, circles indicate the locations of cytoplasmic serines and threonines; each × indicates an alanine substitution.

range of MOR expression (peak DAMGO elicited currents ranging from 50 to 2000 nA), the normalized desensitization rate calculated after 5–10 min of DAMGO perfusion in β-ARK2- and β-arr2-expressing oocytes was never significantly different from control (data not shown). The amount of desensitization evident after 5–10 min of treatment with a higher DAMGO concentration (1 μM) was also not significantly different from control (data not shown). These results indicate that the intrinsic molecular properties of DOR confer a greater sensitivity than MOR to modulation by β-ARK2 and β-arr2.

The putative first, second, and third intracellular loops of MOR and DOR are nearly identical in their primary structure (Fig. 3). However, the amino acid sequences diverge considerably in the cytoplasmic carboxyl-terminal tail. Agonist-dependent desensitization of G protein-coupled receptors mediated by the G protein-coupled receptor kinases follows from the phosphorylation of serine and threonine residues in either the third cytoplasmic loop or the carboxy-terminal tail (15–17). Consequently, we tested a truncated DOR construct in which all residues beyond Arg339 in the carboxy-terminal tail were deleted (DOR TT). Similar to the wild type DOR, this construct coupled efficiently to GIRK. However, agonist-dependent desensitization upon coexpression of β-ARK2 and β-arr2 was significantly less than wild type DOR and was similar to the desensitization rate of MOR (Fig. 4). To confirm that the rapid desensitization of DOR followed from phosphorylation of specific threonine and serine residues in the carboxy-terminal tail, a mutant DOR was constructed in which the five serine and threonine phosphorylation sites at the end of the tail were substituted with alanines (DOR TS) (Fig. 3B). The desensitization rate for DOR TS in the presence of β-ARK2 and β-arr2 was also markedly reduced (Fig. 4).

Receptor kinase-mediated desensitization of the Gαi-coupled m2-muscarinic acetylcholine receptor and the α2-adrenergic receptor is thought to occur after phosphorylation of serine and threonine residues in the third cytoplasmic loop (36–38). The desensitization rate of the DOR LS mutant, however, in which all of the serines in the third cytoplasmic loop were mutated to alanines was not measurably different from that of wild type DOR (Fig. 4).

The DOR mutants tested were not different from wild type DOR in their ability to activate GIRK. DPDPE dose response curves were generated for each of the δ receptor mutants expressed as described in Fig. 4. Control oocytes (injected with 0.1 ng of receptor mRNA but lacking β-ARK2 and β-arr2) were exposed to cumulatively increasing doses of DPDPE in 16 mM K+ recording buffer. The EC50 of DPDPE for the wild type receptor was 4.2 nM (3.4–4.9 nM), and the EC50 was 1.6 nM (1.2–2.0 nM) for DOR TS, 3.3 nM (2.8–3.9 nM) for DOR TT, and 2.7 nM (2.0–3.4 nM) for the DOR TS mutant. Numbers in parentheses indicate the 95% confidence intervals (n = 3 independent measures). The mean (± S.E.) peak responses measured at 32 nM DPDPE for wild type DOR, DOR LS, DOR TT, and DOR TS were 863 ± 51, 813 ± 81, 700 ± 185, and 986 ± 86 nA, respectively.

Whereas the desensitization of MOR-GIRK coupling produced by coexpression of β-ARK2 and β-arr2 after treatment with DAMGO for 4 min was small (Fig. 2), prolonged agonist treatment revealed a more dramatic effect of these modulatory proteins on MOR coupling (Fig. 5). After 2–3 h of exposure to 40 nM DAMGO, the agonist was washed off by perfusion with normal oocyte saline for 5 min, and the response to DAMGO was tested. In control oocytes, expressing neither β-ARK2 nor β-arr2, pretreatment with 40 nM DAMGO did not affect the subsequent MOR response as compared with untreated oocytes (Fig. 5B and C). However, in cells coexpressing both β-ARK2 and β-arr2, DAMGO pretreatment almost completely abolished the subsequent response to MOR activation. Again, cells expressing either β-ARK2 or β-arr2 alone did not demonstrate a significant agonist-dependent MOR desensitization (Fig. 5B).

The desensitization of the response to MOR activation by DAMGO was homologous in β-ARK2- and β-arr2-coexpressing cells; in experiments in which oocytes were co-injected with DOR cRNA, subsequent DPDPE activation of DOR was not altered by prolonged DAMGO exposure (Fig. 5A and C).
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**DISCUSSION**

The principal findings of this study are 3-fold. First, we have directly demonstrated that β-ARK and β-arr2 were both required to produce homologous desensitization of the opioid receptors. Second, δ receptors may be more sensitive than μ receptors to β-ARK2 and β-arr2 modulation. Third, we have identified particular residues in the COOH terminus of the δ receptor that confer increased sensitivity to phosphorylation by β-ARK2. The coexpression of GIRK1 and GIRK4 improved the sensitivity of the system and allowed us to observe receptor-d GIRK coupling without heterologous desensitization. We exploited the lack of desensitization to demonstrate that exogenous β-ARK and β-arr act synergistically to desensitize δ and μ opioid receptors differentially.

Previously, we reported that for cells in which GIRK channel expression is greatly limiting, as in oocytes injected with cRNA for only the GIRK1 channel subunit, opioid receptor-elicited GIRK currents desensitize heterologously (25). Subsequently, a mechanism for the observed heterologous desensitization was proposed by Schreibmayer and co-workers, who demonstrated that activated G_{i1}1, but not G_{i2}2 or G_{i3}3, potently inhibited GIRK1-induced GIRK activity in excised membrane patches of *Xenopus* oocytes expressing GIRK1 (39). In addition, antisense to *Xenopus* G_{i1}1 injected into oocytes attenuated the heterologous desensitization of muscarinic receptor (39) and opioid

**Fig. 4.** Effect of DOR mutations on β-ARK2- and β-arr2-facilitated desensitization. Control oocytes were injected with 0.1 ng of wild type DOR, DOR LS, DOR TT, or DOR TS and with 0.02 ng each of GIRK1 and GIRK4. Some of the control oocytes were also injected with 0.5 ng of β-ARK2 and 1 ng of β-arr2. A, representative traces depicting base line-subtracted responses to 40 nM DPDPE (horizontal line) were followed for at least 4 min. To quantify the amount of response desensitization, DPDPE treatment was followed with 1 μM naloxone. The short vertical bars through the current traces indicate the time agonist treatment was discontinued and antagonist perfusion began. All responses were measured in 24 mM K^+ buffer. Calibration scales are the same for each trace: 100 nA, 2 min. B, comparison of the desensitization rates of the different DOR mutants. The normalized desensitization rates expressed as a percentage of the peak response were calculated as described in the Fig. 1 legend. The desensitization rates represent a summary of measurements collected from three separate experiments performed on oocytes from three separate donors. All values are means ± S.E. calculated from at least six oocytes (*, p < 0.001 compared with control; **, p < 0.001 compared with wild type DOR).

**Fig. 5.** Effect of β-ARK2 and β-arr2 on the response of the μ opioid receptor and the δ opioid receptor to prolonged treatment with the μ opioid agonist DAMGO. Control oocytes were injected with the following cRNAs: 0.02 ng of MOR, 0.1 ng of DOR, 0.02 ng of GIRK1, and 0.02 ng of GIRK4. Some of the control oocytes were injected with cRNA for β-arr2 (1 ng), β-ARK2 (0.5 ng), or both. All experiments were performed 3–4 days postinjection. Oocytes from each group were incubated in separate Petri dishes for 2–3 h with 40 nM DAMGO in normal oocyte saline buffer or in saline buffer alone. Oocytes were then placed in the recording chamber, and DAMGO was washed off with saline buffer perfused at a flow rate of 5 ml/min for 5 min. Oocyte membrane potential was then clamped at ~80 mV, and the recording chamber was equilibrated with 16 mM K^+ buffer. To test for washout of DAMGO, a few of the DAMGO-pretreated oocytes were directly perfused with naloxone, which produced no change in the basal GIRK activity (data not shown). After equilibration of the chamber with the 16 mM K^+ buffer, either 40 nM DAMGO or 40 nM DPDPE was applied to generate a peak response followed immediately by the application of 1 μM naloxone. The recovery to baseline after the peak agonist response was caused by antagonist treatment, not desensitization. The short vertical bars through the current traces indicate the time agonist treatment was discontinued and antagonist perfusion began. Calibration scales are the same for each trace: 100 nA, 1 min. Note that the *horizontal scale* has been expanded compared with the previous figures. Once a peak response was measured, the oocyte was not used again. A, representative responses to DAMGO and DPDPE in the different oocyte groups. B, summary of the peak responses obtained in a single experiment from one donor. Values are expressed as means ± S.E., n = 4. C, the peak response to DAMGO or DPDPE in an oocyte that was pretreated with DAMGO was expressed as a percentage of the mean response to DAMGO or DPDPE, respectively, of the identical oocyte group that was not pretreated with DAMGO. The graph presents a summary of the percentage response remaining calculated with data from four separate donors. All values are means ± S.E. from 4–15 oocytes (*, p < 0.05; ***, p < 0.0001 compared with the corresponding controls).
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receptor–GIRK coupling. These results may suggest that in neurons expressing sufficient quantities of $G_{\alpha}$, the activation of this G protein isoform by opioid receptors could lead to a heterologous desensitization of the GIRK response. Similar mechanisms may be responsible for the heterologous desensitization of opioid receptors observed in vivo (14, 40).

The reasons for abolition of the heterologous desensitization of receptor–GIRK coupling by coexpression of GIRK1 and GIRK4 subunits in the oocyte have not been established. It may be due to an inability of $G_{\alpha}$, 1 to inhibit the GIRK1–GIRK4 channel complex. Alternatively, the levels of endogenously expressed $G_{\alpha}$, 1 may be unable to significantly affect the greatly enhanced expression of functional GIRK channel complexes. Additionally, when GIRK expression is low, as in oocytes injected with only the GIRK1 channel subunit, the high external K$^+$ concentration required for reliably measuring GIRK activity itself strongly inhibited GIRK channels (25). In contrast, the elevated GIRK expression in GIRK1- and GIRK4-injected oocytes allowed opioid receptor activity to be reliably measured in low K$^+$-containing external buffer and monitored for an extensive period without a large rundown.

The membrane-delimited activation of GIRK channels by G protein-coupled receptors is thought to be mediated by $G_{\beta\gamma}$ dimers, probably by direct binding to the channel protein (41, 42). $\beta$-ARK1 and $\beta$-ARK2 interact with $G_{\beta\gamma}$, and a fusion protein constructed with a portion of $\beta$-ARK1 was shown to inhibit basal GIRK activity when applied to membrane patches (41). Therefore, $\beta$-ARK2 could potentially desensitize receptor-elicited GIRK responses by sequestering $G_{\beta\gamma}$. However, the $\beta$-ARK2- and $\alpha$-2-medicated receptor uncoupling reported here is likely to be a result of catalytic phosphorylation of the receptor by $\beta$-ARK2 rather than a sequestration of $G_{\beta\gamma}$ subunits. First, desensitization of the receptor response required the concomitant expression of $\beta$-arrestin. Second, the expressed levels of $\beta$-ARK2 did not significantly inhibit the basal GIRK activity. The observed homologous desensitization observed with coinjected MOR and DOR indicated that the desensitization was the result of actions localized to the receptor rather than through a common postreceptor event such as $G_{\beta\gamma}$ sequestration. Finally, the $\beta$-ARK2- and $\alpha$-2-mediated desensitization of DOR was abolished by specific point mutations of serine and threonine phosphorylation sites in the COOH-terminal tail.

For other $G_{i}$/$G_{o}$-coupled receptors such as the m2-muscarinic acetylcholine receptor and the $\alpha_{2}$-adrenergic receptor, agonist-dependent desensitization by the G protein-coupled receptor kinases has been shown to follow from the phosphorylation of serine and threonine amino acids in the third cytoplasmic loop (36–38). Therefore, our data mapping the $\beta$-ARK2- and $\alpha$-2-mediated desensitization of the $G_{i}$-$G_{o}$-coupled $\delta$ opioid receptor to the last five serines and threonines in the COOH-terminal tail provides an example of a $G_{i}$-$G_{o}$-coupled receptor that is regulated differently. The importance of the COOH terminus in $\mu$ opioid receptor modulation is also suggested by Zimprich and co-workers (43), who report that an alternatively spliced variant of the $\mu$ opioid receptor that differs from MOR only in the amino acid composition of the very COOH-terminal tail is much more resistant to agonist-induced desensitization. It is not clear why the COOH-terminal tail of the MOR should confer greater sensitivity to modulation by $\beta$-ARK2 and $\beta$-arr2. Two possibilities are that either the COOH-terminal tail of DOR is a better substrate for $\beta$-ARK2 or that $\beta$-arr2 has a higher affinity for the phosphorylated DOR.

Recent experiments suggest that in addition to uncoupling the $\beta$-AR from G proteins, phosphorylation of the $\beta$-AR by receptor kinases followed by $\beta$-arrestin binding is also important for receptor internalization (44, 45). Traapайдзе et al. (46) report that the serine and threonine residues in the COOH-terminal tail of the $\delta$ opioid receptor are important for agonist-mediated internalization when transfected into CHO cells. Hence, our results support the hypothesis that phosphorylation by $\beta$-ARK and binding of $\beta$-arr may be important for the internalization of $\delta$ opioid receptors.

The concept that $\mu$ and $\delta$ opioid receptors may be regulated differently has precedence in vivo. Noble and Cox (14) report that in the rat nucleus accumbens and caudate putamen, chronic morphine treatment resulted in a desensitization of $\delta$ but not $\mu$ opioid receptors as measured by the ability of specific agonists to inhibit adenylyl cyclase activity. Our data demonstrate that $\delta$ opioid receptors are much more sensitive to regulation by $\beta$-ARK2 and $\beta$-arr2. Therefore, one hypothesis is that neurons in the above regions express low levels of $\beta$-ARK2 and $\beta$-arr2 or related proteins and may explain the selective desensitization of $\delta$ responses in these regions. The $\mu$ opioid receptor-activated potassium conductance in the locus coeruleus desensitizes homologously such that 70% of the response decayed after 5 min of treatment with high concentrations of agonist (47). The rapid homologous desensitization of the $\delta$ opioid receptor response in these cells may reflect an expression of a G protein-coupled receptor kinase specific for the $\mu$ opioid receptor. Thus, Xenopus oocytes provide an accessible system to characterize the molecular mechanisms of homologous opioid receptor desensitization following prolonged agonist exposure, which may be used to elucidate the role of receptor desensitization in tolerance phenomenon in vivo.

Acknowledgments—We thank Jeremy Celver and Dr. Doug Henry for assistance. We thank Dr. Robert Lefkowitz for the $\beta$-arr2 clone and for permission to use the $\beta$-ARK2 clone; Dr. Shaun Coughlin for the rat $\beta$-ARK2 clone; Dr. John Adelman for GIRK 4 cDNA; Dr. Lei Yu for the rat MOR cDNA, and Drs. Henry Lester and Cesar Lebarca for the GIRK1 and $\beta$-ARK-Clones.

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