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Bradykinin-Induced Functional Competence and Trafficking of the δ-Opioid Receptor in Trigeminal Nociceptors

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Peripheral opioid analgesia is increased substantially after inflammation. We evaluated the hypothesis that an inflammatory mediator, bradykinin (BK), evokes functional competence of the δ-opioid receptor (DOR) for inhibiting trigeminal ganglia (TG) sensory neurons. We also evaluated whether BK evokes trafficking of the DOR to the plasma membrane. Rat TG cultures were pretreated with BK (10 μM) or vehicle, and the effects of DOR agonists ([D-Pen^{2,5}]-enkephalin or [D-Ala^2, D-Leu^5]-enkephalin) on BK (10 μM)/prostagladin E2 (PGE2; 1 μM)-stimulated immunoreactive calcitonin gene-related peptide (iCGRP) release or PGE2 (1 μM)-stimulated cAMP accumulation were measured. The effect of BK treatment on opioid receptor trafficking was evaluated by DOR immunohistochemistry, cell-surface DOR biotinylation, and live imaging of neurons transfected with mDOR–green fluorescent protein. BK pretreatment rapidly and significantly increased DOR agonist inhibition of evoked iCGRP release and cAMP accumulation. These effects of BK pretreatment were blocked by a B_2 receptor antagonist (HOE-140; 10 μM) or a protein kinase C (PKC) inhibitor [bisindolymaleimide (BIS); 1 μM]. Moreover, BK treatment rapidly and significantly increased the accumulation of DOR in the plasma membrane. However, BK-induced trafficking of DOR was not reversed by pretreatment with BIS, nor was trafficking evoked by application of a PKC activator PMA (200 nM). These data suggest that BK, in a PKC-dependent manner, induces rapid functional competence of DOR for inhibiting TG nociceptors and in a PKC-independent manner rapidly induces trafficking of DOR to the plasma membrane. These findings indicate that exposure to certain inflammatory mediators rapidly alters the signaling properties and neuronal localization of DOR, possibly contributing to peripheral opioid analgesia.

Keywords: **bradykinin; δ-opioid receptor; trafficking; pain; trigeminal; nociceptor**

Introduction

Peripherally administered opioids have higher efficacy for inhibiting hyperalgesia in inflammatory conditions compared with uninjured tissue (Joris et al., 1987; Stein et al., 1989). Several mechanisms have been proposed to explain this phenomenon, and those include migration of opioid containing immune cells to the inflamed site, increased axonal trafficking of opioid receptors to the peripheral terminals in the inflamed tissue, upregulation of opioid receptors, and alteration of efficiency of G-protein coupling, etc. (Stein et al., 2003). However, a direct consequence of signaling mediated by receptors of inflammatory mediators on the functional competence of opioid receptor for inhibiting nociceptor function has not been evaluated.

The δ-opioid receptor (DOR), a member of a large family of G-protein-coupled receptors (GPCRs), signals, in part, via activation of the $G_{i/o}$ G-protein and inhibits stimulated adenylyl cyclase activity and voltage-gated calcium channels with an increase in potassium conductance (Satoh and Minami, 1995). Interestingly, studies have demonstrated either no effect (Schroeder et al., 1991), stimulatory actions (Crain and Shen, 1998; Bao et al., 2003), or inhibitory effects (Acosta and Lopez, 1999) of DOR agonists applied to sensory neurons. These differences are consistent with the hypothesis that DOR couples to different signaling pathways under different cellular conditions. Although the cellular mechanisms that regulate DOR activation of various signaling pathways are presently unknown, previous studies have demonstrated heterologous regulation in which the activation of one GPCR alters the signaling functions of another GPCR (Berg et al., 2003). Because inflammation increases peripheral opioid efficacy, we proposed that the inflammatory mediator bradykinin (BK) evokes DOR competence for inhibiting sensory neurons via activation of the B_2 receptor.

In the present study, we addressed three major hypotheses: first, that application of BK rapidly induces functional DOR competence as measured by inhibition of evoked neuropeptide exocytosis and by inhibition of stimulated adenylyl cyclase activity; second, that application of BK induces DOR competence by insertion of receptor into the plasma membrane; and third, that these actions of BK are mediated by protein kinase C (PKC)-dependent signaling pathways.

Materials and Methods

Animals. Adult male Sprague Dawley (Charles River Laboratories, Wilmington, MA) rats weighing 250–300 g were used in this study. All animal study protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio and conformed to the International Association for the

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Study of Pain and federal guidelines. Animals were housed for 1 week before the experiment with food and water available *ad libitum*.

Compounds. [D-Pen^{2,5}]-enkephalin (DPDPE), [D-Ala^2, D-Leu^5]-enkephalin (DADLE), naltrindole, BK, HOE-140 (all from Sigma, St. Louis, MO), and deltorphin (Bachem California, Torrance, CA) were all made up in stock solutions of double-distilled H_2O on the day of the experiment. Bisindolylmaleimide (BIS) and phorbol-12-myristate-13-acetate (PMA; Calbiochem, La Jolla, CA) were dissolved in DMSO and diluted in buffer (final, 0.05% DMSO). Prostagladin E2 (PGE2) (Cayman Chemical, Ann Arbor, MI) was made in stock solutions with ethanol (EtOH) and diluted by buffer on the day of the experiment (final, 0.01% EtOH).

Rat trigeminal ganglia primary culture. Trigeminal ganglia (TG) were removed quickly after decapitation, placed in ice-cold calcium- and magnesium-free HBSS (Invitrogen, San Diego, CA), and washed twice with HBBS. Ganglia were then treated with 5 mg/ml collagenase (Sigma) for 30 min and 0.1% trypsin (Sigma) for 15 min before homogenization. The TG were then treated with 10 U of DNase I (Roche, Indianapolis, IN), centrifuged at 2000 rpm for 2 min, and resuspended in DMEM (Invitrogen), which also contained 1× penicillin–streptomycin (Invitrogen), 1× glutamine (Invitrogen), 3 μg/ml 5-fluoro-2-deoxyuridine, 7 μg/ml uridine (Sigma), 10% fetal calf serum (Invitrogen), and 100 ng/ml nerve growth factor (NGF; Harlan, Indianapolis, IN). The tissue was triturated gently, and cells from six ganglia were plated on one 48-well poly-D-lysine-coated plate (BD Biosciences, Bedford, MA), yielding ~4000 cells per well. For the immunohistochemical and cell-surface biotinylation experiments, cells were plated on poly-D-lysine-coated coverslips or 10 cm plates, respectively. The media were replaced at the end of 24 h and then 48 h later.

CGRP release assay. All culture experiments were performed on days 5–7, at 37°C, using modified HBSS (Invitrogen) (10.9 mM HEPES, 4.2 mM sodium bicarbonate, 10 mM dextrose, and 0.1% bovine serum albumin were added to 1× HBSS). After two initial washes, a 15 min baseline sample was collected. The cells then were exposed to either vehicle or BK (10 μM) for 15 min; samples were collected, and cells were exposed to either vehicle or test opioid (15 min) and stimulated with the combination of BK (10 μM)/PGE2 (1 μM) for 15 min. In experiments using B_2 antagonist or PKC inhibitor, the cells were pre-exposed to HOE-140 (10 μM) or BIS (1 μM) for 15 min before BK application. In experiments using the DOR antagonist, the cells were pre-exposed to naltrindole (2 μM) before the opioid application. All the supernatants were collected for analysis of immunoreactive calcitonin gene-related peptide (iCGRP) content by radioimmunoassay (RIA). The basal release was typically 4–6 fmol per well.

cAMP accumulation. The experimental protocol was identical to that of the CGRP release assay except for the fact that all these experiments included the phosphodiesterase inhibitor rolipram (10 μM). At the end of the experiments, neurons were exposed to 500 μl of ice-cold ethanol to extract cAMP. The EtOH-treated cells were kept at −20°C overnight. The day after evaporation and resuspension in RIA buffer in RIA tubes, cAMP levels were measured using RIA, as described previously (Berg et al., 1994).

Superfusion of acutely isolated rat TG. As described previously (Ulrich-Lai et al., 2001), TG were quickly isolated after decapitation and kept in ice-cold oxygenated Krebs' buffer (135 mM NaCl, 3.5 mM KCl, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 0.1% BSA, 3.3 mM dextrose, 0.1 mM ascorbic acid, 10 mM HEPES, and 16 μM thiorphan, pH 7.4) until groups of four ganglia were collected. Then, 200 μm slices of TG were prepared (McIlwain tissue chopper; Gomshall, Surrey, UK), and slices from each group of four ganglia were placed in a superfusion chamber and washed for 45 min with oxygenated Krebs' buffer at 37°C at a rate of 0.35 ml/min. After collection of three fractions (7 min) for measuring basal iCGRP release, the acutely isolated ganglia were treated with either vehicle or BK (10 μM) for 7 min, followed by vehicle or opioid for 7 min, and then stimulated with BK (10 μM)/PGE2 (1 μM) for 7 min. iCGRP release was measured by RIA. The basal release was typically 8–10 fmol per chamber.

iCGRP RIA. A previously used (Garry et al., 1994) primary antibody against CGRP (final dilution, 1:1,000,000; kindly donated by Dr. M. J. Iadarola, National Institutes of Health, Bethesda, MD) was added in the tubes containing superfusate either from cultured rat TG or acutely isolated rat TG and incubated at 4°C for 24 h. Then, 100 μl of [I-125]-Tyr^0-$CGRP_{28-37}$ (~20,000 cpm) and 50 μl of goat anti-rabbit antisera coupled to ferric beads (PerSeptive Diagnostics, Cambridge, MA) were added to these tubes. The tubes were incubated for another 24 h at 4°C. The assay was stopped using immunomagnetic separation of bound from free tracer. All compounds used in experiments were tested for interference with the RIA. The minimum detectable levels for CGRP for this assay are ~3 fmol and the 50% displacement at 28 fmol.

In situ *hybridization and immunohistochemistry.* In studies with combined *in situ* hybridization (ISH)/immunohistochemistry (IHC) analysis, cryosections of TG or coverslips containing cultured TG neurons were fixed with 4% formaldehyde, permeabilized with 0.5% Triton X-100, acylated in acetic anhydride, dehydrated in alcohol, and delipidated in chloroform. *In situ* hybridizations (55°C) were performed using DIG-cRNA (containing digoxigenin-UTP) probe against bradykinin receptor 2 (position 102–500; GenBank accession number NM_173100). After RNase treatment, slides/coverslips were washed with decreasing concentration of SSC buffer (final wash, 0.1× SSC at 55°C), and hybridization was detected using standard alkaline phosphate-based reaction (substrates BCIP-NBT; Roche). Slides/coverslips were then incubated with already characterized (Cahill et al., 2001b; Bao et al., 2003) rabbit primary antibodies against δ-opioid receptor (1:100; Santa Cruz Biotechnology, Santa Cruz, CA or 1:1000; Chemicon, Temecula, CA) overnight at 4°C and detected using a Alexa-488-conjugated fluorescent secondary antibody (1:300; Molecular Probes, Eugene, OR). Experiments evaluating DOR trafficking were conducted by double IHC using the above mentioned DOR antibody and an already characterized (Cesare et al., 1999) monoclonal antibody against PKCε (1:1000; Molecular Probes) to identify BK-responsive neurons (Cesare et al., 1999). In these experiments, TG cultures were treated with vehicle or BK (10 μM) using conditions identical to that in CGRP release experiments. Experiments evaluating coexpression of B_2 protein with DOR protein or CGRP were conducted by double IHC using an already characterized (Allen et al., 2002; Mukhin et al., 2003) mouse monoclonal B_2 antibody (1:500; Research Diagnostics, Flanders, NJ), the above-mentioned DOR antibody, or previously used (Price et al., 2003) rabbit polyclonal CGRP antibody (1:750; Peninsula Laboratories, San Carlos, CA). The IHC protocol included 4% formaldehyde fixation, 0.2% Triton X-100 permeabalization, 10% goat serum block, and PBS washes. The specificity of the DOR and B_2 antibodies was confirmed by double ISH/IHC method using specific riboprobes against DOR (position 40–290; GenBank accession number U00475) and B_2 mRNA (position 102–500; GenBank accession number NM_173100). Moreover, to further validate the specificity, both DOR antibodies were preincubated with respective immunogenic peptides, and this lead to lack of specific labeling. DOR trafficking mages were collected by a confocal microscope at the University of Texas Health Science Center at San Antonio core facility, and *in situ*/IHC images were taken using a Nikon (Melville, NY) E600 microscope. Images were analyzed using MetaMorph software (version 4.5 r6; Universal Imaging, West Chester, PA)

Plasma membrane DOR labeling. Cultured TG neurons were treated with BK or BIS followed by BK and then biotinylated with EZ-Link Sulfo-NHS-LC-Biotin (0.5 mg/ml; Pierce, Rockford, IL) in PBS for 30 min at 4°C. Cells were rinsed three times with 50 mM glycine in PBS, two times with PBS, and lysed in general lysis buffer by 20 passes through a 25 ga needle. Lysates were clarified by centrifugation, and equal aliquots were retained for either immunoprecipitation with the above-mentioned antibody specific to DOR (1:1000; Chemicon) or precipitation with streptavidin-bound agarose beads. Precipitates were resolved by 12.5% SDS-PAGE, transferred to polyvinylidene difluoride, and visualized by Western blot analysis with antibodies specific to DOR. Results were quantified using NIH Image 1.62, with streptavidin-pecipitated bands normalized to respective immunoprecipitated bands. The specificity of the DOR antibody was confirmed by preincubating the antibody with the appropriate blocking peptide.

Live imaging of DOR–green fluorescent protein trafficking. mDOR–green fluorescent protein (GFP) fusion protein was constructed by sub-

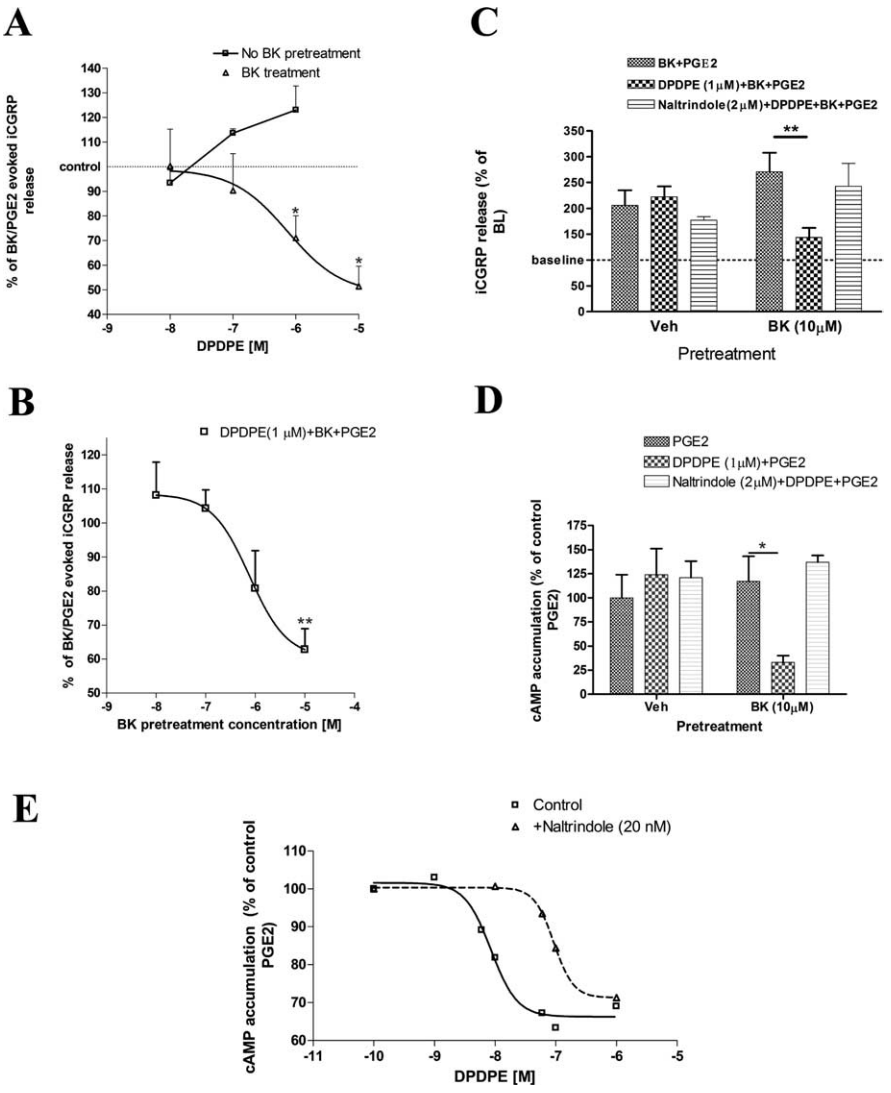

Figure 1. Bradykinin pretreatment rapidly evokes competence in DOR to inhibit TG nociceptors. ***A***, Cultured TG neurons were treated with either vehicle (no BK pretreatment; squares; $n = 6–8$) or BK (10 μM; triangles; $n = 6–8$) for 15 min and aspirated, and DPDPE (10 nM to 10 μM) modulation of BK (10 μM)/PGE2 (1 μM)-evoked iCGRP release was observed. Data are presented as mean ± SEM. ANOVA with Bonferroni's *post hoc* test (*$p < 0.05$). ***B***, In a similar paradigm, BK pretreatment concentration varied from 10 nM to 10 μM with DPDPE (1 μM) (**$p < 0.01$). ***C***, After vehicle or BK pretreatment, an effect of naltrindole (2 μM) pretreatment on DPDPE (1 μM) modulation of BK/PGE2-evoked iCGRP release was observed ($n = 8$; **$p < 0.01$). ***D***, After appropriate pretreatments (15 min vehicle or BK, followed by vehicle or naltrindole), DPDPE (1 μM) modulation of PGE2- (1 μM) evoked cAMP accumulation was observed ($n = 8$; *$p < 0.05$). ***E***, After BK (10 μM) pretreatment, DPDPE (100 pM to 1 μM) modulation of PGE2-evoked cAMP accumulation was observed in the presence (squares; solid line) or absence (triangles; dotted line) of naltrindole (20 nM). Veh, Vehicle; BL, baseline.

cloning of the entire coding sequence of mDOR (apart from start codon) into pEGFP-N1 vector (BD Biosciences) between *Eco*RI and *Bam*HI restriction sites. Cultured TG neurons (density, 50–75 cells per coverslip) were transfected using the PDS-1000/He Biolistic particle delivery system (Bio-Rad, Hercules, CA) according to the manufacturer's protocols. Twenty-four hours after transfection, we typically observed one or two cells per coverslip demonstrating successful transfection. Fluorescent microscopy was performed with an oil-immersion 40×/1.30 numerical aperture objective and Polychrome IV monochromator (TILL Photonics, Pittsfield, MA). Drugs were prepared in standard external solution [containing the following (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose, and 10 HEPES, pH 7.4] and applied (37°C) through a recording chamber inlet. Images were collected every 5 s with equal exposure time of 50 ms and stored/analyzed with TILLvisION 4.0 software (TILL Photonics). The experiment was repeated nine independent times on independently transfected cultures, and a total of 20 transfected cells were observed. The statistical calculations were done using change in initial versus post-BK application fluorescence intensity in membrane and cytosolic areas of the tested cells.

Data analysis. All experiments were conducted with $n = 8$ or more wells to determine the experimental observation and then repeated at least three times to conduct the statistical analysis. The CGRP data are presented as percentage of basal release (mean ± SEM), and cAMP accumulation data are presented as percentage of stimulus (PGE2; mean ± SEM). Data were analyzed using GraphPad (San Diego, CA) Prism software version 4. The DPDPE-treated groups (with or without BK pretreatment) were compared with respective (with or without BK pretreatment) control BK/PGE2 groups. Multifactorial experimental data were analyzed using single-factor, two-way-ANOVA, multiple treatment data were analyzed using one-way-ANOVA, and individual groups were compared using a Bonferroni's *post hoc* test, whereas experiments examining the difference between two groups were analyzed by using a two-tailed *t* test. The statistical significance was tested at 0.05.

Results

Bradykinin pretreatment rapidly evokes competence in DOR to inhibit TG nociceptors

To test our hypothesis of BK modulation of DOR function, we exposed cultured TG neurons to either vehicle or BK (10 μM) for 15 min and then determined DPDPE (10 nM to 10 μM) modulation of BK (10 μM)/PGE2 (1 μM)-evoked iCGRP release (Fig. 1*A*). In the absence of BK (i.e., vehicle pretreatment), DPDPE did not inhibit BK/PGE2-evoked iCGRP release at any of the concentrations tested; indeed, a trend toward potentiation was observed. However, after BK pretreatment, DPDPE significantly inhibited BK/PGE2-evoked iCGRP release in a concentration-dependent manner with a maximal inhibition of 49% ($p < 0.05$). We next evaluated the concentration dependence of BK for altering DPDPE (1 μM) inhibition of BK/PGE2-evoked iCGRP release (Fig. 1*B*). The results demonstrated that BK pretreatment (10 nM to 10 μM) initiates DOR competence in a concentration-dependent manner ($p < 0.01$). The post-BK pretreatment inhibitory effect of DPDPE (1 μM) on BK/PGE2-evoked iCGRP release was completely blocked by treatment with the DOR antagonist naltrindole (2 μM) (Fig. 1*C*).

We then tested our hypothesis by evaluating the effect of BK pretreatment on DOR inhibition of stimulated adenylyl cyclase activity (Fig. 1*D*). Pretreatment with BK (10 μM), but not vehicle, resulted in the rapid development of a significant DPDPE (1 μM) inhibition of PGE2 (1 μM)-stimulated cAMP accumulation by 67% ($p < 0.01$). This effect was blocked by treatment with naltrindole (2 μM). After BK pretreatment, the application of DPDPE (100 pM to 1 μM) produced a concentration-dependent

inhibition of PGE2-stimulated cAMP accumulation (Fig. 1*E*). This DPDPE concentration–response curve was right-shifted in cultures treated with the DOR antagonist naltrindole (20 nM).

BK pretreatment produces DOR competence regardless of the DOR agonist used

Because agonist-specific signaling of GPCRs has been proposed (Berg et al., 1998), we decided to evaluate whether BK pretreatment is necessary to evoke DOR competence using another DOR agonist, DADLE (Fig. 2). Similar to our previous observations with DPDPE, the application of DADLE (100 nM) significantly ($p < 0.05$) inhibited BK/PGE2-evoked iCGRP release (Fig. 2*A*) and PGE2-evoked cAMP accumulation (Fig. 2*B*) by a mechanism requiring BK (10 μM) pretreatment. The inhibitory effect of DADLE was completely reversed by a pretreatment with naltrindole (1 μM).

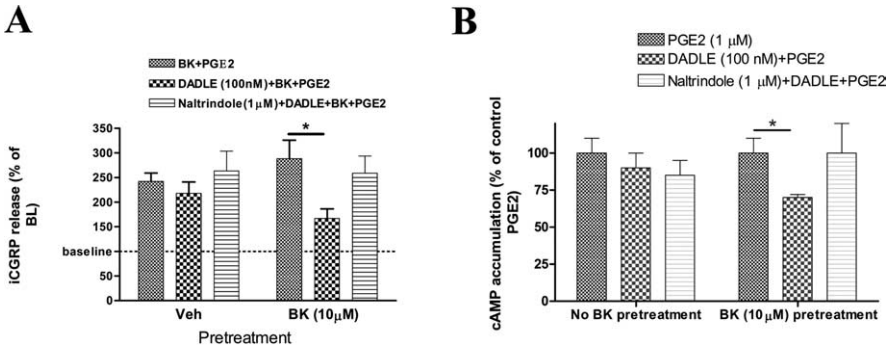

Figure 2. BK pretreatment produces DOR competence regardless of the DOR agonist used. ***A***, The effect of pretreatment with vehicle (Veh) or BK (10 μM) on DADLE (100 nM) modulation of BK/PGE2-evoked iCGRP release was determined using the paradigm of Figure 1*C* ($n = 8$; $^*p < 0.05$). ***B***, The effect of BK pretreatment on DADLE (100 nM) modulation of PGE2-evoked cAMP accumulation was determined using the paradigm of Figure 1*D* ($n = 8$; $^*p < 0.05$). BL, Baseline. Error bars represent SEM.

BK pretreatment is necessary for the inhibitory effect of DPDPE in acutely isolated and superfused rat TG

To verify that the BK pretreatment effect is not an artifact of culturing conditions, we next evaluated the requirement for BK pretreatment on DPDPE modulation of BK/PGE2-evoked iCGRP release using acutely isolated and superfused rat TG slices (Ulrich-Lai et al., 2001). Under these conditions (Fig. 3), DPDPE (1 μM) significantly ($p < 0.05$) inhibited BK/PGE2-evoked iCGRP release with an absolute requirement for BK pretreatment.

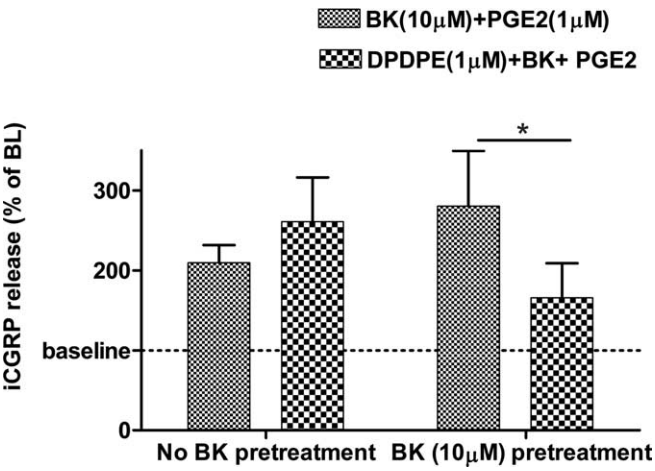

Figure 3. BK pretreatment is necessary for the inhibitory effect of DPDPE in acutely isolated and superfused rat TG. In acutely dissociated rat TG, after a washout time and collection of three fractions for basal release of iCGRP, tissue was superfused with BK (10 μM) or vehicle for two 7 min fractions, and DPDPE (1 μM) modulation of BK (10 μM)/PGE2 (1 μM)-evoked iCGRP release was observed ($n = 8$; $^*p < 0.05$). BL, Baseline. Error bars represent SEM.

BK-evoked DOR inhibitory competence is mediated by activation of bradykinin B_2 receptor that is coexpressed with DOR in rat TG

Because many actions of BK on NGF treated sensory neurons are mediated via B_2 receptor (Vellani et al., 2004), we evaluated whether pretreatment with the B_2 antagonist HOE-140 blocks the development of BK-evoked DOR inhibitory competence (Fig. 4*A*). Pretreatment with HOE-140 (10 μM) completely reversed BK evoked DOR competence. In addition, the coexpression of B_2 mRNA (*in situ* hybridization) with DOR protein (immunohistochemistry) is observed in both cultured TGs (Fig. 4*B*) as well as in native TGs (Fig. 4*C*). In cultured TG neurons, B_2 receptor protein is coexpressed with DOR protein (Fig. 4*D*) and CGRP (Fig. 4*E*). The B_2 receptor expression was observed mainly in small-diameter (0–20 μm; 33%) and medium-diameter (20–40 μm; 64%) TG neurons, and ~73% of B_2-expressing cells also coexpress DOR.

BK evoked DOR competence is mediated via activation of PKC

BK-induced activation of the B_2 receptor leads to PKC signaling in many sensory neurons (Cesare et al., 1999; Premkumar and Ahern, 2000); therefore, we evaluated whether BK-evoked DOR competence occurs via activation of PKC. Pretreatment with a pan-PKC inhibitor BIS (1 μM) completely blocked the effect of BK pretreatment on DOR competence as measured by inhibition of evoked iCGRP release (Fig. 5*A*) and evoked cAMP accumulation (Fig. 5*B*). Moreover, pretreatment with PKC activator PMA (200 nM, 15 min) alone was sufficient to evoke functional DOR competence (Fig. 5B). We could not perform PKC activator pretreatment experiment in the CGRP release assay because, as shown by previous studies (Barber and Vasko, 1996; Kessler et al., 1999), PMA by itself evoked substantial amount iCGRP release that interfered with our additional test stimulus.

BK evokes trafficking of DOR to the plasma membrane in a PKC-independent manner

Because inflammation induces axonal trafficking of DOR toward inflamed tissue (Hassan et al., 1993) and stimulation of cultured sensory neurons by capsaicin, P2Y agonist, or high potassium leads to trafficking of DOR to the plasma membrane (Bao et al., 2003), we hypothesized that the endogenous inflammatory mediator BK also induces trafficking of DOR. To evaluate whether DOR trafficking was associated with BK-induced DOR competence, we examined whether BK-induced trafficking is also PKC dependent. The results demonstrated (Fig. 6*A*) that treatment of cultured TG neurons with BK (10 μM, 15 min) leads to a significant (23.5%; $p < 0.01$) increase in levels of DOR in the plasma membrane as evaluated by cell-surface biotinylation of the receptor (Fig. 6*A*). This increase was not reversed by pretreatment with BIS (1 μM). Preincubation of the antibody with the immunogenic peptide resulted in lack of DOR-specific bands on the Western blot. Cultured TG neurons are a heterogeneous population including BK-responsive and -nonresponsive cells. Therefore, we

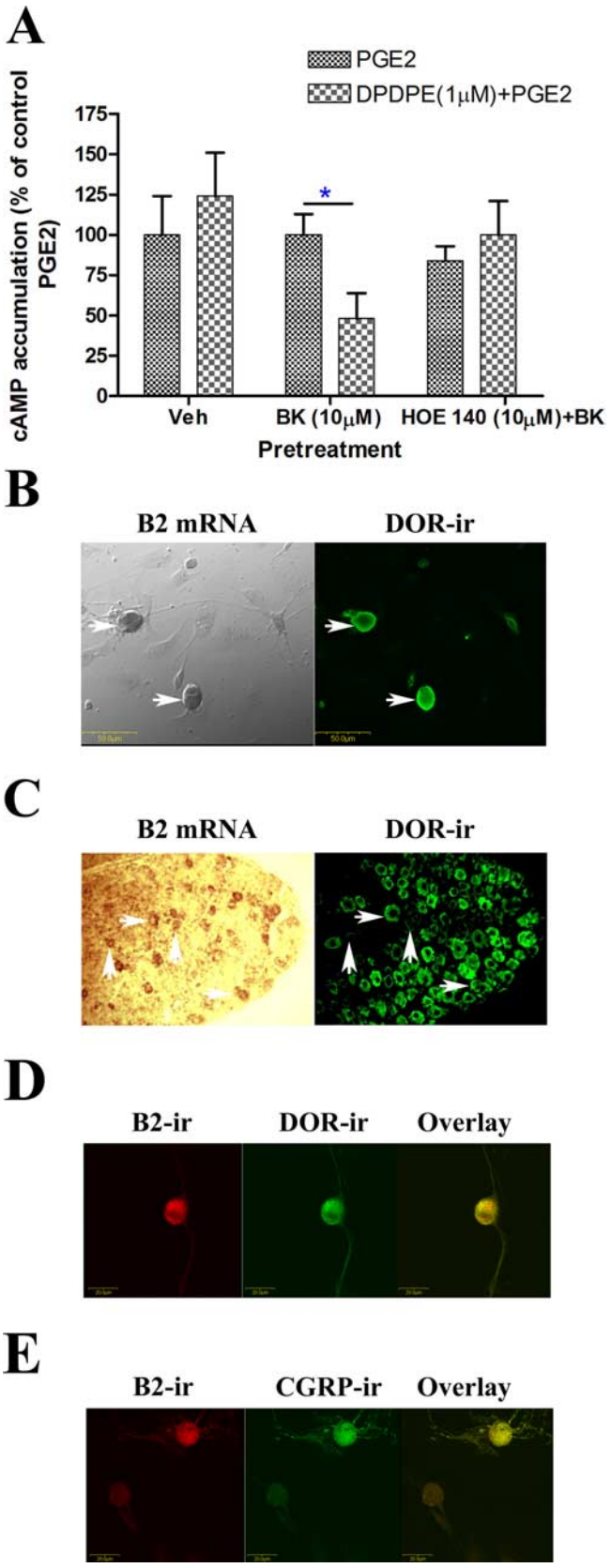

Figure 4. BK-evoked DOR inhibitory competence is mediated by activation of bradykinin B_2 receptor that is coexpressed with DOR in rat TG. ***A***, Effect of pretreatment with B_2 antagonist HOE-140 (10 μM) on BK-evoked DPDPE modulation of PGE2-stimulated cAMP accumulation ($n = 8$; $^*p < 0.05$). Error bars represent SEM. ***B***, Coexpression of B_2 mRNA (left) and DOR immunoreactivity (right) in cultured TG neurons. Pictures were taken at 60×. The horizontal arrows denote examples of coexpressing cells. The vertical arrows denote examples of B_2-expressing cells that do not express DOR. ***C***, Coexpression of B_2 mRNA (left) and DOR immunoreactivity (right) in native TG neurons. Pictures were taken at 20×. Arrows denote examples of coexpressing cells. ***D***, Coexpression of B2 immunoreactivity (left) and DOR immunoreactivity (right) in cultured TG neurons. Pictures were taken at 60×. ***E***, Coexpression of B_2 immunoreactivity (left) and CGRP immunoreactivity (right) in cultured TG neurons. Pictures were taken at 60× (picture shows example of 1 cell coexpressing both or another cell coexpressing neither). Veh, Vehicle; -ir, immunoreactivity.

conducted separate experiments evaluating DOR trafficking at a single-cell level by identifying BK-responsive and -nonresponsive cells using translocation of PKCε as a dependent measure (Cesare et al., 1999). The cells were treated with vehicle/BK (10 μM), BIS (1 μM)/BK, or PMA (200 nM) for 15 min, and DOR immunoreactivity (DOR-IR) and PKCε-IR were detected using double immunohistochemistry (Fig. 6*B*). Inclusion of PMA in the experiment served as both a positive control for PKC activation and directly activated PKC to independently assess the role of this signaling pathway in mediating DOR trafficking. In vehicle-treated cells, DOR-IR and PKCε-IR were mainly observed in the cytoplasm. However, in BK-responsive cells (as defined by translocation of PKCε), DOR-IR was mainly observed in the region of plasma membrane. Moreover, application of PMA did not change cellular localization of DOR-IR, although translocation of PKCε was observed in the same cells. To characterize these data, we conducted cell counts by blinded observers and calculated the percentage of cells demonstrating DOR trafficking in BK-responsive cells and PMA-responsive cells (Fig. 6*C*). Most (89%) of BK-responsive cells exhibited DOR trafficking after BK application. A similar (86%) proportion of cells exhibited DOR trafficking despite BIS (1 μM) pretreatment. Moreover, only 8% of PMA-responsive cells exhibited DOR trafficking. To further validate our results, we repeated our experiments using another well characterized (Cahill et al., 2001a,b, 2003) DOR antibody and replicated our results (supplemental Fig. 1*A*, available at www.jneurosci.org as supplemental material). Both antibodies were preincubated with respective blocking peptides, and this lead to lack of any specific signal (supplemental Fig. 1*B*,*C*, available at www.jneurosci.org as supplemental material).

Live imaging of sensory neurons expressing DOR–GFP fusion protein confirms BK-induced trafficking of DOR

We evaluated BK-evoked trafficking of DOR using a third independent method. We transfected cultured TG neurons with a DOR–GFP-containing vector and observed changes in green fluorescence intensity after BK treatment. Because in rat TGs, B_2 receptor is predominantly expressed in small- to medium-sized neurons, we evaluated BK-induced trafficking of DOR only in cells in that diameter range. We observed two types of neurons in these cultures. One group of cells did not show any change in fluorescence intensity in plasma membrane or cytosol after either vehicle treatment or BK treatment (Fig. 7*A*). However, another group of cells responded to BK application with a gradual increase in fluorescent signal in the plasma membrane accompanied with a gradual decrease in intensity in the cytosol (Fig. 7*B*). Of the total 20 cells tested, eight cells showed DOR trafficking to the plasma membrane. In a digitally magnified image of the plasma membrane of a BK-responsive cell, the membrane fluorescence becomes well defined only after application of BK suggestive of insertion of additional DOR to the plasma membrane (Fig. 7*C*). Additional quantification of change in cytosolic and membrane fluorescence in BK-responsive cells showed that BK application resulted in a significant (17.6%; $p < 0.01$) increase in

plasma membrane intensity accompanied by a 7.2% decrease in cytosolic intensity.

Discussion

The results from this study demonstrate that signaling events occurring via activation of the B_2 receptor rapidly lead to a functional competence in the DOR for inhibition of TG nociceptors. This BK-evoked DOR inhibitory competence was concentration dependent and was observed regardless of the DOR agonist used, dependent measure (iCGRP and cAMP) used, or cellular model (cultured and acutely dissociated TG) studied. Moreover, using three independent methods, we demonstrate that BK induces rapid trafficking of DOR to the plasma membrane. Although the priming effect of BK on DOR competence is PKC dependent, BK-induced DOR trafficking effect is independent of PKC signaling.

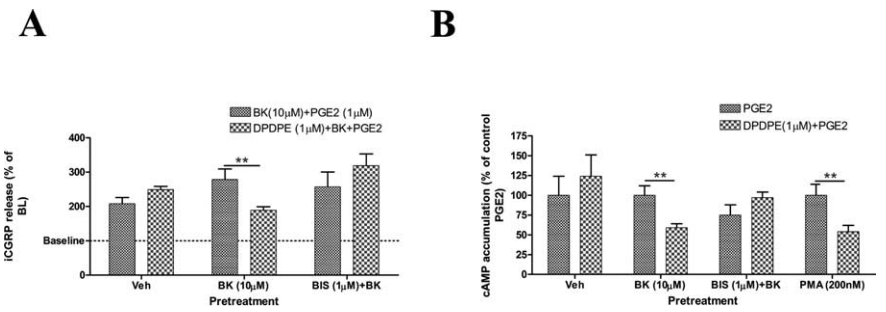

Figure 5. BK-evoked DOR competence is mediated via activation of PKC. ***A***, Effect of pretreatment with BIS (1 μM) on BK-evoked DPDPE inhibition of BK/PGE2-evoked iCGRP release ($n = 8$; $^{**}p < 0.01$). ***B***, Effect of pretreatment with BIS (1 μM) on BK-evoked DPDPE inhibition of PGE2-evoked cAMP accumulation ($n = 8$; $^{**}p < 0.01$). Separate cultures were exposed to pretreatment with PMA (200 nM) to evaluate DPDPE modulation of PGE2-evoked cAMP accumulation ($n = 8$; $^{**}p < 0.01$). BL, Baseline; Veh, vehicle. Error bars represent SEM.

Peripherally administered opioid agonists display increased efficacy after inflammation. Several studies suggest multiple mechanisms could account for this phenomenon, and they include release of endogenous opioid ligands, increased opioid receptor expression, and axonal trafficking, sensory nerve sprouting, disruption of perineural barrier, and alternation in agonist efficacy (Cahill et al., 2003; Stein et al., 2003). Inflammation induces the release of mediators such as BK that profoundly alter nociceptor function. The bradykinin B_2 receptor is a G_q-coupled GPCR that regulates the function of several ion channels on nociceptors (Chuang et al., 2001; Shin et al., 2002; Bandell et al., 2004). However, although heterologous regulation of GPCR signaling has been observed for other receptor systems (Hur and Kim, 2002), including opioid receptors (Jordan et al., 2001, 2003; Evans, 2004; Zhang et al., 2004), such regulation of GPCRs such as DOR by B_2 in nociceptors has not been addressed previously.

The present study demonstrates that activation of B_2 signaling via the PKC pathway leads to rapid functional competence of DOR for inhibiting TG nociceptors. In the absence of BK pretreatment, we observed either no or slight excitatory effects of DOR activation in agreement with other groups (Bao et al.,

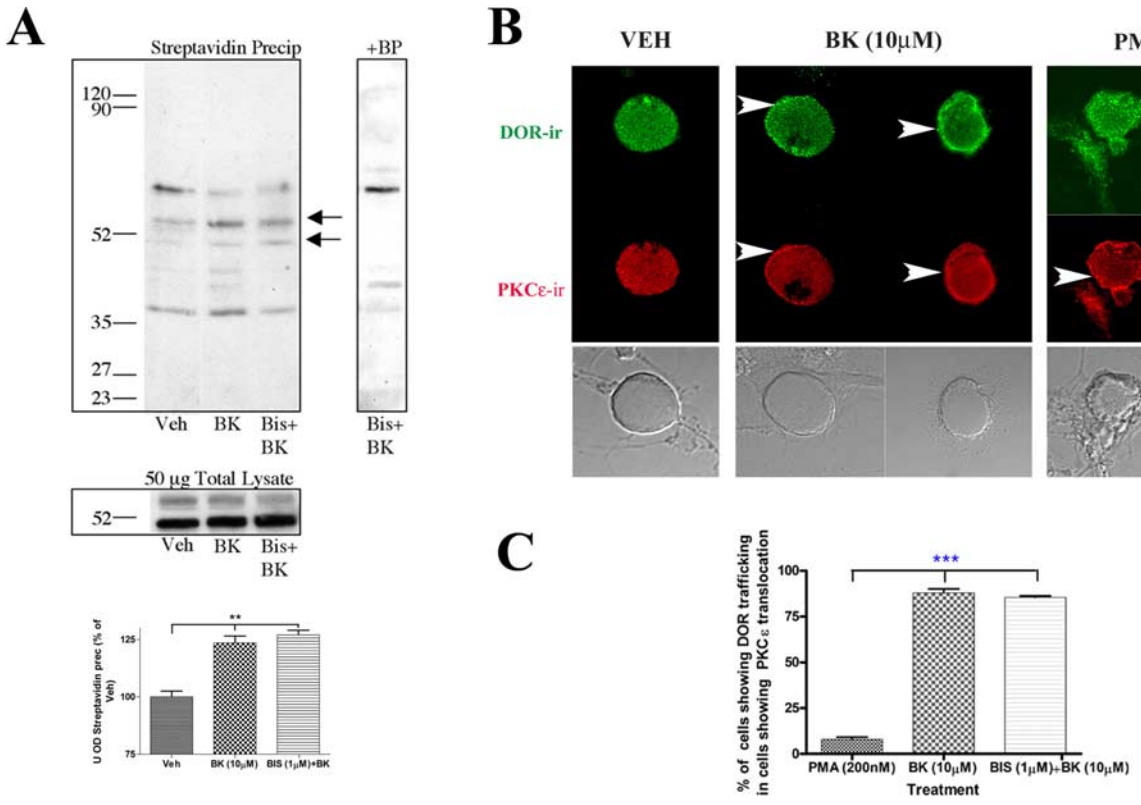

Figure 6. BK evokes trafficking of DOR to the plasma membrane in a PKC-independent manner. ***A***, Cultured TG cells were treated with vehicle (Veh)/BIS (1 μM), followed by Veh/BK (10 μM). Cells were biotinylated and lysed. Streptavidin-precipitated (Precip) bands normalized to respective immunoprecipitated bands (Western blot shown at the top with the blocking peptide control). Finally, data were presented as a percentage of vehicle-treated cells ($n = 3$; $^{**}p < 0.01$). ***B***, Cultured TG cells were treated with vehicle (VEH)/BK (10 μM)/PMA (200 nM) for 15 min. Cells were then fixed, and double immunohistochemistry was performed for DOR-ir (green; top) and PKCε-ir (red; middle). Pictures were taken at 90×. Arrows denote either DOR trafficking to the plasma membrane or PKCε translocation. ***C***, Quantification of cells showing DOR trafficking in cells showing PKCε translocation ($n = 4$; $^{***}p < 0.001$). Error bars represent SEM.

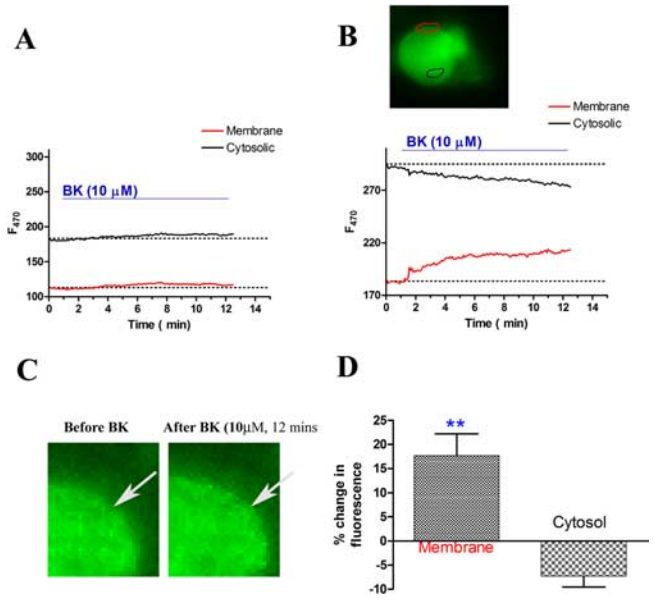

Figure 7. Live imaging of sensory neurons expressing DOR–GFP fusion protein confirms BK induced trafficking of DOR. ***A***, A representative cell showing change in green fluorescence (DOR–GFP) in cytoplasm (black) and plasma membrane (red) over time after BK (10 μM) application (blue line shows time point of BK application). ***B***, A representative cell expressing DOR–GFP fusion protein is shown at top. Changes in green fluorescence (DOR–GFP) in cytoplasm (black) and plasma membrane (red) over time after BK (10 μM) application (blue line shows time point of BK application). ***C***, Membrane close-up of a representative cell showing that after BK (10 μM) application, membrane DOR–GFP fluorescence becomes more defined (arrow). ***D***, Quantification of change in fluorescence in plasma membrane and cytosol after BK (10 μM) application ($n = 8$; $^{**}p < 0.01$). Error bars represent SEM.

2003). BK pretreatment sensitizes nociceptors to the next BK/PGE2 challenge possibly through multiple mechanisms such as phospholipase C–phosphatidylinositol biphosphate removal, activation of PKC, and production of prostaglandins or hydroperoxyeicosatetraenoic acid (Cesare et al., 1999; Chuang et al., 2001; Petho et al., 2001; Shin et al., 2002). However, the activation of the PKC pathway rapidly switches DOR coupling to inhibitory pathways. Previous studies have shown that PKC can selectively modulate DOR coupling to one signaling pathway over another in cell lines (Zhang et al., 1999). Studies done in sensory neurons show either no effect (Schroeder et al., 1991), excitatory effect (Crain and Shen, 1998; Bao et al., 2003), or inhibitory effect (Acosta and Lopez, 1999) of DOR agonists. This is consistent with statistically insignificant trend toward excitation or inhibition of different DOR agonists we observed under non-BK primed condition. The possible explanation for this discrepancy could be differential lipid microdomain localization of DOR under such conditions. Indeed, GPCR signaling preference is dependent on their lipid microdomain localization (Ostrom and Insel, 2004). Importantly, after BK pretreatment, both DOR agonists show a significant inhibitory effect on nociceptor stimulation, suggesting effective coupling to inhibitory pathways. In sensory neurons, DOR can signal via both G_s- and G_i-proteins, depending on the microenvironment of plasma membrane glycolipids (Crain and Shen, 1998). Hence, it is possible that BK-evoked activation of PKC leads to movement of DOR within the cell membrane to a specific lipid microdomain that allows for more effective coupling of DOR with $G_{i/o}$-protein versus G_s-protein. Alternatively, PKC has been shown recently to inhibit G-protein-coupled receptor kinase (GRK) activity (Lorenz et al., 2003). Because GRK is known to abolish DOR signaling, activation of PKC could lead to enhanced DOR signaling by inhibiting GRK. However, it should be recognized that this action of PKC might be altered in cell systems overexpressing DOR (Hayashi et al., 1995; Ueda et al., 1995; Xiang et al., 2001). Although the signaling downstream to PKC that governs heterologous modulation of DOR by B_2 is not known, the finding is replicated in both cultured and acutely isolated sensory neurons and has physiological implications. Thus, BK–PKC-dependent functional competence of DOR could be a novel pathway [in addition to those proposed by Stein et al. (2003)] by which other inflammatory mediators that signal via the PKC pathway such as proteases (Amadesi et al., 2004; Dai et al., 2004), ATP (Moriyama et al., 2003), or 5-HT (Sugiuar et al., 2004) could alter peripheral opioid efficacy.

Inflammation leads to increased axonal trafficking of DOR toward terminals innervating inflamed tissue (Hassan et al., 1993). Although studies have demonstrated both upregulation (Cahill et al., 2003) and downregulation (Ji et al., 1995) of DOR in the presence of inflammation, there seems to be an agreement about increased trafficking of DOR under such conditions. Moreover, DOR trafficking toward plasma membrane was also observed when cultured DRG neurons were stimulated with capsaicin or high potassium (Bao et al., 2003). Stimulus-dependent trafficking of μ-opioid receptors in brainstem (Browning et al., 2004) and κ-opioid receptors in pituitary (Shuster et al., 1999) have also been documented. Therefore, in this study, we evaluated the effect of the endogenous inflammatory mediator BK (stimulus) on trafficking of DOR in nociceptors. We show that BK rapidly induces DOR trafficking to the plasma membrane in a PKC-independent manner, as confirmed by the inability of BIS to block this effect and inability of PMA to mimic this effect. This would suggest that in TG sensory neurons, trafficking of DOR induced by BK is probably a calcium influx-sensitive phenomenon as is the case in DOR trafficking induced by capsaicin and purinergic receptor agonist (Bao et al., 2003). However, a PKC activator, PMA, mimics the effect of BK for evoking functional DOR competence. This suggests that DOR, either already present on plasma membrane or increased after agonist directed trafficking, is sufficient to inhibit nociceptor function if effectively coupled to inhibitory pathways (e.g., after PKC activation). Although BK-induced trafficking of DOR has no effect on the immediate ability of BK to produce functional competence in DOR, it is possible that BK-induced trafficking of DOR to the plasma membrane could mitigate the internalization of DOR that occurs after chronic agonist exposure. Hence, BK-induced DOR trafficking might be relevant for DOR agonists to produce sustained analgesia in persistent inflammation.

In summary, the present study is consistent with the hypothesis that the inflammatory mediator BK rapidly evokes functional DOR competence to inhibit nociceptor function. Moreover, BK evokes DOR trafficking in a PKC-independent manner. The findings of this study might help in developing a better analgesic strategy in conditions such as neuropathic pain in which peripheral opioid administration is less efficacious.

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