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Rapid Communication

Interleukin-1β-Mediated Regulation of μ-Opioid Receptor mRNA in Primary Astrocyte-Enriched Cultures

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Abstract: Opioids have been found to modulate the immune system by regulating the function of immunocompetent cells. Several studies suggest that the interaction between immune and opioid systems is not unidirectional, but rather reciprocal, in nature. In the CNS, one cellular target of immune system activation is the astrocytes. These glial cells have been shown to produce the opioid peptide, proenkephalin, to express the μ-, δ-, and κ-opioid receptors, and to respond to the immune factor interleukin-1β (IL1β) with an increased proenkephalin synthesis. To characterize more completely the astrocytic opioid response to immune factor stimulation, we examined the effect of IL1β (1 ng/ml) on the μ-receptor mRNA expression in primary astrocyte-enriched cultures derived from rat (postnatal day 1–2) cortex, striatum, cerebellum, hippocampus, and hypothalamus. A 24-h treatment with IL1β produced a 70–80% increase in the μ-receptor mRNA expression in the striatal, cerebellar, and hippocampal cultures but had no effect on this expression in the cortical and hypothalamic cultures. This observation represents one of the few demonstrated increases in levels of the μ-receptor mRNA in vitro or in vivo, since the cloning of the receptor. The enhanced μ-receptor mRNA expression, together with the previous observation that IL1β stimulates proenkephalin synthesis in astrocytes, supports the IL1β-mediated regulation of an astroglial opioid peptide and receptor in vitro, a phenomenon that may be significant in the modulation of the gliotic response to neuronal damage. Therefore, the astroglial opioid "system" may be important in the IL1β-initiated, coordinated response to CNS infection, trauma, or injury. **Key Words:** Astrocytes—Interleukin-1β—μ-Opioid receptor—Immune system activation—Immune–opioid system interactions.
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Opiates, such as morphine and heroin, as well as endogenous opioid peptides, such as endorphins, enkephalins, and dynorphins, exert their biological effects via the activation of specific opioid receptors, classified as μ, δ, and κ (Goldstein, 1987). Opioids have been found to modulate the immune system by regulating the growth, proliferation, and activity of various immunocompetent cells (Sibinga and Goldstein, 1988). However, studies suggest that the interaction between the opioid and immune systems is not unidirectional, but rather reciprocal, in nature (Blalock, 1989). In the CNS, one cellular target of immune system activation is the astrocytes, glial cells that are believed to play a critical role in the response to CNS infection and to neuronal trauma or injury.

Astrocytes have been shown to synthesize proenkephalin (Vilijn et al., 1988; Shinoda et al., 1989) and to possess opioid binding sites (Hendrickson and Lin, 1980; Lightman et al., 1983). Two separate investigations of the regulation of astroglial enkephalin by interleukin-1β (IL1β) have demonstrated that this immune factor enhanced the proenkephalin mRNA level in a time-, concentration-, and region-dependent fashion (Low et al., 1992; Negro et al., 1992), suggesting that the IL1β-mediated elevation in proenkephalin gene expression may be important in CNS infection or injury. More recently, cultured astrocytes have also been found to express the mRNA for all three opioid receptors in a manner dependent on the receptor type and the brain region from which the culture was derived (Ruzicka et al., 1995). However, no evidence exists regarding the regulation of astrocytic opioid receptors by immune factors. The objective of the present study was to characterize more completely the nature of the astrocytic opioid response to immune factor exposure, by examining the effects of IL1β on the μ-receptor mRNA expression in several different astrocyte-enriched cultures.

EXPERIMENTAL PROCEDURES

Cell culture

Astroglial cells were isolated and cultured as described previously (Ruzicka et al., 1995). In brief, mixed neuronal–glial cell suspensions were generated from the cortex, striatum, cerebellum, hippocampus, and hypothalamus of 1–2-day-old rat pups. A fixed cell number (5×10^5 per plate) was seeded onto each uncoated 10-cm-diameter plastic culture dish. The growth medium (Eagle's minimal essential medium with D-valine and Earle's salts plus 10% fetal calf serum and 3.2 g of glucose/500 ml) was completely replaced with ice-cold medium the next day (day 1 in vitro). Thereafter, half the growth medium was replaced every 3 days. Cells reached confluence by day 8–10 in vitro and were maintained for a total of 11 days in culture.

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Abbreviations used: GFAP, glial fibrillary acidic protein; IL1β, interleukin-1β; NSE, neuron-specific enolase.

On day 10 in vitro, the growth medium was replaced by medium supplemented with glucose but lacking fetal calf serum and containing IL1β (human recombinant IL1β, 1 ng/ml; Bachem, Torrance, CA, U.S.A.) or vehicle (10 m*M* acetic acid). The 1 ng/ml dose of IL1β was previously shown to elicit a maximal up-regulation of astroglial proenkephalin mRNA (Negro et al., 1992). Cells were incubated with IL1β or under control conditions for 24 h and were then lysed directly on the culture dishes with Trizol reagent (GibcoBRL, Grand Island, NY, U.S.A.) to extract total RNA, as described previously (Ruzicka et al., 1995).

Immunohistochemistry for glial fibrillary acidic protein (GFAP) and neuron-specific enolase (NSE)

Immunohistochemical analysis was performed as detailed previously (Ruzicka et al., 1995). In brief, cells grown on glass coverslips were fixed within their culture wells with Zamboni's fixative. Primary antibody incubations were conducted with rabbit anti-GFAP and anti-NSE, whereas secondary antibody incubations were carried out with biotinylated goat anti-rabbit IgG. After adequate washing, the cells were incubated with avidin–biotin complex (Vector Elite kit; 1:50). The staining reaction, conducted in 0.1 *M* sodium acetate with diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, U.S.A.), 2.5% $NiSO_4$ (Sigma), and 0.01% H_2O_2, was terminated by adding excess potassium phosphate-buffered saline. Coverslips were sequentially dehydrated through graded alcohols, xylene-cleared, and then mounted onto slides for viewing under a light microscope. Under the cell culture conditions described, no neurons were detected following NSE immunohistochemistry, whereas >95% of the cells were immunohistochemically positive for GFAP, an astrocyte-specific marker (Fig. 1). In this regard, the remaining cells presumably represented other glial cell types, namely, oligodendrocytes and microglia.

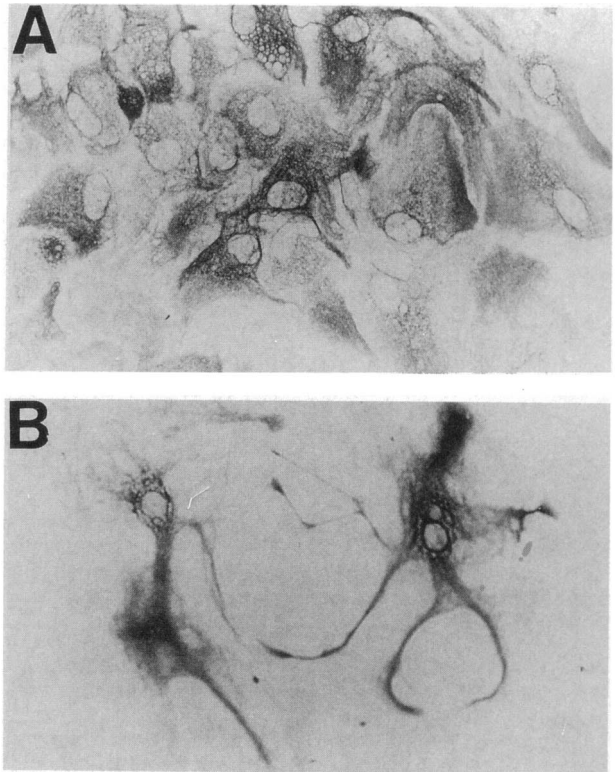

FIG. 1. GFAP immunohistochemical staining of a cortical glial cell culture. After 11 days in culture, the GFAP-positive cells exhibited either a flat, polygonal morphology (**A**) or a stellar, process-bearing morphology (**B**). Such a phenotype was typical of all cultures used in the study, regardless of the brain region from which they were derived or the presence or absence of IL1β.

Solution hybridization/RNase protection assays

Solution hybridization/RNase protection assays for μ-opioid receptor mRNA were carried out as reported previously (Ruzicka et al., 1995). In brief, hybridizations were conducted with a [^{32}P]UTP-labeled cRNA probe generated from a 432-bp PCR fragment designated 5A1. This fragment was cloned into a modified *Eco*RV site of PT7 Blue (Novagen, Madison, WI, U.S.A.). Linearization with *Eco*RI and complete transcription of the linearized insert yielded a cRNA of 514 bp in length. The hybridization reaction was performed at 60°C for an overnight period and was then terminated by addition of RNase A (40 μg/ml at room temperature for 1 h). Following proteinase K treatment and organic extraction, the RNA was precipitated by addition of ethanol.

The obtained cRNA:mRNA fragments were heat-denatured and fractionated on a 4% polyacrylamide denaturing (7 *M* urea) gel in 1× TBE (89 m*M* Tris, 89 m*M* boric acid, and 2 m*M* EDTA). Following electrophoresis, the gel was dried under vacuum and then exposed to x-ray film (Kodak XAR) with two enhancer screens for a period ranging from 24 h to 7 days.

Dot-blot hybridizations

To provide an index of total RNA quantity in each sample, 10% of each RNA sample analyzed by RNase protection assay was concomitantly analyzed by dot-blot hybridization as previously described (Ruzicka et al., 1995). In brief, the RNA samples were heat-denatured and then directly applied, under vacuum, to a Nytran membrane (Schleicher and Schuell, Keene, NH, U.S.A.) contained within a hybridization manifold. The RNA was fixed to the membrane by UV cross-linking using a Stratagene UV cross-linker.

Following prehybridization of the membrane, a [^{32}P]-UTP-labeled cRNA probe for 1B15 (cyclophilin; a ubiquitous "housekeeping" gene product) was added to a final concentration of 1×10^6 cpm/ml. Following an overnight hybridization at 60°C, the membrane was washed at 70°C in 0.1× saline–sodium citrate and 0.1% sodium dodecyl sulfate and then exposed to x-ray film (Kodak XAR) at room temperature for 5–24 h. Previous studies using northern blot analysis of astroglial RNA revealed that the 1B15 cRNA probe hybridized to a single RNA species of the expected and apparent molecular size of 1.4 kb (data not shown).

Data expression and analysis

Autoradiogram signals generated from [^{32}P]cRNA:mRNA hybrids during the solution and dot-blot hybridizations were analyzed for the gray level intensity using computerized image analysis (NIH Image). Regarding the solution hybridization, only those signals generated from the appropriately sized protected mRNA fragments were analyzed. The gray value determined for the signal generated by each protected fragment was corrected for autoradiogram exposure time and standardized with respect to the level of 1B15 expression. Differences between control and IL1β treatment

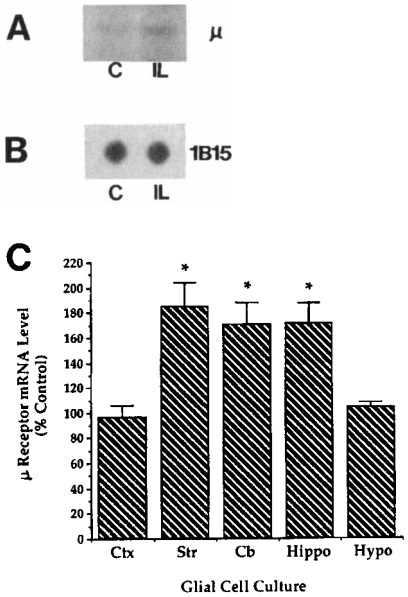

FIG. 2. Effects of IL1β (1 ng/ml) on μ-receptor mRNA expression in primary astrocyte-enriched cultures. Representative autoradiograms were generated from striatal cultures following an RNase protection assay for μ-receptor mRNA (**A**), and a dot-blot analysis for 1B15 mRNA (**B**). Exposures shown are for illustrative purposes only and are not necessarily those used for quantitative analysis. **C:** The standardized data for all five astrocyte-enriched cultures, expressed as percentages of the control value. Data are mean ± SEM (bars) values of five to 10 observations obtained in four separate studies. C, control; IL1β, IL1β treatment for 24 h; Ctx, cortex; Str, striatum; Cb, cerebellum; Hippo, hippocampus; Hypo, hypothalamus. $^*p < 0.05$ compared with control.

values were compared using a paired Student's t test and were considered significant if $p < 0.05$. Results obtained from four separate studies have been expressed as percentages (mean ± SE) of control values.

RESULTS AND DISCUSSION

A 24-h exposure of the astrocyte-enriched cultures to IL1β produced a significant elevation in the μ-receptor mRNA level in those cultures derived from the striatum, cerebellum, and hippocampus (Fig. 2). The enhanced μ-receptor mRNA expression in these cultures, which represented an ~70–80% increase beyond control levels, was observed only after a 24-h incubation with IL1β and not after a 4-h incubation with the cytokine (data not shown). In contrast, a 24-h treatment with IL1β did not produce any apparent change in the μ-receptor mRNA level in astrocyte-enriched cultures derived from either the cortex or the hypothalamus (Fig. 2), suggesting a brain region-dependent action of the cytokine.

The enhanced μ-receptor mRNA expression by IL1β represents one of the few demonstrated increases in this RNA species since the cloning of the μ-receptor (Chen et al., 1993; Thompson et al., 1993; Wang et al., 1993). In addition, it is, to our knowledge, the first demonstration that opioid receptor mRNA can be modulated by this cytokine.

The region-dependent nature of the IL1β effect raises the question of whether this specificity results from differential IL1β receptor expression in these cultures. Although IL1β receptors have been localized to astrocytes (Ban et al., 1993) and IL1β receptors have been found to be heterogeneously distributed among brain regions (Yabuuchi et al., 1994), their relative abundance in these brain regions may not account for the observed IL1β action. Indeed, significant levels of the IL1β receptor have been demonstrated in the rodent cortex (Takao et al., 1990), a brain region generating astrocyte-enriched cultures that were unresponsive to the IL1β treatment. In contrast, relatively low levels of the IL1β receptor have been observed in the cerebellum (Cunningham et al., 1992), a brain region generating astrocyte-enriched cultures that responded to the IL1β exposure with an almost doubling in their μ-receptor mRNA levels. It is unclear whether the IL1β receptor distributions described in these studies represent predominantly neuronal, glial, or both types of localization, but it is interesting that the region-dependent nature of the IL1β-elicited elevation in μ-receptor mRNA level may not be associated with the relative abundance of the IL1β receptor in these cultures. This issue awaits further study.

The IL1β-mediated elevation in μ-receptor mRNA expression apparently also is not associated with the basal μ-receptor mRNA levels in these cultures. We have previously shown that cortical astrocyte-enriched cultures contain approximately twice the level of μ-receptor mRNA as each of the striatal, cerebellar, hippocampal, and hypothalamic cultures (Ruzicka et al., 1995). Although the latter four cultures produce comparable levels of μ-receptor mRNA, only those derived from the striatum, cerebellum, and hippocampus responded to the IL1β with an increased μ-receptor mRNA expression. Thus, the basal level of μ-receptor mRNA expression did not appear to influence the capacity of the astrocytes to respond to the cytokine.

The mechanism of the IL1β action on the μ-receptor mRNA expression is currently not known and is complicated by the fact that the cytokine can activate both astrocytes and microglia, is produced by both these glial cell types, and may be modulated in vivo by the presence of soluble interleukin-1 receptors and an interleukin-1 receptor antagonist (Dinarello, 1991). The astrocyte-enriched cultures in this study may contain a small proportion of microglia. Thus, the possibility that the mechanism of IL1β action may involve, at least in part, the activation of microglia and/or the activity of endogenously-derived IL1β cannot be dismissed. Similarly, the presence of other endogenous factors such as soluble interleukin-1 receptor or receptor antagonist, and their potential IL1β-modulating effects, should also be considered.

Biochemically, cyclic AMP, prostaglandin E_2, and Ca^{2+} have all been implicated as intracellular signaling molecules in the actions of IL1β (Dinarello, 1991; Rothwell, 1991). In addition, IL1β has been shown to induce activity of the nuclear factor AP-1 via increases in the expression of c-*jun* mRNA (Muegge et al., 1989). It is interesting that the μ-receptor gene promoter has also recently been characterized, by sequence analysis, to contain a consensus sequence for the cytokine response element NF-IL6 (nuclear factor-interleukin 6) (Min et al., 1994). Therefore, the possibility exists that IL1β may mediate its effects on μ-receptor mRNA expression either indirectly through an intracellular signaling cascade involving immediate-early gene expression (c-*fos* and c-*jun*) or directly through its own binding to a stimulatory cytokine response element on the μ-receptor gene. Alternatively, IL1β may up-regulate μ-receptor mRNA expres-

sion via an increased stability, i.e., half-life, of the RNA transcript.

The physiological significance of the IL1β-mediated elevation in astroglial μ-receptor mRNA expression is not clear and is confounded by the fact that the cytokine effects on the μ-receptor protein levels are currently unknown. The effects of IL1β on the remainder of the μ-receptor biosynthetic pathway await further study. However, it is interesting to note that both IL1β and opioids have been implicated in the modulation of cell proliferation in the CNS. CNS infection, trauma, or injury has been shown to enhance IL1β synthesis and secretion in immunocompetent cells, neurons, and astrocytes (Dinarello, 1991; Rothwell, 1991). The secreted IL1β has numerous effects, including the stimulation of astrocyte proliferation (gliosis) and activity (IL1β and nerve growth factor synthesis) (Dinarello, 1991; Rothwell, 1991), components of the neuroprotective response. IL1β has also been shown to enhance astroglial proenkephalin synthesis (Low et al., 1992; Negro et al., 1992). Enkephalins, in turn, have been demonstrated to exert antimitogenic effects on both neurons and astroglial cells (Stiene-Martin and Hauser, 1991; Zagon and McLaughlin, 1991). It is therefore intriguing to speculate that the concomitant increase in astroglial proenkephalin and μ-receptor mRNA expression by IL1β may result in the autoregulation of the gliotic response via the enkephalinergic activation of the μ-receptors. Such a modulation of the astroglial opioid "system" (both peptide and receptor) by IL1β would permit this cytokine to regulate finely the intensity of its own actions on astrocytes, particularly with respect to cell proliferation. A regulation of the gliotic response would minimize possible neuropathological events resulting from excessive immune system activation or chronic inflammation. Therefore, the astroglial opioid "system" may be important in the IL1β-initiated, coordinated response to CNS infection, trauma, or injury.

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