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• BASIC RESEARCH •

A new cytokine: the possible effect pathway of methionine enkephalin

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Abstract

AIM: To investigate experimentally the effects of methionine enkephalin on signal transduction of mouse myeloma NS-1 cells.

METHODS: The antigen determinate of delta opioid receptor was designed in this lab and the polypeptide fragment of antigen determinate with 12 amino acids residues was synthesized. Monoclonal antibody against this peptide fragment was prepared. Proliferation of Mouse NS-1 cells treated with methionine enkephalin of 1×10^{-6} mol· L^{-1} was observed. The activities of protein kinase A (PKA) and protein kinase C (PKC) were measured and thereby the mechanism of effect of methionine enkephalin was postulated.

RESULTS: The results demonstrated that methionine enkephalin could enhance the proliferation of NS-1 cells and the effect of methionine enkephalin could be particularly blocked by monoclonal antibody. The activity of PKA was increased in both cytosol and cell membrane. With reference to PKC, the intracellular activity of PKC in NS-1 cells was elevated at 1×10^{-7} mol· L^{-1} and then declined gradually as the concentration of methionine enkephalin was raised. The effects of methionine enkephalin might be reversed by both naloxone and monoclonal antibody.

CONCLUSION: Coupled with the findings, it in-dicates that the signal transduction systems via PKA and PKC are involved in the effects of methionine enkephalin by binding with the traditional opioid receptors,and therefore resulting in different biological effects.

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INTRODUCTION

A number of studies have documented the involvement of endogenous opioid peptide on the cellular functions. It has been known that opioid receptors exist on the surface of cells pertinent to immune function, and that the activation or inhibition of these receptors may enhance or down regulate some cell activities. Methionine enkephalin, the native opioid peptide, has been identified and defined as a cytokine because of its non-neurotransmitter function and sharing all of the major properties of cytokines[1-4]. Although numerous studies have shown that opioid-induced alteration of cellular function can be mediated indirectly via the central nervous system (CNS) or through direct interaction with cells, the precise cellular mechanisms underlying the immunomodulatory effects of opioids are largely unknown.

It is especially true that the opioid receptors contain consensus sites for phosphorylation by numerous protein kinases. Protein kinase C (PKC) has been shown to catalyze the *in vitro* phosphorylation of delta-opioid receptors and to potentiate agonist-induced receptor desensitization[5,6]. On the other hand, studies suggest that acute and chronic opioid can regulate the cAMP-dependent protein kinase (PKA) signaling pathway and the changes in this pathway may be involved in opioid tolerance[7-9]. It has been documented that increased PKA activity can maintain cellular tolerance to opioid receptor agonist by chronic opioid treatment[10].

Although there is mounting evidence supporting the concept that opioids are members of the cytokine-like family, the relative contribution of the opioids to immunoregulation remains unclear. Furthermore, little has been studied how methionine enkephalin acts as binding with the receptor of cell surface and trigger the intracellular biological events via some kinases. In this series of experiments, we analyzed the effect of methionine enkephalin, the endogenoue opioid, on the activities of PKA and PKC at various dosages. The effects of monoclonal antibody and naloxone were also determined so that the mechanism of effect of methionine enkephalin on the signal transduction will be further clarified.

MATERIALS AND METHODS

Materials

Methionine enkephalin, naloxone, dithiothreitol (DTT), leupeptin, histone, phosphatidyl serine (PS), diacylglycerol (DG) and ATP were purchased from SIGMA; DMEM medium, bovine serum albumin(BSA) and 1-eth-3-3-dim-ethylaminopioply carbodimde-HCl (EDC) were purchased from GIBCO; phenylmethyl sulfonyl fluoride (PMSF) and egtazic acid (EGTA) were from SERVAL.

Methods

Design of polypeptide fragment of delta opioid receptor Delta opioid receptor is composed of 372 amino acids residues. Hydrophilic analysis and structure prediction were performed in our laboratory by typing the sequence of opioid receptor into a special computer program designed according to the principle of prediction of antigen determinants, and by which fragment of antigen determinant was selected[11]. The amino acids residues sequence of antigen determinant was selected from the 3rd hydrophilic peak based on the feature of possibility of antigen determinant and listed as follows: NH_2-Gly-Ser-Leu -Arg-Arg-Pro-Arg-Gln-Ala-Thr-Thr-Arg-COOH.

Synthesis of polypeptide fragment and preparation of monoclonal antibody Polypeptide fragment with 12 amino

acids residues of delta opioid receptor was synthesized in North West University, USA. Monoclonal antibody against this polypeptide fragment was prepared according to the routine procedure. In short, BABL/C mice was immunized with synthesized polypeptide fragment conjugated with bovine serum albumin in complete Freunds adjuvant at 2- to 3-week intervals. The splenic cells separated from mice were fused with myeloma cells to form a stable antibody-producing hybridoma cell line. Positive clones were screened by the method of ELISA and inoculated into BALB/C mice. The antibody was harvested from ascitic fluid and purified with affinity chromatography. The titers of monoclonal antibodies were higher than 3 000. The specificity and effects of monoclonal antibody were verified in this experiment.

Effect of methionine enkephalin on proliferation of NS-1 cell lines NS-1, Mouse myeloma cell line, was cultured in DMEM medium containing 10 % fetal calf serum at 37 ℃ in a humidified atmosphere of 5 % CO_2. After the cell growth occupied full of the bottom of the flasks, the cells were washed once and resuspended in medium at a density of 5×10^4 cells per ml. 1 ml of the cells per well was liquated in a 24 wells plate. When the cells were grown about 70 % full of wells, the supernatant was taken out. Then the cells were resuspended in 1 ml of medium without serum and cultured for one more day. After 1 d, supernatants in all wells were removed and the cells were resuspended in 1 ml of medium (with 10 % fetal calf serum). The cells were administrated with 1×10^{-6} mol· L^{-1} of methionine enkephalin. Different concentrations of monoclonal antibody ($0.1\text{-}10\times10^{-9}$ mol· L^{-1}) were used to block the effect of methionine enkephalin. The culture was continued for 2 d and then pulsed with 18.5×10^3 Bq of ^{3}H-TdR in each well. 4 h later, the cells were harvested onto glass microfiber filter using a multiple sample harvester. The incorporation of ^{3}H-TdR was measured by using LKB 1209 Rackbeta liquid scintillation counter.

Determination of protein kinase A activity NS-1 cells were adjusted to 5×10^4 cells per ml with DEME medium (containing 10 % fetal calf serum) and aliquot into 24 well plate at 1 ml cell suspension per well. When the cells were grown about 70 % full of the wells, the supernatant was taken out. Then the cells were resuspended in 1 ml of medium without serum, cultured for one more day and added 1×10^{-6} mol· L^{-1} of methionine enkephalin. For the blocking assay, the different concentration of monoclonal antibody ($0.1\text{-}10\times10^{-6}$ mol· L^{-1}) and naloxone ($0.1\text{-}10\times10^{-6}$ mol· L^{-1}) were added at the same time. After 24 h, the cells were collected, resuspended in 500 μl of buffer A (containing 200 mmol· L^{-1} Tris-HCl pH 7.5, 0.25 mol· L^{-1} sucrose, 2 mmol· L^{-1} edetic acid, 2 mmol· L^{-1} dithiothreitol, 10 mg· L^{-1} leupeptin and 0.5 mmol· L^{-1} PMSF) and destroyed by supersonic instrument for 2 min in ice bath. The supernatants were collected following a spin at 10 000 g for 45 min and defined as the cytosol fraction. The pellet was resuspended in 400 μl of buffer A containing 0.5 % Triton X-100, supersonically destroyed for 2 min and defined as the membrane fraction. The measurement of PKA activity was carried out as described with modifications[12]. In short, 40 μl of extracted enzyme fractions were mixed with 160 μl of the solution at the final concentration of 20 mmol· L^{-1} Tris-HCl pH 7.5, 5 mmol· L^{-1} $MgCl_2$, 0.25 g· L^{-1} BSA, 0.5 g· L^{-1} histone, 2×10^{-7} mol· L^{-1} ATP (γ-^{32}P ATP, 3.7×10^4 Bq) and 8.0 μmol· L^{-1} of cAMP at 37 ℃ for 10 min. After followed by incubation in ice bath for 5 min to terminate the reaction,150 μl of the solution from each sample was collected onto Whatmen GF/C filter paper. After washing 2× with 10 % TCA-2 % phosphoric acid for 30 min at room temperature followed by 2× wash with 5 % TCA for 30 min, the activities of PKA were measured by using liquid scintillation counter and expressed as pmol value of ^{32}P in histone catalyzed by per mg protein per min.

Determination of protein kinase C activity The procedures of cell treatment and enzyme extraction were similar to that described in the determination of PKA instead of cells resuspended in 500 μl of buffer B (buffer A+10 mmol· L^{-1} egtazic acid). The final volume was 200 μl with final concentration of 20 mmol· L^{-1} Tris-HCl pH 7.5, 5 mmol· L^{-1} $MgCl_2$, 0.25 g· L^{-1} BSA, 0.5g· L^{-1} histone, 2×10^{-5} mol· L^{-1} ATP ([γ-^{32}P] ATP, 3.7×10^4 Bq), and 40 μl of extracted enzyme fraction. The measurement of activity of PKC was performed as described by Choi *et al*. with modification[13]. Briefly, 5 mmol· L^{-1} $CaCl_2$, 80 mg· L^{-1} PS and 3 mg· L^{-1} DG were added into reaction system. The collection and assay of samples and the addition of methionine enkephalin, also monoclonal antibody and naloxone, were according to the measurement of PKA. The activities of PKC were expressed with pmol value of ^{32}P in histone catalyzed by per mg protein per min (pmol· mg^{-1}· min^{-1}).

Statistical analysis

The data were expressed as $\bar{x}\pm s$ obtained from at least 3 independent experiments and analyzed by *t* test.

RESULTS

Effects of methionine enkephalin on the proliferation of NS-1 cells

Methionine enkephalin could stimulate the proliferation of NS-1 cells. When 1×10^{-6} mol· L^{-1} of methionine enkephalin was added into the cultured cells, the cells could proliferate up to 109 %. Monoclonal antibody at a lower concentration of 0.1×10^{-9} mol· L^{-1} could not block the effect of methionine enkephalin. Whereas 1 and 10×10^{-9} mol· L^{-1} of monoclonal antibody could reverse the enhancing effect of methionine enkephalin on the cell proliferation that showed significantly the differences as compared with treatment group of methionine enkephalin alone (Figure 1).

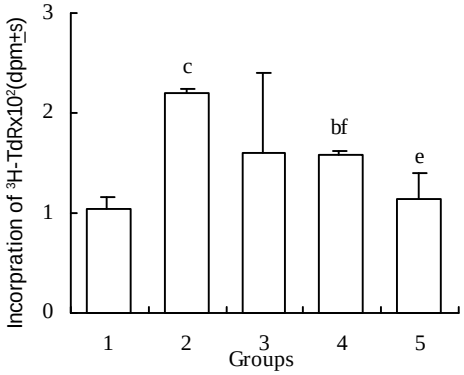

Figure 1 Blockage of monoclonal antibody to the effect of methionine enkephalin on the proliferation of NS-1 cells. Treatment groups; 1: control group; 2; 1×10^{-6} mol·L^{-1} methionine enkephalin; 3; 1×10^{-6} mol·L^{-1} methionine enkephalin plus 1×10^{-10} mol·L^{-1} monoclonal antibody. 4; 1×10^{-6} mol·L^{-1} methionine enkephalin plus 1×10^{-9} mol·L^{-1} monoclonal antibody. 5; 1×10^{-6} mol·L^{-1} methionine enkephalin plus 1×10^{-8} mol·L^{-1} monoclonal antibody. n=3 from 3 independent experiments. $^bP<0.05$ and $^cP<0.01$ *vs* control group, $^eP<0.05$ and $^fP<0.01$ *vs* group 2

Effects of methionine enkephalin on the activity of PKA

Methionine enkephalin at various concentrations could enhance the level of activity of PKA in cytosol and cell membrane. The effects could be observed at the concentration of $0.1\text{-}10\times10^{-6}$ mol· L^{-1} in cytosol and $0.01\text{-}10\times10^{-6}$ mol· L^{-1} in cell membrane. The effects of methionine enkephalin on the activity of PKA were consistent in the cytosol and the cell membrane (Figure 2).

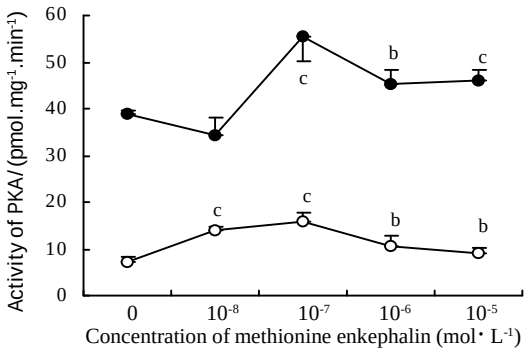

Figure 2 The influences of different concentrations of methionine enkephalin on the activity of PKA in cytosol (●) and cell membrane (○) of NS-1 cells. n=3 from 3 independent experiments. ^{b}P<0.05 and ^{c}P<0.01 *vs* control (0 mol·L^{-1})

Antagonism of monoclonal antibody and naloxone on the activity of PKA

1×10^{-6} mol· L^{-1} of methionine enkephalin was used for the blocking assay of monoclonal antibody on the activity of PKA. The reversed effects could be observed at the concentration of 1×10^{-10} and 1×10^{-9} mol· L^{-1} of antibody (Table 1). After administration of different concentrations of naloxone in the case of cytosol, the reversed effects were also obvious (Figure 3).

Table 1 Antagonism of different of concentrations of monoclonal antibody (MAb) to methionine enkephalin (MENK) at 1×10^{-6} mol·L^{-1} on the activity of PKA in cytosol and membrane of NS-1 cells. n=3 from 3 independent experiments. ^{b}P<0.05 and ^{c}P<0.01 *vs* control, ^{e}P<0.05 and ^{f}P<0.01 *vs* group 2

MENK+MAb	Activity of PKA in cytosol /pmol·mg^{-1}·min^{-1}	Activity of PKA in membrane /pmol·mg^{-1}·min^{-1}
Control	21.10±1.09	6.81±1.63
MENK	24.69±0.49^{c}	10.42±0.71^{b}
MENK+10^{-11} mol·L^{-1} MAb	29.98±0.59^{cf}	13.73±1.84^{ce}
MENK+10^{-10} mol·L^{-1} MAb	20.03±1.47	7.59±0.35^{e}
MENK+10^{-9} mol·L^{-1} MAb	12.99±0.95^{cf}	7.11±2.04^{e}

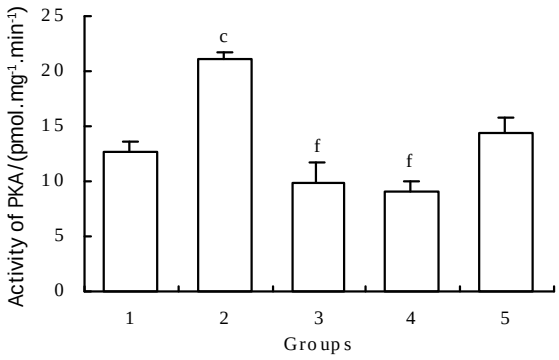

Figure 3 The effects of different concentration of naloxone on the activity of PKA in cytosol of NS-1 cells. Treatment groups: 1: control; 2: 1×10^{-6} mol·L^{-1} methionine enkephalin; 3: 1×10^{-6} mol·L^{-1} methionine enkephalin plus 1×10^{-7} mol·L^{-1} naloxone; 4: 1×10^{-6} mol·L^{-1} methionine enkephalin plus 1×10^{-6} mol·L^{-1} naloxone; 5: 1×10^{-6} mol·L^{-1} methionine enkephalin plus 1×10^{-5} mol·L^{-1} naloxone. n=3 from 3 independent experiments. ^{c}P<0.01 *vs* control, ^{f}P<0.01 *vs* group 2

Effects of methionine enkephalin on the activity of PKC

In Figure 4, a narrow effective range of methionine enkephalin was displayed. At the concentration of 1×10^{-7} mol· L^{-1}, methionine enkephalin could enhance the intracellular activity of PKC (Figure 4), but the higher concentration (10^{-6}-10^{-5} mol· L^{-1}) of methionine enkephalin showed a suppressive effect compared with control (0 mol· L^{-1}).

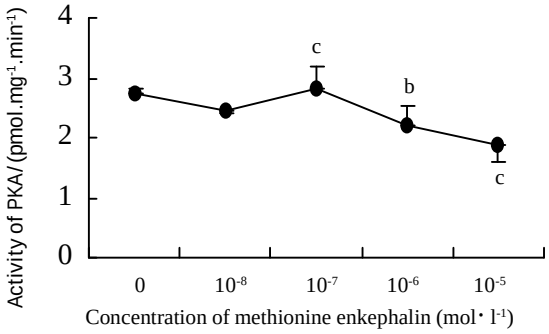

Figure 4 The influences of different concentrations of methionine enkephalin on the intracellular activity of PKC in NS-1 cells. n=3 from 3 independent experiments. ^{b}P<0.05 and ^{c}P<0.01 *vs* control (0 mol·L^{-1}).

Antagonism of monoclonal antibody and naloxone on the activity of PKC

Based on the data in Figure 4, 1×10^{-6} mol· L^{-1} of methionine enkephalin was used to inhibit the PKC. Like observed in the case of PKA, the effect of methionine enkephalin could be blocked by different concentrations of monoclonal antibody (Table 2). Naloxone at concentrations of 1×10^{-6} and 1×10^{-5} mol· L^{-1} could also reverse the suppressed effect of methionine enkephalin in the cytosol (Figure 5).

Table 2 Antagonism of different of concentrations of monoclonal antibody (MAb) to methionine enkephalin (MENK) at 1×10^{-6} mol·L^{-1} on the intracellular activity of PKC in NS-1 cells. n=3 from 3 independent experiments. ^{b}P<0.05 *vs* control, ^{e}P<0.05 and ^{f}P<0.05 *vs* group 2

MENK+MAb	Activity of PKC (pmol·mg^{-1}·min^{-1})
Control	3.06±0.19
MENK	2.26±0.03^{b}
MENK+10^{-11} mol·L^{-1} MAb	2.92±0.02^{e}
MENK+10^{-10} mol·L^{-1} MAb	2.80±0.4^{e}
MENK+10^{-9} mol·L^{-1} MAb	3.13±0.45^{ff}

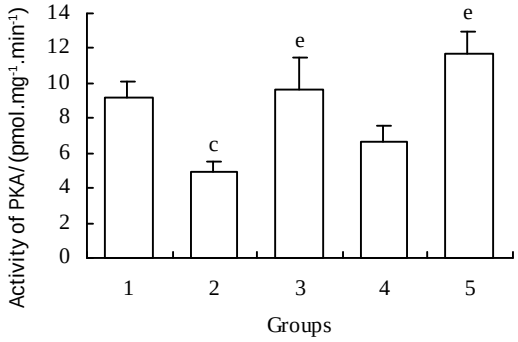

Figure 5 The effects of different concentration of naloxone on the intracellular activity of PKC in NS-1 cells. Treatment groups: 1: control; 2: 1×10^{-6} mol·L^{-1} methionine enkephalin 3: 1×10^{-6} mol·L^{-1} methionine enkephalin plus 1×10^{-7} mol·L^{-1} naloxone; 4: 1×10^{-6} mol·L^{-1} methionine enkephalin plus 1×10^{-6} mol·L^{-1} naloxone; 5: 1×10^{-6} mol·L^{-1} methionine enkephalin plus 1×10^{-5} mol·L^{-1} naloxone. n=3 from 3 independent experiments. ^{c}P<0.01 *vs* control, ^{e}P<0.05 *vs* group 2

DISCUSSION

The biological and clinical effects of opiate interaction with immune cells are well appreciated. In recent years, investigations from several laboratories have indicated that opioids can operate as cytokines, the principal communication signals of the immune system[1,2]. Our previous studies have also proved the cellular modulation of methionine enkephalin[3,4]. In this experiment, the results that methionine enkephalin could enhance the proliferation of NS-1 cells and perform the effect of growth factor-like, were consistent with the conclusion.

One possible component in the receptor signal cascade that can be responsible for these differences is the ligand-receptor interaction site. Receptor chimera studies followed by mutational analysis have revealed that functions of receptor domains were different for various opioid alkaloids and opioid peptides[14-19]. Based on the principle of antigen determinant, we prepared the monoclonal antibody against delta opioid receptor. Our data showed that the effects of methionine enkephalin were reversible in the presence of different concentration of monoclonal antibody, which indicating the existence of a functional domain at the peptides segment.

Although some laboratories have provided evidences that supporting agonist-induced down-regulation of opioid receptors appear to require the phosphorylation of the receptor protein[20-24], the identities of the specific protein kinases that perform this task remain uncertain. Moreover, it is unknown whether the change of protein kinase activation was dependent on the effect of methionine enkephalin. Our data showed that the activities of PKA were up-regulated in both cytosol and membrane of NS-1 cells in a variety of concentrations of methionine enkephalin. The elevation of PKA activity showed dose-independent and the most efficient concentration of methionine enkephalin was at 10^{-7} mol· L^{-1}. It had been shown that increased PKA activity related to the maintenance of cellular tolerance to opioid receptor agonists[10, 25,26].

However, in the case of PKC, the enzyme activity was elevated when methionine enkephalin at the concentration of 1×10^{-7} mol· L^{-1} and declined gradually at 1×10^{-6} to 10^{-5} mol· L^{-1}. The coincident results have also been observed in other laboratory. It had been reported that a biphasic response of opioid on expression of some cytokines had been demonstrated that nanomolar concentration of opioid augmented the secretion of both IL-6 and TNF-alpha, whereas micromolar concentration inhibited their synthesis[27]. It had also been reported that opioid-induced PKC translocation followed a time-dependent and biphasic pattern beginning 2 h after opioid addition, when a pronounced translocation of PKC to the plasma membrane occurred. When exposure to opioids was lengthened to >12 h, both cytosolic and particulate PKC levels dropped significantly below those of control-treated cells because of the decrease of membrane-bound PKC density[5,28].

From our data, the effect of methionine enkephalin could be reversed by a lower concentration of monoclonal antibody. Although little previous information was available to compare the usage of antibody, it was postulated that a complex ligand-receptor interaction was involved. The results that an antagonism of monoclonal antibody to the effect of methionine enkephalin on the activity of PKA or PKC indicated that the antigen determinant of the receptor fragment was also the functional domain of the receptor. The postulate was reinforced by studies involving μ/δ receptor chimeras that investigated the function of each domain[14,15]. Likewise, the same results could be observed in the assay of naloxone, the antagonist of opioid receptor. The effect of naloxone abolishing the effect of opioid on the activity of PKA had been reported[29,30]. Thus, a traditional opioid mechanism on signaling pathway of PKA and PKC was thereby involved.

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