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Morphine Side Effects in β-Arrestin 2 Knockout Mice

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ABSTRACT

Morphine is a potent analgesic, yet, like most opioid narcotics, it exerts unwanted side effects such as constipation and respiratory suppression, thereby limiting its clinical utility. Pharmacological approaches taken to preserve the analgesic properties, while eliminating the unwanted side effects, have met with very limited success. Here, we provide evidence that altering μ opioid receptor regulation may provide a novel approach to discriminate morphine's beneficial and deleterious effects in vivo. We have previously reported that mice lacking the G protein-coupled receptor regulatory protein, β-arrestin 2, display profoundly altered morphine responses. β-Arrestin 2 knockout mice have enhanced and prolonged morphine analgesia with very little morphine tolerance. In this report, we examine whether the side effects of morphine treatment are also augmented in this animal model. Surprisingly, the genetic disruption of opioid receptor regulation, while enhancing and prolonging analgesia, dramatically attenuates the respiratory suppression and acute constipation caused by morphine.

G protein-coupled receptors (GPCRs) are subject to regulation, and the balance of GPCR activation and desensitization can act in concert to determine the overall degree of receptor responsiveness. Upon activation, GPCRs are phosphorylated by GPCR kinases (GRKs) and subsequently bind β-arrestins, which prevent further coupling of the receptor and the G protein (Luttrell and Lefkowitz, 2002). GPCRs can then be internalized and are either recycled to the plasma membrane or degraded. We have previously demonstrated the importance of the actions of GRKs and β-arrestins for determining GPCR responsiveness in vivo (for review, see Bohn et al., 2004b; Gainetdinov et al., 2004). For example, in the absence of GRK5, mice display enhanced responsiveness to muscarinic agonists, and GRK6-knockout (KO) mice are supersensitive to dopaminergic agents (Gainetdinov et al., 1999, 2003; Walker et al., 2004). Furthermore, morphine effects are dramatically altered in mice lacking β-arrestin 2 (βarr2), but not βarr1, indicating a degree of specificity among the six GRKs and two β-arrestins in the regulation of certain classes of GPCRs (Bohn et al., 2004a,b).

The altered morphine responses in the βarr2-KO mice have been extensively studied by our group, and the most prominent behavioral distinction is the overall enhancement of physiological responsiveness to morphine. Morphine-induced hypothermia, analgesia (tail-flick and hot-plate), dopamine release, and drug reinforcement (conditioned place preference) have been well documented, and these behaviors correlate with our observations that the μ opioid receptor (μOR) displays more agonist-induced G protein coupling in the βarr2-KO mice (Bohn et al., 1999, 2000, 2002, 2003). Recently, we have found that the basal level of μOR-G protein coupling in certain brain regions is also elevated in mice lacking β-arrestin 2 (D. Wang, L. Bohn, and W. Sadée, unpublished data). Antinociceptive tolerance to morphine did not occur in the hot-plate test in these mice yet did develop, although to a lesser extent, in the tail-flick studies (Bohn et al., 2002). The lack of morphine tolerance in the hot-plate test could be correlated to a loss of μOR desensitization in brainstem and periaqueductal gray brain regions (Bohn et al., 2000).

Although many of the augmented morphine-induced behaviors observed in the βarr2-KO mice might be explained by enhanced μOR activity or lack of μOR desensitization, other behaviors do not fit this scenario. Morphine-induced locomotor activity is actually decreased in the βarr2-KO mice, even though striatal extracellular dopamine levels are simultaneously increased (Bohn et al., 2003). Furthermore, upon chronic morphine treatment, both wild-type (WT) and βarr2-KO mice display a similar extent of naloxone-precipitated withdrawal, indicating that both

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ABBREVIATIONS: GPCR, G protein-coupled receptor; GRK, GPCR kinase; KO, knockout; βarr, β-arrestin; μOR, μ opioid receptor; WT, wild type; GI, gastrointestinal; ANOVA, analysis of variance.

groups of mice develop morphine-induced physical dependence (Bohn et al., 2000). These observations indicate that not all of morphine's actions are enhanced in the βarr2-KO mice; therefore, in this study, we asked whether the severity of morphine-induced side effects would be altered in these animals.

Morphine induces several side effects in humans as well as in rodents. At low to moderate doses, the inhibition of gastrointestinal transit occurs, and constipation is a concurrent complaint among patients treated with opiates. At higher doses, morphine induces a decrease in respiratory frequency, and this can lead to critical consequences, especially in the case of overdose or when opiates are used postsurgically. Here, we have evaluated the ability of morphine to inhibit gastrointestinal transit and to induce respiratory suppression in the βarr2-KO mice compared with their WT counterparts. At several doses tested in each paradigm, it becomes clear that the side effects of morphine are not enhanced in mice lacking β-arrestin 2, but rather, they are diminished.

Materials and Methods

Mice

Male mice (20–30 g), between the ages of 3 to 6 months, were used only once for each dose and each drug tested. βarr2-KO mice and their littermate control WT mice were generated by heterozygote breeding that has been maintained over the last 10 years wherein efforts have been taken to avoid breeding of closely related mice (first described by Bohn et al., 1999). To increase mouse numbers, some studies employed a small number (less than 30%) of animals derived from first generation homozygous crossing (homozygous breeders are offspring of heterozygous parents). The data obtained from these animals did not differ from those obtained in heterozygously bred animals and were combined with this population. All experiments were conducted in accordance with the National Institutes of Health guidelines for the care and use of animals and with approved animal protocols from the Duke University Animal Care and Use Committee as well as from The Ohio State University Animal Care and Use Committee.

Drugs

Morphine sulfate (Sigma-Aldrich, St. Louis, MO) was prepared in sterile saline (0.9%). Loperamide (Sigma-Aldrich) was prepared in 20% (2-hydroxypropyl)-β-cyclodextrin (Sigma-Aldrich) in water for solubility; in loperamide studies, the vehicle was 20% (2-hydroxypropyl)-β-cyclodextrin in water. All compounds were injected s.c. at 10 μl/g at the back of the neck.

Gastrointestinal Transit Studies

Fecal Boli Accumulation. Mice were provided food and water ad libitum prior to the test. Mice were treated with saline or morphine and then individually placed in a Plexiglas box with a wired mesh or grid floor. Fecal boli were collected in a metal tray and weighed at 1-h intervals.

Small Intestinal Transit. Gastrointestinal transit of the small intestine was measured using the charcoal meal test previously described with some modification (Roy et al., 1998). Forty-eight hours prior to testing, a mesh wire insert was placed in the bottom of each cage to suspend the mice above their bedding and prevent the ingestion of feces or bedding. Animals were first habituated to the modified cage for 24 h in the presence of food and water and then were fasted for 24 h with free access to water. Mice were given an injection of saline (10 μl/g s.c.) or morphine (1, 3, or 10 mg/kg s.c.) 20 min prior to an oral gavage of a charcoal meal containing a 5% aqueous suspension of charcoal (Sigma-Aldrich) in a 10% gum arabic (Acros Organics, Fairlawn, NJ) solution at a volume of 10 μl/g b.wt. At 30 min, animals were sacrificed by cervical dislocation, and the small intestine, from the jejunum to the cecum, was dissected and the mysentry removed. The distance traveled by the leading edge of the charcoal meal was measured relative to the total length of the small intestine, and the percentage of gastrointestinal transit for each animal was calculated as follows: percentage transit = [(charcoal distance)/(small intestine length)] × 100.

Large Intestinal Transit. Gastrointestinal transit of the colon was measured using the bead expulsion test as previously described with some modification (Raffa et al., 1987). Mice were habituated and fasted in the same manner as described for the small intestinal transit studies above. Mice were then given an injection of vehicle, morphine (1, 3, 10, or 20 mg/kg s.c.), or loperamide (0.3, 0.6, or 1.0 mg/kg s.c.). At 20 min postinjection, a 3-mm glass bead (Fisher Scientific Co., Pittsburgh, PA) was inserted 2 cm into the distal rectum using 2-mm round, flexible, plastic tubing. Mice were individually placed into small, Plexiglas chambers (5.5 inches × 5 inches × 6 inches) for observation, and the time to bead expulsion was recorded for each animal. On the rare occasion that mice did not expel their bead without manipulation or produced feces before expelling the bead the subject was excluded from the study.

Respiratory Studies

Whole-body plethysmography was performed in a noninvasive manner similar to methods previously described (Drorbaugh and Fenn, 1955; Hamelmann et al., 1997; Matthes et al., 1998; Walker and Jennings, 1998; Dahan et al., 2001; Romberg et al., 2003). The barometric plethysmograph apparatus (Buxco, Troy, NY) has 12 chambers and allowed for the simultaneous monitoring of several animals of each genotype in parallel. The integrated software analysis was used for calculation of the respiratory frequency and tidal volumes (BioSystem XA software, PLY3211 version 2.1; Buxco Electronics, Sharon, CT). For the calculation of respiratory frequency, rejection criteria were set so that only pressure changes due to respiration were used. For the calculation of tidal volume, mouse body temperature was measured in a separate cohort of mice. Although βarr2-KO mice display more hypothermia at 10 mg/kg morphine than WT mice (Bohn et al., 1999), these genotypic differences were not preserved at the higher doses of morphine, presumably due to a ceiling effect (data not shown). Therefore, the average body temperatures at each dose, along with chamber temperature, were supplied to the software for calculations of tidal volumes. Mice were habituated to the chamber for 30 min prior to injection. Each dose was assessed in five WT and five βarr2-KO mice simultaneously. Analysis of respiratory frequency over the 30-min habituation period revealed that the last 15 min produced relatively steady respiratory frequency. Therefore, breathing rates in this period were used to normalize the drug-induced effects over the 2.5-h test period for calculation of average respiratory frequency to be compared at several doses.

Statistical Analysis

Data were analyzed using GraphPad Software version 3.0 for Windows (GraphPad Software Inc., San Diego, CA). The specific tests used are indicated within the text of the figure legends.

Results

Opioid receptors have been shown to be critical in mediating the inhibition of gastrointestinal transit (Reisine and Pasternak, 1996). Therefore, we asked whether morphine-induced acute constipation is enhanced or prolonged in the βarr2-KO mice. Morphine's effect on gastrointestinal function was initially assessed by measurement of fecal boli production over time wherein the boli were collected and weighed over a 6-h period. Mice were housed together prior to

the test and were provided food and water ad libitum. To assure that both genotypes were eating, food consumption was monitored for grams of food consumed in 24 h normalized per mouse when a single cage housed three to five mice per cage, and the data were then averaged for three cages containing each genotype (WT, 2.71 ± 0.26; KO, 2.99 ± 0.41 g/mouse/24 h). Food consumption was monitored on several occasions, and no significant differences were determined between the genotypes (additional data not shown). Saline treatment resulted in a similar profile of fecal production in both genotypes (Fig. 1A), suggesting that the two genotypes are not intrinsically different in their normal gastrointestinal function. Morphine (10 mg/kg s.c.) induced an initial suppression of defecation in both groups of mice; however, the βarr2-KO mice fully recover after 2 h, whereas the WT mice continue to produce less defecation throughout the test period (Fig. 1B) relative to the saline treatment. At each of the doses tested, the βarr2-KO mice defecate more than the WT mice in the 6-h interval (Fig. 1C), indicating that morphine produces less constipation in the absence of β-arrestin 2.

To further study the gastrointestinal transit in response to morphine, we assessed small intestinal transit times by measuring the distance traveled of an orally administered charcoal meal. The nature of this assay dictates that the GI tract must be empty; therefore, the mice were fasted 24 h prior to the test. Mice were treated with saline or morphine; 20 min later, they received the charcoal meal by oral gavage. After an additional 30 min, mice were euthanized by cervical dislocation, and the small intestine was dissected out from the duodenum to the jejunum. The length of this portion of the tract was measured, and the distance traveled by the leading edge of the charcoal bolus was normalized to the total length of the intestinal tract for each mouse as previously described (Ward and Takemori, 1982; Raffa and Porreca, 1986; Roy et al., 1998). Morphine treatment led to a significant decrease in charcoal transit in both genotypes in a dose-dependent manner (Fig. 2). Interestingly, we did not see a significant difference between the genotypes at any of the doses tested. Thus, morphine equally delays small intestinal transit in WT and βarr2-KO mice, suggesting that β-arrestin 2 is not limiting in the regulation of this portion of the GI tract.

Since significant differences were apparent in overall fecal boli production, we next asked whether morphine differentially affected colon motility in βarr2-KO mice. Therefore, a simple assay of colonic propulsion in conscious, freely moving mice was adapted from previously described studies (Porreca et al., 1984; Raffa et al., 1987). The nature of these experiments necessitates an evacuated colon; therefore, mice were once again fasted for 24 h prior to the study. Mice were injected with morphine or saline, and 20 min later, a 3-mm glass bead was inserted 2 cm into the rectum of each mouse. Mice were observed, and the time was recorded when the

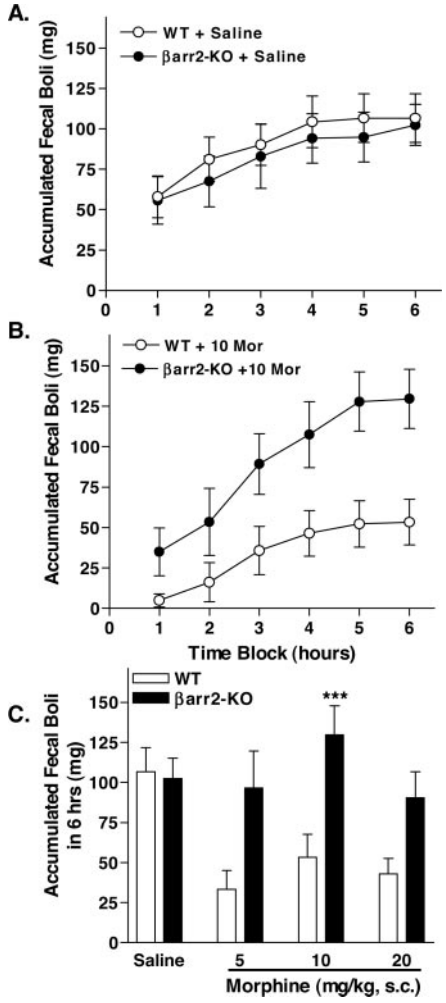

Fig. 1. Morphine effects on fecal boli accumulation. Mice were provided food and water ad libitum before the test period, and both genotypes consumed comparable amounts of food prior to the test as measured over a 24-h period in the test environment. No food or water was available during the test. Mice were caged in acrylic boxes with grid floors suspended over filter paper. Fecal boli were collected from each mouse every hour for 6 h following the injection of saline or morphine. Mice were only used once. A, amount of feces accumulated over time was recorded by weight following saline (WT versus βarr2-KO, saline, $P > 0.05$ two-way ANOVA; $n = 9$) or B, morphine (10 mg/kg s.c.; WT versus βarr2-KO, morphine, $P < 0.001$, two-way ANOVA; $n = 11$). C, total mass of defecation produced over the entire 6-h test period was recorded for saline or morphine (5, 10, or 20 mg/kg s.c.) treatment (for genotype, $F_{1,58} = 15.65$, $P = 0.0002$; for dose, $F_{3,58} = 2.812$, $P = 0.0472$, two-way ANOVA; WT versus KO, ***, $P < 0.001$; Bonferroni post hoc analysis; $n = 6–11$).

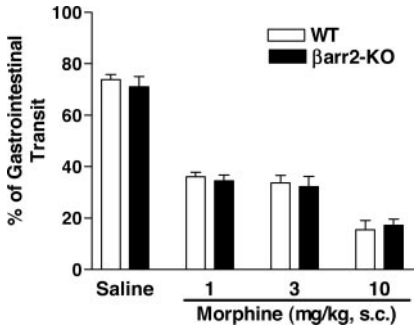

Fig. 2. Morphine inhibition of small intestinal transit. Mice were fasted for 24 h prior to the test and had free access to water. Mice were treated with saline or morphine and 20 min later given a charcoal gavage (5% aqueous suspension of charcoal in a 10% gum Arabic solution at a volume of 10 μl/g b.wt. At 30 min, animals were sacrificed by cervical dislocation, and the small intestine from the jejunum to the cecum was dissected and the mysentry removed. The distance traveled by the leading edge of the charcoal meal was measured relative to the total length of the small intestine, and the percentage of gastrointestinal transit for each treatment group was calculated as follows: percentage transit = [(charcoal distance)/(small intestine length)] × 100. Data represent the mean ± S.E.M. There were no significant differences between the two genotypes at any dose tested (two-way ANOVA for genotype, $F_{1,28} = 0.2263$, $P = 0.6380$; for dose, $F_{3,28} = 124.21$, $P < 0.0001$; $n = 4–6$).

glass bead was expelled. Saline treatment resulted in bead expulsion in approximately 5 min in both genotypes and morphine treatment produced a dose-dependent increase in the bead retention time (Fig. 3). In this assay, βarr2-KO mice displayed significantly shorter delays in bead expulsion times at the lower doses of morphine (1, 3, and 10 mg/kg s.c.), suggesting that the βarr2-KO mice are less affected by morphine-induced inhibition of colonic propulsion than their WT counterparts.

Morphine acts at opioid receptors both centrally and peripherally to affect GI function. To ascertain whether the differences in the colonic motility were due to peripheral site of action, the μOR agonist, loperamide, was used. Loperamide (Imodium) does not cross the blood-brain barrier, acts to reverse diarrhea, and acts primarily at the μOR (Mackerer et al., 1976; Stahl et al., 1977; Schulz et al., 1979). Although it is more of an antidiarrheal drug than a constipatory agent, loperamide has been shown to effectively inhibit both small intestinal transit as well as colonic motility. Mice were treated in the same manner as in the morphine bead expulsion studies. Loperamide delayed colonic transit times in the WT mice, yet had no significant effect in the βarr2-KO mice (Fig. 4).

A clear difference between genotypes regarding morphine-induced constipation is apparent; therefore, we extended our studies to ask whether other morphine-induced side effects are also altered in βarr2-KO mice. Of all of morphine's side effects, the most acutely detrimental is the onset of respiratory suppression, which is generally the cause of death in cases of opiate overdose. The suppression of respiration elicited by morphine occurs via the activation of opioid receptors (Santiago and Edelman, 1985; Reisine and Pasternak, 1996), and mice lacking the μOR do not experience this side effect of morphine (Matthes et al., 1998; Dahan et al., 2001; Romberg et al., 2003). To determine whether morphine-induced respiratory suppression is altered by β-arrestin 2 deletion, we analyzed the breathing frequency of the βarr2-KO mice and their WT controls using whole-body plethysmography following administration of saline or relatively high doses of morphine. Resting breathing frequency was not different between WT and βarr2-KO mice, and saline treatment did not alter breathing frequency in either genotype (Fig. 5A). Morphine administration at a dose of 50 mg/kg s.c. caused a significant and sustained decline in breathing frequency in WT mice but not in βarr2-KO mice. The lack of morphine-induced respiratory suppression in βarr2-KO mice was apparent at 20 and 50 mg/kg doses of morphine wherein respiratory frequency did not fall below basal levels (Fig. 5, B and C). At higher doses of morphine (100 and 150 mg/kg s.c.), βarr2-KO mice did experience respiratory suppression; however, this effect was significantly less than that observed in WT mice (Fig. 5C). Since opiates have been shown to affect tidal volume as well as respiratory frequency (Borison, 1977; Mather and Smith, 1999), we analyzed tidal volume levels and found no differences between the two genotypes at any of the doses tested (data not shown). Therefore, changes in tidal volume could not account for the genotype differences observed in breathing frequency. These studies demonstrate that morphine produces significantly less suppression of respiratory frequency in βarr2-KO mice.

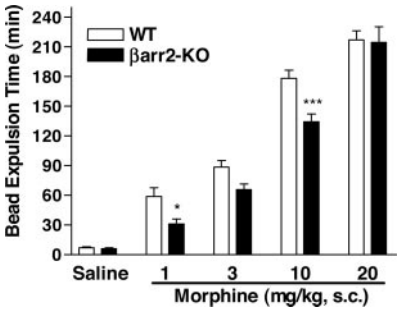

Fig. 3. Morphine effects on colonic propulsion. Morphine dose-dependently inhibited colon transit in both genotypes. However, the βarr2-KO mice are less adversely affected compared with their WT counterparts (two-way ANOVA for genotype, $F_{1,65} = 11.98$, $P = 0.0010$; for dose, $F_{4,65} = 178.96$, $P < 0.0001$; WT versus KO, *, $P < 0.05$; ***, $P < 0.001$ Bonferroni post hoc analysis; $n = 4–11$).

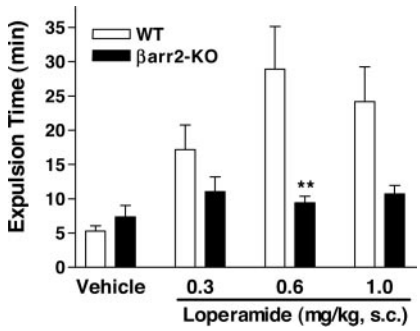

Fig. 4. Loperamide effects on colonic propulsion. Loperamide inhibits colonic propulsion times in WT mice in a dose-dependent manner (WT for dose, $P = 0.0067$, one-way ANOVA); however, loperamide does not inhibit colonic propulsion in the βarr2-KO mice at any of the doses (βarr2-KO for dose, $P = 0.3772$ one-way ANOVA). Therefore, the βarr2-KO mice are less responsive to loperamide than their WT counterparts (for genotype, $F_{1,45} = 12.12$, $P = 0.0011$; for dose, $F_{3,45} = 4.58$, $P = 0.0070$, two-way ANOVA; WT versus KO, **, $P < 0.01$; Bonferroni post hoc analysis; $n = 5–8$).

Discussion

Disruption of μOR regulation, by removal of β-arrestin 2, changes the relative efficacy of morphine in mice wherein morphine produces greater antinociception at lower doses while simultaneously precipitating less severe side effects. As a mediator of GPCR desensitization, β-arrestin 2 regulates the degree of coupling between the μOR and G proteins, and this has been demonstrated in certain brain regions in the βarr2-KO mice (Bohn et al., 1999, 2000). However, this simple scenario, in which β-arrestin 2 only acts as a desensitizing element, would indicate that all behavioral responses to morphine, including respiratory suppression and constipation, should be enhanced in the βarr2-KO mice. In contrast, here we show that the morphine-induced side effects are not worsened and are actually diminished in a mouse model that displays enhanced morphine analgesia.

Although previous studies support a role for β-arrestin 2 as a negative regulator of opioid receptor G protein-mediated cell signaling, we must also consider that β-arrestins can mediate GPCR cell signaling that is independent of G proteins (Lefkowitz and Shenoy, 2005). Furthermore, GPCRs can activate mitogen-activated protein kinase cascades via β-arrestin-Src kinase scaffolds (Luttrell et al., 2001). This signaling paradigm has been demonstrated for several GPCRs but has not yet been shown for the opioid receptors. However, it is possible that the opioid receptors that lead to

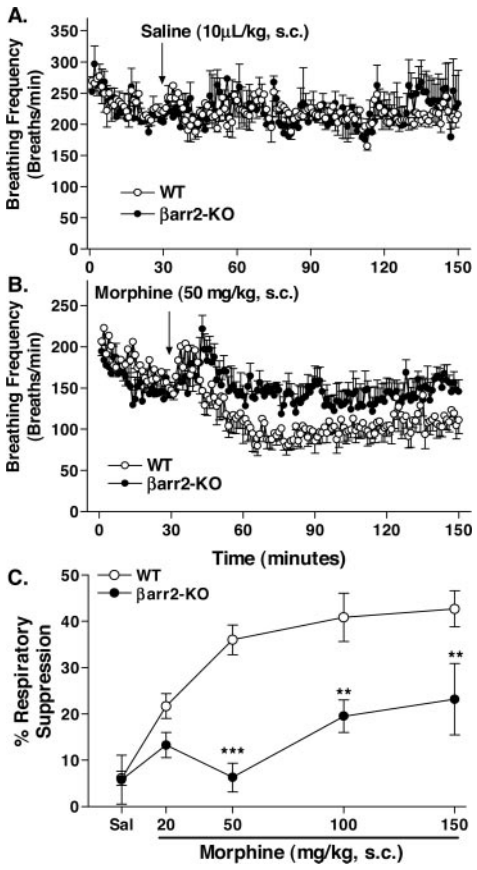

Fig. 5. Respiratory suppression as determined by whole-body plethysmography. WT and βarr2-KO mice were treated with saline or morphine following a 30-min habituation period. Measurements were performed with a 12-chamber Buxco whole-body plethysmograph, and WT and βarr2-KO mice were assessed simultaneously. Breathing frequency was recorded electronically by computer software. WT and βarr2-KO mice were injected with saline or morphine as indicated. Measurements were taken over 2 h and are presented as the average number of breaths/min. A, saline did not suppress respiratory frequency ($F_{119,701} = 0.8656$, $P = 0.8358$), nor was there a difference in response between the genotypes ($F_{1,701} = 1.790$, $P = 0.1814$, two-way ANOVA, $n = 5$ per dose and genotype). B, morphine (50 mg/kg s.c.) significantly suppressed respiratory frequency ($F_{119,949} = 2.622$, $P < 0.0001$), but βarr2-KO mice were less affected ($F_{1,949} = 253.1$, $P < 0.0001$, two-way ANOVA, $n = 5$ per dose and genotype). C, dose response data reflect percent suppression based on the average number of breaths/min measured during a 2-h period following morphine treatment, as normalized by each mouse's breathing rate in the last 15 min of the habituation period (two-way ANOVA for dose, $F_{4,45} = 15.11$, $P < 0.0001$; for genotype, $F_{1,45} = 40.90$, $P < 0.0001$; WT versus βarr2-KO, **, $P < 0.01$, ***, $P < 0.001$, Bonferroni post hoc analysis; $n = 5$ per dose and genotype).

gastrointestinal transit inhibition or respiratory suppression are in cellular environments in which the β-arrestin molecule plays an important role in initiating G protein-independent signal transduction via the receptor. In such a scenario, removal of the β-arrestin molecule could prevent the downstream signaling and the subsequent biological response. For example, it was recently demonstrated that the βarr2-KO mice responded less to an α adrenergic 2 receptor agonist in the rotorod test, suggesting that β-arrestin 2 may be positively mediating signal transduction via these receptors in this particular behavioral response (Wang et al., 2004). Another attractive hypothesis is that other neurotransmitter systems, such as noradrenaline and serotonin, are known to alter gastrointestinal function and respiration, also act at GPCRs and therefore may display altered receptor responses in the absence of β-arrestin 2 (Manzke et al., 2003). Further studies assessing the function of these receptors and their contribution to respiratory regulation and gastrointestinal transit are also warranted in the βarr2-KO mice.

Gastrointestinal transit function was assessed at three physiologically distinct levels: small intestinal transit, colonic propulsion, and overall production of fecal boli following morphine treatment. Interestingly, although genotypic differences were seen for fecal boli production over time and colonic bead propulsion, we did not detect differences in the measures of small intestinal transit. The fecal boli accumulation studies may be the ultimate test for morphine-induced constipation because the animals had free access to food and water prior to the test and were simply monitored for their ability to produce fecal waste following drug treatment compared with saline treatment. At each of the doses tested in this paradigm, the βarr2-KO mice consistently recovered from the morphine-induced constipation more rapidly and to a greater extent than the WT mice. The food deprivation could potentially confound the effects on the small intestinal transit times; however, the colonic propulsion studies, also performed under fasting conditions, paralleled the findings in total fecal accumulation at the lower doses. At the highest dose, 20 mg/kg, the delay in colonic propulsion was not significantly different between the genotypes. However, this high dose may have produced a ceiling effect, especially under the fasting conditions of this particular test. A compelling interpretation of the differences seen between the two gastrointestinal regions is that the effects on colon and small intestine may represent distinct sites of morphine's actions in regulating these individual components of gastrointestinal transit. Our initial observations suggest that μOR levels are not different between the WT and βarr2-KO mice in the colon (data not shown); however, further studies investigating receptor signaling as well as other ex vivo assessments of gastrointestinal function are ongoing.

Morphine and other opiate drugs act at opioid receptors expressed both within the central nervous system as well as in the periphery. Furthermore, opiate agonists act at receptors directly in the gut wall and through central opioidergic mechanisms to effect gastrointestinal transit. Although there is evidence to suggest that δ and κ opioid receptors can play a role in inhibiting gastrointestinal transit (Ward and Takemori, 1982; Porreca et al., 1984; Shook et al., 1989; Broccardo and Improta, 1992), it appears that the μOR plays a prominent role in this action since mice lacking the μOR experience, no delay in morphine inhibition of gastrointestinal motility (Roy et al., 1998). Furthermore, μOR-KO mice do not display respiratory suppression following high doses of morphine (Matthes et al., 1998; Dahan et al., 2001; Romberg et al., 2003), suggesting that both of these side effects are mediated through activation of the μOR. Our study with the μOR agonist, loperamide, which is limited to peripheral sites of action, recapitulates the finding with morphine in the colonic propulsion studies, suggesting that the differences in genotype may be due, to some extent, to receptor regulation in the periphery. Evaluation of μOR coupling and signaling in the gastrointestinal tract of the βarr2-KO mice will provide greater insight into the role of β-arrestin 2 in regulating the receptors in these tissues.

It is not clear why respiratory suppression and constipation are not enhanced in the βarr2-KO mice. Since morphine

acts at many sites, both on neurons and on other cell types, the μOR in certain regions may be subject to different cellular complements of regulatory proteins and may hence show different sensitivities to the loss of βarr2. For example, it has been demonstrated in vitro that although the morphine-bound μOR is a poor substrate for β-arrestin 2 binding, this limitation can be overcome by simply expressing more GRK2 (Zhang et al., 1998; Bohn et al., 2004a). Therefore, if morphine-activated receptors were sufficiently phosphorylated by a greater complement of GRK, then β-arrestin 1 may suffice for regulation of the receptor in that cell type. In such a scenario, the absence of β-arrestin 2 might not have an impact on downstream signaling.

In addition to targeting multiple cell types, morphine may act at multiple μOR subtypes. A number of studies have suggested that opiate control of respiration might be due to activation of a different subset of μORs (μ_2OR, type 2 μOR) as opposed to those that are believed to mediate antinociception (μ_1OR, type 1 μOR) (Ling et al., 1985, 1989). Others have also noted this difference, finding less correlation between antinociception and respiratory suppression with highly selective μOR agonists (Pick et al., 1991; Stott and Pleuvry, 1991). This concept of differential regulation may serve to ratify the concept of pharmacologically distinct μOR subtypes because the aforementioned subtypes have yet to be disseminated on a genetic basis. For example, the cellular environment that determines the scaffolding or regulation of the μOR in the neurons that mediate analgesia may require the inhibitory action of the β-arrestin 2 to dampen signaling and G protein coupling, and this could reflect the μ_1OR subtype. In the neurons or peripheral cells wherein morphine acts to regulate either respiration or gastrointestinal transit, the cellular environments might be such that β-arrestin 2 is a regulatory factor that initiates, rather than dampens, receptor signaling. This difference in receptor regulation could manifest pharmacologically as a difference in relative opiate efficacy (Bohn et al., 2004a), supporting the pharmacological differentiation between receptor subtypes such as the μ_2OR, which is implicated in regulating gastrointestinal transit and respiratory suppression (Ling et al., 1985, 1989; Pick et al., 1991).

Taken together with our previous findings, these observations suggest that although the analgesic properties of morphine are enhanced in β-arrestin 2 knockout mice, the removal of β-arrestin 2 may actually be protective against morphine-induced constipation and respiratory suppression. Therefore, developing a modulator of morphine-mediated μOR desensitization, or μOR-β-arrestin interactions, may prove to have beneficial therapeutic value in enhancing and prolonging the analgesic effects of morphine in the absence of antinociceptive tolerance, while at the same time preventing constipation and respiratory suppression.

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