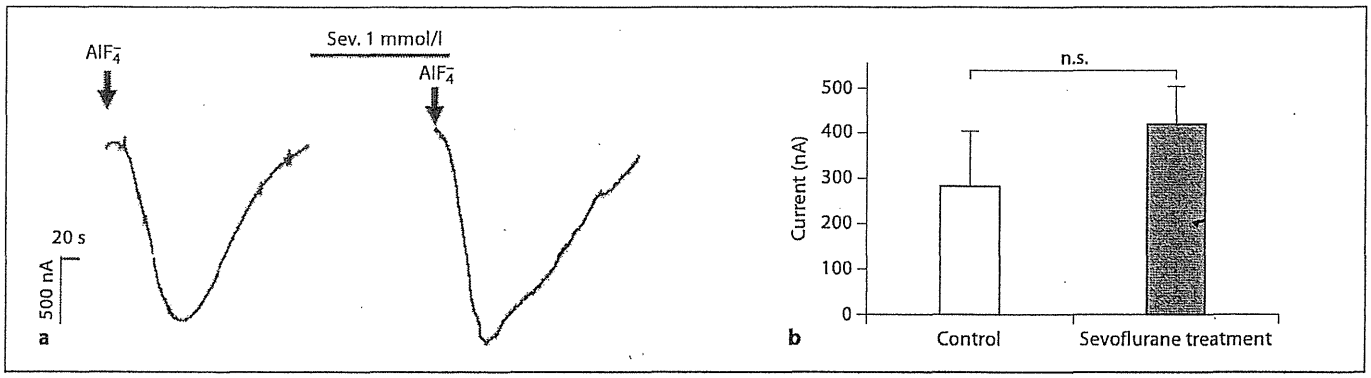


Fig. 3. Effects of sevoflurane (Sev.) on AlF_4^--induced currents in *Xenopus* oocytes. **a** Tracings were obtained from a single oocyte showing the effect of sevoflurane on AlF_4^--induced currents in oocytes expressing $\mu OR\text{-}G_{qi5}$. **b** Oocytes were injected with 30 nl test solution (20 mmol/l NaF and 60 μmol/l $AlCl_3$) in the presence (Sev. treatment) (n = 6) or absence (control) (n = 6) of 1 mmol/l sevoflurane. Data are expressed as means ± SEM of peak currents (nA).

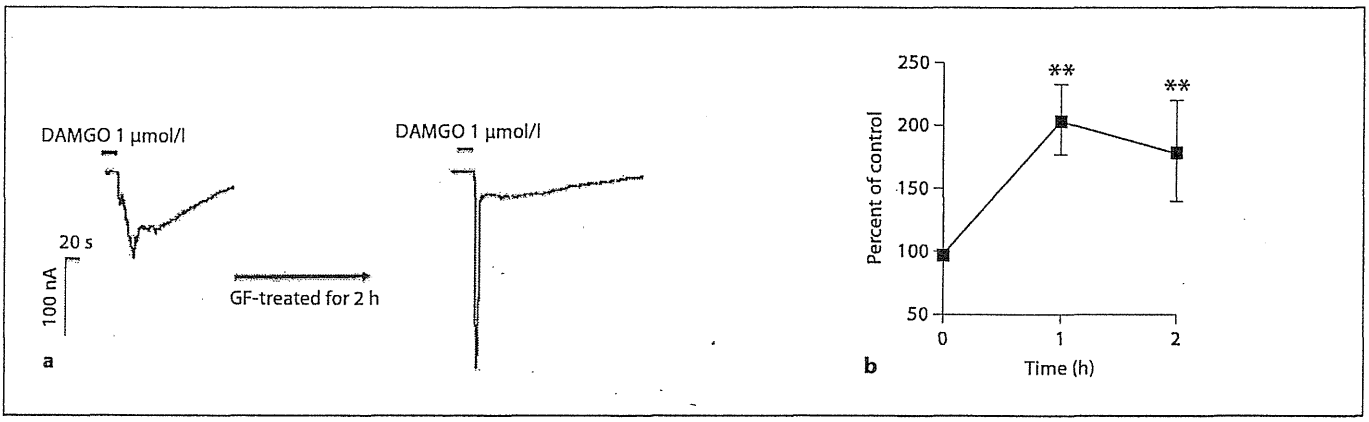
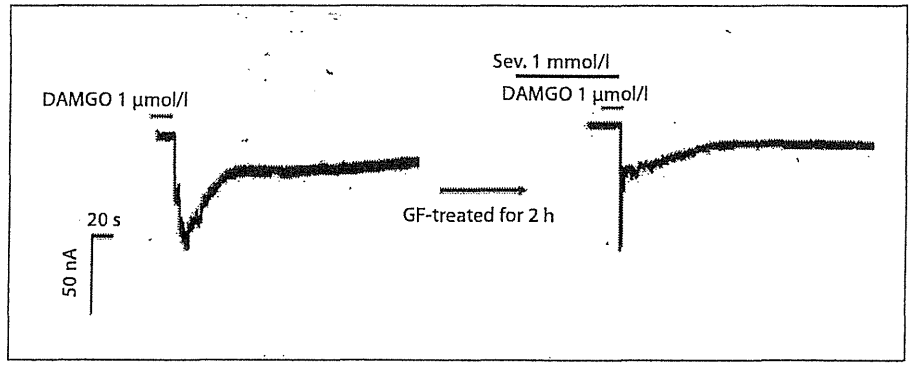


Fig. 4. Effects of bisindolylmaleimide I (GF109203X) on DAMGO-induced Cl^- current in oocytes expressing $\mu OR\text{-}G_{qi5}$ receptor. **a** Tracings were obtained from a single oocyte showing the DAMGO (1 μmol/l)-induced currents in oocytes expressing $\mu OR\text{-}G_{qi5}$ receptors before and after treatment with GF109203X (GF). Oocytes were incubated with 200 nmol/l GF for 2 h and were then stimulated by DAMGO. **b** Time course of effects of GF on DAMGO-induced Cl^- current in oocytes expressing $\mu OR\text{-}G_{qi5}$ receptor. Oocytes were incubated with GF (200 nmol/l) for 120 min. DAMGO (1 μmol/l) was applied at 60 and 120 min during treatment of GF. Data represent means ± SEM of 6 oocytes. ** $p < 0.01$ vs. time at starting incubation with 200 nmol/l GF (0 h).

Fig. 5. Effects of bisindolylmaleimide I (GF109203X) on the inhibitory effects of sevoflurane (Sev.) on DAMGO (1 μmol/l)-induced currents. Tracings were obtained from a single oocyte showing the effect of sevoflurane on 1 μmol/l of DAMGO-induced currents in oocytes expressing $\mu OR\text{-}G_{qi5}$ receptor before and after treatment with GF109203X (GF). Oocytes were incubated with 200 nmol/l GF for 2 h, and were then stimulated by DAMGO (1 μmol/l) in the presence of sevoflurane (1 mmol/l).



Discussion

We showed that sevoflurane had inhibitory effects on DAMGO-induced Cl^- currents in oocytes expressing μOR-G_{qi5}. In clinical situations, the free plasma concentration of sevoflurane was approximately 0.5 mmol/l [22, 23]. Sevoflurane suppressed DAMGO-induced Cl^- currents in oocytes expressing μOR-G_{qi5} at concentrations more than 0.5 mmol/l. Consistent with these reports, our present results suggest that anesthetic concentrations of sevoflurane would have inhibitory effects on μOR.

Our study raises the question of how sevoflurane inhibits μOR function. In our results, sevoflurane had little effect on AlF_4^--induced currents, suggesting that sevoflurane may not interfere with the signaling pathways downstream of activation of G proteins, such as phospholipase C activation, intracellular Ca^{2+} release, and Ca^{2+}-activated Cl^- channels. From these results, the action site of sevoflurane would be OR.

There is considerable evidence that PKC plays an important role in the regulation of OR function. A number of studies have reported that PKC is involved in morphine-induced tolerance in vivo [24–27]. In our present results, the PKC inhibitor GF109203X enhanced DAMGO-induced currents. These results suggested that PKC would inhibit the OR function. Moreover, the PKC inhibitor GF109203X abolished the inhibitory effects of sevoflurane on μOR function, suggesting that sevoflurane would inhibit μOR function by PKC-mediated pathways. In our study, unfortunately, we could not study how sevoflurane activates the PKC because of difficulties in measuring the activities of PKC in *Xenopus* oocyte preparation. However, there are several lines of evidence which reveal that sevoflurane activated PKC [28, 29]. To confirm this hypothesis, it could be required to investigate the region of μOR responsible for PKC action by using mutated μOR whose serine/threonine sites were point mutated.

In conclusion, we demonstrated that sevoflurane has significant inhibitory effects on the function of μOR at clinically relevant concentrations, and the inhibition might be mediated via PKC pathways. Although several investigations have reported the effects of opioids on sevoflurane anesthesia, the nature of the interaction between opioids and sevoflurane remains unclear. Our present results showed the inhibitory effects on μOR. To clarify the interaction between sevoflurane and opioid in the clinical situation, further study would be necessary.

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Short Communication

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

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KEY WORDS 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

M.N and Y.U contributed equally to this work.

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modified eagle medium (DMEM: Invitrogen™) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Transient transfection was then performed with Effectene transfection reagent (Qiagen, Tokyo, Japan) in 0.2 μg 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 μM morphine, 100 nM fentanyl or 10 μM oxycodone. To investigate the resensitization of MORs, the cells were incubated with 100 nM fentanyl or 10 μM 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 × 100/[plasma membrane + cytosol (total 100 cells)]. The drugs used in this study were fentanyl citrate (Hisamitsu Pharmaceutical, Tokyo, Japan), morphine hydrochloride

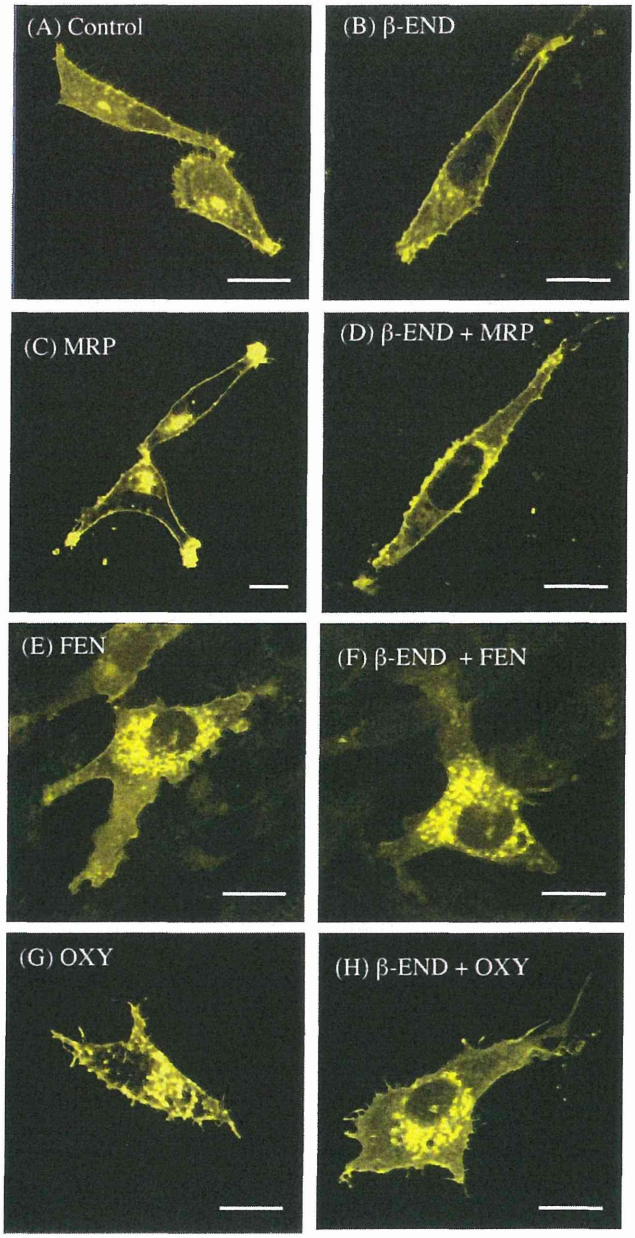


Fig. 1. Confocal imaging of agonist-induced internalization of MORs in BHK cells expressing Venus-fused MORs. The cells were incubated in the absence (**A**, **C**, **E**, and **G**) or presence (**B**, **D**, **F**, and **H**) of 100 nM β-endorphin (β-END) for 30 min at 37°C and then treated with 10 μM morphine (MRP; C, D), 100 nM fentanyl (FEN; E, F), or 10 μM 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 μm.

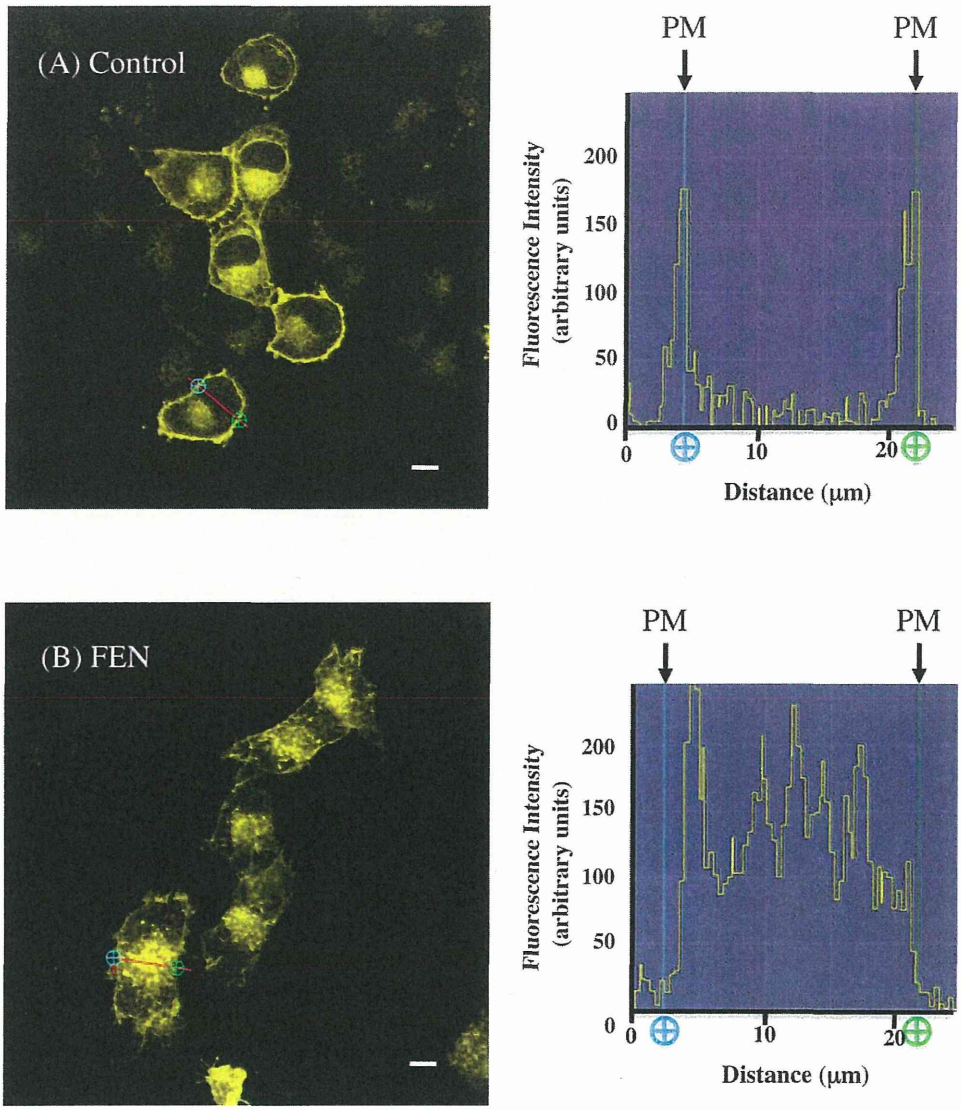
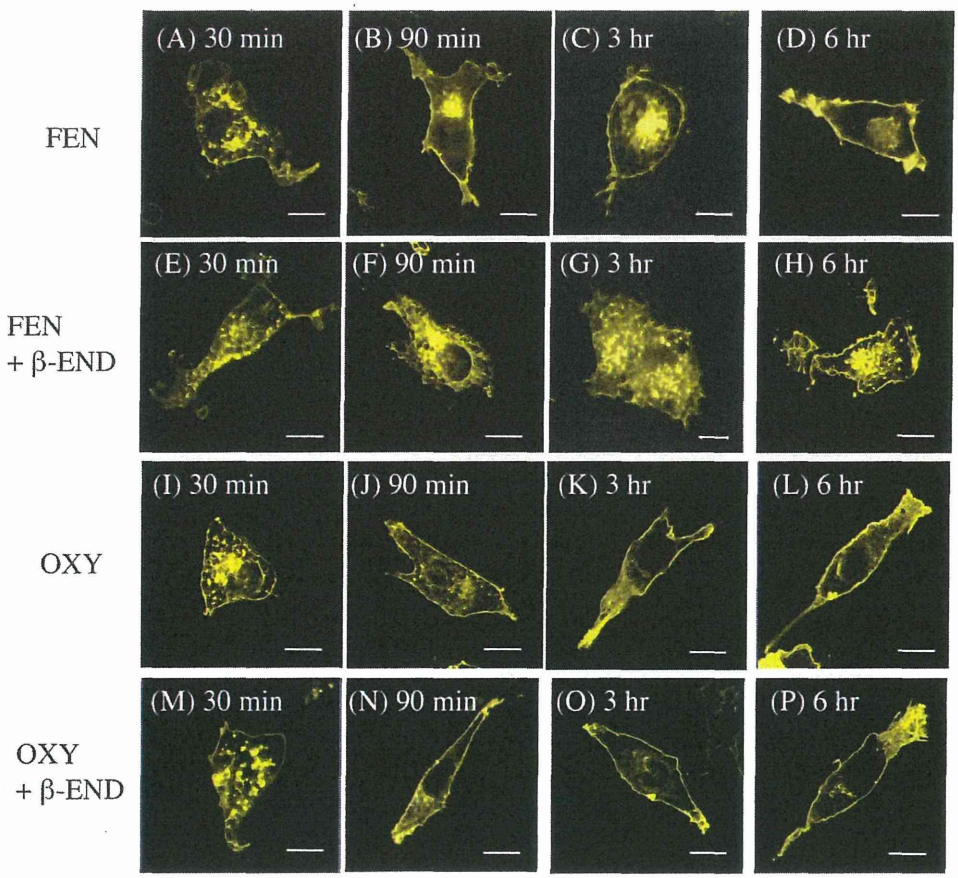


Fig. 2. Confocal imaging of agonist-induced internalization of MORs in BHK cells expressing Venus-fused MORs. Typical cells where most of MOR-Venus intensity was at the plasma membranes, [**A**, control cells (Control)] or in the cytosolic fraction [**B**, 100 nM fentanyl-stimurated for 30 min (FEN)].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

(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 μM 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 μM 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



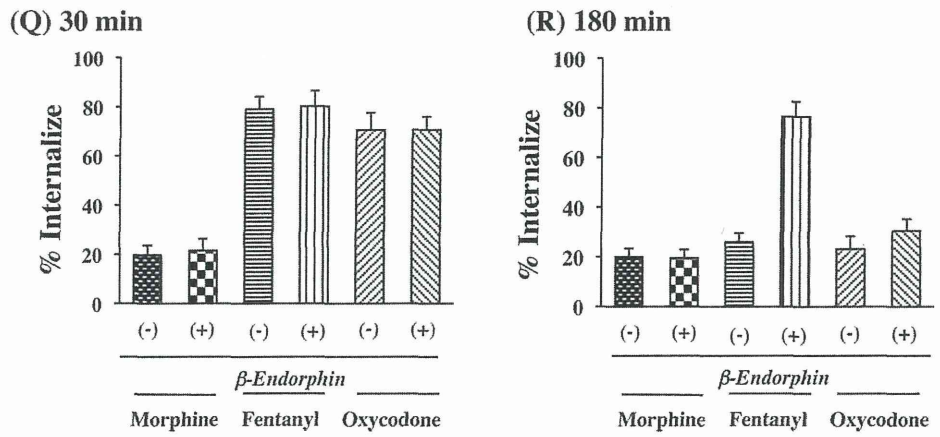


Fig. 3. Confocal imaging of resensitization of MORs in BHK cells expressing Venus-fused MORs. Cells were incubated with 100 nM fentanyl (**A–H**) or 10 μM oxycodone (**I–P**) in the absence (A-D and I-L) or presence (E-H and M-P) of β-endorphin, and then apposed for 30 min, 90 min, 3 h, or 6 h at 37°C. 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 μm. 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 ± SEM of six separate experiments.

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 ± 5.14%, β-endorphin fentanyl: 80.2 ± 3.7%, oxycodone: 70.5 ± 7.09%, β-endorphin oxycodone: 70.7 ± 5.35%), which was not seen in morphine-treated cells (morphine: 19.67 ± 3.93%, β-endorphin morphine: 21.5 ± 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 ± 5.12%, β-endorphin oxycodone: 30.5 ± 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 ± 5.47%, β-endorphin fentanyl: 76.5 ± 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 (Claing 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 form that of morphine or oxycodone.

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基礎医学セミナー　連載 第4回

がん患者の症状緩和のために
—がん悪液質の予防,症状改善をめざす基礎医学研究

独立行政法人国立がん研究センター研究所がん患者病態生理研究分野分野長
上園　保仁

はじめに

2009年より始まった基礎医学セミナーでは，基礎医学研究が臨床医学にどのように結びついているか，そしてがん患者のために活かされているかについて紹介させていただいています。第1回セミナーでは，「がん患者の生活の質(quality of life：QOL)の向上をめざして，基礎医学研究者も積極的にがんの痛みなどの基礎研究に携わり，そこで明らかとなった知見が臨床の現場で活かされるようになれば。その具体的な研究を今後のセミナーでご紹介いたします」と結びました[1]。そのなかで，がん患者のQOLを下げるものは痛みに止まらず疲労感，倦怠感，食思不振，不眠，便秘，嘔気嘔吐などたくさんの症状があることをお伝えしました。倦怠感，衰弱感，食思不振といった一連の症状は，進行がん患者の「悪液質」と呼ばれる症状で多くみられます。

今回は，進行がん患者の約50～60%にみられる「がん悪液質」について，がん悪液質の病態の説明，そしてがん悪液質の予防ならびにその症状改善のためにどのような基礎 - 臨床医学の橋渡し研究が行われているのかについてご紹介いたします。

がん悪液質とは

悪液質は，食思不振，体重減少，特に筋肉量の減少を主症状として，疲労感，倦怠感を伴い，また血中炎症性サイトカインレベルなどに異常がみられる消耗性の疾患です。体重減少に関しては「飢餓」の状態と変わらないのですが，飢餓では基礎代謝，糖代謝が低下しているのに対して悪液質ではむしろ基礎代謝や糖代謝が亢進すること，また飢餓では脂肪組織の減少が主にみられるのに対し，悪液質では筋肉量の低下が著明であることなど，悪液質は単なる食思不振や栄養不足のために体重減少を伴う症状ではないことが知られています。この悪液質は，がん患者以外に慢性呼吸器疾患や慢性心臓病，慢性腎臓病の患者などでもみられます。がん悪液質は終末期のがん患者では50～60%に認められますが，近年明らかになってきたのは，がん悪液質患者は明らかに予後が悪く，さらにがん悪液質自体が原因で死亡する患者ががん死亡の20%を占めるということです。すべてのがん腫でがん悪液質の報告がみられますが，特に膵臓がん，胃がん患者にがん悪液質の傾向が高く(両がん患者とも約80%)，ほかに食道がん，頭頸部がん，大腸がん患者もがん悪液質を伴うことが多いことが報告されています[2]。

がん悪液質の研究報告を調べますと，がん悪液質を予防し症状の改善を行うことは，がん患者のQOLを向上させるのみならず生命予後を長くすることが示されています。つまり，がん悪液質にならない，あるいは発症時期を遅らせる，発症してもできるだけ症状を軽くするという治療は，患者のために有効であるということです。ところが，がん悪液質の予防，治療には決定的なものがないのが現状です。

そもそもがん悪液質の成因，素因といったものは，がん細胞自体が出す何らかのファクターによるもの，がん細胞からのファクターに反応して起こる宿主の免疫，代謝異常などの二次性反応によるものなど，さまざまな原因が報告されてはいるものの，その本質はほとんどわかっていません。したがって，原因がはっきりしないのでそれに対する予防法，治療法もわからないということです。

がん悪液質の診断基準

2008年に悪液質の診断基準が示されました。それによると，12ヵ月以内に5％以上体重が減少し，加えて筋力低下，疲労などの症状の5つの基準のうち3つ以上を満たすこと，と定義されています(図1)[3]。

また，ごく最近新たな分類も提唱されています。それによると，がん悪液質が前悪液質(pre-cachexia)，悪液質(cachexia)，治療不応性悪液質(refractory cachexia)の3種に分類されています[4]。がん患者の悪液質は早期に対応すればするほどその改善が顕著であることを考えると，がん悪液質の早期診断，そして早期介入はとても重要です。前悪液質として定義される基準が導入されると着実な早期診断ができると思われるので，がん患者にとっても介入を行う医療サイドとしても，この基準の導入は望まれるところです。また，がん悪液質への薬効を検討する臨床治験においても，どのがん患者に対してどのタイミングでどのような薬剤の治験を行うかという詳細な基準を設定できることにもなり，正確な薬効評価が生まれるものと期待されます。

がん悪液質治療の具体例(研究中であるものも含む)

悪液質を有しているがん患者にはその基礎疾患であるがんが存在するのは明白であり，もちろんその基礎疾患が治癒されれば悪液質は消失します。しかし，がん本体の治癒は，手術で取り除けない例や，また抗がん剤治療を含む内科的治療はまだ完璧ではないこともあり，とても困難な課題です。がん悪液質の患者に対しては食思不振の改善や体重減少を防止する薬物療法が行われることになりますが，栄養面での工夫，改善もまた重要な治療介入ポイントです。

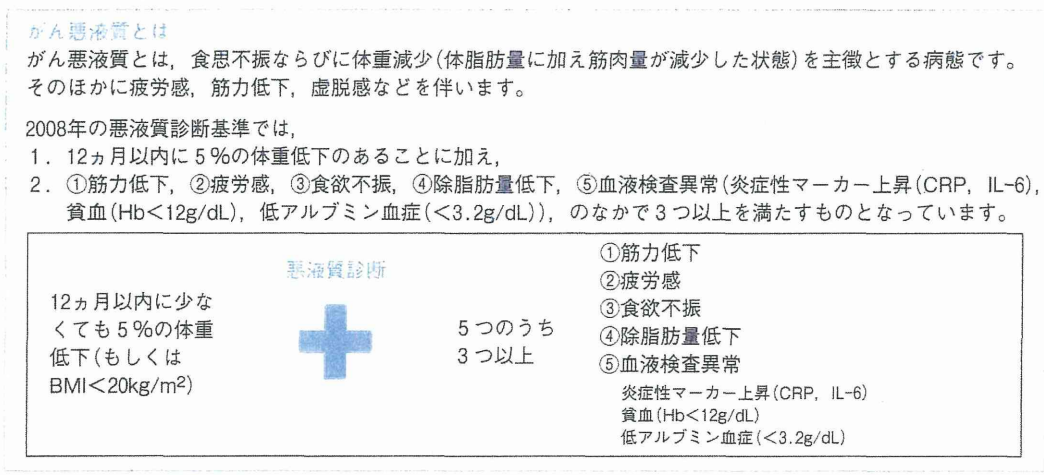

図1　がん悪液質の診断基準
CRP：C反応性蛋白，IL：インターロイキン，Hb：ヘモグロビン

(文献3より一部引用)