Activation of μ-Opioid Receptor Induces Expression of c-fos and junB via Mitogen-activated Protein Kinase Cascade

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Background: Opioid-induced long-term functional alterations of the nervous system, such as tolerance, addiction, and dependence, conceivably involve changes in gene expression. The authors have previously reported that opioid receptors are functionally coupled to extracellular signal–regulated kinase, a class of the mitogen-activated protein kinase. To address whether activation of the opioid receptor induces changes in gene expression via the activation of extracellular signal–regulated kinase, the authors examined μ-opioid receptor (MOR)–induced immediate early gene expression.

Methods: Chinese hamster ovary cells stably expressing MOR were used. Cells were stimulated by MOR agonists after 24-h serum starvation. Expression of c-fos and junB genes was analyzed by RNA blot hybridization. To explore the mechanism of MOR–mediated c-fos and junB expression, activity of a transcription factor, Elk-1, was assessed by reporter assay. Furthermore, to investigate the functional consequences of c-fos and junB induction, MOR–mediated formation of the functional transcription factor complex AP-1 was examined by reporter assay and electrophoretic mobility shift assay.

Results: μ-Opioid receptor activation induced c-fos and junB messenger RNAs, which were inhibited by pretreatment of the cells with pertussis toxin and PD98059, an inhibitor of extracellular signal–regulated kinase cascade. MOR stimulation elevated Elk-1–mediated transcriptional activity by about 10-fold. AP-1–mediated transcriptional activity was stimulated by MOR agonists by about twofold. Electrophoretic mobility shift assay revealed that AP-1 binding activity in the nuclear extract was elevated by MOR activation and further showed that products of c-fos and junB genes are involved in formation of AP-1 complex.

Conclusions: μ-Opioid receptor activation induces c-fos and junB expression and elevates AP-1–mediated transcriptional activities via the mitogen-activated protein kinase cascade.

The opioid receptors, classified into μ, δ, and κ types on the basis of their interactions in the binding affinities for ligands, mediate a variety of biologic effects of opioids. In particular, recent studies using knock-out mice show that the μ-opioid receptor (MOR) plays a major role in the pharmacologic effects of morphine, the most popular opioid analgesic, such as analgesia, respiratory depression, addiction, physical dependence, and neuroendocrine effects. At the cellular level, pharmacologic, physiologic, and biochemical studies have shown that activation of the opioid receptors induces inhibition of adenylate cyclase, inhibition of voltage-dependent Ca2+ channels, and activation of inward rectifier K+ channels. These cellular responses are inhibited by pretreatment with pertussis toxin (PTX), indicating that the opioid receptors are coupled with the PTX-sensitive G-protein (G, or G, or both).

Molecular biologic techniques, including cloning of the complementary DNAs (cDNAs) and expression of the cloned cDNAs in cultured cells, have enabled us to further examine the intracellular signal-transduction mechanisms activated by the opioid receptors. We and other groups have previously demonstrated that extracellular signal–regulated kinase (ERK), a class of the mitogen-activated protein kinases, is activated by the opioid receptors expressed from cloned cDNAs in cultured cells. Mitogen-activated protein kinases transmit various stimuli from the cell surface to the cytoplasm and nucleus and are known to be involved in cell proliferation, differentiation, and long-term potentiation in neurons. ERK is a serine/threonine kinase that has been shown to affect many aspects of cellular functions by phosphorylating a number of intracellular proteins, including transcription factors, protein kinases, and cytosolic phospholipase A2. Previously, we reported that opioid receptor activation in the presence of A23187, a calcium ionophore, resulted in an increase in arachidonate release, suggesting that cPLA2 is activated by the opioid receptors, possibly through phosphorylation by ERK. However, the other opioid-activated cellular responses mediated by mitogen-activated protein kinases have not been thoroughly analyzed.

In the current study we examined opioid-induced immediate early gene expression in cultured cells stably transfected with the cloned MOR cDNA. Our data demonstrate that MOR activation induces expression of the immediate early genes c-fos and junB, via the PTX-sensitive G-protein and ERK cascade.

Materials and Methods

Materials

The CHO cell line stably expressing the cloned rat MOR (CROR-B22 cells) was described previously. The following materials were purchased: [D-Ala2,N-Me-Phe4,Gly-ol5]enkephalin (DAMGO; Peninsula Laborato-

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School of Medicine, Baltimore, MD), respectively. The probes were labeled with \[^{32}P\]dCTP and \[^{32}P\]ATP (Amersham-Pharmacia, Uppsala, Sweden); PathDetect reporter assay system (Roche, Indianapolis, IN); and other reagents (Wako, Osaka, Japan; Nacalai Tesque, Kyoto, Japan; and Sigma, St. Louis, MO).

**Cell Culture**

CROR-B22 cells were maintained in minimum essential medium \(\alpha\) containing deoxyribonucleosides and ribonucleosides supplemented with 6% bovine calf serum, penicillin G (50 units/ml), streptomycin sulfate (50 \(\mu\)g/ml) and G418 (300 \(\mu\)g/ml) in the humidified atmosphere of 95% air and 5% \(\mathrm{CO}_2\) at 37°C.

**RNA Blot Hybridization Analysis**

CROR-B22 cells were grown to confluence in 60-mm culture dishes, deprived of serum for 24 h, and then stimulated with agonists for the indicated times. After stimulation, total RNA was extracted with use of TRizol reagent according to the manufacturer’s instruction. Total RNAs were analyzed essentially as described previously.15 The RNAs were electrophoresed in 1% agarose gel and transferred to a nylon membrane (Pall). The hybridization probes for \(c\)-fos and \(junB\) were the 0.5-kb pair AclI/Aval fragment in the fourth exon of the human \(c\)-fos genome14 (TaKaRa, Kyoto, Japan) and 1.5-kb pair EcoRI fragment in the mouse \(junB\) cDNA (kindly provided by Daniel Nathans, M.D., Johns Hopkins University School of Medicine, Baltimore, MD).15 The hybridization probe for \(\beta\)-actin was the 0.4-kb pair Hinfl fragment of the human \(\beta\)-actin gene16 (Wako, Osaka, Japan). The probes were labeled with \[^{32}P\]dCTP by the random primer method.17 Autodigestion was performed at \(-80^\circ\text{C}\) with an intensifying screen for 2 days.

**Electrophoretic Mobility Shift Assay**

CROR-B22 cells were plated at 2 or 3 \(\times\) \(10^6\) cells per well in 6-well-plates and incubated for about 24 h before transfection. Transfection was performed with use of the FuGene 6 transfection reagent according to the manufacturer’s instruction.

For the Elk-1-mediated transcriptional reporter assay, CROR-B22 cells were transfected with the three expression plasmids of the PathDetect Trans-Reporting system (Stratagene), pFA2-Elk1 (encoding a fusion protein of Gal4 DNA binding domain and Elk-1 transactivation domain), pFR-Luc (constructed by cloning the entire coding sequence of the firefly luciferase downstream of a basic promoter element, TATA box, and joined to five tandem repeats of the 17-bp Gal4 binding element), and pSV\(\beta\)gal (encoding the Escherichia coli \(\beta\)-galactosidase).

To perform the AP-1-mediated transcriptional reporter assay,19 cells were transfected with the two expression plasmids of the PathDetect Cis-Reporting system, pAP1-Luc (constructed by cloning the entire coding sequence of the firefly luciferase downstream of a TATA box and joined to seven tandem repeats of the 7-bp AP-1 binding element) and pSV\(\beta\)gal.

At 24 h after transfection, incubation medium was replaced with serum-free minimum essential medium \(\alpha\), and the cells were incubated for a further 24 h. After serum starvation, cells were stimulated with 1 \(\mu\)M MOR agonists for 5 h at 37°C and washed twice with phosphate-buffered saline. The luciferase and \(\beta\)-galactosidase activity in the cell lysate was measured by the Luciferase Assay System and \(\beta\)-galactosidase reporter assay system, respectively. The luciferase activity reflects Elk-1- or AP-1-mediated transcriptional activity. The \(\beta\)-galactosidase activity is measured to compare the transcription efficiency between experiments. The luciferase activity was normalized to each \(\beta\)-galactosidase activity.

**Electrophoretic Mobility Shift Assay**

CROR-B22 cells were grown to confluence in 100-mm dishes, serum-starved for 24 h, and stimulated by 1 \(\mu\)M agonists for 60 min at 37°C. Then cells were washed twice with phosphate-buffered saline, suspended in hypotonic buffer A (10 mM HEPES (pH, 7.6), 15 mM KCl, 2 mM MgCl\(_2\), 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 \(\mu\)g/ml leupeptin, 5 \(\mu\)g/ml aprotinin, and 1 \(\mu\)g/ml pepstatin A), and centrifuged for 20 s at 800g. The pellet was lysed with buffer A added with 0.2% Nonidet P-40 and was centrifuged for 20 s at 800g. The resulting pellet was resuspended in buffer A added with 250 mM sucrose and was centrifuged for 20 s at 800g to collect nuclei. From the nuclear pellet, nuclear extracts were eluted in buffer B (50 mM HEPES (pH, 7.9), 400 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 5 \(\mu\)g/ml leupeptin, 5 \(\mu\)g/ml aprotinin, and 1 \(\mu\)g/ml pepstatin A).

The electrophoretic mobility shift assay was carried out essentially as described previously.20 The AP-1 binding probe was created by annealing synthetic oligonucleotides, forward (5’-CGCTTGTAGTACGCCGGGAA-3’) and reverse (5’-TTCCGGCTGACTCATCAAGCG-3’). The annealed probe was 5’-end-labeled with T4 polynucleotide kinase and [\(^{32}P\)]ATP. The nuclear extract was incubated with the \(^{32}P\)labeled probe at room temperature for 15 min. For supershift assay, the nuclear extract was preincubated with c-Fos-, JunB- or c-Jun-specific antibody for 60 min on ice before addition of the \(^{32}P\)labeled probe. The samples were electrophoresed in 4% acrylamide gel. The gel was then dried and autoradiographed with an intensifying screen at \(-80^\circ\text{C}\) for 2 days.

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Fig. 1. μ-Opioid receptor (MOR)–mediated c-fos and junB mRNA expression. Intensities of the β-actin mRNA bands indicate that a nearly equal amount of RNA was loaded in each lane. (A) Time course of c-fos and junB mRNA expression after 1 μM agonist exposure. (B) Dose–response relation for c-fos and junB mRNA expression induced by 30-min exposure to the agonists. Lane c is nonstimulation control. (C) Pharmacologic analysis of MOR-mediated c-fos and junB mRNA expression. After pretreatment with vehicle (lanes 1 and 4), PTX (100 ng/ml; 24 h) (lane 2), or PD98059 (50 μM; 2 h) (lane 3), cells were stimulated with 1 μM agonists for 30 min in the absence (lanes 1, 2, and 3) or presence of 1 μM naloxone (lane 4). DAMGO = [D-Ala²,N-Me-Phe⁵,Gly-ol³]enkephalin.

**Statistical Analysis**

Data are expressed as mean ± SD. Statistical analyses of data were performed by one-way analysis of variance, with post boc comparison by means of the Dunnett test. Values of *P* < 0.05 were considered statistically significant.

**Results**

**MOR-mediated Immediate Early Gene Expression**

First, we tested whether MOR activation induces immediate early gene expression by RNA blot hybridization analysis. CROR-B22 cells, CHO cells transformed to express the cloned rat MOR, were stimulated with morphine, an opioid alkaloid agonist acting primarily on MOR, and DAMGO, a synthetic peptide agonist selective for MOR. After agonist stimulation, MOR-mediated c-fos messenger RNA (mRNA) expression appeared at 10 min, peaked at 30 min, thereafter declined gradually, and returned to the control level by 90 min (fig. 1A). Expression of junB was also induced by activation of MOR, although the time course of expression was quite different from that of c-fos expression. The band of junB mRNA appeared at 10 min and peaked at 30 min but was still remarkable at 90 min after agonist exposure (fig. 1A). The c-fos and junB expression was dependent on agonist concentration (fig. 1B), antagonized by naloxone, and inhibited by pretreatment with PTX or PD98059, an inhibitor of the mitogen-activated protein kinase/ERK kinase (MEK)-1, which activates ERKs by phosphorylation (fig. 1C). Thus, our results demonstrate that MOR activation induces transcription of c-fos and junB genes via the PTX-sensitive G-protein (G₁ or G₉, or both) and ERK cascade.

**MOR-mediated Elk-1 Activation**

To elucidate the mechanism of MOR-mediated c-fos and junB transcription via ERK cascade, we tested the involvement of a transcription factor, Elk-1, in the MOR signaling pathway, because it has been suggested that Elk-1 phosphorylated by ERK contributes to growth hormone-stimulated c-fos and junB expression.²¹ CROR-B22 cells were transiently cotransfected with the plasmid encoding Gal4-Elk-1 fusion protein and the plasmid containing luciferase-coding DNA sequence linked with Gal4 binding region. If a serine residue (383Ser) in the Elk-1 region of the Gal4-Elk-1 fusion protein is phosphorylated in the cells, the fusion protein binds to the Gal4 binding region upstream of the luciferase-coding sequence, resulting in an increase in production of luciferase mRNA and protein. Figure 2 shows that stimulation of MOR by agonists, DAMGO and morphine, enhanced Elk-1-mediated transcriptional activation of the luciferase reporter gene by about 10-fold, and this activation was inhibited by pretreatment of the cells with PTX and PD98059. This result indicates that stimulation of MOR induces activation of Elk-1, through the action of the PTX-sensitive G-protein and ERK cascade.

**MOR-mediated AP-1 Formation**

Immediate early gene products belonging to the Fos and Jun families form the dimer AP-1 complex, which activates transcription of a variety of genes by binding to the specific AP-1 binding site located in the 5' flanking region of the gene. Therefore, we expected that MOR activation could stimulate AP-1–mediated transcriptional activation. CROR-B22 cells were transiently transfected with the plasmid containing luciferase reporter gene linked with seven tandem repeats of AP-1 binding ele-
Discussion

In the current investigation we demonstrated that MOR activation induces expression of the immediate early genes c-fos and junB via the PTX-sensitive G-protein and ERK cascade, and possibly through the action of the transcription factor Elk-1. Furthermore, the induced immediate early gene products, c-Fos and JunB, are shown to participate in formation of functional AP-1 complex, which can induce expression of other genes. These results are schematically depicted in figure 5. Although further study is necessary to elucidate which genes are activated by this MOR-mediated signal transduction mechanism in vivo and in CHO cells, it is possible that MOR-mediated gene expression takes part in long-term pharmacologic effects of opioids, such as tolerance and addiction.

We used a CHO cell line, CROR-B22, permanently transfected with the cloned rat MOR cDNA. This cell line expresses MOR at the level of approximately 10 pmol/mg protein, which is much higher than the expression level of MOR in the brain. The native opioid receptors are predominantly expressed in neuronal cells in the central nervous system. In contrast, CHO cells, which do not endogenously express opioid receptors, are derived from Chinese hamster ovary and do not have neuronal lineage. Therefore, it may be difficult to completely exclude the possibility that MOR expressed in CHO cells is coupled with the signal transduction mechanism, different from that activated by the opioid receptor in the neuronal cells. However, a heterologous expression system using cloned cDNAs has provided a powerful tool for studying the signal transduction mechanism activated by a receptor existing poorly in the neuronal cells, because we can use cultured cells expressing the receptor in a high density. As a next step, we should test whether the MOR-mediated re-

Fig. 2. μ-Opioid receptor (MOR) activation induces Elk-1-mediated transcription. Cells were pretreated with pertussis toxin (PTX; 100 ng/ml; 24 h) or PD98059 (50 μM; 2 h), and then stimulated with 1 μM [D-Ala²,N-Me-Phe⁴,Gly-ol⁵]enkephalin (DAMGO) or 1 μM morphine. The ratio of normalized luciferase activity to that of nonstimulated control cells is expressed as mean ± SD from three separate experiments done in duplicate (*P < 0.05 vs. nonstimulated control; #P < 0.05 vs. cells stimulated by the same agonist without pretreatment).

Fig. 3. μ-Opioid receptor (MOR) activation induces AP-1-mediated transcription. Cells were pretreated and stimulated in a manner similar to that described in the figure 2 legend. The ratio of normalized luciferase activity to that of nonstimulated control cells is expressed as mean ± SD from three separate experiments done in duplicate (*P < 0.05 vs. nonstimulated control; #P < 0.05 vs. cells stimulated by the same agonist without pretreatment). DAMGO = [D-Ala²,N-Me-Phe⁴,Gly-ol⁵]enkephalin; PTX = pertussis toxin.
Our data demonstrate that stimulation of MOR induces transcriptional activation mediated by Elk-1, a transcription factor belonging to the ternary complex factor family.26 Phosphorylated Elk-1 forms a complex with the serum response factor, another transcription factor, and binds to the serum response element of the genes, resulting in transcriptional activation. Serum response elements exist in the promoter of the c-fos gene27 as well as in that of the junB gene.15,28 Therefore, it is possible that MOR-induced expression of c-fos and junB mRNAs involve activation of Elk-1–mediated transcription. However, involvement of transcription factors other than Elk-1 cannot be excluded. For example, serine phosphorylation, possibly mediated by ERKs, is thought to contribute to transcriptional activation mediated by a transcription factor, STAT (signal transducer and activator of transcription),29 which can bind to the sis-inducible element in the c-fos promoter.30 Further study will be necessary to clarify whether the transcription factor STAT is involved in opioid-activated gene expression via ERK cascade.

There have been several reports that morphine induces immediate early gene expression in vivo. Chang et al.31 demonstrated that c-fos mRNA levels in rat caudate putamen were increased at 45 min and returned to control level at 90 min after injection with morphine sulfate (10 mg/kg). Garcia et al.32 showed induction of immediate early gene products, c-Fos, JunB, c-Jun, and a Jun-related antigen, in specific regions of the rat forebrain by acute morphine administration (10 mg/kg). However, they neither investigated the molecular mechanism of the induction nor clarified whether the immediate early gene induction was the result of direct or indirect effect of morphine. In fact, it was reported that...
intraperitoneal injection of morphine (10 mg/kg) induces expression of c-fos and junB in rat striatum and nucleus accumbens and that this immediate early gene induction by MOR is mediated by dopamine D1 and N-methyl-D-aspartate receptors. In contrast, we showed in the current investigation that immediate early gene expression can be directly induced by acute opioid exposure in MOR-expressing cells. It remains to be elucidated whether the mechanism we showed here is relevant in the mammalian central nervous system.

AP-1 complex is a transcriptional activator dimer composed of transcriptional factors belonging to the Fos (c-Fos, FosB, Fra-1, and Fra-2) and Jun (c-Jun, JunB, and JunD) families. Our data showed that MOR stimulation induces AP-1-mediated transcriptional activity and increases binding of nuclear protein to the AP-1 probe. Moreover, we demonstrated that c-Fos, JunB, and c-Jun participate in forming the AP-1 complex. Together, it is conceivable that c-Fos and JunB, the expression of which is induced by MOR activation via the PTX-sensitive G-protein and ERK cascade, can form functional AP-1 complex and induce expression of other proteins. The involvement of AP-1 complex in opioid-induced alteration of gene expression in vivo has not been shown previously. However, it is possible that opioid-induced AP-1 complex formation contributes to physiologic responses to opioids in vivo. The genes encoding opioid peptide precursors, including proopiomelanocortin, preproenkephalin, and prodynorphin, are known to induce AP-1 complex and induce expression of other proteins. (c-Fos, FosB, Fra-1, and Fra-2) and Jun (c-Jun, JunB, and JunD) are transcriptional factors belonging to the Fos family and play important roles in various biological processes, including cell proliferation, differentiation, and apoptosis. The AP-1 complex is a dimer of these transcription factors and is known to be involved in a variety of cellular responses.

Moreover, we demonstrated that c-Fos, JunB, and c-Jun participate in forming the AP-1 complex. Together, it is conceivable that c-Fos and JunB, the expression of which is induced by MOR activation via the PTX-sensitive G-protein and ERK cascade, can form functional AP-1 complex and induce expression of other proteins. The involvement of AP-1 complex in opioid-induced alteration of gene expression in vivo has not been shown previously. However, it is possible that opioid-induced AP-1 complex formation contributes to physiologic responses to opioids in vivo. The genes encoding opioid peptide precursors, including proopiomelanocortin, preproenkephalin, and prodynorphin, are known to contain AP-1 binding sites. It has been shown that expression of preproenkephalin and prodynorphin is suppressed during prolonged morphine administration. On the other hand, Chang et al. demonstrated that repeated morphine exposure causes significant induction of proopiomelanocortin mRNA in SH-SY5Y human neuroblastoma cells. Opioid-induced AP-1 formation, which was demonstrated in the current study, might be the molecular basis of this phenomenon. Furthermore, chronic morphine administration increases expression of the β-adrenergic receptor kinase (ßARK) in the rat locus coeruleus. ßARK promotes desensitization of the opioid receptors by phosphorylation and may contribute to morphine tolerance. It will be interesting to examine the involvement of morphine-induced immediate early gene expression and AP-1 formation in the induction of ßARK expression by morphine.

Opioid-induced changes in gene expression may be manifested also in the immune system. Hedin et al. reported that activation of the ß-opioid receptor expressed by transfection with the cloned cDNA leads to increased c-fos mRNA and AP-1 complex in Jurkat T lymphocytes and results in enhancement of interleukin-2 secretion. On the other hand, it was reported that some sets of immune cells endogenously express MOR and that administration of morphine causes an enhanced release of interleukin-2. It is likely that MOR activation induces interleukin-2 release via c-fos induction and AP-1 formation, similar to that with the cloned ß-opioid receptor, because it has been shown that the intracellular signal transduction mechanism activated by MOR is essentially the same as that activated by ß-opioid receptor. Thus, it may be possible that administration of opioids could affect the immune system via AP-1-mediated transcription.

In conclusion, we showed that MOR activation induces immediate early gene expression and elevates AP-1-mediated transcriptional activity via ERK cascade in CHO cells. Our findings suggest that administration of opioids induces a wide range of gene-expression changes, not only in the central nervous system but also in the immune system.

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