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The Stimulatory Effect of Opioids on Mitogen-Activated Protein Kinase in Chinese Hamster Ovary Cells Transfected to Express μ-Opioid Receptors

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SUMMARY

The mitogen-activated protein (MAP) kinase of Chinese hamster ovary cells (CHO μ66 cell line) transfected to express μ-opioid receptors was markedly activated by μ agonists. The rank order of effectiveness of agonists was approximately the same as the rank order of their binding affinities to the μ receptor. The δ and κ receptor-specific agonists cyclic[D-Pen^2, D-Pen^5]enkephalin and U69,593 showed a very weak stimulatory effect. The μ agonist-stimulated MAP kinase activity peaked at ~4-8 min and lasted almost 1 hr. The stimulatory effect of μ agonists was antagonized by the opioid receptor antagonist naltrexone and inhibited by pretreatment of cells with pertussis toxin. This opioid-induced activation of MAP kinase activity may have a role in the long term effects of opioids.

Opioids exert their effects through three major types of opioid receptors: μ, δ, and κ. These have been well characterized in pharmacological and biochemical studies (1–4), and their cDNAs were recently cloned (5–9). Structurally, the opioid receptors belong to the superfamily of heterotrimeric G proteins. Opioid receptors are predominantly coupled by G_i and G_o proteins to the inhibition of adenylyl cyclase activity and reduction of cAMP levels in neuronal cells (10–12), the modulation of ion channel activity (13–15), and the inhibition of neurotransmitter release. Chronic use of opiates produces dependence in humans and animals, but the molecular mechanisms underlying this phenomenon are poorly defined. Recent studies indicated that some G_i-coupled receptor agonists have a stimulatory effect on MAP kinase (16, 17). This effect seems to be mediated by $G_{\beta\gamma}$ subunits of the G_i heterotrimeric protein (18). The MAP kinase pathway is now well known as a major pathway for signal transduction from cell surface growth factor receptors to nuclear transcriptional activation (19–22). We demonstrate profound stimulation of MAP kinase activity by opioids in a Chinese hamster ovary cell line that was transfected to express rat μ-opioid receptors.

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Materials and Methods

MAP kinase activity was assessed by an immune complex kinase assay method described by Wu *et al.* (23). Chinese hamster ovary cells (designated as CHO μ66) that were stably transfected with μ cDNA to express μ-opioid receptors (24) (a gift of Dr. L.-Y. Liu-Chen, Temple University, Philadelphia, PA, and Dr. L. Yu, Indiana University, Indianapolis, IN) were cultured to confluence in 100-mm-diameter culture dishes in Dulbecco's modified Eagle's medium/F-12 (1:1) medium (GIBCO BRL, Baltimore, MD) with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT). Fetal bovine serum was removed by changing the medium to Dulbecco's modified Eagle's medium ~2 hr before experiments. Cells were incubated with opioid agonists with or without the μ antagonist naltrexone or the uncoupling agent pertussis toxin. The opioid receptor agonists that were used were PL017 ([*N*-methyl-Phe^3,D-Pro^5]morphiceptin), methionine-enkephalin, morphine, DAMGO, etorphine, cyclic[D-Pen^2,D-Pen^5]enkephalin, and U69593 {(5α,7α,8β)-(+)-*N*-methyl-*N*-(7-[1-pyrrolidinyl]-1-oxaspiro[4,5]dec-8-yl)benzeneacetamide)}. After different incubation times, the cells were washed with cold phosphate-buffered saline and solubilized with 1 ml of lysis buffer [10 mM Tris, pH 7.5, 40 mM β-glycerophosphate, 1.5 mM EGTA, 0.5 mM EDTA, 25 mM NaF, 1 mM sodium pyrophosphate, 0.5 mM sodium orthovanadate, 1 mM benzamidine, 20 mM 4-nitrophenylphosphate (Boehringer-Mannheim Biochemicals, Indianapolis, IN), 1 mM dithiothreitol, 0.1 M NaCl, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 0.1 mg/ml phenylmethylsulfonyl fluoride, and 1%

ABBREVIATIONS: MAP, mitogen-activated protein; Erk1, extracellular signal-regulated kinase 1; Erk2, extracellular signal-regulated kinase 2; aFGF, fibroblast growth factor-acidic; DAMGO, [D-Ala^2,*N*-methyl-Phe^4,Gly-ol^5]enkephalin; MBP, myelin basic protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary.

Triton X-100] and then centrifuged. The protein concentration of the supernatant was determined according to the rose bengal method (25). Three hundred microliters of the lysate was added to 3 μl of agarose conjugated anti-MAP kinase (Erk2) antibody (Santa Cruz Biochemicals, Santa Cruz, CA) and shaken in a cold room for 3 hr. The agarose was washed twice with lysis buffer with 0.5% Triton X-100 and once with lysis buffer without Triton X-100. The activity of MAP kinase immunoprecipitated onto agarose beads was assayed by adding 5 μl of 10× kinase buffer (1× = 20 mM HEPES, 10 mM MgCl, 1 mM dithiothreitol, 10 mM nitrophenylphosphate), 10 μl of MBP solution (containing 25 μg of MBP), 5 μl of ATP (500 μM), and 2.5 μCi of [γ-^{32}P]ATP. The total volume was brought to 50 μl by the addition of water. The samples were vortexed, incubated at 30° for 15 min, and centrifuged. Twenty microliters of the supernatant was spotted onto P81 filter paper, washed five times with 360 mM phosphoric acid, rinsed with ethanol, dried, and counted with a scintillating counter. The activation of MAP kinases after stimulation by opioids was also demonstrated by their mobility changes on SDS-PAGE (15% acrylamide); phosphorylated MAP kinases move slower than their corresponding nonphosphorylated forms. For this purpose, aliquots of cell lysates prepared from stimulated CHO μ66 cells by the above method were added with equal volumes of SDS-PAGE sample buffer (100 mM Tris·HCl, ph 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue and 20% glycerol). After being heated at 100° for 5 min, the samples were subjected to SDS-PAGE and blotted onto nitrocellulose membrane. MAP kinases were stained with Erk2 or Erk1 antibody (Santa Cruz Biochemicals) by enhanced chemiluminescence according to the protocol of the assay kit (ECL; Amersham Life Science, Clearbrook, IL). All of the compounds used were from Sigma Chemical (St. Louis, MO) unless otherwise specified.

Results and Discussion

This study demonstrated that μ-opioid receptors expressed in CHO cells are functionally coupled to the stimulation of MAP kinase. When CHO μ66 cells were challenged with μ agonists such as etorphine, DAMGO, or morphine, a profound increase in MAP kinase activity occurred (Fig. 1).

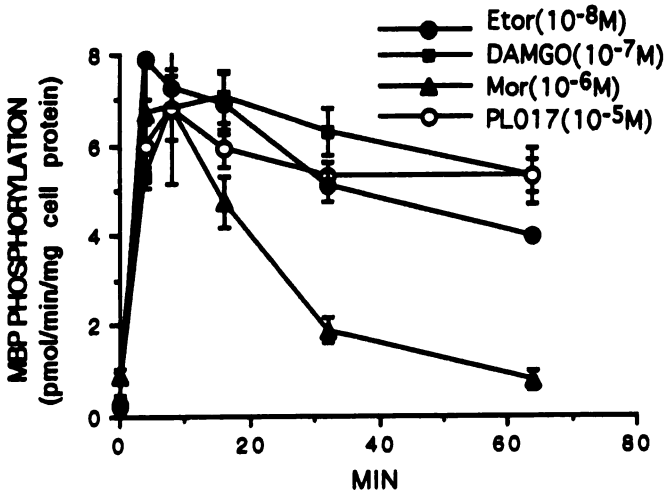

Fig. 1. The time course of stimulatory effects of μ agonists on MAP kinase activity of CHO μ66 cells. CHO μ66 cells were serum starved for 2 hr and stimulated by the addition of opioids. The reaction was stopped by washing cells with cold phosphate-buffered saline at the indicated time, and cells were lysed with Triton X-100 for the MAP kinase assay. The MAP kinase in the lysate was immunoprecipitated by incubation with Erk-2 antibody coupled to agarose. The activity of MAP kinase was assessed by the incorporation of ^{32}P into MBP from [γ-^{32}P]ATP. Results are the average ± standard deviation of triplicate experiments.

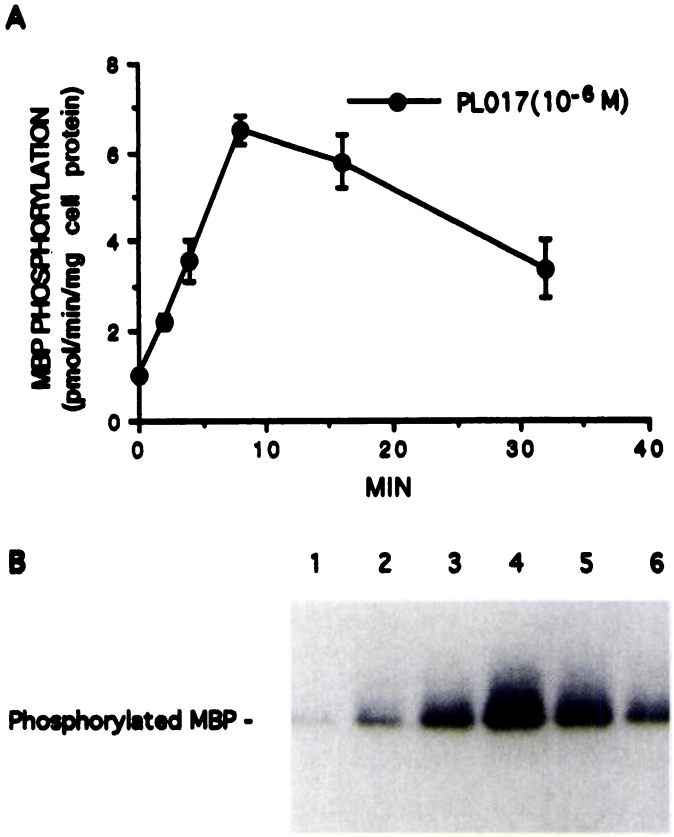

Fig. 2. *Top*, time course of stimulatory effects of PL017 on MAP kinase activity of CHO μ66 cells. The method used was the same as described in the legend to Fig. 1. Results are the average ± standard deviation of triplicate experiments. *Bottom*, autoradiographic results of MBP phosphorylation of the above experiment. Aliquots (5 μl) of the samples from the above experiments were subjected to 10% SDS-PAGE, blotted onto nitrocellulose paper, and autoradiographed. *Lane 1*, basal MAP kinase activity; *lanes 2–6*, MAP kinase activities at 2, 4, 8, 16, and 32 min after the addition of PL017, respectively.

Marked increase in MAP kinase activity was observed 4 min after the addition of 1 μM morphine, 0.1 μM DAMGO, or 10 nM etorphine. Similar results were observed for the highly selective μ agonist PL017 (Figs. 1 and 2, *top*). An autoradiograph of the ^{32}P-phosphorylated MBP after 10% SDS-PAGE of MAP kinase assay mixture confirmed the stimulatory effects of opioids on MAP kinase (Fig. 2, *bottom*). The stimulatory effect gradually declined after 8 min of incubation and returned to near basal activity after 1 hr of morphine incubation (the effect was much longer for etorphine, DAMGO, and PL017), suggesting that tolerance to opioids also developed for this MAP kinase effect. The untransfected control CHO cells did not show a stimulatory response to opioids on MAP kinase activity (data not shown).

Concentration-response curves were obtained for a series of opioid agonists (Fig. 3). All μ agonists (etorphine, DAMGO, methionine-enkephalin, morphine, and PL017) showed concentration-dependent stimulation of MAP kinase activity. The rank order of potency was similar to the known order of potency of μ receptor binding affinity for these μ agonists (26). In contrast, the δ agonist cyclic[D-Pen^2,D-Pen^5]enkephalin and κ agonist U69593 had little effect on MAP kinase activity; this was expected because the cells expressed only transfected μ receptors. The stimulatory ef-

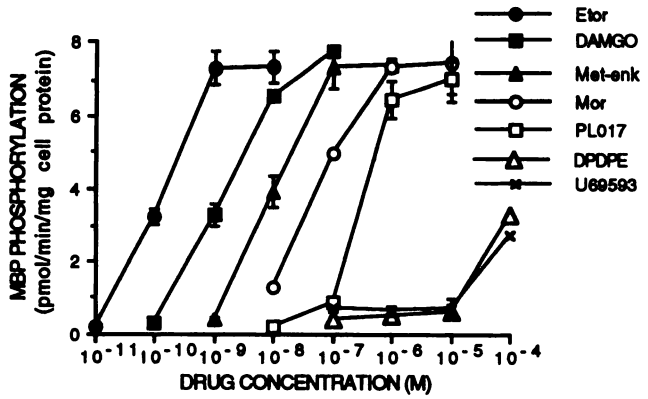

Fig. 3. The concentration-stimulation curves of various μ agonists on MAP kinase activity of CHO μ66 cells. The MAP kinase activity was determined 5 min after the addition of opioids. Results shown are the average ± standard deviation of triplicate experiments.

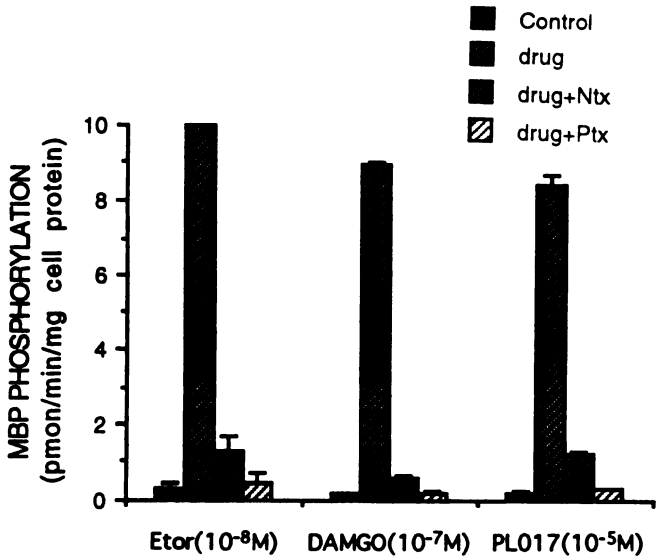

Fig. 4. Antagonistic effects of naltrexone and pertussis toxin on μ agonist-stimulated MAP kinase activity in CHO μ66 cells. The method used was the same as described in the legend to Fig. 1. The MAP kinase activity was determined 5 min after the addition of opioid agonists. For inhibition experiments, 5×10^{-8} M naltrexone was added 10 min and 500 ng/ml pertussis toxin was added 1 hr before the addition of opioid agonists. Results are the average ± standard deviation of triplicate experiments.

fect of μ-opioid agonists on MAP kinase was antagonized by the opioid antagonist naltrexone (Fig. 4).

All opioid-induced biological responses are also known to be coupled by pertussis toxin-sensitive G_i or G_o proteins (1–4, 10, 27). To confirm that opioid stimulation of MAP kinase activity is also mediated by pertussis toxin-sensitive G proteins, CHO μ66 cells were pretreated with 0.5 μg/ml pertussis toxin for 1 hr; MAP kinase activity was then determined after challenge with μ agonists. The stimulatory effects of μ-opioids on MAP kinase activity were completely inhibited in cells pretreated with pertussis toxin (Fig. 4).

aFGF also stimulated the MAP kinase activity in this same cell line (Fig. 5A). The maximum stimulations by aFGF and μ agonists are similar (Fig. 5A). MAP kinases exist as dephosphorylated forms in unstimulated cells and become activated when both tyrosine and threonine residues are phosphorylated (19, 20). The mobility of the phosphorylated forms of MAP kinases in SDS-PAGE is slower than their corresponding inactive nonphosphorylated forms. Fig. 5B confirms that both aFGF and etorphine activated the MAP kinase activity and changed the mobility of Erk2 in SDS-PAGE. The Erk2 antibody used here is highly specific to Erk2. One band of protein corresponding to the molecular mass of 42 kDa was detected by this antibody. Two bands of proteins were detected by the less-selective Erk1 antibody. Their mobilities in SDS-PAGE are consistent with the molecular masses of Erk1 and Erk2. Similar changes after stimulation in the mobility of Erk1 and Erk2 in SDS-PAGE were also detected by this less-selective Erk1 antibody (data not shown), suggesting that Erk1 is also stimulated by both aFGF and μ-opioids.

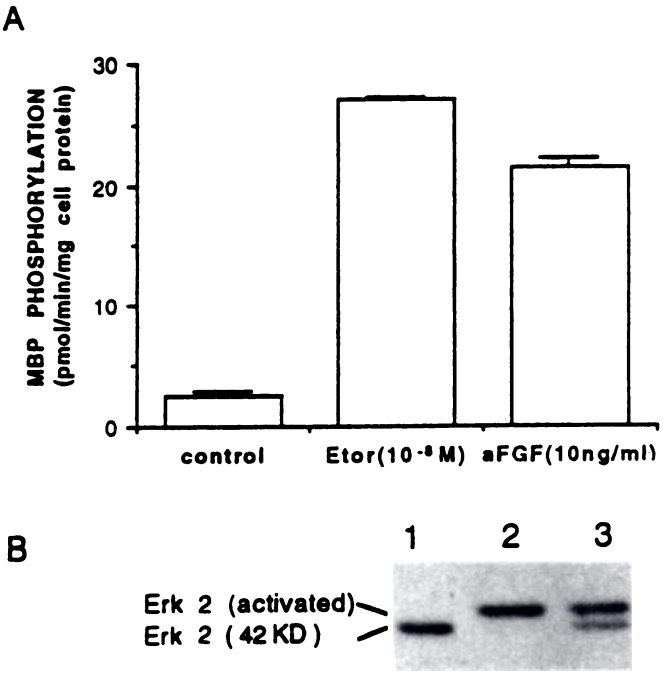

Fig. 5. A, Stimulation of MAP kinase activity of CHO μ66 cells by etorphine and aFGF. The activation of MAP kinase was determined according to the immune complex kinase assay method: CHO μ66 cells were serum starved for 2 hr, stimulated with etorphine (10^{-8} M) or aFGF(10 ng/ml) for 5 min, washed two times with cold phosphate-buffered saline, and lysed with lysis buffer. The method of determination of the MAP kinase activity in the lysate was the same as that described in legend to Fig. 1. B, Activation of MAP kinase shown by SDS-PAGE was determined in the following manner: aliquots of the above lysates of control unstimulated cells (*lane 1*), etorphine-stimulated cells (*lane 2*), and aFGF-stimulated cells (*lane 3*) were added with equal volumes of SDS-PAGE sample buffer, heated at 100° for 5 min, subjected to SDS-PAGE on a 15% gel, and transferred onto a nitrocellulose membrane. Erk2 was detected with specific rabbit Erk2 antibody by enhanced chemiluminescence. The activated MAP kinase (Erk2) was shown by a slower moving band above unactivated Erk2 band. The specific band of 42-kDa Erk2 was not seen if Erk2 antibody was preincubated with the antigen peptide.

Several G_i-coupled receptor agonists were recently shown to stimulate MAP kinase activity in various cell types. These include the agonists of m2 muscarinic receptor, lysophosphatidic acid receptor, α_2-adrenergic receptor, and platelet-activating factor receptor (16–18, 20). We have demonstrated that μ agonists can also stimulate MAP kinase activity through μ-opioid receptors and pertussis toxin-sensitive G protein. It is pertinent to discuss the consequences of MAP

kinase stimulation induced by opioids. MAP kinases are a family of serine/threonine protein kinases that play a major role in signal transduction downstream of mitogenic receptor tyrosine kinases, ras and raf proteins. MAP kinases are known to phosphorylate cytoskeleton proteins and regulate many serine/threonine kinases such as ribosomal S6 kinase, casein kinases, and phospholipase A_2. MAP kinases also regulate a variety of proto-oncogenic transcription factors, such as c-Jun, c-Myc, Elk-1, and ATF-2 (20–22). Thus, it is conceivable that long term effects (e.g., dependence) of opioids may be mediated through this MAP kinase pathway. Another major effect of opioids is the inhibition of adenylyl cyclase (see review in Ref. 10). This MAP kinase pathway cascade was recently shown to be negatively regulated by the cAMP/protein kinase A system in a variety of cells (23, 28, 29). The long term effects of opioids might be primarily mediated by the MAP kinase pathway. The opioid-induced inhibition of adenylyl cyclase activity could further strengthen or be necessary for the signals transmitted down to the nucleus at the transcriptional level. Finally, the increased basal adenylyl cyclase activity in neurons after continuous opioid treatment may result from the activation of the MAP kinase pathway by opioids.

Among heterotrimeric G_i-coupled receptors, both $G_{i\alpha}$-GTP and $G_{\beta\gamma}$ subunits are now believed to carry signals downstream (30). Evidence has accumulated to support the suggestion that $G_{\beta\gamma}$ subunits mediate the signal generated by G_i-coupled receptors to activate the MAP kinase pathway (18, 31, 32). Opioid-induced stimulation of MAP kinase is sensitive to pretreatment with pertussis toxin, suggesting that G_i or G_o is the coupling protein. Similar to muscarinic m2, α_2-adrenergic, and lysophosphatidic acid receptors, in μ-opioid receptor, the agonist-activated $G_{\beta\gamma}$ subunits may also stimulate the activity of MAP kinase indirectly via the activation of ras/raf proteins (16, 17).

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