**Communication**

**Induction and Activation of Mitogen-activated Protein Kinases of Human Lymphocytes as One of the Signaling Pathways of the Immunomodulatory Effects of Morphine Sulfate**

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Morphine sulfate causes immunomodulatory and immunosuppressive effects in human. In this study, the signaling pathway involved in these morphine effects was studied. Addition of morphine sulfate to human CEMx174 lymphocytic cells resulted in increased expression of mitogen-activated protein kinase cascade proteins. Morphine enhanced the cellular levels of ERK1 (44 kDa), ERK2 (42 kDa), a 54-kDa ERK, MEK1 (45 kDa), and MEKK (78 kDa). A time-dependent increase in the activated (Thr and Tyr dually phosphorylated) state of ERK1 and ERK2 was also observed. Naloxone, a morphine antagonist, reversed the observed morphine effects, implicating a μ opioid receptor-mediated process. These findings suggest that mitogen-activated protein kinases are important intermediates in signal transduction pathways initiated by morphine receptors in immune cells.

Morphine and related opioids have been shown to cause immunological disturbances (1). Opioid users are reported to be more susceptible to opportunistic infections than non-users, and they also have a faster progression of disease after infection with the AIDS virus (1–3). In a previous study (4) we injected morphine or saline (as a control) to rhesus monkeys and isolated the peripheral blood mononuclear cells (PBMCs)† from the animals to assess their T helper cell functions. From those studies, we found that treatment with morphine significantly influenced the ability of the quiescent lymphocytes in nine of eleven monkeys to proliferate. Five of these nine animals showed a transient increase in [3H]thymidine incorporation. Furthermore, it was found that at the early stage of morphine administration, a number of animals had a transient enhanced response to mitogens. In addition, four of six morphine-treated monkeys had an elevated interleukin 2 production, ranging from 1.3- to 34-fold increases in stimulation indexes. However, prolonged treatment of these animals with morphine revealed an immunosuppressive effect, and the immunosuppression was nalozone-irreversible (4). These studies suggested that morphine receptors may be present on lymphocytic cells, and a cause of opioid tolerance in these monkeys may have been the saturation of these receptors.

Recently, evidence has shown that monkey and human immune cells indeed possess brain-like opioid receptors (5–7). The discovery of the expression of three types of opioid receptors, δ, κ, and μ, in lymphocytes (5–7) provides a mechanism by which opioids such as morphine, in addition to altering various neuroendocrine mediators of immunosuppression, may cause immune disturbances by directly exerting their effects on the cells of the immune system.

The present study was undertaken to explore the signaling pathways involved in morphine-induced alteration of T cell function. Because protein phosphorylation represents a major mechanism of signal transduction, we have focused our study on the effects of morphine treatment on protein kinases. Considering the mitogen-like characteristics of morphine (4), we began to investigate the effect of morphine on the levels of various mitogen-activated protein (MAP) kinases in morphine-treated lymphocytes using CEM x174, a human lymphocytic cell line highly susceptible to simian AIDS viral infection.

**EXPERIMENTAL PROCEDURES**

Materials—Morphine sulfate was obtained from Mallinckrodt, TRizol Reagent for protein isolation was obtained from Life Technologies, Inc. Antibodies to various MAP Kinases were purchased from Transduction Laboratories (Lexington, KY). Antibody to the tumor suppressor protein, p53, was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-active MAPK polyclonal antibodies and horseradish peroxidase-conjugated secondary antibodies used to detect the primary antibodies in the immunoblot (Western blot) assays were purchased from Promega (Madison, WI).

Cell Culture and Morphine Sulfate Treatment—CEM x174 cells were maintained in suspension cultures at 37 °C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cell viability was determined by the trypan blue dye exclusion method. The cells were diluted 1:3 with fresh medium every 3–4 days. 16–24 h after the dilution, CEM x174 cells were treated with morphine sulfate or H2O (as control) for the time periods specified. The cells were harvested by centrifugation, washed once with phosphate-buffered saline solution, and stored at –20 °C until use.

Extraction of Proteins from CEM x174 Cells—Proteins were isolated from CEM x174 cells using the TRizol Reagent by a procedure specified by the manufacturer.

SDS/Polyacrylamide Gel Electrophoresis and Immunoblot Analysis of MAP Kinases—Samples of 5 or 15 μg of protein were analyzed by 10% SDS/polyacrylamide gel electrophoresis (PAGE) using the Mighty Small II Slab Gel Electrophoresis Unit SE 250 (Hoefer Science Institute, San Francisco, CA). Prestained SDS/PAGE protein standards (Bio-Rad) were run in parallel to reveal the molecular mass of the test proteins. Polypeptide bands resolved in SDS/PAGE gels were electroblotted to nitrocellulose filters, immunoblotted with antibodies to MAP kinases, and detected by incubation with horseradish peroxidase-conjugated secondary antibodies. Immunocomplexes resolved by electrophoresis were visualized by incubation of the filters with ECL (Amerham Corp.) or the SuperSignal substrate (Pierce) followed by exposure to an autoradiography film NEF (NEN Life Science Products).

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‡ The abbreviations used are: PBMC, peripheral blood mononuclear cell; MAP, mitogen-activated protein; MAPK, MAP kinase; PAGE, polyacrylamide gel electrophoresis; HIV, human immunodeficiency virus; SIV, simian form of HIV.
Morphine and MAP Kinases

RESULTS

Morphine Induces MAP Kinases in Immune Cells—The MAP kinases (MAPKs, also referred to as extracellular signal-regulated protein kinases, or ERKs) are the terminal enzymes in a three-kinase cascade (see Ref. 8 for review). The three kinases are a MAPK kinase kinase (or MEKK) that activates a MAPK kinase (or MEK) which, in turn, activates a MAPK/ERK enzyme (Fig. 1). This MAPK superfamily of enzymes is a critical component of a central switchboard that coordinates incoming signals generated by a variety of extracellular and intracellular mediators. Specific phosphorylation and activation of enzymes in the MAPK module transmits the signal down the cascade and results in the phosphorylation of many proteins with substantial regulatory functions throughout the cell. These include other protein kinases or transcription factors that subsequently control the expression and function of many cellular and nuclear genes (8).

ERK1 (44 kDa) and ERK2 (42 kDa) were the first members of the MAPK subfamily whose cDNAs have been cloned (9–12). In addition, the signaling cascades that lead to their activation are the best characterized to date (8). Our studies showed that the syntheses of these two protein kinases were significantly enhanced in morphine-treated CEM x174 cells. Results of a representative experiment were shown in Fig. 2. A 2.18-fold increase and a 1.58-fold increase in the amounts of ERK1 and ERK2 were observed at 12 and 42 h post-morphine treatment, respectively (Fig. 2). In addition to ERK1 and ERK2, a 54-kDa ERK and a 85-kDa MAP kinase could also be identified in the CEM x174 cells. The presence of a 62-kDa MAPK (ERK3), which is expressed in high abundance in the nervous system (12, 13), was otherwise not detected in CEM x174 cells (data not shown). Our immunoblot analysis revealed that the amount of 54-kDa ERK was increased by 13.9-fold after 12 h of morphine treatment, whereas the 85-kDa MAP kinase was not shown. Our immunoblot analysis revealed that the percentage of the total areas of the four bands. Results were quantitated by densitometry, and experiments were repeated at least three times.

Morphine Activates MAP Kinases in Immune Cells—Because activation of enzymes of the MAP kinases requires dual phosphorylation of the catalytic core of the enzyme, we used an antibody against the active form of ERK1 or ERK2, an antibody that targets the dually phosphorylated peptide encompassing the Thr183 and Tyr185 residues of the enzymes (Promega), for this study. The results (Fig. 4) showed that morphine indeed activated MAP kinases by increasing the cellular concentration of the active (phosphorylated) form of the enzyme in a time-dependent (Fig. 4A) and naloxone-reversible (Fig. 4B) manner. These studies indicated that morphine induced both the active and basal forms of ERK enzymes. These findings suggest that activation of MAP kinases is one of the signaling pathways morphine uses to modulate a variety of cellular and nuclear events in immune cells.
**FIG. 4. Immunoblot detection of ERK1 and ERK2 activation in CEM x174 cells using anti-active MAPK polyclonal antibody.** A, a time-dependent phosphorylation. 24 h after dilution, CEM x174 cells were treated with 10 μM morphine sulfate. Cells were withdrawn at 2.5 (lane 1), 9.5 (lane 2), or 19.5 (lane 3) h for analysis for active MAP kinases. The remaining culture was diluted again, and morphine treatment was repeated (to a final concentration of 10 μM) for 3 h before the cells were harvested for analysis (lane 4) of the presence of active MAP kinases. The negative results in lane 4 suggest the importance of the cell growth kinetics for MAPK activation induced by morphine. B, the naloxone effect. 24 h after dilution, CEM x174 cells were treated with H2O (lane 1), 10 μM morphine sulfate (lane 2), 10 μM naloxone (lane 3), or 10 μM morphine sulfate plus 10 μM naloxone (lane 4). The treatment was for 19.5 h before analysis was made for active MAP kinases. Lane 5, activated human fibroblast cell extract as a positive control. Each gel lane in A or B contained 15 μg of total protein.

**DISCUSSION**

The finding that immune cells contain opioid receptors, just like the neuronal cells (5–7), prompted us to explore the G-protein-coupled signaling pathways involved in morphine-treated lymphocytes. CEM x174, a human T and B hybrid cell line susceptible to SIV infection, was used for this analysis. Because protein phosphorylation represents a major mechanism of signal transduction, we have focused our studies on the effects of morphine on G-protein-regulated protein kinases. Previously we have found that administration of morphine to monkeys may cause an activation of monkey lymphocytes, an effect similar to that of “mitogens” (4). We thus initiated the study by assaying the amounts of various MAP (mitogen-activated protein) kinases in morphine-treated CEM x174 cells and found that morphine caused a time-dependent and naloxone-reversible activation of MAP kinase module. This suggests that morphine, as an extracellular signal, may trigger initial events upon association with its cell surface receptors (possibly μ receptors due to naloxone antagonist effects), and this signal is then transmitted to the interior of the cell, where it activates various cellular and nuclear events as illustrated in Fig. 1.

Drug abuse has been identified as a significant factor contributing to the AIDS epidemic. It has been recognized (21) that T helper/inducer lymphocytes of the CD4+ phenotype are the primary target cells of HIV. To assess a possible primary opiate effect on the immune system, Peterson et al. (22) studied HIV-infected human PBMCs and found that morphine promoted the growth of HIV-1 and the effect of morphine could be blocked by the opioid receptor antagonist naloxone. Using SIV, a simian form of HIV, we found that morphine also stimulated SIV replication both in CEM x174 cells (23) and in the PBMCs of morphine-treated monkeys (3). Because activation of T cells is a prerequisite for HIV/SIV infection (24), the morphine-induced mitogenic effect, as revealed by the present study, may be an underlying mechanism for the induced virus replication seen in morphine-treated human or animal cells.

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