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Relative Opioid Efficacy Is Determined by the Complements of the G Protein-Coupled Receptor Desensitization Machinery

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ABSTRACT

G protein-coupled receptor regulation by G protein-coupled receptor kinases and β-arrestins can lead to desensitization and subsequent internalization of the receptor. In in vitro and cellular systems, β-arrestins do not seem to play a major role in regulating μ opioid receptor (μOR) responsiveness. Removal of the *βarrestin2* (*βarr2*) gene in mice leads paradoxically to enhanced and prolonged μOR-mediated antinociception. The βarr2 knockout (βarr2-KO) mice also fail to develop morphine antinociceptive tolerance in the hot-plate test, further indicating that the βarr2 protein plays an essential role in μOR regulation in vivo. In this study, the contribution of βarr2 to the regulation of the μOR was examined in both human embryonic kidney 293 cells and in βarr2-KO mice after treatment with several opiate agonists. A green fluorescent protein tagged βarr2 was used to assess receptor-βarr2 interactions in living cells. Opiate agonists that induced robust βarr2-green fluorescent protein translocation produced similar analgesia profiles in wild-type and βarr2-KO mice, whereas those that do not promote robust βarr2 recruitment, such as morphine and heroin, produce enhanced analgesia in vivo. In this report, we present a rationale to explain the seemingly paradoxical relationship between β-arrestins and μOR regulation wherein morphine-like agonists fail to promote efficient internalization and resensitization of the receptor.

Morphine is the prototypical example of the opiate class of drugs, which regulate analgesia by binding to the μOR. The regulation of this heptahelical GPCR is of particular relevance to pain research and represents a potentially beneficial pharmaceutical target. The μOR is subject to regulation by many different means, including classic desensitization mechanisms. Most GPCR agonists rapidly induce desensitization of their cognate receptors by promoting a receptor conformation susceptible to phosphorylation by GPCR kinases (GRKs). This in turn facilitates the subsequent binding of β-arrestins to the phosphorylated receptors. In this desensitization paradigm, the β-arrestin binding acts as a damper for further signaling by preventing further G protein coupling, which ultimately leads to waning of receptor signaling and a diminished physiological response (Bohn et al., 1999; Kohout et al., 2001; Ahn et al., 2003). Many μOR agonists promote this pattern of regulation; however, the most historical and commonly used agonist, morphine, seems to be the exception to the rule.

Several reports over the past decade have focused on this aspect of μOR regulation. Keith et al., (1996) observed that murine μORs, when transfected into human embryonic kidney (HEK) 293 cells, rapidly internalized upon addition of etorphine to the media but did not when morphine was the agonist. The failure of morphine, an agonist with moderately high affinity and efficacy to the μOR, to promote receptor internalization was an unusual observation, not only because many other members of the GPCR family undergo internalization in the presence of agonist but also because other agonists, such as etorphine and the enkephalin analog [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin, do promote the robust internalization of the same receptor (Arden et al., 1995; Keith et al., 1996, Sternini et al., 1996; Keith et al., 1998; Whistler and von Zastrow, 1998; Zhang et al., 1998). Further studies have shown that the association of β-arrestins with the μOR is also specific for the agonists used; morphine did not lead to a detectable translocation of GFP-tagged β-arrestins to the plasma membrane in HEK cells transfected

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ABBREVIATIONS: μOR, μ-opioid receptor; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; WT, wild-type; KO, knockout; βarr, β-arrestin; GFP, green fluorescent protein; GRK, G protein-coupled receptor kinase; MEM, minimal essential medium; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; MEF, mouse embryonic fibroblast.

with the rat μOR, whereas etorphine promoted recruitment of β-arrestins to the receptor (Zhang et al., 1998). The translocation of β-arrestins to the membrane after agonist stimulation has been shown to be an indicator of activation of many different GPCRs (Barak et al., 1997; Oakley et al., 1999; Mundell and Benovic, 2000). Therefore, it is surprising that morphine, which has been known since the discovery of the μOR to be a potent agonist for this receptor, does not seem to induce this robust response.

Morphine has been shown to mediate its analgesic effects, as well as many of its other physiological effects in mice, via the μOR receptor, because μOR knockout mice no longer respond to the drug (Kieffer, 1999). When mice that lack β-arrestin-2 were tested for their responses to morphine, one might have expected, based on the cell culture observations, that the removal of this regulatory element, which did not seem to interact with the morphine-activated μOR, would have little impact on the actions of morphine in these mice. It was surprising that the opposite proved to be true in that all of the physiological effects of morphine tested so far have revealed differences between the WT and βarr2-KO mice. In particular, morphine-induced antinociception is enhanced in both the hot plate and tail-flick tests (Bohn et al., 1999, 2002). The βarr2-KO mice experience less locomotor activation after morphine and greater morphine-paired reinforcement compared with WT control mice (Bohn et al., 2003). The coupling of the μOR to G proteins was also enhanced in several regions of the nervous system (periaqueductal gray, brainstem, and spinal cord) (Bohn et al., 1999, 2000, 2002). Therefore, although βarr2 seems in cellular assays to show very little interaction with the μOR, the physiological evidence suggests otherwise. In this study, we have sought to address the apparent paradoxical relationship between μOR activation and β-arrestin–mediated desensitization in the presence of different opiate agonists. The βarr2-KO mice were treated with agonists that lead to the translocation of βarr2-GFP, and their antinociceptive responses were compared with those induced by agonists that do not promote translocation (i.e., morphine). Herein, we provide evidence that the morphine-activated μOR does indeed interact with βarr2, but not βarr1, and although this interaction possesses a low affinity, it is very essential for the regulation of the morphine-bound receptor.

Materials and Methods

Mice. βarr2-KO mice and their littermate control WT mice were generated by heterozygote breeding that have been maintained over the last 9 years (Bohn et al., 1999). βarr1-KO mice were originally generated on a mixed strain background (Conner et al., 1997) and were backcrossed for 7 generations onto C57BL6 mice (Jackson Labs, Bar Harbor, ME) before use in these experiments. βarr1-KO mice and their WT littermates were also generated by heterozygous breeding. Male mice (20–30 g), between the ages of 3 and 6 months, were used only once for each dose and each drug tested. All narcotics were provided by the National Institute on Drug Abuse and were prepared in sterile saline and injected s.c. at 10 μl/g. All experiments were conducted in accordance with the National Institutes of Health guidelines for the care and use of animals and with an approved animal protocol from the Duke University Animal Care and Use Committee.

Antinociception. The standard hot plate test was used to determine antinociceptive responses as described previously (Bohn et al., 1999). The plate was kept at 56°C and the maximum time allowed on the plate was limited to 30 s. The "maximum possible effect" is expressed as: 100% × [(drug response time – basal response time)/(30-s basal response time)] = percentage of maximum possible effect. Dose response curves were generated by single doses of each drug on mice that had not been previously used.

Plasmid DNA. Construction of plasmids containing the N-terminal hemagglutinin epitope-tagged mouse μOR were generated from mouse μOR-1 cDNA provided by Dr. G. Pasternak (Pan et al., 1999). β-arrestin-2 or β-arrestin-1 with GFP conjugated to the carboxyl terminus was described previously (pS65TGFP-N3-βarr1, Zhang et al., 1999; pS65TGFP-N3-βarr2 (Barak et al., 1997).

Cell Culture and Transfection. HEK-293 cells were from the American Type Culture Collection (Manassas, VA). Cells were grown in Eagle's minimal essential medium (MEM; Mediatech, Herndon, VA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum and gentamicin (100 μg/ml). Cells stably expressing GRK2 express roughly 5-fold the GRK2 as normal HEK-293 cells (Bektas et al., 2003; L. Bohn, unpublished observations). Transient transfections were performed by electroporation using the Gene Pulser II system (Bio-Rad, Hercules, CA). Cells were resuspended in MEM + 10% fetal bovine serum + 5 mM BES at a concentration of 4×10^6 cells in 0.5 ml per 0.4-cm cuvette with 6 μg of mμOR cDNA + 1 μg of βarr2-GFP cDNA. A single pulse at 220 V, 1 μF, was used and produced a time constant around 20 ms. Additional complete media without BES was added immediately to the cells, and then cells were plated in collagen-coated 35-mm glass-bottomed culture dishes at approximately 0.5×10^6 cells/dish. Thirty minutes before confocal microscopy, media was replaced with MEM lacking phenol red and serum. Mouse embryonic fibroblasts (MEFs) were generated from double-knockout βarr1/βarr2 mouse embryos that were derived by breeding mice heterozygous for both βarr1 and βarr2 as previously characterized (Kohout et al., 2001). The MEFs were transiently transfected by electroporation in a manner similar to that described for HEK cells (10 μg mμOR cDNA + 1 μg βarr2-GFP cDNA per 3×10^6 cells), and Dulbecco's modified Eagle's media (DMEM; Mediatech) + 10% fetal bovine serum was used for maintenance of the line.

Confocal Microscopy and β-Arrestin-GFP Translocation. Confocal microscopy was performed on a Zeiss laser-scanning confocal microscope (LSM-510 Meta) as described previously. Agonists were added directly to the culture media. Images were collected sequentially using single-line excitation (488 nm). The β-arrestin-GFP translocation experiments have been designed such that the endogenous arrestin complement is comparable with the β-arrestin-GFP complement and the receptor complement is larger than both of them. Because the complement of endogenous arrestins remains fixed throughout all the different drug treatments the behavior of the receptor reflects the efficacy of the drug in inducing β-arrestin-GFP translocation to the plasma membrane in the presence of a particular complement of GRKs in a qualitative manner (Oakley et al., 2000; Barak et al., 2003).

Statistical Analysis. Animal behavior responses were analyzed by two-way analysis of variance and compared for factors of genotype as well as the effect of either time or dose on the experiment. Interactions are also indicated. For dose response analyses, upon achieving significance in the two-way analysis of variance, a Bonferroni post hoc analysis was performed at individual doses. Statistics were performed using Prism software (GraphPad Software, Inc., San Diego, CA).

Results

The translocation of βarr2-GFP to the plasma membrane GPCRs has been routinely used as an indicator of agonist-induced receptor activation and desensitization (Barak et al., 1997; Oakley et al., 1999; Johnson et al., 2003). HEK-293 cells were transfected with mouse μOR and expression levels

ranged between 500 and 700 fmol/mg of protein in whole-cell binding assays using [^{3}H]naltrexone. As reported previously for the rat μOR (Zhang et al., 1998), morphine does not provoke a robust translocation of βarr2-GFP to the plasma membrane, whereas etorphine, fentanyl, and methadone promote the rapid and robust recruitment of βarr2 to the cell surface (Fig. 1). Heroin, which is structurally similar to morphine and is spontaneously hydrolyzed to 6-monoacetomorphine in solution (Inturrisi et al., 1983; Selley et al., 2001), does not lead to βarr2-translocation in HEK cells. Morphine and heroin were unable to produce greater translocation when the incubation period was extended to 30 or 60 min (data not shown). The concentrations of agonist chosen for these experiments were based upon the previous literature as well as to account for the differences in drug efficacy and to approach an equipotent treatment profile.

These findings, which have been reported previously (Whistler and von Zastrow, 1998; Zhang et al., 1998), might lead to the assumption that the βarr2 molecule plays very little role in the regulation of the morphine-activated μOR. However, when we observed that βarr2-KO mice display profound behavioral and biochemical phenotypes upon morphine treatment, a reassessment of this initial possibility proved necessary. The most pronounced phenotype observed in the βarr2-KO mice described to date is the enhanced antinociception after morphine treatment (Bohn et al., 1999, 2000, 2002). Therefore, other opioid agonists that promoted robust βarr2-GFP translocation in vitro were evaluated in the βarr2-KO for their antinociceptive properties. For comparison, we have repeated the hot plate antinociceptive tests with morphine that we reported previously (Bohn et al., 1999; Fig. 2A). The βarr2-KO mice display enhanced and prolonged antinociception after morphine treatment, and this enhanced sensitivity is reflected in a dose-dependent manner as well (Bohn et al., 1999, 2000; Fig. 2A). It was surprising that, upon administration of equipotent doses of etorphine, fentanyl, or methadone, the WT and βarr2-KO mice responded to the same extent, suggesting that the loss of βarr2 has no influence on the responsiveness of the βarr2-KO mice to these drugs (Fig. 2, B–D). Heroin, however, which can produce morphine upon metabolism in brain, generated a response profile very similar to that of morphine's in the βarr2-KO mice, where again there is a prolonged and enhanced antinociceptive response (Fig. 2E). Although the pharmacokinetics of fentanyl and etorphine differ greatly from morphine, methadone has a very similar pharmacokinetic profile and therefore can be most directly compared with the effects induced by morphine.

Morphine can induce βarr2-GFP translocation when GRK2 is overexpressed in HEK-293 cells, presumably by overriding the low degree of phosphorylation of the receptor that occurs upon binding morphine (Zhang et al., 1998). The overexpression of GRK2 promotes more robust translocation of each of the agonists used in the present study, as well as leading to morphine- and heroin-induced translocation (Fig. 3). Although the overexpression of GRK is sufficient to induce the translocation, the overexpression of βarr2-GFP alone (as seen in Fig. 1A, where βarr2-GFP is expressed; Zhang et al., 1998) is not sufficient, suggesting that the limiting step in μOR/βarr2 interactions is the phosphorylation of the receptor. GRK2-HT, GRK3-KO, GRK4-KO, GRK5-KO, and GRK6-KO mice have not revealed enhanced antinociceptive profiles after morphine treatment, suggesting either that GRK2 is the specific kinase involved and that heterozygotes express enough of the kinase for normal function or that more than one of the GRK enzymes are responsible for regulating the μOR (Bohn et al., 2004).

In HEK-293 cells, the translocation of βarr2-GFP to the morphine-activated μOR is barely detectable; however, we must consider that these cells express both βarr1 and βarr2 endogenously. Therefore, the endogenous β-arrestins could potentially compete with the recruitment of the GFP-labeled βarr2 that is being assessed. To determine the translocation of βarr2-GFP in the absence of endogenous competitive unlabeled β-arrestins, translocation studies were carried out in cells lacking both endogenous β-arrestins. Mouse embryonic fibroblasts were generated from double-knockout βarr1/βarr2 mouse embryos that were derived by breeding mice heterozygous for both βarr1 and βarr2, as characterized previously (Kohout et al., 2001). These cells were transfected with the μOR and either βarr2-GFP or βarr1-GFP, and cells were then treated with either etorphine or morphine. In cells that lack an endogenous population of β-arrestins, the translocation of βarr2-GFP is preserved upon etorphine treatment and now becomes detectable after morphine treatment (Fig. 4, left). It is interesting that although βarr1-GFP translocates to the etorphine-stimulated μOR, morphine does not lead to the recruitment of βarr1-GFP (Fig. 4, right). The figures shown are representative of more than five different transfections (at least three plates per transfection) and extensive scanning of each plate in search of cells manifesting the translocation. Although transfection efficiencies were

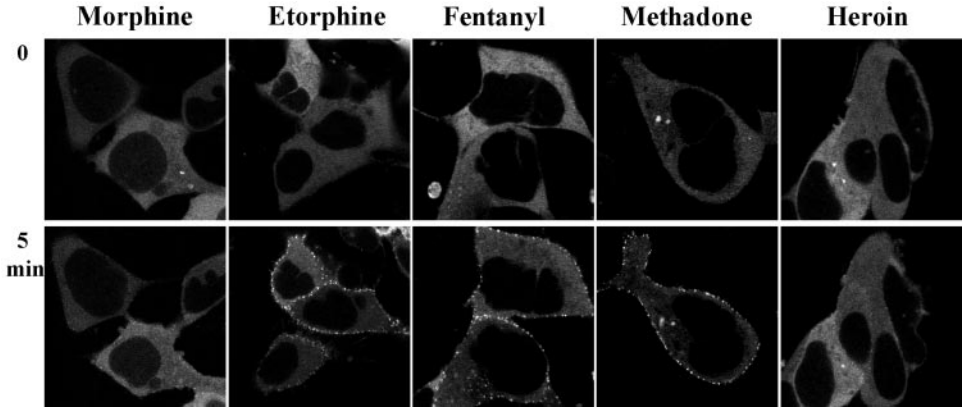

Fig. 1. βarr2-GFP translocation to μORs in HEK-293 cells. HEK-293 cells expressing approximately 400 to 700 fmol/mg of protein mouse μOR were grown collagen-coated, 35-mm, glass-bottomed culture dishes. Drugs were added to serum and phenol-red free MEM in the following concentrations: morphine (5 μM), etorphine (100 nM), fentanyl (100 nM), methadone (1 μM), and heroin (5 μM). A warming stage was used to maintain the cells at 37°C. The translocation of βarr2-GFP from cytosol to membrane is shown at 5 min. The same cells are shown before an after stimulation for comparison.

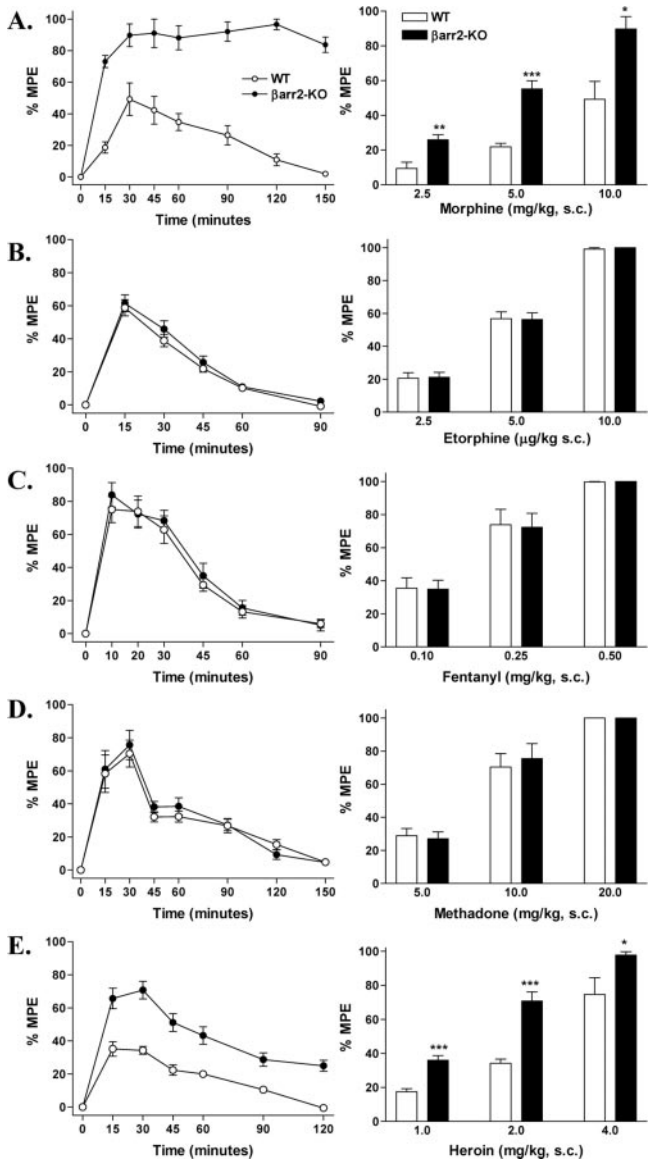

Fig. 2. Hot plate antinociception in WT and βarr2-KO mice. The hot plate analgesia meter was set at 56°C, and an artificial ceiling of 30 s was used to prevent damage to tissue. Mice were assessed for their paw withdrawal latency (paw licking) over time (left side) and at different drug doses (right side). A, left, morphine (10 mg/kg, s.c.) induced enhanced and prolonged antinociception in the βarr2-KO mice ($p < 0.0001$; $n = 5$ WT, KO). Right, morphine, after 30 min, induces enhanced antinociception at different doses (2, 5, and 10 mg/kg, s.c,) ($p < 0.0001$, $n = 5$–8 WT, KO) A Bonferroni post hoc analysis reveals significant differences between the genotypes at the individual doses of 5 and 10 mg/kg, s.c.: *,$p < 0.05$; **, $p < 0.001$. B, left, etorphine (5 μg/kg, s.c) did not reveal difference between the genotypes over time ($p = 0.1130$, $n = 13$ WT, 14 KO). Right, there were no differences between the genotypes at different doses of etorphine when assessed after 15 min ($p = 0.8697$; $n = 5$–13 WT, 5–14 KO). C, left, fentanyl (0.25 μg/kg, s.c) did not reveal difference between the genotypes over time ($p = 0.3875$, $n = 6$ WT, KO). Right, at 20 min, there were no differences between the genotypes at different doses of fentanyl ($p = 0.9089$, $n = 5$–6 WT, KO) D, left, methadone (10 mg/kg, s.c) did not reveal difference between the genotypes over time ($p = 0.5337$, $n = 5$ WT, KO). Right, at 30 min, there were no differences between the genotypes at different doses of methadone ($p = 0.8045$, $n = 5$ WT, KO). E, left, heroin (2 mg/kg, s.c.) induced prolonged antinociception in the βarr2-KO mice ($p < 0.0001$, $n = 13$ WT, 14 KO). Right, heroin, after 30 min, induces enhanced antinociception at different doses (1, 2, and 4 mg/kg, s.c,) ($p < 0.0001$, $n = 6$–13 WT, 6–12 KO) A Bonferroni post hoc analysis reveals significant differences between the genotypes at the individual doses of 2 and 4 mg/kg, s.c.: *, $p < 0.05$; **, $p < 0.001$.

very low in the MEFs, the positive results obtained with etorphine in each of the transfected batches of cells served as an internal control to demonstrate that receptor is expressed. Note also that the βarr1/βarr2-KO MEFs do not detectably express more of any particular GRK than found in the HEK-293 cell line (data not shown).

These transfected cell studies suggest that the μOR interacts with βarr2 only when morphine is the agonist; when etorphine is used, however, the receptor seems to interact with both β-arrestin-1 and -2. The lack of differences observed in the etorphine-treated mice supports this concept if we assume that in the mice lacking βarr2, βarr1 suffices to regulate the receptor. To test the contribution of βarr1 to the regulation of the morphine-bound μOR, we tested antinociceptive responses in mice lacking βarr1 upon morphine treatment. Both the βarr1-KO and their littermate control animals displayed the same antinociceptive profiles, suggesting that unlike βarr2, the removal of βarr1 has very little effect on morphine-induced antinociception (Fig. 5). This further supports the concept that βarr1 is essential in regulating the μOR under many conditions yet seems to play very little role in regulating the morphine-bound receptor.

Discussion

In this study, we examined the ability of several different μOR agonists to induce antinociception in βarr2-KO mice and have correlated this with the agonist's ability to recruit βarr2-GFP to the receptor. Although morphine and heroin lead to very little βarr2 recruitment under normal levels of GRK expression in HEK-293 cells, the importance of βarr2 in regulating the behavioral response to morphine is readily apparent when the molecule is genetically ablated in mice. For ligands that lead to the robust recruitment of βarr2 to the receptor (i.e., etorphine, fentanyl, and methadone; Fig. 1), the loss of βarr2 does not have an effect on the behavioral responsiveness in vivo after administration of these drugs. The overexpression of GRK2 can promote βarr2-GFP translocation to the morphine or heroin bound receptor. Furthermore, the removal of the endogenous complement of β-arrestins facilitates the visualization of the recruitment of βarr2-GFP but not βarr1-GFP to the morphine-bound receptor. When morphine is tested for antinociceptive properties in the mice lacking βarr1, no difference between the genotypes can be detected. Taken together, these data suggest that although the μOR may bind βarr2 in the presence of all the agonists studied to desensitize, βarr2 becomes a limiting component of the system when morphine is bound. Coupled with previous observations that morphine does not lead to μOR internalization (Arden et al., 1995; Keith et al., 1996; Sternini et al., 1996; Whistler and von Zastrow, 1998; Zhang et al., 1998), it would seem that this weak interaction with βarr2, and lack of interaction with βarr1, may not allow the receptor to traffic to coated pits as efficiently when morphine or heroin is bound compared with etorphine, fentanyl, and methadone.

The results suggest a model of μOR behavior in the presence of heroin and morphine in which receptors remain intermittently desensitized at the plasma membrane and become incapable of trafficking-dependent resensitization. These conclusions are a consequence of the following. The canonical description of GPCR desensitization depicts GRK phosphorylated receptors as associating tightly enough with

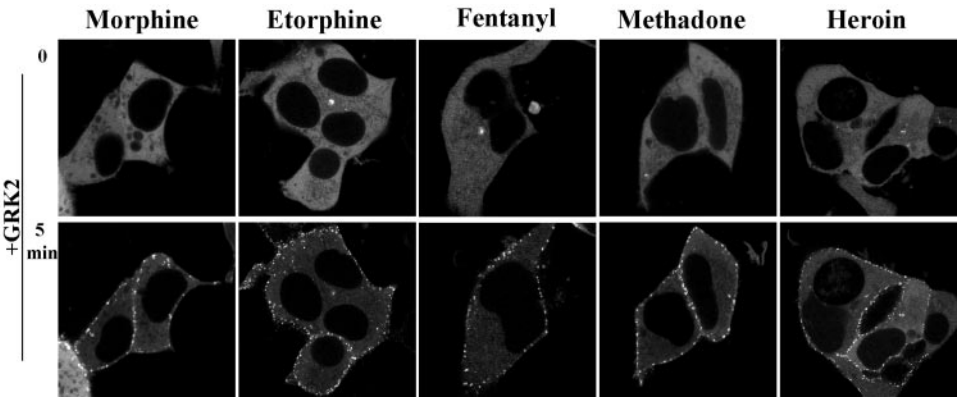

Fig. 3. βarr2-GFP translocation to μORs in HEK-293 cells stably overexpressing GRK2. HEK-293 stably transfected with GRK2 and expressing between 500 and 700 fmol/mg of protein μOR were treated as described in Fig. 1. Unstimulated cells are shown on the left and stimulated translocation is shown after a 5-min stimulation at 37°C on the right.

β-arrestins to redistribute with them from signaling complexes to clathrin-coated pits, and this redistribution is correlated with a depletion of βarr2-GFP from the cytosol. Despite the observations that the μOR does not phosphorylate well in the presence of morphine and that β-arrestin remains predominantly cytosolic, the enhanced signaling of the μOR in βarr2 knockout mice indicates that agonist-activated μORs do interact with βarr2 well enough to uncouple them from G-protein signaling. The relative inability of the μOR to translocate β-arrestins upon exposure to morphine compared with etorphine suggests this interaction is relatively weak and reversible at the plasma membrane.

Reversibility of the β-arrestin–μOR interaction would have major consequences on short- (seconds to minutes) and long-term (hours to days) signaling. Compared with more efficacious agonists such as etorphine, morphine leads to very little down-regulation of μORs (Yabaluri and Medzihradsky, 1997; Stafford et al., 2001) and receptors, therefore, probably remain trapped at the plasma membrane. After long-term morphine treatment, however, μORs have been shown to become uncoupled from their G proteins (Sim et al., 1996; Elliott et al., 1997; Yabaluri and Medzihradsky, 1997; Bohn et al., 2000) and to lose their ability to inhibit adenylyl cyclase activation (Noble and Cox, 1996), demonstrating that morphine does lead to μOR desensitization. Our observation that the μORs remain coupled after long-term morphine treatment in mice that lack βarr2 suggests that although receptor numbers do not decrease, βarr2 plays a role in desensitizing the morphine-bound receptor (Bohn et al., 2000).

The limitation of the receptor-β-arrestin interaction may ultimately be responsible for the receptor fate after activation. If the receptor is able to internalize, a process that may be facilitated by its interaction with βarr1 or βarr2, it then has the potential to be recycled back to the plasma membrane. This would result in less overall desensitization because there would be a continuous replenishment of active receptor at the membrane. Therefore, agonists that could promote more robust internalization and, importantly, resensitization, would be likely to lead to a less profound state of desensitization or "tolerance". In both cell culture and animal studies, the lower efficacy agonists (morphine-like) induce more desensitization and tolerance, respectively, than the high efficacy agonists (etorphine) (Duttaroy et al., 1995; Yabaluri and Medzihradsky, 1997; Law et al., 2000). These observations are directly correlated with the agonists ability to recruit β-arrestin and internalize the receptors whereby agonists such as methadone and fentanyl, which promote

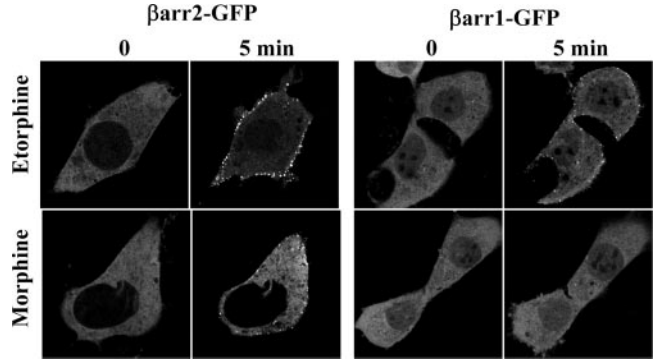

Fig. 4. βarr2-GFP translocation in βarr1/βarr2 double-knockout mouse embryonic fibroblasts. Double KO MEFs were transfected with mouse μOR as well as either βarr2-GFP (left) or βarr1-GFP (right). Cells were treated with etorphine (100 nM, top) or morphine (5 μM, bottom) for 5 min at 37°C as indicated. The relative level of β-arrestin-GFP fluorescence (in intensity per pixel) was measured using the "range of interest" analysis provided with the Zeiss LSM-510 confocal microscope software. Settings on the microscope (laser power, pinhole size, detector gain, amplifier offset, amplifier gain, etc.) were held constant within and between experiments to ensure that cells expressing similar amounts of the different β-arrestin isoforms were compared (Oakley et al., 2000).

strong associations with β-arrestins and lead to μOR internalization, have a much lower tolerance liability than agonists such as morphine or heroin, which do not lead to receptor internalization yet do lead to the rapid development of tolerance (Duttaroy et al., 1995; Zhang et al., 1998; Finn and Whistler, 2001).

Morphine and its derivatives are unusual among GPCR agonists in their ability to signal robustly without producing an equally similar robust phosphorylation of the receptor. GPCR homologous desensitization normally should occur over a period of seconds to a few minutes at most. Morphine however, despite its potent agonist properties in vivo, fails in vitro to promote efficient homologous desensitization of its cognate receptor on similar time scales through GRK/β-arrestin mediated mechanisms. Although morphine lacks the ability in the short term to robustly activate these mechanisms at the μOR, our data demonstrating an induction of βarr2 translocation with GRK overexpression and a blunting of analgesia in wild-type mice compared with βarr2-KO animals indicate that persistent morphine exposure must ultimately induce a regulatory GRK-mediated β-arrestin response. Thus, in wild-type animals, a short-term insensitivity but long-term susceptibility of the μOR to morphine-induced GRK phosphorylation might be expected to produce compensatory physiological responses unlike or

more extreme than those observed with conventional desensitizing agonists. For instance, there is evidence that long-term opiate treatment can lead to the up-regulation of GRK levels in brain (Terwilliger et al., 1994, Ozaita et al., 1998; Hurle, 2001). The compensatory elevations in levels of GRK secondary to extended receptor activation may have a relatively greater dampening effect on morphine-induced μOR-signaling than methadone- or etorphine-induced signaling, because GRK phosphorylation does not seem to be rate-limiting for these latter two compounds. Therefore, a relatively greater decrease in receptor signaling or a tolerance to repeated challenges of morphine (see Fig. 6), may be caused in part by elevations in the complement of GRKs secondary to the unusual kinetics of morphine-regulated μOR phosphorylation.

This would cover both direct GRK-dependent (signaling side more turned off) and indirect (resensitization side more turned on, GRK-dependent or not) mechanisms of tolerance. In addition, the very limited β-arrestin/receptor interaction obtained with morphine can easily be enhanced to resemble that of etorphine by simply overexpressing GRK2, thereby increasing the phosphorylation of the receptor and facilitating its internalization and its ability to be resensitized. Therefore, we propose that the failure of morphine-like agonists to effectively enable receptor/β-arrestin trafficking in coated pits, and subsequent resensitization, except in the presence of elevated GRKs, contributes to the induction of opiate tolerance.

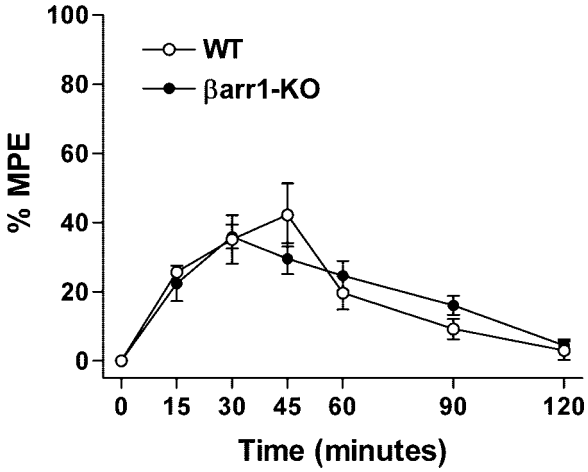

Fig. 5. Morphine antinociception in mice lacking βarr1. Male βarr1-KO mice were treated with morphine (10 mg/kg, s.c.), and hot plate antinociception was assessed over time as described in Fig. 2. Although morphine induced antinociception in both groups of mice, there was no difference in the extent of this response between genotypes (p = 0.9144, n = 5 WT, 6 KO).

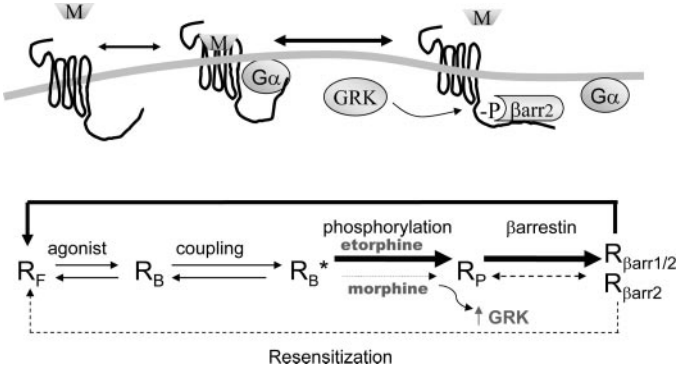

Fig. 6. Model depicting the agonist-specific fate of μOR regulation. The unbound receptor (free receptor, R_F) binds agonist to become bound receptor (R_B). This activation triggers the coupling of receptor and G protein to obtain the "activated receptor" state (R*). The activated receptor then recruits GRK and becomes phosphorylated (R^*_P). However, the extent of the phosphorylation is agonist-dependent; agonists like etorphine, fentanyl, or methadone produce much greater phosphorylation (indicated by larger arrow) than morphine-like compounds. Concurrent with the degree of phosphorylation, the receptor binds β-arrestins ($R\beta_{arr}$), which determines the extent of resensitization—a process that is probably dependent upon internalization, dephosphorylation, and recycling of the receptor back to the plasma membrane.

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