Proinflammatory Chemokines, Such as C-C Chemokine Ligand 3, Desensitize μ-Opioid Receptors on Dorsal Root Ganglia Neurons

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Pain is one of the hallmarks of inflammation. Opioid receptors mediate antipain responses in both the peripheral nervous system and CNS. In the present study, pretreatment of CCR1;µ-opioid receptor/HEK293 cells with CCL3 (MIP-1α) induced internalization of µ-opioid receptors and severely impaired the µ-opioid receptor-mediated inhibition of cAMP accumulation. Immunohistochemical staining showed that CCR1 and µ-opioid receptors were coexpressed on small to medium diameter neurons in rat dorsal root ganglion. Analysis of ligand-induced calcium flux showed that both types of receptors were functional. Pretreatment of neurons with CCL3 exhibited an impaired [d-Ala², N-MePhe⁴, Gly-ol⁵]enkephalin-elicited calcium response, indicative of the heterologous desensitization of µ-opioid receptors. Other chemokines, such as CCL2, CCL5, and CXCL18, exhibited similar inhibitory effects. Our data indicate that proinflammatory chemokines are capable of desensitizing µ-opioid receptors on peripheral sensory neurons, providing a novel potential mechanism for peripheral inflammation-induced hyperalgesia. The Journal of Immunology, 2004, 173: 594–599.

Opioids have long been used to suppress pain. Their analgesic effects are mediated by a family of three closely related G protein-coupled receptors: µ-, δ-, and κ-opioid receptors (1–4). The µ-opioid receptor (MOR) plays a critical role in modulating the sensation of pain. Disruption of the µ-opioid receptor in mice results in loss of morphine-induced analgesia, reward, and physical dependence (5). µ-Opioid receptors exert their analgesic effects by activating Gi/o signaling pathways (6–8). Binding of [d-Ala², N-MePhe⁴, Gly-ol⁵]enkephalin (DAMGO), a MOR-specific agonist, elicits a spectrum of downstream signaling events, including inhibition of forskolin-induced cAMP accumulation, activation of G protein-coupled inward rectify potassium channels (GIRK), and inhibition of calcium channels (3, 9–13). Agonist-induced transient calcium flux has also been reported in both primary neurons and cell lines transfected to express MORs (12, 14). Inhibition of calcium channels impairs the release of neurotransmitters, while activation of GIRK hyperpolarizes neuron membranes, thereby preventing the excitation and propagation of the action potential. This results in the suppression of the sensing and propagation of pain.

Chemokine receptors, by coupling to Gi/o protein signaling pathways, play a pivotal role in inflammation (15). They orchestrate cell trafficking, angiogenesis, and wound healing (16–20). Recently, chemokines and their receptors have been detected on cells of both the CNS and peripheral nervous system (21–23). The expression of chemokine receptors is not limited to microglial cells, and their function is indicative of neuronal-dependent effects. Neuronal cells from both the hippocampus and dorsal root ganglia respond to chemokines with a transient calcium flux, suggesting the expression of various chemokine receptors on neuronal cells (23). Disruption of CXCR4 causes many proliferating granule cells to invade the cerebellar anlage, indicating a critical role of this chemokine receptor during brain development (23, 24). Mice lacking CCR2 exhibit impairment in development of mechanical allodynia, suggesting a role of chemokine receptors in modulating the sensing of pain (25). We have reported that introduction of either CXCL12 or CCL5 (RANTES) into rat central periaquaductal gray matter impaired DAMGO-induced analgesic effects, suggesting that MORs could be inhibited by chemokines (26). These studies suggest that chemokines are involved in development of the nervous system as well as in the CNS sensory processes.

Desensitization of MORs provides a means of suppressing their analgesic effects. Persistent activation by opioids results in a gradual loss of receptor function through homologous desensitization, mainly by activating G protein-coupled receptor kinase and arrestin pathways (27). Heterologous desensitization is the other fundamental mechanism governing the function of G protein-coupled receptors. In a cell expressing multiple types of G protein-coupled receptors, activation of one type of receptor often results in suppression of other types through activation of protein kinase A or protein kinase C (PKC) (27). Phosphorylation of MORs by kinases results in decoupling of the receptors to G proteins, accompanied in some cases by internalization of receptors, resulting in the impairment of MOR-mediated analgesic effects. Previous studies have shown that chemokines are capable of inhibiting MOR activity in leukocytes (26). We hypothesized that, during inflammation, persistent activation of chemokine receptors expressed by peripheral sensory neurons can also desensitize the analgesic activity.

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of MOR, and thus directly contributes to inflammation-induced hyperalgesia.

**Materials and Methods**

** Constructs and cell lines **

pCCR1 and pMOR were constructed, as described by Zhang et al. (28). pCCR1 was linearized and electroporated into HEK293 cells. After G418 selection, a single positive colony was selected based on Western blotting analysis of CCR1 to be CCR1/HEK293 cells. CCR1/MOR/HEK293 cells were obtained by transiently transfecting CCR1/293 cells with pMOR. Briefly, CCR1/HEK293 cells were plated in 75-cm² culture flask at a 50% confluence. On the next day, cells were cultured in 10 ml of fresh medium for 1 h, and 0.5 μg of Eugenol 6 (Roche Diagnostic Systems, Somerville, NJ) was used to deliver 10 μg of pMOR to cells. Cells were allowed to incubate for another 48 h before experiments. Immunohistochemical staining showed that >85% of cells were MOR positive. Both CCR1/MOR/HEK293 and CCR1/HEK293 cell lines were cultured in DMEM supplied with 10% FCS, 400 μg/ml G418, and antibiotics (BioWhittaker, Walkersville, MD).

** Western blotting analysis of MOR and CCR1 **

Western blotting was conducted as described in Ref. 33 with minor modification. Polyvinylidene difluoride membrane was substituted for nitrocellulose; the transfer was performed at 150 mA at 4°C for 2 h; rabbit anti-CCR1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1000-fold, and rabbit anti-MOR Ab (Santa Cruz Biotechnology) was diluted 3000-fold; the secondary Ab was a HRP, anti-rabbit IgG conjugate (Sigma-Aldrich, St. Louis, MO). Band detection was achieved with the use of ECL.

** Assays for GPCR-mediated inhibition of cAMP accumulation **

Assays of GPCR-mediated inhibition of cAMP accumulation were conducted, as described by Zhang et al. and Amersham’s protocol (Piscataway, NJ) (29). Briefly, cells were starved for 3 h in serum-free medium before the experiments, harvested by trypsin treatment, and resuspended in 0.1% DE, and unlabeled DAMGO was used to determine nonspecific bindings. Cells were incubated with 10 μg/ml CCL3 for 30 min, then fixed, permeabilized, and stained by rabbit anti-CCR1 and anti-MOR antisera. The same areas of the two stained slices were compared to map the identical cells. After recording fluorescent images, the slides were counterstained with hematoxylin (Vector Laboratories, Burlingame, CA; H3401).

** Calcium flux analysis by microscope-based ratiometric imaging **

Microscope-based ratiometric analysis was performed, as described by Caterina et al. (31), with minor modifications. Primary neurons from rat DRG were cultured in eight-well chamber cover glass slides (Nunc) for 24–48 h. For desensitization experiments, CCL3, CCL2, CCL5, or CXCL8 was added while loading fura 2 for 45 min (Molecular Probes, Eugene, OR). Ratiometric calcium imaging was performed using a Nikon Eclipse TE2000 fluorescence microscope equipped with a variable filter wheel (Sutter Instruments, Novato, CA), a Spot charge-coupled device camera, and a Nikon S Fluor 40x objective lens (Nikon, Melville, NY). Dual images (340 and 380 nm excitation, 510 nm emission) were collected by Openlab System 3.14 (Improvision, Lexington, MA), and pseudocolor ratiometric images were monitored every 4 s. The ratio of emission340 nm vs emission380 nm was defined as 1 U of calcium flux. On the twentieth second, DAMGO was added to the chamber wells and the time-lapse recording continued for another 60 s.

** Results **

** Pretreatment with CCL3 impairs DAMGO-elicted inhibition of cAMP production in MOR:CCR1/HEK293 cells **

Using HEK293 cells doubly transfected with CCR1 and MOR, we assessed the possibility of heterologous desensitization of MOR by activated CCR1. Western blotting analysis showed two bands of 43 and 55 kDa, corresponding to CCR1 and MOR, respectively, expressed only in the extracts from doubly transfected cells, but not from control cells (Fig. 1A). DAMGO elicited a dose-dependent inhibition of forskolin-induced cAMP production, indicative of the functional coupling between MOR and G/o proteins (Fig. 1B). CCL3, a proinflammatory CCR1/5 ligand, also elicited a marked inhibition of cAMP production, suggesting that CCR1 was functional in doubly transfected cells (Fig. 1B). Pretreatment with CCL3, as well as CCL5, at 1 μg/ml for 30 min decreased the inhibitory effects of DAMGO, consistent with heterologous desensitization mediated by CCR1 (Fig. 1C). The heterologous desensitization effects of CCL3 were not detected on HEK293 cells transfected with MOR only (data not shown). Pretreatment with CCL3 also inhibited DAMGO-mediated chemotaxis of MOR:CCR1/HEK cells, providing additional evidence of heterologous desensitization (data not shown). In controls, DAMGO induced homologous desensitization in both MOR/HEK293 and CCR1: MOR/HEK293 cells, consistent with previous reports (data not shown; Fig. 1C). CXCL8, a ligand for CXCR1/2, did not interfere with MOR-mediated inhibition of adenylyl cyclase in doubly transfected cells (Fig. 1C).

Extensive reports have demonstrated that PKC plays a critical role in heterologous desensitization of Gi protein-coupled receptors (27). We assessed the role of PKC by using staurosporine, a potent inhibitor for classical and novel PKC isozymes. Treatment with staurosporine blocked heterologous desensitization of MOR function by CCL3 (Fig. 1D). Our data suggest that, when coexpressed on HEK293 cells, prolonged activation of CCR1 resulted in impairment of MOR function in a PKC-dependent manner.
Pretreatment with CCL3 induces a loss of surface MOR expression

We assessed the level of MOR expression on the cell surface by the binding of radioactive-labeled ligand, [3H]DAMGO. MOR-expressing cells, but not CCR1/HEK293, bound [3H]DAMGO (Fig. 2A) (data not shown). After 30 min of pretreatment with DAMGO, the cells lost 49 ± 11% of their DAMGO binding sites, indicative of homologous desensitization, and preincubation with CCL3 also resulted in a 78 ± 1% decrease in DAMGO binding sites. To examine the possibility that the decrease in DAMGO binding sites is due to receptor internalization, we directly visualized the expression of MOR using immunohistochemical staining. As shown in Fig. 2B, two representative cells on the left panels show MOR on the plasma membrane of MOR:CCR1/HEK293 cells. After 30 min of treatment with CCL3 at 37°C, the level of cell membrane MOR diminished, and, in 32% of cells, an enrichment of MOR staining was detected intracellularly, as shown by two representative cells on the right panels of Fig. 2B. Taken together, our data showed a marked decrease in MOR level on the cell surface, probably due to the CCL3-induced internalization of MOR.

Coexpression of functional MOR and CCR1 on rat dorsal root ganglia

We examined the expression of MOR and CCR1 in rat dorsal root ganglia using immunohistochemical staining of two adjacent tissue slices. As shown in Fig. 3A, two sets of representative tissue slices on the right panels, MOR is expressed on small to medium diameter neurons, consistent with previously published observations (32). CCR1 exhibited a similar expression pattern. MOR-positive cells also coexpressed medium to high levels of CCR1. To assess the function of these two receptors, we cultured acutely dissociated dorsal root ganglion neurons and examined the ligand-induced calcium flux. CCL3 and DAMGO induced a transient calcium flux in 21 ± 4 and 17 ± 3% neurons, respectively, indicating that both receptors were capable of activating G protein signaling pathways. The calcium flux returned to the basal level within 60 s, a typical characteristic of Gi protein-mediated transient response. These data suggest that rat dorsal root sensory neurons coexpress functional MOR and CCR1.

Pretreatment with CCL3 desensitizes MOR function on DRG

To assess the function of MOR on primary cells, we examined DAMGO-induced transient calcium flux on acutely dissociated rat DRG neurons. The numbers of primary neurons were too limited to be used for cAMP analysis. Microscope-based calcium imaging recorded the responses of multiple cells simultaneously, providing more data than single cell-based electrophysiological assays of potassium or calcium channels. A representative neuronal cell, upon stimulation by DAMGO, responded by 12 s with a transient calcium flux, indicating that the MOR was functional. Among the 50 neuronal cells examined, 8 responded to stimulation by DAMGO, consistent with the immunohistochemical staining of MOR (Fig. 4A). After CCL3 pretreatment, only 3 of 50 neurons were activated by DAMGO, and the responses were significantly lower than those of untreated cells, suggesting that MOR was heterologously desensitized. To determine whether the copy numbers of surface MOR were decreased after CCL3 treatment, we visualized MOR by using immunohistochemical staining. In freshly isolated primary neuronal cells, 17 ± 3% of neurons were positive for MOR.
staining and the fluorescent signals were strongly associated with the cell surface (Fig. 4B). After CCL3 pretreatment, only 4 ± 3% of neurons retained predominant staining for MOR on their cell surface, and 8 ± 4% of neurons showed an enrichment of MOR intracellularly (Fig. 4B). Taken together, our data showed that prolonged incubation with CCL3 impaired the function of MOR of sensory neurons, possibly due to a marked decrease in the copy numbers of cell surface MOR.

Chemokines desensitize MOR

We assessed the capacity of other chemokines to desensitize MOR. CCL2, CCL5, and CXCL8 were capable of eliciting transient calcium flux in a subpopulation of DRG neurons, consistent with previous reports (23) (data not shown). To assess the responses of MOR, 50 neurons were randomly picked in each experiment and positive responses were recorded. In the control experiments, DAMGO induced calcium flux in 15 ± 1% neurons (Fig. 5). Pretreatment with CCL2 impaired 62% of these responding neurons to various degrees (Fig. 5A). The lack of CCL2-induced inhibitory effects on the rest of the neurons may be due to the absence of CCR2 on those neurons or the fact that the activity of CCR2 was too low to desensitize MOR. Pretreatment with CCL5 or CXCL8, primary ligands for CCR5 and CXCR1/2, respectively, also impaired DAMGO-elicited calcium flux by DRG neurons. Not all chemokines exhibited strong inhibitory effects on MOR. For example, stromal-derived factor-1α/CXCL12, a ligand for CXCR4, didn’t induce a significant desensitization effect (data not shown). Taken together, our data suggest that MOR on DRG neurons can be desensitized by a number of proinflammatory chemokines.

Discussion

In previous studies, we have shown that prolonged activation of opioid receptors inhibits the function of chemokine receptors on leukocytes by a calcium-independent PKC pathway (28). In this study, our data from CCR1:MOR/HEK293 cells indicated that the
heterologous desensitization was bidirectional because activation of CCR1 resulted in internalization of µ-opioid receptors and a marked inhibition of MOR function. Furthermore, long-term exposure to proinflammatory chemokines compromises the function of MORs on sensory neurons of dorsal root ganglia. MORs are preferentially expressed on small to medium diameter neurons (32). Our data from immunohistochemical staining clearly showed that CCR1 and µ-opioid receptors were coexpressed on the same neuronal cells. Pretreatment with CCL3 reduced the numbers of DAMGO-responsive neurons and impaired the magnitude of DAMGO-elicited calcium flux. Other proinflammatory chemokines, such as CCL2, CCL5, and CXCL8, exhibited similar inhibitory effects. Although we did not directly monitor analgesia-related GIRK and calcium channel activity, the impairment in DAMGO-induced calcium flux reflected suppression of MOR function. Moreover, internalization of MORs is another clear indication of the CCR1-induced desensitization. Unless analgesia activities require extremely low levels of MOR activation, long-term exposure to chemokines is likely to be sufficient to suppress opioid-induced antipain responses. Thus, activation of chemokine receptors on neurons desensitizes MORs, which may block opioid-induced analgesic effects.

The chemokines tested in our study varied in their desensitization effects on MORs. One possible explanation is that the expression level and pattern of each type of chemokine receptor on sensory neurons differ. For example, RT-PCR data show that the expression level of CCR1 is higher than that of CCR5 (our unpublished results). We also detected a greater number of neuronal cells responding to CCL3 than to CCL2, suggesting that CCR1/5 is expressed at a higher level on these neurons than CCR2 (data not shown). These data are consistent with a previous report that different chemokines showed a range of potency in inducing calcium flux in dorsal root ganglia neurons (23). The ability of different chemokine receptors to inhibit MORs may also result from the intrinsic potency of each receptor in stimulating downstream signaling events (27, 28). Indeed, in the immune system, a hierarchy of heterologous desensitization has been well documented (27). For example, studies using CCR1-MOR/HEK293 cells showed that CCR1 was a potent inhibitor of MOR function, while conversely, the inhibitory effects of MOR on CCR1 were moderate (28). Extensive reports have suggested that the hierarchy of heterologous desensitization may stem from the intrinsic capacity of each receptor to activate phospholipase C and PKC (27, 28).

Previous studies have shown that injection of RANTES into the periaqueductal grey region of a rat blocks DAMGO-elicited analgesic effects, suggesting cross talk between chemokine and µ-opioid receptors (26). In this study, we clearly showed the coexpression of CCR1 and MOR on peripheral sensory neurons. Thus, coexpression of CCR1 and/or 5 along with MOR on CNS neurons was presumably responsible for the hyperalgesia effects of RANTES.
The phenomenon of inflammation-induced hyperalgesia has been recognized for thousands of years. However, only recently has an understanding begun to emerge of the molecular basis for heightened sensitivity to painful stimuli following inflammation. A repertoire of cellular mediators (e.g., bradykinin, PG, and nerve growth factor) has been shown to enhance the sensitivity of nociceptive (i.e., pain-sensing) neurons to noxious stimuli. They appear to do so, in part, by augmenting the responsiveness of excitatory ion channels, such as voltage-gated sodium channels and the capsaicin- and heat-gated channel, transient receptor potential vanilloid 1, to their respective stimuli. Recent studies have suggested that chemokines, pivotal mediators of innate and adaptive immunity, also participate in inflammation-induced hyperalgesia. For example, Oh et al. (23) proposed that upon binding to their receptors on nociceptors, chemokines stimulate these neurons directly. Along similar lines, our recent studies indicate that chemokines can sensitize transient receptor potential vanilloid 1 on nociceptive neurons (our unpublished data). Abbadie et al. (25) suggested that the chemokine-mediated recruitment and activation of macrophages and microglia in skin and nerve tissue might contribute to both inflammatory and neuropathic pain states. The data from this study reveal yet another facet of the function of chemokine receptors: they can inhibit analgesic effects by heterologously desensitizing coexpressed opioid receptors, and as a result promote pain signals centrally as well as in the peripheral nervous system.

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**References**