

Agonist Induced Homologous Desensitization of μ -Opioid Receptors Mediated by G Protein-Coupled Receptor Kinases Is Dependent on Agonist Efficacy

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ABSTRACT

Using *Xenopus laevis* oocytes coexpressing mammalian μ -opioid receptors (MORs), β -adrenergic receptor kinase 2 (β -ARK2) [also called G protein-coupled receptor kinase (GRK3)], and β -arrestin 2 (β -arr 2), we compared the rates of β -ARK2 (GRK3)- and β -arr 2-mediated homologous receptor desensitization produced by treatment with opioid agonists of different efficacies. The response to MOR activation was measured using two-electrode voltage clamp as an increase in the conductance of the coexpressed G protein-coupled inwardly rectifying potassium (heteromultimer of $K_{IR}3.1$ and $K_{IR}3.4$) channels. Treatment with opioids of high efficacy, either [D-Ala², N-MePhe⁴, Gly-ol⁵]-enkephalin, fentanyl, or sufentanyl, produced a GRK3- and β -arr 2-dependent reduction in response in <20 min, whereas treatment with the partial agonist morphine pro-

duced receptor desensitization at a significantly slower rate. Because GRK3 requires activation and membrane targeting by free G protein $\beta\gamma$ subunits released after agonist-mediated activation of G proteins, a low efficacy agonist such as morphine may produce weak receptor desensitization as a consequence of poor GRK3 activation. To address this hypothesis, we substituted GRK5, a GRK that does not require activation by G protein $\beta\gamma$. In oocytes expressing GRK5 instead of GRK3, both [D-Ala², N-MePhe⁴, Gly-ol⁵]-enkephalin and fentanyl, but not morphine, produced desensitization of MOR-activated potassium conductance. Thus, μ -opioid agonists produced significant receptor desensitization, mediated by either GRK3 or GRK5, at a rate dependent on agonist efficacy.

The processes underlying the clinically observed tolerance to opioid drugs are complex and may involve learning mechanisms, compensatory changes in neuronal circuits, and desensitization of signal transduction mechanisms (Nestler and Aghajanian, 1997; Ramsay and Woods, 1997). Studies both *in vivo* and *in vitro* have documented that the response to opioids desensitize after prolonged exposure to opioids (Cox, 1978). One of the important molecular events that has been shown to be involved in the desensitization of the response to opioids is opioid receptor desensitization. The molecular basis for opioid receptor desensitization was shown to be a reduction in opioid agonist efficacy (Chavkin and Goldstein, 1982, 1984; Porreca and Burks, 1983) that manifests as a reduction in the rate of G protein activation by the agonist-bound receptor complex. For example, continuous infusion of guinea pigs or rats with morphine results in an uncoupling of μ -opioid receptors from associated G proteins as measured biochemically (Werling *et al.*, 1989; Tao *et al.*, 1993), cyto-

chemically (Sim *et al.*, 1996), or electrophysiologically (Christie *et al.*, 1987). The uncoupling of MOR from G proteins is likely to result from the agonist-dependent phosphorylation of MOR mediated by a GRK (Kovoor *et al.*, 1997). Because clinically useful opioids differ in their intrinsic efficacies, the rates of tolerance development for different opioid agonists might be expected to differ (Stevens and Yaksh, 1989a). However, the relationship between agonist efficacy and opioid tolerance is controversial. Stevens and Yaksh (1989a, 1989b) reported that tolerance to the analgesic effects of opioids measured by the hot-plate response of rats was greater for agonists with lower efficacy when continuously infused at equieffective doses. Duttaroy and Yoburn (1995) confirmed that the amount of analgesic tolerance after continuous infusion with opioids was inversely proportional to agonist efficacy but that intrinsic efficacy had no effect on the magnitude of tolerance produced by intermittent administration of opioid agonist to mice.

The complexity of the pharmacological parameters controlling the *in vivo* situation confounds the analysis of the relationship between efficacy and tolerance because differences in receptor occupancy, drug distribution, metabolism, and

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ABBREVIATIONS: MOR, μ -opioid receptor; DAMGO, [D-Ala², N-MePhe⁴, Gly-ol⁵]-enkephalin; β -ARK, β -adrenergic receptor kinase; β -arr, β -arrestin; GRK, G protein-coupled receptor kinase; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

receptor selectivity affect the measured response and subsequent changes after prolonged drug administration. Stevens and Yaksh (1989a, 1989b) explained their results by suggesting that agonists with higher efficacy had a larger fraction of spare receptors and, hence, tolerance development to the more efficacious agonists was slower. However, this assumes that agonists with different efficacies produce receptor desensitization at the same rate when they bind the same fraction of receptors. Thus, to better elucidate the molecular mechanisms underlying the relationship between efficacy and receptor desensitization, we used *Xenopus laevis* oocytes coexpressing the MOR and the G protein-gated inwardly rectifying potassium channel complex ($K_{IR}3.1$ and $K_{IR}3.4$) to reconstitute a typical neuronal opioid response (North *et al.*, 1987). To reconstitute a homologous receptor desensitization mechanism, we also coexpressed GRKs and β -arr 2. Using this gene expression system, we previously showed that coexpression of β -ARK2 (or GRK3) and β -arr 2 synergistically produced an agonist-dependent homologous desensitization of the β_2 -adrenergic receptor, the δ -opioid receptor, and the MOR activation of the $K_{IR}3$ response (Kovoor *et al.*, 1997). Recently, Yu and colleagues (1997) reported that opioids with high receptor efficacies were better able to stimulate MOR phosphorylation than were opioids with low efficacies. Similarly, partial agonists were previously reported to be less effective at promoting β -adrenergic receptor phosphorylation by β -ARK (i.e., GRK2) than full agonists (Benovic *et al.*, 1988). In the current study, we asked whether μ -opioid agonists with different efficacies produce GRK/ β -arr 2-mediated desensitization at different rates.

Materials and Methods

Chemicals. DAMGO was obtained from Peninsula Laboratories (San Carlos, CA). Fentanyl, buprenorphine, and naloxone were from Research Biochemicals International (Natick, MA). Sufentanyl was a gift from Janssen Pharmaceuticals (Brussels, Belgium). All other chemicals were from Sigma Chemical (St Louis, MO).

Complementary DNA clones and cRNA synthesis. Except for GRK5, the cDNA clones used and cRNA synthesis methods have been described previously (Kovoor *et al.*, 1997). The rat MOR clone was obtained from Dr. Lei Yu (GenBank accession No. L13069). cDNA for the $K_{IR}3.1$ (GIRK1) channel was obtained from Dr. Henry Lester (GenBank accession No. U01071). The $K_{IR}3.4$ (GIRK4) clone provided by Dr. John Adelman (GenBank accession No. X83584) and the β -arr 2 cDNA (clone provided by Dr. Robert Lefkowitz, GenBank accession No. M91590) were first amplified by the use of Amplitaq DNA Polymerase (Perkin-Elmer Cetus, Norwalk, CT) in a standard polymerase chain reaction using oligonucleotides designed to add a T7 promoter region and a 45-base poly(A)⁺ tail. The rat GRK3 cDNA (GenBank accession No. M87855) was provided by Dr. Shaun Coughlin in the pFROGY vector. The human GRK5 clone (GenBank accession No. L15388) was obtained from Dr. Jeffrey Benovic (Kunapuli *et al.*, 1993). Plasmid templates for all constructs were linearized before cRNA synthesis, and mMESSAGE MACHINE kits (Ambion, Austin, TX) were used to generate capped cRNA from the cDNA templates, which contained either T7, T3, or SP6 promoters to direct synthesis of sense transcripts.

Oocyte culture and injection. Stage IV oocytes were prepared as described previously (Kovoor *et al.*, 1995). cRNA was injected (50 nl/oocyte) using a Drummond automatic microinjector, and then oocytes were incubated at 18° for 3–7 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) solution supplemented with sodium pyruvate (2.5 mM) and gentamycin (50 μ g/ml).

Electrophysiology. Oocytes were clamped at -80 mV with two electrodes filled with 3 M KCl having resistances of 0.5–1.5 M Ω using a Geneclamp 500 amplifier and pCLAMP 6 software (Axon Instruments, Foster City, CA). Data were digitally recorded (Digidata 1200, Axon Instruments, and Intel 386PC) and filtered. Membrane current traces also were recorded using a chart recorder. To facilitate the inward potassium current flow through the $K_{IR}3$ channels, normal oocyte saline buffer was modified to increase KCl concentration to 16 mM as stated in Results. All drug responses were evaluated in this high K⁺-containing solution. In the experiments in which sufentanyl desensitization was evaluated, the high K⁺ solution in which drug responses were measured also contained 0.1% bovine serum albumin (Sigma). The concentration of NaCl was correspondingly decreased to maintain isoosmolarity. Opioid activation of $K_{IR}3$ conductance was measured as described previously (Kovoor *et al.*, 1997).

Statistical analysis. The Student's *t* test (with two-tailed *p* values) was used for comparison of the independent mean values. Dose-response curves were fitted to a simple E_{max} model using the nonlinear regression analysis PCNONLIN v4.2 (SCI Software) to determine the EC₅₀ values and 95% confidence intervals.

Results

Dose-response relationship of μ -opioid agonists in *X. laevis* oocytes expressing MOR and $K_{IR}3$. As reported previously (Kovoor *et al.*, 1995, 1997), activation of MOR by DAMGO, a selective MOR agonist, elicited a G protein-activated inwardly rectifying potassium conductance in *X. laevis* oocytes injected with cRNA for MOR and the $K_{IR}3$ channel (Fig. 1). MOR-activated $K_{IR}3$ channel response was measured as the increase in inward current produced in oocytes clamped at -80 mV. The opioid-induced increase in potassium conductance was blocked by naloxone and required oocyte injection with both MOR and $K_{IR}3.1$ cRNA. As reported, coexpression of $K_{IR}3.1$ and $K_{IR}3.4$ resulted in the formation of heteromultimeric channels, enhanced the agonist-activated response, reduced the amount of channel cRNA required, and greatly attenuated the amount of heterologous desensitization produced on prolonged agonist treatment (Krapivinsky *et al.*, 1995; Kovoor *et al.*, 1997). In this study, we generated cumulative concentration-response curves for activation of $K_{IR}3$ by the MOR ligands DAMGO, fentanyl, sufentanyl, buprenorphine, and morphine. To normalize for daily differences in receptor expression, dose-response data are presented as a percentage of the average maximal stimulation produced by DAMGO as measured in the same oocyte batch and under the same conditions.

Sufentanyl was the most potent opioid tested followed with fentanyl and DAMGO (Fig. 1, Table 1). As expected, morphine was found to be a less efficacious agonist than DAMGO, sufentanyl, or fentanyl; the maximal response to morphine was smaller than that to the other agonists tested. In oocytes injected with a low dose of cRNA for MOR (0.08 ng), morphine was unable to elicit any measurable $K_{IR}3$ currents (Fig. 1) but instead was a competitive antagonist of the DAMGO-activated response. Schild analysis of the antagonist potency of morphine under these conditions gave a K_d value of 98 nM (data not shown). For morphine to produce a MOR-mediated $K_{IR}3$ response, receptor expression was increased by increasing the amount of MOR cRNA injected/oocyte to 0.5 ng (Fig. 1). At this dose of MOR cRNA, the EC₅₀ value of morphine was 90 nM (Table 1), a value consistent with the K_d value determined by Schild analysis. A second, weak opioid receptor agonist, buprenorphine, also was tested

under these conditions in an attempt to extend the analysis, but 10 μM buprenorphine did not produce detectable MOR activation at the MOR cRNA amounts injected (0.08–0.5 ng).

In oocytes injected with 0.08 ng of MOR cRNA, the maximal responses produced by sufentanyl, DAMGO, or fentanyl were not significantly different. For oocytes injected with 6.3-fold higher (0.5 ng) MOR cRNA, the maximal stimulation produced by DAMGO was increased (Fig. 1, *inset*). Similarly, increasing the amount of MOR injected to 1 ng also increased the maximal receptor-stimulated currents (data not shown). These results suggest that in oocytes injected with either 0.08 or 0.5 ng of MOR cRNA, there were no spare MORs for DAMGO, fentanyl, or sufentanyl because the maximal receptor-stimulated currents were limited by receptor expression. Thus, although sufentanyl was more potent than either DAMGO or fentanyl, all three drugs were equally efficacious in this expression system.

Effect of DAMGO, fentanyl, and morphine treatment on GRK3- and β -arr 2-mediated homologous desensitization of MOR. In oocytes expressing only MOR and the $K_{\text{IR}}3$ channel subunits $K_{\text{IR}}3.1$ and $K_{\text{IR}}3.4$, no significant homologous desensitization of the receptor-elicited $K_{\text{IR}}3$ current was observed (Fig. 2A). However, as demonstrated previously (Kovoov et al., 1997), coexpression of both GRK3 (i.e., β -ARK 2) and β -arr 2 led to a significant homologous desensitization of the DAMGO-activated $K_{\text{IR}}3$ current (Fig. 2A). Using oocytes coexpressing GRK3 and β -arr 2 with the receptor and channel, we compared the DAMGO-induced desensitization with the desensitization induced by either 1 μM fentanyl or 1 μM morphine, each at the receptor-saturating concentration of agonist. Responses elicited by DAMGO or fentanyl desensitized by $\sim 50\%$ during 8 min of continuous agonist exposure. In the same group of oocytes, the mor-

phine-activated responses showed no desensitization over the same period. To test whether the lack of acute desensitization by morphine was a result of its lower activity, we tested a concentration of DAMGO that evoked a response similar to the maximal morphine response and found that it also produced little receptor desensitization (data not shown). In the absence of either GRK3 or β -arr 2, neither fentanyl nor DAMGO treatment produced acute homologous receptor desensitization (Fig. 2C and data not shown).

Effect of GRK5 substitution for GRK3 on MOR desensitization. We also tested whether another member of the GRK family, GRK5, was able to modulate MOR activity (Fig. 3). Brief treatment (8 min) with either DAMGO, fentanyl, or morphine (1 μM) did not produce significant desensitization in MOR-activated $K_{\text{IR}}3$ responses in oocytes that had been coinjected with cRNA for GRK5 and β -arr 2. However, prolonged DAMGO or fentanyl treatment did produce significant desensitization in oocytes coexpressing both GRK5 and β -arr 2. In this experiment, oocytes were exposed for 12–14 hr to either DAMGO, fentanyl, or morphine in normal oocyte saline buffer and then the drug was removed by perfusion with saline buffer for 5 min. The amount of receptor desensitization produced by each agonist was assessed by measuring the response to a challenge with 1 μM DAMGO; this is important because the degree of desensitization may otherwise vary depending on the efficacy of the test agonist.

In oocytes expressing GRK5 and β -arr 2, pretreatment with DAMGO or fentanyl significantly diminished the amplitude of the inward current elicited by subsequent DAMGO application compared with oocytes not injected with GRK5 and β -arr 2 (Fig. 3, A and B). In contrast, pretreatment with morphine did not significantly diminish the inward current elicited by DAMGO application. The data demonstrate that

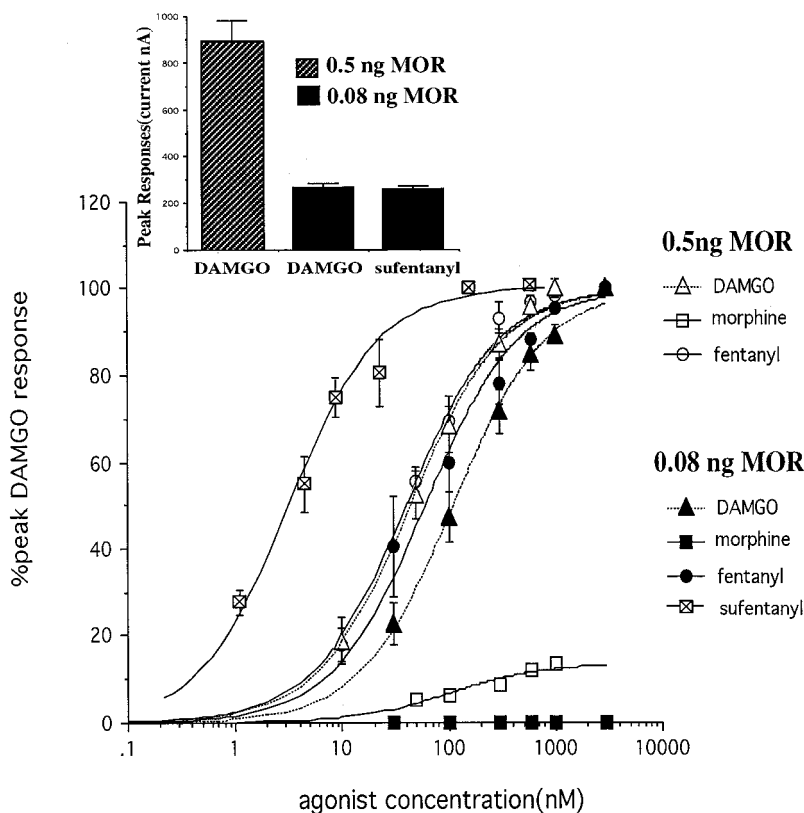


Fig. 1. Dose-dependent MOR activation by DAMGO, fentanyl, sufentanyl, and morphine of $K_{\text{IR}}3$ expressed in *X. laevis* oocytes. Dose-response curves for DAMGO, fentanyl, sufentanyl, and morphine were constructed by testing increasing concentrations of agonist in *X. laevis* oocytes expressing $K_{\text{IR}}3$ and MOR. Oocyte membrane potential was clamped at -80 mV while bathed in normal oocyte saline buffer containing 2 mM KCl, as described in the text. Oocytes then were superfused with a saline buffer in which the KCl concentration was increased to 16 mM, enabling a basal inward current to flow through the inwardly rectifying K^+ channels. Cumulatively increasing doses of MOR agonists were applied to the bath, followed by perfusion with the opioid receptor antagonist naloxone (1 μM), which reversed the activation of MOR. The response to each dose of agonist was defined as the increase in inward current over the basal current. The agonist response at each dose of opioid was normalized as a percentage of the average maximal DAMGO response obtained in separate oocytes from the same group. Each point represents the mean response measured in three or four oocytes. Once a set of dose-response measurements for an agonist was performed, the oocyte was discarded. Oocytes were injected with 0.5 or 0.08 ng of cRNA for MOR, and 0.02 ng of each G protein-activated inwardly rectifying potassium channel subunit $K_{\text{IR}}3.1$ and $K_{\text{IR}}3.4$. Higher amounts of receptor cRNA produced a greater opioid response for each agonist. This is particularly evident for morphine, which was a pure antagonist at 0.08 ng of MOR cRNA and a partial agonist at 0.5 ng of MOR cRNA. *Inset*, maximal response to DAMGO (1 μM) at MOR cRNA concentrations of 0.08 and 0.05 ng of MOR cRNA and the maximal response to sufentanyl (300 nM) at MOR cRNA concentrations of 0.08 ng. EC_{50} values at 0.5 and 0.08 ng of MOR cRNA injected for each drug along with confidence intervals are summarized in Table 1. Error bars, mean \pm standard error and are smaller than the plotted symbols when not shown.

GRK5 also can mediate agonist-dependent MOR desensitization after treatment with DAMGO or fentanyl and can substitute for GRK3 in this system. However, the kinetics of GRK5-mediated desensitization were slower than that of GRK3 under these expression conditions. The results obtained showed further that morphine pretreatment was significantly less effective than DAMGO or fentanyl pretreatment

at producing GRK5-mediated receptor desensitization. Because GRK5 does not require $G_{\beta\gamma}$ activation, the difference between morphine- and DAMGO- or fentanyl-mediated desensitization did not result from the weaker production of $G_{\beta\gamma}$ by the partial agonist morphine.

Although acute treatment of oocytes with morphine did not produce opioid receptor desensitization (Fig. 2), we next tested whether a longer treatment with morphine was effective. After the protocol described in Fig. 3, but with GRK3 instead of GRK5, oocytes were treated for 12–14 hr with either 1 μ M DAMGO or 1 μ M morphine (Fig. 4). As expected, oocytes coexpressing GRK3 and β -arr 2 showed complete desensitization after 12–14-hr treatment with 1 μ M DAMGO. Significantly less desensitization was produced by DAMGO in oocytes not expressing GRK3 and β -arr 2; however, in this group of oocytes, the responses observed were significantly smaller than evident in control oocytes not pretreated with DAMGO. Interestingly, prolonged treatment with morphine also produced significant desensitization in oocytes coexpressing GRK3 and β -arr 2, and significantly less desensitization was produced by morphine in oocytes not expressing

TABLE 1

Data from dose-response determinations

Data from Fig. 1 were fitted to a simple, single-site E_{\max} model using the nonlinear regression analysis package PCNONLIN v4.2 (SCI SOFTWARE) to determine EC_{50} values and confidence intervals for each drug with oocytes injected with 0.5 or 0.08 ng of MOR cRNA along with 0.02 ng each of cRNA for the G protein-activated inwardly rectifying potassium channel subunits $K_{IR} 3.1$ and $K_{IR} 3.4$.

Drug	0.08 ng of MOR cRNA		0.5 ng of MOR cRNA	
	EC_{50}	95% CI	EC_{50}	95% CI
DAMGO	110	66–133	43	34–66
Fentanyl	54	31–76	39	33–46
Morphine			90	47–133
Sufentanyl	2.8	1.9–3.4		
Buprenorphine				

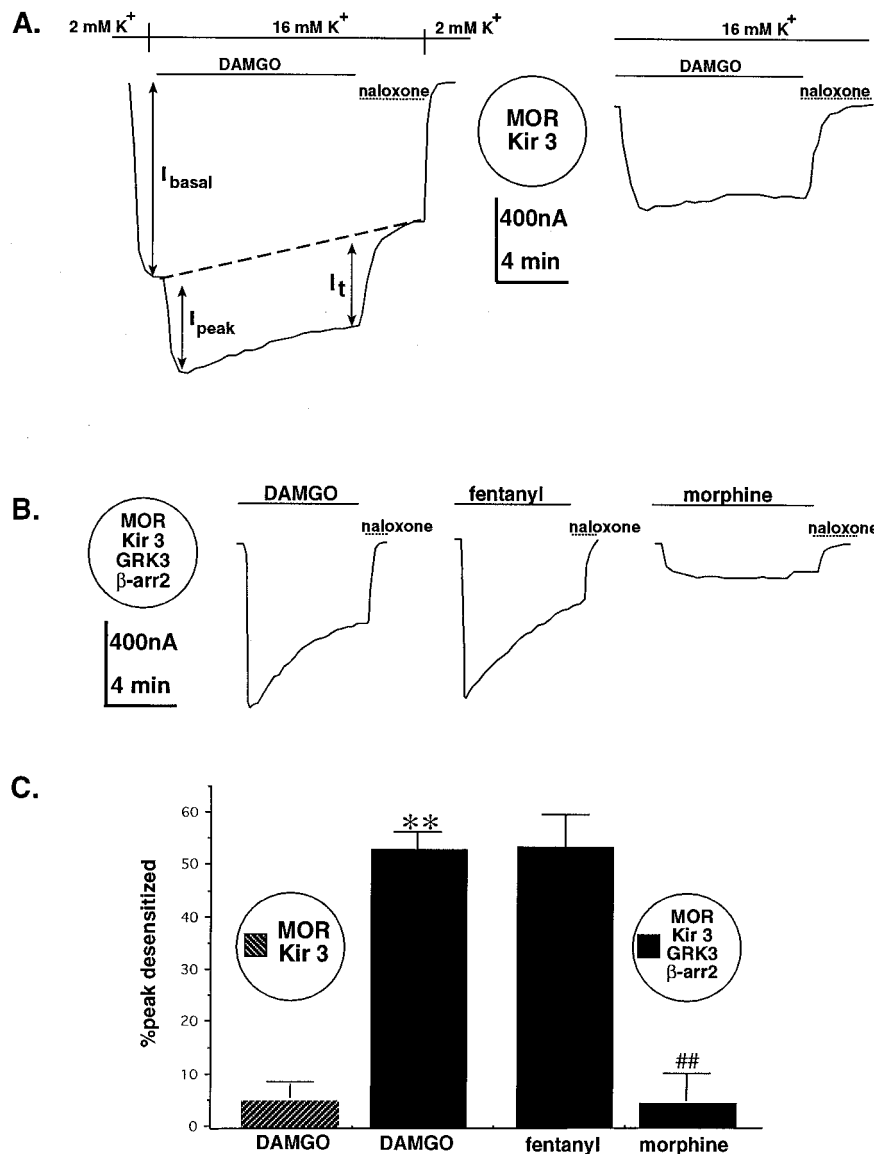


Fig. 2. Effect of GRK3 on desensitization of DAMGO-, fentanyl-, or morphine-elicited MOR responses. **A, Left,** a representative current trace from an oocyte injected with 0.5 ng of MOR cRNA and 0.02 ng of each $K_{IR} 3.1$ and $K_{IR} 3.4$ shown to illustrate the data acquisition and measurement methods used in this study. Initially, the oocyte was bathed in normal oocyte saline buffer containing 2 mM KCl and clamped at -80 mV. The oocyte was then superfused with a saline buffer containing 16 mM KCl. The elevation in potassium concentration facilitates the measurement by increasing the basal current through the inwardly rectifying potassium channels while the cell is clamped at -80 mV. Subsequent application of 1 μ M DAMGO further increased the inward current. Application of the MOR antagonist naloxone (1 μ M) reversed the effects of DAMGO and enabled detection of a change in basal current (dashed line). Finally, decreasing the potassium concentration from 16 to 2 mM brought the current back to the initial value. **Inset (circle),** Respective cRNA injections. **Right,** identical current trace after base-line subtraction showing only the agonist-induced current. The subtraction was performed by taking the difference between the total inward current and the base-line inward current (dashed line on the left trace). **B,** Representative traces showing base-line-subtracted responses to sustained application of DAMGO, fentanyl, or morphine (1 μ M each), respectively. Oocytes were injected with 0.5 ng of MOR, 0.02 ng of $K_{IR} 3.1$, 0.02 ng of $K_{IR} 3.4$, 0.5 ng of GRK3, and 2 ng of β -arr 2. To determine the amount of response desensitization, agonist treatment was followed by 1 μ M naloxone perfusion. The amount of desensitization was calculated as the change in response to agonist after 8 min expressed as a percentage of the peak response. Calibration for each trace: 400 nA, 4 min. **C,** Summary of the percent change in the peak response after acute agonist treatment of oocytes. Treatment of oocytes injected with MOR and $K_{IR} 3$ with 1 μ M DAMGO did not produce a significant amount of response desensitization, whereas DAMGO treatment of oocytes from the same batch that also were injected with 0.5 ng of GRK3 and 2 ng of β -arr 2 showed a substantial amount of desensitization. Treatment of oocytes coexpressing GRK3/ β -arr 2 with 1 μ M fentanyl, but not with 1 μ M morphine, also produced significant desensitization of the agonist response. Error bars, mean \pm standard error for four to eight independent determinations. **, $p < 0.01$ compared with oocytes not injected with GRK3 and β -arr 2; ##, $p < 0.01$ compared with DAMGO or fentanyl.

GRK3 and β -arr 2. These results suggest that morphine can produce GRK/ β -arr 2-dependent receptor desensitization in this expression system, but it does so at a significantly slower rate than does the more efficacious agonists, DAMGO and fentanyl.

Finally, we also compared the GRK3- and β -arr 2-mediated desensitization produced by treatment with DAMGO with that produced by treatment with the more potent but equally efficacious MOR ligand sufentanyl. After 20 min of agonist pretreatment, the amounts of homologous, GRK/ β -arr 2-dependent desensitization produced by DAMGO and sufentanyl were not significantly different (Fig. 4). These results suggest that the rate of homologous desensitization of the MOR depends on agonist efficacy.

Discussion

Here, we report that in addition to regulation by GRK3, MOR also may be uncoupled in an agonist-dependent manner by a GRK with different properties, GRK5. We also show that the highly efficacious μ -opioid agonists DAMGO, sufentanyl, and fentanyl produce a GRK- and β -arr-mediated un-

coupling of the MOR. In our investigation, efficacy directly correlated with GRK3- and β -arr 2-mediated receptor uncoupling. The highly efficacious ligands DAMGO, fentanyl, and sufentanyl caused a greater amount of receptor desensitization than morphine at saturating concentrations of agonist.

The faster rate of GRK-mediated desensitization caused by treatment with either DAMGO, fentanyl, or sufentanyl compared with that produced by morphine suggests that the more efficacious agonists induce a conformation of the receptor that is a better substrate for the kinase. Because $G_{\beta\gamma}$ subunits are required for recruitment of GRK3 to the plasma membrane and activation of the kinase (Pitcher *et al.*, 1995, Muller *et al.*, 1997), the more efficacious ligands would be expected to activate the GRK3 more effectively than does morphine. However, we demonstrated here that GRK5, which differs from GRK3 in that it is not bound or activated by $G_{\beta\gamma}$, also produces a faster rate of MOR desensitization after DAMGO or fentanyl treatment. The more efficacious agonists thus allow more effective desensitization of the receptor independent of $G_{\beta\gamma}$ release. The slower rate of GRK5-mediated desensitization evident in our study is consistent with the results of Kunapuli *et al.* (1994), who showed that

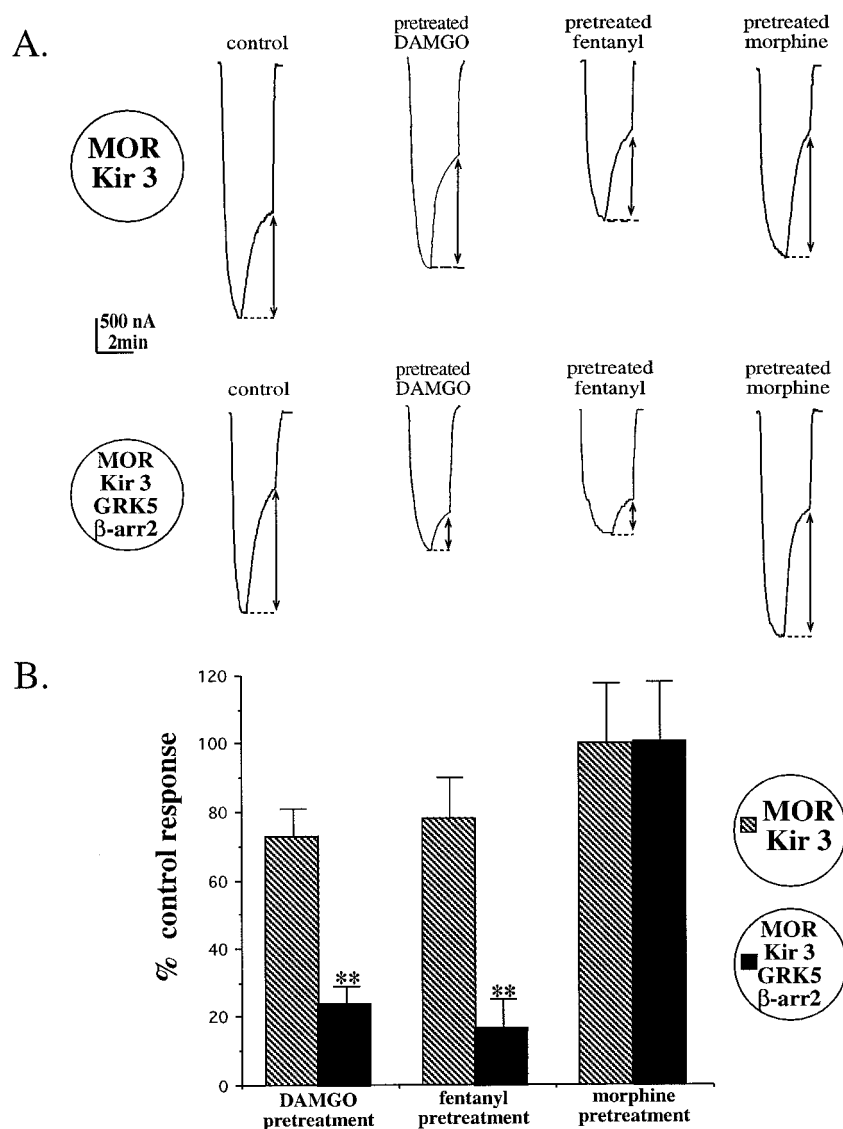


Fig. 3. Effects of pretreatment with DAMGO, fentanyl, or morphine on GRK5-mediated desensitization of MOR responses. Control oocytes were injected with a mixture of the cRNAs: 0.5 ng of MOR, 0.02 ng of $K_{IR}3.1$, and 0.02 ng of $K_{IR}3.4$. Some of the oocytes also were injected with 1 ng of GRK5 and 3 ng of β -arr 2. Insets (circles), cRNA injected; the $K_{IR}3.1/K_{IR}3.4$ complex is represented as $K_{IR}3$, and all recordings were performed 3–4 days after injection. A, Representative current traces from oocytes untreated with agonist (control) or pretreated with DAMGO, fentanyl, or morphine (1 μ M) for 12–14 hr. Top set of four traces, from oocytes expressing MOR and $K_{IR}3$. Bottom set of traces, from oocytes also coexpressing GRK5 and β -arr 2. For these traces, oocytes were clamped at -80 mV in normal oocyte buffer and then perfused with a challenge dose of 1 μ M DAMGO in oocyte buffer containing 16 mM KCl. Dashed lines, application of 1 μ M naloxone in the same buffer. The amplitude of the DAMGO response was defined as the portion reversed by naloxone (arrow to dashed line). The $K_{IR}3$ currents were reversed by perfusion with high Na^+ -containing normal oocyte buffer. Calibration for each trace is 500 nA, 2 min. B, Summary of the desensitization of DAMGO-elicited MOR responses after 12–14-hr pretreatment with DAMGO, fentanyl, or morphine compared with matched, untreated oocytes. Error bars, mean \pm standard error for three or four independent determinations. **, $p < 0.01$ compared with oocytes not coexpressing GRK5 and β -arr 2.

purified GRK5 had a lower V_{\max} than GRK2 at other G protein-coupled receptor substrates.

Sternini *et al.* (1996) and Keith *et al.* (1996) reported that in the HEK 293 cell line and in neurons *in vivo*, high efficacy agonists such as etorphine produced MOR internalization. However, morphine, which effectively activated the receptor, did not produce MOR internalization in HEK 293 cells or *in vivo*. Recent experiments suggest that in addition to uncoupling the β_2 -adrenergic receptor from G proteins, phosphorylation of the β_2 -adrenergic receptor by receptor kinases followed by β -arr binding also is important for receptor internalization (Goodman *et al.*, 1998). β -Arr serves as an adapter protein that links the agonist-bound receptor to the cellular internalization machinery. Using the *X. laevis* oocyte

expression system, we have shown a synergistic action of the GRKs and β -arr on MOR desensitization. If phosphorylation of the MOR by receptor kinases followed by β -arr binding to MOR also is important for MOR internalization, then our demonstration that morphine only very weakly stimulates GRK-mediated arrestin binding to MOR may provide a mechanistic explanation for the observations of Keith and colleagues. Under conditions in which the levels of receptor expression are high or when the amount of receptor activation is greatly amplified by the effector system used to monitor receptor activity, poorly efficacious agonists such as morphine may be expected to produce maximal responses. However, for poorly efficacious ligands, the fraction of bound receptors that are in the active state at any time would be small, and this would reduce the probability for GRK phosphorylation and subsequent β -arr binding. In support of that hypothesis, we observed that the concentration of DAMGO that produced a response similar to that evoked by saturating doses of morphine also produced little receptor desensitization. Our results predict that the minimal dose of etorphine or DAMGO producing the same response as the dose of morphine tested also would fail to produce receptor internalization.

There are two discrepancies between the conclusions presented in the current study and the recent report by Yu *et al.* (1997). They found that sufentanyl was more efficacious than DAMGO in activating MORs expressed in oocytes and concluded that agonist-dependent receptor phosphorylation occurred in oocytes without adding GRK-cRNA. Under the conditions in which no cRNA for GRK or β -arr is injected (Yu *et al.*, 1997), we previously established that the response desensitization that occurs is heterologous and occurs via adaptive changes downstream of the receptor (e.g., without receptor phosphorylation) (Kovoor *et al.*, 1995). In oocytes coexpressing MOR and the 5-hydroxytryptamine_{1A} receptor, stimulation of either receptor resulted in heterologous desensitization of the subsequent response to the other and injection of the nonhydrolyzable GTP analogue guanosine-5'-O-(3-thio)triphosphate did not affect the rate of desensitization (Kovoor *et al.*, 1995). Furthermore, the heterologous desensitization of receptor/ $K_{IR}3$ coupling was greatly attenuated by the coexpression of the $K_{IR}3$ channel subunits $K_{IR}3.1$ and $K_{IR}3.4$ (Kovoor *et al.*, 1997). After testing a series of agonists with differing efficacies at MOR in the *X. laevis* oocyte expression system, they determined that the amount of desensitization of the agonist-induced responses was related directly to the efficacy of the agonist and concluded that the more efficacious agonists produced a greater receptor desensitization. However, Yu *et al.* (1997) did not show that receptor phosphorylation resulted in homologous receptor desensitization because their results simply indicate that heterologous desensitization (e.g., changes occurring downstream of the receptor, presumably at the channel) also is dependent on agonist efficacy.

Furthermore, under the conditions used by Yu *et al.*, the high levels of MOR cRNA and low levels of channel expression (caused by the limiting expression of endogenous $K_{IR}3.5$) are likely to have resulted in spare opioid receptors for DAMGO, sufentanyl, and fentanyl. In *X. laevis* oocytes injected with cRNA for only the $K_{IR}3.1$ subunit (as done by Yu *et al.*), the expression of functional channels is dependent on the formation of heteromultimers between the exogenously

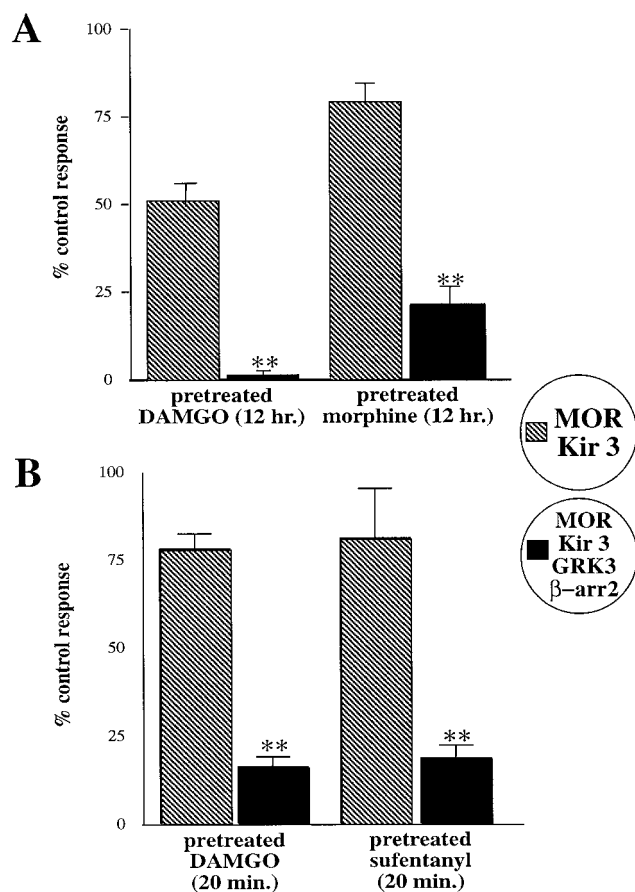


Fig. 4. Effect of pretreatment with DAMGO, sufentanyl, or morphine on GRK3-mediated desensitization of DAMGO-elicited MOR. **A**, Comparison of DAMGO and morphine treatment on GRK3-mediated desensitization of MOR. Oocytes were treated with either 1 μ M DAMGO or 1 μ M morphine for 12–14 hr and tested for MOR desensitization as described in Fig. 3. Conditions were the same as described above except 1 ng of GRK3 cRNA was substituted for GRK5 cRNA. *Insets (circles)*, summarize the cRNA injected. After 12–14-hr pretreatment with either DAMGO or morphine, oocytes were clamped at -80 in normal oocyte buffer for 5 min to wash out residual agonist and then perfused with a challenge dose of 1 μ M DAMGO in oocyte buffer containing 16 mM KCl. The amplitude of the DAMGO response was defined as the portion reversed by naloxone. *Bars*, mean amplitude of the naloxone-reversible DAMGO response compared with the DAMGO response in matched oocytes not pretreated with either DAMGO or morphine (percent control response). **B**, Comparison of DAMGO and sufentanyl treatment on GRK3-mediated desensitization of MOR. The experiment was performed exactly as described above except that oocytes were pretreated for only 20 min. with either 400 nM DAMGO or 300 nM sufentanyl. *Error bars*, mean \pm standard error for four to seven independent replicate determinations. **, $p < 0.01$ compared with oocytes not injected with GRK3 and β -arr 2.

expressed K_{IR}3.1 subunit and the endogenously expressed K_{IR}3.4 homologue K_{IR}3.5 (Krapivinsky et al., 1995; Hedin et al., 1996). In oocytes injected only with cRNA for the K_{IR}3.1, it has been shown that channel, and not receptor, is limiting (Hedin et al., 1996). This conclusion is supported by the lower EC₅₀ values obtained (Yu et al., 1997). Under conditions in which there are spare opioid receptors, it thus is surprising that sufentanyl produced greater responses than DAMGO (Yu et al., 1997). We failed to replicate that finding.

Previously, Blake et al. (1997) stated that treatment of MORs expressing HEK 293 cells with buprenorphine abolished the ability of other μ opioids to inhibit adenylyl cyclase, and they interpreted these results to argue that buprenorphine produced a profound receptor desensitization. This conclusion is in direct contrast to our results, which indicate that less efficacious agonists produce less receptor desensitization. To reconcile this discrepancy, we tested 10 μ M buprenorphine and found that it did not activate K_{IR}3 channels at any of the levels of functional MOR. Buprenorphine is an agent that binds the MOR with very high affinity but very low efficacy, and we found that 1 μ M buprenorphine acted as a pseudoirreversible antagonist consistent with previous findings (Thomas and Hoffman, 1993). Thus, the reduction in response previously reported may have resulted from receptor antagonism rather than desensitization.

There is an apparent contradiction in the relationship between ligand efficacy and receptor desensitization observed in this study and the opposite relationship between opiate tolerance and ligand efficacy observed *in vivo* (Stevens and Yaksh, 1989a, 1989b; Duttaroy and Yoburn, 1995). Highly efficacious agonists produced less tolerance *in vivo* while producing greater GRK-mediated receptor phosphorylation and receptor desensitization *in vitro*. The discrepancy can be reconciled if we assume that a very large fraction of spare receptors mediates the analgesic response to the highly efficacious opioids but that morphine has relatively few, if any, spare receptors *in vivo*. That assumption is supported by previous studies demonstrating a large fraction of spare opioid receptors *in vivo* (Chavkin and Goldstein, 1981; Perry et al., 1982). Furthermore, a drug with low efficacy such as morphine may act as a full agonist in tissues that express an excess of MOR and act as a partial agonist in tissues with lower receptor expression. After the desensitization or inactivation of an identical number of receptors, a less efficacious drug will show a greater shift in the dose-response curve than a more efficacious drug with an extremely large receptor reserve based on mass action kinetic principles. The *in vivo* tolerance studies were confounded because the amount of tolerance produced by prolonged exposure to an agonist was evaluated by examining the shift of the dose response curve to the same agonist. Under these conditions, a reduction in receptor reserve would be expected to produce a greater shift in the dose response of a partial agonist than a full agonist. A clearer measure of the degree of change at the MOR after prolonged treatment with various toleragens would have been obtained by evaluating the shifts in the dose-response curves for a single, receptor-selective test agonist.

In studies using established cell lines, heterologous gene expression systems, or real neurons, efforts to define the relationship between agonist efficacy and tolerance or desensitization have been hindered by the confounding presence of

spare opioid receptors. In the current study, we deliberately used a system in which there were no spare receptor receptors for any of the opioids tested (increases in MOR cRNA injection gave proportional increases in agonist-activated maximal response). Thus, further work is required to define the molecular changes in opioid signaling after prolonged *in vivo* exposure to drug. Nevertheless, the results obtained using *in vitro* model systems predict that highly efficacious opioid agonists produce a higher rate of receptor phosphorylation in animals treated with drug for extended times.

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