Published in final edited form as:

Eur J Pharmacol. 2012 June 15; 685(1-3): 133–140. doi:10.1016/j.ejphar.2012.04.001.

Differences in the characteristics of tolerance to μ -opioid receptor agonists in the colon from wild type and β -arrestin2 knockout mice

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Abstract

Drawbacks to opioid use include development of analgesic tolerance and persistent constipation. We previously reported that tolerance to morphine develops upon repeated exposure in the isolated ileum but not the isolated colon. The cellular mechanisms of antinociceptive tolerance vary among μ -opioid receptor agonists. In this study, we assess β -arrestin2 deletion on the development of tolerance to different opioids in ileum and colon circular muscle. Tolerance was determined by assessing the ability of repeated *in-vitro* opioid exposure to induce contraction of the circular muscle from C57BL/6 wild type (WT) and β -arrestin2 knockout (KO) mice. Repeated exposure every 30 min with in-between washes resulted in tolerance to all agonists in the ileum of both WT and KO mice. However, in the colon of WT mice, comparison of the contractions between the 4th exposure and 1st response was similar to DAMGO ($100 \pm 10\%$; $N = 5$) but reduced to fentanyl ($62 \pm 13\%$; $N = 8$) and etorphine ($38 \pm 4\%$; $N = 7$) indicative of tolerance to fentanyl and etorphine but not DAMGO. In contrast, all agonists produced tolerance in the colon of KO: DAMGO response at the 4th exposure decreased to $52 \pm 10\%$ ($N = 5$), fentanyl to $20 \pm 5\%$ ($N = 6$) and etorphine $33 \pm 7\%$ ($N = 6$). Differences in tolerance among opioid agonists in the colon suggest ligand bias. The deletion of β -arrestin2 in colon appears to be necessary for tolerance to DAMGO but not fentanyl or etorphine. β -arrestin2 potentially represents an important target for treating opioid-induced bowel dysfunction and warrants further exploration of its ligand bias.

Keywords

μ -Opioid receptor; β -arrestin2; opioid; circular smooth muscle; tolerance

1. INTRODUCTION

Chronic use of opioids is associated with the rapid development of tolerance. Increasing the dose to compensate for the loss in efficacy raises the risk of adverse events including overdose, withdrawal and dependence. The extent of tolerance varies significantly

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Disclosure: The authors disclose no conflicts

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depending on the pharmacological effect e.g. opioid tolerance develops quicker to urinary retention and respiratory depression effects compared to the analgesic effects, whereas little manifests to constipation in humans. It is for this reason that constipation is a major side effect observed in opioid-induced bowel dysfunction (Holzer et al., 2009; Thomas, 2008).

Several mechanisms have been proposed for the development of opioid tolerance depending on the ligand and tissue preparation. Classical mechanisms include decreased cell surface receptor expression, prolonged signaling resulting in desensitization, receptor phosphorylation, etc. It is however becoming increasingly clear that functional selectivity manifests with different ligands targeting the same opioid receptor (for review, see Raehal et al., 2011). Ligands that are less efficacious at promoting desensitization and internalization such as morphine produce significant antinociceptive tolerance while high efficacy agonists including etorphine and fentanyl produce less (Pawar et al., 2007; Raehal et al., 2011; Sirohi et al., 2008). In contrast, we recently reported the lack of tolerance to morphine in the colon from mice and guinea pigs: However, tolerance developed in the colon of β -arrestin2 knockout (KO) mice following repeated administration of morphine (Kang et al., 2011). Based on these findings, we compared the effects of other opioids towards tolerance development in the mouse colon. While numerous studies have focused on the effects of various opioids on antinociceptive tolerance, to our knowledge, no studies have investigated the effects of high efficacy ligands such as etorphine on tolerance in the colon. Etorphine, DAMGO and fentanyl desensitization pathways have been observed to be dependent on β -arrestin2, an integral protein which facilitates receptor desensitization, whereas morphine and levorphanol show minimal recruitment of the moiety (McPherson et al., 2010; Whistler and von Zastrow, 1998). To delineate possible differential development of tolerance among opioid agonists, we assessed the ability of repeated opioid exposure to induce contraction of isolated ileum and colon circular muscle from C57BL/6 wild-type and β -arrestin2 KO mice. In this preparation, enhanced spontaneous contractions occur as a result of suppression of inhibitory tone by μ -opioid receptors located on nitrergic and VIP-ergic myenteric neurons (Grider and Makhlof, 1987; Iwata et al., 2007).

Our results demonstrate lack of tolerance to repeated administration of DAMGO in the colon similar to that previously observed with morphine. Partial tolerance occurs to fentanyl while significant tolerance manifests with etorphine. However, tolerance occurs to all agonists in the β -arrestin2 KO mouse colon. The differences between highly potent agonists like fentanyl and etorphine and the less potent DAMGO and morphine, point to potential ligand biased signaling. Our data demonstrate the downregulation of β -arrestin2 to be necessary for development or enhancement of tolerance to specific agonists and further illustrate differences between CNS models where tolerance is abrogated, in contrast to the colon where it is enhanced in the absence of β -arrestin2.

2. MATERIALS AND METHODS

2.1 Drugs and Chemicals

Morphine (morphine sulfate pentahydrate salt), fentanyl (as citrate salt) and DAMGO ([D-Ala², N-Me-Phe⁴, Gly⁵-ol]-Enkephalin acetate) were procured from Sigma-Aldrich Co. (St. Louis, MO), whereas etorphine (as hydrochloride salt) was acquired from the National Institute on Drug Abuse (NIDA), Bethesda, MD and CTAP (H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂) was procured from Tocris Biosciences (Ellisville, MO). Fentanyl and morphine were dissolved in water and etorphine and DAMGO were dissolved in DMSO to make 10 μ M stock solutions which were further diluted with distilled water to make the targeted concentration.

2.2. Subjects

All experimental procedures employing animals were reviewed and approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and were conducted in accordance with the guidelines for the humane use of animals in research (NIH “Public Health Service Policy on Humane Care and Use of Laboratory Animals” [revised 2002]). The breeding pairs for the β -arrestin2 KO mice were obtained from Dr Lefkowitz (Duke University, Durham, NC) and housed within the transgenic facility at Virginia Commonwealth University. The animals were housed up to five per cage with access to food and water *ad libitum*. The mice were kept in the animal facility for one week to permit acclimation prior to the experiments. Every effort was made to reduce the use of animals to the minimum number required to achieve sufficient statistical power.

2.3 Procedures

2.3.1. Preparation of colon and ileum circular muscle—Mice were sacrificed by cervical dislocation. Segments of the distal colon (approximately 1 cm from anus) and terminal ileum (2 cm from ileo-cecal junction) were removed and placed in a dissecting dish containing Krebs solution (118 mM NaCl, 4.6 mM KCl, 1.3 mM NaH_2PO_4 , 1.2 mM MgSO_4 , 25 mM NaHCO_3 , 11 mM glucose, and 2.5 mM CaCl_2) bubbled with 95% O_2 and 5% CO_2 . The segments of ileum and colon were dissected, flushed of their contents, and trimmed of mesentery. The tissues, 1 cm in length, were suspended vertically along the axis of the circular muscle with a metal triangle tied to a hook under 1 g of passive tension in 15 ml siliconized organ baths. The tissues were allowed to equilibrate for 60 minutes prior to drug exposure, with the Krebs solution changed every 15 minutes. Isometric contractions were recorded by a force transducer (GR-FT03; Radnoti, Monrovia, CA) connected to a personal computer using Acknowledge 382 software (BIOPAC Systems, Inc., Santa Barbara, CA). Acetylcholine-induced (3 μM) contractions were measured at the beginning of the experiments as a reference control.

2.3.2 In vitro drug exposure schedules - assessment of tolerance—We first attempted to determine a challenge dose with the following characteristics: non-supramaximal, provide a quantifiable response from which tolerance could be assessed and equipotent to the previously used morphine concentration (3 μM - equated to ED90). The concentration response curves for DAMGO (1.0 nM – 30 μM), fentanyl (0.1 nM – 1 μM) and etorphine (0.01 nM – 0.1 μM) were determined cumulatively using an isolated circular muscle ring preparation in the organ bath. Successive drug concentrations were added separated by a 2 min lapse. Tolerance was then assessed based on the predetermined ED90 values for each agonist; 3 μM for DAMGO, 0.1 μM for fentanyl and 0.01 μM for etorphine. Tolerance was assessed as previously described (Ross et al 2008). In brief, following the initial 60 minute equilibration period, a reference dose of acetylcholine 3 μM , was added to the bath followed by a washout 5 minutes later. Further washouts were instituted over a 30 minute span to washout the acetylcholine. Thereafter, the first exposure (15 min duration) to the opioid agonist was instituted followed by a washout using Krebs. This series of steps was repeated until the forth exposure and the ability of each agonist to induce a neurogenic contraction was determined and calculated as percent inhibition from the original amplitude.

2.3.3 Assessment of circular muscle sensitivity to acetylcholine—To determine whether genetic deletion of β -arrestin2 had altered the contractile response of the tissues we assessed the sensitivity of the circular muscle ring preparation to acetylcholine. Following an equilibration period during which washes are performed every 15 minutes, the tissues were exposed to increasing concentrations of acetylcholine (1.0 nM - 100 μM) added in a cumulative fashion with a 2 min lapse between successive drug concentrations. Responses were calculated as grams of tension per unit tissue weight or as percent of the maximum

contraction for that tissue. The values were used to determine the EC₅₀ (i.e. concentration required to produce 50% of the maximum response) and to calculate the maximum tension produced. Computer assisted analysis of each concentration-response curve using GraphPad Prism software (GraphPad Software Inc.) was employed to determine the EC₅₀.

2.3.4 Functional assessment of μ -opioid receptor distribution in neurons—

Though there has been controversy regarding the distribution of μ -opioid receptors responsible for eliciting contractions of the circular muscles, overwhelming evidence points to selective neuronal expression of receptors on the myenteric neurons and not directly on the smooth muscle. To confirm this assertion, we used two methods. One method assessed the effect of a 15 min pre-exposure to tetrodotoxin (TTX – 1 μ M), a selective neuronal sodium channel blocker, on the capacity of morphine to induce a contractile response in the circular muscle preparation. A second method studied the effect of cold denervation by incubating the colonic and ileal tissue in Krebs buffer at 4°C for 48 hours. The sensitivity of the circular muscle preparation to an opioid agonist was then assessed after the incubation (Akbarali et al., 1987). In this experiment, the viability of the smooth muscle after the 48 hr cold incubation was verified by a robust response to acetylcholine (3 μ M). We further verified that the contractile response was due to stimulation of μ -opioid receptors and not other opioid receptor subtypes by assessing the impact of 15 minute pre-exposure to the μ -opioid receptor selective blocker, CTAP, on the response to morphine (3 μ M).

2.4 Data analysis

The percent contraction was calculated using the mean contraction height divided by the initial contraction. Contractile responses after repeated administrations of the agonist were analyzed by repeated measures ANOVA followed by Bonferroni post hoc test. Data are presented as mean $\pm$ S.E.M. Values of $P < 0.05$ were considered to be statistically significant. All data was analyzed using appropriate statistical tools using the GraphPad Prism software (GraphPad Software Inc.).

3. RESULTS

3.1 Development of tolerance in circular muscle

Tolerance was determined by assessing the ability of repeated transient opioid exposure to induce contraction of the ileum and colon circular muscle ring preparations *in vitro*. The drug concentrations used (DAMGO - 3 μ M, fentanyl - 0.1 μ M, and etorphine – 0.01 μ M) were based on preliminary *in vitro* concentration response studies performed to determine equipotent concentrations that produced significant quantifiable contractions from which tolerance could be assessed. Fig. 1A shows the concentration response curve for DAMGO, fentanyl and etorphine. ED values close to ED90 were used to perform the development of tolerance. For DAMGO, 3 μ M equated to ED92.6, for fentanyl 0.1 μ M equated to ED87.1 and for etorphine 0.01 μ M equated to ED87.8. These values correlate to the potency of the agonists as antinociceptive agents with etorphine being the most potent followed by fentanyl, and DAMGO being the least potent. We further verified that the contractile response was due to the stimulation of μ -opioid receptors and not other opioid receptors subtypes. Fig. 1B illustrates a complete blockade of morphine (3 μ M)-induced contractile effect following 15 minute pretreatment with CTAP (3 nM). The morphine-induced contractile response was restored after washing out using Krebs.

Fig. 2 illustrates representative isometric tension recording tracing from colon and ileum circular muscle ring preparation from WT and β -arrestin2 KO mice showing the effect of repeated exposure with DAMGO 3 μ M. Data from these tracing was used to plot linear graphs (Fig. 3) outlining changes in efficacy following repeated exposure to the opioid

agonists using the predetermined equipotent agonist concentrations. Significant tolerance to DAMGO, fentanyl and etorphine was observed in the ileum of WT and β -arrestin2 KO mice upon 4 repeated exposures separated by a washout period. Compared to the 1st exposure, DAMGO induced contraction was decreased to $70.0 \pm 13.6\%$ ($N = 4$) in the WT and $43.5 \pm 9.5\%$ ($N = 5$) in β -arrestin2 KO mice during the 4th exposure whereas fentanyl induced contractile response was decreased to $28.1 \pm 9.1\%$ ($N = 5$) in the WT and $27.0 \pm 6.6\%$ ($N = 6$) in β -arrestin2 KO mice during the 4th exposure; in a similar fashion, etorphine induced contraction in the ileum was reduced to $34.4 \pm 7.2\%$ ($N = 6$) in the WT and $26.7 \pm 5.3\%$ ($N = 5$) in β -arrestin2 KO mice. In contrast, the colon of WT mice exposed to DAMGO did not develop tolerance with the 4th exposure producing a $100.1 \pm 9.6\%$ ($N = 5$) contraction compared to the 1st exposure; interestingly, tolerance occurred in the colon of the β -arrestin2 KO mice with the 4th exposure showing significant reduction as evidenced by the $52.2 \pm 9.9\%$ contraction ($N = 5$; $P < 0.05$). For fentanyl, tolerance was observed in colon preparations from both animal strains with the 4th contractions being reduced to $61.6 \pm 13.1\%$ ($N = 8$) in WT mice and more acutely to $20.2 \pm 4.6\%$ ($N = 6$) in KO mice. It should be noted that though tolerance was observed in both tissues, the extent was significantly greater in the β -arrestin2 KO compared to the WT mice. For etorphine, tolerance was observed in colon preparations from both animal strains with the 4th contractions being reduced to $37.8 \pm 3.6\%$ ($N = 7$) in WT mice and more acutely to $33.2 \pm 6.9\%$ ($N = 6$) KO mice. There was no statistically significant difference at any of the time points between the two groups.

3.2 Effect of β -arrestin2 deletion on the sensitivity to acetylcholine or μ -opioid receptor agonists

In order to determine whether the genetic deletion of β -arrestin2 affected the initial sensitivity to the opioid agonists, we compared magnitude of the contraction elicited by the first exposure to each opioid. As depicted in Fig. 4, the initial contractile force revealed no statistically significant difference produced by DAMGO in the colon (53.8 ± 2.0 vs. 85.8 ± 24.5 mg/mg tissue weight; $P = 0.21$) and ileum (48.8 ± 20.0 vs. 58.7 ± 8.0 mg/mg tissue weight; $P = 0.63$) in tissues obtained from a wild type and the β -arrestin2 KO mice respectively. Similar results were observed with etorphine where the tension observed in the colon (48.0 ± 8.6 vs. 66.0 ± 13.1 mg/mg tissue weight; $P = 0.26$) and ileum (79.8 ± 13.8 vs. 109.7 ± 9.7 mg/mg tissue weight; $P = 0.12$) was not statistically different between the wild type and the β -arrestin2 KO groups respectively. Interestingly, a statistically significant reduction in the maximum inhibitory response to fentanyl was observed in the colon (27.5 ± 4.2 vs. 79.4 ± 20.8 mg/mg tissue weight; $P = 0.02$) but there was no difference observed in the ileum (59.9 ± 18.0 vs. 83.3 ± 20.1 mg/mg tissue weight; $P = 0.42$) of wild type compared to the β -arrestin2 KO groups respectively. Though high variation was observed with the colonic data, there appears to be a trend for all the agonists to increase responsiveness in the β -arrestin2 KO group; however, statistical significance was only observed with fentanyl.

We further assessed whether genetic deletion of β -arrestin2 would affect the ability of acetylcholine to produce a contraction in the circular muscle preparation. As illustrated in Fig. 5, the impact of genetic deletion of β -arrestin2 on the response of the ileum circular muscle to acetylcholine revealed no statistically significant difference in the sensitivity to acetylcholine. The respective $-\log EC_{50}$ values between the wild type and β -arrestin2 KO groups were also comparable (5.7 ± 0.1 vs. 5.9 ± 0.1 ; $P > 0.05$). As illustrated in Fig. 5, the ability of acetylcholine to induce a maximal response in the ileum (WT, 72.8 ± 6.2 vs. KO, 76.7 ± 3.8 mg/mg tissue weight; $P > 0.05$) was not altered between the two groups. Similar results were observed in the colon where the sensitivity to acetylcholine, assessed by comparing the respective $-\log EC_{50}$ values, appeared to be similar between the WT and the

KO mice group (5.9 ± 0.2 vs. 6.2 ± 0.2 respectively $P > 0.05$). The efficacy and ability to induce a maximal response was not altered in the colon (WT, 89.5 ± 9.4 vs. KO, 86.9 ± 9.6 mg/mg tissue weight; $P > 0.05$).

3.3 Assessment of μ -opioid receptor distribution in the circular muscle preparation neurons

Fig. 6A illustrates a typical isometric recording showing the effect of 15 min pre-exposure to tetrodotoxin. After addition of tetrodotoxin, the spontaneous contractions were enhanced due to the removal of the inhibitory neural pathways that inhibit the myogenic properties of the muscle cells. The presence of tetrodotoxin also completely blocked morphine's ability to contract the circular muscle. The effect is however restored after washing out the tetrodotoxin. In Fig. 6B we show that denervation through 48 hr incubation at 4°C results in blocking both morphine and tetrodotoxin contractile effects, which are normally imparted through neuronal stimulation. The muscle activity remains viable since acetylcholine managed to induce a concentration dependent contraction.

4.0 DISCUSSION

In this study we compared the ability of three different opioid analgesics to induce tolerance in the ileum and colon of wild type and β -arrestin2 KO mice. While DAMGO, fentanyl and etorphine all produced tolerance upon repeated administration in the isolated ileum of WT and KO mice, there was differential development of tolerance in the colon. Similar to our previous findings with morphine (Kang et al., 2011; Ross et al., 2008), tolerance did not develop to DAMGO in the colon of the WT but was observed in the β -arrestin2 KO mice. In contrast, fentanyl and etorphine both produced significant tolerance in the WT mice. It is noteworthy that tolerance to fentanyl was exaggerated in the KO mice while tolerance to etorphine was similar in the absence and presence of β -arrestin2. The differences in tolerance observed among the agonists in the WT mouse colon could be due to ligand biased receptor desensitization resulting from differential regulation by kinases, β -arrestin2 and assembly of adaptor and scaffolding protein entities that determine the nature and mechanism of the tolerance. *In vitro* studies using HEK-293 cells have shown that DAMGO induces greater μ -opioid receptor internalization and desensitization in a GRK2-dependent fashion whereas morphine confers negligible internalization and its desensitization is PKC dependent (Johnson et al., 2006; Shukla et al., 2008). Furthermore Hull et al. (2010) have reported that tolerance observed *in vivo* with low and moderate efficacious opioids like morphine, meperidine and fentanyl is dependent on PKC whereas that of high efficacy agonists like DAMGO is dependent on GRK. It has been proposed that this difference in spectrum of actions and downstream signaling could be due to differences in the receptor configuration attained upon receptor activation (Violin and Lefkowitz, 2007). The specific receptor configuration attained upon agonist activation may also in turn dictate the phosphorylation pattern on the receptor. One current hypothesis suggests that specific agonist-induced phosphorylation patterns on the C-terminal of the receptor, representing a "bar code", dictate the type and extent of modulation/recruitment of downstream entities including β -arrestin2 (Nobles et al., 2011). Indeed several opioid agonists like etorphine and fentanyl have been shown to be highly efficacious recruiter of β -arrestin2 whereas morphine and levorphanol are less efficacious (McPherson et al., 2010).

As a follow up to our previous studies in which we reported that the genetic deletion of β -arrestin2 resulted in the development of tolerance to morphine response in the colon (Kang et al., 2011), we investigated the impact of β -arrestin2 knockout on the development of tolerance to other opioid agonists in the ileum and the colon circular muscle. Results comparing the colon of β -arrestin2 KO and WT mice clearly show that the genetic deletion of β -arrestin2 results in either the development or reinforcement of tolerance to

DAMGO and fentanyl respectively. However no differences between the WT and β -arrestin2 KO mice were observed with etorphine suggesting a ligand bias component in which etorphine appears to induce tolerance independent of β -arrestin2. In previous studies using the guinea pig longitudinal muscle myenteric plexus (LMMP) preparation we have shown that 2 hr *in vitro* exposure to morphine results in a time-dependent downregulation of β -arrestin2 in the ileum that is associated with tolerance. In contrast, no tolerance was observed in the colon where the β -arrestin2 levels remained unaltered (Kang et al., 2011). This data strengthens our proposal that abrogation of β -arrestin2 through either downregulation or genetic deletion may be crucial to the development of tolerance in the gastrointestinal tract. Data from the current study suggest that β -arrestin2 is required to maintain and sustain the efficacy though it is not directly required for smooth muscle contraction since the efficacy of acetylcholine, DAMGO and etorphine remained unaltered. One possibility is that β -arrestin2 may play an important role in the resensitization process such that loss of β -arrestin2 prevents resensitization and leads to tolerance development. β -arrestin2 is a key component required for the internalization of the kinase- (GRK, PKC or PKA) modified/inactivated G protein-coupled receptor. The fate of the internalized receptor is either degradation or endosome based dephosphorylation and subsequent recycling which avails the receptor for further activation thus sustaining signaling. Since it has been reported that 80% of the internalized receptors are recycled, β -arrestin has been suggested to be a primary component required for resensitization and maintaining efficacy of the recycled receptors (Bailey and Connor, 2005; Koch et al., 1998; Zhang et al., 1998). We speculate that the genetic deletion of β -arrestin2 disrupts receptor resensitization of the inactivated receptors which results in the development of tolerance as was observed in the colon of β -arrestin2 KO mice. The tolerance observed in the ileum of WT mice corresponds to our previously reported data that shows selective downregulation of β -arrestin2 in the ileum following 2 hr exposure to morphine and resembles that observed in the β -arrestin2 KO mice.

Interestingly, there appears to be a correlation between the ability to recruit β -arrestin2 and the type of tolerance observed in the colon of WT mouse. In this study we report that fentanyl and etorphine, which are highly efficacious recruiters of β -arrestin2, induce tolerance in the colon of WT animals whereas the less efficacious recruiter morphine does not (McPherson et al., 2010). The recruitment of β -arrestin2 may result in terminal signaling leading to a reduction of β -arrestin2 levels. One proposed pathway may involve co-internalization of β -arrestin2 with the activated receptor into lysosomes where both are degraded. Another pathway may involve agonist induced downregulation of β -arrestin2 through ubiquitination (Perroy et al., 2004; Shenoy and Lefkowitz, 2003). DAMGO, however, does not fit this model since it is a highly efficacious recruiter of β -arrestin2 but does not induce tolerance. Further correlative evidence using other opioid agents will be important to confirm this finding.

Tolerance studies using CNS analgesia models such as the hot plate test in β -arrestin2 KO mice have shown contrasting results to those observed in the gastrointestinal tract. The development of tolerance to morphine analgesic effects is attenuated by β -arrestin2 deletion (Bohn et al., 2000; Bohn et al., 2002) whereas β -arrestin2 deletion in the enteric nervous system appears to enhance tolerance development in the colon without affecting the magnitude of tolerance observed in the ileum (Kang et al., 2011). These differences in manifestation of tolerance may suggest tissue/model specificity due to different levels of a particular protein or protein isoforms involved in the tolerance development, namely β -arrestins, GRK, PKA, PKC, MAPK and/or other auxiliary proteins (Bloom and Dewey, 1978; Hine, 1985). The results from this study reflect acute tolerance possibly involving immediate receptor phosphorylation by PKA, PKC, and GRK leading to β -arrestin2 recruitment. Further studies would need to be performed over a longer time course *in vivo* to

elucidate long term adaptive desensitization since a CNS component is also involved in the regulation of gastrointestinal peristalsis (Bernstein and Welch, 1998; Holzer, 2009; Narita et al., 1996; Smith et al., 2006).

Since tetrodotoxin- and cold storage- induced denervation attenuated the response to morphine in the circular muscle preparation, this confirms the opioid response in the circular muscle preparation to be neurogenic in origin. Although morphine and etorphine have partial affinity for the kappa (κ) and delta (δ) receptors, we however exclude significant involvement of these receptor subtypes since the morphine response in the circular muscle preparation was completely blocked by the selective μ -opioid receptor agonist, CTAP. Furthermore studies in rat ileum have shown that κ -opioid receptors are not involved in the contraction of circular and longitudinal muscles (Gray et al., 2005).

The data presented in this study provide support for the concept of ligand functional selectivity affirming that the phenomenon is not only agonist specific but also tissue specific since the agonist response observed in the ileum is considerably different from that in the colon or CNS. Furthermore, we show that the regulation of β -arrestin2 plays a key role in the development of tolerance in the gastrointestinal tract. This could be a function of several converging influences subject to considerable variation dependent upon the agonist employed to elicit the adaptive response. Delineation of specific pathways altered by the absence of β -arrestin2 will represent an important advance in the knowledge of the functional changes. Furthermore, the data can be used as basis for designing “biased” ligands that attenuate β -arrestin2 which could potentially be employed to modify the therapeutic management of patients suffering from constipation associated with opioid bowel dysfunction. The rapid development of tolerance observed with etorphine and fentanyl in the colon could theoretically be used advantageously in chronically ill patients prone to constipation associated with morphine use.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH DA024009.

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Fig. 1. Mean concentration-response curves for DAMGO, fentanyl and etorphine in whole ileum circular muscle preparation from drug naive C57BL/6 wild type mice. Contraction of the ileum circular muscle induced by DAMGO (1.0 nM – 30 μM), fentanyl (0.1 nM – 1 μM) and etorphine (0.01 nM – 0.1 μM). The values are expressed as a percentage of the maximum response. Points represent mean percent contraction ± S.E.M. The concentration response curve was used to determine the closest value to the ED90 for all the agonists. The N values for the experimental sets are between 3 and 4. Panel (B) illustrates a complete blockade of morphine (3 μM) induced contractile effect by pretreatment using the μ-opioid receptor selective agonist CTAP (3 nM). The morphine contractile response is restored after washing out using Krebs.

Fig. 2. Representative isometric tension recording tracing from colon and ileum circular muscle ring preparation showing the effect of repeated exposure with DAMGO 3 μ M separated by a washout period. A and B show tracings from C57BL/6 wild type ileum and colon tissue respectively; tracing C and D are for β -arrestin2 KO mice

Fig. 3.

Graphs depicting mean percent contraction for circular muscles following repeated exposure to, DAMGO 3 μ M (A and B), fentanyl 0.1 μ M (C and D) or etorphine 10 nM (E and F) using colon and ileum circular muscle ring preparations obtained from drug naïve C57BL/6 wild type and β -arrestin2 KO mice. The values are expressed as a percentage of the maximum response. Statistically significant differences (P < 0.05) between the WT and β -arrestin2 KO mice are identified by *. The N value for each experimental ranges from 4 – 8.

Fig. 4.

Bar graphs comparing maximal contractile response for colon and ileum circular muscle ring preparations obtained from C57BL/6 wild type and β -arrestin2 KO mice after first exposure to DAMGO 3 μ M (A) fentanyl 0.1 μ M (B) and etorphine 0.01 μ M (C). Statistically significant differences ($P < 0.05$) are identified by *. The N value for each experimental ranges from 4 – 8.

Fig. 5. Mean concentration-response curves comparing sensitivity to and efficacy of acetylcholine in ileum (A and B) and colon (C and D) circular muscle ring preparations obtained from C57BL/6 wild type and β -arrestin2 KO mice. Contraction of the ileum circular muscle was induced by acetylcholine (1.0 nM – 0.1 mM). (A) and (C) show the sensitivity expressed as a percentage of the maximum response for ileum and colon circular muscle respectively. (B) and (D) show the efficacy expressed as the amount of force generated (mg/mg tissue weight) in ileum and colon circular muscle respectively. Points represent mean contraction $\pm$ S.E.M. The N value for each experimental ranges from 3 – 7.

Fig. 6. Representative isometric tension tracing from circular muscle of ileum assessing the neuronal distribution of μ -opioid receptors in the ileum circular muscle preparation. Panel (A) illustrates a complete blockade of morphine (3 μ M) induced contractile effect by tetrodotoxin (1 μ M) pretreatment. The morphine contractile response is restored after washing out using Krebs. Panel (B) illustrates that denervation by incubation at 4°C for 48 hrs results of in a complete block of the neuronal contractile responses typically observed with tetrodotoxin (1 μ M) and morphine (3 μ M). The acetylcholine induced contractile response observed confirms the viability of the smooth muscle.