Discrimination between peptide and non-peptide opioid agonists on the transcription of opioid receptors in two cell lines

Yun Wang, Xiao-Min Wang, Ji-Sheng Han*

Neuroscience Research Institute, Peking University, Beijing, 100083, People’s Republic of China

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Abstract

The aim of the present study was to characterize the effects of prolonged use of peptide μ- and δ-receptor agonists [D-Ala², N-me-phe, Gly⁵-ol]-enkephalin (DAMGO) and [D-Pen², D-Pen⁵]-enkephalin (DPDPE) and non-peptide agonists ohmefentanyl (OMF) and BW373U86 on the transcription of opioid receptors of cultured NG108-15 cell and SHSY5Y cells, respectively using the method of reverse transcription-polymerase chain reaction (RT-PCR). It was found that (1) The abundance of μ- and δ-receptor mRNA decreased significantly up to 48h after the administration of DAMGO and DPDPE, respectively; whereas the inhibitory effect of OMF and BW373U86 lasted only for 24h; (2) DAMGO and DPDPE produced a significant decrease of the mRNA coding for μ-receptor and δ-receptor at concentrations as low as 10⁻⁸ mol/L and 10⁻⁶ mol/L, respectively, whereas OMF and BW373U86 were effective at concentrations one order of magnitude higher, respectively. These results suggested that (1) Long-term administration of either peptide or non-peptide opioid agonist to cultured cell line produced a significant decrease of the gene expression of opioid receptor at transcription level. (2) The effect of peptide agonists was stronger and lasted longer than that of corresponding non-peptide agonists. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Peptide opioid agonist; Non-peptide opioid agonist; Long-term exposure; Gene expression; Opioid receptor

Introduction

Prolonged exposure to morphine and other opioid leads to tolerance and dependence. Tolerance is manifested by a reduced effect upon repeated exposure to a drug, and in the need for increased amount of drug to achieve the same effect. Despite intensive research, very little is known about the underlying cellular mechanisms leading to opioid tolerance.
ations at the level of opioid receptors after prolonged morphine treatment are ambiguous, whereas the alterations at the number of opioid receptors after prolonged highly selective opioid receptor agonist treatment are unanimous [1–3].

The cloning of the δ [4] and μ [5] opioid receptors cDNA has made it possible to investigate whether the expression of opioid receptor genes is altered after prolonged opioid exposure.

In the present study, we measured the levels of mRNA coding for μ and δ opioid receptors in SHSY5Y and NG108-15 cells respectively after the two cell lines were sustainedly exposed to peptide and non-peptide opioids to address the possible changes in the transcription of genes coding for μ and δ opioid receptors.

Materials and Methods

Chemicals

Chemicals and reagents were obtained from the following sources: DAMGO, DPDPE, 8-bromo-cAMP, guanidinium, agarose and retinoic acid from SIGMA, DMEM and RPMI1640 from GIBCO Company, PPO from PACKARD, POPP from SERVA, AMV reverse transcriptase, RNase inhibitor and dNTPs from PROMEGA. Omefentanyl (OMF) was a generous gift from Professor Z. Q. Chi of the Institute of Materia Medica Chinese Academy of Sciences and BW373U86 from Dr. K.R. Chang of B. Welcome Co.

Cell culture

SHSY5Y cells were cultured in RPMI1640 containing 15% (v/v) fetal bovine serum; NG108-15 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal bovine serum and hypoxanthine/aminopterin/thymidine. Cells were maintained in a humidified incubator at 5% CO₂ and 95% O₂.

Cell differentiation

SHSY5Y cells and NG108-15 cells were induced to differentiate by culturing with 10 μmol/L Retinoic acid (RA) for 6 days and 8-bromo-cAMP for 3 days respectively. When cells were between 80% and 100% confluent, the medium was changed to that containing RA or 8-bromo-cAMP. Medium change was performed every other day for the duration of the treatment (totaling 3 times for RA, twice for 8-bromo-cAMP)

Drug administration and cell collection

To observe the time-response relationship, differentiated SHSY5Y cells were exposed to μ opioid receptor agonists DAMGO (peptide) and OMF(non-peptide), whereas NG108-15 cells were exposed to δ opioid receptor agonists DPDPE (peptide) and BW373U86 (non-peptide), respectively. Cells were collected at 0, 1, 3, 12, 24 and 48 h after the administration of the agonists. To observe the dose-response relationship, cells were incubated in the medium containing different doses of opioid agonists (0, 10⁻⁹, 10⁻⁸, 10⁻⁷, 10⁻⁶, 10⁻⁵ mol/L) and the cells were collected at 24h after the drug administration.
RNA isolation

Total RNA was extracted from the collected cells using the single-step method [6] and the RNA was quantified with UV spectrophotometer.

Oligonucleotide primers

Primers were synthesized according to sequences published elsewhere [4,7,8]. The oligonucleotide primers complementary [downstream primers (1)] and identical [upstream primers (2)] to mRNA sequences were chosen in different exons as shown in table 1.

Semi-quantitative reverse transcription-PCR

Specific first-strand cDNA synthesis

(1) cDNA complementary to μ receptor mRNA: 1.5 μg of total RNA from each sample was heated for 3 min, and then reverse transcribed by incubating with 100 pmol/L of specific downstream primers of μ opioid receptor and β-actin, 5 mmol/L MgCl₂, 10 mmol/L Tris-HCl, 0.1% TrionX-100, 1 mmol/L each of dATP, dGTP, dCTP, dTTP, 1U/μl RNAasin and 15 U AMV reverse transcriptase (final volume 20 μl) for 90 min at 42°C.

(2) cDNA complementary to δ receptor mRNA: The procedure of reverse transcription of δ receptor was the same as μ receptor, except that the downstream primers were changed to that of δ receptor and the internal control GAPDH. The samples were then heated at 95°C for 5 min to terminate the reverse transcription.

PCR amplification

(1) Single amplification of μ receptor and internal control β-actin cDNA: μ receptor and β-actin cDNA were amplified in different tubes. 10μl resulting cDNA was added into each tube, overlaid with 50 μl liquid paraffin, denatured at 94°C for 20 min, then added 4 μl 5× Taq Polymerase buffer, 5 μl 25 mmol/L MgCl₂, 0.5 μl 10 mmol/L dNTP, 1 μl [α-32P]dCTP(1 μCi), 100 pmol μ receptor or β-actin 5′ primer, 3U Taq Polymerase into each tube, the final reaction volume was 100 μl. They were mixed and centrifuged. Amplification of μ receptor cDNA was performed in sequential cycles at 94°C, 1 min, 5°C, 1 min, and 72°C 1.5 min. Amplification was proceeded for 28 cycles. The condition of β-actin PCR was: 94°C, 1 min, 65°C, 1 min, 72°C, 1 min, for 20 cycles. After the last cycle, all samples were incubated for

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Primer sequence (5’—3’)</th>
<th>Predicted length of the amplified fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ opioid receptor, rat</td>
<td>1:atc ttc acc acc ccg ttc gaa ccg</td>
<td>356bp</td>
</tr>
<tr>
<td>μ opioid receptor, rat</td>
<td>1:tgg gcc acc gcc ag</td>
<td>525bp</td>
</tr>
<tr>
<td>β-actin</td>
<td>1:tgg aga gct atg gac tgc ctg</td>
<td>201bp</td>
</tr>
<tr>
<td>GAPDH</td>
<td>1:ttc ttc aag att gtc gac aa</td>
<td>309bp</td>
</tr>
</tbody>
</table>

Table 1
Sequence of synthetic oligonucleotide primers used for the analysis of cDNA-PCR kinetics and mRNA
an additional 10 min at 72°C. 10 μl of the products were electrophoresed in 2% agarose gels. Bands were cut from gel and put into the scintillation bottles, dried at 50°C for 5~6 h. Scintillation counting was performed in 5 ml of scintillation liquid. Ratio of μ receptor and β-actin cpm was taken as the relative content of μ receptor mRNA of each sample.

(2) Co-amplification of δ receptor and the internal control GAPDH: The procedure was similar as the single amplification, except that δ receptor and GAPDH were amplified in one tube. Ratio of δ receptor and GADPH cpm was taken as the relative content of δ receptor mRNA of each sample.

**Data processing and statistical analyses**

The results were expressed as mean±SEM. Continuous data were processed with analysis of variance (ANOVA), discrete data were treated with Kruscal-Wallis ANOVA. A P value of less than 0.05 was considered statistically significant.

**Results**

*The relationship between PCR cycles and the yield of final products*

Under above mentioned experimental condition, samples were taken every other two cycles and bands were cut after the samples were electrophoresed and the content of amplified DNA was detected. The results are shown in Fig.1 and Fig. 2. Fig.1 shows the real amplification efficiency of μ opioid receptor and β-action cDNA fragments. It can be seen that the yield of cDNA increased with the number of cycle within 26 (β-actin) and 30 (μ receptor) cycles. So we chose 20 and 26 cycles for β-actin and μ opioid receptor, respectively. Fig. 2

![Fig. 1. The relationship between PCR cycles and the yield of products of μ opioid receptor and internal control β-actin. A. Electrophoretogram of the yield of products of β-actin and μ receptor. Numerals indicate the number of cycles. M: PBR322/MSPI marker. B. The results of scintillation counting.](image-url)
shows the real amplification efficiency of δ opioid receptor and GAPDH cDNA fragments. The yield increased along with the increase of the number of cycles within 32 cycles, so 28 cycles was chosen for the amplification both δ receptor and GAPDH.

**Effect of long-term administration of μ opioid receptor agonists on the transcription of opioid receptor**

*The time course of the effect of peptide (DAMGO) and non-peptide (ohmfentanyl, OMF) μ agonists on the transcription of μ opioid receptor*

In this experiment, μ−receptor enriched cell line SHSY5Y was used. Differentiated cells were exposed to 10⁻⁵ mol/L DAMGO and OMF respectively for various time periods (0, 1, 3, 12, 24, 48h), then the abundance of μ receptor mRNA of every sample was detected using RT-PCR method. Fig.3A shows that the level of μ receptor mRNA did not change at 1 and 3h, but decreased significantly 12h, 24h and 48h after the administration of DAMGO. The inhibit rate was 24%, 45% and 65% respectively (P<0.01). Fig.3B shows that the non-peptide μ agonist OMF produced a significant decrease in μ-receptor mRNA 12h (−57%, P<0.01) and 24h (−70%, P<0.01) after its administration, but not at 48h. The results suggest that exposure of either DAMGO or OMF (10⁻⁵ mol/L) for 12h and 24h produces a significant decrease of the mRNA coding for μ-receptor.

*The dose-effect relationship of DAMGO and OMF on the transcription of μ-receptor*

Based on the findings mentioned above, we chose 24h as the time point to observe the dose-effect relationship. The result shown in Fig.3C indicate that DAMGO produced a significant decrease of the μ-receptor mRNA at 10⁻⁸~10⁻⁵ mol/L, with inhibition rate of 46%, 48%, 80% and 73% respectively, but not at 10⁻⁹ mol/L. In contrast, OMF produced a significant decrease of the μ-receptor mRNA at 10⁻⁷~10⁻⁵ mol/L, with inhibition rate of 36%, 32% and 51% respectively, but not at 10⁻⁹~10⁻⁸ mol/L. (Fig. 3D).
Effect of long-term administration of δ opioid receptor agonists on the transcription of δ receptor

The time course of the effect of peptide (DPDPE) and non-peptide (BW373U86) δ-receptor agonists on the transcription of δ opioid receptor

The effect of DPDPE and BW373U86 on the transcription of δ opioid receptor was observed in a δ receptor enriched cell line NG108-15. It was found (Fig. 4A) that the δ-receptor mRNA did not change at 1, 3 and 12h, but decreased significantly (P<0.01) 24h and 48h after the administration, with the inhibition rate of 51% and 21%, respectively. The non-peptides δ-agonist BW373U86 produced a significant decrease of δ-receptor mRNA 24h (−20%) but not 48h (−14%) after its administration.

The dose-effect relationship of DPDPE and BW373U86 on the transcription of δ opioid receptor

The effect of δ opioid agonists was observed 24h after its administration. DPDPE produced a significant decrease of the abundance of δ-receptor mRNA at 10^{-6} mol/L and 10^{-5} mol/L, with inhibition rate of 27% and 29%, respectively (P<0.01), but not at 10^{-9}~10^{-7} mol/L. The non-peptide BW373U86 did not produce any significant decrease in δ-receptor mRNA at the concentration ranging from 10^{-9} mol/L~10^{-7} mol/L. It was only at the concentration of 10^{-5} mol/L when the abundance of δ-opioid receptor mRNA was decreased by 40% (P<0.01) (Fig. 4D).
Discussion

It remains controversy whether the number and affinity of opioid receptors manifest significant changes during morphine tolerance. Some author reported no change in the density of \( \mu \) opioid receptors during tolerance, while others have shown an increase or decrease in receptor number. Results became unanimous when highly selective \( \mu \)- and \( \delta \)-opioid agonists were used instead of morphine the number of opioid receptors decreased significantly with the declining of the analgesic effect of opioid agonists [2,9]. In contrast to the in vivo studies, results from the cultured cell lines were rather consistent. The number of opioid receptors decreased when the cells enriched with \( \mu \)- or \( \delta \)-opioid receptor were exposed to selective opioid agonists for a period of more than 12h [1,3,10,11].

Concerning the possible mechanisms of opioid receptor down-regulation, it could be due either to the internalization of receptors or to the blockade of receptor biosynthesis. While a decrease in receptor transcription may result in a lowering of the receptor number, this may not apply in all the cases. Kim et al reported that in a study on NG108-15 cells immersed in a etorphine solution, a decrease in the number of \( \delta \) opioid receptor occurred 24h prior to a decrease in \( \delta \) receptor mRNA [12], suggesting that opioid agonist-induced down-regulation of opioid receptor may occur at protein level (e.g. internalization) prior to the changes at gene expression level. Similar results were obtained in vivo and in vitro studies using the method of in situ hybridization [13] and solution hybridization respectively [14]. The level of \( \delta \) and \( \mu \) receptor mRNA decreased obviously in NG108-15 cells and central nervous system after long term administration of \( \delta \) agonist etorphine, DADLE and \( \mu \) agonist morphine respectively.
The change in opioid receptor gene expression during prolonged opioid agonist exposure can be studied with different approaches, for example, Northern Blotting [12], *in situ* hybridization [13] and solution hybridization [14] etc. Compared to in vivo studies, study on cultured cell lines may generate more clear and precise data. However, even in cultured cells, results can still be heterogeneous and difficult to compare when different cell lines, cells in different status of differentiation and different opioid agonists are used. For example, Kim et al observed significant changes of δ opioid receptor expression in NG108-15 cells 24 h after the administration of etorphine (a non-peptide agonist) [12], whereas Bazus et al, using the same cell line, could not find any decrease of the abundance of δ opioid receptor mRNA 5 h or 24 h after the exposure to the δ opioid agonist DSLET (a peptide agonist) [15]. The discrepancy may have been resulted from difference in the characteristics of the two δ opioid agonists.

In the present study, we focused on attention to the effects produced by peptide or non-peptide opioid agonist, respectively on the transcription of opioid receptor in the same cell line with same degree of cell differentiation. NG108-15 cell contains only δ opioid receptor, whereas SHSY5Y cells are equipped with both μ and δ opioid receptor. Theoretically it is possible to use SHSY5Y cells for the study of μ and δ opioid receptor. However, since in differentiated SHSY5Y cells, the number of μ opioid receptor is 5 times as much as δ receptors, the putative cross talk existing between the two types of receptors may result in a substantial effect of μ opioid receptor or δ opioid receptor. Therefore we chose to use two different cell lines with unique δ opioid receptor (NG108-15) and highly enriched μ opioid receptor (SHSY5Y), respectively.

The results showed a very clear agonist-induced suppression of the expressions of opioid receptor mRNA. This suppression was time dependent and dose dependent, and was applicable for both μ and δ receptors.

The most important findings in the present study were that effect of peptide agonists on opioid receptor gene transcription seems to be more potent and longer lasting than the corresponding non-peptide agonists. The difference was at least one order of magnitude, and was statistically very significant.

A survey of the literature revealed that 25 nmol/L opioid agonist etorphine was reported to cause a decrease of the affinity of δ opioid receptor toward peptide opioid agonists or antagonists but not toward that of non-peptide ligands [16]. In another study, cholecystokinin (CCK) was shown to inhibit the excitatory effect produced by non-peptide agonist on hippocampal pyramidal cells, but not that produced by peptide agonist [17]. To our knowledge, there is no report so far concerning the discrimination between peptide and non-peptide on agonist which of the transcription of opioid receptors. This is therefore the first to report this phenomenon.

Concerning the possible mechanisms underlying the differential effects of the peptide and non-peptide opioid agonists in modulating opioid receptor gene expression, the following may be taken into consideration: (1) Peptide and non-peptide agonists may interact with different binding sites on the same receptor. Gether found that NK₁ (SP) receptor possesses different binding sites for peptide and non-peptide agonists [18]. Similar findings were obtained in CCK-B receptor [19]. It was also reported that different binding sites for peptide and non-peptide opioid agonists exist on the μ- and κ-opioid receptor [20]. (2)
efficacy of peptide agonists may be stronger than that of non-peptide agonists. (3) The interaction between opioid receptors and endogenous opioid peptides in central nervous system may adapt reciprocally in the long evolution process, whereas exogenous non-peptide agonists may just mimic all the regulatory process of endogenous opioid peptides. That non-peptide opioid agonists are not as potent and long lasting as that of peptide agonist in suppressing opioid receptor transcription may just reflect an evolutional reminiscent of the two kinds of agonists. In addition, the extent of receptor phosphorylation, coupling to effectors induced by peptide and non-peptide agonists may be different at least quantitatively, if not qualitatively.

Acknowledgments

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