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European Journal of Pharmacology 414 (2001) 289–294

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# Opioid receptors on bone marrow neutrophils modulate chemotaxis and CD11b/CD18 expression

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Received 17 August 2000; received in revised form 18 December 2000; accepted 22 December 2000

**Abstract**

Opiates impair neutrophil-mediated host defense, but the involvement of κ-opioid receptors in this action has not been defined. The selective κ-opioid receptor agonist [*trans*-(+)3,4-dichloro-*N*-methyl-*N*[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide methanesulfonate inhibited macrophage inflammatory protein-2-induced chemotaxis of bone marrow neutrophils from C57BL/6 mice. Its effects were concentration-dependent ($pIC_{50} = 10.40 \pm 0.61$) and inhibited by naloxone ($K_e = 0.27$ nM). The κ-opioid receptor agonists bremazocine and ICI-204, 488 also inhibited chemotaxis, as did the respective μ- and δ-opioid receptor agonists [D-Ala$^2$, *N*-methyl-Phe$^4$, Gly$^5$-ol]enkephalin and [D-Pen$^{2,5}$]enkephalin albeit with lower potencies. U-50,488H also decreased neutrophil expression of the $\beta_2$ integrin CD11b/CD18 (Mac-1) and adhesion to plastic in a naloxone-reversible manner. The results indicate that κ-opioid receptors expressed by neutrophils rapidly modulate chemotaxis and adhesion in vitro. 

*Keywords:* Innate immunity; Integrin; Macrophage inflammatory protein; Adhesion; U-50,488H

## 1. Introduction

The administration of morphine and other opiate drugs has been associated with immunosuppression and decreased resistance to infection (Eisenstein and Hilburger, 1998; Risdahl et al., 1998). The effects of opioids are mediated by three major types of G protein-coupled receptors, namely the δ-, μ- and κ-opioid receptors. These receptors are expressed on neurons contained within the central and peripheral nervous systems and may mediate the neuroimmunomodulatory actions of opioids (Mellon and Bayer, 1998). A growing body of evidence suggests that these receptors, and κ-opioid receptors in particular, may be expressed by a variety of immune cells (Sharp et al., 1998). Messenger RNA transcripts for κ-opioid receptors (Belkowski et al., 1995; Chuang et al., 1995; Gaveriaux et al., 1995; Wick et al., 1996), κ-opioid receptor immunoreactivity (Buchner et al., 1997) or binding sites for fluorescent κ-opioid receptor ligands (Lawrence et al., 1995; Ignatowski and Bidlack, 1999) have been detected in thymocytes, splenocytes, and both primary and transformed monocytes and lymphocytes.

Neutrophils play a major role in non-specific host defense against pathogen invasion. The infiltration of neutrophils into sites of inflammation and infection is dependent upon specific adhesive interactions with endothelial cells and the expression of cytokines at the target site (Jutila et al., 1991; Ley, 1996). Although κ-opioid receptors have not been previously characterized in neutrophils, morphine and other drugs capable of interacting with opioid receptors can alter chemotaxis, adhesive interactions and pathogen removal processes in these cells (Sharp et al., 1985; Van Epps and Kutvirt, 1987; Mazzone et al., 1990; Liu et al., 1992; Qu et al., 1993; Grimm et al., 1998; Miyagi et al., 2000).

In the present study, we tested the hypothesis that opioid receptor agonists alter the migratory and adhesive functions of murine bone marrow neutrophils through a κ-opioid receptor-dependent mechanism. Our results indicate that U-50,488H and other selective κ-opioid receptor agonists inhibit chemotaxis and downregulate the expression of the $\beta_2$-integrin CD11b/CD18 (Mac-1) on neutrophils in a naloxone-reversible manner in vitro.

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0014-2999/01/$ - see front matter 
PII: S0014-2999(01)00727-0

## 2. Materials and methods

### 2.1. Reagents

[D-Pen$^{2,5}$]enkephalin (DPDPE); [D-Ala$^2$, *N*-methyl-Phe$^4$, Gly$^5$-ol]enkephalin (DAMGO); and nociceptin were purchased from Peninsula Laboratories (Belmont, CA). Naloxone HCl; naltrindole HCl; [*trans*-(+)3,4-dichloro-*N*-methyl-*N*[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide methanesulfonate (U-50,488H); (±)-6-ethyl-1,2,3,4,5,6-hexahydro-3-[(1-hydroxycyclopropyl)methyl]-11,11-dimethyl-2,6-methano-3-benzazocin-8-ol (bremazocine HCl); and *R*,*S*-[3-[1-[[(3,4-dichlorophenyl)acetyl]-methylamino]-2-(1-pyrrolidinyl)ethyl]phenoxy]-acetic acid hydrochloride (ICI 204,488 HCl) were purchased from Research Biochemicals International (Natick, MA). These substances were dissolved in Hank's balanced salt solution (HBSS; Celox, St. Paul, MN) prior to use. (6*aR*)-*trans*-3-(1,1-dimethylheptyl)-6*a*,7,10,10*a*-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[*b*,*d*]pyran-9-methanol (HU-210) was purchased from Tocris Cookson (Ballwin, MO) and dissolved in 2-hydroxypropyl-β-cyclodextrin (Sigma, St. Louis, MO). All other reagents were dissolved in nanopure water and stored at −20°C until use.

Murine recombinant macrophage inflammatory protein-2 (MIP-2) was purchased from R&D Systems (Minneapolis, MN), and bovine serum albumin was purchased from Sigma. Monoclonal antibodies M1/70 and MEL-14, which have been previously shown to recognize mouse CD11b/CD18 and L-selectin respectively, were raised in rats (Holmberg et al., 1981; James et al., 1991). A monoclonal anti-mouse CD45 (CD45.2) antibody was purchased from Pharmingen (San Diego, CA). Goat anti-rat and goat anti-mouse immunoglobulin G heavy and light chain $F(ab')_2$ antibodies that were conjugated to fluorescein isothiocyanate (FITC) were purchased from Jackson Immuno Research Laboratories (West Grove, PA).

### 2.2. Animals and neutrophil isolation

Six- to eight-week-old, male C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were used as cell donors. Mouse bone marrow neutrophils were isolated as previously described (Jutila et al., 1991). Briefly, femurs were harvested from euthanized mice and flushed with phosphate-buffered saline (PBS; pH 7.4) to retrieve bone marrow leukocytes. Neutrophils were separated from mononuclear cells by centrifugation through Ficoll 1.077. Erythrocytes were removed by hypotonic lysis and neutrophils were washed twice in HBSS and resuspended in HBSS containing 0.5% bovine serum albumin. This method yielded a neutrophil population of >95% purity as verified by trypan blue staining and flow cytometry. In initial experiments, neutrophils were stained with propidium iodide to verify that opioid receptor agonists and antagonists had no effects on cell viability.

### 2.3. Measurement of chemotaxis

Neutrophil chemotaxis towards MIP-2, a potent neutrophil chemoattractant that acts through murine interleukin-8Rh (Sherry et al., 1992), was measured in 48-well microchemotaxis chambers (Neuroprobe, Cabin John, MD). The upper and lower chambers of each well were separated by a polycarbonate polyvinylpyrrolidone-free filter with a pore size of 5 μm (Corning, Acton, MA). A concentration of 100 ng/ml MIP-2 was found to produce maximum neutrophil chemotaxis in preliminary experiments. Therefore, in all experiments, MIP-2 dissolved in HBSS with bovine serum albumin was added at this concentration in a volume of 29 μl to the lower chamber of each well. In control experiments, HBSS with bovine serum albumin was substituted for MIP-2 in lower chamber or an identical concentration of MIP-2 was added to the upper chamber containing neutrophils.

Drugs or their solvent vehicles serving as controls were added to neutrophil suspensions immediately prior to each chemotaxis assay. In some cases, an opioid receptor antagonist (either the non-selective opioid receptor antagonist naloxone or the δ-opioid receptor selective antagonist naltrindole) was added to neutrophil suspensions at a concentration of 100 nM immediately prior to vehicle or agonist exposure. Aliquots of drug- or vehicle-treated neutrophil suspensions ($1.5 \times 10^5$ cells/50 μl) were immediately added to the upper chamber of duplicate wells. The chambers were maintained at 37°C in air containing 5% $CO_2$ for 45 min. At the end of each incubation period, filters were removed, and washed on the upper side with PBS; the lower migratory side of the filter was fixed in acetone and stained using a Leukostat kit (Fisher Scientific, Chicago, IL) following the manufacturer's protocol. Cells that had migrated through each filter were counted microscopically at high (40×) magnification across five randomly selected fields. All assays were performed at least three times. A chemotaxis index (CI) was calculated as the quotient between the number of cells that migrated towards the chemoattractant and the number of cells that migrated towards HBSS with bovine serum albumin; this parameter was further multiplied by 100. The effects of drugs on neutrophil chemotaxis was determined as the quotient between the number of drug-treated cells and the number of vehicle-treated or untreated cells migrating towards the MIP-2 gradient; drug treatment effects were expressed as a percentage of chemotaxis towards MIP-2 relative to control conditions.

### 2.4. Fluorescence-activated cell sorting (FACS) analysis

To determine the effects of opioids on neutrophil adhesion molecules involved in cell migration, cells were incubated with a drug or its vehicle at 37°C for 45 min. Cells were washed twice in HBSS to remove drugs and stained

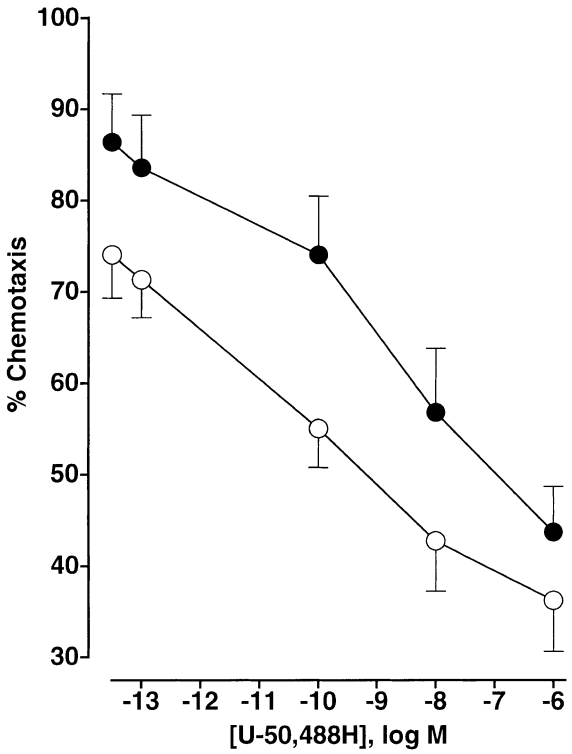


Fig. 1. Inhibitory effect of U-50,488H on MIP-2-directed chemotaxis of bone marrow neutrophils. The antichemotactic action of U-50,488H was determined for 45 min after the application of the agonist alone (open circles) or in the cells pretreated with 100 nM naloxone (filled circles). Data represent the means ± S.E.M. of the percentage of cells migrating in response to MIP-2 (100 ng/ml) compared to vehicle-treated controls ($n = 6$ concentration–effect curves for each condition).

with monoclonal antibodies to CD11b/CD18 and L-selectin (1:100 dilution) or CD45 (1:200 dilution) prior to flow cytometric analysis as previously described (Alexander et al., 2000). Briefly, Fc receptor and nonspecific antibody binding sites on neutrophils were blocked by an initial incubation of the cells with FACS wash buffer (1% normal goat serum and 5 mM sodium azide in PBS, pH 7.4). Incubations of neutrophils ($10^6$ cells) with primary antibodies were carried out at 4°C for 20 min in FACS wash buffer. Bound primary antibodies were revealed by secondary immunofluorescence using FITC-conjugated $F(ab')_2$ goat anti-mouse or anti-rat immunoglobulin G secondary antibodies (1:200 dilution) diluted in FACS wash buffer. Negative controls consisted of omission of primary antibodies from the staining protocol. For each sample, $10^4$ antibody-labeled cells were analyzed by flow cytometry on a FACS Caliber instrument (Becton Dickson, San Jose, CA). Mean relative fluorescence intensity distribution was measured on a logarithmic scale; a histogram statistic was determined using CellQuest software (Becton Dickinson). Mean fluorescence intensity of cells was expressed as a percentage of the mean fluorescence intensity observed under control conditions.

### 2.5. Adhesion assay

To test the hypothesis that U-50,488H can affect neutrophil adhesion, neutrophil adherence to plastic Petri dishes (35 mm diameter) was examined. Neutrophils were exposed to 1 μM U-50,488H alone or in the presence of 1 μM naloxone; an equivalent volume of HBSS was added to suspended cells serving as controls. Cells were then added to Petri dishes and allowed to adhere for 45 min at 37°C. After rinsing twice with HBSS, neutrophils were fixed in acetone and stained using a Leukostat kit. The number of neutrophils remaining on the plastic surface in each condition was counted microscopically in five randomly chosen fields under high (40 ×) magnification.

### 2.6. Data analysis

Prism software (version 2; GraphPad Software, San Diego, CA) was used to determine the concentration of each drug producing half-maximal inhibition of neutrophil chemotaxis by nonlinear regression of drug concentration–effect relationships. The antichemotactic potencies of opioid receptor agonists are expressed as the negative logarithm of the 50% inhibitory concentration ($pIC_{50}$) obtained in experiments with cells from three to seven mice. An equilibrium constant ($K_e$) for naloxone was calculated according to the method of Kosterlitz and Watt (1968). Comparisons between a control mean and multiple treatment means were made by one-way analysis of variance (ANOVA) followed by a multiple comparison test when appropriate. A $P$ value of less than 0.05 was chosen as the limit for statistical significance.

## 3. Results

### 3.1. Effects of opioid receptor agonists on neutrophil chemotaxis

The selective κ-opioid receptor agonist (±)U-50,488H inhibited neutrophil chemotaxis to MIP-2 in a concentra-

Table 1
Inhibitory potencies of opioid receptor agonists on neutrophil chemotaxis in vitro

| Agonist | $pIC_{50}$ for chemotaxis inhibition (mean ± S.E.M.) | Maximum chemotaxis inhibition (% of control) | $n$[a] |
|---|---|---|---|
| (±)U-50,488H | 10.40 ± 0.61[b] | 36.3 ± 5.6 | 6 |
| Bremazocine | 9.10 ± 0.93 | 47.9 ± 6.7 | 3 |
| ICI-204, 488 | 9.07 ± 0.85 | 41.2 ± 6.2 | 3 |
| DAMGO | 8.84 ± 0.48 | 40.7 ± 3.7 | 5 |
| DPDPE | 6.93 ± 0.78[c] | 25.7 ± 11.8 | 4 |

[a] Number of concentration–effect curves.
[b] Mean $pIC_{50}$ value in presence of 100 nM naloxone was 7.83 ± 0.72 ($n = 6$).
[c] $P < 0.01$ vs. U-50,488H $pIC_{50}$ value, Tukey's multiple comparison test, $df$ (2, 12).

tion-related manner (Fig. 1). Co-incubation of cells with 100 nM naloxone produced a significant 372-fold reduction in the inhibitory potency of U-50,488H ($P = 0.022$ vs. mean agonist $pIC_{50}$ in absence of antagonist, unpaired two-tailed *t*-test, $n = 6$). The chemically unrelated κ-opioid receptor agonists bremazocine and ICI-204, 488 and the respective μ- and δ-opioid receptor agonists DAMGO and DPDPE also inhibited neutrophil chemotaxis with maximal antichemotactic effects similar to U-50,488H (Table 1).

Neither nociceptin/orphanin FQ (10 pM–30 nM) nor the cannabinoid agonist HU-210 (1 μM) altered neutrophil chemotaxis (data not shown).

### *3.2. Effect of opioids on expression of CD11b/CD18, L-selectin and CD45*

U-50,488H, at a concentration of 1 μM, significantly reduced the constitutive expression of CD11b/CD18 immunoreactivity on neutrophils. Its effect was reversed by an equimolar concentration of naloxone (Fig. 2) At 1 μM, DAMGO, DPDPE, naloxone or naltrindole alone or in combination had no significant effect on expression of CD11b/CD18 immunoreactivity (Fig. 2). No significant changes in L-selectin or CD45 expression were detected in cells exposed to either U-50,488H or naloxone at 1 μM.

### *3.3. Effect of U-50,488H on neutrophil adhesion to plastic*

In comparison to vehicle-treated cells serving as controls, the number of neutrophils adhering to plastic was significantly reduced in the presence of 1 μM U-50,488H.

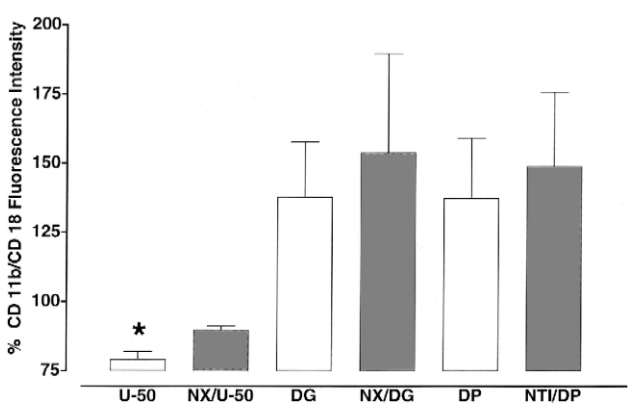


Fig. 2. Effects of selective opioid receptor agonists on expression of CD11b/CD18 immunoreactivity in murine bone marrow neutrophils. Cells were incubated with 1 μM of either U-50,488H (U-50), [D-Ala$^2$, *N*-methyl-Phe$^4$, Gly$^5$-ol]enkephalin (DG) or [D-Pen$^{2,5}$]enkephalin (DP) alone (open bars) or in the presence (filled bars) of 1 μM naloxone (NX) or naltrindole (NTI). Data represent the mean percent ± S.E.M. ($n = 3$ mice) of CD11b/CD18-immunoreactive fluorescence intensity in drug-treated cells relative to control cells treated with Hank's balanced salt solution. * $P = 0.03$ vs. NX/U-50 condition, Kruskal–Wallis test.

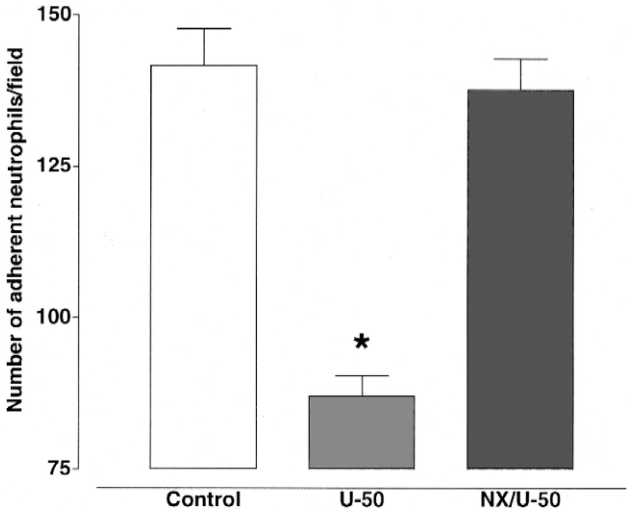


Fig. 3. Effect of U-50,488H on neutrophil adherence to a plastic surface. Neutrophils were incubated for 45 min after administration of Hank's balanced salt solution (control; open bar), 1 μM U-50,488H alone (U-50; light gray bar) or 1 μM U-50,488H in the presence of an equimolar concentration of naloxone (NX/U-50; dark gray bar). Data represent the means ± S.E.M. of observations across five fields of the number of cells adhering to plastic surface in each condition ($n = 3$ mice). * $P < 0.05$ vs. control condition, Dunnett's test.

The agonist had no effect on neutrophil adherence in the presence of 1 μM naloxone (Fig. 3).

## 4. Discussion

In the present report, U-50,488H, an opioid receptor agonist that exhibits a relatively high selectivity for κ-opioid receptors (VonVoightlander et al., 1983), rapidly inhibited neutrophil chemotaxis to MIP-2 in a concentration-related manner. Its effects were competitively antagonized by the classical opioid antagonist naloxone with a $K_e$ value of 0.27 nM, which is slightly lower than its dissociation constants at cloned μ- and κ-opioid receptors (Raynor et al., 1994). The κ-opioid receptor-selective agonist ICI-204,488 as well as bremazocine, a less selective κ-opioid receptor ligand, mimicked the antichemotactic actions of U-50,488H. Although the selective μ-opioid receptor agonist DAMGO exhibited an antichemotactic potency that did not differ significantly from that of U-50,488H, the δ-opioid receptor agonist DPDPE was considerably less potent in reducing neutrophil chemotaxis. Similar results have been obtained in murine lymphocytes (Taub et al., 1991) and macrophages (Guan et al., 1994). The κ-opioid receptor specificity of U-50,488H action was underscored by the lack of effect of nociceptin/orphanin FQ, which interacts with a naloxone-insensitive receptor closely related to κ-opioid receptors. Moreover cannabinoids, like opioids, have been shown to decrease immune function (Cabral and Dove-Pettit, 1998). However, the

potent cannabinoid agonist HU-210 (Pop, 1999) was also without significant effect on neutrophil chemotaxis. These results provide evidence for κ-opioid receptor involvement in U-50,488H activity and support the hypothesis that κ-opioid receptors, and possibly μ-opioid receptors are expressed by neutrophils.

The immunofluorescence intensity for CD11b/CD18, a $\beta_2$-integrin that is involved in neutrophil adhesion, degranulation, and receptor-mediated phagocytosis (Li, 1999), was reduced on neutrophils by U-50,488H. This effect appears to involve opioid receptors because it was absent in neutrophils pretreated with naloxone. In contrast, U-50,488H had no effect on cellular expression of L-selectin or CD45 immunoreactivities indicating that the drug exhibits a high degree of specificity for its cellular targets. Decreases in CD11b/CD18 expression have been previously reported in neutrophils of heroin abusers (Mazzone et al., 1994). However, both β-endorphin and enkephalin increase CD11b/CD18 expression in human neutrophils (Pasnik et al., 1999). U-50,488H also reduced the adherence of neutrophils to a plastic surface. This effect, which was reversed by naloxone, may be related to decreased cellular Mac-1 expression. Impairments in CD11b/CD18 expression with a consequent decrease in neutrophil adhesion would be expected to reduce the ability of neutrophils to participate in host defense processes. Indeed, κ-opioid receptor agonists have been shown to possess antiinflammatory properties (Qu et al., 1993; Antic et al., 1996).

Although the results of this study suggest a role for κ-opioid receptors in mediating aspects of neutrophil chemotaxis and adhesion in vitro, additional definition of these receptors awaits experiments with selective antagonists at the different opioid receptor types. Moreover, studies are necessary to elucidate the cellular mechanisms by which κ-opioid receptor agonists act, and the potential importance of neutrophil opioid receptors in modulating innate immunity in healthy and diseased hosts should be examined.

## Acknowledgements

This investigation was supported by NIH grant DA-10200. A. K.-N. is a postdoctoral fellow supported by NIH Psychoneuroimmunology Training grant T32-07239. The authors thank Mrs. Sheila Alexander for her excellent help and advice in FACS analyses.

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