Biochemical Pharmacology, Vol. 56, pp. 397–404, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00161-0

κ-Opioid Potentiation of Tumor Necrosis Factor-α-Induced Anti-HIV-1 Activity in Acutely Infected Human Brain Cell Cultures

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ABSTRACT. Opioids have been postulated to play an immunomodulatory role in the pathogenesis of HIV-1. Synthetic κ-opioid receptor (KOR) ligands have been found to inhibit HIV-1 expression in acutely infected microglial cell cultures. We recently found that interleukin(IL)-1β and tumor necrosis factor(TNF)-α have antiviral effects in acutely infected mixed glial/neuronal cell cultures. In the present study, we investigated whether selective KOR ligands would exert antiviral effects in acutely infected brain cell cultures. While the KOR ligand *trans*-3,4-dichloro-*N*-methyl-*N*[2-(1-pyrolidinyl)cyclohexyl]benzeneaceamide methanesulfonate (U50,488) alone had little anti-HIV-1 activity, this opioid potentiated in a concentration-dependent manner the antiviral activity of TNF-α, but not of IL-1β. The potentiating effect of U50,488 was detected after a 6-hr pretreatment and peaked at 24 hr. The KOR antagonist nor-binaltorphimine completely blocked the potentiating effect of U50,488, suggesting the involvement of a KOR-mediated mechanism. Antibodies to TNF-α completely blocked the potentiating effect of U50,488, suggesting a critical role for TNF-α. Antibodies to IL-1β blocked the potentiating effect of U50,488, suggesting that IL-1β was released following U50,488 treatment, which might contribute to the potentiating effect of U50,488. These *in vitro* findings support the notion that synthetic κ-opioids could be considered as potential adjunctive therapeutic agents in HIV-1-related brain disease. BIOCHEM PHARMACOL **56**;3:397–404, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. AIDS; HIV; cytokines; brain cell cultures; neuroimmunology; opioids

Abundant studies have demonstrated that endogenous opioid peptides modulate the function of the endocrine, cardiovascular, respiratory, gastrointestinal, and nervous systems [1, 2] via seven-transmembrane G-protein-coupled opioid receptors [3, 4]. There is also considerable evidence that opioids affect the immune system [5, 6]. KOR† ligands, for example, have been shown to alter bone-marrow cell proliferation [7], macrophage phagocytosis [8] or tumoricidal activity [9], and to modulate the inflammatory response in animal models of arthritis [10] and multiple sclerosis [11]. KOR ligands also have direct effects within the CNS on the release of serotonin from the hippocampus [12] and presynaptic release of glutamate [13] and are able to protect against certain forms of injury due to cerebral ischemia [14–18].

In HIV-1 infection of the CNS, microglia are the only cell type permissively infected in patients with AIDS encephalopathy [19, 20]. We recently developed an *in vitro* model of HIV-1 brain infection, using human fetal brain cell cultures comprised of glial and neuronal cells, that resembles the composition of cells within the cerebral cortex. The proinflammatory cytokines TNF-α and IL-1β have been found to profoundly suppress HIV-1 expression in this mixed glial/neuronal brain cell culture model [21].

We have shown recently that the synthetic KOR ligands exert an antiviral effect in acutely infected cultures of highly enriched microglia, the resident macrophages of the brain [22]. We previously found that dynorphin stimulates the release of TNF-α in brain cell cultures [23]. In the present study, we tested the hypothesis that synthetic KOR ligands would also have an antiviral effect in this brain cell culture model of acute HIV-1 infection. Surprisingly, the synthetic κ-ligand *trans*-3,4-dichloro-*N*-methyl-*N*[2-(1-pyrolidinyl)cyclohexyl]benzeneaceamide methanesulfonate (U50,488), which markedly suppresses HIV-1 expression in acutely infected highly enriched microglial cell cultures [22], only slightly attenuated viral expression in acutely infected brain cell cultures. In the presence of small amounts of TNF-α, however, U50,488 markedly potentiated TNF-α-induced antiviral activity.

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† *Abbreviations:* Ag, antigen; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IL, interleukin; KOR, κ-opioid receptor; MOR, μ-opioid receptor; nor-BNI, nor-binaltorplumine; and TNF, tumor necrosis factor.

Received 16 February 1998; accepted 23 April 1998.

MATERIALS AND METHODS

Reagents

The monocytotropic HIV-1SF_{162} strain was provided by the National Institutes of Health AIDS Research and Reference Reagent Program (National Institute of Allergy and Infectious Diseases). The selective KOR ligands U50,488 and (5α,7α,8β)-(+)-*N*-methyl-*N*-(7-[1-pyrrolidinyl]-1-oxaspiro[4,5]dec-8-yl)-benzeneacetamide (U69,593) were gifts of the Upjohn Co. Dynorphin $A_{1\text{-}17}$ was purchased from Peninsula Laboratories, Inc. The κ-selective antagonist nor-BNI was provided by P. S. Portoghese (University of Minnesota). Morphine was obtained from the Pharmacy Department in our institution. Other reagents were purchased from the indicated sources: TNF-α and IL-1β (R&D Systems Inc.); antibodies to microglial cell CD68 antigen and astrocyte glial fibrillary acid protein (Dako); anti-neuron specific enolase antibodies (Polysciences); FBS (HyClone Laboratories); DMEM, uridine, fluorodeoxyuridine, penicillin (100 units/mL), streptomycin, and all other culture reagents (Sigma Chemical Co.).

Brain Cell Cultures

Primary human fetal mixed glial/neuronal brain cell cultures were prepared using a recently developed technique [23] under a protocol approved by our institutional Human Subjects Research Committee. Briefly, brain cortices from 14- to 18-week-old abortuses were treated with 0.25% trypsin for 30 min. After extensive washing, dispersed brain cells were plated onto collagen-coated, 24-well plates at a concentration of 5×10^5 cells/500 μL and cultured in DMEM containing 10% FBS at 37° in a humidified 10% CO_2 atmosphere. On day 5, the culture medium was replaced with DMEM containing 10% FBS, uridine (33.6 μg/mL), and fluorodeoxyuridine (13.6 μg/mL) to prevent the overgrowth of astroglial cells. Culture medium with 10% FBS was replaced on day 6 and every 4 days thereafter. On day 12, the neuronal cell cultures contained differentiated neurons growing on a supporting layer of astrocytes. The brain cell cultures consisted of about 50% neurons, 45% astrocytes, and 2–3% microglia, as determined by staining with specific antibodies to these cell types. These 12-day brain cell cultures were used in all experiments.

HIV-1 p24 Ag Assay

HIV-1 p24 Ag levels were measured using an enzyme-linked immunosorbent assay (Abbott Laboratories), as previously described [21]. A standard curve derived from known amounts of p24 Ag was used to quantify the Ag levels in culture supernatants. The sensitivity of this assay is 30 pg/mL.

Statistical Analysis

Where appropriate, data are expressed as means ± SEM. To compare the means of two groups, Student's *t*-test was used. For means from multiple groups, ANOVA was performed followed by Scheffe's F-test.

RESULTS

Effects of Opioids on TNF-α Antiviral Activity

Using acutely infected brain cell cultures, we found in three separate experiments that treatment with U50,488 alone at concentrations between 10 fM and 1 μM resulted in only slight inhibition of p24 Ag production, with a maximal inhibition of 17 ± 1% at a concentration of 1 nM (data not shown). When these brain cell cultures were incubated with 200 pg/mL of TNF-α (which by itself suppressed about 20% p24 Ag production), U50,488 markedly potentiated the anti-HIV-1 activity of this cytokine (Fig. 1). The potentiating effect of U50,488 was concentration-dependent with a maximal inhibition of 77% detected at a concentration of 1 nM. In four separate experiments, we found that treatment of acutely infected brain cell cultures with 1 nM U50,488 in the presence of 200 pg/mL of TNF-α induced a potent inhibition ($P < 0.01$) of p24 Ag production (mean ± SEM: 56 ± 3% inhibition) as observed at 7 days postinfection. Similar findings were detected at a later time point (i.e. 14 days postinfection) in four separate experiments (i.e. 61% inhibition), suggesting a long-lasting effect of this opioid.

To investigate the influence of pre-exposure of brain cell cultures to U50,488 on TNF-α anti-HIV-1 activity, cultures were pretreated with 1 nM U50,488 for the indicated periods prior to infection and TNF-α treatment. We found that pretreatment of brain cell cultures for 24 hr with U50,488 resulted in a maximal potentiating effect (Fig. 2). A 6-hr pretreatment also appeared to be effective but pretreatment for 3 hr was not sufficient for U50,488 to exert its potentiating effect. Again, pretreatment of mixed brain cell cultures with U50,488 in the absence of TNF-α had only a minimal effect on HIV-1 expression (Fig. 2).

Next, we investigated the effects of other selective KOR ligands, i.e. the synthetic U69,593 and the endogenous κ-opioid $dynorphin_{1\text{-}17}$, as well as the selective MOR ligand morphine. Treatment of brain cell cultures with the KOR selective ligand U69,593 and $dynorphin_{1\text{-}17}$, each alone at concentrations between 1 fM and 1 μM, did not alter HIV-1 expression significantly (data not shown). U69,593 and dynorphin, however, both enhanced TNF-α-mediated antiviral effects at concentrations of 1 nM for U69,593 and 100 pM for dynorphin (p24 Ag levels in the control group: 1135 ± 71 pg/mL; TNF-α group: 877 ± 15 pg/mL; U69,593 + TNF-α group: 700 ± 25 pg/mL [15% potentiation]; and dynorphin + TNF-α group: 684 ± 3 pg/mL [17% potentiation]). Treatment of acutely infected brain cell cultures with morphine, at concentrations between 10 fM and 100 μM, alone or in the presence of 200 pg/mL or 2 ng/mL of TNF-α did not alter p24 Ag production (data not shown).

To evaluate receptor specificity, the selective KOR antagonist nor-BNI was used. Pretreatment of brain cell

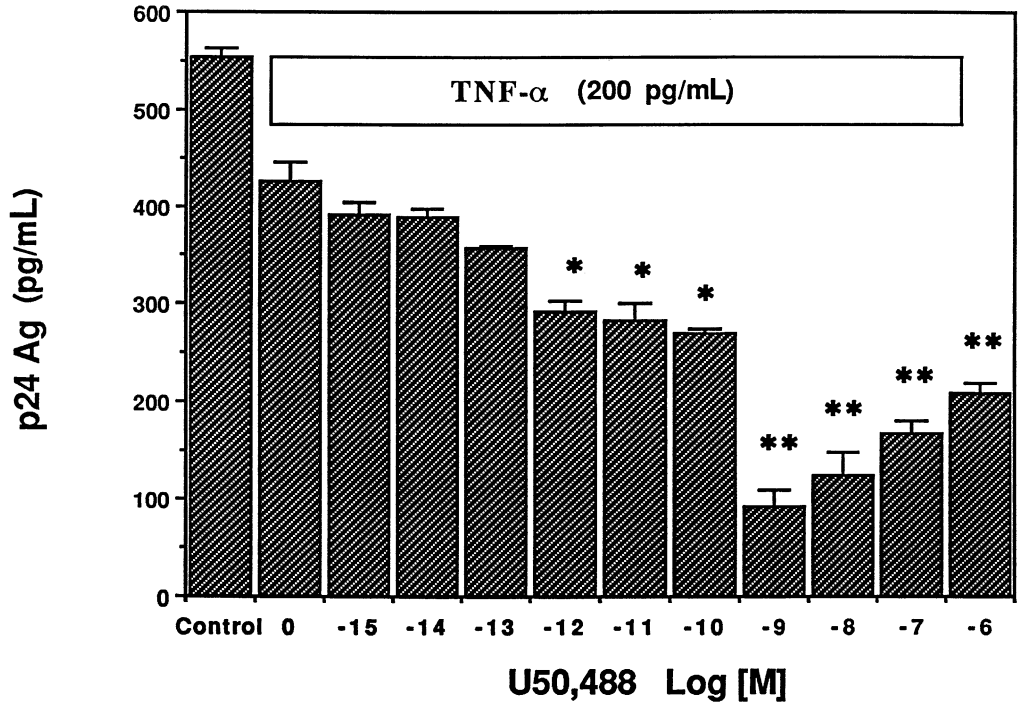

FIG. 1. Effects of U50,488 on TNF-α-induced inhibition of HIV-1 expression. Human brain cell cultures were incubated with medium (control) or medium containing the indicated concentrations of U50,488 for 24 hr prior to the addition of 200 pg/mL of TNF-α and HIV-1 and incubation for another 24 hr. After washing and replacing culture medium with U50,488 and TNF-α, supernatants were harvested on day 7 postinfection and assayed for p24 Ag. Data (mean ± SEM) are representative of four separate experiments. (*) $P < 0.05$ and (**) $P < 0.01$ vs non-U50,488-treated group.

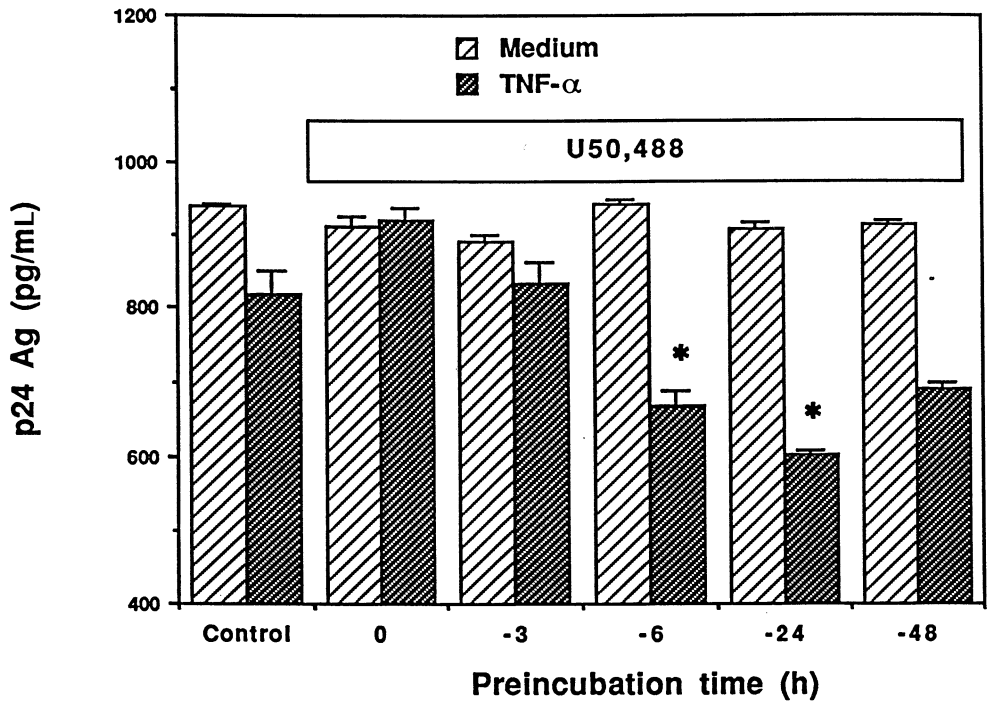

FIG. 2. Effect of pretreatment duration on the potentiating effect of U50,488. Brain cell cultures were incubated with medium (control) or medium containing 1 nM U50,488 for the indicated periods prior to the addition of medium or 200 pg/mL of TNF-α and infection with HIV-1. After washing and replacing culture medium with U50,488 and TNF-α, supernatants were harvested on day 7 postinfection and assayed for p24 Ag. Data (means ± SEM of triplicates) are representative of two separate experiments. (*) $P < 0.05$ vs control-TNF-α group.

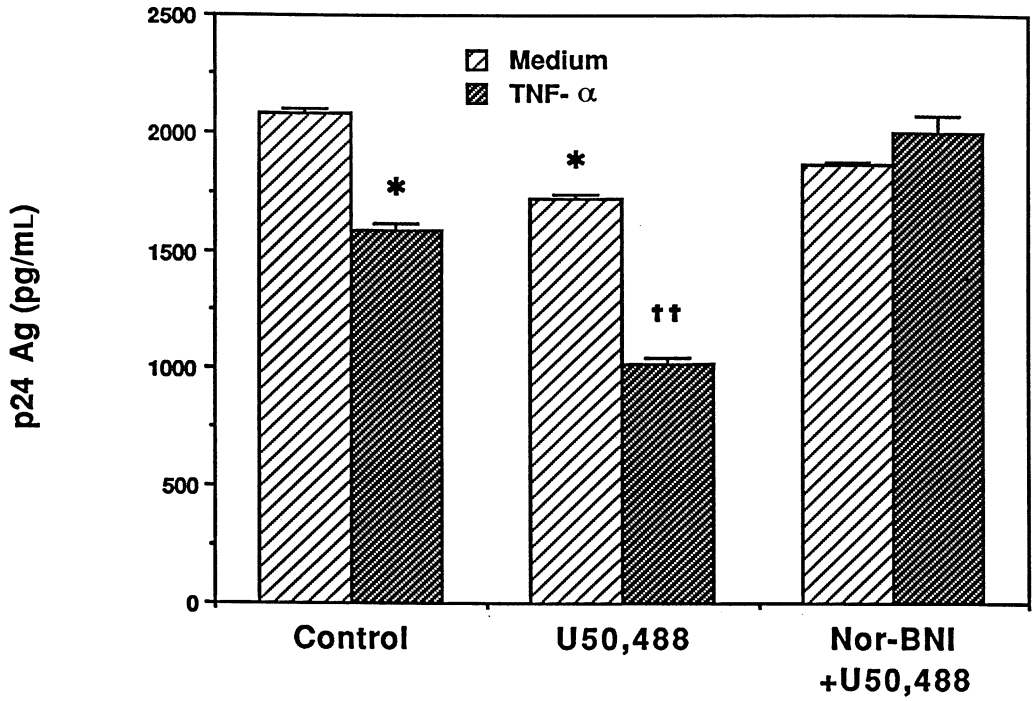

FIG. 3. Effect of nor-BNI on the potentiating effect of U50,488 on TNF-α-induced inhibition of HIV-1 expression. Brain cell cultures were incubated with medium (control) or medium containing 1 nM nor-BNI for 30 min prior to the addition of 1 nM U50,488 for 24 hr, followed by the addition of 200 pg/mL of TNF-α and infection with HIV-1. After washing and replacing culture medium with nor-BNI, U50,488, and TNF-α, supernatants were harvested on day 7 post-infection and assayed for p24 Ag. Data (means ± SEM) are representative of three separate experiments. (*) $P < 0.05$ vs medium-control and (††) vs TNF-α-control.

cultures for 30 min with an equal concentration (1 nM) of nor-BNI totally reversed U50,488-induced antiviral effects (Fig. 3). By itself, nor-BNI slightly blocked p24 Ag production by approximately 10% (Fig. 3). These findings suggest that activation of KOR is involved in U50,488-mediated potentiation of the antiviral effect of TNF-α in human brain cell cultures.

Involvement of Cytokines in the Potentiating Effects of U50,488

To further characterize the relationship between the antiviral effects of U50,488 and TNF-α, a concentration–response study of TNF-α in the absence or presence of 1 nM U50,488 was performed. We found that U50,488 markedly enhanced TNF-α-mediated inhibition of HIV-1 expression at a wide range of TNF-α concentrations, i.e. between 8 pg/mL and 1 ng/mL (Fig. 4). At concentrations of 5 ng/mL, TNF-α by itself inhibited >80% p24 Ag production, and U50,488 did not further potentiate this potent antiviral effect.

To investigate the potential role of TNF-α in the potentiating effects of U50,488, antibodies specific to TNF-α were used. We found that pretreatment of brain cell cultures with anti-TNF-α antibody (2 μg/mL) by itself for 30 min did not alter p24 Ag production. However, this pretreatment, prior to addition of TNF-α (200 pg/mL), completely abrogated the inhibitory effect of TNF-α by itself as well as the potentiating antiviral effect of U50,488 (Fig. 5). This finding indicates that the potentiating effect of U50,488 is dependent upon TNF-α.

Because IL-1β also has antiviral activity in acutely infected human brain cell cultures [21], we evaluated whether the antiviral effects of U50,488 would also be observed in the presence of this proinflammatory cytokine. Treatment of acutely infected brain cells with 200 pg/mL of IL-1β resulted in 45 ± 1% inhibition of p24 Ag production (N = 3). Pretreatment of brain cell cultures with U50,488 for 24 hr did not affect IL-1β-induced inhibition of HIV-1 expression (Fig. 6), suggesting that the potentiating effect of U50,488 is not related to IL-1 receptors. Pretreatment of brain cell cultures with morphine for 24 hr also did not alter IL-1β-induced suppression of HIV-1 expression (Fig. 6).

Because IL-1β also has antiviral activity in human brain cell cultures [21], we investigated whether this proinflammatory cytokine plays a role in the potentiating effect of U50,488. Treatment of brain cells with U50,488 in the presence of anti-IL-1β antibody but not control antibody significantly blocked the potentiating effect of U50,488 (Fig. 7), suggesting that IL-1β may be released, which contributes to the potentiating effect of U50,488 on TNF-α antiviral activity.

DISCUSSION

The KOR ligand U50,488 has been shown previously to have potent direct anti-HIV-1 effects in acutely infected

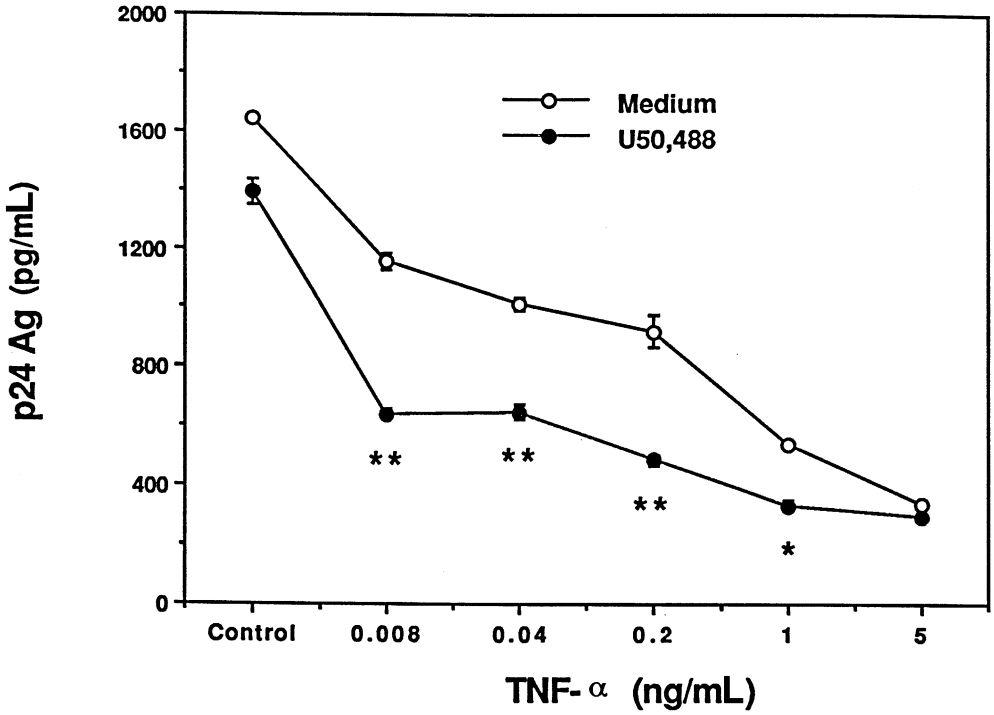

FIG. 4. Effect of various concentrations of TNF-α on the potentiating effect of U50,488. Brain cell cultures were incubated with medium or medium containing 1 nM U50,488 for 24 hr prior to the addition of the indicated concentrations of TNF-α and infection with HIV-1. After washing and replacing culture medium with U50,488 and TNF-α, supernatants were harvested on day 7 postinfection and assayed for p24 Ag. Data (means ± SEM) are representative of three separate experiments. (*) $P < 0.05$ and (**) $P < 0.01$ vs corresponding medium group.

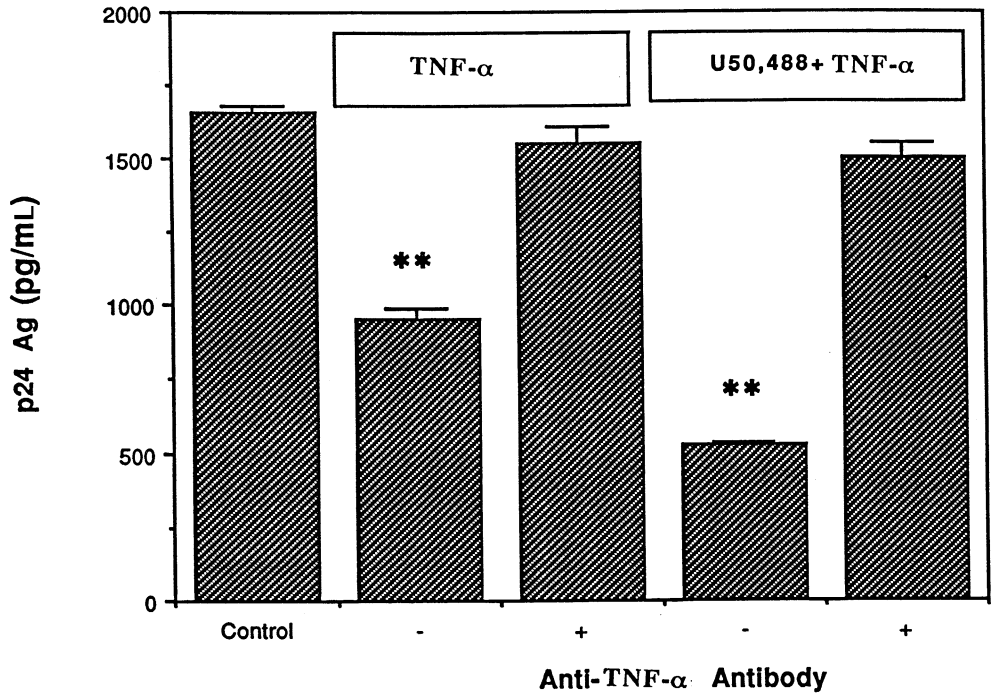

FIG. 5. Effect of anti-TNF-α antibody treatment on the potentiating effect of U50,488. Brain cell cultures were incubated with medium (control) or medium containing 1 nM U50,488 for 24 hr, prior to the addition of 2 μg/mL of anti-TNF-α antibody for 30 min followed by addition of 200 pg/mL of TNF-α and infection with HIV-1. After washing and replacing culture medium with U50,488, anti-TNF-α antibody, and TNF-α, supernatants were harvested on day 7 postinfection and assayed for p24 Ag. Data (means ± SEM) are representative of three separate experiments. (**) $P < 0.01$ vs corresponding anti-TNF-α antibody.

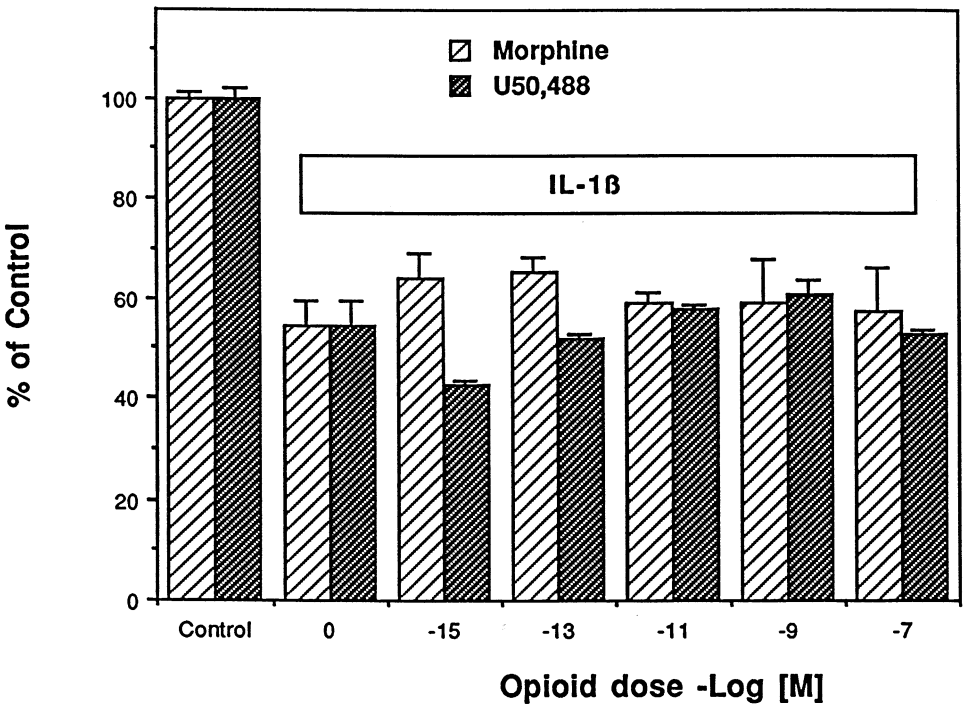

FIG. 6. Effect of U50,488 and morphine treatment on IL-1β-induced inhibition of HIV-1 expression. Brain cell cultures were incubated with medium (control) or medium containing the indicated concentrations of U50,488 for 24 hr prior to the addition of 200 pg/mL of IL-1β in the presence of HIV-1 for another 24 hr. After washing and replacing culture medium with U50,488 and IL-1β, supernatants were harvested on day 7 postinfection and assayed for p24 Ag. Data (means ± SEM), expressed as percent of control (2354 ± 135 pg p24 Ag/mL), are representative of three separate experiments.

microglial cell cultures [22]. In the present study, this same opioid was also found to exert only a modest antiviral effect when added alone to acutely infected mixed glial/neuronal cell cultures. U50,488, however, was found to potently enhance the antiviral activity of TNF-α, but not of IL-1β, in these brain cell cultures. These *in vitro* findings support the hypothesis that activation of KOR with synthetic kappa opioid ligands is associated with reduction of HIV-1 infection in the brain.

Although U50,488 exerts antiviral effects in both highly enriched microglial cell [22] and mixed glial/neuronal cell cultures (the present study), a major difference was observed in the concentrations of U50,488 that maximally inhibited HIV-1 in these two types of brain cell cultures. While U50,488 was maximally effective at 1 pM in microglial cells [22], optimal antiviral activity of U50,488 in mixed glial/neuronal cell cultures occurred at 1 nM. Because glial cells (microglia and astrocytes) and neurons constitutively express an identical KOR [6], it is possible that interactions of U50,488 with astrocytes or neurons in the mixed brain cell cultures not only alter the direct antiviral effect in microglial cells interspersed within these cultures but also shift the effective concentration to the right, i.e. higher concentrations are needed to see the antiviral effect. The potentiating effects of other KOR ligands (i.e. U69,593 and dynorphin$_{1\text{-}17}$), however, were relatively modest. It is unclear why the structurally related ligand U69,593, which has an identical maximal antiviral effect in highly enriched microglial cell cultures [22], was less potent in mixed glial/neuronal cell cultures as compared with U50,488. The modest effect of dynorphin could be associated with its peptidergic property, which is susceptible to degradation by proteases present on cell membranes [24].

The bell-shaped concentration–response property of U50,488 observed in the present study is not uncommon. Opioids have been known to exert a bell-shaped concentration–response curve in several immunomodulatory systems, including cytokine release [25–27] and an antiviral activity [22] in highly enriched primary microglial cell or macrophage cultures. The explanation of this bell-shaped concentration–response activity of U50,488 is unknown. Several possibilities may explain this phenomenon. Because activation of different classes of opioid receptors may trigger opposite effects [28], it is possible that higher concentrations of U50,488 are associated with a loss of KOR selectivity and cross-reaction with other classes of opioid receptors, thus triggering antagonism. Because opioid receptors are tightly linked to G proteins [3, 4], it is possible that higher concentrations of U50,488 induce a differential effect on G protein activation, which may result in desensitization to the anti-HIV-1 effect of U50,488.

The antiviral effect of U50,488 appears to be mediated via KOR, because the selective KOR antagonist nor-BNI totally blocked the antiviral effects of U50,488 in brain cell cultures in the present study as well as in microglial cell

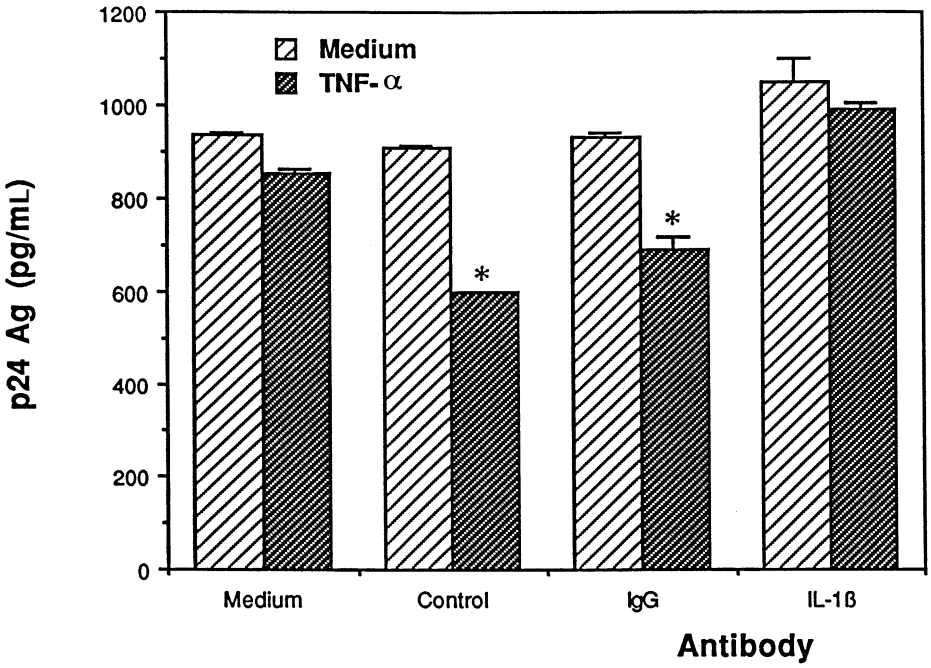

FIG. 7. Effect of anti-IL-1β antibody on the potentiating effect of U50,488. Brain cell cultures were incubated with medium (control) or medium containing 1 nM U50,488 for 24 hr, prior to the addition of 2 μg/mL of anti-IL-1β antibody or nonspecific mouse IgG for 30 min followed by addition of 200 pg/mL of TNF-α and infection with HIV-1. After washing and replacing culture medium with U50,488, respective antibody, and TNF-α, supernatants were harvested on day 7 postinfection and assayed for p24 Ag. Data (means ± SEM) are representative of three separate experiments. (*) $P < 0.05$ vs corresponding medium group.

cultures [22]. Also, the presence of TNF-α in the mixed brain cell cultures is critical for U50,488 to exert its immunomodulatory activity, because neutralization of TNF-α action by anti-TNF-α antibody completely blocked the potentiating effects of U50,488.

Although the precise mechanisms associated with the immunomodulatory effect of U50,488 are currently unknown, several explanations may be considered. Pretreatment of mixed brain cells with U50,488 for 24 hr could increase the expression of TNF-α protein or TNF-α receptors on microglial cells, and thus increase the sensitivity of TNF-α-mediated antiviral effects. The finding that dynorphin stimulates an expression of TNF-α in brain cell cultures [23] supports this notion. It is also possible that U50,488 enhances TNF-α-induced release of other antiviral cytokines, such as IL-1β, which may add to the antiviral effect of TNF-α [21]. To support this possibility, we found that antibodies specific to IL-1β blocked the potentiating effect of U50,488, suggesting that this cytokine may be released into cell cultures upon U50,488 exposure.

Opiates like morphine have been shown to modulate HIV-1 expression using an *in vitro* brain cell culture model containing chronically infected promonocytes (U1 cells) [25]. Also, morphine up-regulates viral expression in acutely infected peripheral blood mononuclear cell cocultures [29]. Moreover, we have found that morphine potentiates lipopolysaccharide-stimulated TNF-α production by enriched microglial cell cultures, which directly up-regulates HIV-1 expression in U1 cells [25, 26]. These proviral effects of morphine are bell-shaped and concentration-dependent, are maximal at opiate concentrations of 1 pM, and are mediated via MOR activation. Morphine, however, did not modulate HIV-1 expression significantly in acutely infected brain cell cultures in the present study.

Abused opiates have been proposed to play a role as a cofactor in accelerating progression of AIDS by altering cell-mediated immune function [29]. Different cell culture systems, using acutely or chronically infected primary cells or cell lines, have shown that morphine can increase HIV-1 expression. The findings in the present study of the KOR ligand U50,488 in mixed glial/neuronal cell cultures, taken together with previous studies of enriched microglial cell cultures [22], suggest that κ-opioids have opposite effects from MOR ligands. Because of the potential therapeutic implications of these findings, further explorations of the antiviral effects of a broader array of KOR ligands and testing in an *in vivo* model seem warranted.

This work was supported, in part, by U.S. Public Health Service Grants DA09924 and DA04381 from the National Institute on Drug Abuse.

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