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Possible Involvement of β -Endorphin in a Loss of the Coordinated Balance of μ -Opioid Receptors Trafficking Processes by Fentanyl

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Keywords

internalization/recycling pathway; opioids; receptor trafficking; fentanyl

BACKGROUND

It has been considered that opioid tolerance is, in part, the end result of a coordinated balance between processes that govern the desensitization, internalization, and resensitization of μ -opioid receptors (MOR) (Claing et al., 2002; Gainetdinov et al., 2004). However, a several line of evidence suggests that the trafficking properties of MORs driven by MOR agonists may depend on intrinsic characters of each agonist, and are still complicated. Previous biochemical studies on cultured enteric neurons have indicated that fentanyl induces either the functional desensitization or internalization of MORs (Minnis et al., 2003). In contrast, under the same condition, morphine does not promote the detectable internalization of MORs in cultured cells after prolonged or acute treatment in healthy animals, although it has been well-established that morphine causes the development of tolerance to its pharmacological actions (Minnis et al., 2003). However, recent studies have demonstrated that morphine activates MORs with promoting internalization of MORs via β -arrestin-2-dependent mechanisms in striatal neurons (Haberstock-Debic et al., 2005).

In the previous study, we demonstrated that repeated treatment with fentanyl, but not morphine, causes a rapid desensitization to its ability to block the hyperalgesia associated

with the attenuation of MOR resensitization in mice with inflammatory pain (Imai et al., 2006). Based on this study, we hypothesized that released β -endorphin within the spinal cord under a chronic pain-like state may be implicated in the rapid development of tolerance to fentanyl, but not morphine and oxycodone. Namely, these findings raise the possibility that β -endorphin could attenuate the resensitization of MOR after the treatment with fentanyl, resulting in the high degree of tolerance to fentanyl-induced antihyperalgesic effects under long-lasting pain state. To further address this issue, this cell culture study was performed to investigate the effects of fentanyl on MOR internalization and resensitization in the presence or absence of β -endorphin.

MATERIALS AND METHODS

Baby hamster kidney (BHK) cells (Riken Cell Bank, Tsukuba, Japan) were grown in Dulbecco's modified eagle medium (DMEM: Invitrogen™) supplemented with 10% fetal bovine serum (FBS), penicillin (100 μ /ml), and streptomycin (100 μ m/ml) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Transient transfection was then performed with Effectene transfection reagent (Qiagen, Tokyo, Japan) in 0.2 μ g of each cDNA according to the protocol provided by the manufacturer. Cells were used in confocal microscopy 16–24 h after transfection. cDNA for rat MOR was kindly provided by Dr. Dascal (Tel Aviv University). Venus, a brighter variant of yellow fluorescent protein (Nagai et al., 2002) was obtained from Dr. T. Nagai (Riken, Wako, Japan). Primers (5'-GGG GTA CCC CAT GGA CAG CAG CAC-3') and (5'-GCG GCC GCG GGG CAA TGG AGC AGT-3') were engineered to ligate the N-terminus of MOR by using standard molecular approaches with the polymerase chain reaction (PCR). Venus-fused MOR was created by ligating the MOR cDNA sequences into the *NotI* site of the corresponding Venus site. cDNA for transfection in BHK cells was subcloned into pcDNA3.1 (Invitrogen™ Life Technologies, CA). cDNA for rat β -arrestin 2 was generously provided by Dr. Y. Nagayama (Nagasaki University, Japan). For the analysis of the agonist-induced internalization of MORs, BHK cells that had been transfected with Venus-fused MORs and β -arrestin-2 were incubated in the absence or presence of 100 nM β -endorphin for 30 min at 37°C, and then treated with 10 μ M morphine, 100 nM fentanyl or 10 μ M oxycodone. To investigate the resensitization of MORs, the cells were incubated with 100 nM fentanyl or 10 μ M oxycodone in the presence or absence of β -endorphin, and then apposed for 30 min, 90 min, 3 h, or 6 h at 37°C. The cells were subsequently fixed and examined by confocal microscopy as previously reported (Corbani et al., 2004). Venus was excited by a 488-nm laser was used to detect Venus fluorescence with a 505- to 530-nm band-pass filter, and images were obtained by placing the dish on the stage of an inverted Zeiss LSM510 META confocal microscope (Carl Zeiss, Jena, Germany). Data were stored on the hard disc with and analyzed with the Zeiss LSM software Zen 2009. For the quantitative analysis of agonist-induced internalization of MORs, BHK cells were fixed with 4% paraformaldehyde in PBS and stored at 4°C. The numbers of cells expressing Venus-fused MORs were counted. For counting cells whether Venus fluorescence was at the plasma membrane or in cytosol (internalization), we basically followed by Corbani et al. (2004). Localization of Venus-fused MORs in BHK cells was categorized as “mainly expressed at the plasma membrane,” “not detected in plasma membrane but detected in cytosol,” or “not detected” (whose localization was not belong to the former category), separated with a software Zen 2009 equipped with Zeiss LSM510 META confocal microscope, with reference to the control, not stimulated BHK cells. A total of 100 cells (counted mean 200–250 cells in sum of “the plasma membrane,” “in the cytosol,” plus “not detected”) in six independent each dish. % Internalization was described as cytosol $\times$ 100/[plasma membrane + cytosol (total 100 cells)]. The drugs used in this study were fentanyl citrate (Hisamitsu Pharmaceutical, Tokyo, Japan), morphine hydrochloride (Daiichi-Sankyo, Tokyo, Japan), oxycodone hydrochloride

(a kind gift from Shionogi Pharmaceutical, Osaka, Japan), and β -endorphin (Sigma-Aldrich, St Louis, MO), which were dissolved in assay buffer.

RESULTS AND DISCUSSION

In this study, we assessed whether β -endorphin could affect the trafficking properties of MORs using immunocytochemical methods in BHK cells with confocal microscope. Confocal imaging of the BHK cells expressing Venus-fused MOR with β -arrestin-2 revealed that the yellow fluorescence was largely confined to the plasma membrane (Figs. 1A and 2A). In both the presence and absence of 100 nM β -endorphin, at which concentration there did not cause any internalization of MORs (Figs. 1B and 1C), cells expressing MORs treated with 10 μ M morphine (Figs. 1C and 1D) showed little internalization of MORs, while the cells treated with 100 nM fentanyl (Figs. 1E, 1F, and 2B) and 10 μ M oxycodone (Figs. 1G and 1H) showed robust internalization of the receptor. These findings were consistent with previous reports that fentanyl and etorphine caused partial internalization, while morphine failed to induce detectable MOR endocytosis (Koch et al., 2005). We next investigated the resensitization properties of MORs after the washing-out of agonists. In the absence of β -endorphin, internalized MOR returned to the plasma membrane from 90 min after the washing-out of fentanyl (Figs. 3B–3D). However, in the presence of β -endorphin, the internalized MOR induced by fentanyl remained in the cytosolic fraction at 3–6 h after the washing-out of β -endorphin and fentanyl (Figs. 3F–3H). However, in both the presence and absence of β -endorphin, the internalized MOR induced by oxycodone returned to the plasma membrane after the washing-out of agonist in a time-dependent manner (Figs. 3I–3P). We performed quantitative analysis of the agonist-induced internalization of MORs after the washing-out of each agonist shown in Materials and Methods. At 30 min after the washing-out of agonists, cells treated with fentanyl or oxycodone showed robust internalization of MORs (fentanyl: $79.0 \pm 5.14\%$, β -endorphin fentanyl: $80.2 \pm 3.7\%$, oxycodone: $70.5 \pm 7.09\%$, β -endorphin oxycodone: $70.7 \pm 5.35\%$), which was not seen in morphine-treated cells (morphine: $19.67 \pm 3.93\%$, β -endorphin morphine: $21.5 \pm 4.76\%$; Fig. 3Q). However, while there was no difference in the degree of oxycodone-induced MOR internalization between the presence and absence of β -endorphin 3 h after washing-out (oxycodone: $23.17 \pm 5.12\%$, β -endorphin oxycodone: $30.5 \pm 4.72\%$), in fentanyl-treated cells, β -endorphin caused the prolonged internalization of MORs and fluorescence was stayed in the cytosolic fraction (fentanyl: $27.67 \pm 5.47\%$, β -endorphin fentanyl: $76.5 \pm 6.02\%$; Fig. 3R).

It has been widely accepted that receptor desensitization, internalization and trafficking appear to play a key role in the development of opioid tolerance (Clainig et al., 2002; Gainetdinov et al., 2004). The initial process in these events is the phosphorylation of intracellular domains of MOR. Phosphorylated MORs are mostly internalized via clathrin-coated pits into early endosomes and subsequently dephosphorylated by intracellular protein phosphatases. The dephosphorylated MORs might either be recycled to the plasma membrane or transported to lysosomes for degradation. A growing body (Smalheiser and Lugli) of evidence suggests that among diverse serine/threonine (Thr) residues of the intracellular domain of MOR, the phosphorylation of Ser 375 in the mouse MOR is essential for the internalization of MORs (Schulz et al., 2004). In a previous study, we found that repeated treatment with fentanyl, but not morphine, resulted in an increase in the levels of phosphorylated-MOR (Ser 375) associated with the enhanced inactivation of protein phosphatase 2A and a reduction in Rab4-dependent MOR resensitization in the spinal cord of mice that showed inflammatory pain (Imai et al., 2006). However, several lines of evidence indicate that, in response to pain stimulus, endogenous β -endorphin is released within some brain regions (Zubieta et al., 2001). We previously reported that β -endorphin released in the ventral tegmental area is a key factor in regulating the dysfunction of MOR to negatively modulate opioid reward under a neuropathic pain-like state (Niikura et al.,

2008, 2010). Taken together, although further studies are still needed, these findings support the idea that inhibition of the resensitization system of MOR following chronic treatment with fentanyl in the presence of β -endorphin may be associated with antihyperalgesic tolerance to fentanyl under a chronic pain-like state.

In conclusion, we demonstrated here that unlikely morphine, either fentanyl or oxycodone induced a robust MOR internalization and, in turn, its resensitization. In the presence of β -endorphin, the internalized MOR induced by fentanyl, but not oxycodone, remained within the cytosolic fraction even after washing out. These findings strongly support that idea that fentanyl has different pharmacological profile from that of morphine or oxycodone.

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Fig. 1.

Confocal imaging of agonist-induced internalization of MORs in BHK cells expressing Venus-fused MORs. The cells were incubated in the absence (**A**, **C**, **E**, and **G**) or presence (**B**, **D**, **F**, and **H**) of 100 nM β -endorphin (β -END) for 30 min at 37°C and then treated with 10 μ M morphine (MRP; **C**, **D**), 100 nM fentanyl (FEN; **E**, **F**), or 10 μ M oxycodone (OXY; **G**, **H**). The cells were subsequently fixed and examined by confocal microscopy. Yellow fluorescence from Venus indicates the localization of MORs in BHK cells. Scale bars, 10 μ m.

Fig. 2. Confocal imaging of agonist-induced internalization of MORs in BHK cells expressing Venus-fused MORs. Typical cells where most of MOR-Venus intensity was at the plasma membranes, [A, control cells (Control)] or in the cytosolic fraction [B, 100 nM fentanyl-stimulated for 30 min (FEN)]. PM; plasma membranes in BHK cells. Scale bars, 10 μm.

Fig. 3. Confocal imaging of resensitization of MORs in BHK cells expressing Venus-fused MORs. Cells were incubated with 100 nM fentanyl (A–H) or 10 μ M oxycodone (I–P) in the absence (A–D and I–L) or presence (E–H and M–P) of β -endorphin, and then apposed for 30 min, 90 min, 3 h, or 6 h at 37°C. The cells were then fixed and counted by confocal microscopy. Yellow fluorescence from Venus indicates the cellular localization of MOR in BHK cells. Scale bars, 10 μ m. Quantitative analysis of the % of the internalized cells expressing Venus-fused MORs treated with the drugs for 30 min (Q) or 180 min (R), respectively. The agonist concentrations represent the dose required to induce the maximal effect on receptor endocytosis for each drug. Each value represents the mean $\pm$ SEM of six separate experiments.