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Ubiquitination of the μ-opioid receptor regulates receptor internalization without affecting $G_{i/o}$-mediated intracellular signaling or receptor phosphorylation

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ABSTRACT

Opioids are highly potent analgesics but develop tolerance. Previous studies have focused on phosphorylation of the μ-opioid receptor as it is involved in maintaining cellular sensitivity via desensitization, recycling, and degradation of the activated receptor. Recently, ubiquitination, another form of posttranslational modification has attracted attention in terms of triggering intracellular signaling and regulation of the activated receptor. Here, we generated a ubiquitination-deficient mutant of the μ-opioid receptor to investigate whether ubiquitination is involved in driving $G_{i/o}$-mediated analgesic signaling, receptor desensitization or subsequent receptor internalization. Our study shows that the $G_{i/o}$ pathway and receptor phosphorylation do not require ubiquitination. Instead, ubiquitination regulates the internalization efficiency and might help in promoting internalization of the desensitized MOP.

1. Introduction

Opioids are highly potent analgesics but inevitably develop tolerance. Recently, long-term survival rates for various kinds of cancer have improved significantly, hence opioids are expected to provide adequate pain relief for extended periods [1]. Therefore, elucidating the molecular mechanisms underlying tolerance development might help in discovering more effective opioid use in various clinical settings. One possible explanation for opioid tolerance is that the opioid receptors undergo diverse posttranslational modifications upon ligand binding [2]. For example, the activated μ-opioid receptor (MOP) is rapidly phosphorylated by GPCR kinases (GRKs) and gets desensitized [2]. Then, the phosphorylated MOP recruits β-arrestins to drive intracellular signaling in cooperation with $G_{i/o}$ and, at the same time, promote internalization of the desensitized receptors and sort them to recycling or degradation pathways [3,4]. These processes are considered to negatively regulate the cellular sensitivity to prevent excessive activation of the MOP-mediated signaling. Due to the importance of posttranslational modifications of MOP in tolerance development [5], previous reports have extensively investigated the molecular mechanisms of MOP phosphorylation and their physiological significances. For example, the extend and pattern of multisite-phosphorylation occur hierarchically based on the ligand types [6,7]. Besides, a recent report showed that MOP mutant mice that do not undergo phosphorylation did not develop tolerance, clearly demonstrating in vivo the physiological significance of MOP phosphorylation for tolerance development [8].

G protein-coupled receptors (GPCR) are known to undergo diverse types of posttranslational modifications other than phosphorylation [5]. Ubiquitination is a type of posttranslational modifications that has recently attracted attention for its role in activating intracellular signaling and regulation of receptor functions. In general, the role of ubiquitination in regulating the degradation of substrate molecules [9], signal transduction [10], and DNA repair [11,12] is well known. Several reports have suggested that MOP also undergoes robust ubiquitination upon activation [13,14], and different ligands ubiquitinate it differently upon activation [15]. The ubiquitination of μ- or δ-opioid receptors functions like a barcode facilitating transfer of the substrate to the

Abbreviations: MOP, μ-opioid receptors; DAMGO, [D-Ala^2, NMe-Phe^4, Gly-ol^5]-Enkephalin; DADLE, [D-Ala^2, D-Leu^5]-Enkephalin; HA, hemagglutinin; ERK, extracellular signal-regulated kinase; HEK293 cells, human embryonic kidney 293 cells; MEXT, Ministry of Education, Culture, Sports, Science and Technology-Japan.

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target organelle such as lysosome or proteasome [16]. A recent study has demonstrated that ubiquitination of MOP by DADLE, a δ-opioid ligand, functions as a checkpoint during clathrin-coated pit formation [17]. Importantly, ubiquitination of some receptors serves as a scaffold for recruiting various enzymes that drive effective intracellular signaling and determine receptor function, although studies on GPCRs has not yet identified any such case [18,19]. Therefore, we wanted to know whether MOP ubiquitination is involved in activating the $G_{i/o}$-mediated analgesic pathway or the subsequent MOP phosphorylation in receptor internalization that regulates cellular sensitivity toward external stimuli. Investigating the processes that require ubiquitination would help in elucidating the molecular mechanisms of tolerance development to opioids.

Here, we generated ubiquitination-deficient mutants where lysine residues in the intracellular region were substituted to arginine and then investigated which processes require MOP ubiquitination; the $G_{i/o}$-mediated analgesic pathway, phosphorylation of MOP, or subsequent receptor internalization.

2. Materials and methods

2.1. Cell culture

HEK293 cells were kindly provided by the RIKEN BRC, Tsukuba, Japan (Cat# RCB1637) through the National BioResource Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. Following the distributor's instructions, HEK293 cells were cultured in DMEM (high glucose) containing L-glutamine, phenol red, and sodium pyruvate (Cat# 043–30085; FUJIFILM Wako), 10% fetal bovine serum at 37 °C in 5% CO_2 incubator. The Lenti-X™ 293T cell line (HEK293T) was purchased from Clontech Laboratories, Mountain View, CA, USA (Cat# 632180) and cultured the same as HEK293 cells.

2.2. Lentiviral expression

CSII-CMV-MCS-IRES2-Bsd was kindly provided by the RIKEN BRC (Cat# RDB04385) through the National BioResource Project of MEXT, Japan. As described previously [20], Lenti-X™ 293T cells were transfected with Lentiviral Hight Titer Packaging Mix (Cat# 6955; TaKaRa, Kusatsu, Japan) using TransIT-293 Transfection Reagent (Cat# MIR2704; Mirus, Madison, WI, USA) according to the manufacturer's instructions. The lentivirus-containing supernatant was collected after 48 h of incubation and filtered through a 0.45 μm polyvinylidene difluoride filter (Cat# SLHVR33RS; Merck, Darmstadt, Germany). The supernatant was concentrated using Lenti-X Concentrator (Cat# 631231; Clontech Laboratories) to infect HEK293 cells in the culture medium containing 9 μg/ml of polybrene (Cat# H9268; Sigma Aldrich, St. Louis, MO, USA). The stably transduced cells were selected with use of blasticidin (10 μg/ml) or puromycin (3 μg/ml).

2.3. Generation of ubiquitination-deficient mutant

The open reading frame of human MOR1 was amplified by RT-PCR. The MOR1 mutants were generated by multiple-step polymerase chain reaction using amplified ORFs (TaKaRa PrimeSTAR HS DNA Polymerase R010A). Each gene was cloned into the lentiviral expression vector as described above (Cat# RDB0438, RIKEN BRC). The lysine residues that are potential ubiquitination targets in the intracytoplasmic region: K100, K102, K176, K187, K262, K271, K273, and K346 were mutated to arginine. Next, to identify which part of the lysine residues are important for receptor regulation, the MOR-8K/R mutant was partially reverted to arginine.

2.4. Cell lysis and immunoblotting

All the samples were lysed on ice in lysis buffer consisting of 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, and the protease inhibitor cocktail (Cat# 160–26071; Fujifilm Wako). Lysates were clarified by centrifugation at 15,000 rpm for 20 min at 4 °C. To 20 μg of the lysate, SDS sample buffer containing 2-mercaptoethanol was added and then incubated at either 95 °C for 5 min for detecting phospho-p44/42 MAPK, p44/42-MAPK, and α-tubulin, or 30 °C for 30 min for detecting MOP. Then, the proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane. After blocking with Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% (wt/vol) non-fat milk for 2 h at room temperature, the membrane was incubated with the appropriate antibody for 16 h at 4 °C. After washing with TBST, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies against mouse or rabbit IgG for 2 h at room temperature or for 16 h at 4 °C. Bound antibodies were detected using enhanced chemiluminescence and visualized with ChemiDoc XRS+ (Bio-Rad). Intensity of bands were quantified by densitometry using the Image Lab (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. Antibodies

For western blotting, following antibodies were obtained from commercial sources and used at the indicated dilutions: anti-phospho-p44/42 MAPK (ERK1/2) (1:5,000, Cat# 9101; Cell Signaling Technology, Danvers, MA, USA), anti-p44/42 MAPK (ERK1/2) (1:2,000, Cat# 9102; Cell Signaling Technology), anti-phospho-μ-Opioid Receptor (Ser375 of mouse MOR1, Ser 377 of human MOR1) (1:2000, Cat#3451; Cell Signaling Technology), anti-α-tubulin (1:5,000, Cat# 3873; Cell Signaling Technology), and purified anti-HA.11-epitope tag (1:2000, Cat# 901501; BioLegend, San Diego, CA, USA). For flow cytometry analysis, the antibodies used were PE anti-HA.11 Epitope Tag Antibody (1:100, 901518; BioLegend) and PE Mouse IgG1, κ Isotype Control Antibody (1:100, 400111; BioLegend).

2.6. Flow cytometry analysis

The cultured cells were trypsinized and collected in tubes, and the pellet was suspended in 1,000 μL culture medium. Then, the indicated ligands were added to a final concentration of 10 μM and incubated at 37 °C. The reaction was stopped by keeping the tubes on ice, and then the cell pellets were washed with PBS containing 2% bovine serum (blocking buffer). The cells were stained with antibodies (1:100) in blocking buffer (50 μL per sample) for 30 min on ice. After rinsing with blocking buffer, cells were resuspended in 500 μL PBS containing 5 mM EDTA. Flow cytometry data were collected using a BD FACSCalibur™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The fluorescence signal derived from PE was recorded in the FL2 channel (488 nm laser). We analyzed the data using the FCSalyzer ver.09.15-alpha (Slashdot Media, San Diego, CA, USA). We used the values of mean fluorescence intensity (MFI) for quantification. We used samples stained with the isotype control antibody as negative controls to subtract nonspecific signals and calculated the change in fluorescence intensity using the following formula as described previously [20]:

$$\text{Receptor fluorescence (\% of initial value)} = \frac{MFI(value\ after\ stimulation) - MFI(negative\ control)}{MFI(initial\ value) - MFI(negative\ control)} \times 100$$

The data were presented as the percentage of the initial value.

2.7. MOP ligand and inhibitors

We used the MOP ligand: [D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin (DAMGO) (Cat# ab120674; Abcam, Cambridge, UK). We purchased $G_{i/o}$ inhibitor and pertussis toxin from Fujifilm (Cat# 168–22471), the E1 inhibitor, TAK243 (MLN7243) from Selleck (Cat# S8341), and MG132 from ChemScene (Cat# CS-0471). For analysis with these inhibitors, cells were pre-incubated with pertussis toxin for 18 h, MG132 for 4 h, TAK2433 for 90 min prior to the ligand stimulation following the manufacture's instruction.

2.8. Statistical analysis

Quantitative data were expressed as the mean. The error bars represent the standard deviation unless indicated otherwise. Statistical significance was analyzed by Student's *t*-test or one-way analysis of variance with Dunnett's multiple comparison test ($\alpha = 0.05$) using Prism 9.0 (GraphPad Software, San Diego, CA, USA).

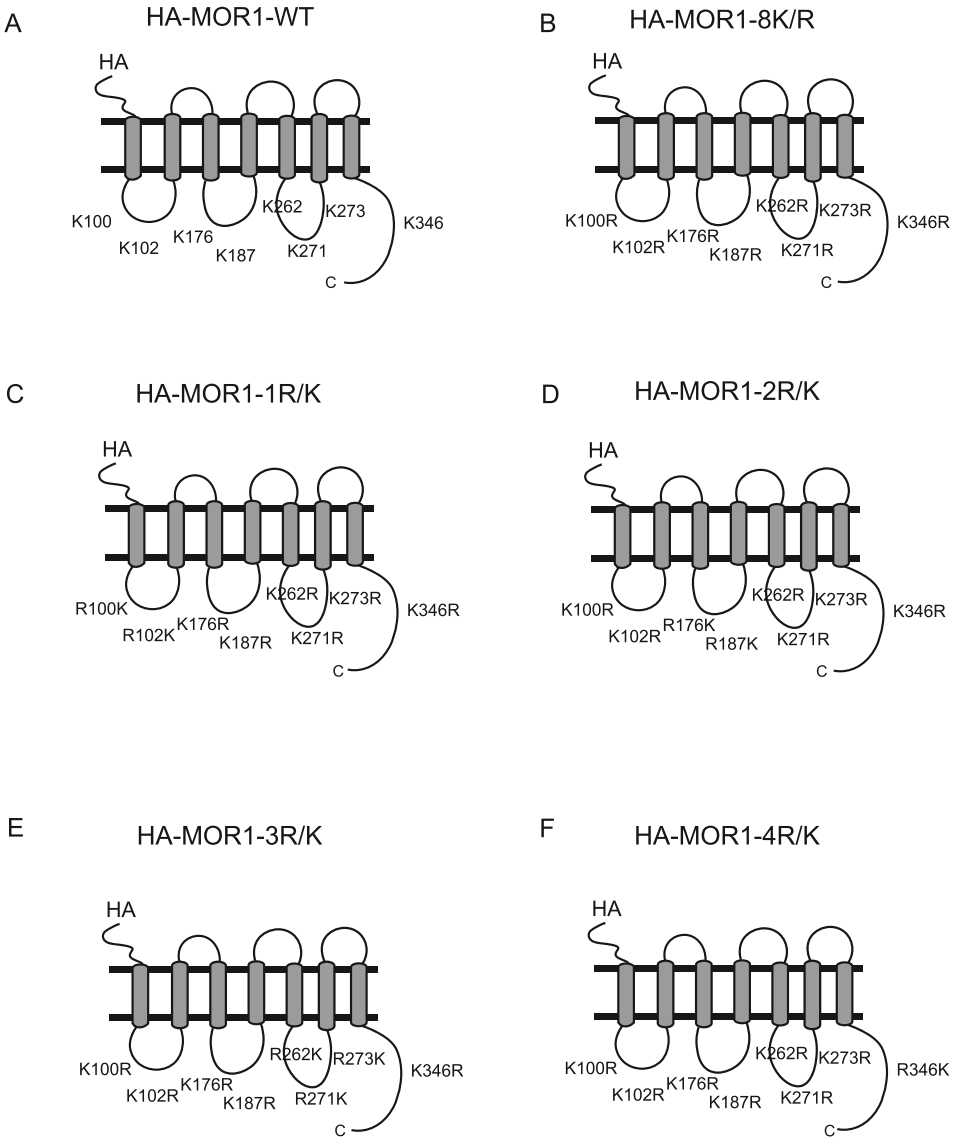

Fig. 1. Generation of ubiquitination-deficient MOR1 mutant.
(A–F) Schema of generating MOR1-WT (A) and ubiquitination-deficient mutant, MOR1-8 K/R (B) in which all eight intracellular lysine residues were substituted for arginine. C–F The substituted arginine in the MOR1-8 K/R mutant were partially reverted back to lysine. (C) First cytoplasmic loop. (D) Second cytoplasmic loop. (E) Third cytoplasmic loop. (F) Carboxyl-terminus.

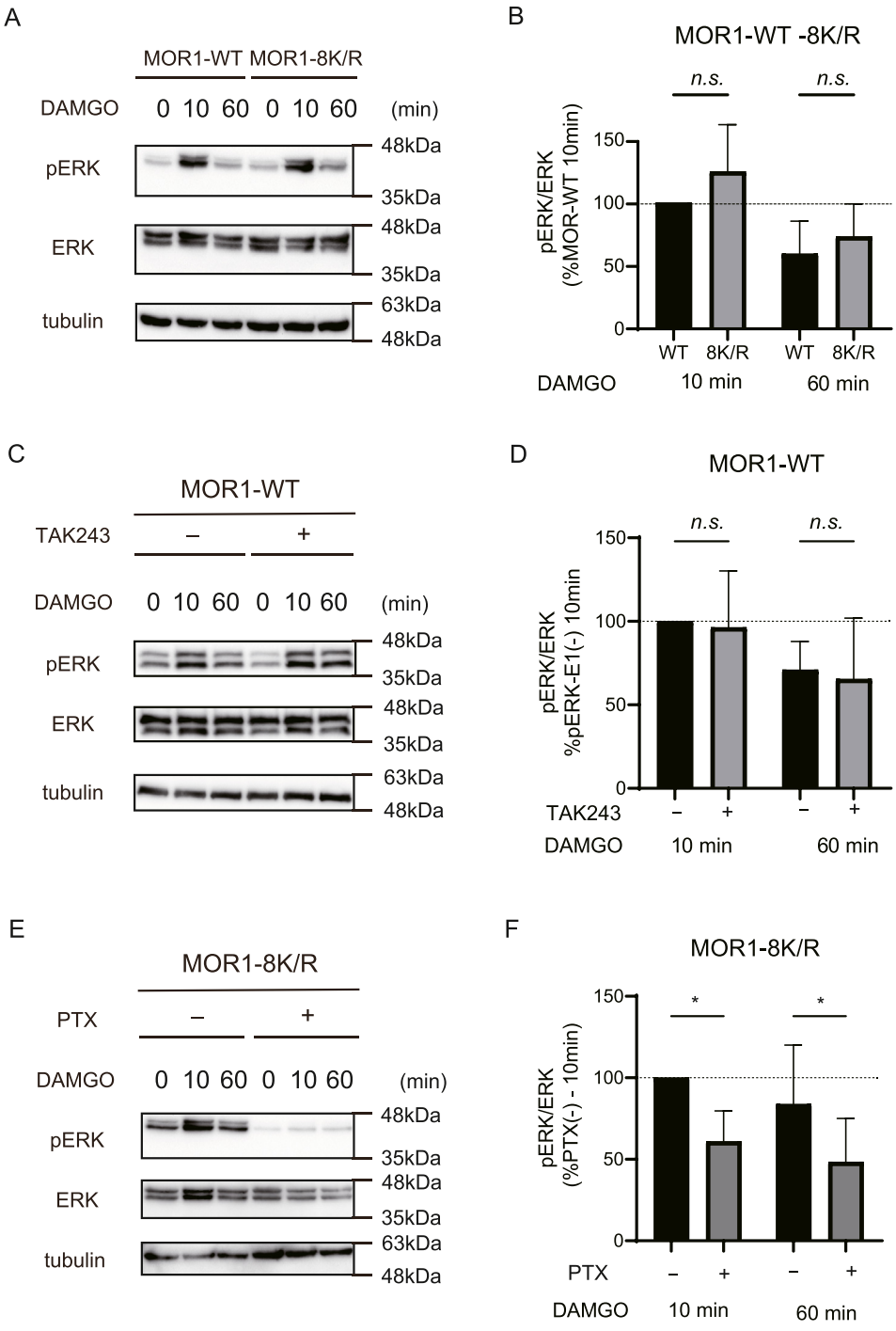

Fig. 2. Ubiquitination is not prerequisite for $G_{i/o}$-mediated MAPK activation.
(A and B) Immunoblotting analyses of MAPK activation in whole-cell lysates from HEK293 cells either expressing MOR1-WT or ubiquitination-deficient MOR1-8 K/R mutant following stimulation with 10 μM DAMGO for the indicated time. (C and D) Immunoblotting analysis of MAPK activation in whole-cell lysates from HEK293 cells expressing MOR1-WT, followed by stimulation with 10 μM DAMGO after pretreatment with TAK243, an E1 inhibitor. (E and F) Immunoblotting analysis of MAPK activation in whole-cell lysates from HEK293 cells expressing MOR1-8 K/R mutant, followed by stimulation with 10 μM DAMGO after pretreatment with 250 ng/mL of PTX for 18 h. The annotation indicates the results of statistical analyses with multiple comparison (*, $P < 0.05$; n.s. no significant difference).

Statistical significance was defined as a *P* value or adjusted *P* value less than 0.05. All experiments showing representative data were repeated at least three times independently, and similar results were obtained.

3. Results

3.1. Generation of ubiquitination-deficient μ-opioid receptor

As ubiquitin is attached to the lysine residues of the target protein [21], first we generated ubiquitination-deficient MOR1-8 K/

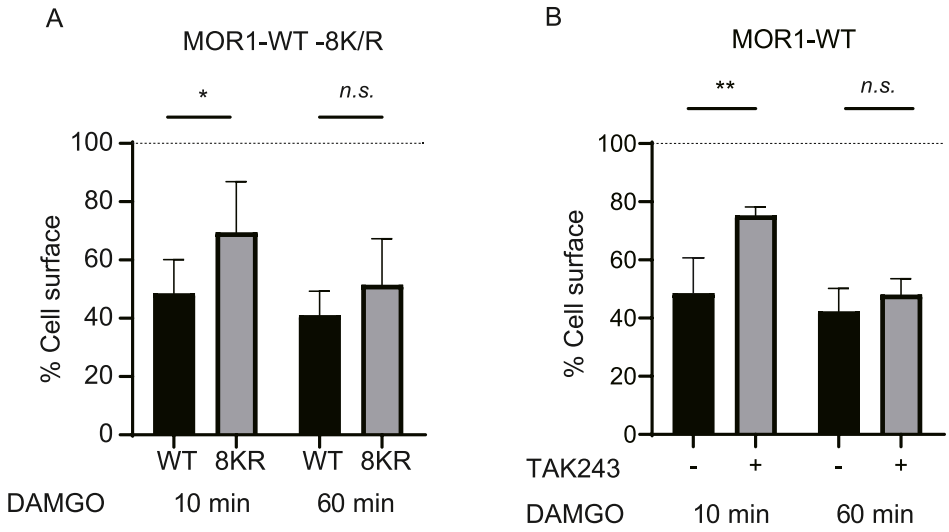

Fig. 3. Ubiquitination-deficient MOP decelerate receptor internalization
(A) Flow cytometry analysis of hemagglutinin (HA)-tagged MOR1 internalization with the indicated genotypes introduced into HEK293 cells upon stimulation with 10 μM DAMGO for the indicated time period. MOR1 internalization was determined by calculating the mean fluorescence intensity of the PE anti-HA tag on the cell surface after stimulation, as the percentage of the initial value using a flow cytometer. (B) Both HEK293 cells expressing MOR1-WT and MOR1-8 K/R mutant were pretreated with TAK243, an E1 inhibitor, followed by stimulation with 10 μM DAMGO for the indicated time period. The annotation indicates the results of statistical analyses with multiple comparison (*, $P < 0.05$; **, $P < 0.01$; n.s., no significant difference).

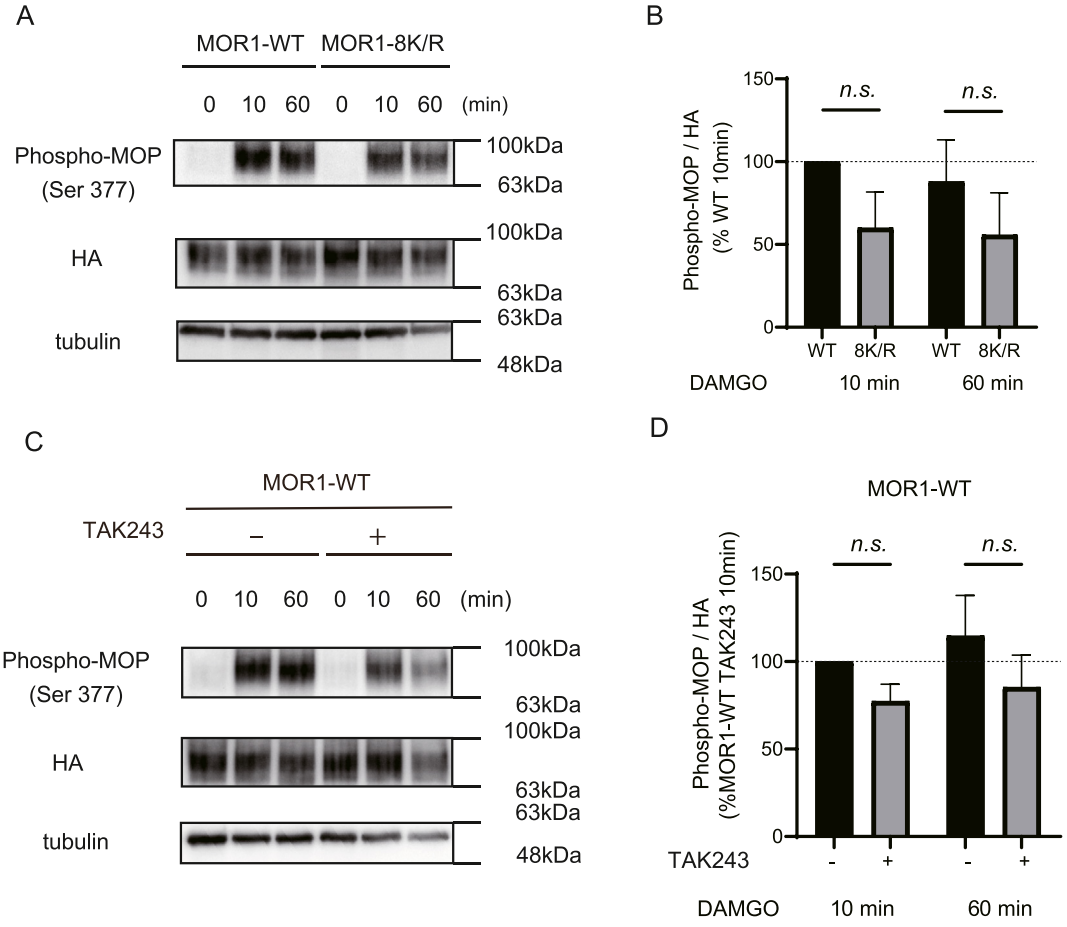

Fig. 4. Ubiquitination of MOP is not required for its phosphorylation.
(A and B) Immunoblotting analyses of MOR1 phosphorylation in the whole-cell lysates from the cell lines expressing either MOR1-WT or ubiquitination-deficient MOR1-8 K/R mutant following stimulation with 10 μM DAMGO for 10 min. (C and D) Both HEK293 cells expressing MOR1-WT and MOR1-8 K/R mutant were pretreated with TAK243, an E1 inhibitor, followed by stimulation with 10 μM DAMGO for 10 min. The annotation indicates the results of statistical analyses with multiple comparison (n.s., no significant difference).

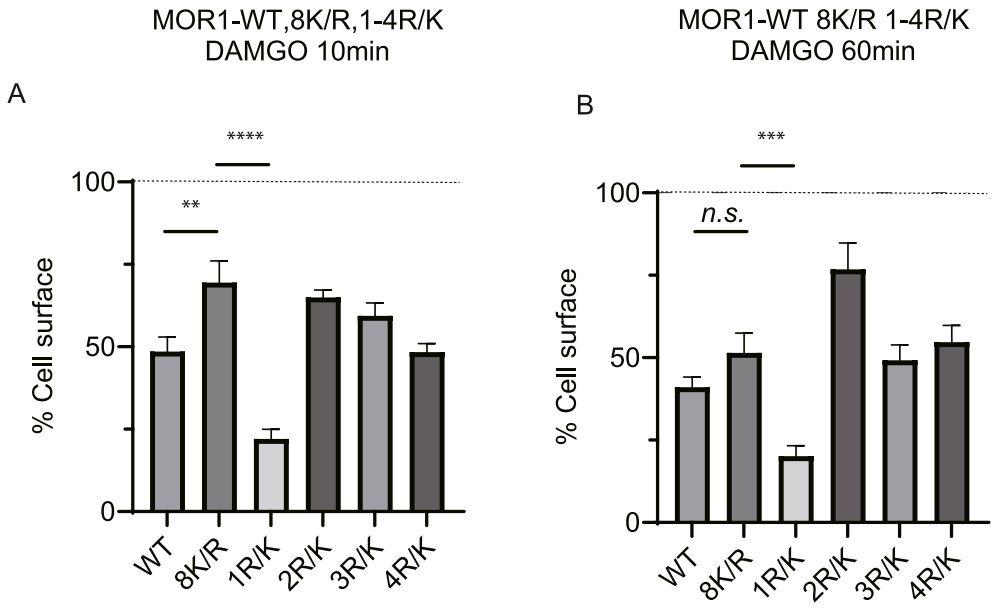

Fig. 5. Lysine residues at the first cytoplasmic loop are involved in the regulation of receptor internalization.
(A and B) Flow cytometry analysis for hemagglutinin (HA)-tagged MOR1 internalization with the indicated genotypes introduced into HEK293 cells upon stimulation with 10 μM DAMGO for 10 min (A) and 60 min (B). The annotation indicates the results of statistical analyses with multiple comparison (**, $P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

R mutants, by mutating all of the eight lysine residues to arginine in the intracellular region of MOR1, which is the most widely studied OPRM1 gene: K100R, K102R, K176R, K187R, K262R, K271R, K273R, and K346R mutants (Fig. 1A and 1B). We then generated mutants in which the residues replaced by arginine were partially reverted to lysine to verify the significance of these lysine residues. These mutants were R100K and R102K in the first cytoplasmic loop region (Fig. 1C), R176K and R187K in the second cytoplasmic loop region (Fig. 1D), R262K, R271K and R273K in the third intracellular loop region (Fig. 1E), and R346K in the carboxyl-terminus region of MOR1-8 K/R mutant (Fig. 1F). We introduced either wild-type or above mentioned MOR1 mutants in HEK293 cells to evaluate the physiological roles of MOR1 ubiquitination.

$G_{i/o}$-mediated intracellular signaling does not require MOP ubiquitination.

Ubiquitination targeting the activated receptor often functions as an essential scaffold for effectively recruiting various enzymes or scaffolding molecules to promote intracellular signaling [18,19]. Therefore, we were interested in investigating whether ubiquitination targeting MOR1 is involved in the activation of $G_{i/o}$-mediated analgesic pathway. Recent studies have indicated that G proteins and β-arrestins cooperatively act to activate $G_{i/o}$-mediated analgesic pathways [20,22], therefore we evaluated the phosphorylation of ERK upon stimulation with a ligand as a hallmark of MAPK activation. First, we stimulated HEK293 cells expressing either MOR1-WT or MOR1-8 K/R mutant with DAMGO. The MOR1-8 K/R mutant activated MAPK pathway as effectively as MOR1-WT, indicating that ubiquitination of MOR1 does not play an essential role in activation of $G_{i/o}$-mediated intracellular signaling (Fig. 2A and 2B). We then used E1 inhibitor, TAK243 [23], to further confirm the effect of ubiquitination of MOR1 on $G_{i/o}$-mediated signaling and found that inhibition of ubiquitin conjugating cascade did not interfere with MAPK signaling (Fig. 2C and 2D). Further, we treated MOR1-8 K/R mutant with pertussis toxin (PTX), a selective $G_{i/o}$ inhibitor, and found out that PTX successfully inhibited MAPK signaling downstream of MOR1-8 K/R mutant (Fig. 2E and 2F). This showed that signaling observed above were derived from the $G_{i/o}$-mediated pathway. Therefore, our results indicated that $G_{i/o}$-mediated analgesic pathway does not require ubiquitination of MOR1.

3.2. Ubiquitination-deficient MOP mutant delays receptor internalization

The activated MOP is rapidly phosphorylated and desensitized by GPCR kinases [23]. The phosphorylation of the receptor plays an essential role in receptor internalization, which negatively regulates excessive activation of intracellular signaling [24]. Therefore, next we investigated whether ubiquitination of MOR1 is involved in receptor internalization. For this, we introduced MOR1-WT and MOR1-8 K/R mutants into HEK293 cells and analyzed receptor internalization with flow cytometry. Although receptor internalization was significantly impaired in MOR1-8 K/R mutant at early time point, i.e., 10 min, it showed the tendency to internalize to almost the same degree as MOR1-WT (Fig. 3A). Use of E1 inhibitor, TAK243, also showed similar results (Fig. 3B). These data collectively indicated that ubiquitination of MOR1 is involved in regulating the efficiency of MOR1 internalization.

4. MOP phosphorylation does not require ubiquitination

Phosphorylation of MOP plays a crucial role in recruitment of β-arrestins leading to receptor internalization [25]. Since some types of ubiquitination serve as a scaffold for recruitment of various enzymes [26], we wanted to know whether ubiquitination of MOP is required for MOP phosphorylation, for example, helping GPCR kinases (GRKs) access activated MOP. Therefore, we evaluated the phosphorylation of MOR1 Ser377, the first residue to get phosphorylated [27] to see whether MOP ubiquitination is involved in recruitment of GRKs. MOR1-8K/R was phosphorylated to same extent as MOR1-WT upon ligand stimulation, indicating that ubiquitination of MOR1 is not required for MOR1 phosphorylation

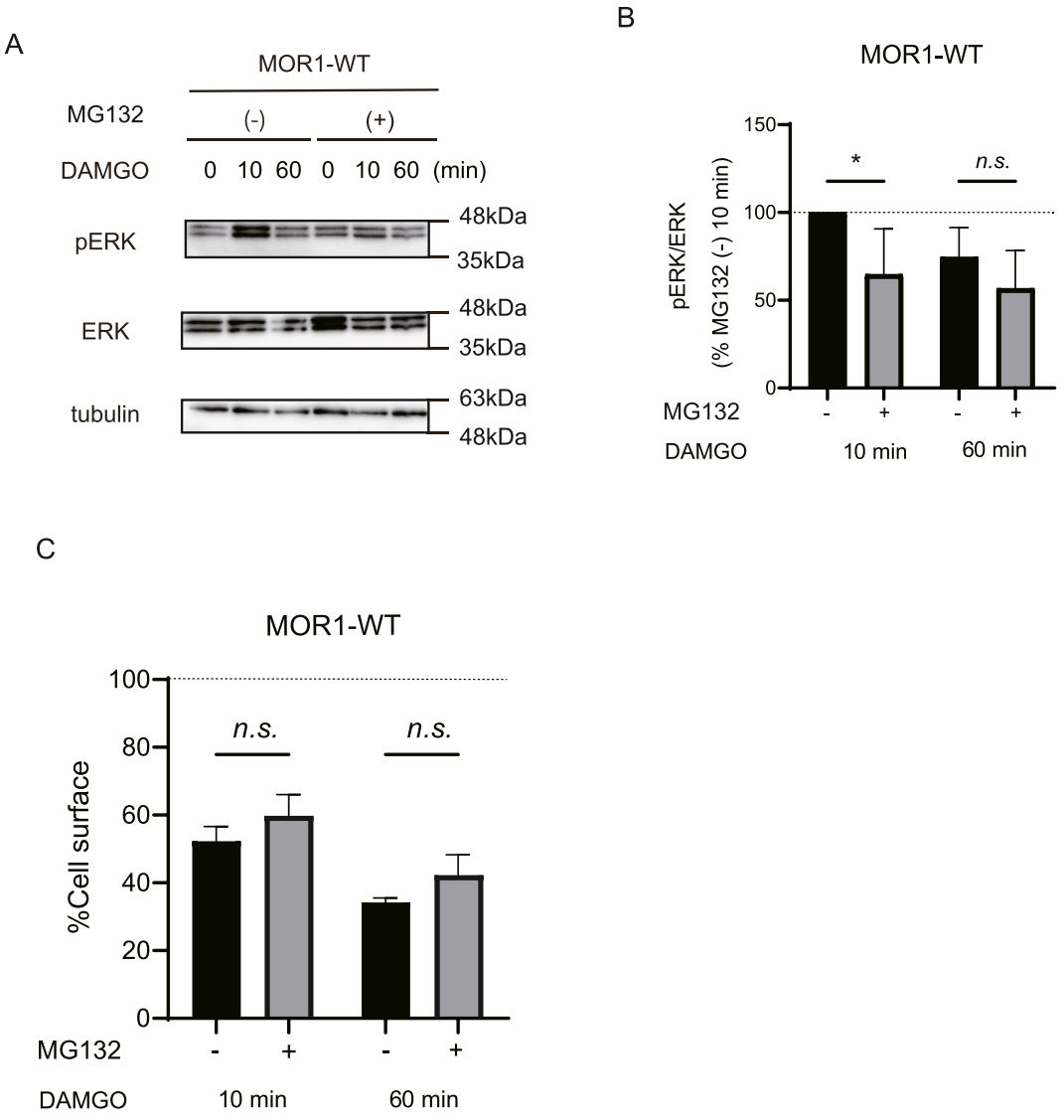

Fig. 6. Non-degradative ubiquitination regulates receptor internalization.
(A) Immunoblotting analysis of MAPK activation in the whole-cell lysates from HEK293 cells expressing MOR1-WT followed by stimulation with 10 μM DAMGO after pretreatment with proteasome inhibitor, MG132. (B) Flow cytometry analysis for hemagglutinin (HA)-tagged MOR1-WT internalization upon stimulation with 10 μM DAMGO for the indicated time period after pretreatment with proteasome inhibitor, MG132. The annotation indicates the results of statistical analyses with multiple comparison (*, $P < 0.05$; n.s. no significant difference).

(Fig. 4A and 4B). The E1 inhibitor, TAK243, confirmed that ubiquitination is not prerequisite for MOR1 phosphorylation (Fig. 4C and 4D). These data collectively indicated that phosphorylation of MOR1 does not depend on ubiquitination of MOR1.

Ubiquitination of the first cytoplasmic loop plays an essential role in MOP internalization.

We determined lysine residues that were targeted by ubiquitination by using MOR1-8 K/R mutants in which substituted arginine resides were partially reverted to lysine. The MOR1-8 K/R mutant in which substituted arginine in the first cytoplasmic loop were reverted to lysine residue significantly recovered receptor internalization (Fig. 5A). The data suggested that ubiquitination of the first cytoplasmic loop is important for effective receptor internalization. Of note, mutants in which substituted arginine residues were reverted to lysine showed a tendency to be internalized to almost the same degree as MOR1-WT after 60 min, whereas they internalized only weakly after 10 min of ligand stimulation (Fig. 5B).

4.1. Non-degradative ubiquitination plays an essential role in MOP internalization

Previous reports indicated that MOR1 undergo proteasomal degradation, indicating that K48-linked polyubiquitin chains are conjugated to MOP in some conditions [14]. Therefore, we evaluated whether intracellular signaling or receptor internalization are affected by proteasome inhibition. We treated MOR1-WT and MOR1-8 K/R mutant expressing HEK293 cells with MG132, a proteasome inhibitor [28], to evaluate the effect of K48-linked polyubiquitin chain that might be potentially attached to MOR1. While MG132 showed a tendency to slightly impair MOR1-mediated intracellular signaling (Fig. 6A and 6B), it did not affect receptor internalization (Fig. 6C). These data indicated that non-degradative ubiquitination is involved in regulating the efficiency of receptor internalization.

5. Discussion

In this study, we showed that ubiquitination of MOP at the first intracellular loop regulates the efficiency of receptor internalization. We also showed that MOP ubiquitination is not required for both $G_{i/o}$-mediated analgesic signaling and desensitization of MOP via phosphorylation.

Ubiquitin is a small about 8.5-kDa protein with four large hydrophobic patches. Therefore, ubiquitination, under some conditions, acts as a scaffold for recruiting various enzymes or scaffolding proteins timely and selectively to activate intracellular signaling or regulating receptor functions [21]. Indeed, ubiquitination of some types of receptors has been shown to play essential roles in the recruitment of various enzymes for driving intracellular signaling as well as the regulation of receptor functions [19,21,29,30]. Therefore, we were particularly interested in which of these processes require MOP ubiquitination: $G_{i/o}$ activation, MOP phosphorylation, or receptor internalization. Besides, we tried to identify the relationship between MOP phosphorylation and ubiquitination, as phosphorylation and ubiquitination are closely interrelated. For example, in some cases, phosphorylation of a molecule is required for its recognition as the substrate by E3 ubiquitin ligase [29]. Also, ubiquitination might help the appropriate kinases to access their substrate [31]. Because previous reports have identified phosphorylation of MOP as a barcode for desensitization to negatively regulate cellular sensitivity, we tried to investigate whether ubiquitination plays an important role in this desensitization process.

Our results indicated that ubiquitination is not a prerequisite for recruiting $G_{i/o}$ to the activated MOP (Fig. 2). Importantly, ubiquitination-deficient mutant was phosphorylated to same extent as MOR1-WT (Fig. 4), indicating that receptor phosphorylation does not require ubiquitination. On the other hand, although receptor internalization did proceed without ubiquitination, ubiquitination-deficient mutants showed significant delay in receptor internalization, indicating that MOP ubiquitination regulates the efficiency of MOP internalization (Fig. 3). β-arrestins, member of GPCR family, act as a scaffolding protein to recruit E3 ubiquitin ligase to the receptor [32]. We also indicated that ubiquitination follows receptor phosphorylation. Therefore, β-arrestins might acts as scaffolding for recruiting E3 ubiquitin ligase that target MOP as β2-adrenergic receptors. If so, since the extent of MOP phosphorylation is known to vary with ligand, whether the extent of ubiquitination varies with ligand and whether this affects the regulation of cellular sensitivity will be an important question for future studies. Additionally, determining whether E3 ubiquitin ligase targets MOP is an essential question that remain to be answered.

Our data suggests that the ubiquitination of MOP may be involved in maintaining cellular sensitivity by promoting the internalization of the desensitized MOP. Besides, considering that in some types of receptor intracellular signaling remain active after endocytosis [33], analyses for longer period might reveal physiological significance of ubiquitination in the intracellular signaling. Our results are similar to a previous report that showed that ubiquitination of the first cytoplasmic loop of the MOP function as a checkpoint of clathrin-coated pit formation for regulating the efficiency of receptor internalization (Fig, 5) [32]. However, the previous study used DADLE, a δ-agonist, which is not a μ-selective agonist. Here, we have used DAMGO, which is a μ-opioid receptor selective agonist. Therefore, our result might help in revealing the role of ubiquitination in more physiological settings.

A large number of in vitro studies on MOP phosphorylation have deepened our understanding of physiological effect of MOP. Furthermore, most recent reports using phosphorylation-deficient MOP mutant mice have clearly demonstrated that receptor phosphorylation is not involved in the analgesic pathway and is essential for tolerance development [17]. Generation of ubiquitination-deficient mutant mice would help in further clarifying the physiological significance of MOP ubiquitination.

In the present study, we showed that ubiquitination of MOP regulates the efficiency of MOP internalization. Our result provide insight into a part of molecular mechanism underlying tolerance development.

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Declaration of competing interest

The authors have no conflicts of interest to declare.

Acknowledgement

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