Painful Inflammation-Induced Increase in $\mu$-Opioid Receptor Binding and G-Protein Coupling in Primary Afferent Neurons

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ABSTRACT

Opioids mediate their analgesic effects by activating $\mu$-opioid receptors (MOR) not only within the central nervous system but also on peripheral sensory neurons. The peripheral analgesic effects of opioids are best described under inflammatory conditions (e.g., arthritis). The present study investigated the effects of inflammation on MOR binding and G-protein coupling of full versus partial MOR agonists in dorsal root ganglia (DRG) of primary afferent neurons. Our results show that Freund’s complete adjuvant (FCA) unilateral hindpaw inflammation induces a significant up-regulation of MOR binding sites (25 to 47 fmoles/mg of protein) on DRG membranes without affecting the affinity of either full or partial MOR agonists. In our immunohistochemical studies, the number of MOR-immunoreactive neurons consistently increased. This increase was mostly caused by small-diameter nociceptive DRG neurons. The full agonist DAMGO induced MOR G-protein coupling in DRG of animals without FCA inflammation ($EC_{50} = 56 \text{nM};$ relative $E_{max} = 100\%$). FCA inflammation resulted in significant increases in DAMGO-induced MOR G-protein coupling ($EC_{50} = 29 \text{nM};$ relative $E_{max} = 145\%$). The partial agonist buprenorphine hydrochloride (BUP) showed no detectable G-protein coupling in DRG of animals without FCA inflammation; however, partial agonist activity of BUP-induced MOR G-protein coupling was detectable in animals with FCA inflammation ($EC_{50} = 1.6 \text{nM};$ relative $E_{max} = 82\%$). In behavioral studies, administration of BUP produced significant antinociception only in inflamed but not in noninflamed paws. These findings show that inflammation causes changes in MOR binding and G-protein coupling in primary afferent neurons. They further underscore the important differences in clinical studies testing peripherally active opioids in inflammatory painful conditions.

Materials and Methods

Guanosine-5'-O-(3-[35S]thio)-triphosphate ([35S]GTP\(\gamma\)S) (1250 Ci/mlmol) was purchased from PerkinElmer Life Sciences (Boston, MA). [\(\text{3H}\)]\(\text{d-Ala}^2,\text{N-Me-Phe}^4,\text{Gly}^\text{\beta\text{-}}\text{ol}\)-enkephalin (DAMGO; 56 Ci/mlmol) was purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). DAMGO, buprenorphine hydrochloride, naloxone, and d-Phe-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr-NH\(_2\) (CTOP) were

ABBREVIATIONS: FCA, Freund’s complete adjuvant; MOR, $\mu$-opioid receptor; DRG, dorsal root ganglion; GTP\(\gamma\)S, guanosine-5'-O-(\(\text{y}\)-thio)-triphosphate; BUP, buprenorphine hydrochloride; DAMGO, [\(\text{d-Ala}^2,\text{N-Me-Phe}^4,\text{Gly}^\text{\beta\text{-}}\text{ol}\)]-enkephalin; CTOP, d-Phe-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr-NH\(_2\); NLX, naloxone; PPT, paw pressure threshold; IR, immunoreactivity.
purchased from Sigma RBI (Taufkirchen, Bayern, Germany). Scin- 
tillation fluid was obtained from PerkinElmer Wallac (Turku, Fin-
land). Antibodies for immunohistochemistry were obtained from Vec-
tor Laboratories (Burlingame, CA). Synthetic peptide for MOR was 
obtained from Gramesch Laboratories (Schwabhausen, Bayern, Ger-
many). Dibutylphthalate polystyrene xylene was provided by Merck 
(Darmstadt, Hessen, Germany). Tissue Tek compound (OCT) was 
provided by Miles (Elkhart, IN). Anesthesia was performed with 
halothane from Willy Rüsch GmbH (Böblingen, Baden Württemberg, 
Germany). FCA was obtained from Calbiochem (San Diego, CA).

**Subjects.** Experiments were performed in male Wistar rats (180– 
200 g) individually housed in cages lined with sawdust. Standard 
laboratory rodent chow and water were available ad libitum. Room 
temperature and relative humidity were maintained at 22 ± 0.5°C and 
60%, respectively. A 12-h:12-h light/dark cycle was used. All 
testing was conducted in the light phase, employing separate groups 
of animals. The guidelines on ethical standards for investigations of 
experimental pain in animals were followed (Zimmermann, 1983).

**Induction of Inflammation.** Unilateral hindpaw inflammation 
was induced by injection of 0.15 ml of FCA into the right hindpaw 
under brief halothane anesthesia. A detailed description of the time 
course and magnitude of the inflammatory reaction is given else-
where (Stein et al., 1988b). The inflammation remained confined to 
the inoculated paw and all experiments were performed 96 h (4 days) 
after FCA inoculation.

**Membrane Preparations.** Rats were killed by halothane anes-
thesia after 96-h treatment with saline or FCA and lumbar (L3–L5) 
DRGs were removed. In animals treated with FCA, DRG on the 
inflamed and contralateral sites were removed separately. The tis-
sue was placed immediately on ice in cold assay buffer (50 mM 
Tris-HCl, 1 mM EGTA, 5 mM MgCl2, pH 7.4). Membrane prepara-
tions were made by pooling DRG tissue from 10 rats. Tissue was 
homogenized with a Polytron homogenizer (Kinematica AG, Littau, 
Switzerland) and centrifuged at 48,000 g at 4°C for 20 min. The pellet 
was resuspended in assay buffer followed by a 10-min incubation at 
37°C to remove endogenous ligands. The homogenate was centri-
 fuged again at 48,000 g and resuspended in assay buffer. Membranes 
were aliquoted and stored at −80°C.

**Preparation of Sciatic Nerves.** Rats were anesthetized with 
halothane 48 h after FCA or saline injections. The right sciatic nerve 
was surgically exposed, dissected away from the surrounding tissue, 
and ligated with nonabsorbable silk at the midterminal position (5 
mm below the sciatic notch) in animals with FCA inflammation or 
saline treatment. The incision was then closed with wound clips. 
After 96 h of FCA inflammation, rats were killed, and the proximal 
part of sciatic nerve was removed and membranes prepared as de-
scribed above.

**Opioid Receptor Binding.** Membranes were diluted in assay 
buffer. Saturation analysis of [3H]DAMGO binding was performed 
by incubating 50 μg of membrane protein with 0.02 to 2 nM 
[3H]DAMGO in the presence and absence of 10 μM unlabeled nal-
oxone (NLX) to determine nonspecific binding. Affinity (inhibition 
constants, Ki) of DAMGO and BUP at DRG membranes of animals 
with and without FCA anesthesia. A detailed description of the time 
course and magnitude of the inflammatory reaction is given else-
where. In animals treated with FCA, DRG on the 
inflamed and contralateral sites were removed separately. The tis-
sue was placed immediately on ice in cold assay buffer (50 mM 
Tris-HCl, 1 mM EGTA, 5 mM MgCl2, pH 7.4). Membrane prepara-
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37°C to remove endogenous ligands. The homogenate was centri-
 fuged again at 48,000 g and resuspended in assay buffer. Membranes 
were aliquoted and stored at −80°C.

**Immunohistochemistry.** Four days after FCA treatment, six 
rats were deeply anesthetized with halothane and transcranially 
perfused with 80 ml of warm saline, followed by 300 ml of 4% (w/v) 
paraformaldehyde with 0.2% (w/v) picric acid in 0.16 M phosphate 
buffer solution, pH 6.9. The ipsilateral and contralateral L5 DRG 
were removed, postfixed in the same fixatives for 90 min, and then 
placed in 15% (w/v) sucrose solution at 4°C overnight. The tissue was 
embedded in Tissue Tek compound (OCT; Miles), frozen and cut in 
14-μm sections. The sections were incubated overnight with anti-
MOR (1:1000) (kindly provided by Drs. Stefan Schulz and Volker 
Höllt, Department of Pharmacology and Toxicology, Otto-von-Gu-
ricke University, Magdeburg, Germany). The sections were incu-
brated for 90 min with the appropriate biotinylated secondary anti-
body and with avidin-biotin-conjugated peroxidase. Finally, the 
sections were washed and stained with 3,3′-diaminobenzidine tet-
rahydrochloride containing 0.01% H2O2 in 0.05 M Tris-buffered sa-
line, pH 7.6, for 3 to 5 min. After the enzyme reaction, the sections 
were washed in tap water, mounted onto gelatin-coated slides, de-
hydrated in alcohol, cleared in xylene, and mounted in dibutylpha-
late polystyrene xylene. To demonstrate specificity of staining, the 
following controls were included: 1) preabsorption of diluted anti-
body against MOR with a synthetic peptide for MOR (Gramesch 
Laboratories, Schwabhausen, Germany) for 24 h at 4°C and 2) omiss-
on of either the primary antiserum, the secondary antibodies, or the 
avidin-biotin complex. These control experiments did not show MOR 
staining.

The method of quantification for DRG staining has been described 
previously (Ji et al., 1995). Briefly, we stained every fourth section of 
DRG that was serially cut at 14 μm. The total number of MOR-
containing neurons was counted by an observer blinded to the ex-
perimental protocol. This number was divided by the total number 
of neurons in each DRG section, and the percentage of MOR immu-
no reactive neurons was calculated. Percentages from four sections of 
each DRG were averaged. Five rats per group (inflamed and non-
flamed) were used for analysis. The cell body diameter was measured 
with the nucleus in the focal plane and was estimated from the 
average length and width determined with a calibrated micrometer. 
A total number of 30 immunoreactive neurons with nucleus were 
measured in each animal.

**Measurement of Agonist Efficacy and Potency at MOR in DRG Membranes.** Membranes were thawed, homogenized, and 
centrifuged at 48,000g for 10 min. Membranes were resuspended in 
[35S]GTPγS assay buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 0.2 
M EDTA, 100 mM NaCl, and 1 mM DTT). The buffer condition 
was similar to that used by Newman-Tonge et al. (2000). Concen-
tration-effect curves were generated by incubating the appropriate 
concentration of membranes (50 μg) in assay buffer with 0.1% bovine 
serum albumin, various concentrations of BUP or DAMGO (10−12– 
10−4 M), with 50 μM GDP and 0.05 nM [35S]GTPγS in a total volume 
of 800 μl. Basal binding was assessed in the absence of agonist, and 
nonspecific binding was measured in the presence of 10 μM unlabeled 
GTPγS. The reaction was incubated for 2 h at 30°C. 

[35S]GTPγS Saturation Binding at MOR of DRG Mem-
branes. Saturation analysis of DAMGO and BUP-stimulated 
[35S]GTPγS binding to DRG membranes was performed. In the pre-

cence (DAMGO or BUP 10 μM) or absence (H2O) of agonists, mem-
branes were incubated with 0.05 to 2 nM [35S]GTPγS in assay buffer 
for 2 h at 30°C. Unstimulated [35S]GTPγS binding was subtracted 
from agonist-stimulated binding at each measurement point. The 
incubations for all experiments were terminated by filtration under 
vacuum through Whatman GF/B glass fiber filters, followed by four 
washes with cold buffer (50 mM Tris-HCl, pH 7.4). Bound radioac-
tivity was determined by liquid scintillation spectrophotometry after 
evacuation overnight in scintillation fluid.

**Measurement of Paw Pressure Threshold.** Four days after 
FCA inoculation, nociceptive thresholds were assessed before (base-
line) and after drug administration using the paw pressure algesi-
ometer (modified Randall-Selitto test; Ugo Basile, Comerio, Italy).
The pressure required to elicit paw withdrawal, the paw pressure threshold (PPT) (cut-off at 250 g), was determined by averaging three consecutive trials separated by 10 s (Stein et al., 1988b). The sequence of left and right paws was alternated between animals to avoid bias. Drugs were administered intraplantarly (100 μl), and antagonists were given concomitantly with agonist in a total volume of 200 μl. Control animals received saline in the same volume. The experimenter was blind to the treatment.

Data Analysis. All ligand binding and [35S]GTPγS binding data are reported as mean ± S.E. values of at least three experiments, each of which was performed in duplicate. [3H]DAMGO ligand binding experiments and [35S]GTPγS saturation binding experiments were fitted to a one-site binding hyperbola using Prism (GraphPad, San Diego) to determine Kd and Bmax values. For saturation analysis of stimulated [35S]GTPγS binding, basal [35S]GTPγS binding was subtracted from agonist (10 μM DAMGO or 10 μM BUP)-stimulated [35S]GTPγS binding. Stimulated [35S]GTPγS binding is defined as agonist-stimulated minus basal [35S]GTPγS binding. Efficacy (Emax) is defined as the maximum percentage stimulation by an agonist, as determined by nonlinear regression analysis of concentration-effect curves. Relative Emax values are expressed as a percentage of maximal stimulation with DAMGO in animals without inflammation. Non-specific binding was subtracted from all [35S]GTPγS binding data. Statistical differences between animals with and without FCA inflammation were determined by the nonpaired Student’s t test and Mann-Whitney rank sum tests. Amplification factors were defined as % of G-protein coupling (Table 1, Fig. 3B).

Results

Binding Affinities and Opioid Receptor Numbers in DRG Membranes. Saturation binding of [3H]DAMGO displaced by naloxone showed similar binding affinities (Kd) in DRG membranes of animals without (Kd, 0.2 ± 0.03 nM) and with (Kd, 0.3 ± 0.04 nM) FCA inflammation. The number of opioid receptors (Bmax) increased significantly in animals with (Bmax, 47 ± 2.1 fmol/mg of protein) compared to animals without (Bmax, 25 ± 1.1 fmol/mg of protein) FCA inflammation (t test, p < 0.05) (Fig. 1A). This increase in MOR binding sites was restricted to the inflamed side and was not detectable on the contralateral side of inflammation (data not shown). Competitive inhibition experiments of [3H]DAMGO by unlabeled DAMGO and BUP were performed to determine the inhibition constant Ki. There was no significant difference detectable between DRG of animals with and without FCA inflammation (t test, p > 0.05) (Fig. 1B). In animals with inflammation, the accumulation of binding sites was shown proximal to the ligature (7 ± 1.3 fmol/mg of protein). However, after 96 h of FCA inflammation, a significantly higher accumulation of MOR-specific binding sites was detected in proximal parts of the ligature (17 ± 2.5 fmol/mg of protein, t test, p < 0.05).

Immunohistochemistry. In nontreated rats, some DRG neurons contained MOR-immunoreactivity (MOR-IR) (Fig. 2A). These neurons were mainly of small diameter. The mean cell body diameter of MOR-positive neurons was 37.4 ± 1.1 μm; the majority lay between small- and medium-diameter neurons (23–51 μm). Of all neurons, 17.2 ± 0.9% were MOR-IR. Four days after FCA, there was a noticeable increase in the number of MOR-positive DRG neurons on the inflamed side (Fig. 2B) and this increase in MOR was not detectable on the contralateral side of inflammation (data not shown). Of all DRG neurons, 25 ± 1.3% were MOR-positive, which represents a 45.3% relative increase (p < 0.01, Mann-Whitney rank sum test). There was no significant difference in the mean diameter of MOR-positive neurons between animals with and without FCA inflammation (p > 0.05), suggesting that any increase in MOR synthesis was not caused by a change in cell size.

Potencies and Efficacies of DAMGO and BUP for [35S]GTPγS Binding in DRG. It has been shown that agonist efficacy for stimulation of [35S]GTPγS binding is dependent on the concentration of GDP (Traynor and Nahorski, 1995; Selley et al., 1997; Newman-Tancredi et al., 1999). Among various concentrations (5–200 μM) of GDP tested, 50 μM GDP achieved the maximal percentage stimulation by DAMGO in DRG membranes and was used in all subsequent studies. EC50 and Emax values are shown in Table 1 and Fig. 3. Relative Emax values were expressed as a percentage of maximal DAMGO stimulation in DRG membranes of animals without FCA inflammation. After 96 h of FCA inflammation, DAMGO induced a significant increase in efficacy (Emax) (Mann-Whitney rank sum test, p < 0.05) and a non-significant, leftward shift in potency in DRG membranes (Table 1, Fig. 3A). In contrast, the partial agonist BUP did not induce any detectable G protein activation in DRG membranes of animals without FCA inflammation. However, after 96 h of FCA inflammation, BUP showed effective G-protein coupling (Table 1, Fig. 3B).

[35S]GTPγS Saturation Binding Experiments. [35S]GTPγS saturation binding exhibited high affinities for G-proteins at MOR (Table 2) after DAMGO (10 μM) stimulation, but no significant differences were detectable between Kd Gprotein in DRG membranes of animals with and without FCA inflammation (p > 0.05) (Table 2). In animals with FCA inflammation, a significant increase in DAMGO-stimulated Bmax Gprotein was detected (Fig. 4, Table 2) (p < 0.05). After stimulation with the partial agonist BUP, Bmax Gprotein was only measurable in animals with FCA inflammation (Table 2 and Fig. 4). Bmax determination of G-proteins in animals with FCA inflammation revealed that after BUP stimulation, only 34% (145 fmol/mg) of G-proteins were activated compared with 100% (425 fmol/mg) of G-proteins activated by the full agonist DAMGO (Table 2, Fig. 4). The amplification factors (amount of G-protein bound/number of opioid receptors expressed on the surface) was calculated according to Selley et al. (1998). No significant difference in amplification factors was detectable between animals with (amplification factor 9) and without (amplification factor 11) FCA inflammation.

Behavioral Studies. Intraplantar injection of BUP in a dose of up to 5 μg in normal rats did not show any significant changes in PPT (p > 0.05) (Fig. 5B). Injection of higher doses...
of BUP, such as 10 μg, increased PPT not only in the injected paw, but also in the contralateral paw of rats with and without FCA inflammation, indicating a systemic (central) site of action (data not shown). In contrast, administration of BUP into inflamed paws resulted in significant elevations of PPT ($p < 0.05$) (Fig. 5A). No PPT changes were observed in the contralateral noninflamed paws ($p > 0.05$) indicating that the site of action is restricted to the inflamed paw (data not shown). PPT elevations in inflamed paws increased dose dependently. BUP in a dose of 1 μg showed a peak effect at 5 min, whereas BUP in a dose of 3 and 5 μg showed maximum effect at 30 min. This antinociception was very long-lasting (up to 2 h), and by 4 h, the PPT returned to baseline values (Fig. 5A). The peripheral antinociceptive effect produced by BUP in inflamed paws (5 μg at 30 min) was dose-dependently antagonized by intraplantar coadministration of naloxone (30 μg, $p < 0.05$) (Fig. 6A) and CTOP (120 μg, $p < 0.05$) (Fig. 6B).

**Discussion**

Our results show that inflammation enhances the efficacy of G-protein coupling of full and partial MOR agonists by an increased binding and G-protein coupling at MOR of primary

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**Fig. 1.** A, saturation binding was performed with [3H]DAMGO to DRG membranes of animals with and without inflammation. Nonspecific binding was determined with 10 μM naloxone. Data shown are means of duplicates from at least three independent experiments. B, displacement of [3H]DAMGO binding to DRG membranes of animals with and without inflammation. Nonradioactive DAMGO and BUP was tested as a displacer at $10^{-11}$ to $10^{-4}$ M concentrations. One representative curve of four independent experiments is shown.
afferent neurons. First, FCA inflammation induces an up-regulation of MOR binding sites on DRG membranes without affecting binding affinities. Consistently, the number of MOR per DRG as well as the number of MOR-ir neurons that are predominantly of small diameter increases after FCA inflammation. Second, the full agonist DAMGO but not the partial agonist BUP induces MOR G-protein coupling in DRG of animals without FCA inflammation. FCA inflammation results in significant increases in DAMGO-induced MOR G-protein coupling and in partial agonist activity of BUP-induced MOR G-protein coupling. Third, BUP injection into inflamed, but not normal hindpaws elicits potent and long-lasting antinociceptive effects.

Peripheral opioid receptors are localized and expressed on primary sensory neurons. Primary sensory neurons offer the advantage of characterizing receptors in their native environment. In addition, the possibility to induce a locally applied inflammation allows study of the effects of MOR binding and signaling under pathological conditions. In animals with FCA inflammation, we found a significant increase in the number of MOR binding sites on DRG membranes, although the affinity of DAMGO to MOR remained unchanged. Displacement experiments of \(^{3}H\)DAMGO with either non-labeled DAMGO or the partial agonist BUP revealed similar inhibitory constants \((K_i)\) in DRG membranes of animals with and without FCA inflammation. These results suggest that an inflammatory stimulus can increase the number of MOR on DRG membranes but does not change the affinity of MOR to opioids.

Axonal transport has been demonstrated for various neuroreceptors, including MOR in peripheral nerves (Laduron and Castel, 1990). We performed a set of experiments to show that an increase in MOR specific binding sites in the DRG is accompanied by an increase of the axonal transport of MOR to the periphery. Almost no MOR binding sites were detectable in the unligated sciatic nerve preparations. However, ligation of the sciatic nerve in the absence of inflammation resulted in an accumulation of MOR at the proximal part of the ligation, indicating an anterograde transport from the DRG to the noninflamed paw. In rats with inflamed paws, a significant increase in MOR-specific binding sites at the proximal part of the ligation was detected. Together with our binding data in DRG membranes, this indicates that inflammation can cause an increase in MOR-specific binding sites in both DRG and the sciatic nerve. This strongly suggests that an increase in MOR levels in the DRG would also be seen in the peripheral portions of the nerve after axonal transport to the periphery. This also confirms previous neuroanatomical evidence for the existence of MOR in the sciatic nerve and peripheral cutaneous nerve fibers (Hassan et al., 1993). It was shown recently that an increase in MOR number might be related to mediators of inflammation (e.g., IL-4, tumor necrosis factor) (Kraus et al., 2001).

Our immunohistochemical results confirmed an up-regulation of MOR after FCA inflammation. However, this increase was restricted to DRG on the inflamed side and was not detected in DRG on the contralateral side. An increase in \(B_{max}\) (90%, as determined with \(^{3}H\)DAMGO binding) and an

Table 1

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<th>(EC_{50})</th>
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<td>nM</td>
<td>%</td>
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<tr>
<td>DRG(_{Control})</td>
<td>56 ± 18</td>
<td>20 ± 0.9</td>
<td>100 ± 5.0</td>
<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>DRG(_{FCA})</td>
<td>29 ± 12</td>
<td>31 ± 1.9*</td>
<td>148 ± 5.6*</td>
<td>1.6 ± 0.4</td>
<td>16 ± 1.5</td>
<td>82 ± 4.2</td>
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N.D., not detected.

\(^*\) \(P < 0.05\) different from animals without inflammation.

Fig. 2. Bright-field micrographs showing MOR positive neurons in L5 DRGs of rats without FCA inflammation (A) and in DRGs of rats with FCA inflammation (B). MOR-IR is mainly seen in small DRG neurons. Scale bar, 20 \(\mu m\).
increase in the number of MOR-positive DRG neurons (45%, as determined in our immunohistochemical studies) indicates that this up-regulation is caused by an increase in the number and density of MOR-positive neurons. Consistent with previous studies, under both normal and inflammatory conditions, MOR immunoreactive DRG neurons were mainly of small diameter, suggesting that the increase in MOR immunoreactivity is mainly restricted to nociceptive neurons (Ji et al., 1995; Mousa et al., 2001).

An important finding of the present study is that the efficacy of DAMGO-stimulated G-protein activation increased significantly in animals with FCA inflammation. This observation might explain why the application of exogenous opioids in peripheral antinociception is enhanced under inflammatory conditions (Stein et al., 2001). In addition, the mechanisms of μ-opioid agonist efficacy and inflammation were investigated using agonist-stimulated [35S]GTPγS binding. The advantages and disadvantages to this measurements have been described earlier (Newman-Tancredi et al., 1997a; Selley et al., 1997): Scatchard analysis of [35S]GTPγS binding measures the competition of a radiolabeled ligand ([35S]GTPγS) for a nonlabeled ligand (GDP) only under non-equilibrium conditions. At equilibrium, the [35S]GTPγS binding is expressed as percentage of maximal stimulation with DAMGO (100%) obtained in non-inflamed animals. Nonspecific binding was determined using 10 μM unlabeled GTPγS and was subtracted from each data set. Basal [35S]GTPγS binding in the absence of added drugs was 4000 to 6000 cpm in both groups. Each value represents the mean ± S.E.M. of at least three independent experiments.

The characterization of G-protein binding to MOR is given in Table 2. The affinity (Kd) and number (Bmax) of G-proteins for net agonist-stimulated [35S]GTPγS binding in DRG membranes of animals without (Control) and with (FCA) inflammation after DAMGO and BUP stimulation. Membranes were incubated with varying concentrations of [35S]GTPγS as described under Materials and Methods. Data are mean Bmax and Kd values ± S.E.M., obtained from at least three independent experiments.

Table 2

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<th>DAMGO</th>
<th>BUP</th>
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<tr>
<td>Kd (nM)</td>
<td>Bmax (fmol/mg)</td>
<td>Kd (nM)</td>
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<td>Bmax (fmol/mg)</td>
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<tr>
<td>DRG_control</td>
<td>0.8 ± 0.1</td>
<td>266 ± 16</td>
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<tr>
<td>DRG_FCA</td>
<td>0.8 ± 0.2</td>
<td>425 ± 54*</td>
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*P < 0.05 different from animals without inflammation.
would displace as much of the GDP as possible, and no agonist-stimulated binding could be observed. This assay is therefore not quantitatively accurate in the sense that a given $B_{\text{max}}$ G protein represents the exact maximal number of G proteins; however, relative comparisons between inflamed and noninflamed tissue are possible. We found that the number of $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding sites that can be occupied after DAMGO stimulation increased 1.6-fold (from 266 to 425 fmol/mg of protein) in animals with FCA inflammation. The amplification factor (or number of G-proteins activated per MOR) decreased, suggesting that an increase in MOR during inflammation is not proportional with an increase in G-protein coupling. In addition, we found that DAMGO-occupied receptors on DRG membranes in animals with and without FCA inflammation revealed no differences in the DAMGO-induced guanine nucleotide affinities (measured as the $K_{d}$ G protein value of $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding). Taken together, these results suggest that inflammation can cause an increase in receptor density per cell, which results in an increased number of activated G-proteins. Consistent with this notion, it was shown earlier that a drop in MOR in SH-SYSY cells and in cannabinoid receptors in certain areas in the brain could cause an equivalent drop in the level of $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding (Sim et al., 1996; Remmers et al., 2000; Selley et al., 2001). These results appear in contrast to other studies in the 5-HT$_{1A}$ system, where an increase in receptor density did not change the activated G-protein number (Newman-Tancredi et al., 1997b). However, those studies were performed in highly expressing Chinese hamster ovary cells in which the number of G-proteins might be limited in comparison with the number of receptors. DRG membranes of noninflamed animals expressed 25 fmol/mg of protein MOR, which is clearly below Chinese hamster ovary cells expressing 1600 fmol/mg 5-HT$_{1A}$ receptors (Newman-Tancredi et al., 1997b). Therefore, an increase of MOR in animals with inflammation might explain the observed increase in G-protein activation. Although animals with FCA inflammation exhibited a 90% increase in expression of MOR, maximal stimulation of $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding by DAMGO was only 48% higher and therefore did not increase proportionally with receptor levels.

Surprisingly, BUP did not induce any detectable G-protein coupling in DRG membranes of animals without FCA inflammation. In contrast, BUP stimulated G-protein coupling in DRG membranes of animals with FCA inflammation. The extent of stimulation of $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding (EC$_{50}$) in animals with FCA inflammation was lower for BUP than for DAMGO, as in many other in vitro (Huang et al., 2001; Zaki et al., 2000) and in vivo (Gopal et al., 2002; Traynor et al., 2002) systems. It has been suggested previously that a given biological effect requires the switching on (or off) of a certain number of effector molecules (Chavkin and Goldstein, 1984). Due to stoichiometric interactions between receptors and effectors, it might be that the partial agonist BUP could not activate a detectable amount of G-proteins in animals without FCA inflammation. However, because of the increase of MOR in DRG membranes of animals with FCA inflammation, the number of receptors appears sufficient to activate the pool of G-proteins.

Scatchard analysis of basal and agonist-stimulated $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding confirmed that BUP (low efficacy partial agonist) produced a lower affinity GTP-binding state in DRG of animals with inflammation (presumably in the guanine nucleotide binding site of $\mu$ receptor-coupled G protein $\alpha$ subunits) than DAMGO (higher efficacy agonist). Partial agonists do not fully shift the affinity of the G-proteins into a GTP-prefering state (Selley et al., 1998; Traynor et al., 2002), and this was clearly evident for BUP in DRG membranes of animals with FCA inflammation: agonist-induced guanine nucleotide affinity for BUP ($K_{d}$ G protein, 1.8 nM) compared to DAMGO ($K_{d}$ G protein, 0.8 nM) was different and the catalytic activation of G proteins, as measured by the agonist-stimulated $B_{\text{max}}$ G protein of $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding, was lower for BUP ($B_{\text{max}}$ G protein, 145 fmol/mg) than for DAMGO ($B_{\text{max}}$ G protein, 425 fmol/mg).

It should be noted that an increase in maximal $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding as a function of receptor density does not necessarily result in a similar increase in the magnitude of a downstream response (Law et al., 1994; Prather et al., 1994). Therefore, we performed a set of behavioral experiments to test whether the results obtained with $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding and BUP showed functional consequences in antinoceception.

We found that intraplantar injection of the partial agonist BUP in noninflamed paws did not change paw pressure thresholds compared with intraplantar saline injections. In contrast, local BUP injections in animals with inflamed paws produced PPT elevations (i.e., antinoceception). These results
indicate that BUP can act as an effective peripheral antinociceptive agent only in the presence of inflammation. The MOR-selective antagonists NLX and CTOP could block this antinociceptive effect of BUP, which clearly indicates that BUP mediates its antinociceptive activity through MOR. The contralateral paws showed no changes in PPT, suggesting that low doses of BUP induce only peripheral, not central, opioid analgesic effects. It was already shown earlier that opioid full agonists (e.g., fentanyl) produce dose-dependent elevations of PPT in animals with and without FCA inflammation; however, antinociception is smaller in noninflamed hindpaws compared with inflamed hindpaws (Antonijevic et al., 1995). There are many steps between MOR binding and antinociception (e.g., inhibition of cAMP, inhibition of calcium channel conductance) that can modulate the downstream responses. However, the observed antinociceptive action after BUP injection only in animals with FCA inflammation might be related to the lack of G-protein coupling observed in DRG membranes. This supports the hypothesis that in animals without FCA inflammation, there are not enough receptors present to develop opioid analgesia by the partial MOR agonist BUP.

In conclusion, inflammation is associated with an up-regulation of MOR, mainly in small-sized primary afferent neurons, and enhances the efficacy of full and partial MOR agonists in G-protein coupling. These changes might contribute to the occurrence of peripheral antinociceptive effects of the partial MOR agonist BUP, which are not present under normal conditions. These adaptive changes underscore the important differences in opioid receptor binding and signaling between normal and inflamed tissue. They strongly indicate that clinical studies testing peripherally active opioids are much more likely to yield positive results when they are performed in inflammatory painful conditions.

**Fig. 5.** Time course and dose-response of the antinociceptive effects of BUP in injected paw of (A) rats with inflamed hindpaws and (B) normal rats. *, significantly different from saline treated group (related to 1, 3, and 5 µg BUP).

**Fig. 6.** Blocking effect of Naloxone (A) and CTOP (B) on the antinociceptive action of BUP. *, significantly different from saline treated group; !, significantly different from BUP (5 µg)-treated group.
References


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