Receptor desensitization by G-protein receptor kinases (GRK) and arrestins is likely to be an important component underlying the development of tolerance to opioid drugs. Reconstitution of this process in Xenopus oocytes revealed distinct differences in the kinetics of GRK and arrestin regulation of the closely related opioid receptors μ (MOR), δ (DOR), and κ (KOR). We demonstrated that under identical conditions, GRK and arrestin-dependent desensitization of MOR proceeds dramatically slower than that of DOR. Furthermore, GRK3 phosphorylation sites required for opioid receptor desensitization also greatly differ. The determinants for DOR and KOR desensitization reside in the carboxy-terminal tail, whereas MOR depends on Thr-180 in the second intracellular loop. Although this later finding might indicate an inefficient phosphorylation of MOR Thr-180, increasing the amount of arrestin expressed greatly increased the rate of MOR desensitization to a rate comparable with that of DOR. Similarly, coexpression of a constitutively active arrestin 2 (R169E) with MOR and DOR desensitized both receptors in an agonist-dependent, GRK-independent manner at rates that were indistinguishable. Together, these data suggest that it is the activation of arrestin, rather than its binding, that is the rate-limiting step in MOR desensitization. In addition, mutation of Thr-161 in DOR, homologous to MOR Thr-180, significantly inhibited the faster desensitization of DOR. These results suggest that DOR desensitization involves phosphorylation of both the carboxy-terminal tail and the second intracellular loop that together leads to a more efficient activation of arrestin and thus faster desensitization.

Opiates are clinically used for the treatment of pain act through opioid receptors that are members of the superfamily of G-protein-coupled receptors (GPCR). Although opioid receptors share only 30% homology with other GPCRs, the three subtypes, μ (MOR), δ (DOR), and κ (KOR), share 65–75% homology with each other, and the opioid receptors differ most in the extracellular domains responsible for ligand binding and the more distal regions of their carboxyl-terminal tails (1). As with many other GPCRs, prolonged agonist treatment leads to a reduction in effector response. Such tolerance has been attributed to decreased receptor function, receptor down-regulation, and compensatory changes in gene expression (2). One of the leading molecular mechanisms underlying this tolerance is believed to be G-protein uncoupling of the receptor due to G-protein receptor kinase (GRK) phosphorylation and arrestin binding.

The proposed model of GPCR desensitization, initially determined for the β2-adrenergic receptor, involves first agonist activation of the receptor stimulating GTP binding to and activation of the G-protein. The activated G-protein dissociates into the Gα and Gβγ subunits that can then go on to activate multiple effectors, including the inward rectifying potassium channel (K\(_r\)3) that is stimulated by Gβγ binding following opioid receptor activation (3–5). The Gβγ subunit also recruits GRK to the membrane where it can phosphorylate the agonist-occupied receptor (6–8). The negative charge of the phosphate group is believed to induce a conformational change in arrestin, thereby exposing an additional receptor-binding site (9, 10). The activated arrestin can then bind to the receptor sterically interfering with further G-protein coupling to the receptor, effectively attenuating receptor signaling. Arrestin can also serve as an adapter linking the receptor to adaptin AP2 and other components of the clathrin-mediated internalization machinery (11). The internalized receptor can then either be recycled back to the plasma membrane as has been shown for MOR (12, 13) or targeted to lysosomes for degradation as has been shown for DOR (14). We reported previously (15) that reconstitution of this system in Xenopus oocytes requires exogenous expression of both GRK3 and β-arrestin to produce homologous desensitization of both DOR and MOR, although MOR required prolonged agonist treatment to show a significant effect. In this study, we examined this suggested difference in receptor regulation in greater detail by coexpressing MOR and DOR in the same oocytes with equal levels of GRK and β-arrestin expressed. Under identical conditions, we demonstrated that DOR desensitized within minutes of agonist exposure, whereas MOR desensitization required more than an hour of agonist treatment. Other expression studies in HEK-293 cells have also suggested that MOR is less responsive to arrestin-mediated desensitization than either DOR or KOR (16). In order to identify the step in the desensitization process that was contributing to this difference in rate, we manipulated the levels of GRK and arrestin protein. We further sought to identify the regions of the receptor responsible for the differential regulation by GRK and arrestin. For most GPCRs, the carboxy-terminal tail or third cytoplasmic loop has been implicated specifically in GRK and
arrestin-mediated desensitization. We showed previously (15) that the carboxyl-terminal tail was critical for DOR desensitization; however, elimination of all the potential phosphorylation sites in either the carboxyl-terminal tail or the third cytoplasmic loop had no effect on MOR desensitization in oocytes (17). Instead, we found that a single threonine in the second cytoplasmic loop was required for a GRK and arrestin effect (17). Because the sequence of this domain is nearly identical in MOR and DOR, we sought to determine whether this region was also important in DOR desensitization.

**EXPERIMENTAL PROCEDURES**

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

**Mutagenesis of DOR—**The mouse cDNA was subcloned into the HindIII/BamHI sites of pcDNA/Amp (Invitrogen). The DOR carboxyl-terminal tail was truncated at Arg-339 by excision of a sense or antisense oligonucleotide along with an oligonucleotide targeted to either the 3′ or 5′ end of the DOR cDNA, respectively. The DOR T161A sense oligonucleotide was as follows: cctggactttcgggcctggcaagcgagctg) introduced an SP6 transcriptional recognition site, whereas the 3′-oligonucleotide (ttaagccggggcgcccctggg) added a poly(A) tail. The resulting PCR product was subcloned into pGEM (Invitrogen), and the mutation was confirmed by sequencing.

**Complementary DNA Clones and cRNA Synthesis—**All cDNA clones used in this study were described previously (18, 19). Capped cRNA was generated from linearized plasmid templates for K3.1, K3.4, rat GRK3, and the truncated DOR TT using T7, T3, or SP6 mMessage Machine kits (Ambion, Austin, TX). The SP6 kit was used for RNA synthesis from PCR templates, with introduced SP6 promoter sites and poly(A) tails, of wild type DOR, DOR (T161A), rat MOR, bovine Arr3, wild type Arr2, and Arr2 (R169E).

**Oocyte Culture and Injection—**Defollicated stage IV oocytes were prepared as described previously (15). cRNA was injected (50 nl/oocyte) using a Drummond automatic microinjector. Oocytes were then incubated at 18 °C for 3–4 days in normal oocyte saline buffer (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 5 mM HEPES, pH 7.5) supplemented with sodium pyruvate (2.5 mM) and gentamycin (50 μg/ml).

**Electrophysiology—**Oocytes were clamped at −80 mV with two electrodes filled with 3 M KCl having resistance of 0.5–1.5 megohms using a GeneClamp 500 amplifier and pCLAMP6 software (Axon Instruments, Foster City, CA). Data were digitally recorded and filtered (Digitica 1200, Axon Instruments, and Intel 386 PC). Membrane current traces were also recorded using a chart recorder. To facilitate the inward potassium current flow through the K₃ channels, normal oocyte saline buffer (ND96) was modified to increase the KCl concentration to 16 mM, and the NaCl concentration was decreased correspondingly to maintain osmolality.

**Statistical Analysis—**Student’s t test (with two-tailed p values) was used for comparison of independent mean values. Dose-response curves were fitted to a simple Emax model using NFIT software (Island Products, Galveston, TX).

**RESULTS**

**Kinetics of GRK3 and Arr3-mediated Opioid Receptor Desensitization—**The two-electrode voltage clamp technique was used to measure inward potassium currents in oocytes expressing MOR, DOR, and both K₃ channel subunits K₃.3 and K₃.4. Either DAMGO activation of MOR or DPDPE activation of DOR led to an increase in the K₃ current, which remained stable under conditions where there were no spare opioid receptors. We have shown previously (15) that when the opioid response was limited by the amount of channel expressed (i.e. spare receptors present), the response diminished with prolonged agonist treatment but that this desensitization was mostly heterologous resulting from changes in signaling downstream of the receptor. Thus, to ensure that we measured homologous desensitization at the level of the receptor in these studies, the absence of spare receptors was confirmed by showing that doubling the amount of receptor cRNA injected also significantly increased the agonist response. For example, increasing DOR cRNA injected from 1 to 2 ng per oocyte increased the current activated by 500 nM DPDPE from 76 ± 16 to 151 ± 21 nA, and increasing MOR cRNA injected from 0.04 to 0.08 ng per oocyte increased the current activated by 1 μM DAMGO from 97 ± 17 to 200 ± 42 nA (n = 5 for each group). Because we sought to compare GRK and arrestin regulation of MOR and DOR, it was necessary to express them in the same oocytes such that they were exposed to equal levels of GRK and arrestin. Lower receptor expression also helped to maintain agonist specificity when both receptors were expressed, because at higher receptor levels an additive effect of nonspecific ligand interactions could produce a small response. In a separate group of oocytes expressing only channel and DOR at equal levels to the group expressing both MOR and DOR, a maximal dose of DAMGO (1 μM) was unable to increase the K₃ current. Similarly, a maximal dose of DPDPE (500 nM) did not potentiate the K₃ current in oocytes expressing only channel and MOR. Thus under the conditions used in these studies, the two opioid agonists retained their expected receptor selectivities.

As described previously, GRK3 and Arr3 coexpression led to a rapid decrease in DOR responsiveness upon continuous DPDPE activation (Fig. 1). MOR was also sensitive to GRK3...
and Arr3 coexpression, but the desensitization was significantly slower during DAMGO treatment (Fig. 1). Peak MOR responses were measured from oocytes expressing MOR, DOR, K_r3.1, K_r3.4, along with either GRK3, Arr3, or both GRK3 and Arr3. These oocytes were then treated with 1 \mu M DAMGO for 15, 30, or 45 min, followed by a 10-min wash in normal oocyte buffer, ND96. The MOR response to 1 \mu M DAMGO remaining after DAMGO treatment was again measured and compared with the initial response. Oocytes injected with only MOR, DOR, and K_r3 cRNA showed almost no desensitization after a 45-min treatment with DAMGO. Additional expression of only GRK3 or Arr3 did not significantly increase MOR desensitization rate, whereas simultaneous addition of both GRK3 and Arr3 enabled significant MOR desensitization with nearly 50% of the initial response desensitized after a 45-min DAMGO exposure (Fig. 1). DOR desensitization in the same group of oocytes, expressing identical amounts of GRK3 and Arr3, proceeded much more rapidly with less than 25% of the initial response remaining after only 8 min of DPDPDE treatment (Fig. 1).

Independent Function of Coexpressed DOR and MOR—Recent reports (1, 20) have suggested that the different classes of opioid receptors may heterodimerize to form a receptor complex that exhibits functional differences and a distinct pharmacological profile from the single receptor. To test for a possible functional interaction between MOR and DOR when were coexpressed in the same oocyte along with GRK3 and Arr3, we first confirmed that the desensitization rate difference between untreated and pretreated groups of oocytes. Error bars, means ± S.E. from 8 to 15 independent determinations. Right, maximal currents obtained with 2 \mu M DAMGO were not significantly different between untreated and pretreated groups of oocytes. Error bars, means ± S.E. from 15 independent determinations.

Desensitization of DOR and MOR by a Dominant Positive Arrestin—Overexpression of Arr3 was not able to fully overcome the discrepancy between MOR and DOR desensitization kinetics. Although Arr3 seemed to underlie the difference, there are believed to be two components to arrestin interaction with the receptor: first, activation by the receptor attached phosphates, and second, high affinity binding to the receptor. In order to elucidate which of these two steps might contribute to the slower regulation of MOR, we expressed MOR and DOR with a preactivated or constitutively active form of arrestin, Arr2(R169E). Arr2(R169E) has been shown previously to desensitize G-protein-coupled receptors, including both DOR and MOR, in an agonist-dependent but phosphorylation-independent manner (19). Because there has been recent evidence that Arr2 and Arr3 may distinguish certain classes of GPCRs (21), we first confirmed that the desensitization rate difference between MOR and DOR was also evident for wild type Arr2. In oocytes expressing MOR, DOR, GRK3, and Arr2, DAMGO treatment for 4 min was unable to elicit significant MOR desensitization, whereas DPDPDE treatment left less than 50% of the DOR response remaining (Fig. 4). Expression of the constitutively active Arr2(R169E) in the absence of GRK3 desensitized both MOR and DOR to similar levels after 4 min of respective agonist treatment (Fig. 4). These results strongly suggest that once activated, arrestin can bind to and inactivate...
either receptor equally as well. This conclusion further implies that MOR must be less efficient in its activation of arrestin, thereby leading to slower desensitization.

**Desensitization of DORs Lacking Potential GRK3 Phosphorylation Sites**—MOR and DOR contain highly homologous sequences in their cytoplasmic domains, with the most variance in the carboxyl-terminal tail. Although the carboxyl-terminal tail has been implicated as being crucial for desensitization of many GPCR including the DOR, we have shown previously (17) that in oocytes, deletion of the carboxyl-terminal tail of MOR does not affect GRK3 and Arr3-mediated desensitization. Instead, we found previously that a single threonine residue in the second intracellular loop, Thr-180, is required for such desensitization. Because MOR and DOR have nearly complete sequence identity in this putative second intracellular loop, we constructed a mutant DOR having an alanine substitution for the homologous residue Thr-161. As we have shown previously (15), truncation of the carboxyl-terminal tail at Arg-339 (DOR TT), which eliminates the potential serine and threonine phosphorylation sites in the tail domain, completely blocked GRK3 and Arr3-mediated desensitization (Fig. 5B). Coexpression of DOR T161A with GRK3 and Arr3 also blocked DOR desensitization but only partially. The amount of desensitization of DOR T161A produced by 4 min of agonist treatment was significantly less than that of DOR wild type, but also significantly more than that of oocytes expressing DOR Thr-161 in the absence of GRK3 and Arr3 (Fig. 5B). Thus, whereas the carboxyl-terminal tail of DOR was found to be critical for GRK3 and Arr3-mediated desensitization, Thr-161 also seemed to play an important role. Perhaps these two potential regions of phosphorylation work synergistically to allow for the rapid activation of arrestin and thus rapid desensitization of DOR. MOR, limited by a single phosphorylation site Thr-180, may be less efficient at activating arrestin, thereby slowing the desensitization process.

To ensure that the inhibition of desensitization of DOR TT or DOR Thr-161 was not an artifact of reduced binding affinity or intrinsic efficacy due to truncation or mutation of the receptor, we constructed cumulative dose-response curves for these receptors compared with wild type DOR. Neither the curves nor the EC50 values for the mutant receptors were significantly different from wild type DOR, suggesting there was no gross change in receptor function (Fig. 5C, EC50 DORWT = 7.3 ± 0.70 nM, DOR TT = 8.6 ± 1.5 nM, DOR L2 = 8.6 ± 1.5 nM). We were also careful to keep the amount of mutant receptor expressed close to the amount of wild type DOR expressed to avoid the receptor reserve issues discussed previously. Peak DPDPE current was our measure of the levels of DOR expression and was not significantly different between the different receptor groups.

**DISCUSSION**

In this study, we demonstrated that when expressed in the same cell with equal levels of GRK3 and arrestin expression, DOR was more rapidly desensitized than MOR. The principal findings were as follows: 1) there was no evidence of functional interaction between the two receptors; 2) manipulating the levels of arrestin expression and using a constitutively active form of arrestin suggested that the arrestin interaction was the rate-limiting component of MOR desensitization; and 3) desensitization of arrestin was represented as the percentage of the effect measured for the respective control group. Although the GRK and Arr effects were significantly different between MOR and DOR, at the higher levels of Arr expression, MOR desensitization was significantly different from control oocytes without Arr. Error bars, means ± S.E. from 7 to 8 independent determinations.
DOR Desensitization Is Faster Than MOR

The activation of arrestin is rate-limiting for MOR desensitization. Oocytes were injected with 1 ng of DOR cRNA, 0.04 ng of MOR, 0.02 ng of K₃.1 and K₃.4, alone or with either 16 ng of Arr2 wt and 0.3 ng of GRK3 or 16 ng of Arr2 (R169E) alone. Oocytes were perfused with either 1 μM DAMGO or 500 nM DPDPE in HK for 4 min for activation of MOR and DOR, respectively. The amount of current remaining after treatment is represented as the percentage of the initial current. Arr2 (R169E) desensitization of MOR was not significantly different from DOR. Error bars, means ± S.E. from 5 independent determinations. For the data without S.E., the error bar was too small to be evident. *, p < 0.01 compared with DAMGO effect.

The lack of cross-desensitization confirmed that the desensitization measured was homologous, occurring at the receptor rather than at a downstream effector of both MOR and DOR. Desensitization in this system also seems to be a measure of receptor uncoupling from the G-protein. Despite the fact that the receptor was not desensitized in the absence of an internalization process, it seems that the receptor uncoupling can be separated from the internalization process and that these two events may not be required for the differential regulation of the two receptors in this system. Overexpression of Arr3 increased the rate of MOR desensitization, whereas rate of DOR desensitization was minimally accelerated. Moreover, expression of the constitutively active Arr2R169E in the absence of GRK desensitized both MOR and DOR at indistinguishable rates. These data strongly suggest that the activation of arrestin by GRK-phosphorylated MOR is slower than for DOR because once activated arrestin can bind equally well to either receptor.
was observed with both Arr2 and Arr3. Moreover, the pre-activated Arr2(R169E) was able to rapidly desensitize MOR. This suggests that like MOR, this second class of receptors may have a reduced ability to activate Arr2 compared with Arr3.

GRK-phosphorylated serine and threonine residues in the receptor are believed to interact with the putative phosphate-sensing polar core in the arrestin molecule, thereby inducing a change from the basal conformation to an activated state that shows high affinity binding to the activated receptor (19, 34). A difference in receptor phosphorylation seems a likely candidate in reconciling the inefficient activation of arrestin by MOR compared with DOR. For DOR, two domains seem critical for GRK/arrestin-mediated desensitization, one in the carboxyl-terminal tail (15, 35) and one in the second cytoplasmic loop (this study). In contrast, a single threonine in the second intracellular loop was required for MOR desensitization (17). Cen et al. (36) also demonstrated that neither the carboxyl-terminal tail nor the third loop of MOR interact with arrestin, whereas arrestin could bind both the third loop and tail of DOR. Our results suggest that the more rapid desensitization of DOR by GRK and arrestin may be due to a synergistic action of these two sites leading to a more efficient activation of arrestin.

**FIG. 5.** Thr-161 in the second intracellular loop is important in DOR desensitization. A, schematic of DOR is represented with potential serine and threonine phosphorylation sites highlighted. DOR TT removes all serines and threonines in the carboxyl-terminal tail by truncating the receptor at Arg-339. DOR T161A removes the only threonine in the second cytoplasmic loop. B, oocytes were injected with 0.02 ng of K,3.1 and K,3.4 with either 1 ng DOR wt, DOR TT, or DOR (T161A) alone or with 0.3 ng of GRK3 and 2.3 ng of Arr3. Desensitization was measured by continual perfusion of 500 nM DPDPE for 4 min. The amount of current remaining after treatment is represented as the percentage of the initial response. Error bars, means ± S.E. from 5 to 15 independent determinations. *p < 0.01 compared with oocytes injected with that receptor construct without GRK3 and Arr3. Ψp < 0.01 compared with oocytes injected with DOR WT and both GRK3 and Arr3. C, oocytes expressing only K,3.1 and K,3.4 and either DOR wt or DOR (T161A) were exposed in increasing concentrations of DPDPE. The current measured at each dose is represented as a percentage of the maximal DPDPE response for that oocyte. Error bars, means ± S.E. from 5 to 15 independent determinations.
Instead, MOR has only the one site in the second intracellular loop that alone seems to be less efficient at activating arrestin. Visual arrestin has been shown to require more than one receptor-attached phosphate for effective activation (37). Based on the similarity in structure, it seems likely that this may apply to all arrestins (38). Therefore, in agreement with our data, MOR, phosphorylated at only one site, should less efficiently activate arrestin than MOR. Consistent with this hypothesis, Cheng et al. (16) have demonstrated that when the carboxy-terminal of DOR was substituted for the tail of MOR, they were able to see greatly increased arrestin-mediated desensitization of MOR.

In spite of the remarkable sequence homology, MOR and DOR are apparently regulated very differently. Immunoelectron microscopy studies have shown that MOR shows a predominant plasmamembrane localization, whereas DOR is more often associated with intracellular membranes (39). Interestingly, Cahill et al. (40) showed that prolonged morphine treatment increased the levels of DOR in the membrane, without affecting total DOR expression. They were further able to link this increase in agonist-accessible DOR to an increase in DOR-mediated analgesia. However, our results would suggest that once at the membrane DOR would be quickly desensitized by GRK and arrestin. In HEK-293 cells, once internalized, DOR is targeted to lysosomes where it undergoes proteolytic degradation (14). In oocytes, we showed that MOR desensitization proceeds much more slowly, whereas others have shown that in mammalian cell lines, MOR recycles back to the plasma membrane after internalization (12, 13). Similarly, the 5HT2A and β2-adrenergic receptors have also been shown to be relatively resistant to arrestin regulation (28, 41, 42). Thus, it seems that the overall bioavailable lifetime of DOR in the membrane may be much shorter than that of MOR. Although the physiological significance for such a differential regulation of these two closely related receptors remains to be elucidated, the design of MOR might ensure a longer lasting response.

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μ-Opioid Receptors Desensitize Less Rapidly than δ-Opioid Receptors Due to Less Efficient Activation of Arrestin

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