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Full Paper

μ-Opioid Receptor Forms a Functional Heterodimer With Cannabinoid CB_1 Receptor: Electrophysiological and FRET Assay Analysis

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Abstract. Interactions between μ-opioid receptor (μOR) and cannabinoid CB_1 receptor (CB_1R) were examined by morphological and electrophysiological methods. In baby hamster kidney (BHK) cells coexpressing μOR fused to the yellow fluorescent protein Venus and CB_1R fused to the cyan fluorescent protein Cerulean, both colors were detected on the cell surface; and fluorescence resonance energy transfer (FRET) analysis revealed that μOR and CB_1R formed a heterodimer. Coimmunoprecipitation and Western blotting analyses also confirmed the heterodimers of μOR and CB_1R. [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO) or CP55,940 elicited K^+ currents in *Xenopus* oocytes expressing μOR or CB_1R together with G protein activated-inwardly rectifying K^+ channels (GIRKs), respectively. In oocytes coexpressing both receptors, either of which was fused to the chimeric Gα protein G_{qi5} that activates the phospholipase C pathway, both DAMGO and CP55,940 elicited Ca^{2+}-activated Cl^- currents, indicating that each agonist can induce responses through G_{qi5} fused to either its own receptor or the other. Experiments with endogenous $G_{i/o}$ protein inactivation by pertussis toxin (PTX) supported the functional heterodimerization of μOR/CB_1R through PTX-insensitive $G_{qi5(m)}$ fused to each receptor. Thus, μOR and CB_1R form a heterodimer and transmit a signal through a common G protein. Our electrophysiological method could be useful for determination of signals mediated through heterodimerized G protein–coupled receptors.

Keywords: μ-opioid receptor, cannabinoid CB_1 receptor, receptor heterdimerization, fluorescence resonance energy transfer (FRET), electrophysiology

Introduction

G protein–coupled receptors (GPCR) were previously considered to have monomeric structures. However, recent evidence suggests that some or even most of the GPCR are oligomeric structures formed by GPCR homodimers, heterodimers, multimers, and also different types of proteins that intercommunicate at the plasma membranes (1 – 3). It is well known that opioids that bind to G protein–coupled μ-opioid receptors (μOR) and cannabinoids that bind to G protein–coupled cannabinoid receptors (CB_1R) expressed in the central nervous system have common pharmacological effects, such as antinociception, hypothermia, inhibition of locomotor activity, hypotension, and sedation (4, 5). The synergy in the analgesic effects of opioids and cannabinoids is attributed to a cross-talk between these two signaling pathways mediated by simultaneous activation of opioid and cannabinoid receptors. Furthermore, delta 9-tetrahydrocannabinol, a cannabinoid CB_1R agonist, can enhance the potency of opioids such as morphine (6). Recent studies reported that μOR and CB_1R form a heterodimer in the heterologous expression system (7). However, confirmation of direct functional signaling through such heterodimerized receptors remains to be elucidated.

In the present study, several experiments were conducted to confirm the heterodimerization of μOR and

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CB_1R. Morphological analysis was employed using fluorescence resonance energy transfer (FRET) assay and baby hamster kidney (BHK) cells expressing receptors fused to fluorescent proteins. Whether μOR and CB_1R form heterodimers was determined using BHK cells expressing CB_1R fused to Cerulean, a brighter variant of cyan fluorescent protein (CFP) (8) and μOR fused to Venus, a brighter variant of yellow fluorescent protein (YFP) (9). We also conducted coimmunoprecipitation with subsequent western blot assay in BHK cells expressing FLAG-tagged μOR and CB_1R. Electrophysiological assay was also conducted to confirm the formation of functional heterodimers of μOR and CB_1R. To this end, we developed an electrophysiological assay using the *Xenopus* oocyte expression system with μOR and CB_1R fused to a chimeric $G\alpha_q$ protein, G_{qi5}. The chimeric G_{qi5} protein, whose last five amino acid residues in the C-terminus was replaced with the corresponding portion of G_i protein, is useful for monitoring the responses mediated by stimulation of $G_{i/o}$ protein-coupled receptors in the *Xenopus* oocyte expression system, as reported previously by our laboratory (10, 11). Chimeric G_{qi5} allows $G_{i/o}$-coupled receptors to couple to the phospholipase C (PLC)-mediated signal pathway (12, 13). Agonists for $G_{i/o}$ protein-coupled receptors can elicit Ca^{2+}-activated Cl^- currents in such oocytes only through the chimeric G_{qi5}, but not through $G_{i/o}$ endogenously expressed in oocytes (10, 11). Based on the results, we proposed that our electrophysiological method for functional analyses could be useful for determination of signals mediated through heterodimerized G protein–coupled receptors.

Materials and Methods

Drugs and chemicals

Baclofen, gentamicin, sodium pyruvate, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO), and pertussis toxin (PTX) were purchased from Sigma (St. Louis, MO, USA). CP55,940 was from Tocris Bioscience (Bristol, UK). Other chemicals used in the study were of analytical grade and obtained from Nacalai Tesuque (Kyoto).

Construction of cDNAs and preparation for cRNA

cDNAs for the rat μOR was kindly provided by Dr. N. Dascal (Tel Aviv University, Ramat Aviv, Israel), human CB_1R was from Dr. M. Parmentier (Universite Libre de Bruxelles, Belgium), and rat G protein activated-inwardly rectifying K^+ channel 1 (GIRK1) and mouse GIRK2 were donated by Dr. H.A. Lester (Caltech, Pasadena, CA, USA). $GABA_{B1a}R$ and $GABA_{B2}R$ subunits were from Dr. N. Fraser (Glaxo Wellcome, UK). Cerulean, a brighter variant of CFP (8) was from Dr. D.W. Piston (Vanderbilt University, TN, USA), and Venus, a brighter variant of YFP (9) was from Dr. T. Nagai (Hokkaido University, Sapporo). The chimeric $G\alpha_{qi5}$ was a kind gift from Dr. B.R. Conklin (University of California at San Francisco, CA, USA). PTX-insensitive chimeric $G\alpha_{qi5}$ protein $G_{qi5(m)}$, whose cysteine residue at –4 from the C-terminus end was changed to isoleucine, was created with a site-directed mutagenesis kit (QuickChange TM site-directed mutagenesis Kit; Stratagene, Tokyo). The CB_1R-Cerulean/Venus, μOR-Cerulean/Venus, and $GABA_BR$-Cerulean/Venus were generated by ligating the receptor cDNAs into *Not*I or *Bam*HI sites into the corresponding site of Venus or Cerulean cDNAs. The tandem cDNAs of μOR-G_{qi5}/$G_{qi5(m)}$, CB_1R-G_{qi5}/$G_{qi5(m)}$, and $GABA_{B2}R$-G_{qi5}/$G_{qi5(m)}$ were created by ligating the receptor cDNA sequences into the *Nhe*I site of G_{qi5} or $G_{qi5(m)}$ cDNAs. FLAG-tagged μOR was constructed by PCR with the 5'-FLAG sequence–containing open reading frame primer and the 3'-end of the receptor sequence primer. The sequences of all PCR products were confirmed by sequencing with ABI3100 (Applied BioSystems, Tokyo). All cDNAs for transfection in BHK cells were subcloned into pcDNA3.1 (Invitrogen, Tokyo). All cDNAs for the synthesis of cRNAs were subcloned into the pGEMHJ vector, which provides the 5'- and 3'-untranslated region of the *Xenopus* β-globin RNA (14), ensuring a high level of protein expression in the oocytes. Each of the cRNAs was synthesized using the mCAP mRNA Capping Kit, with T7 RNA polymerase in vitro Transcription Kit (Ambion, Austin, TX, USA) from the respective linearized cDNAs.

Cell culture and transfection

BHK cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere at 95% air and 5% CO_2. For transfection experiments, BHK cells were seeded at a density of 1×10^5 cells/35-mm glass-bottomed culture dish (World Precision Instrument, Sarasota, FL, USA) for 24 h. For western blot assay, cells were seeded at a density of 1×10^6 cells/35-mm dish. Transient transfection was then performed with Effectene transfection reagent (Qiagen, Tokyo) containing 0.2 μg each of cDNAs as described previously (11, 15). Cells were used in confocal microscopy analysis, FRET analysis, and Western blotting assay 16 – 24 h after transfection.

Confocal microscopy for FRET analysis

For the analysis of heterodimerization of CB_1R and

μOR with FRET assay, CB_1R, μOR, and $GABA_BRs$ were fused at their carboxy terminus to Cerulean or Venus. Both Cerulean and Venus were excited with 458-nm laser, and images were obtained by placing the dish onto a stage in a Zeiss LSM510 META confocal microscope (Carl Zeiss, Jena, Germany).

Photobleaching and calculation of FRET efficiency

To confirm FRET between Cerulean and Venus, we monitored acceptor photobleaching analysis in BHK cells that coexpressed Cerulean- and Venus-fused receptors. FRET was measured by imaging Cerulean before and after photo-bleaching Venus with the 100% intensity of 514-nm argon laser for 30 – 60 s, a duration that efficiently bleached Venus with little effect on Cerulean (11, 15). An increase of the donor fluorescence (Cerulean) was interpreted as evidence of FRET from Cerulean to Venus. For each experiment, at least six cells were analyzed, with three independent regions of interest. As a control, we examined the FRET efficiency of the unbleached area of the membranes or the cytosol in the same cell in at least three areas.

FRET efficiency was calculated using emission spectra before and after acceptor photobleaching. In our study as well as others, this protocol had almost no effect on Cerulean in the absence of Venus (11, 16). According to this procedure, if FRET is occurring, then photobleaching of the acceptor (Venus) should yield a significant increase in fluorescence of the donor (Cerulean). Increase of donor spectra due to desensitized acceptor was measured by taking Cerulean emission (at 488 nm) from spectra before and after acceptor photobleaching (see Fig. 1). FRET efficiency was then calculated using the equation $E = 1 - I_{DA} / I_D$, where I_{DA} is the peak of donor emission in the presence of the acceptor and I_D is the peak in the presence of the sensitized acceptor, as described previously (11, 15, 17). Before and after this bleaching, Cerulean images were collected to assess changes in donor fluorescence.

Coimmunoprecipitation and Western blotting

Monoclonal anti-FLAG M2, anti-μOR, and anti-CB_1R antibodies were obtained from Sigma. BHK cells were transiently cotransfected with each of the FLAG-tagged rat μOR cDNA and human CB_1R cDNA, alone or both as described previously (15). Briefly, 24 h after transfection, BHK cells were harvested, sonicated, and solubilized in the PROREP protein extraction buffer containing a protease inhibitor cocktail (iNtRON Biotechnology, Sungnam, Korea) for 1 h at 4°C. The mixtures were centrifuged (15,000 rpm, 30 min) and the supernatants were incubated with rabbit polyclonal antibodies against FLAG at 5 μg/ml overnight at 4°C. The mixture was centrifuged and the pellets were washed five times by centrifugation and resuspension. Immunoprecipitated materials were dissolved in Lammeli sample buffer containing 0.1 M DTT subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and membranes were treated with blocking solution and incubated with a monoclonal antibody against FLAG (dilution, 1:10,000), μOR (dilution, 1:250), or CB_1R (dilution, 1:250) and then bovine mouse anti-IgG conjugated with horseradish peroxidase at 1:5,000 and reacted with chemiluminescence western blotting detection reagents (Nacalai Tesuque).

Oocyte preparation and injection

Immature V and VI oocytes from *Xenopus* were enzymatically dissociated as described previously (18, 19). Isolated oocytes were incubated at 18°C in ND-96 medium (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.4) containing 2.5 mM sodium pyruvate and 50 μg/ml gentamicin. For measurement of GIRK currents induced by DAMGO or CP55,940, cRNAs for GIRK1/2 (0.2 ng each) were coinjected into the oocytes together with or without μOR, CB_1R, $GABA_{B1a}R$, μOR-Venus/Cerulean, or CB_1R-Cerulean/Venus (5 ng each). For the Ca^{2+}-activated Cl^- current assay, cRNA for G_{qi5}- or $G_{qi5(m)}$-fused μOR, CB_1R, or $GABA_BR$ (5 ng each) were injected. The final volume of the injectate was less than 50 nl in all cases. Oocytes were incubated in ND-96 and used 3 – 8 days after injection as reported previously (11, 18, 19).

Electrophysiological recordings

Electrophysiological recordings were performed using the two-electrode voltage clamp method with a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA, USA) at room temperature. Oocytes were clamped at −60 mV and continuously superfused with ND-96 or 49 mM high K^+ (HK) solution (48 mM NaCl, 49 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.4) in a 0.25 ml chamber at a flow rate of 5 ml/min, and test compounds were added to the superfusate. Voltage recording microelectrodes were filled with 3 M KCl and their tip resistance was 1.0 – 2.5 MΩ. Currents were continuously recorded and stored with a MacLab (AD Instruments, Castle Hill, NSW, Australia) and a Macintosh computer, as described previously (18, 19). All test compounds applied to oocytes were dissolved into ND-96 or HK (49 mM K^+) medium.

Statistical analyses

Data are expressed as mean ± S.E.M. Differences between two groups were examined for statistical significance by using the paired *t*-test. For comparisons between multiple groups, one-way analysis of variance (ANOVA) was used followed by Scheffe's test. GraphPad Prism (GraphPad, La Jolla, CA, USA) software was used for data analysis. A *P* value of less than 0.05 denoted the presence of a statistically significant difference.

Results

FRET and acceptor bleaching analysis of Venus-fused μOR and Cerulean-fused CB_1R expressed in BHK cells

In order to confirm FRET, we performed acceptor photobleaching analysis (Fig. 1). As a control study, we constructed CB_1R tandemly fused to both Cerulean and Venus (CB_1R-Cerulean-Venus) and expressed it in BHK cells. As shown in the upper panels of Fig. 1A, fluorescence from Cerulean and Venus was detected by

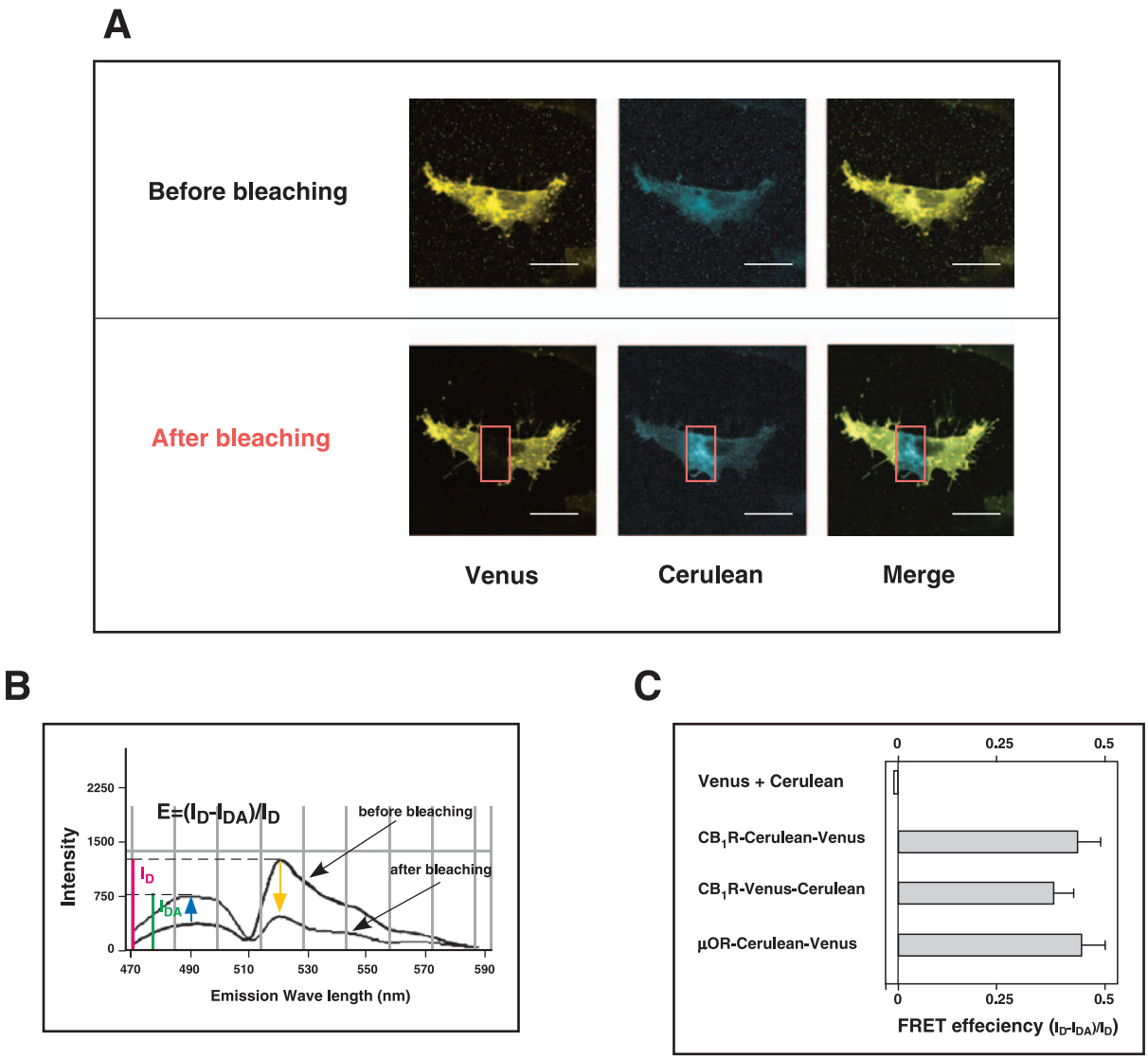


Fig. 1. Confocal imaging and fluorescence resonance energy transfer (FRET) analysis of the cannabinoid CB_1 receptor tandemly fused to Cerulean and Venus (CB_1R-Cerulean-Venus) in baby hamster kidney (BHK) cells. BHK cells were transiently transfected with the plasmid DNA for CB_1R-Cerulean-Venus and fixed 24 h after transfection. A: Visualization of the CB_1R-Cerulean-Venus by excitation with 458-nm wavelength laser and acceptor photo-bleaching (within the red box in the lower panel). Calibration bar = 10 μm. B: Emission spectra from a BHK cell before and after Venus photo-bleaching as indicated in panel A. Note the increase of the Cerulean peak emission following photo-bleaching emission in the presence of sensitized acceptor (Venus). C: FRET efficiency of Venus + Cerulean, CB_1R-Cerulean-Venus, CB_1R-Venus-Cerulean, and μOR-Cerulean-Venus after photo-bleaching of Venus (acceptor). Each column represents the mean ± S.E.M. of the FRET efficiency from independent experiments on 6 BHK cells.

scanning while exciting with the 458 nm laser line. The use of acceptor photobleaching increased Cerulean fluorescence (donor) and decreased Venus fluorescence (acceptor) (Fig. 1A, lower panel, and Fig. 1B). As shown in Fig. 1C, FRET efficiency was significantly higher in BHK cells expressing CB_1R-Ceruelan-Venus, CB_1R-Venus-Cerulean, and μOR-Cerulean-Venus than in the negative control (Cerulean + Venus).

As shown in the upper panels of Fig. 2A, fluorescence of Venus and Cerulean was observed in the cell membrane and cytoplasm of BHK cells coexpressing μOR-V + CB_1R-C, at pre-photobleaching (Fig. 2A). The photo-bleaching results showed that Cerulean fluorescence increased with a decrease in Venus fluorescence in both the cell membranes and the cytosol (Fig. 2A, lower panel). As shown in Fig. 2B, the FRET efficiency was significantly higher in BHK cells coexpressed with μOR-V + CB_1R-C compared with the negative control [pairs of Venus (V) + Cerulean (C), μOR-V + $GABA_{B2}R$-C or CB_1R-C + $GABA_{B2}R$-V], as well as a heterodimer pair of $GABA_BR$-V/C, known to form an obligatory heterodimer to be functional (11). When the FLAG-tagged μOR-V was coexpressed with CB_1R-C in BHK cells, the FRET efficiency was not different from BHK cells expressed non-FLAG-tagged μOR-V (Fig. 2B).

Coimmunoprecipitation and Western blotting of FLAG-μOR and CB_1R

Immunoprecipitation with the anti-FLAG was performed with proteins extracted from BHK cells. Samples immunoprecipitated with FLAG antibody were blotted with anti-FLAG, anti-μOR, and anti-CB_1R antibodies (Fig. 3, left). The immune complexes blotted with anti-FLAG were identified in precipitants containing FLAG-tagged μOR (Fig. 3, top left). Furthermore, immune complexes blotted with anti-μOR were identified in precipitants containing FLAG-μOR (Fig. 3, middle left). The immune complexes blotted with anti-CB_1R antibody were found in precipitants containing CB_1R and FLAG-tagged μOR, but not CB_1R alone (Fig. 3, bottom left).

In experiments using total cell lysates, the immune complexes blotted by anti-CB_1R antibody were detected in CB_1R-expressing lysates such as in CB_1R alone or in combination of CB_1R with FLAG-μOR (Fig. 3, right).

Responses to DAMGO or CP55,940 in Xenopus oocytes expressing μOR or CB_1R together with GIRK1 and GIRK2 (GIRK1/2)

A selective μOR agonist DAMGO or a selective CB_1R agonist CP55,940 elicited inward rectifying K^+ currents in oocytes coexpressing μOR or CB_1R together with GIRK1/2, respectively (Fig. 4: A and B) in a

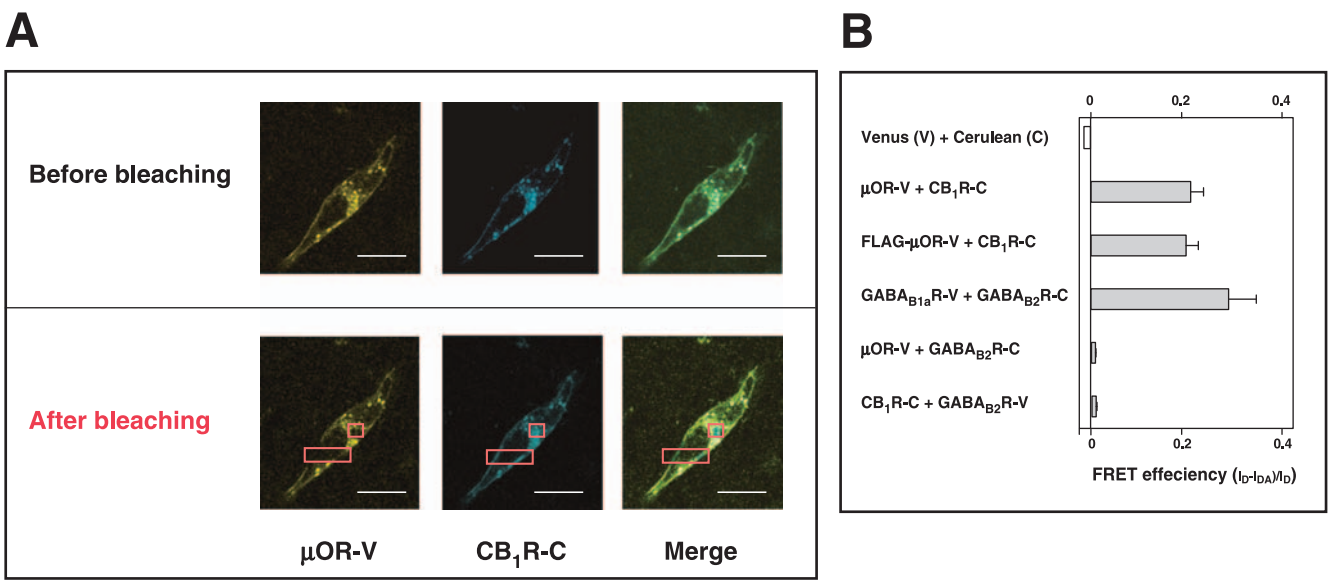


Fig. 2. Confocal imaging and FRET analysis of the Venus-fused μOR (μOR-V) and Cerulean-fused CB_1R (CB_1R-C) coexpressed in a BHK cell. A: Visualization of the μOR-V and CB_1R-C in a cotransfected BHK cell and Cerulean and Venus fluorescences by photobleaching (after 30-s sustained application of 514-nm wavelength laser, lower panel). Fluorescence readings were performed 24 h after transfection as described in Materials and Methods. Calibration bar = 10 μm. B: Summary of FRET efficiency for the pair μOR-V and CB_1R-C and the pair FLAG-μOR-V and CB_1R-C. The pair $GABA_{1a}R$-V + $GABA_{B2}R$-C and the pair μOR-V + $GABA_{B2}R$-C or CB_1R-C + $GABA_{B2}R$-V were used as a positive and negative control for interacting receptors, respectively. Each column represents the mean ± S.E.M. of the FRET efficiency from independent experiments of 6 BHK cells.

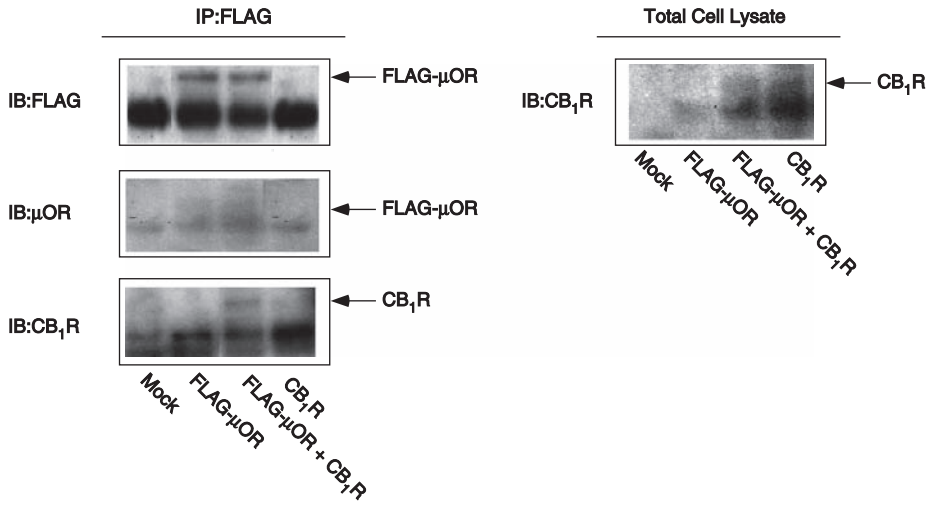


Fig. 3. Western blot analysis of FLAG-tagged μOR (FLAG-μOR) and CB_1R coexpressed in BHK cells. Left: Western blotting of FLAG-μOR and/or CB_1R using immunoprecipitation with anti-FLAG. Immunoblotting was then performed with anti-FLAG (upper panel), anti-μOR (middle panel), and anti-CB_1R (bottom panel). Right: Western blotting of FLAG-μOR and/or CB_1R using total cell lysate. Immunoblot was performed with anti CB_1R. Representative data of five experiments with similar results.

concentration-dependent manner (Fig. 4C). On the other hand, no responses were recorded to DAMGO or CP55,940 in oocytes coexpressing μOR or CB_1R without GIRK1/2 (Fig. 4: A and B). The EC_{50} of DAMGO was 10^{-7} M in oocytes expressing μOR with GIRK1/2 and that of CP55,940 was 3×10^{-7} M in oocytes expressing CB_1R with GIRK1/2.

Responses to DAMGO or CP55,940 in Xenopus oocytes expressing μOR fused to G_{qi5} or CB_1R fused to G_{qi5}

Electrophysiological experiments were performed in oocytes expressing μOR and CB_1R, either of which was fused to G_{qi5} protein. Since the chimeric $G\alpha_{qi5}$ protein contains the carboxyl-terminal five amino acids of $G\alpha_i$, the signal mediated by $G_{i/o}$-coupled receptors is transferred to a PLC/IP_3/Ca^{2+}-mediated signal, and thus oocytes expressing G_{qi5}-fused receptors can elicit Ca^{2+}-activated Cl^- currents in response to each agonist (10 – 13). As expected, oocytes expressing G_{qi5}-fused μOR (μOR-$G\alpha_{qi5}$) or G_{qi5}-fused CB_1R (CB_1R-G_{qi5}) elicited Ca^{2+}-activated Cl^- currents in response to DAMGO or CP55,940, respectively (Fig. 5: A and B) in a concentration-dependent manner (Fig. 5C). The EC_{50} of DAMGO was ~10^{-7} M in oocytes expressing μOR-G_{qi5} and that of CP55,940 in oocytes expressing CB_1R-G_{qi5} was ~3×10^{-7} M. There were almost no differences in EC_{50} of DAMGO and CP55,940 between oocytes expressing each receptor-G_{qi5} and oocytes coexpressing each receptor with GIRK1/2 (Figs. 4 and 5), as reported previously (10).

Responses to DAMGO and CP55,940 in Xenopus oocytes coexpressing μOR and CB_1R either of which was fused to G_{qi5}

To investigate whether μOR and CB_1R can form a functional heterodimer, we coexpressed receptor-G_{qi5} and the non-fused receptor counterpart in the oocytes. DAMDO and CP55, 940 were sequentially applied to the oocytes and vice versa. Oocytes coexpressing non-fused μOR + CB_1R-G_{qi5} elicited Ca^{2+}-activated Cl^- currents in response to the 1st and 2nd application of DAMGO and CP55,940 (Fig. 6: A and D). In turn, when CP55,940 and DAMGO were sequentially applied to the oocytes coexpressing μOR + CB_1R-G_{qi5}, they elicited currents of almost the same size as the currents in oocytes applied with DAMGO followed by CP55,940 (Fig. 6: A and D). Similarly, oocytes coexpressing CB_1R + μOR-G_{qi5} elicited Ca^{2+}-activated Cl^- currents in response to the 1st application of CP55,940 followed by DAMGO and also to the 1st application of DAMGO followed by CP55,940; sizes of the currents of the 1st and 2nd application of each agonist were almost similar (Fig. 6D). These results show that both DAMGO and CP55,940, regardless of whether it was the 1st or 2nd sequential application, induced Ca^{2+}-activated Cl^- currents in oocytes coexpressing μOR and CB_1R, either of which was fused to G_{qi5} (Fig. 6: A, B and D). $GABA_BRs$ form obligatory functional heterodimers (11). As shown in Fig. 6C, when $GABA_{B1a}R$ and $GABA_{B2}R$-G_{qi5} were expressed in oocytes, the $GABA_BR$ agonist baclofen elicited Ca^{2+}-activated Cl^- currents, as reported previously (11). In oocytes coexpressing $GABA_{B1a}R$ + $GABA_{B2}R$-G_{qi5} + μOR, baclofen but not DAMGO elicited the currents. Furthermore, in oocytes coexpressing $GABA_{B1a}R$ + $GABA_{B2}R$ + μOR-G_{qi5}, DAMGO but not baclofen elicited Cl^- currents (Fig. 6C). No responses to DAMGO and CP55,940 were elicited in oocytes coexpressing μOR and CB_1R, both of which were not fused to G_{qi5} (Fig. 6D).

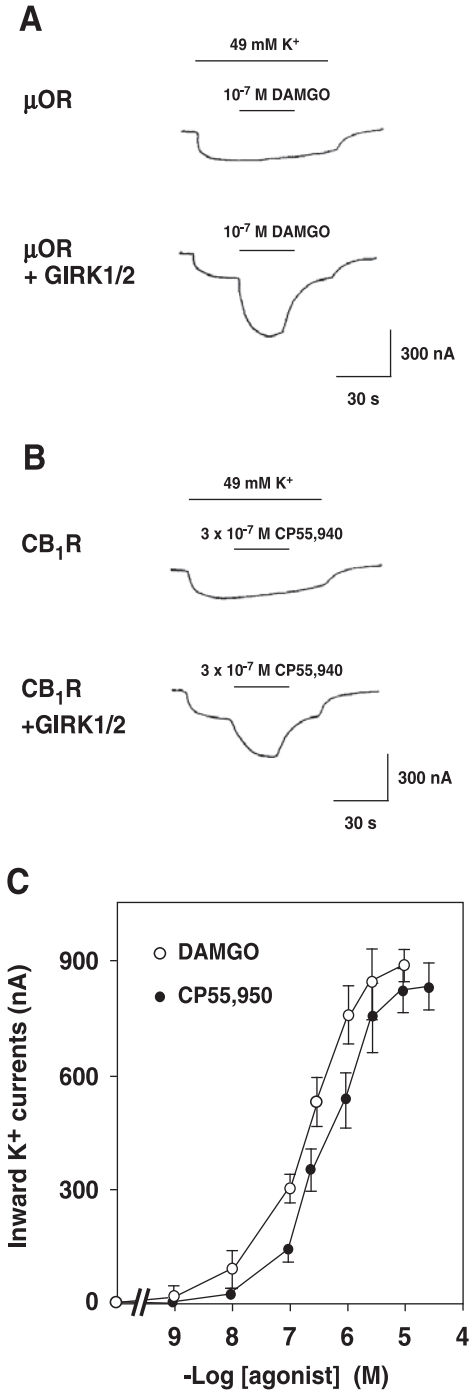


Fig. 4. DAMGO- and CP55,940-induced GIRK currents in *Xenopus* oocytes coexpressing μOR and CB_1R together with GIRK1/2 channels. A and B: Representative trace of each agonist-induced GIRK currents. Varying concentrations of DAMGO and CP55,940 were applied separately to the oocytes for 30 s as indicated by the bar. C: Concentration-response curves of DAMGO (open circle) and CP55,940 (closed circle) in oocytes expressing μOR or CB_1R together with GIRK1/2. Varying concentrations of DAMGO and CP55,940 were applied separately to the oocytes for 30 s. Each point represents the mean ± S.E.M. of the peak GIRK currents expressed as percentage of the maximum response from 8 oocytes.

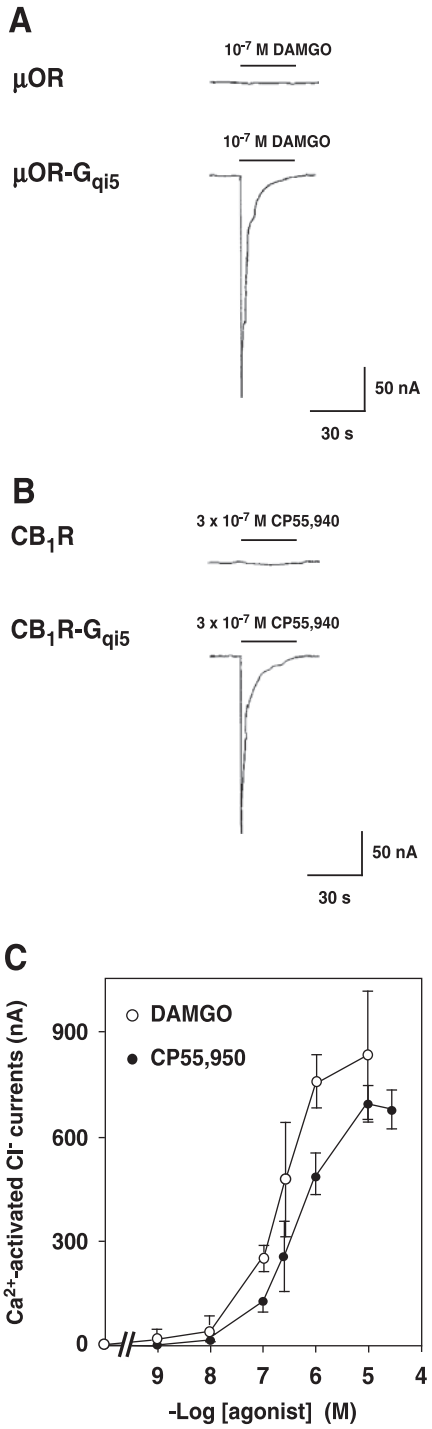
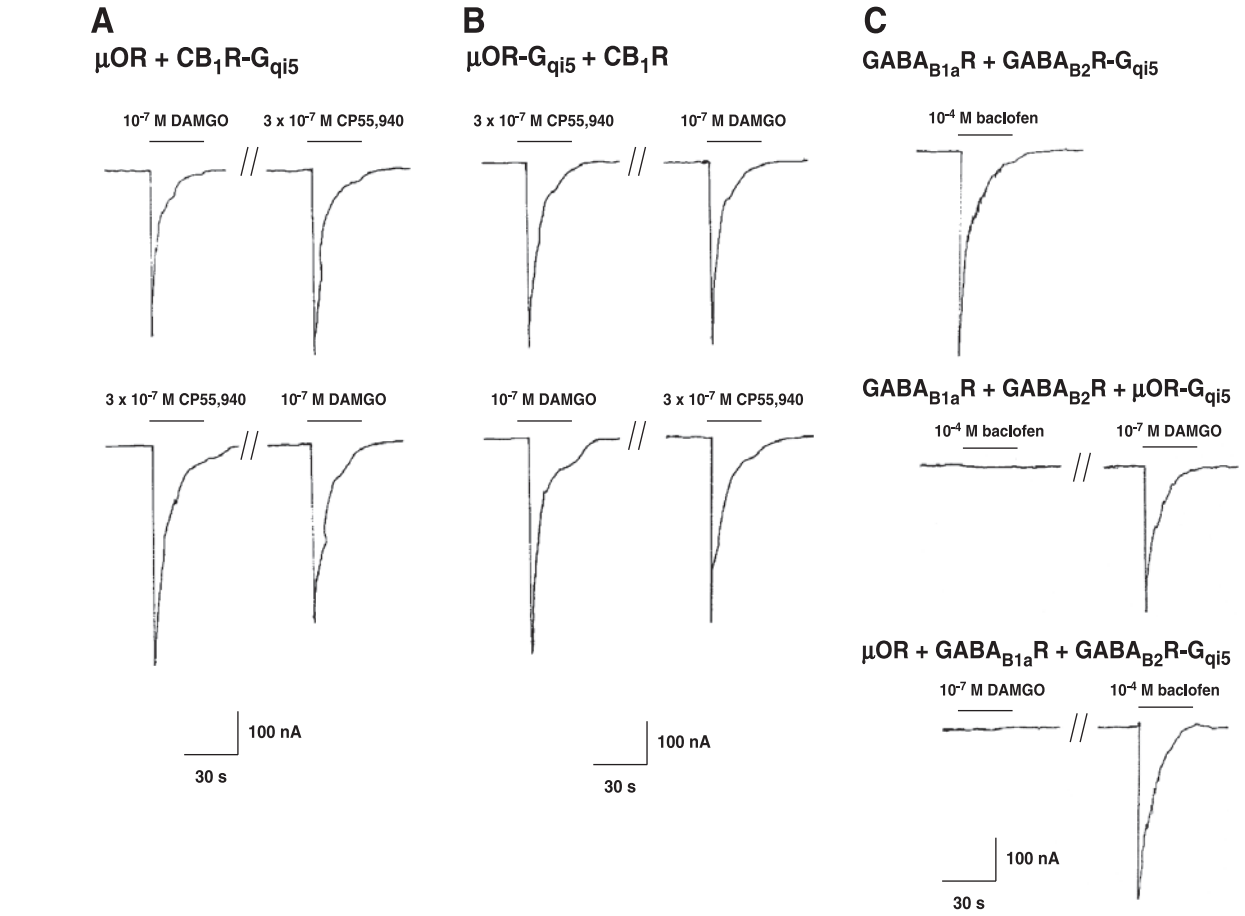


Fig. 5. DAMGO- and CP55,940-induced Cl^- currents in μOR-expressing oocytes fused to G_{qi5} (μOR-G_{qi5}) and cannabinoid CB_1R fused to G_{qi5} (CB_1R-G_{qi5}). A and B: Representative trace of each agonist-induced Ca^{2+}-activated Cl^- current. C: Concentration-response curves of DAMGO (open circle) or CP55,940 (closed circle) in oocytes expressing μOR-G_{qi5} or CB_1R-G_{qi5}. Varying concentrations of DAMGO or CP55,940 were applied to the oocytes for 30 s. Each point represents the mean ± S.E.M. of the peak Cl^- currents expressed as a percentage of the maximum response from 8 oocytes.



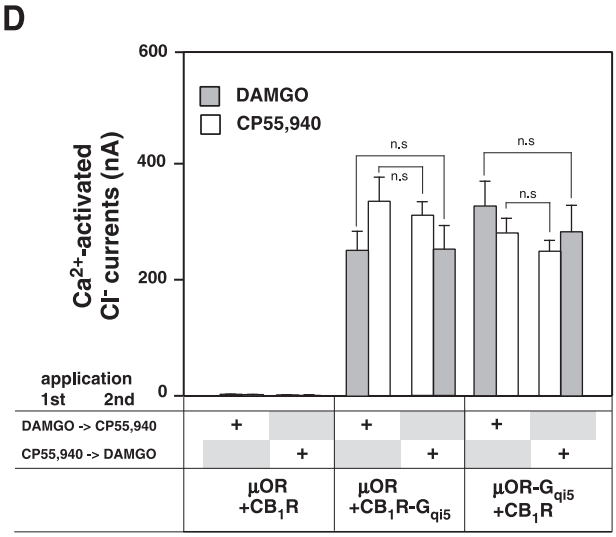
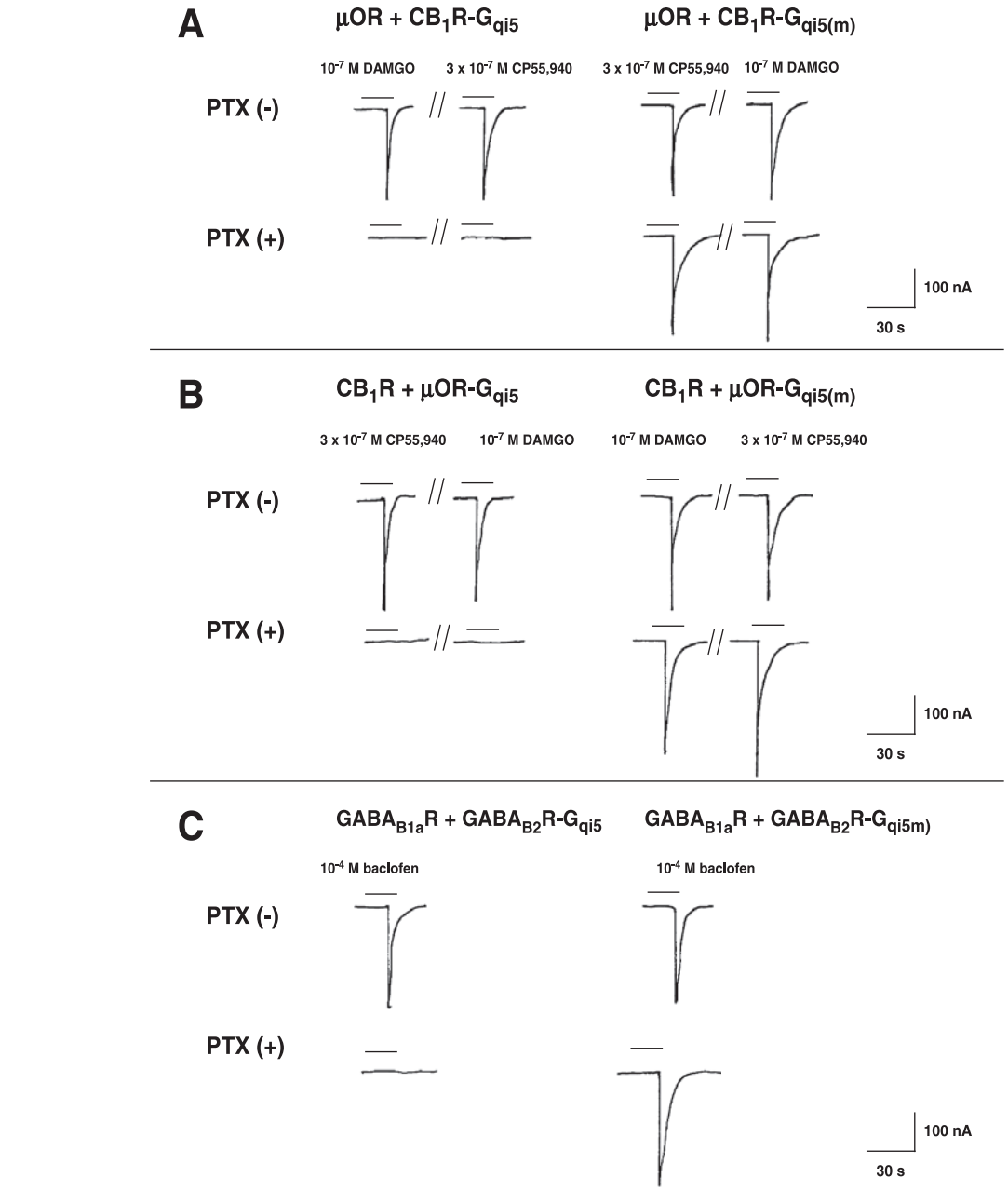


Fig. 6. DAMGO- and CP55,940-induced Cl^- currents in oocytes coexpressing μOR and CB_1R, one of which was fused to G_{qi5}. Effects of sequential application of DAMGO and CP55,940 or baclofen in oocytes coexpressing μOR-G_{qi5} with CB_1R (A); μOR with CB_1R-$G\alpha_{qi5}$ (B); and $GABA_{B1a}R + GABA_{B2}R$-G_{qi5}, μOR + $GABA_{B1a}R + GABA_{B2}R$-G_{qi5}, or $GABA_{B1a}R + GABA_{B2}R + \mu$OR-$G_{qi5}$ (C). DAMGO (10^{-7} M), CP55,940 (3×10^{-7} M), and baclofen (10^{-4} M) were applied separately to the oocytes for 30 s. In sequential application of DAMGO and CP55,940, DAMGO was first applied followed by CP55,940, and vice versa. D: Summary of the effects of coexpression of G_{qi5}-fused and non-fused receptors on the agonist-induced Ca^{2+}-activated Cl^- currents, and the effects of sequential application of each agonist to the same oocytes on the Ca^{2+}-activated Cl^- currents. Each bar represents the mean ± S.E.M. of peak Cl^- currents from each of 8 oocytes. n.s.: not significant difference between the sizes of currents in the first and second application of DAMGO or CP55,940.

Properties of transactivating responses induced by DAMGO or CP55,940 in oocytes coexpressing PTX-insensitive receptor-$G_{qi5(m)}$

As shown in Fig. 6, both DAMGO and CP55,940 elicited Ca^{2+}-activated Cl^- currents in oocytes coexpressing μOR-G_{qi5} + CB_1R or μOR + CB_1R-G_{qi5}, suggesting functional heterodimer formation in oocytes. It has been reported that $G_{i/o}$ protein and G_{qi5} are targets of ADP ribosylation of PTX at –4 cysteine from the C-terminus, and PTX treatment abrogates signals from $G_{i/o}$-coupled receptors to $G_{i/o}$ as well as those to G_{qi5} (11, 19 – 21). Another chimeric $G_{qi5(m)}$, whose cysteine residue was changed to isoleucine, was reported to be insensitive to PTX (21). Accordingly, we pretreated oocytes coexpressing μOR + CB_1R-G_{qi5} or μOR + CB_1R-$G_{qi5(m)}$ with PTX (2 μg/ml) for 16 h (Fig. 7A). The concentra-



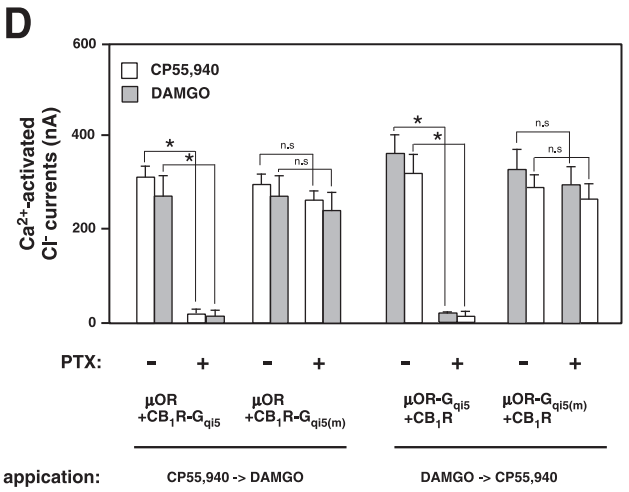


Fig. 7. Properties of DAMGO- and CP55,940-induced Cl^- currents in oocytes coexpressing μOR and CB_1R, one of which was fused to PTX-insensitive $G\alpha_{qi5(m)}$ or PTX-sensitive G_{qi5}. Effects of DAMGO, CP55,940, or baclofen in oocytes coexpressing CB_1R-G_{qi5} or CB_1R-$G_{qi5(m)}$ with μOR (A), coexpressing μOR-G_{qi5} or μOR-$G_{qi5(m)}$ with CB_1R (B), or coexpressing $GABA_{B2}R$-G_{qi5} or $GABA_{B2}R$-$G_{qi5(m)}$ with $GABA_{B1a}R$ (C). Oocytes were pretreated with PTX (0.2 mg/ml) for 16 h before electrophysiological measurement. In sequential application of DAMGO and CP55,940, DAMGO was first applied followed by CP55,940, and vice versa. D: Summary of the effects of PTX treatment on Ca^{2+}-activated Cl^- currents. Each bar represents the mean ± S.E.M. of peak Cl^- currents from each of 8 oocytes. *P<0.05, compared to the Cl^- currents in oocytes not pretreated with PTX. n.s: not significant compared to the currents in oocytes not pretreated with PTX.

tions and durations of PTX treatment used in the present study were reported to produce almost complete inactivation of the $G_{i/o}$ proteins in oocytes (11, 19). Under these conditions, oocytes coexpressing μOR + CB_1R-G_{qi5} showed no responses to DAMGO or CP55,940, whereas both agonists caused Cl^- currents in oocytes coexpressing μOR + CB_1R-$G_{qi5(m)}$ (Fig. 7: A and D). Similarly, oocytes coexpressing μOR-$G_{qi5(m)}$ + CB_1R responded to both DAMGO and CP55,940 pretreated with PTX (Fig. 7: B and D). In addition, PTX-pretreated oocytes coexpressing $GABA_{B1a}R$ + $GABA_{B2}R$-$G_{qi5(m)}$ also responded to baclofen (Fig. 7C).

Discussion

The present morphological and functional studies demonstrated that μOR and CB_1R form functional heterodimers. FRET and acceptor bleaching analysis demonstrated that μOR and CB_1R form heterodimers in BHK cells coexpressing μOR-Venus and CB_1R-Cerulean both in the plasma membrane and in cytosol. Coimmunoprecipitation followed by western blot analysis further supported the heterodimerization of μOR and CB_1R. Recent reports have shown that CB_1R and μOR form heterodimers as determined by optical bioluminescence resonance energy transfer (BRET) assay (7). Our results are in agreement with the above reports in that both receptors exist as heterodimers on the membrane or cytosol. In addition, we presented direct evidence for the first time that μOR and CB_1R form a functional heterodimer, by our electrophysiological experiments.

In the electrophysiological study, *Xenopus* oocytes coexpressing $G_{i/o}$-coupled μOR or CB_1R together with GIRK1/2 caused inward rectifying K^+ currents in response to the selective μOR agonist DAMGO and CB_1R agonist CP55,940, respectively, as reported previously (19, 22). The signals generated by stimulation of each receptor are transmitted to GIRK via G$\beta\gamma$ subunits liberated from $G_{i/o}$ endogenously expressed in oocytes (10, 19). The G_{qi5} is a chimeric Gα_q protein containing the last five amino acid residues from the C-terminus of G_i protein, which enables coupling of $G_{i/o}$-coupled receptors to the PLC signaling pathway (10, 11). By using fused receptors with G_{qi5}, we demonstrated that oocytes expressing either μOR-G_{qi5} or CB_1R-G_{qi5} alone activated PLC to subsequently elicit Ca^{2+}-activated Cl^- currents in response to their specific agonists. Such a G_{qi5}-mediated signaling pathway induced by receptors that originally couple to $G_{i/o}$ proteins has been demonstrated in oocytes coexpressing other $G_{i/o}$-coupled receptors, namely muscarinic M_2, serotonin 5-HT_{1A}, somatostatin type 2, and $GABA_B$ receptors with coexpression of G_{qi5}, which was conducted in our laboratory (10, 11). The EC_{50} values of these agonists were similar to those obtained in oocytes expressing the receptor together with GIRK1/2 and the receptor fused to G_{qi5}, suggesting that the efficacy is similar between the receptors/endogenous $G_{i/o}$ and the receptors/G_{qi5}, as reported previously (10). In oocytes coexpressing μOR and CB_1R, only one of which was fused to G_{qi5}, the agonist for the fused receptor elicited Ca^{2+}-activated Cl^- currents, as expected. Moreover, both DAMGO and CP55,940, which are agonists for fused receptors and for non-fused receptors, could elicit Ca^{2+}-activated Cl^- currents in those oocytes, which is a novel finding in our present study. One study using a three-dimensional structure model suggested that the homo- or heterodimeric signaling molecules of the GPCR-G protein complex were pentamers composed of two dimerized GPCRs with one trimeric G protein (23). Considered together, the present findings and those of the above study (23) suggest that μOR and CB_1R functionally interact on the plasma membrane and form a heterodimer and transmit the signal downstream through a common G_{qi5} protein involved in the pentameric complex, but not through G proteins endogenously expressed in oocytes. Selective responsiveness in this interaction was revealed by the fact that oocytes coexpressing μOR + $GABA_{B1a}R$ + $GABA_{B2}R$-G_{qi5} elicited Cl^- currents only in response to baclofen but not DAMGO. Furthermore, μOR-G_{qi5} + $GABA_{B1a}R$ + $GABA_{B2}R$ responded to only DAMGO but not baclofen, indicating that μOR did not form a μOR/$GABA_BR$ heterodimer. We also previously reported that μOR and $GABA_BR$ did not form heterodimers with each other by FRET analysis (11).

The specific transactivating signal among the heterodimerized receptor complex was further confirmed by the PTX treatment experiments. In oocytes pretreated with PTX, most G$\alpha_{i/o}$ proteins were modified through their cysteine residue located at the 4th position from the C-terminus, and consequently, the signal from $G_{i/o}$-coupled GPCRs to G$\alpha_{i/o}$ was prevented (19 – 21). Even under PTX-pretreatment condition, $G_{qi5(m)}$ is insensitive so that signals from receptors can be transmitted through the $G_{qi5(m)}$. We clearly showed that both DAMGO and CP55,940 elicited Ca^{2+}-activated Cl^- currents in PTX-pretreated oocytes coexpressing μOR + CB_1R-$G_{qi5(m)}$ or μOR-$G_{qi5(m)}$ + CB_1R. Taken together, our results suggest that μOR and CB_1R form a functional heterodimer on the plasma membrane.

Several studies reported that μOR forms homo- and heterodimeric structures with several types of receptors including opioid μOR and δOR, adrenergic β_2R, and neurokinin NK1 receptor [see review (24)]. Furthermore, CB_1R has also been reported to form homo- and

heterodimers with CB_1R itself, dopamine D_2, and orexin OR1 receptors (25 – 27). In general, heterodimeric receptors show distinct pharmacological profiles compared with their parental receptors [see review (3)]. The synergy in the analgesic effects of opioids and cannabinoids can be attributed to a cross-talk between these two signaling pathways mediated by simultaneous activation of opioid and cannabinoid receptors. In rat nucleus accubens core where μOR and CB_1R are abundantly expressed, both receptors may physically associate and simultaneous stimulation of the receptors resulted in non-additive glutamate release and synergistic GABA release, suggesting the existence of functional $\mu OR/CB_1R$ heterodimers distinct from their parental receptors in the tissue (28). Electron microscopic studies on localization of CB_1R and μOR in rat nucleus accumbens also supported coexistence of these receptors within the same neuron (29). The results reported by Rios et al. (7) and those of the present study showed that μOR formed a heterodimer with CB_1R. In their report, simultaneous activation of $\mu OR/CB_1R$ heterodimer leads to a significant attenuation of the extracellular receptor-regulated kinase (ERK) activity compared to the responses seen upon activation of the individual receptor (7). Our results showed that separate application of each agonist elicited Ca^{2+}-activated Cl^- currents, and the sizes of the currents observed were almost similar to those obtained with oocytes expressing $\mu OR\text{-}G_{qi5}$ or $CB_1R\text{-}G_{qi5}$ alone (data not shown). Although we did not conduct experiments to determine the effects of simultaneous application of both μOR and CB_1R agonists on the cellular responses, we plan to do such experiments soon as part of our ongoing studies.

In summary, we demonstrated that μOR and CB_1R form a functional heterodimer and may transmit a signal through a common G protein. It is important to clarify the physiological roles of $\mu OR/CB_1R$ heterodimer and to develop specific agonists/antagonists that act specifically on the dimerized forms of $\mu OR/CB_1R$. Our novel approach using the heterologous expression system may be useful for such development. We propose that the electrophysiological method used in this study can be suitable for investigation and analysis of signals mediated through heterodimerized G protein–coupled receptors.

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