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Regulation of kappa opioid receptor signaling in peripheral sensory neurons *in vitro* and *in vivo*.

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Abbreviations:

AA, arachidonic acid; **BK**, bradykinin; **CGRP**, calcitonin gene-related peptide; **COX**, cyclooxygenase; **G protein**, guanine nucleotide binding protein; **MOR**, µ opioid receptor; **KOR**, k opioid receptor; **DOR**, • opioid receptor; **5'-GNTI**, 5'-guanidinonaltrindole; **nor-BNI**; *nor*-Binaltorphimine dihydrochloride; **NAL**, naltrindole; **NGF**, nerve growth factor; **PBS**, phosphate buffered saline; **PLA_2**, phospholipase A_2; **PLC**, phospholipase C; **PKC**, protein kinase C; **TG,** trigeminal ganglion

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Abstract

There is considerable interest in understanding the regulation of peripheral opioid receptors to avoid central nervous system side effects associated with systemically administered opioid analgesics. Here we investigated the regulation of the kappa opioid receptor (KOR) on rat primary sensory neurons *in vitro* and in a rat model of thermal allodynia. Under basal conditions, application of the KOR agonist, U50488, did not inhibit adenylyl cyclase (AC) activity nor release of calcitonin gene-related peptide (CGRP) *in vitro* and did not inhibit thermal allodynia *in vivo*. However, following 15 min pre-treatment with bradykinin (BK), U50488 became capable of inhibiting AC activity, CGRP release and thermal allodynia. Inhibition of AC by 5-HT_1 or neuropeptide Y_1 receptor agonists and stimulation of ERK activity by U50488 did not require BK pre-treatment. The effect of U50488 in BK-primed tissue was blocked by the KOR antagonist nor-BNI both *in vitro* and *in vivo*. The effect of BK *in vitro* was blocked by either indomethacin or bis-indolylmaleimide, suggesting that an arachidonic acid (AA) metabolite and protein kinase C (PKC) activation mediate BK-induced regulation of the KOR system. Further, the effect of U50488 in BK-treated tissue was blocked by a soluble integrin-blocking peptide (GRGDSP), but not the inactive reverse sequence peptide (GDGRSP), suggesting that in addition to AA and PKC, RGD-binding integrins participate in the regulation of KOR signaling in response to U50488. Understanding of the mechanisms by which peripheral KOR agonist efficacy is regulated may lead to improved pharmacotherapy for treatment of pain with reduced adverse effects.

Introduction

Drugs that act via opioid receptors, such as morphine and its analogs, are considered a “gold-standard” for the treatment of pain. However, the management of pain with opioid analgesics is complicated by adverse effects that limit clinical dosages to below those needed for adequate pain relief. Many of these adverse effects are associated with opioid actions within the CNS and include respiratory depression, dysphoria, and addiction. Consequently, there is considerable interest in understanding the function of opioid receptors located in the periphery on primary sensory neurons. Targeting these receptors with peripherally-restricted opioid drugs would avoid centrally-mediated adverse effects.

Opioid receptors are expressed on primary sensory neurons, however the reported analgesic efficacy of peripherally-restricted opioid drugs is highly variable. Although in animal studies local, peripheral administration of opioids is antihyperalgesic/antiallodynic when administered to injured or inflamed tissue, peripheral opioids are much less effective, or ineffective, when administered to normal tissue (Joris et al., 1987; Przewlocki and Przewlocka, 2001; Stein and Zollner, 2009). This has led to the hypothesis that some stimulus derived from inflamed or damaged tissue is required to induce functional competence of quiescent opioid receptor systems. Although many studies with animals demonstrate analgesic efficacy, studies in human indicate that the efficacy of peripherally-

restricted opioids on inflammatory pain is often mild to moderate, with many studies reporting no efficacy over placebo at all (Gupta et al., 2001; Kalso et al., 2002). This variability may be due to clinical pain conditions that differ in their ability to evoke and/or maintain opioid receptor system functional competence. Consequently, it is important to understand the mechanisms by which opioid receptor system function in the periphery is regulated since this may yield insight into developing adjunctive agents to maximize peripheral opioid receptor system competence.

Recently, we have found that μ and δ opioid receptors (MOR and DOR, respectively) are expressed in primary cultures of rat sensory neurons but appear to be functionally quiescent in that opioid agonists are ineffective at inhibiting neuropeptide release and adenylyl cyclase activity. However, brief exposure to an inflammatory mediator, such as bradykinin (BK), rapidly induces functional competence for both inhibition of neuropeptide release and inhibition of adenylyl cyclase activity. This effect of BK is mediated by a cyclooxygenase-dependent metabolite of arachidonic acid that is downstream from activation of protein kinase C (PKC) (Patwardhan et al., 2005; Patwardhan et al., 2006; Berg et al., 2007a). Moreover, DOR agonists injected locally into the hindpaw do not alter prostaglandin E_2- (PGE_2) induced thermal allodynia unless the injection is preceded by an intraplantar injection of BK (Rowan et al., 2009).

Historically, k opioid receptors (KOR) have not been considered viable targets for development of analgesic drugs since systemic administration results in CNS-mediated adverse effects including hallucinations and dysphoria (for reviews see Kivell and Prisinzano, 2010; Vanderah, 2010). However, very little is known about analgesic responses mediated by peripheral KOR. Here we studied regulation of responses to the KOR agonist, U50488, in primary cultures of sensory neurons and in a rat model of thermal allodynia.

Methods

Drugs and chemicals: The following compounds were purchased from Cayman Chemicals (Ann Arbor, MI): prostaglandin E_2, arachidonic acid and indomethacin. The PKC inhibitor, bisindolylmaleimide I was obtained from Calbiochem (San Diego, CA). [^{125}I]-cAMP was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). (-)U50488, Naltrindole, nor-BNI and 5'-GNTI were purchased from Sigma-Aldrich (St Louis, MO). Fetal bovine serum was from Atlanta Biologicals (Atlanta, GA). Collagenase was from Worthington Biochemical Corp. (Lakewood, NJ). All other tissue culture reagents were purchased from Invitrogen Corp (Carlsbad, CA). All other drugs and chemicals (reagent grade) were purchased from Sigma-Aldrich (St. Louis, MO).

Animals: Adult male Sprague-Dawley rats (Charles River, Wilmington, MA, USA), weighing 250-300 gm, were used in this study. The animal study protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio and conformed to International Association for the Study of Pain (IASP) and federal guidelines. Animals were housed for one week, with food and water available *ad libitum*, prior to experimentation.

Rat trigeminal ganglia culture: Primary cultures of rat trigeminal ganglion (TG) cells were prepared as described previously ((Berg et al., 2007a; Berg et al., 2007b; Patwardhan et al., 2005; Patwardhan et al., 2006). Fresh TG were washed with

Hank's balanced salt solution (HBSS; Ca^{++}, Mg^{++} free), digested with 3 mg/ml collagenase for 30 min at 37° C, and centrifuged (1000 rpm; 1 min). The pellet was further digested with 0.1% trypsin (15 min) and 167 µg/ml DNase (10 min) at 37° C in the same solution. Cells were pelleted by centrifugation (2 min at 2000 rpm) and resuspended in Dulbecco's Modified Eagle Medium (high glucose) containing 100 ng/ml nerve growth factor (Harlan), 10% fetal bovine serum, 1x Pen/Strep, 1x L-glutamine and the mitotic inhibitors: 7.5 µg/ml uridine and 17.5 mg/ml 5-fluoro-2'-deoxyuridine. After trituration to give a single cell suspension, cells were seeded on polylysine-coated 48-well plates (BD Bioscience). Media was changed 24h and 48h after plating. On the 5th day of culture, cells were re-fed with serum-free DMEM without NGF. Cells were used on the 6th day of culture. When present, pertussis toxin (400 ng/ml) was added during the 24h serum free culture period.

Measurement of cellular cAMP accumulation: Opioid agonist-induced inhibition of PGE_2-stimulated adenylyl cyclase activity was measured as described previously (Berg et al., 2007a; Berg et al., 2007b). Briefly, TG cultures in 48-well plates were washed twice with HBSS containing 20 mM HEPES, pH 7.4 (wash buffer). Cells were pre-equilibrated in 250 µl wash buffer per well for 30 min at 37°C (room air). Cells were then incubated with the KOR agonist, U50488 (various concentrations), in the presence of the phosphodiesterase inhibitor, rolipram (100 µM) for 15 min at 37°C. A maximal concentration of PGE_2 (1 µM) was added and cells incubated for a further 15 min. As appropriate, BK (10 µM) was added during the pre-equilibration period, 15 min before U50488. Incubations were terminated by

aspiration of the wash buffer and addition of 500 µl ice cold absolute ethanol. The ethanol extracts from individual wells were dried under a gentle air stream and reconstituted in 100 µl 50 mM sodium acetate, pH 6.2. The cAMP content of each 100 µl sample was determined by radioimmunoassay.

Measurement of iCGRP release: Opioid agonist-induced inhibition of BK/PGE_2-stimulated immunoreactive calcitonin gene-related peptide (iCGRP) release from TG cultures was measured as we have described previously (Patwardhan et al., 2005; Berg et al., 2007a). Briefly, TG cells were harvested and grown in 48-well plates for 5 days as described above. Cells were washed twice in release buffer (HBSS supplemented with 10.9 mM HEPES, 4.2 mM sodium bicarbonate, 10 mM dextrose and 0.1% bovine serum albumin, pH 7.4) and then pretreated with vehicle or BK (10 µM). Fifteen min later, cells were treated with opioid ligands or vehicle (15 min), followed by PGE_2/BK (1 µM / 10µM) or vehicle for an additional 15 min. Levels of iCGRP obtained from the supernatant (500 µL) were measured with radioimmunoassay.

ERK1/2 phosphorylation: TG cells were harvested and grown in 48-well plates culture for 5 days as described above. Cells were washed twice with HBSS containing 20 mM HEPES pH 7.4 (wash buffer) and pre-equilibrated in 250µl wash buffer per well for 30 min at 37°C (room air). Cells were then incubated with the KOR agonist, U50488 (100 nM), in the absence or presence of nor-BNI (3 nM) for 0-15 min at 37°C. BK (10 µM) or vehicle was added during the pre-equilibration

period 15 min prior to U50488. Incubations were terminated by aspiration of buffer and addition of 50 µl lysis buffer supplied with the SureFire phospho-ERK (pERK) assay kit (PerkinElmer, Waltham, MA). Samples were processed according to the manufacturer's directions. The fluorescence signal from pERK was measured in duplicate using a Fluostar microplate reader (BMG Labtech, Offenburg, Germany) with AlphaScreen settings.

Behavioral assay: Opioid agonist-mediated changes in paw withdrawal latency (PWL) to a thermal stimulus was measured with a plantar test apparatus (Hargreaves et al., 1988) as described previously (Rowan et al., 2009). Briefly, rats were placed in plastic boxes (10 cm x 20 cm) with a glass floor. After a 30 min habituation period, the plantar surface of the hindpaw was exposed to a beam of radiant heat through the glass floor. The rate of increase in temperature of the glass floor was adjusted so that baseline PWL values were approximately 10 ± 2 s; cut-off time was 25 s to prevent tissue damage. PWL measurements were taken in duplicate at least 30 s apart at 5 min intervals and the average was used for statistical analysis. After baseline PWL was determined, animals were pre-treated with BK (25 µg) or Veh via intraplantar (i.pl.) injection 15 min prior to co-injection with U50488 (with indicated doses) and PGE_2 (0.3 µg). Where indicated, antagonists were injected i.pl. with BK or Veh pre-treatment. Data are expressed as the change (sec) from individual PWL baseline values. All drugs, prepared in PBS, were administered via i.pl. injection at a final volume of 50 µl. Observers were blinded to the treatment allocation.

Data analysis: For TG cell culture experiments, concentration-response data were fit to a logistic equation (Equation #1) using non-linear regression analysis to provide estimates of maximal response (R_{max}), potency (EC_{50}) and slope factor (n).

$$R = R_o - \frac{R_o - R_i}{1 + \left({[A]}/{EC_{50}} \right)^n}$$

Equation #1

where R is the measured response at a given agonist concentration (A), R_o is the response in the absence of agonist, R_i is the response after maximal inhibition by the agonist, EC_{50} = the concentration of agonist that produces half-maximal response, and n = slope index. R_{max} (the maximal inhibition produced by the agonist) was calculated as R_o-R_i. Experiments were repeated at least 3 times. Statistical differences in concentration-response curve parameters between groups were analyzed with Student's paired t-test. When only a single concentration was used, statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc or Student t-test (paired) using Prism software (Graphpad Software, Inc., San Diego, CA). $p<0.05$ was considered statistically significant.

For behavioral experiments, time-course data were analyzed with repeated measures two-way analysis of variance, followed by Bonferroni's post-hoc test using Prism. Area under the time-response curve was calculated and analyzed with one-way ANOVA followed by Bonferroni's post-hoc test using Prism. Statistical inference was made when $p<0.05$ and data are presented as mean $\pm$ SEM.

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Results

Characterization of KOR responses in primary cultures derived from TG.

In primary cultures derived from adult rat TG, incubation with the selective KOR agonist, U50488, did not alter PGE_2-stimulated adenylyl cyclase activity. After fifteen minutes of pretreatment with the inflammatory mediator, BK, U50488 inhibited adenylyl cyclase activity by approximately 60% in a pertussis toxin-sensitive manner (Figure 1A). Figure 1B shows concentration-response curves for U50488 in which the EC_{50} was 2.3 nM (pEC_{50} 8.63 ± 0.53) with a maximal inhibition of 51% ± 2%. The response to U50488 was blocked by the selective KOR antagonist, nor-binaltorphimine (nor-BNI), but not by the DOR antagonist, naltrindole. Similarly, U50488 did not alter stimulated iCGRP release unless TG neurons were first pre-exposed to BK (Figure 2).

Unlike the results from the adenylyl cyclase and the iCGRP experiments, U50488-mediated stimulation of extracellular signal-regulated kinase (ERK) activation (measured as production of pERK) was not dependent upon prior treatment with BK. Figure 3 shows the time-course of pERK production over 15 min in response to U50488. Neither the time-course, nor the maximal response (•130% basal), were altered by BK. Stimulation of pERK production by U50488 was completely blocked by nor-BNI (3 nM).

In addition to opioid receptors, cells of the trigeminal ganglion express other Gi protein-coupled receptors, such as $5\text{-}HT_{1B/1D}$ receptors (Ahn and Basbaum, 2006)

and receptors for neuropeptide Y (NPY) (Gibbs et al., 2007). Figure 4 shows that inhibition of PGE_2-stimulated adenylyl cyclase activity occurred without BK pretreatment in response to maximal concentrations of the $5\text{-}HT_1$ agonist, 5-carboxamidotryptamine (5-CT), or by the NPY_1 receptor agonist, Leu-Pro NPY (L,P-NPY). The response to either agonist was not altered by BK and was sensitive to inhibition mediated by treatment with pertussis toxin.

Mechanism of BK-mediated competency of KOR function in TG primary cultures.

Figure 5 shows that the effect of BK to promote functional competency of KOR to inhibit PGE_2-stimulated adenylyl cyclase activity was blocked by the inhibitor of protein kinase C (PKC), bisindolylmaleimide I (BIS), and by the cyclooxygenase inhibitor, indomethacin, suggesting that PKC and cyclooxygenase are involved in mediating the action of BK on KOR signaling. Furthermore, the BK effect was also blocked by an antagonist of the arginine-glycine-asparagine (RGD)-binding integrins, soluble Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) peptide, but not by the inactive, reverse sequence peptide, Gly-Asp-Gly-Arg-Ser-Pro (GDGRSP) (Figure 6).

KOR-mediated inhibition of PGE_2-induced thermal allodynia.

Administration of PGE_2 to the hindpaw of rats by intraplantar injection reduces PWL in response to a thermal stimulus (thermal allodynia) for at least 20

min (Rowan et al., 2009). Intraplantar injection of BK produces a transient thermal allodynia that lasts less than 15 min (Rowan et al., 2009). Co-administration of U50488 to the hindpaw, at doses up to 10 µg, with PGE_2 does not alter PWL (Figure 7). However, if U50488 is co-administered at a dose as low as 0.1 µg 15 min after administration of BK, the thermal allodynia is significantly reversed (Figure 7). Interestingly, after BK administration the dose-response curve for U50488 for anti-allodynia was an inverted-U shape. The largest anti-allodynic effect of U50488 (as assessed by the area under the time-response curve) occurred for the 0.1 µg dose, whereas the response to 10 µg U50488 was less (Supplementary Figure 1). The effect of U50488 (1 µg) in BK-pretreated hindpaws was blocked by the KOR antagonists, nor-BNI and 5'-guanidinonaltrindole (5'-GNTI) (Figure 8A). The antagonists did not alter PGE_2-induced thermal allodynia when administered to BK-pre-treated hindpaws (data not shown, $p > 0.05$, two-way ANOVA with Bonferroni's post test). The effect of U50488 was restricted to the hindpaw ipsilateral to the BK injection (Figure 8B) indicating the effect of U50488 was mediated by peripheral KOR.

Discussion

An understanding of the mechanisms by which peripheral opioid receptor system function is regulated may be of value for improving the efficacy of peripherally-acting opioid drugs and limiting the adverse effects that restrict doses used to treat pain. We recently found that MOR and DOR agonists do not inhibit PGE_2-stimulated adenylyl cyclase activity nor BK/PGE_2-stimulated neuropeptide release from cultures of adult rat sensory neurons unless the cells are pretreated with BK or the protease-activated receptor 2 agonist, Ser-Leu-Ile-Gly-Arg-Leu-NH_2, both of which activate Gq/11-mediated PLC signaling (Patwardhan et al., 2005; Patwardhan et al., 2006; Berg et al., 2007a). Moreover, DOR agonists do not reduce PGE_2-stimulated thermal allodynia when administered to the rat hindpaw at peripherally-restricted doses unless BK is first pre-administered to the hindpaw (Rowan et al., 2009).

As outlined in Figure 9, the cellular mechanism by which MOR system functional competence is induced involves an increase in receptor-G protein coupling efficiency (assessed as an increase in GTP[γ^{35}S] binding to Gi/o proteins) in response to a cyclooxygenase-dependent metabolite of arachidonic acid that is downstream from PKC (Berg et al., 2007a). BK induction of functional competence of opioid receptor systems in both in vitro and *in vivo* studies is blocked by inhibitors of PKC and cyclooxygenase (Patwardhan et al., 2005; Berg et al., 2007a; Rowan et al., 2009). The effect of BK on MOR signaling also is mediated by the RGD-binding class of integrins as RGD-binding integrin inhibitors block the

induction of MOR functional competence to inhibit adenylyl cyclase activity (Berg et al., 2007b). Here we found that the KOR agonist, U50488, was ineffective at inhibition of adenylyl cyclase activity and iCGRP release from primary cultures of sensory neurons and did not inhibit PGE_2-induced thermal allodynia when administered via intraplantar injection unless cells or tissue were pre-treated with BK. Additionally, the effect of BK in sensory neurons was blocked by inhibitors of PKC (BIS), cyclooxygenase (indomethacin) and RGD-binding integrins (soluble RGD peptide). These data suggest that all three peripheral opioid receptor systems require stimuli associated with inflammation to become capable of inhibiting adenylyl cyclase activity, neuropeptide release and thermal allodynia.

The requirement of a stimulus, like BK, to enable opioid receptor inhibition of adenylyl cyclase appears to be somewhat specific to peripheral opioid receptors and to the adenylyl cyclase signaling pathway. Central opioid receptors do not require a pre-treatment stimulus to be functional to inhibit adenylyl cyclase nor to promote analgesia (Waldhoer et al., 2004; Corbett et al., 2006). Moreover, BK pre-treatment was not required for, and did not modify, inhibition of adenylyl cyclase activity produced by other PTx-sensitive Gi-coupled receptor agonists, such as 5-CT and L,P-NPY, which act upon $5\text{-}HT_1$ and NPY_1 receptors, respectively. This suggests that the target of the BK effect may be at the opioid receptors themselves leading to increases in receptor-G protein coupling efficiency (Berg et al., 2007a). BK pre-treatment was also not required for, and did not modify, the stimulation of the ERK pathway by U50488. However, BK pre-treatment was necessary for U50488 to

inhibit thermal allodynia, suggesting that the adenylyl cyclase signaling pathway and not the ERK pathway plays a role in mediating the anti-allodynic effect of U50488. Interestingly, ERK activation in dorsal root ganglion primary afferents that express transient receptor potential channel V1 (TRPV1) occurs rapidly (2-5 min) in response to noxious heat stimulation and capsaicin injection into the skin (Dai et al., 2002). This suggests that stimulation of ERK activity in primary sensory neurons is associated with increased pain transmission. Recently, KOR ligands with differential activity toward kinase signaling pathways have been identified (Bruchas and Chavkin, 2010) and it is interesting to speculate that KOR ligands which display functional selectivity toward inhibition of adenylyl cyclase activity versus activation of ERK may have a higher degree of analgesic efficacy.

The duration of the anti-allodynic effect of U50488 was inversely related to its dose. Analgesic efficacy of U50488 was maintained for at least 15 min when administered at a dose of 0.1 µg (i.pl.). However at a dose of 1 µg, the anti-allodynic effect lasted for less than 15 min and for the dose of 10 µg, the duration of the effect was less than 10 min. Similarly, the area under the time-response curve was significantly less for the 10 µg dose than for the 0.1 µg dose of U50488. These results suggests that in addition to its anti-allodynic effects, U50488 may also initiate pro-nociceptive mechanisms that compete temporally with anti-nociceptive mechanisms. It is interesting that this profile required BK pretreatment as U50488 did not alter paw withdrawal latency in the absence of BK pretreatment. Many studies have shown that acute administration of opioids can enhance pain sensation

("paradoxical pain") via a variety of mechanisms (for review see Ossipov et al., 2004), and MOR, DOR and KOR agonists have been shown to prolong action potentials of dorsal-root ganglion neurons maintained in long-term organotypic explants (Crain and Shen, 1990). The reduced duration of the anti-allodynic action of U50488 could also result from rapid desensitization of U50488-mediated KOR signaling. Opioid receptor systems, like many 7 transmembrane-spanning receptor systems, can undergo rapid, agonist-induced desensitization that can involve receptor phosphorylation, internalization, and downregulation as well as post-receptor effects (Marie et al., 2006; Kelly et al., 2008). MOR signaling to voltage-gated calcium channels in dorsal root ganglion neurons desensitizes rapidly in response to agonist pre-treatment of cultures (Nomura et al., 1994; Samoriski and Gross, 2000; Tan et al., 2003; Konig et al., 2010), however, prolonged (4 day) morphine pre-treatment of rats did not reduce morphine-induced receptor-G protein coupling nor the inhibition of adenylyl cyclase activity in dorsal root ganglia innervating inflamed hindpaw (Zollner et al., 2008). It was found that the lack of MOR system desensitization required the availability of endogenous opioids in the inflamed tissue, which may explain differences when agonist treatment is applied to cells in culture versus the intact animal. Additional experiments are required to determine the mechanism underlying the shortened duration of the anti-allodynic effect of high doses of U50488.

Many studies have demonstrated analgesic effects mediated by opioid receptors located in the periphery following inflammation or tissue damage (see

Joris et al., 1987; Przewlocki and Przewlocka, 2001; Stein and Zollner, 2009), although peripheral efficacy of opioids in humans is variable and weak (Gupta et al., 2001; Kalso et al., 2002). Additionally, there is a prominent role for endogenous opioids released from circulating leukocytes of the immune system that migrate into sites of inflammation in regulating pain sensitivity (Stein and Zollner, 2009; Busch-Dienstfertig and Stein, 2010). As a result there has been strong interest in developing new pharmacological approaches to treat pain that involve either direct activation of opioid receptors on sensory neurons or indirect opioid receptor activation by stimulating release of endogenous opioids. Efforts to directly activate opioid receptors focus on developing drugs with low permeability to penetrate into the CNS via the blood-brain barrier (DeHaven-Hudkins and Dolle, 2004). By limiting access to the CNS, adverse effects mediated by CNS opioid receptors would be expected to be reduced or eliminated. Although drugs that target MOR are currently the “gold-standard” for treatment of pain, activation of peripheral MOR leads to adverse effects such as constipation and itch (Manara et al., 1986; Yamamoto and Sugimoto, 2010). In various animal models and clinical studies, there is evidence that peripherally-restricted DOR and KOR agonists can be effective in reducing inflammatory pain (for reviews see Riviere, 2004; Stein and Zollner, 2009; Kivell and Prisinzano, 2010). It is expected that the adverse effect profile of DOR or KOR peripherally-restricted ligands would be less than that of MOR, consequently approaches that target peripheral DOR or KOR may provide for a better therapeutic index.

In summary, we found that regulation of KOR-mediated inhibition of adenylyl cyclase activity and neuropeptide release from primary sensory TG neurons by U50488 and KOR-mediated anti-allodynia *in vivo* by U50488 is similar to regulation of MOR and DOR. However, BK pre-treatment was not required for U50488-mediated stimulation of ERK activity nor for 5-$HT_{1B\backslash 1D}$ or NPY_1 receptor agonists to inhibit adenylyl cyclase activity. Further understanding of the mechanisms by which inflammation and/or tissue damage regulates the efficacy of opioid drugs along with the roles and regulation of each of the multiple signaling pathways coupled to peripheral opioid receptors may lead to improved pharmacotherapy for treatment of pain with reduced adverse effects.

Authorship contributions:

Participated in research design: Berg, Milam, Patwardhan and Clarke.

Conducted experiments: Berg, Rowan, Patwardhan, Sanchez and Silva.

Performed data analysis: Berg, Rowan, Patwardhan and Clarke.

Wrote or contributed to the writing of the manuscript: Berg, Hargreaves and Clarke.

Other: Berg and Clarke acquired funding for the research.

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Footnotes:

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Figure Legends

Figure 1: **Effect of the KOR agonist, U50488, on PGE_2-stimulated cAMP accumulation in TG cultures**. **A)** Pre-treatment with BK is necessary for U50488 to inhibit cAMP accumulation. TG primary cultures were incubated with or without PTx (400 ng/ml, 24 h). Cells were then pre-treated with or without BK (10 μM) for 15 min. After pre-treatment, cells were incubated with U50488 (100 nM) for 15 min followed by addition of PGE_2 (1 μM) and further incubation for 15 min. Cellular levels of cAMP were determined by RIA. Neither BK nor PTx significantly affected PGE_2-stimulated cAMP accumulation (p=0.72, one way ANOVA). Basal levels of cAMP (mean pmol ± SEM) were 1.09 ± 0.21(n=3) for vehicle conditions, 2.28 ± 0.29 (n= 7) for BK pre-treated cells, and 1.22 ± 0.19 (n=4) for cells treated 24h with PTx. The data shown represent the mean percentage above basal cAMP accumulation ± SEM of 3-7 experiments. *p<0.05 Veh vs. U50488, one-way ANOVA with Bonferroni's post test. **B)** Concentration-response curve to U50488 for inhibition of PGE_2-stimulated cAMP accumulation in TG cultures pre-treated with BK (10 μM, 15 min). The U50488 response is blocked by the KOR antagonist, nor-BNI (3 nM), but not by the DOR antagonist, naltrindole (Nal, 20 nM). Antagonists were added during the BK pre-treatment period. The data shown represent the mean percentage of PGE_2-stimulated cAMP accumulation ± SEM of 4 experiments.

Figure 2. **Effect of the KOR agonist, U50488, on BK/PGE_2-evoked iCGRP release in TG cultures.** Pre-treatment with BK is necessary for U50488 to inhibit

iCGRP release. Cells were pre-treated with or without BK (10 μM) for 15 min, washed and incubated for 15 with or without U50488 (100 nM) and then further incubated with PGE_2 (1 μM) + BK (10 μM) for 15 min. Samples of the media were assayed for iCGRP with RIA. Data are expressed as the percentage over basal iCGRP release and represent the mean ± SEM of 3-5 experiments. Horizontal line at 100% represents baseline release. *p< 0.05, Veh vs. BK pre-treatment conditions. **p< 0.01, Veh vs. U50488 conditions, one-way ANOVA with Bonferroni's post test.

Figure 3. U50488 stimulation of ERK activity in TG cultures does not require BK pre-treatment. TG cultures were pre-treated with or without BK (10 μm) for 15 min. Cells were then treated with U50488 (100 nM) for the times indicated and the level of pERK was measured using the pERK *Surefire* assay kit from PerkinElmer, according to the manufacturer's protocol. Nor-BNI was present 15 min before addition of U50488. Data are expressed as the percentage increase in pERK levels over basal (no ligand) activity and represent the mean ± SEM of 4 experiments. *p< 0.05, nor-BNI vs. Veh; ***p< 0.0001, U50488 vs. basal, two-way ANOVA with Bonferonni post-test.

Figure 4. Inhibition of PGE_2-stimulated cAMP accumulation by the $5\text{-}HT_1$ receptor agonist, 5-CT, or the NPY_1 receptor agonist, L,P-NPY, in TG cultures does not require BK pre-treatment. TG primary cultures were incubated with or without PTx (400 ng/ml, 24 h). Cells were then pre-treated with or without BK (10 μM) for 15 min. After pre-treatment, cells were incubated with 5-

CT (100 nM) or L,P-NPY (1 μM) for 15 min followed by addition of PGE_2 (1 μM) and further incubation for 15 min. Cellular levels of cAMP were determined by RIA. Basal levels of cAMP (mean pmol ± SEM) were 1.07 ± 0.13 (n=11) for vehicle conditions, 1.3 ± 0.13 (n= 7) for BK treated cells, and 1.14 ± 0.23 (n=4) for cells treated 24h with PTx. The data shown represent the mean percentage above basal cAMP accumulation ± SEM of 3-7 experiments. BK pretreatment did not significantly alter the inhibition response of either 5-CT (p=0.94) or L,P-NPY (p=0.72) as compared to vehicle, one-way ANOVA with Bonferroni's post test. ***p< 0.001 Veh vs. 5-CT or Veh vs. L,P-NPY.

Figure 5. Effect of inhibition of PKC or cyclooxygenase on U50488-mediated inhibition of PGE_2-evoked cAMP accumulation. TG cultures were treated with vehicle or either the cyclooxygenase inhibitor, indomethacin (Indo, 2 μM), or the PKC inhibitor, bisindolylmaleimide (Bis, 1 μM), for 15 min before pre-treatment with BK (10 μM) for 15 min. Following pre-treatment, TG cells were incubated with or without U50488 (100 nM) for 15 min followed by addition of PGE_2 (1 μM) and further incubation for 15 min. Cellular cAMP levels were determined by RIA. Data shown are the mean ± S.E.M. of four experiments. Data are expressed as the mean percentage above basal cAMP levels ± SEM. Neither Indo nor Bis altered PGE_2-stimulated cAMP levels, p> 0.05 vs vehicle, one way ANOVA. ***p< 0.001, Veh vs. U50488, one way ANOVA with Bonferroni's post test.

Figure 6. **BK effects on KOR-mediated inhibition of PGE_2-stimulated cAMP accumulation is dependent on RDG-binding integrin activation.** Soluble RGD peptides (G**RGD**SP), but not the inactive, reverse sequence peptides (G**DGR**SP) block BK pre-treatment effects on U50488-mediated inhibition of PGE_2-stimulated cAMP accumulation. TG cultures were incubated with vehicle, soluble G**RGD**SP peptides (100 µM) or the inactive soluble G**DGR**SP (100 µM) for 30 min, followed by addition of BK (10 µM) and further incubation for 15 min. After BK pre-treatment, cells were incubated with vehicle or U50488 (100 nM) for 15 min, followed by addition of PGE_2 (1 µM) and further incubation for 15 min. Cellular cAMP levels were determined by RIA. Data shown are the mean ± S.E.M. of four experiments. Data are expressed as the mean percentage above basal cAMP levels ± SEM. PGE_2-stimulated cAMP levels were not significantly altered by either RGD or DGR treatment, $p > 0.05$ vs vehicle, one way ANOVA. ***$p < 0.001$, Veh vs. U50488, one way ANOVA with Bonferroni's post test.

Figure 7. The anti-allodynic effect of U50488 on PGE_2-mediated thermal allodynia requires pre-treatment with BK. Separate groups of animals were injected (i.pl.) with BK (25 µg) or vehicle 15 min prior to co-injection with U50488 (doses indicated) and PGE_2 (0.3 µg). PWL was measured in duplicate every 5 min for 20 min after the last injection. Data are expressed as change from individual baselines and represent mean ± SEM of 6 animals per group. Baseline PWLs were 10.06 ± 0.21. **$p < 0.01$, ***$p < 0.001$, U50488 vs. corresponding Veh time point, two way repeated ANOVA with Bonferroni's post test.

Figure 8. The anti-allodynic effect of U50488 on PGE_2-mediated thermal allodynia is blocked by KOR antagonists and is peripherally mediated. A) Separate groups of animals were injected (i.pl.) with BK (25 µg) with or without the KOR-selective antagonists, nor-BNI (10 µg) or 5'-GNTI (2 µg), 15 min prior to co-injection with U50488 (1 µg) and PGE_2 (0.3 µg). PWL was measured in duplicate every 5 min for 20 min after the last injection. Data are expressed as change from individual baselines and represent mean ± SEM of 6 animals per group. Baseline PWLs were 9.74 ± 0.26 s. **p< 0.01, ***p< 0.001 versus Veh, two way repeated ANOVA with Bonferroni's post test. **B)** Administration of U50488 to the contralateral hindpaw did not alter PGE_2-induced thermal allodynia in the hindpaw ipsalateral to the BK injection. Fifteen min after ipsilateral BK injection (25 µg), rats received single injections into both the ipsilateral and contralateral hindpaws. Animals received either co-injection of PGE_2 (0.3 µg) with U50488 (10 µg) in the ipsilateral hindpaw ("Ipsi"), along with injection of vehicle in the contralateral hindpaw ("Contra"), or co-injection of PGE_2 (0.3 µg) with vehicle ipsilaterally, along with injection of U50488 (10 µg) contralaterally. PWLs of the ipsilateral hindpaw were measured at 5 min intervals for 20 min after the last injection. Baseline PWLs were 9.93 ± 0.23 s. Data are expressed as change from individual baselines and represent mean ± SEM of 6 animals per group. *p< 0.05, **p< 0.01, ***p< 0.001 versus "Contra", two way repeated ANOVA with Bonferroni's post test.

Figure 9: **Proposed mechanism for the regulation of opioid receptor functional competence by activation of BK signaling**, based upon

(Patwardhan et al., 2005; Patwardhan et al., 2006; Berg et al., 2007a; Berg et al., 2007b; Rowan et al., 2009) and the present study. BK, acting via bradykinin B2 receptors, activates PLC to produce inositol trisphosphate (IP_3) and diacylglycerol (DAG). The B2 selective antagonist, HOE 140, but not the B1 selective antagonist, Lys-[Leu^8]des-Arg^9-BK, prevents BK from eliciting functional competence. Agonists at other Gq-coupled receptors, such as the PAR-2 receptor, will also induce functional competence. DAG activates PKC. Inhibitors of PKC, such as bisindolylmaleimide I (Bis) or Go 6976, prevent the induction of functional competence by BK and activators of PKC, such the phorbol ester, phorbol dibutyrate (PdBu), promote functional competence. Activation of PKC in turn increases PLA_2-mediated arachidonic acid (AA) release which is metabolized by a cyclooxygenase (COX). Inhibitors of COX, such as indomethacin (Indo), prevent induction of functional competence by BK and by PdBu. Application of exogenous AA induces functional competence which is not blocked by inhibitors of PKC however, inhibition of COX blocks functional competence induced by PdBu, suggesting that PLA_2-AA-COX pathway is downstream from PKC. As indicated by the dashed lines, the COX-generated AA metabolite that promotes functional competence is not known. Inhibitors of the RGD-binding class of integrins also block induction of functional competence by BK, suggesting that RGD-binding integrins are necessary for production of functional competence by BK. It is not known whether integrins are an intermediary in BK signaling or act in parallel with BK to promote functional competence of opioid receptors systems. The effect of BK on the opioid receptor

signaling system appears to be at the receptor-G protein level since BK pretreatment enhances opioid agonist-stimulated GTP[γ^{35}S] binding.

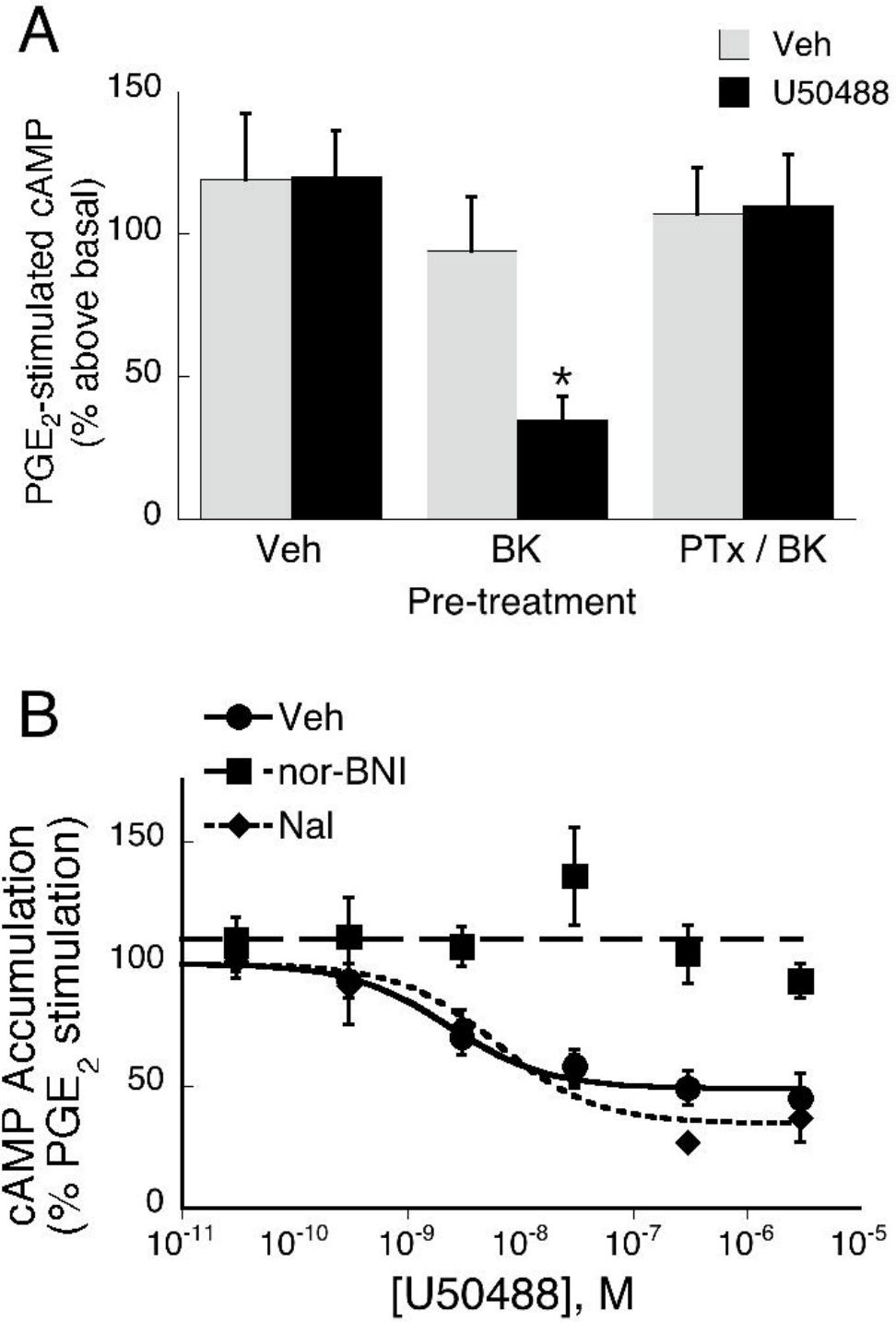
A
Veh
U50488
150
100
50
0
PGE2-stimulated cAMP
(% above basal)
*
Veh
BK
PTx / BK
Pre-treatment
B
Veh
nor-BNI
Nal
150
100
50
0
cAMP Accumulation
(% PGE2 stimulation)
10-11
10-10
10-9
10-8
10-7
10-6
10-5
[U50488], M

Figure 1

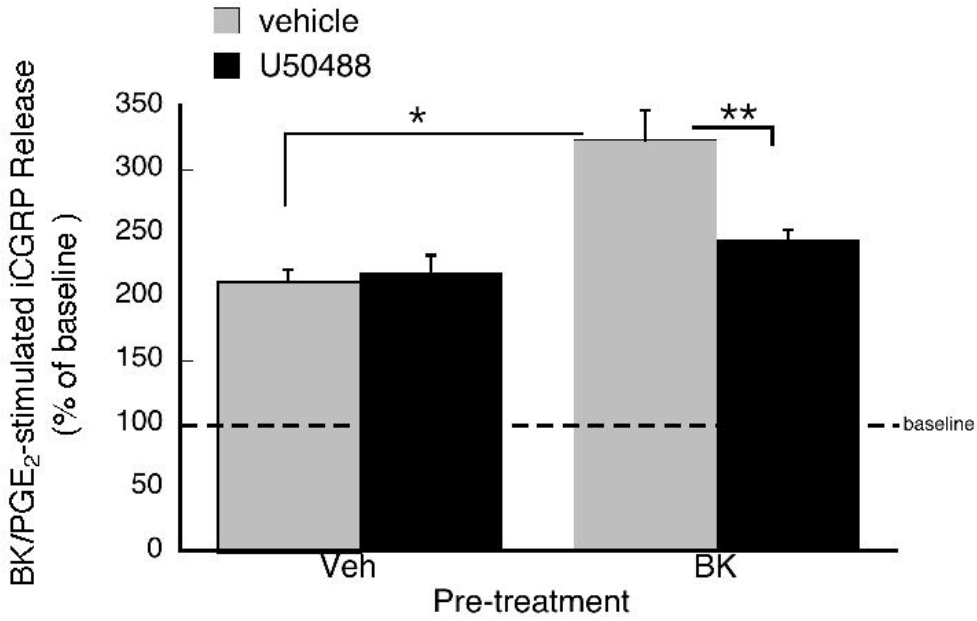
vehicle
U50488
BK/PGE2-stimulated iCGRP Release
(% of baseline)
350
300
250
200
150
100
50
0
*
**
baseline
Veh
BK
Pre-treatment

Figure 2

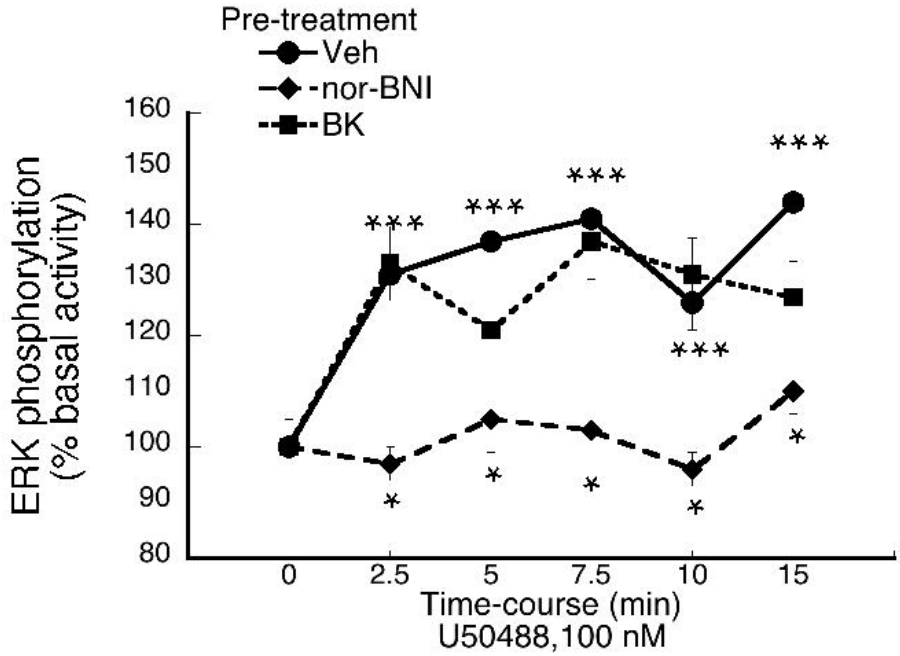
Pre-treatment
Veh
nor-BNI
BK
ERK phosphorylation
(% basal activity)
160
150
140
130
120
110
100
90
80
0
2.5
5
7.5
10
15
Time-course (min)
U50488,100 nM

Figure 3

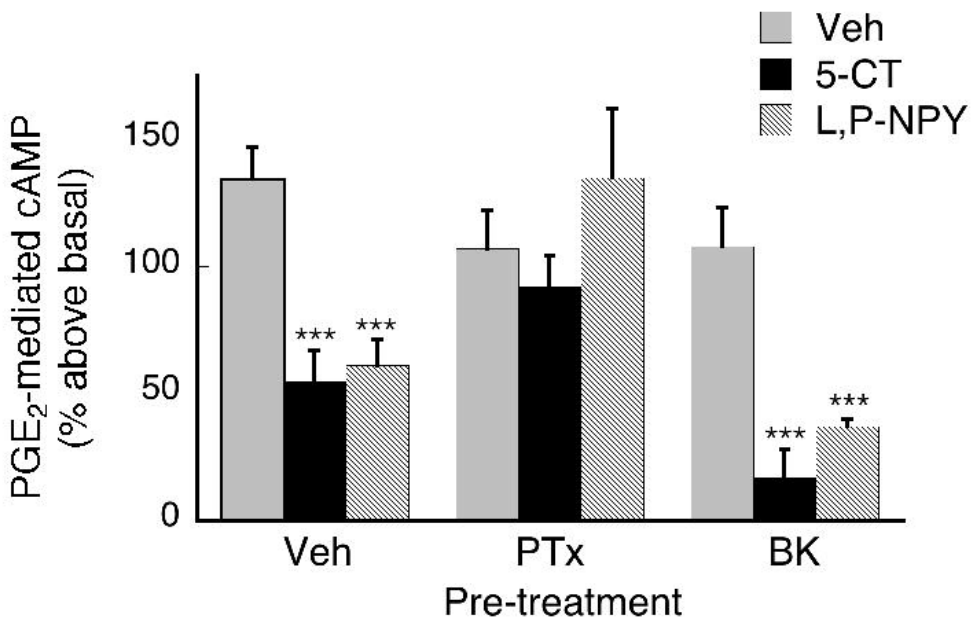
Veh
5-CT
L,P-NPY
PGE2-mediated cAMP
(% above basal)
150
100
50
0
***
***
***
***
Veh
PTx
BK
Pre-treatment

Figure 4

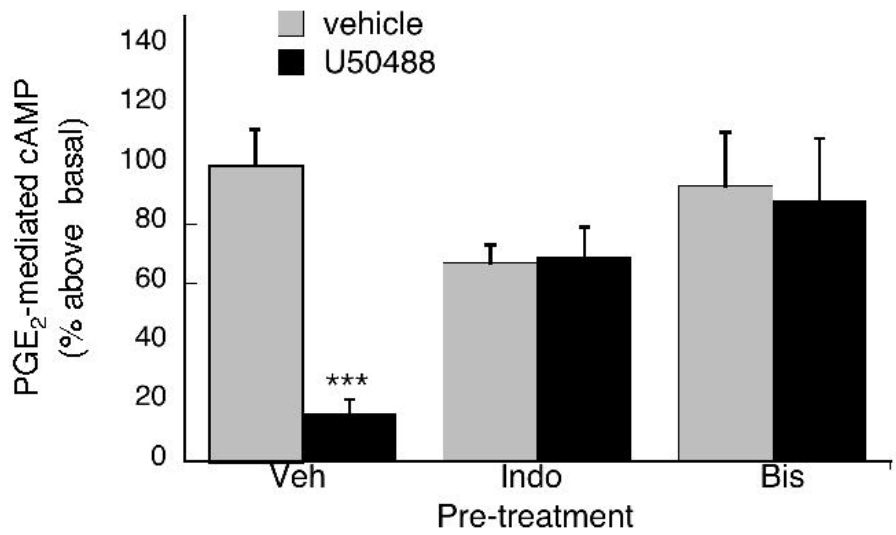
vehicle
U50488
140
120
100
80
60
40
20
0
PGE2-mediated cAMP
(% above basal)
***
Veh
Indo
Bis
Pre-treatment

Figure 5

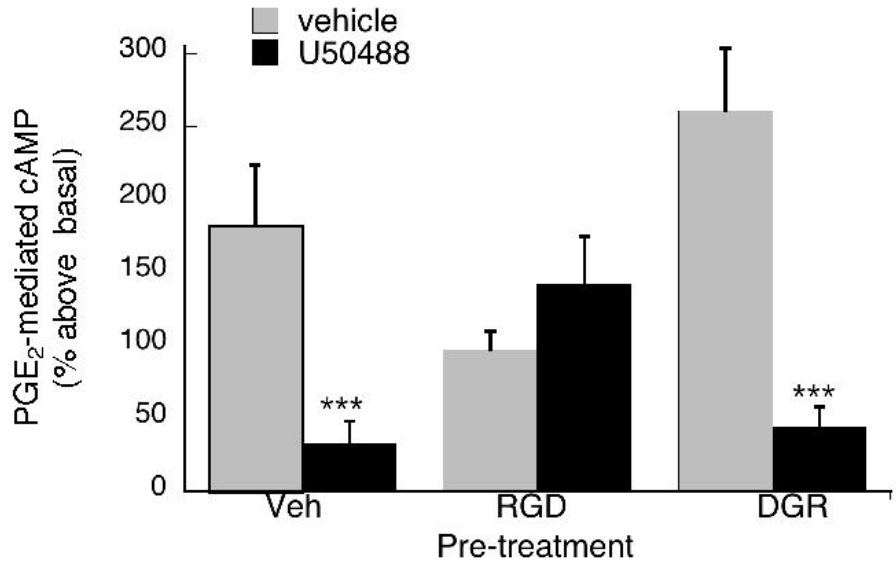
vehicle
U50488
300
250
200
150
100
50
0
PGE2-mediated cAMP
(% above basal)
***
***
Veh
RGD
DGR
Pre-treatment

Figure 6

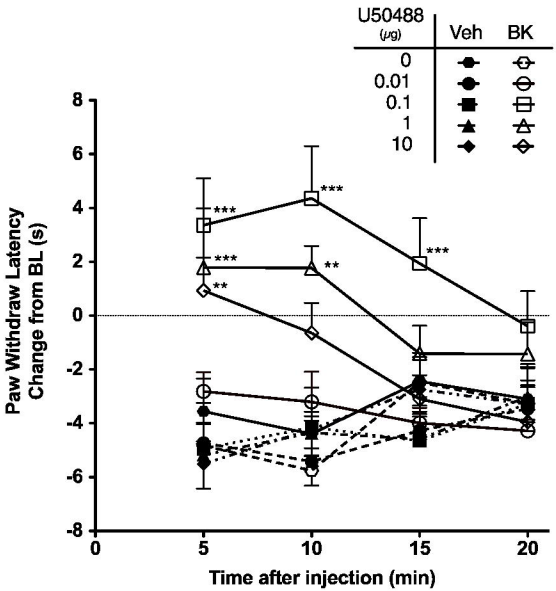
U50488
(μg)
Veh
BK
0
0.01
0.1
1
10
Paw Withdraw Latency
Change from BL (s)
8
6
4
2
0
-2
-4
-6
-8
0
5
10
15
20
Time after injection (min)
***
***
***
**
**

Figure 7

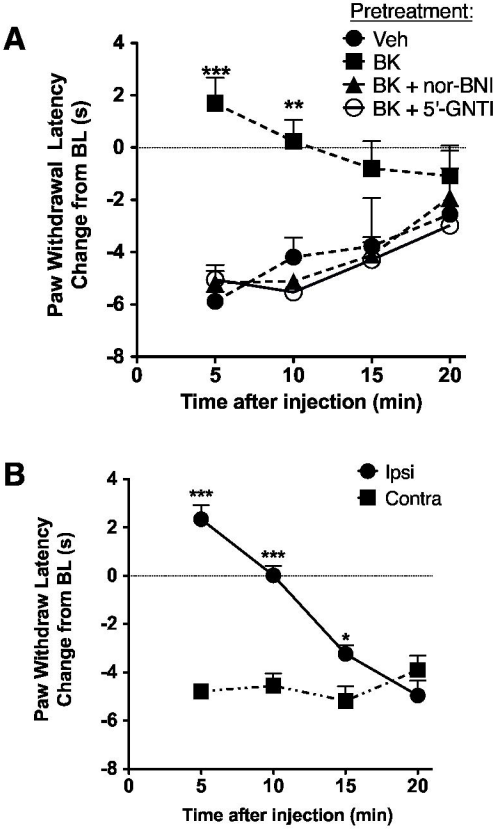
A
Pretreatment:
Veh
BK
BK + nor-BNI
BK + 5'-GNTI
***
**
Paw Withdrawal Latency
Change from BL (s)
Time after injection (min)
B
Ipsi
Contra
***
***
*
Paw Withdraw Latency
Change from BL (s)
Time after injection (min)

Figure 8

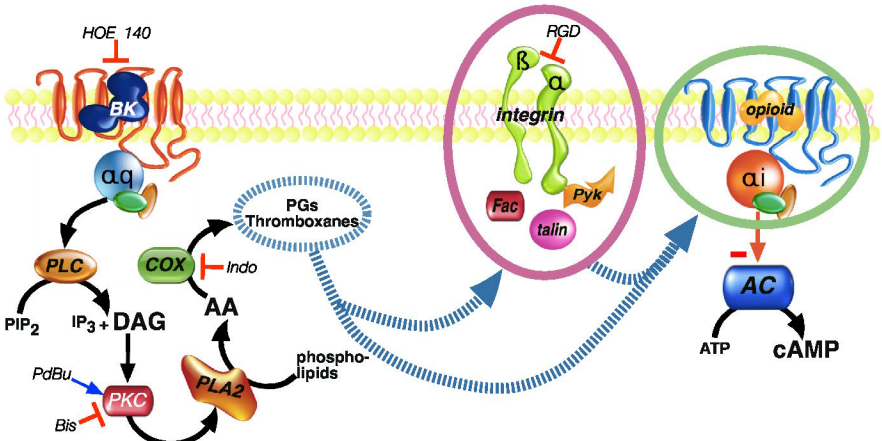
HOE 140
BK
αq
PLC
PIP2
IP3 + DAG
PdBu
PKC
Bis
COX
Indo
PGs
Thromboxanes
AA
PLA2
phospho-
lipids
RGD
ß
α
integrin
Fac
talin
Pyk
opioid
αi
AC
ATP
cAMP

Figure 9