Mu-Opioid Receptor-Mediated Phosphorylation of I κ B Kinase in Human Neuroblastoma SH-SY5Y Cells

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Key Words

Mu-Opioid receptor • Nuclear factor- κ B • Inhibitor- κ B kinase • Mitogen-activated protein kinase • Calmodulin-dependent protein kinase • Src kinase

Abstract

Opioid receptors are involved in regulating neuronal survival. Here we demonstrate that activation of the μ -opioid receptor in human neuroblastoma SH-SY5Y cells led to the phosphorylations of I κ B kinase (IKK) and p65, denoting the stimulation of the nuclear factor- κ B (NF κ B) transcription factor. This response was mediated through pertussis toxin-sensitive G proteins. The μ -opioid-induced IKK phosphorylation required extracellular signal-regulated protein kinase, phosphatidylinositol 3-kinase and c-Src. Moreover, c-Jun N-terminal kinase and calmodulin-dependent kinase II also participated in the IKK activation, despite the lack of involvement of phospholipase C β and protein kinase C. These data suggest that the μ -opioid receptor is capable of simulating NF κ B signaling via the phosphorylation of IKK and p65 in human neuroblastoma SH-SY5Y cells.

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Introduction

Narcotic analgesics such as morphine and other opiates exert diverse physiological responses through the multiple G protein-coupled opioid receptors present in both central and peripheral nervous systems. Each of the three types of opioid receptors (μ , δ and κ) is capable of regulating diverse intracellular effectors through their associated G proteins [1]. Prolonged activation of opioid receptors invariably leads to the development of opiate tolerance and dependence. The molecular mechanism of opiate addiction is extremely complex and involves changes in the activity as well as expression of different types of cellular proteins in target neurons [2]. Increasing evidence indicates that the opioid receptors can regulate transcription in different cell types. Opioid-induced transcriptional regulation can proceed via multiple pathways including those involving the mitogen-activated protein kinases [3–5] and signal transducer and activator of transcription (STAT) [6, 7].

The nuclear factor- κ B (NF κ B) is a ubiquitously expressed mammalian transcription factor which translocates from the cytosol to the nucleus upon phosphorylation and activation by upstream regulators. In SH-SY5Y human neuroblastoma cells, NF κ B can be activated by a wide variety of cell surface receptors and signaling mol-

ecules via complex pathways. Depending on the nature of the primary signal, activation of NF κ B can be either pro- or anti-apoptotic. NF κ B activation mediates cell death induced by doxorubicin [8], yet its activation promotes nerve growth factor-induced survival of SH-SY5Y cells [9].

Possible linkage between the opioid receptors and the NF κ B pathway has been reported. Chronic morphine treatment results in inhibition of binding of NF κ B to the interferon- γ promoter element in primary activated murine T cells [5]. Long-term treatment of cortical neurons with a μ -selective agonist increases the activity of the NF κ B [10]. The ability of opioid receptors to regulate NF κ B may provide a mechanism to modulate complex biological processes including neuronal survival and the development of opiate tolerance and dependence. In the present study, we explored the μ -opioid receptor-regulated NF κ B activity in human neuroblastoma SH-SY5Y cells. The possible involvement of PTX-sensitive G proteins and other signaling molecules in the μ -opioid receptor-induced activation of NF κ B were also investigated.

Materials and Methods

Materials

Human neuroblastoma SH-SY5Y cells were purchased from American Type Culture Collection (ATCC number: CRL-2266). Cell culture reagents were from Invitrogen (Carlsbad, Calif., USA). Pertussis toxin (PTX) was from List Laboratories (Campbell, Calif., USA). [D-Ala², N-Me-Phe⁴, Gly⁵-ol]enkephalin (DAMGO), D-Phe-Cys-Tyr-D-Trp-Orn-Pen-Thr-NH₂ (CTOP), Tyr-D-Pen-Gly-Phe-D-Pen (DPDPE), ($\pm$)-trans-U-50488 methanesulfonate (U50488), orphanin FQ (OFQ) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, Mo., USA) or CalBiochem (San Diego, Calif., USA). Various antisera were products of Cell Signaling Technology (MA) and Amersham Biosciences (Piscataway, N.J., USA).

Cell Culture and Western Blot

Human neuroblastoma SH-SY5Y cells were maintained at 37°C in a mixture (1:1) of Eagle's minimal essential medium (MEM) and Ham's F-12 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μ g/ml streptomycin. One day before assay, SH-SY5Y cells were seeded into 6-well plates at a density of 4×10^5 cells per well. The cells were serum-starved for 4 h and subsequently incubated with DAMGO at various concentrations and for different periods. Where indicated, PTX (100 ng/ml) treatment was performed 4 h before agonist application. The cells were then lysed in 150 μ l of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 100 mM NaCl, 1% Triton X-100, 40 mM NaP₂O₇, 1 mM dithiothreitol, 200 μ M Na₃VO₄, 4 μ g/ml aprotinin, 100 μ M phenylmethylsulfonyl fluoride, and 2 μ g/ml leupeptin. When kinase inhibitors were examined, the cells were pretreated with different kinase inhibitors for

30 min in serum-free medium. Clarified lysates (40 μ g) were resolved on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membrane (BioRad, Calif., USA). Phospho-p65-Ser⁵³⁶, p65, phospho-IKK α -Ser¹⁸⁰/IKK β -Ser¹⁸¹ and IKK α were detected by specific primary antisera and horseradish peroxidase-conjugated secondary antisera. The immunoblots were visualized by chemiluminescence with the ECL kit (Amersham). Images detected on X-ray films were quantified by densitometric scanning using ImageJ software.

Statistical Analysis

Data were expressed as mean $\pm$ SEM of at least three independent sets of experiments. The probability of observed difference being a coincidence was evaluated by ANOVA and paired t test using the KyPlot software. Differences at values $p < 0.05$ were considered significant.

Results

μ -Opioid Receptor Employs G_{i/o} Proteins to Stimulate NF κ B Phosphorylation in Human Neuroblastoma SH-SY5Y Cells

We began our study by determining the ability of a μ -selective opioid peptide, DAMGO, to induce phosphorylations of IKK α/β and NF κ B (the p65 subunit) in human neuroblastoma SH-SY5Y cells. SH-SY5Y cells were challenged with a saturating concentration of DAMGO (100 nM) for various time periods before lysis. Phosphorylations of IKK α/β and NF κ B in the cell lysates were determined by immunoblot analysis using phosphospecific antisera. Both IKK α/β and p65 became phosphorylated upon treatment with DAMGO for 5 min, reaching a peak at around 15 min (approximately 2-fold increase) and the phosphorylations were sustained up to 30 min (fig. 1). Activation of the μ -opioid receptor in SH-SY5Y cells thus appeared to induce a rapid and sustained activation of IKK α/β and p65. The agonist-induced phosphorylations of IKK α/β and p65 were characterized by a dose-dependency on the concentration of DAMGO, reaching maximum activity at 10 nM (fig. 2). The DAMGO-induced phosphorylation of IKK α/β was more robust than that of NF κ B. Hence, in subsequent experiments we focused on examining DAMGO-induced phosphorylation of IKK α/β in SH-SY5Y cells under optimal conditions (100 nM; 10 min). To examine if the DAMGO-induced phosphorylation of IKK α/β required G_i proteins, we pretreated the SH-SY5Y cells with PTX prior to stimulation with DAMGO. As shown in figure 3a, PTX treatment completely suppressed the DAMGO-induced phosphorylation of IKK α/β . This result indicated that the DAMGO-induced phosphorylation of IKK α/β was pri-

Fig. 1. DAMGO time-dependently activates the phosphorylations of IKK α/β and p65 in SH-SY5Y cells. SH-SY5Y cells were stimulated with 100 nM DAMGO for various durations of time. Cell lysates were immunoblotted with antisera against total or phosphorylated IKK α/β and p65. DAMGO-induced phosphorylations of IKK (solid squares) and p65 (open squares) as a percentage of basal were quantified by densitometric analyses. The blots shown are from one single representative experiment, while the accompanying graphs are derived from three independent experiments.

Fig. 2. DAMGO induces phosphorylations of IKK α/β and p65 in a dose-dependent manner. SH-SY5Y cells were stimulated with various concentrations of DAMGO for 10 min. Cell lysates were collected and analyzed as in legend to figure 1. DAMGO-induced phosphorylations of IKK (solid squares) and p65 (open squares) were quantified and expressed as a percentage of the basal value. The blots shown are from one single representative experiment, while the accompanying graphs are derived from three independent experiments.

marily mediated via endogenous PTX-sensitive $G_{i/o}$ proteins. Moreover, the IKK α/β phosphorylation was blocked in the presence of 10 μ M CTOP (a specific μ -opioid antagonist) or 10 μ M naloxone (a non-selective opiate antagonist), confirming the requirement of activated μ -opioid receptors (fig. 3b).

To investigate whether other opioid receptors also activate NF κ B, we stimulated SH-SY5Y cells with different specific agonists (100 nM): Tyr-D-Pen-Gly-Phe-D-Pen (DPDPE; δ -opioid agonist), trans-($\pm$)-U50,488 methane sulfonate (U50488; κ -opioid agonist) and orphanin FQ (OFQ; ORL agonist). Apart from DAMGO, OFQ was also capable of inducing IKK phosphorylation (fig. 3c). On the other hand, both δ - and κ -opioid receptor ligands failed to activate IKK α/β in SH-SY5Y cells.

DAMGO-Induced Phosphorylation of IKK α/β Required the Participation of Multiple Signaling Molecules

Activation of the μ -opioid receptor has been shown to regulate multiple signaling pathways, several of which in-

volve mitogen-activated protein kinases (MAPKs) such as the extracellular signal-regulated protein kinase (ERK) [3], c-Jun N-terminal kinase (JNK) [4], p38 MAPK [5], protein kinase C (PKC) [11] and Src tyrosine kinase [4]. To determine if any of these kinases are required for DAMGO-induced phosphorylation of IKK α/β , we examined the effects of specific inhibitors on μ -opioid receptor-elicited IKK α/β phosphorylation. The results in figure 4a illustrate the possible involvement of ERK and JNK, but not p38 MAPK, in mediating DAMGO-induced phosphorylation of IKK α/β . Inhibition of ERK kinase 1/2 (also known as MEK1/2) and JNK by U0126 (10 μ M) and SP600125 (30 μ M), respectively, abolished the DAMGO-induced phosphorylation of IKK α/β . In contrast, the inactive analogues of U0126 and SP600125 (U0124, 10 μ M; SP600-ve, 30 μ M) were ineffective. A specific inhibitor of p38 MAPK (SB202190; 10 μ M) and its inactive analogue (SB202474; 10 μ M) were unable to suppress the DAMGO response. These results suggest that ERK and JNK cascades are able to provide a relay from the μ -opioid receptor to the NF κ B pathway.

Fig. 3. DAMGO-induced IKK α/β phosphorylation is suppressed by PTX and co-treatment with antagonists. **a** SH-SY5Y cells, either with or without 4-hour PTX pretreatment (100 ng/ml), were stimulated with 100 nM DAMGO for 10 min. **b** SH-SY5Y cells were co-stimulated with DMSO (vehicle control), 10 μ M naloxone or CTOP in combination with 100 nM DAMGO for 10 min. **c** SH-SY5Y cells were stimulated with other opioid agonists, including DPDPE, U50488 and OFQ (each at 100 nM) for 10 min. Cell lysates were collected and analyzed by immunoblotting for total and phosphorylated IKK α/β . The blots shown are from one single representative experiment, while the accompanying graphs are derived from three independent experiments. * DAMGO significantly induced IKK phosphorylations.

Since activation of NF κ B can proceed via PI3K [12], while activation of μ -opioid receptors can lead to the stimulation of PI3K [4], SH-SY5Y cells were pretreated with two specific PI3K inhibitors, wortmannin (100 nM) or LY294002 (10 μ M) for 30 min before DAMGO stimulation. As shown in figure 4b, the DAMGO-induced IKK α/β phosphorylation was effectively blocked by both inhibitors, supporting the involvement of PI3K in this pathway.

The μ -opioid receptor has previously been demonstrated to regulate phospholipase C β (PLC β) [13], PKC [11], and calmodulin [14]. A panel of specific inhibitors was therefore used to assess the possible involvement of these signaling components in DAMGO-induced phosphorylation of IKK α/β . Inhibition of PLC β and PKC by U73122 (10 μ M) and calphostin C (100 nM), respectively, did not affect the DAMGO-induced phosphorylation of IKK α/β (fig. 4c). However, the DAMGO-induced response was attenuated by treating the SH-SY5Y cells with the calmodulin-dependent kinase II (CaMK-II) inhibitor, KN62 (10 μ M; fig. 4c). The inactive analogue of KN62, KN92, was unable to suppress the DAMGO-induced phosphorylation of IKK α/β .

In the activation of NF κ B by the G $_i$ -coupled dopamine D $_2$ receptor, the participation of c-Src is clearly evident [15]. Interestingly, activation of the μ -opioid receptor has been shown to stimulate c-Src in SH-SY5Y cells [4]. Thus, we sought to investigate if c-Src is also involved in DAMGO-induced phosphorylation of IKK α/β . Blockade of Src family tyrosine kinases by two selective inhibitors, PP1 and PP2, significantly abrogated μ -opioid receptor signaling to IKK α/β (fig. 4d). In contrast, PP3, the inactive analogue of PP1 and PP2, had no effect under identical conditions. These results support the requirement of Src kinases in the pathway.

Discussion

In the central nervous system, many processes including synaptic transmission, neuronal plasticity, as well as neuronal survival and differentiation appear to involve NF κ B [16]. In neuroblastoma SH-SY5Y cells, μ -opioid agonists have protective effects against serum withdrawal-induced cell death. Iglesias et al. [17] have reported that activation of the μ -opioid receptor promotes neuronal survival via a G $_{i/o}$ -coupled, PI3K/Akt-dependent signaling cascade. These anti-apoptotic effects are likely to be regulated by NF κ B. The present study clearly demonstrates that activation of G $_i$ -coupled μ -opioid receptors

Fig. 4. DAMGO-induced IKK α/β phosphorylation requires ERK, JNK, PI3K, c-Src and CaMK-II. **a–d** SH-SY5Y cells were treated with DMSO (vehicle control) and different kinase inhibitors for 30 min. Kinase inhibitors included raf-1 kinase inhibitor (10 μ M), U0126 (10 μ M), U0124 (10 μ M), SP600125 (30 μ M), inactive analogue of SP600125 (SP600-ve; 30 μ M), SB202190 (10 μ M), SB202474 (10 μ M), LY294002 (10 μ M), wortmannin (Wort; 100 nM), U73122 (10 μ M), U73343 (10 μ M), KN62 (10 μ M), KN92 (10 μ M), calphostin C (Cal C; 100 nM), PP1 (25 μ M), PP2 (25 μ M) and PP3 (25 μ M). Pre-treated cells were then stimulated with 100 nM DAMGO for 10 min. Cell lysates were collected and analyzed by immunoblotting for total and phosphorylated IKK α/β . The blots shown are from one single representative experiment, while the accompanying graphs are derived from three independent experiments. * Pretreatment with kinase inhibitor significantly attenuated DAMGO-induced IKK phosphorylations.

can lead to the phosphorylation of IKK and p65 in neuroblastoma SH-SY5Y cells. These results are in agreement with the findings by Hou et al. [10], wherein they showed that DAMGO upregulates the DNA binding activity of NF κ B in primary cultures of rat cortical neurons.

A number of G $_i$ -coupled receptors are known to possess the ability to stimulate NF κ B activity [12, 15], but the precise mechanism by which these receptors modulate NF κ B signaling is poorly understood. The G $\beta\gamma$ -sensitive PI3K appears to be important in G $_i$ -mediated activation of NF κ B. We have previously shown that activation of the μ -opioid receptors in SH-SY5Y cells can indeed lead to the phosphorylation of Akt [4]. This downstream effector, Akt, is fully capable of regulating IKK β and transactivating the p65 subunit [18], and, hence, activating the NF κ B cascade. Indeed, the formyl peptide [12] receptor has been shown to activate NF κ B through

the PI3K/Akt pathway. Our results suggest that the μ -opioid receptor may also employ a PI3K-dependent pathway to regulate IKK.

Besides PI3K/Akt, MAPKs can also participate in the NF κ B signaling cascade [19]. MAPKs are important loci for signal integration [20]. The abilities of opioid receptors to stimulate MAPK activities have clearly been documented [3–5]. Furthermore, Zhao et al. [21] have revealed that ERK can directly activate the IKK complex. Indeed, ERK is involved in the activation of IKK/NF κ B by G $_i$ -coupled dopamine D $_2$ receptor [15]. The participation of ERK is similarly observed in DAMGO-induced IKK phosphorylation in SH-SY5Y cells wherein Raf-1 and MEK1/2 inhibitors significantly blocked the IKK activation.

In SH-SY5Y cells, DAMGO can also activate JNK [4], a MAPK which regulates inflammation and apoptosis. Linkage of JNK to the NF κ B signaling cascade is pro-

vided by the β -transducin repeat-containing protein (β -TrCP), which mediates the ubiquitination of the phosphorylated I κ B α and acts synergistically with IKK on the transcriptional activity of NF κ B [22]. Furthermore, the upstream activator of JNK, mitogen-activated protein kinase 1 (MEKK1), can directly activate the IKK complex [23]. The product of a novel NF κ B-activating gene named CIKS (connection to IKK-complex and SAPK) serves to bring both the IKK complex and JNK into close proximity [24]. Although NF κ B can be activated via the JNK pathway, no GPCR has yet been reported to utilize this mechanism. The ability of SP600125, but not its inactive analogue, to attenuate DAMGO-induced IKK phosphorylation suggests that the μ -opioid receptor may employ JNK to regulate NF κ B cascade in SH-SY5Y cells. Selective involvement of JNK in DAMGO-induced IKK phosphorylation is further illustrated by the ineffectiveness of inhibitors against p38 MAPK. It remains to be demonstrated how signals from ERK and JNK are integrated for the regulation of IKK/NF κ B.

Another interesting aspect of DAMGO-induced IKK phosphorylation in SH-SY5Y cells pertains to the involvement of CaMK-II; KN62, but not KN92, effectively suppressed the response. The involvement of CaMK-II has been demonstrated in phorbol ester-induced activation of IKK [25]. Although activation of the μ -opioid receptor can lead to the stimulation of PLC β [13] and PKC [11], neither appears to be responsible for mediating the CaMK-II dependency of IKK phosphorylation. The μ -opioid receptor has been shown to associate with calmodulin directly [14]. The binding of agonist to the μ -opioid receptor leads to an elevation in cytosolic Ca²⁺ [26] and the subsequent activation of calmodulin [14] and its downstream effectors including CaMK-II. Thus, this feature of the μ -opioid receptor provides an alternative route to activate IKK $\alpha\alpha\beta$, independent of PLC β and PKC. It should also be noted that despite the presence of δ -opioid receptors in SH-SY5Y cells [27] and its ability to bind calmodulin [14], the δ -selective agonist could not induce IKK phosphorylation (fig. 3c). This difference may in part be due to the distinct requirement for PI3K in μ -opioid receptor-mediated signaling, as in the regulation of JNK [4].

We have also clearly identified that DAMGO-induced IKK activation is mediated through c-Src. Both G $\beta\gamma$ [28] and G α_i [29] subunits are capable of inducing c-Src activation. Crosstalk between signaling cascades are also crucial in c-Src activation. CaMK-II is one of many signaling intermediates which can transactivate c-Src [30]. c-Src activation can lead to the tyrosine phosphorylation of

IKK and the subsequent stimulation of NF κ B activity [31]. Moreover, the ability of c-Src to regulate MAPKs [28] and PI3K [32] provides alternative routes for activating IKK/ NF κ B. Hence, c-Src serves as an integrator for several signaling pathways and connects GPCRs with the IKK/NF κ B pathway.

In conclusion, this study provides evidence that the μ -opioid receptor can induce phosphorylation of IKK and p65 via PTX-sensitive G proteins leading to the activation of the NF κ B signaling cascade. Moreover, the activation of IKK/NF κ B by the μ -opioid receptor is channeled through numerous signaling intermediates, including PI3K/Akt, ERK, JNK, CaMK-II, and c-Src. Even though JNK cascade has long been associated with NF κ B signaling, the mechanism of how JNK modulates the IKK activities is still unclear. In addition, the special feature of μ -opioid receptor to interact with calmodulin allows CaMK-II to affect DAMGO-induced activation of IKK/ NF κ B. Since the opioid receptors are involved in regulating neuronal survival [17] and development [33], it remains to be demonstrated if the IKK/NF κ B pathway plays a central role in these processes.

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