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Original Article

Replacement of Serine^{363} and Serine^{375} Codons by Alanine in Rat μ-Opioid Receptor cDNA

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Abstract

The aim of this study was to use site directed mutagenesis technique to construct a vector in which serine^{363} and serine^{375} residues of the COOH-terminal portion of the μ-opioid receptor (MOR) were substituted by alanine. These constructs are essential in studying G-protein coupled receptor kinase-mediated MOR desensitization. The nested PCR carried out for conversion of serine^{363} and serine^{375} to alanine resulted in the production of a band comparable to the expected size of 1400 bp. Restriction analysis of these bands confirmed the integrity of the PCR products. Ligation of the mutated PCR product into pcDNA3 and its digestion with appropriate restriction enzymes further confirmed the integrity of the PCR product and its orientation into the vector.

Keywords: Opioid receptor; pcDNA3; Serine^{363}; Serine^{375}.
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1. Introduction

Morphine and endogenous opioid peptides exert their multiple biological effects including analgesia by interacting with μ-opioid receptors (MOR) [1]. The utility of opiate drugs for treatment of chronic pain is hindered by the development of tolerance [2, 3]. Tolerance to opiate occurs on continued use, such that the amount of drug required to elicit pain relief must be increased to compensate for administrated responsiveness [3]. Decreased responsiveness to agonists has been shown to correlate with desensitization and internalization of G-protein-coupled receptors [4]. Being a member of superfamily of G-protein-coupled receptors, MOR activity, following the repeated exposure to agonists, is also rapidly attenuated by the process of desensitization [5].

To better understand the molecular mechanism of MOR desensitization, specific amino acids of the MOR involved in G-protein recognition and activation must be identified. Molecular cloning of μ, σ, and κ opioid receptors has made it possible to investigate the structural determinants of opioid receptors involved in the signal transduction process and the regulation of receptor function [6, 7]. Site-directed mutagenesis studies have shown that the third intracellular loop or the cytoplasmic C-terminal loop of the G-protein-

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coupled receptor contains critical domains required for recognizing and activating G-proteins [8-10]. In most G-protein-coupled receptors, the receptor desensitization, is mostly initiated by G-protein-coupled receptor kinase (GRK)-mediated phosphorylation of serine and/or threonine residues within the third intracellular loop and the C terminus [11]. Similar to other G-protein-coupled receptors, GRK is likely to induce MOR desensitization by phosphorylating serine and theronine residues within the third intracellular loop of cytoplasmic tail [12, 13]. Recent studies using various C-terminal deletion mutants of rat MOR indicated that Ser^{363} and Ser^{375} residues are phosphorylation sites [5]. The aim of the present study was to use site directed mutagenesis technique to construct receptor mutants in which 363 and 375 Ser residues of the COOH-terminal portion of the MOR were substituted to alanine. These constructs are essential in studying the role of GRK-mediated MOR desensitization.

2. Materials and methods

2.1. Materials

Oligonucleotides were synthesized by Fazabiotech (Tehran, Iran). Taq polymerase and restriction enzymes were obtained from Bio-tools (Tehran, Iran). DNA ligase enzyme and DNA molecular weight marker were from Fermentase (Tehran, Iran). Gel extraction kit was purchased from Qiagen (Tehran, Iran). The rat MOR cDNA subcloned in the $pcDNA_3$ was kindly provided by Professor G. Henderson (Bristol, UK). All other chemicals were purchased from Fazapazho (Tehran, Iran).

2.2. Construction of an expression vector containing mutant MOR cDNA

Rat MOR was mutated by oligonucleotide-directed mutagenesis using the following forward and reverse oligonucleotides with the desired mutations. For conversion of Ser-363 to alanine, forward primer Omid1 (26-mer; 5´GAACAGCAAAACACCACTCG AGTCCG3´; positions 1100 to 1075 of the coding sequence of MOR) and reverse primer Omid2 (26-mer; 5´GAACAGCAAAACTC CACTCGAGTCCG3´ positions 1075 to 1100) were developed. PCR Omid2 with forward Nested1 primers (GTGGATAGCG-GTTTGACT) flanked a 1344-bp fragment of the MOR gene. For the second round of the nested PCR, primer Omid1 with reverse Nested2 primers (5´CTTCTTTCCGCCTCA-GA 3´) flanked a 413-bp fragment. The second round of the PCR was carried out using one pair of primer that were designed to flank the mutated regions, forward FpcDNA3´: 5´ GAACCCACTGCTTACT G3´; Reverse RpcDNA: 5´GGTCAAG GAAGGCACGG3´.

For conversion of Ser^{375} to Ala, forward primer Mem1 (30-mer; 5´ CTAGGGAA CATCCCGCAACGGCTAATACAG 3´; positions 1138 to 1108 of the coding sequence of MOR) and reverse primer Mem2 (26-mer; 5´ CTGTATTAGCCGTTGCGGGATGTTC-CCTAG 3´ positions 1108 to 1138) were developed.

PCR Mem2 with forward Nested1 primers flanked a 1382-bp fragment of the MOR gene. For the second PCR, primer Mem1 with reverse Nested2 primers flanked a 390-bp fragment. The second round of the PCR was carried out using one pair of primer that was designed to flank the mutated regions, forward: 5´ GAACCCACTGCTTACTG3´; Reverse 5´ GGTCAAGGAAGGCACGG 3´. Ten microliters of cDNA of rat MOR (10 ng) was amplified in a 50 µl reaction mixture containing 2.5 µM of each primer, 5 U of Taq DNA polymerase, 0.5 mM (each) deoxyribonucleotide triphosphate, 2 mM $MgCl_2$ and 1×PCR buffer. The thermocycling profile was as follows: denaturation at 94 °C for 5 min.; 35 cycles of 94 °C for 1 min., 55 °C for 2 min., and 72 °C for 3 min.; and a final extension at 72 °C for 20 min. PCR-amplified

DNA fragments were observed by agarose gel electrophoresis in 0.7% agarose gel. Ten microliters of each amplification mixture and the molecular mass marker were subjected to agarose gel electrophoresis and ethidium bromide staining. Gels were run at 90 V for 30 to 45 min. The amplified DNA fragments were visualized by UV illumination and were stored on computer.

All digestions were performed using the suitable restriction enzymes at 37 °C for 1 h. A DNA extraction kit was utilized for isolation of the DNA bands from the agarose gel. The vector and insert (molar ratio of 1/3, respectively), DNA ligase and its buffer were added at a final volume of 20 μl and were incubated at 16 °C overnight. The product of ligation was transformed using HB101 competent cells. The heat shock was performed for 1 min. at 42 °C. The cells were spread over LB plates containing ampicillin and were incubated overnight at 37 °C. The obtained colonies were cultured in LB medium at 37 °C overnight. Plasmid preparation was carried out using manual plasmid preparation [14].

3. Results

3.1. Conversion of Serine^{363} codon to Alanine in MOR

The first round of the PCR carried out with Omid2 + Nested1 and Omid1 + Nested2 primers. Using the product of the above PCR reaction as templates, the second round of the PCR was carried out with FpcDNA3 and RpcDNA3 primers. A band of approximately 1400 bp was observed on 0.7% agarose gel as shown in Figure 1a. Restriction digestion of the mutated MOR with *Xba*I and *Hin*dIII produced a band of 1200 bp which further confirmed the integrity of the nested PCR product (data not shown). Having confirmed the integrity of the MOR DNA on the gel, the corresponding band was purified from the gel and then prepared for ligation into pcDNA3. After the ligation of the insert into the pcDNA3, the plasmid containing the mutated receptor gene was cut with *Nco*I enzyme. The digestion of the recombinant plasmid with *Nco*I restriction enzyme would produce four bands of 590, 730, 1970, 3400 bp. Figure 1b, lane 2 shows that these bands were obtained.

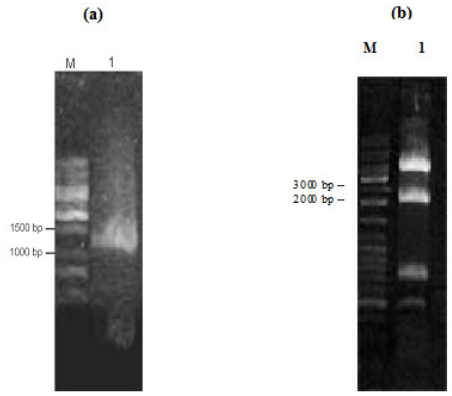

Figure 1. Site directed mutation Ser^{363} to Ala in MOR and cloning of the mutated PCR product into the pcDNA3. (a) Nested PCR amplification of mutated (Ser^{363}) MOR using primers Omid1, Omid2, Nested1 and Nested2. Lanes M, DNA molecular size marker; 1, the nested PCR product of the mutated MOR. (b) Lanes M, DNA molecular size marker; 1, Digestion of the recombinant pcDNA3 containing the mutated MOR gene with *Nco*I enzyme. Ten μl of samples were applied to a 0.7% agarose gel for electrophoretic separation.

3.2. Conversion of Serine^{375} codon to Alanine in MOR

The first round of the PCR carried out with Mem2 + Nested1 and Mem1 + Nested2 primers. Using the product of the above PCR reaction as templates, the second round of the PCR was carried out with FpcDNA3 and RpcDNA3 primers. A band of approximately 1400 bp was observed on 0.7% agarose gel as shown in Figure 2a. Presence of two restriction sites at each end of MOR allowed the digestion of the mutated product with *Xba*I and *Hin*dIII to yield a band of 1200 bp (data not shown). Having confirmed the integrity of the MOR DNA, the corresponding band was purified from the gel and prepared for ligation into pcDNA3. After the ligation of the insert into the pcDNA3, the plasmid containing the mutated receptor gene was cut with NcoI enzyme. The digestion of the recombinant plasmid with *Xba*I and *Hin*dIII restriction enzymes produced a 1200 bp band which corresponds to the MOR insert (Figure 2b).

4. Discussion

It is commonly known that receptor responsiveness decreases following the repeated exposure to agonists. Decreased responsiveness to agonists has been shown to correlate with desensitization and internalization of G-protein-coupled receptors [4]. MOR activity, following the repeated exposure to agonists, is also rapidly attenuated by the process of desensitization [5]. To understand the molecular mechanism of MOR desensitization, two phosphorylation sites on the third intracellular loop of cytoplasmic tail were mutated using site directed mutagenesis technique. These two phosphorylation sites were the serine residues 363 and 375 on MOR that are required for recognizing and activating G-proteins [8, 10]. The C-terminal tail and the third intracellular loop of various G-protein-coupled receptors contain numerous serine and threonine residues that are potential phosphorylation sites for GRKs.

Site directed mutation of the MOR leads to the conversion of Ser^{363} and Ser^{375} to Ala. Restriction analysis of these PCR products

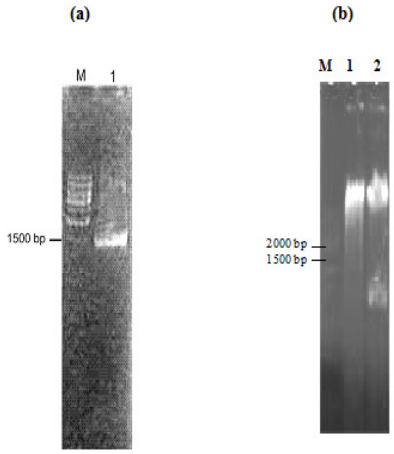

Figure 2. Site directed mutation Ser^{375} to Ala in MOR and cloning of the mutated PCR product into the pcDNA3. (a) Nested PCR amplification of mutated (Ser^{375}) MOR using primers Mem1, Mem2, Nested1 and Nested2. Lanes M, DNA molecular size marker; 1, the nested PCR product of the mutated MOR. (b) Lanes M, DNA molecular size marker; lanes 1, the wild type uncut pcDNA3 plasmid, lane 2 is the samples from recombinant pcDNA3 containing the mutated MOR gene that was digested with *Xba*I and *Hin*dIII restriction enzymes. 10 µl of samples were applied to a 0.7% agarose gel for electrophoretic separation.

with various enzymes confirmed the integrity of the produced bands. Having confirmed the mutation in the MOR, the gene was inserted into the pcDNA3 vector and then again analyzed with restriction enzymes. Restriction site analysis is quite suitable method for confirming the integrity of the products and also the orientation of the insert into the vector. All of the these analysis confirmed the present of mutated product in the vector and it is now possible to conduct a functional assay in order to measure the cAMP accumulation in mammalian cells transfected with these plasmids.

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