Rapid Modulation of \( \mu \)-Opioid Receptor Signaling in Primary Sensory Neurons

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ABSTRACT

Management of pain by opioid analgesics is confounded by central adverse effects that limit clinical dosages. Consequently, there is considerable interest to understand peripheral analgesic effects of opioids. The actions of opioids on peripheral sensory neurons have been difficult to study because of a general lack of effect of opioid agonists on nociceptor function in culture despite documented presence of opioid receptors. In this study, the \( \mu \)-opioid receptor agonist, [\( \alpha \)-Ala\( ^2 \),N-MePhe\( ^4 \),Gly-\( \text{ol} \)\( ^{P} \)]-enkephalin (DAMGO), did not alter guanosine 5'-\( \text{O} \)-(3-\( \text{[35S]} \)thio) triphosphate (GTP\( \gamma \)\( ^{35S} \)) binding, adenylyl cyclase activity, or neuropeptide release in primary cultures of rat trigeminal ganglion (TG). However, after brief exposure to bradykinin (BK), DAMGO stimulated GTP\( \gamma \)\( ^{35S} \) binding and inhibited both prostaglandin \( E_2 \) (PGE\( _2 \))-stimulated adenylyl cyclase activity and BK/PGE\( _2 \)-stimulated neuropeptide release. The effect of BK was blocked by the \( \beta \)-antagonist HOE 140 [\( \alpha \)-Arg\[Hyp\( ^3 \),Thi\( ^5 \),\( \alpha \)-Tic\( ^7 \),Oic\( ^8 \)\]-bradykinin], but not by the \( \beta \)-antagonist, Lys\[Leu\]des-Arg\( ^9 \)BK, and was mimicked by the protease-activated receptor-2 agonist, Ser-Leu-Ile-Gly-Arg-Leu-NH\( _2 \), and by activation of protein kinase C (PKC) or by administration of arachidonic acid (AA). The enhanced responsiveness of \( \mu \)-opioid receptor signaling by BK priming was blocked by both cyclooxygenase and PKC inhibitors; however, the effect of AA was blocked only by a cyclooxygenase inhibitor. The results indicate that \( \mu \)-opioid receptor signaling in primary sensory TG neurons is enhanced by activation of phospholipase C-coupled receptors via a cyclooxygenase-dependent AA metabolite that is downstream of PKC.

Opioids represent a major drug class for the treatment of pain; however, there are substantial drawbacks to their systemic use. In addition to serious adverse effects (e.g., dependence, tolerance, sedation, retention), there are social and legal issues that limit their use by patients and clinicians. Consequently, there has been considerable interest in the peripheral analgesic effects of opioids because this approach may offer improved therapeutic outcomes. However, there are relatively few studies on opioid signaling in sensory neurons, such as those in the trigeminal ganglion (TG), in part perhaps due to the difficulty in obtaining consistent opioid responses in peripheral tissues.

The \( \mu \)-opioid receptor (MOR) is a member of the seven-transmembrane spanning receptor superfamily commonly referred to as G protein-coupled receptors. Many, but notably not all, cellular responses to activation of MOR are blocked by pertussis toxin, indicative of mediation by members of the \( \alpha_i/o \) family of heterotrimeric G proteins (Law et al., 2000). Among the wide variety of signaling systems reported to be activated/inhibited by opioid receptors, perhaps the best studied signaling cascade is the adenylyl cyclase pathway, where activation of MOR typically, but not always (Makman et al., 1988; Chan et al., 1995; Olianas and Onali, 1995), is inhibitory. In addition to adenylyl cyclase, MOR has been shown to regulate a variety of intracellular signaling cascades, including inward-rectifying K\( ^+ \) channels, calcium channels, and mitogen-activated protein kinase (MAPK) signaling cascades (Connor and Christie, 1999; Law et al., 2000).

Although opioid receptors are expressed by primary sen...
sory neurons (Chen et al., 1997; Li et al., 1998), a major issue is that they are functionally inactive under most basal conditions (i.e., peripheral injection of opioids only produces analgesia under conditions of injury or neuronal sensitization) (Stein et al., 2003). Antihyperalgesic/antiallodynic effects of peripheral opioids are observed within 10 to 15 min of injection of bradykinin (BK), prostaglandin E₂ (PGE₂), capsaicin, or other agents (Ferreira and Nakamura, 1979; Levine and Taito, 1989). Thus, it seems that peripheral opioid analgesia does not occur until some stimulus (e.g., inflammation) is present in the peripheral tissue (for review, see Stein et al., 2003). Within a few minutes of this conditioning stimulus, the opioid receptor system becomes “competent” in that analgesic effects can be detected, and opioids become capable of producing substantial inhibition of behavioral or cellular markers of nociception. The mechanisms mediating peripheral opioid receptor system competence are critically important because these processes may influence the potential clinical utility of peripherally selective opioid agonists.

Studies of the regulation of opioid receptor system competence have been hampered due to a lack of an in vitro model system. In our work, we have chosen to study regulation of opioid receptor responsiveness in cultures of trigeminal ganglion derived from adult rats because orofacial pain represents a major category of pain disorders that affects millions of Americans (Welch, 2001; Sarlani et al., 2005). In addition, TG cultures derived from adult animals are functional with respect to membrane excitability, exocytosis, receptor signaling and trafficking, and calcium mobilization (Patwardhan et al., 2005, 2006; Jeske et al., 2006; Gibbs et al., 2007). We recently reported that brief (15 min) activation of inflammatory mediator receptors (BK and PAR-2) on TG neurons promoted signaling of otherwise quiescent δ-opioid receptors (Patwardhan et al., 2005, 2006). Here, we report that, in untreated rat primary TG cultures, MOR agonists are ineffective. However, after brief exposure to BK or the PAR-2 agonist, Ser-Leu-Ile-Gly-Arg-Leu-NH₂ (SL-NH₂), activation of MOR significantly stimulates GTPγ[S] binding and inhibits adenylyl cyclase activity and immunoreactive calcitonin gene-related peptide (iCGRP) release. This action of BK to promote MOR signaling is mediated by a cyclooxygenase-dependent metabolite of arachidonic acid (AA) that lies downstream of protein kinase C (PKC). We hypothesize that this mechanism may contribute to the enhanced peripheral analgesic efficacy of μ-opioids when administered to inflamed tissue.

### Materials and Methods

**Materials.** The following compounds were purchased from Cayman Chemicals (Ann Arbor, MI): PGE₂, arachidonic acid, and indomethacin. SL-NH₂ (PAR-2-activating peptide) was purchased from Tocris (Ellisville, MO). The PKC inhibitors bisindolylmaleimide I and Go6976 were obtained from Calbiochem (San Diego, CA). [3H]myoinositol, [125I]-CAMP, and GTPγ[S] were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Fetal bovine serum was from Gemini Bioproducts (Calabasas, CA). 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), weighing 250 to 300 g, 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 and federal guidelines. Animals were housed for one week, with food and water available ad libitum, before harvesting of TG cells.

**Rat TG Culture.** Primary cultures of rat TG cells were prepared as described previously (Patwardhan et al., 2005, 2006). Fresh TGs were washed with Hanks’ balanced salt solution (HBSS; Ca²⁺, Mg²⁺-free), digested with 3 mg/ml collagenase for 30 min at 37°C, and centrifuged. 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 (5 min at 5000g) and resuspended in Dulbecco’s modified Eagle’s medium (high glucose) containing 100 ng/ml nerve growth factor (Harlan, Indianapolis, IN), 10% fetal bovine serum, 1× penicillin/streptomycin, 1× L-glutamine, and the mitotic inhibitors, 7.5 μg/ml uridine and 17.5 mg/ml 5-fluorodeoxyuridine. After trituration to disrupt tissue, the cell suspension was seeded on polylysine-coated 24- or 48-well plates. Media were changed 24 and 48 h after plating. Cells were used on the 5th or 6th days of culture.

**In Situ Hybridization and Immunohistochemistry.** Coverslips containing cultured TG neurons were fixed with 4% formaldehyde, permeabilized with 0.5% Triton X-100, acetylated in acetic anhydride, and dehydrated in alcohol. In situ hybridizations (55°C) were carried out using the digoxigenin-UTP-ERNA probe against bradykinin receptor 2 (position 102–500, accession no. NM_173101). After RNase (Roche, Indianapolis, IN) treatment (2 mg/ml; 30 min; 37°C), coverslips were washed with a decreasing concentration of saline sodium citrate buffer (substrates 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium; Roche). Coverslips were then incubated with rabbit primary antibody against μ-opioid receptor (1:100; ImmunoStar Inc., Hudson, WI; see Ji et al., 1995) overnight at 4°C and detected using an AlexaFluor-488-conjugated fluorescent secondary antibody (1:300; Molecular Probes, Eugene, OR). Images were generated in the Core Optical Imaging Facility, University of Texas Health Science Center at San Antonio.

To determine the extent of colocalization of the BK₂ receptor with MOR, coverslips containing cultured TG neurons were fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 10% normal goat serum. The coverslips were then incubated with previously characterized mouse monoclonal antibody against BK₂ (1:100; Research Diagnostics Inc., Concord, MA) and rabbit polyclonal antibody against MOR (1:100; ImmunoStar, Inc.) (Arvidsson et al., 1995; Ji et al., 1995; Allen et al., 2002; Mukhin et al., 2003; Patwardhan et al., 2005) overnight at 4°C. The immunoreactivity was detected using appropriate Alexa-488- or Alexa-594-conjugated secondary antibodies (1:300; Molecular Probes). Images were acquired using a Nikon Eclipse 90 microscope with a Nikon C1si confocal scanner (Nikon, Melville, NY). Images were analyzed using Metamorph software (version 4.5 r6; Molecular Devices, Sunnyvale, CA). Nine coverslips were analyzed from three independent cultures for the cell counting experiments. Images were acquired from one or two nonoverlapping areas of each coverslip, and the scaling feature of Metamorph software was used to assign an average pixel value for background staining. All the neurons that demonstrated pixel density above that threshold were considered positive.

**Measurement of Cellular cAMP Levels.** MOR-mediated inhibition of adenylyl cyclase activity was determined by measuring the amount of cAMP accumulated in the presence of the phosphodies- terase inhibitor, rolipram, and the adenylyl cyclase activator, PGE₂, similar to that described previously (Patwardhan et al., 2005). Cultures in 48-well plates were washed twice with HBSS containing 10 mM HEPES and 4 mM sodium bicarbonate, pH 7.4 (wash buffer). Cells were pre-equilibrated in 250 to 500 μl of wash buffer per well.
of a 48-well plate for 30 min at 37°C in 5% CO₂. To determine MOR-mediated effects, cells were incubated with rolipram along with the MOR agonist, Tyr-d-Ala-Gly-N-Me-Phe-Gly-ol enkephalin (DAMGO; 15 min, 37°C) followed by addition of a maximal concentration of PGE₂ (1 μM final concentration) and incubation for a further 15 min. To assess the effects of BK, BK (10 μM, a concentration to produce 100% receptor occupancy) was added during the pre-equilibration period, 15 min before DAMGO. For time course experiments, the length of time of the pretreatment period with BK was varied from 0 to 60 min. In some experiments, the PAR-2 agonist SL-NH₂ (100 μM) was added in place of BK during the 15-min pretreatment period. Incubations were terminated by aspiration of the wash buffer and addition of 500 μl of ice-cold absolute ethanol. The ethanol extracts from individual wells were dried under a gentle air stream and reconstituted in 100 μl of 50 mM sodium acetate, pH 6.2. The cAMP content of each 100-μl sample was determined by radioimmunoassay (RIA).

Measurement of Inositol Phosphate Accumulation. BK-stimulated inositol phosphate (IP) accumulation in TG cultures was measured as described previously (Berg et al., 1998). Cells, grown in 24-well plates, were labeled with 2 μCi/ml [3H]myoinositol for 24 h before experiments. After labeling, cells were rinsed three times with 1 ml of HBSS that contained 20 mM HEPES and 20 mM LiCl and were preincubated in HBSS/LiCl at 37°C in room air for 15 min. Drugs were added to a final volume of 500 μl, and cells were further incubated for 30 min. Where indicated, antagonists were added during the preincubation period. The incubation was terminated by the addition of 200 μl of ice-cold 10 mM formic acid, and total [3H]IPs (i.e., IP₁, IP₂, and IP₃) were separated with ion-exchange chromatography and measured with liquid scintillation spectrometry. Data are expressed as accumulation of total inositol phosphates (disintegrations per minute) or as a percentage of basal.

Measurement of iCGRP Release. iCGRP release from TG cultures was measured as we have described previously (Patwardhan et al., 2005, 2006). TG cells were harvested and grown in 48-well plates for 5 days as described above. Cells were washed two times 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, BK (10 μM), or SL-NH₂ (100 μM). Fifteen minutes later, cells were treated with opioid ligands or vehicle (15 min), in the continued presence of BK, and iCGRP release was stimulated with PGE₂/BK (1/10 μM) or vehicle for 15 min. The combination of PGE₂ and BK to stimulate iCGRP release was chosen because many common inflammatory mediators (e.g., serotonin, proteases, ATP, histamine, etc.) activate the same signaling pathways. PGE₂ (1 μM) or BK (10 μM) was added in place of BK during the 15-min pretreatment period. Where indicated, antagonists were added during the preincubation period. The incubation was terminated by the addition of 200 μl of ice-cold 10 mM formic acid, and total [3H]IPs (i.e., IP₁, IP₂, and IP₃) were separated with ion-exchange chromatography and measured with liquid scintillation spectrometry. Data are expressed as accumulation of total inositol phosphates (disintegrations per minute) or as a percentage of basal.

Data Analysis. Concentration response data for stimulation were fit with nonlinear regression to obtain estimates of maximal response (Eₐ₅₀), potency (EC₅₀), and slope index (n). Where appropriate, analysis of variance followed by Dunnett’s post hoc test or Student’s t test (paired) was used for statistical comparisons. Experiments were repeated at least three times. p Values < 0.05 were considered statistically significant.

Results

Figure 1 shows expression of the BK B₂ receptor mRNA, as assessed with in situ hybridization, and its colocalization with the μ-opioid receptor, assessed with immunocytochemistry, in primary cultures of adult rat TG. The extent of coexpression of the BK B₂ receptor with MOR was 77.4 ± 3.3% (224 cells counted from nine coverslips of three independent cultures). Consistent with expression of the B₂ receptor in TG cultures, BK administration resulted in the activation of phospholipase C (PLC) and increased accumulation of IP (Fig. 2). The EC₅₀ for BK was 2 nM, and the maximal response was 56% over basal. This effect of BK was antagonized by the B₂ antagonist HOE 140 (140 (IC₅₀ = 25 nM) but not by the selective B₁ receptor antagonist, Lys-[Leu₈]des-Arg⁹-BK, at concentrations up to 10 μM (Fig. 2B). In addition, the BK-PLC response was eliminated following treatment of TG cultures with capsaicin (100 μM) for 24 h, which causes death of transient receptor potential ion channel V₁-positive, small sensory neurons (Shin et al., 2003) (Fig. 2C).

The MOR agonist DAMGO did not alter the binding of GTPγS ([35S] (Fig. 3) or that of PGE₂-stimulated adenylyl cyclase activity in TG cultures under basal conditions (Fig. 4). However, after exposure of cultures to BK for 15 min, Figs. 1A and 1B shows expression of BK B₂ receptor mRNA in a 48-well plate for 30 min at 37°C in 5% CO₂. To determine MOR-mediated effects, cells were incubated with rolipram along with the MOR agonist, Tyr-d-Ala-Gly-N-Me-Phe-Gly-ol enkephalin (DAMGO; 15 min, 37°C) followed by addition of a maximal concentration of PGE₂ (1 μM final concentration) and incubation for a further 15 min. To assess the effects of BK, BK (10 μM, a concentration to produce 100% receptor occupancy) was added during the pre-equilibration period, 15 min before DAMGO. For time course experiments, the length of time of the pretreatment period with BK was varied from 0 to 60 min. In some experiments, the PAR-2 agonist SL-NH₂ (100 μM) was added in place of BK during the 15-min pretreatment period. Incubations were terminated by aspiration of the wash buffer and addition of 500 μl of ice-cold absolute ethanol. The ethanol extracts from individual wells were dried under a gentle air stream and reconstituted in 100 μl of 50 mM sodium acetate, pH 6.2. The cAMP content of each 100-μl sample was determined by radioimmunoassay (RIA).

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GTPγS Binding. Guanosine 5’-O-(thio)triphosphate binding to Goα, was done as described previously (Evans et al., 2001). TG cells were grown in 10-cm² plates for 5 days as described above and were treated with BK (10 μM) or vehicle for 15 min before prepara-
DAMGO stimulated GTPγS binding (Fig. 3) and inhibited PGE2-stimulated cAMP accumulation by approximately 50 to 75%, with an EC50 value of 10 nM (Fig. 4). The effect of BK to promote MOR-mediated inhibition of adenylyl cyclase was maximal with 10-min pretreatment and reversed between 30 and 60 min pretreatment. The BK B2 receptor antagonist, HOE 140, blocked the effect of BK to sensitize the DAMGO response and the effect of DAMGO (100 nM and 1 μM) was completely blocked by the MOR antagonist, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Phe-Thr-NH2. Activation of another Gq-PLC coupled receptor, PAR-2, with SL-NH2 also promoted inhibition of adenylyl cyclase activity by DAMGO (Fig. 4). Likewise, in the absence of priming by BK, DAMGO was ineffective in inhibiting BK/PGE2-stimulated iCGRP release in TG cultures. However, after 15-min treatment with BK or SL-NH2, DAMGO inhibited stimulated iCGRP release by approximately 30% (Fig. 5).

Because the BK B2 and PAR-2 receptors are coupled to Gq and PLC, we investigated whether this effect was PKC-dependent. Figure 6 shows that pretreatment of TG cultures with the PKC inhibitor bisindolylmaleimide I or Go 6976 blocked the BK priming effect on MOR-mediated inhibition of adenylyl cyclase activity and iCGRP release, respectively. Activation of BK B2 receptors also is known to activate phospholipase A2 (PLA2) (Leeb-Lundberg et al., 2005), resulting in production of AA that can be metabolized by a variety of enzymes.
enzymes, including cyclo-oxygenase (Folco and Murphy, 2006). In addition, AA can be generated from diacylglycerol derived from activation of PLC (Shears, 1991). Figure 6 shows that the cyclo-oxygenase inhibitor indomethacin also blocked the effect of BK on both MOR-mediated inhibition of adenylyl cyclase activity and iCGRP release. In addition, the effect of BK was mimicked by briefly pretreating the TG cultures with AA, as well as by direct activation of PKC with the phorbol ester PdBu (Fig. 7). To delineate whether AA was downstream from PKC, we attempted to block the effect of AA with a PKC inhibitor. Unlike the BK effect, the enhanced MOR responsiveness elicited by exogenous administration of AA was not blocked by bisindolylmaleimide; however, the PdBu effect was blocked by indomethacin (Fig. 7).

**Fig. 4.** Effect of the MOR agonist DAMGO on PGE₂-stimulated cAMP accumulation in TG cultures. A, pretreatment with the BK (a B₂-selective agonist) or SL-NH₂ (a PAR-2-selective agonist) is necessary for DAMGO to inhibit cAMP accumulation. TG primary cultures were incubated with or without the BK B₂ receptor antagonist HOE 140 (1 μM) for 5 min followed by BK (10 μM) or SL-NH₂ (100 μM) for 15 min. After pretreatment, cells were incubated with or without DAMGO (1 μM) for 15 min, followed by addition of PGE₂ (1 μM) and further incubation for 15 min. Neither BK nor SL-NH₂ significantly affected PGE₂-stimulated cAMP levels. Cellular cAMP levels were determined by RIA. Basal cAMP levels were 0.97 ± 0.21. Data shown are the mean ± S.E.M. of four experiments. Data are expressed as the percentage above basal cAMP levels.

**Fig. 5.** TG cultures were pretreated with or without BK (10 μM) (A) or SL-NH₂ (100 μM) (B) for 15 min, washed, and incubated for 15 min with or without DAMGO (1 μM) and then further incubated with PGE₂ (1 μM) + BK (10 μM) for 15 min. Samples of the supernatant were assayed for iCGRP with RIA. Data are presented as the percentage over basal iCGRP release and represent the mean ± S.E.M. of three experiments. Horizontal line at 100% represents baseline release. ***, p < 0.01 vehicle versus DAMGO.**
BK (10 nM) was added with or without the indicated inhibitors, cells were incubated with or without BK (10 nM) for 15 min followed by addition of PGE\(_2\) (1 \(\mu\)M) for 15 min. A, following pretreatment, TG cells were incubated with or without DAMGO (1 \(\mu\)M) for 15 min followed by addition of PGE\(_2\) (1 \(\mu\)M) and further incubation for 15 min. Cellular cAMP levels were determined by RIA. Data shown are the mean \(\pm\) S.E.M. of five experiments. Data are expressed as the percentage above basal cAMP levels. *, \(p < 0.05\), vehicle versus DAMGO conditions.

**Discussion**

The results of this work show that MOR receptor function, as assessed by stimulation of GTP\(\gamma\text{S}\) binding, inhibition of adenyl cyclase, and inhibition of iCGRP release, is not detectable under basal conditions but rapidly becomes functional after brief activation of receptors for inflammatory mediators (BK \(B_2\) and PAR-2 receptors). This finding of heterologous sensitization of MOR inhibitory signaling is similar to that which we recently described for the \(\delta\)-opioid receptor (Patwardhan et al., 2005, 2006) and may have fundamental significance for understanding the development of peripheral opioid analgesia that occurs after tissue inflammation or injection of inflammatory mediators into the skin (for review, see Stein et al., 2003; Ferreira and Nakamura, 1979; Joris et al., 1987; Levine and Taiwo, 1989).

The priming effect of BK to sensitize MOR signaling in TG cultures was mediated by the BK \(B_2\) receptor. We found that \(B_2\) receptors were coexpressed with MOR in TG cells and that BK, a selective \(B_2\) receptor agonist, stimulated PLC activity, resulting in an increase in accumulation of inositol phosphates that was blocked by the selective \(B_2\) antagonist HOE 140. This finding is in agreement with those of Jenkins et al. (2003), who also demonstrated functional \(B_2\) responses in TG cultures. In addition, treatment of TG cultures with capsaicin for 24 h, which causes death of transient receptor potential ion channel V1-positive, small sensory neurons (Shin et al., 2003), abolished the BK-PLC signal, suggesting that the BK effect occurs in this population of cells. Brief (15 min) pretreatment of TG cultures with BK promoted stimulation of GTP\(\gamma\text{S}\) binding, inhibition of cAMP accumulation, and inhibition of iCGRP release in response to the MOR agonist DAMGO. This BK-induced functional competence of MOR was blocked by HOE 140. Like the \(B_2\) receptor, the PAR-2
receptor couples to the \(G_{\alpha}\) family of G proteins and activates PLC (Trejo, 2003; Patwardhan et al., 2006). Activation of the PAR-2 receptor with SL-NH\(_2\) also promoted MOR signaling, suggesting that a \(G_{\alpha}\)-PLC signaling pathway mediates the priming effect on the MOR system.

The mechanism by which BK priming promotes MOR-mediated inhibition of TG neurons seems to involve a cyclooxygenase-dependent metabolite of AA, derived from activation of PKC. Inhibition of PKC blocked the inhibitory action of DAMGO on adenylyl cyclase activity and on iCGRP release. Moreover, indomethacin, an inhibitor of cyclooxygenase, also blocked these responses to DAMGO, suggesting involvement of the PLA\(_2\)-AA signaling cascade. Both exogenously applied AA and activation of PKC with phorbol ester substituted for BK priming to promote MOR-mediated inhibition of adenylyl cyclase activity. The effect of PKC activation was blocked by indomethacin, but the effect of AA was not blocked by inhibitors of PKC, suggesting that the AA-cyclooxygenase pathway is downstream from PKC. PLA\(_2\) activity can be regulated by phosphorylation of several serine residues in its catalytic domain (Leslie, 2004), and BK \(B_2\) receptor-mediated stimulation of PLA\(_2\) has been shown to be dependent upon PKC- and MAPK-mediated phosphorylation (Lal et al., 1998).

Interestingly, several studies have reported that activation of PKC enhances desensitization of MOR signaling. For example, in studies of locus coeruleus neurons in brain slices, Bailey et al. (2004) reported that the activation of G protein-coupled inwardly rectifying \(K^+\) channels by morphine did not desensitize unless PKC was activated by a phorbol ester or by activation of \(M_3\) muscarinic receptors. Such desensitization promoted by PKC activation would be expected to reduce signaling by MOR. Here, we found that activation of PKC in response to BK priming enhanced DAMGO-mediated inhibition of PGE\(_2\)-stimulated cAMP accumulation. The reason for this discrepancy is not known but could involve differences in neurons of the central nervous system (locus coeruleus) versus those of the peripheral nervous system (trigeminal ganglion). It has been shown that central administration of opioid agonists produces antinociceptive effects that do not occur when the same agonists are applied peripherally (Ferreira and Nakamura, 1979; Levine and Taiwo, 1989; Ko et al., 2000). In addition, differences in the action of PKC on MOR signaling could involve drug-specific effects. Although Bailey et al. (2004) found that PKC activation increased desensitization of MOR signaling to GiRK in slices containing the locus coeruleus, Ueda’s group (Inoue and Ueda, 2000; Ueda et al., 2001) has shown that PKC effects on acute tolerance to peripheral MOR-mediated analgesia and MOR internalization are dependent upon the agonist; tolerance and internalization in response to morphine, but not DAMGO, was PKC-dependent.

Arachidonic acid can be metabolized to a large variety of bioactive compounds, including prostaglandins, thromboxanes, leukotrienes, and hydroperoxyicosatetraenoic acids (Folco and Murphy, 2006). We found that the effect of BK \(B_2\) receptor activation on MOR responsiveness was blocked by indomethacin, suggesting that a cyclooxygenase metabolite of AA mediates the effect on MOR signaling. Possible candidates include prostaglandins (e.g., PGE\(_2\), PGD\(_2\), PGI\(_2\), and PGF\(_{\alpha\_2}\)) and thromboxane \(A_2\). After synthesis, the prostaglandins are rapidly transported out of the cell, where they can act on cell surface G protein-coupled receptors (autocrine or paracrine). The prostaglandin receptor family is composed of five types \([DP, EP_1-4, FP, IP, and TP_{\alpha,\beta}]\) that can stimulate \(IP, DP, EP_2,\) and \(EP_3\) or inhibit \(EP_4\) adenyl cyclase or stimulate PLC (TP, FP, and EP1), with most receptors being promiscuous for multiple G proteins/responses (for review, see Jabbour and Sales, 2004). It has also been reported that some prostaglandin receptors can transactivate receptor tyrosine kinases, such as the epidermal growth factor receptor, leading to activation of MAPK or phosphatidylinositol 3-kinase signaling (Sales et al., 2004). Recently, evidence has been accumulating that points to intracellular actions of prostaglandins. Receptors for prostaglandins have been found on the nuclear membrane, where they can regulate the production of nitric oxide and levels of intranuclear calcium and ultimately regulate gene transcription (Bhattacharya et al., 1999; Gobeil et al., 2002). Future studies are needed to identify the nature of the cyclooxygenase metabolite and its mechanism of action to sensitize MOR signaling.

Previous work has shown that MOR-mediated inhibition of \(GABA_{\alpha}\)-ergic miniature postsynaptic currents in the rat peri-aqueductal gray is produced by an AA metabolite derived from the 12-lipoxygenase pathway (Vaughan et al., 1997). Blockade of cyclooxygenase with indomethacin or aprenin enhances the effect of MOR agonists (morphine and DAMGO), apparently by shunting AA metabolism from COX to the lipoxygenase pathway. This effect has been postulated to underlie the observed synergy between nonsteroidal anti-inflammatory drugs and opioids because nonsteroidal anti-inflammatory drugs may produce their nalozone-sensitive analgesic actions via the periaqueductal gray by inhibition of COX. In our experiments, we found that antagonism of cyclooxygenase with indomethacin blocks induction of functional competence of MOR induced by BK. The reason for this apparent discrepancy in the effect of COX inhibition is not known but could involve differences in the function of central opioid receptors versus those on peripheral neurons or differences in the signaling pathways (voltage-dependent \(K^+\) channels versus adenylyl cyclase) studied.

Interestingly, the time course of enhancement of MOR signaling by BK was biphasic and transient. After 5 min of BK priming, DAMGO increased PGE\(_2\)-stimulated cAMP accumulation. The effect of DAMGO on the cAMP response was inhibitory between 10 and 30 min of BK priming, and after 60 min of BK, DAMGO did not alter PGE\(_2\)-stimulated cAMP levels. Although the mechanism for the effect of BK to enhance DAMGO-mediated inhibition of adenyl cyclase activity involves a PLC-PKC-PLA\(_2\)-COX pathway, the mechanism for the action of BK at the 5-min time point to cause MOR-mediated enhancement of PGE\(_2\)-stimulated cAMP accumulation is not clear. BK \(B_2\) receptors signal to a variety of signaling pathways in addition to the PLC signaling cascade (e.g., phospholipase D, nitric oxide) (Leeb-Lundberg et al., 2005). Although BK \(B_2\) receptor activation can result in increased PGE\(_2\) release in TG cultures (Jenkins et al., 2003), this is probably not involved in the enhancement of cAMP because the concentration of PGE\(_2\) (1 \(\mu\)M) used in this study was maximal, and there was no effect of BK priming on PGE\(_2\)-stimulated cAMP accumulation. Additional studies will be required to define the mechanism for the enhancement of the PGE\(_2\) response. In addition, the mechanism for the reduction in DAMGO-mediated inhibition of adenylyl
cyclic AMP activity after priming with BK for 60 min is not known. It is possible that this represents time-dependent desensitization of the B2 response to continued BK presence.

Our results suggest that the effect of BK priming leads to increased efficiency of coupling of MOR to Go_i because BK priming increased DAMGO-stimulated GTPγ[S] binding. This effect could occur if cell surface expression of functional MOR were increased or if the coupling efficiency of existing receptors to Go_i was increased. Previous studies have shown that inactivation can up-regulate MOR in sensory neurons (Ji et al., 1995; Zolnier et al., 2003). Although we found that cell surface expression of the δ-opioid receptor was increased following BK priming of TG neurons (Patwardhan et al., 2005), this effect was not dependent upon PKC activation and therefore was probably not involved in the mechanism of enhanced function of the δ-opioid receptor in the time-frame (15 min of BK priming) studied. Post-translational modification of MOR and/or its G protein could lead to increased coupling efficiency. Changes in the phosphorylation status of MOR can result in changes in receptor-G protein coupling efficiency (Bohn et al., 2002) or in trafficking of the receptor between the cell membrane and intracellular compartments (Borgland et al., 2003), which can regulate the capacity of the MOR signaling system to respond to agonist.

The lack of responsiveness of MOR to activation by ligands in the absence of BK is reminiscent of the general lack of effect of opioids when injected locally into normal tissue but that produce a peripherally mediated antihyperalgesic/antiallodynic effect within 10 to 15 min after inflammatory mediator injection (for review, see Stein et al., 2003; Ferreira and Nakamura, 1979; Levine and Taivo, 1989; Ko et al., 2000). Likewise, the local injection of morphine into uninflamed tissue does not seem to produce analgesia in humans (Wrench et al., 1996; Atanassoff et al., 1997; Likar et al., 2001), whereas local injection into inflamed or capsaicin-treated tissue produces an analgesic response under double-blind, placebo-controlled randomized conditions (Kinnman et al., 1997; Uhle et al., 1997; Dionne et al., 2001; Likar et al., 2001). Thus, the BK-primed TG culture system used in this study may be a good model for studying mechanisms involved in the regulation of opioid receptor responsiveness in peripheral sensory neurons. Understanding the mechanisms that mediate peripheral opioid receptor competence is critically important because this process may limit the potential clinical utility of peripherally selective opioid agonists. Although numerous animal studies demonstrate nearly 80 to 100% efficacy of peripheral opioids in reversing hyperalgesia/allodynia (Stein et al., 2003), such uniform effectiveness is not the case in clinical trials. Several clinical trials have not detected a significant peripheral component to opioid analgesia (Gupta et al., 1993, 1999; Dionne et al., 2001), and one systematic review of the clinical literature estimated that the overall efficacy of peripheral opioid analgesia is "mild" for reducing clinical pain (see Gupta et al., 2001). Thus, there is a large difference in efficacy estimates between animal and clinical studies on peripheral opioids. We hypothesize that clinical pain conditions vary in their ability to evoke opioid receptor competence and that this contributes to the variability in peripheral opioid analgesic efficacy. Further study of the mechanisms by which BK priming promotes opioid receptor system competence should provide a basis for understanding peripheral mechanisms of opioid analgesia and aid in the development of more effective pain management.

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