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Ligands regulate cell surface level of the human κ opioid receptor (hKOR) by activation-induced down-regulation and pharmacological chaperone-mediated enhancement: differential effects of non-peptide and peptide agonists *

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Running Title: Down-regulation and pharmacochaperone effects of ligands

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Nonstandard abbreviations: BFA, Brefeldin A; CHO, Chinese hamster ovary; CHO-FLAG-hKOR, CHO cell lines stably expressing FLAG-hKOR; DOR, Delta opioid receptor; ER, Endoplasmic reticulum; FBS, Fetal bovine serum; FLAG, DYKDDDDK; FLAG-hKOR, FLAG-tagged human kappa opioid receptor; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; ICI 204,448, [(*RS*)-[3-[1-[[(3,4-Dichlorophenyl)acetyl]methylamino]-2-(1-pyrrolidinyl)ethyl]phenoxy] acetic acid hydrochloride]; MOR, Mu opioid receptor; *Mr*, Relative molecular mass; rKOR, Rat kappa opioid receptor; R(+)-SCH 23,390, [(*R*)-(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride]; TRK-820, [17-cyclopropylmethyl-3,14-dihydroxy-4,5-epoxy-6-[*N*-methyl*trans*-3-(3-furyl) acrylamido] morphinan hydrochloride]; U50,488H, [2-(3,4-dichlorophenyl)-N-methyl-N-[(2R)-2-pyrrolidin-1-ylcyclohexyl]acetamide].

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Abstract

Two peptide agonists, 8 non-peptide agonists, and 5 non-peptide antagonists were evaluated for their capacity to regulate FLAG-tagged hKOR stably expressed in CHO cells following incubation for 4 h with a ligand at a concentration ~1000-fold of its EC_{50} (agonist) or *Ki* (antagonist) value. Dynorphin A and B decreased the fully glycosylated mature form (55-kDa) of FLAG-hKOR by 70%, whereas non-peptide full agonists (U50,488H, TRK-820, ethylketocyclazocine, bremazocine, asimadoline and ICI 204,448) caused 10~30% decreases. In contrast, pentazocine (partial agonist) and etorphine (full agonist) up-regulated by ~15% and 25%, respectively. The antagonists naloxone and Nor-BNI also significantly increased the 55-kDa receptor, whereas selective μ, δ and D_1 receptor antagonists had no effect. Naloxone up-regulated the receptor concentration- and time-dependently and enhanced the receptor maturation extent, without affecting its turnover. Treatment with brefeldin A (BFA), which disrupts Golgi, resulted in generation of a 51-kDa form that resided intracellularly. Naloxone up-regulated the new species, indicating that its action site is in the ER as a pharmacological chaperone. Following treatment with BFA, all non-peptide agonists up-regulated the 51-kDa form, whereas dynorphins A and B did not, indicating that non-peptide agonists act as pharmacological chaperones, but peptide agonists do not. BFA treatment enhanced down-regulation of cell surface receptor induced by non-peptide agonists, but not by the peptide agonists, and unmasked etorphine- and pentazocine-mediated receptor down-regulation. These results demonstrate that ligands have dual effects on receptor levels: enhancement by chaperone-like effect and agonist-promoted down-regulation and the net effect reflects the algebraic sum of the two.

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Introduction

The κ opioid receptor (KOR) is one of the three major types (μ, δ and κ) of opioid receptors that mediate physiological and pharmacological effects of opioids *in vivo*. Stimulation of KOR generates many effects, such as antinociception (especially for visceral chemical pain), dysphoria, water diuresis, hypothermia, modulation of immune responses and alleviation of craving for cocaine in addicts [reviewed in (Liu-Chen, 2004)]. The cDNA clones of KORs have been isolated and characterized from several species including the human, mouse, rat, guinea pig, zebra fish, frog and *C. elegans*. The receptors are coupled preferentially to pertussis toxin-sensitive heterotrimeric Gi/o proteins. Following receptor activation, both α and βγ subunits of G proteins recruit downstream signaling effectors to inhibit adenylyl cyclase and voltage-gated Ca^{2+}-channel, and to stimulate G-protein-activated inwardly rectifying K^+ channel, mitogen-activated protein kinase (ERK1/2) and phospholipase Cβ [for a review, (Law et al., 2000b)]. In addition, activation of the KOR regulates Na^+, H^+-exchanger 3 via interaction with Ezrin-radixin-moesin-binding phosphoprotein-50/Na^+, H^+-exchanger regulatory factor-1 (EBP50/NHERF-1) (Huang et al., 2004).

Peptides derived from prodynorphin, including dynorphin A and dynorphin B, are endogenous ligands for the KOR. Many non-peptide agonists and antagonists have been synthesized. U50,488H, an arylacetamide compound, is the prototypic non-peptide selective κ opioid agonist (von Voigtlander et al., 1983). Several other arylacetamide compounds, including U69,593, ICI 204,448 and asimadoline, were subsequently found to be selective for the KOR (Szmuszkovicz, 1999). In addition, several non-arylacetamide compounds have been reported to be selective KOR agonists, including salvinorin A (Roth et al., 2002) and TRK-820 (Seki et al.,

1999). Norbinaltorphimine (Nor-BNI) is the first selective κ antagonist (Portoghese et al., 1987). There are many non-selective opioids compounds, including bremazocine, etorphine, ethylketocyclazocine, pentazocine, naloxone and naltrexone.

The number and activity of the receptor on cell surface are important factors in determining its capacity to modulate downstream signaling molecules (Law et al., 2000b). The receptor number on cell surface can be regulated through biosynthesis pathway (including transcription, translation, protein folding and transport) and degradation. Agonist-induced adaptative events of the KOR have been extensively investigated. Briefly, following activation by an agonist, the KOR is phosphorylated by G protein-coupled receptor kinases (GRKs) and then non-visual arrestin is recruited which reduces coupling between the receptor and G protein, causing receptor desensitization. Through clathrin- and dynamin-dependent pathway, phosphorylated KOR is endocytosed (internalization) followed by either dephosphorylation and EBP50/NHERF-1-involved recycling (resensitization) or down-regulation via both lysosome and proteasome systems [for a review, (Liu-Chen, 2004)]. In the absence of an agonist, there is a low level of constitutive internalization (Li et al., 1999) and presumably down-regulation and resensitization. On the contrary, not much is known about regulation of the KOR along the protein biosynthesis pathway. We recently found that GEC1, a small tubulin-binding protein, bound to the KOR and enhanced cell surface expression of the receptor by facilitating trafficking from endoplasmic reticulum (ER) to Golgi to plasma membranes (Chen et al., 2006).

As the first intracellular compartment responsible for protein synthesis and processing, ER plays very prominent roles in controlling the fate of cellular proteins. It has been widely accepted that ER functions as the quality control system in cells (Ellgaard and Helenius, 2003). In particular, to assume a natively correct conformation is the prerequisite for proteins to avert

the ER-associated degradation pathway and to exit this quality control system before reaching their action sites (Kleizen and Braakman, 2004).

A growing body of evidence has demonstrated that small-molecule pharmacological chaperones are able to stabilize native conformation, promote ER-to-Golgi export and protein maturation. Generally, these small molecules are selective ligands, substrates or inhibitors of the unstable receptors, enzymes or transmembrane channels. Thus, the concentrations of the pharmacological chaperones required to facilitate the export of their target proteins from the ER are usually low enough to be therapeutically approachable and to minimize adverse effects (Bernier et al., 2004; Ulloa-Aguirre et al., 2004).

It has been demonstrated that some newly-synthesized wild-type and mutant membrane-bound proteins cannot exit from the ER quality control system, including cystic fibrosis transmembrane conductance regulator and several G protein-coupled receptors (Gelman and Kopito, 2002; Bernier et al., 2004; Ulloa-Aguirre et al., 2004). Thus, the wild-type receptors can be the targets of the pharmacological chaperones that can regulate their cell surface expression. In the present study, we explored how KOR ligands regulated cell surface expression of the receptor and whether they function as pharmacological chaperones. CHO cells stably expressing FLAG-hKOR were used as the model system.

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Materials and Methods

Materials

Dynorphin A (1–17) and dynorphin B (1-13) were purchased from Phoenix Pharmaceuticals (Belmont, CA). U50,488H [2-(3,4-dichlorophenyl)-N-methyl-N-[(2R)-2-pyrrolidin-1-ylcyclohexyl]acetamide], ethylketocyclazocine, bremazocine, etorphine, norbinaltorphimine, pentazocine and naltrindole were provided by the National Institute on Drug Abuse (Bethesda, MD). ICI 204,448 [(*RS*)-[3-[1-[[(3,4-Dichlorophenyl)acetyl]methylamino]-2-(1-pyrrolidinyl)ethyl]phenoxy] acetic acid hydrochloride] was purchased from Tocris Cookman (Ballwin, MO). Asimadoline and TRK-820 [17-cyclopropylmethyl-3,14-dihydroxy-4,5-epoxy-6-[*N*-methyl*trans*-3-(3-furyl) acrylamido] morphinan hydrochloride] were generous gifts from Adolor Corporation (Exton, PA). Radioligands, L-[^{35}S]-Methionine/Cysteine (~1175 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Naloxone, naloxonazine, R(+)-SCH 23,390 [(*R*)-(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride], brefeldin A, M_1 mouse anti-FLAG monoclonal antibody, rabbit anti-FLAG polyclonal antibody, normal goat serum, $CaCl_2$, EDTA, bovine serum albumin and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). SuperSignal West Pico Chemiluminescent substrate kit, EZ-Link Sulfo-NHS-SS-Biotin and Immobilized Streptavidin were obtained from Pierce (Rockford, IL). Cell media (DMEM/F-12, 1:1 and DMEM without methionine/cysteine), Opti-MEM I reduced serum and fetal bovine serum (FBS) were acquired from Invitrogen (Carlsbad, CA). Materials for cell counting, including Isoton II diluent, Accuvettes and Coulter Clenz solution, were purchased from Beckman Coulter (Fullerton, CA). The following reagents were bought from the indicated

companies: geneticin (G418) from Cellgro Mediatech (Herndon, VA), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG from New England Biolabs (Beverly, MA), complete EDTA-free protease inhibitor cocktail tablets from Roche Diagnostics (Indianapolis, IN), Immobilon-P PVDF 0.45 μm transfer membrane from Millipore (Bedford, MA), Alexa Fluor 488-conjugated goat anti-mouse IgG from Molecular Probes (Eugene, OR), PANSORBIN cells from Calbiochem (San Diego, CA) and Lab-Tek II Slide Chambers from Lab-Tek (Naperville, IL). .

Cell Lines

Clonal CHO cell line stably expressing the Flag-tagged human kappa opioid receptor (FLAG-hKOR) was generated previously (Li et al., 2002). Cells were cultured in 10-cm Petri dishes in DMEM/F-12 medium supplemented with 10% FBS, 0.2 mg/ml geneticin in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37°C.

SDS-PAGE, Western blotting and ligand-induced regulation of FLAG-hKOR

CHO-FLAG-hKOR cells (~90% confluence) were treated with ligands or vehicle at 37°C for 4 h, harvested with Versene buffer (0.54 mM EDTA, 140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4 and 1 mM glucose, pH7.4) and then cell number was determined by Z1 cell and particle counter (Beckman Coulter). One million cells were solubilized in 200 μl of 2× Laemmli sample buffer [4% SDS, 100 mM DTT, 10% glycerol, 62.5 mM Tris-HCl (pH6.8) and 0.1% Bromophenol Blue] and 2×10^5 cells (40 μl of sample buffer) per lane were subjected to Tricine-SDS-PAGE on 8% separating gel, along with SeeBlue pre-stained protein molecular weight markers (Invitrogen, Carlsbad, CA). The separated protein bands were transferred to

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Immobilon-P PVDF transfer membranes which were then incubated with the blocking solution A [5% nonfat dry milk in TBS-T buffer (25 mM Tris-HCl, 150 mM NaCl and 0.1% Tween 20, pH 7.6)] for at least 1 h and then overnight with rabbit polyclonal anti-FLAG (F7425) antibody (0.8 mg/ml, 1:5000) in the blocking solution at 4°C on a head-over-head shaker. The PVDF membranes were washed three times with the TBS-T buffer and incubated at room temperature for 2 h with HRP-linked goat anti-rabbit IgG (1:1000) in the blocking solution. After washing the membrane three times with TBS-T buffer, the protein bands were visualized by applying SuperSignal West Pico Chemiluminescent substrate and then digitalized with Fuji LAS-1000 Plus Gel Documentation System (Fuji Film, Tokyo, Japan). The densitometric analyses of FLAG-hKOR bands were performed by using ImageGauge 4.1 software (Fuji Film). Quantitative comparison of optical densities of FLAG-hKOR between ligand-treated and vehicle-treated cells was carried out to assess ligand-promoted regulation of FLAG-hKOR. %change of FLAG-hKOR = 100 × (optical density in ligand-treated group - optical density in vehicle-treated group) / (optical density in vehicle-treated group).

Metabolic Labeling, immunoprecipitation and autoradiography

Experiments were performed using a modified procedure as described by Petaja-Repo et al. (2002). For conducting [^{35}S]-Met/Cys labeling, 1.2×10^{6} cells were subcultured into 60-mm Petri dish, grown in complete medium for 24 h (to reach ~90% confluence), and then pre-incubated with 2 ml of Met/Cys-free DMEM depletion medium at 37°C for 1.5 h, and pulse-labeled with 150 μCi/ml of L-[^{35}S] Methionine/Cysteine in fresh depletion medium. After 30-min labeling at 37°C, the pulse phase was terminated by washing the cells one time with the chase medium (complete medium supplemented with 5 mM L-methionine) and then incubated

with the chase medium for specified time periods. If BFA and/or ligands were used, BFA was present from the starting of depletion phase until the end of chase phase, but ligands were added into medium only in chase phase. Following the chase phase, cells were detached using Versene buffer, washed one time with phosphate buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4 and 1 mM glucose, pH 7.4), pelleted by centrifugation at 2500*g* at 4°C for 5 min and stored at -80°C until further studies.

Protein solubilization was accomplished by thawing the cells in 400 µl of TTSEC buffer (2% Triton X-100, 50 mM Tris-HCl (pH7.4), 150 mM NaCl, 5 mM EDTA and protease inhibitor cocktail tablet) with 1-h shaking at 4°C. The supernatant was obtained by centrifugation at 13500g for 15 min. For better signal/noise ratio, two-time antibody-PANSORBIN precipitations were performed in tandem to purify the [^{35}S]-labeled FLAG-hKOR. Firstly, 400 µl of supernatant was incubated with 2 µg of polyclonal anti-FLAG antibody at 4°C for 1 h, and mixed with 20 µl of PANSORBIN at 4°C for 1 more hour. The receptors adsorbed on PANSORBIN cells were washed 3 times by repeated centrifugation and resuspension in TBS-T buffer and then eluted by incubating the pellets in 20 µl of 2× Laemmli sample buffer at room temperature for 15 min. The 20 µl of supernatant was added into 380 µl of TTSEC buffer for the 2nd round of precipitation experiment. Adsorbed receptors were eluted with 50 µl of 2× Laemmli sample buffer for Tricine-SDS-PAGE on 8% separating gel. The gel was dried using a gel dryer (Bio-Rad Laboratories, Hercules, CA) and then exposed to pre-bleached storage phosphor screen for 2 days. The autoradiogram was captured by a Cyclone Storage Phosphor System (PerkinElmer Life and Analytical Sciences). The intensities of radio-labeled receptor bands were quantitated with the OptiQuant software. Background signal of each lane was subtracted before performing quantitative kinetic data analysis.

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Transformation of the immature receptor to the mature receptor and turnover of both receptor forms appear to follow the first-order kinetics (see Fig. 3). All analyses were performed using Prism 3.0 to fit the data to the following equations (Tallarida and Murray, 1987):

$$X = A \times \left[e^{-k_e \times t} - e^{-k_a \times t} \right] \quad \text{(For mature form)}$$

$$X = A \times e^{-k_e \times t} \quad \text{(For immature form)}$$

where X is the amount of FLAG-hKOR, A is a constant for each equation, k_a is the transformation rate constant of the immature to mature receptor, k_e is the turnover rate constant of the mature or immature receptor and t is the time of chase. Based on the amount of mature receptor vs. time and the calculated rate constants, maturation extent of the FLAG-hKOR, peak time of mature receptor and half lives of the mature and immature receptors were computed. Maturation extent is defined as the total amount of the mature FLAG-hKOR transformed from the immature receptor, which is determined by the area under the curve (AUC) in the plot of the amount of the mature receptor against time (Fig. 3B). Turnover rate constant (k_e) means the fraction of the receptor degraded per unit of time. Half life ($t_{1/2}$) is the time for the receptor to reduce by 50%, and it is equal to $0.693/k_e$. Peak time (t_{max}) is the time point at which the amount of the mature receptor is maximal and is calculated according to the following equation (Tallarida and Murray, 1987):

$$t_{\max} = \frac{\ln(k_a / k_e)}{(k_a - k_e)}$$

Immunofluorescence staining of FLAG-hKOR on cell surface

Surface FLAG-tagged hKOR was visualized using an "antibody feeding" method described by Wang et al. (2005). Briefly, FLAG-hKOR-CHO cells grown on Lab-Tek II Slide

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Chambers for 24 h to reach ~90% confluence, and the surface receptors were specifically labeled by incubating cells with monoclonal M_1 anti-FLAG antibody (1:1000) in complete medium at 37°C for 30 min. Labeled cells were then fixed with 4% paraformaldehyde in TBS buffer for 15 min at room temperature. After washed 3 times with TBS buffer, cells were blocked with the blocking solution B (4% normal goat serum in TBS buffer) for 15 min, and then treated with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (1:1000) in TBS buffer containing 1% normal goat serum for 45 min at room temperature. Due to the requirement of Ca^{2+} for binding of M_1 anti-FLAG antibody to the FLAG epitope, $CaCl_2$ (final concentration of 1 mM) was added into all solutions in steps from incubation with M_1 antibody. Immunostained cells were mounted with Slow Fade mounting medium, and coverslips were sealed with nail polish. ELIPSE TE300 fluorescence microscope (Nikon, Tokyo, Japan) equipped with a 60× NA 1.4 objective and fluorescein filter sets was used to examine receptor distribution. Cells were treated in the absence of M_1 anti-FLAG antibody were employed as the control group, which did not show any staining.

Cell surface biotinylation and separation of FLAG-hKOR

Experiments were carried out using a protocol recommended by the manufacturer (Pierce). For biotinylation of cell surface proteins, 10 mM stock solution of Sulfo-NHS-SS-Biotin (cell-impermeable and cleavable) was prepared immediately before use. Cells (~90% confluence in 60-mm Petri dish) were washed 3 times with ice-cold PBS buffer containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS/Ca^{2+}/Mg^{2+} buffer, pH7.4) and incubated with gentle agitation for 30 min at 4°C in PBS/Ca^{2+}/Mg^{2+} buffer containing 1 mM of Sulfo-NHS-SS-Biotin. Excess biotin reagent was quenched by treating the cells with 50 mM of Tris-HCl buffer (pH7.4) for 10

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min at 4°C. The biotin-labeled cells were washed twice and detached with Versene buffer and stored at -80°C until protein solubilization step.

Cells were solubilized after treating thawed cells in 1%TTSEC buffer (TTSEC buffer containing 1% Triton X-100) for 1 h at 4°C with head-over-head shaking, and then precipitated with 30 μl of immobilized streptavidin-agarose beads. After washed 3 times with 1%TTSEC buffer, the streptavidin-bound biotinylated proteins were eluted by incubating the beads in 50 μl of 2× Laemmli sample buffer for 1 h at room temperature. Biotinylated proteins were incubated with polyclonal anti-FLAG antibody and PANSORBIN for immunoprecipitation of FLAG-hKOR as described above.

Fluorescence flow cytometry and ligand-induced regulation of cell surface FLAG-hKOR in the presence of BFA

A fluorescence flow cytometry assay was conducted according to a modified protocol described by Li et al. (2003). CHO-FLAG-hKOR cells (~90% confluence) grown on 100-mm Petri dishes were firstly incubated with 5 μg/ml of BFA for 2 h and then treated with ligands or vehicle in the presence of BFA at 37°C for 4 h. After harvested, 1.5×10^6 cells were used for further flow cytometric assay without permeabilization of cell membranes. Because calcium ion is necessary for M_1 monoclonal anti-FLAG antibody binding with the antigen, all solutions used in the subsequent steps contain 1 mM $CaCl_2$. Cells were washed once with Opti-MEM I and then incubated with M_1 antibody (1 μg/ml, 1:2000) in 1 ml of Opti-MEM I for 1 h. After another three washes with PBS buffer, cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (1 μg/ml, 1:2000) in 1 ml of Opti-MEM I for 1 h, washed three times and then resuspended with 1 ml PBS buffer. All used solutions were ice-cold and all above procedures

were performed in cold room unless specified otherwise. Surface FLAG-hKOR immunofluorescence of 1 x 10^4 cells was quantitated using FACScan (BD Biosciences, San Jose, CA) and mean fluorescence intensity of single cell was calculated. The mean fluorescence intensity of cells stained only with the second antibody was also measured and subtracted from the mean intensity. Ligand-induced % change of cell surface FLAG-hKOR = 100 × (fluorescence intensity in ligand group - fluorescence intensity in vehicle group) / (fluorescence intensity in vehicle group).

Data analysis

All quantitative data were present as Mean ± S.E. if they were acquired from at least three independent experiments. Student's *t*-test was employed for comparing two sets of independent samples. The difference was defined to be significant if the *p* value was less than 0.05. All statistical analyses were performed using Prism 3.0 (GraphPad Software, San Diego, CA).

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Results

Different regulatory effects of KOR ligands on the steady-state level of the mature form of FLAG-hKOR

Immunoblotting data showed that FLAG-hKOR stably expressed in CHO cells migrated as two bands of *Mr* 55 kDa and 45 kDa (Fig. 1A). We have previously demonstrated that the 45-kDa band represents N-linked glycosylated high-mannose intermediates, which mainly distribute in ER and/or *cis*-Golgi, whereas the 55-kDa band represents the fully glycosylated mature forms of FLAG-hKORs, most of which locate in plasma membranes (Chen et al., 2006). In addition, our receptor binding results showed that cell surface receptors accounted for ~85% of the total receptor (Xu et al., 2005). Cell surface receptors are the ones responding to agonists leading to signal transduction. Therefore, to evaluate how different ligands regulated the FLAG-hKOR, we focused on the 55-kDa mature form. The 55-kDa band appears to be a doublet, which may be due to heterogeneity of glycosylation. The heterogeneity may be attributed to different chain lengths of carbohydrate. In addition, there are two N-linked glycosylation sites (Asn^{25} and Asn^{39}) in the N-terminal domain and the observed heterogeneity may result from glycosylation at one *vs.* two Asn residues. Since they both represent fully glycosylated receptors, we did not examine their regulation separately.

Fifteen ligands were examined for their abilities to change expression level of the receptor (Fig. 1). Unless indicated otherwise, the concentration of each drug was ~1000-fold of its EC_{50} (agonists) or *Ki* (antagonists) [(Henry et al., 1995; Zhu et al., 1997; Roth et al., 2000; Wang et al., 2005) and our unpublished data] and the incubation duration was 4 h (Li et al., 2000; Zhang et al., 2002).

Dynorphin A and dynorphin B, endogenous peptides and full agonists for the KOR, reduced the mature FLAG-hKOR by ~70%. By comparison, the non-peptide full agonists U50,488H, TRK-820, bremazocine, ethylketocyclazocine, asimadoline and ICI 204,448, caused much less reduction, ranging from 10% to 30%. In contrast, the non-peptide full agonist etorphine and the non-peptide partial agonist pentazocine up-regulated the mature receptor by ~25% and ~15%, respectively.

Four non-peptide opioid antagonists were also examined. After 4-h incubation, the non-selective opioid antagonist naloxone and the KOR-selective antagonist Nor-BNI significantly enhanced the mature form by ~25% and ~15%, respectively. However, naloxonazine and naltrindole, selective for μ and δ opioid receptors, respectively, did not have significant effects on the receptor level, nor did the selective D_1 dopamine antagonist R(+)-SCH 23,390. These results indicate that receptor binding is required for the regulatory effects of ligands.

Time- and concentration-dependence of ligand-mediated receptor regulation

We then examined whether the effects of the agonist U50,488H and the antagonist naloxone on receptor level were dependent on incubation duration and concentration. As shown in Fig. 2, U50,488H (5 μM) induced KOR down-regulation and naloxone (10 μM) promoted up-regulation in time-dependent manners, reaching the respective plateaus following 16-h treatment. In addition, when cells were treated for 4 h, both naloxone-promoted increases and U50,488H-induced decreases were concentration-dependent, attaining maximal effects at 10 μM and 5 μM, respectively. Thus, these results demonstrate that their effects on FLAG-hKOR level are time- and concentration-dependent, indicating this process observes the law of mass action and requires receptor occupancy.

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Naloxone enhanced conversion of 45 kDa to 55 kDa form of hKOR

Naloxone treatment does not cause activation-dependent receptor internalization and down-regulation (Li et al., 1999; Li et al., 2000). Therefore, it is a good tool to study whether how a ligand up-regulates cell surface receptor. Pulse-chase technique was employed to determine the rates and extents of maturation and turnover of the newly-synthesized receptors. As shown in Fig. 3A, after metabolic labeling with [^{35}S]Met/Cys (pulse) for 30 min, most of the labeled FLAG-hKOR existed as the intracellular immature form (45 kDa). After removal of [^{35}S]Met/Cys and incubation with chase medium, the 45-kDa protein band gradually decreased and, concomitantly, the fully glycosylated 55-kDa protein band gradually increased, reaching the highest level around 2 h. Incubation with 10 μM naloxone expedited turnover of the immature receptors and significantly enhanced maturation extent of FLAG-hKOR (Fig. 3B and Table 1). In addition, this ligand treatment did not alter the turnover rate of the mature receptors. Therefore, naloxone-induced enhanced mature receptor expression is due to increased transformation from immature specie to mature one, but not increased stability of the mature receptor.

Naloxone acted intracellularly in the ER as a pharmacological chaperone

We next examined whether naloxone acted intracellularly along the biosynthesis pathway (from ER to Golgi apparatus to plasma membrane). Following 6-h treatment with brefeldin A (BFA), which disrupts Golgi apparatus, there was a new receptor form with a *Mr* of 51 kDa which migrated between the immature and the mature receptors (Fig. 4A). The incubation time was identical to that used by Petaja-Repo et al. (2002). Pulse chase experiments showed that this

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new form was generated from the 45-kDa species (Fig. 4B). Incubation of cells with naloxone enhanced the 51-kDa form in BFA-treated cells and the 55-kDa form in control cells (Fig. 4C).

We next investigated if the 51-kDa form was located intracellularly or on cell surface by immunofluorescence staining, flow cytometry and cell surface biotinylation. Immunofluorescence staining with anti-FLAG antibody without permeabilization of plasma membranes showed that incubation with BFA resulted in an apparent reduction of cell surface FLAG-hKOR (Fig. 5A). Flow cytometric assay revealed that, following BFA treatment, there was a ~40% decrease in cell surface receptors (Fig. 5B). For cell surface receptor biotinylation (Fig. 5C), cells were incubated with [^{35}S]Met/Cys for 30 min to label newly-synthesized proteins followed by the 4-h chase in the absence or presence of naloxone and/or BFA, cell surface proteins were labeled with biotin and then precipitated with immobilized streptavidin agarose beads followed by anti-FLAG antibody and PANSORBIN to immunoprecipitate biotinylated FLAG-hKOR. While in control cells a broad radiolabeled 55-kDa band was detected, in BFA-treated cells no radioactive protein band was observed. Taken together, these results demonstrate that the 51-kDa species is located intracellularly. Thus, by disrupting Golgi apparatus, BFA interrupts maturation and membrane targeting of the receptor. In addition, in control cells, naloxone treatment up-regulated the mature form (55 kDa); however, in BFA-treated cells, naloxone did not promote trafficking of the 51-kDa species to cell surface or induce generation of the 55 kDa form (Fig. 5C).

The results in Fig. 4 and Fig. 5 indicate that naloxone acts intracellularly, most likely at the ER, to enhance the level of the 55-kDa form in control cells and the 51-kDa form in BFA-treated cells. Thus, naloxone acts as a pharmacological chaperone in the ER to stabilize the receptor and thus to facilitate maturation of the receptor.

Pharmacological chaperone effects of the agonists

BFA has been widely used as a tool to examine whether a ligand has chaperone-like effect on wild-type and mutant receptors (Petaja-Repo et al., 2002; Chaipatikul et al., 2003; Van Craenenbroeck et al., 2005). We then used BFA-treated cells to assess whether the agonists tested in Fig. 1 had similar chaperone-like effects as naloxone. Fig. 6 shows that both hydrophilic peptide agonists (Dyn A and Dyn B) did not up-regulate the 51-kDa species, but all hydrophobic non-peptide agonists did enhance its level. Therefore, non-peptide, but not peptide, ligands behave as pharmacological chaperones to facilitate anterograde trafficking of the receptor along the biosynthesis pathway, indicating that membrane-permeability is required for the chaperone roles.

Ligand-induced regulation of surface FLAG-hKOR following treatment with BFA

Next we examined how the ligands regulated cell surface receptor if membrane targeting of proteins was blocked by BFA. Since both 55-kDa and 51-kDa bands are broad and diffuse, there was overlap of the two bands making it difficult to quantify the 55-kDa band. Fluorescence flow cytometry, instead of immunoblotting, was employed to quantify cell surface receptors for evaluating ligand-mediated receptor regulation. It is reasonable to use cell surface receptor to represent the mature form since most of fully glycosylated proteins are located on cell surface. With BFA treatment, Dyn A and Dyn B (Fig. 7) still caused the highest degrees of down-regulation; the down-regulation extent (65%) was comparable to that (70%) in the absence of BFA determined by immunoblotting (see Fig. 1). On the other hand, in the presence of BFA, all non-peptide full agonists mediated higher levels of down-regulation than they did in the absence

of BFA (see Fig. 1). In particular, etorphine and pentazocine, which up-regulated the receptor in the absence of BFA (see Fig. 1), down-regulated cell surface receptors in the presence of BFA (Fig. 7). Additionally, with BFA treatment, naloxone had no influence on the expression of FLAG-hKOR on plasma membranes, although it up-regulated the receptor without BFA.

These results demonstrate that the effects of ligands on cell surface expression are a combination of activation-dependent down-regulation and pharmacological chaperone effects.

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Discussion

To the best of our knowledge, this is the first report showing that membrane-permeable non-peptide full agonists caused less down-regulation of cell surface receptors than membrane-impermeable peptide full agonists due to their pharmacological chaperone effects counteracting activation-dependent degradation.

Cell-permeable ligands act in the ER to produce chaperone effects

We have shown previously that dynorphin A, U50,488H, U69,593, EKC and tifluadom promote similar levels of hKOR internalization (Li et al., 1999; Li et al., 2003). Therefore, the observed differential down-regulation of cell surface hKOR by peptide and non-peptide agonists is likely due to differences in trafficking in biosynthesis pathway and in receptor degradation following internalization.

BFA causes rapid disintegration of the Golgi apparatus, blockade of ER-to-Golgi vesicular transport and retrograde transport of Golgi resident proteins, such as glycosyltransferases, back into the ER (Rios et al., 1994). For G protein-coupled receptors (GPCRs) of which the sorting of internalized receptor is Golgi-dependent [for example, (Tawfeek and Abou-Samra, 2004)] , it is conceivable that BFA may have negative impacts on post-endocytic recycling of the receptor. However, our results demonstrated that BFA did not affect the extents of Dyn A- and Dyn B-induced reduction of cell-surface hKOR (Fig. 1 and Fig. 7). In addition, Law et al. (2000a) reported that inhibition of receptor recycling with monensin had no effect on reduction of cell surface μ opioid receptor after prolonged agonist treatment (≥ 4

h). Thus, under our experimental conditions, BFA effects are primarily attributed to blockade of ER to Golgi transport.

Following BFA treatment, a new 51-kDa form of FLAG-hKOR was generated, which is localized intracellularly and represents the receptor formed in the ER by retrogradely transported Golgi enzymes. We found that non-peptide KOR ligands, regardless of efficacy, upregulated the 51-kDa form, whereas peptide ligands did not, indicating that non-peptide, but not peptide, ligands act as pharmacological chaperones. Exit from ER to Golgi appears to be a rate-limiting step for many GPCRs. The membrane-permeable KOR ligands most likely act in the ER to bind to the newly synthesized receptors in various states of the folding pathway. The interaction of non-peptide ligands with amino acid side chains within the transmembrane hydrophobic core most likely promotes energy-favorable conformational states of the receptor, leading to increased exit of the receptor from ER to Golgi (Bernier et al., 2004; Ulloa-Aguirre et al., 2004).

Mechanisms of differential effects of ligands

With BFA, all non-peptide agonists decreased cell surface receptor to greater degrees than without BFA, whereas peptide agonists caused receptor down-regulation to similar extents. In addition, after BFA incubation, etorphine and pentazocine caused down-regulation, whereas in the absence of BFA they up-regulated the mature receptor. Moreover, BFA abrogated naloxone-induced up-regulation.

The results are interpreted as follows. Chronic ligand treatment potentially has dual effects on receptor levels: activation-induced receptor degradation and chaperone-mediated enhancement. At two ends of the spectrum are the peptide agonists and non-peptide antagonists. Membrane-impermeable peptide agonists cause activation-induced receptor degradation without

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any chaperone effect. Membrane-permeable antagonists exert chaperone-like actions without activation-promoted degradation. In between the two ends, cell-permeable non-peptide agonists have both effects and their net effects are the algebraic sum of the two. Thus, all non-peptide full agonists, except etorphine, led to smaller reductions of the steady-state mature receptor than peptide full agonists. Etorphine or pentazocine induced receptor up-regulation, due to their chaperone effects and low efficacy to induce receptor degradation.

Down-regulation and chaperone effects appear to have similar time courses *in vitro*

For down-regulation, ligand binding to cell surface receptors causes internalization of the receptor via clathrin-coated pits and internalized receptors are trafficked through endocytic vesicles, early endosomes, late endosomes and finally lysosomes. For chaperone effect, the ligand has to penetrate plasma membranes to reach the ER to stabilize favorable receptor conformations that allow the receptor to exit the ER to Golgi and then to plasma membranes. Thus, both ligand-induced down-regulation and chaperone effects involve a series of vesicle fusion events, therefore their time courses may be similar.

Non-peptide agonists have differential activities in down-regulating the KOR and as pharmacological chaperones

Non-peptide full agonists produced higher degrees of down-regulation with BFA than without BFA. However, BFA did not cause these non-peptide agonists to down-regulate the hKOR to the same levels. These results suggest that non-peptide agonists have different activities in regulating the KOR. This notion is supported by our finding that although etorphine is a full agonist, it does not cause hKOR internalization (Li et al., 1999; Li et al., 2003), which is required

for down-regulation (Li et al., 2000). In addition, internalized receptor may be sorted differently depending on the agonist used. Marie et al. (2003) have reported that compared to DPDPE and deltorphin I, etorphine results in less lysosome-mediated degradation, but more recycling, of the hDOR. This possibility remains to be investigated for the hKOR. Moreover, among non-peptide agonists, there may be differences in their abilities to pass through membranes. Asimadoline and ICI 204,448, two peripherally acting KOR agonists (Shaw et al., 1989; Barber et al., 1994), caused higher degrees of down-regulation (Fig. 1), likely because they are less able to penetrate membrane. Furthermore, this discrepancy may be due to differential stability of these ligands in the incubation medium, which includes 10% FBS that contains numerous enzymes.

Other mechanisms appear not to play important roles in ligand-induced KOR up-regulation

To date, several other mechanisms have been demonstrated for ligand-induced receptor up-regulation, including enhanced transcription and translation and reduced internalization and degradation. In the present study, we used the hKOR cDNA construct, which does not contain the promoter region, and expression of the receptor is driven by the constitutively active cytomegalovirus promoter. Thus, regulation at the level of DNA transcription or mRNA translation does not play a role.

Chronic antagonist-induced decrease in lysosomal enzyme (β-glucuronidase and β-hexoseaminidase) activities and in trafficking proteins (GRK2 and dynamin2) were reported to be important in facilitating receptor expression (Belcheva et al., 1992; Rajashekara et al., 2003). However, our results do not support this explanation because naloxone treatment did not affect the turnover rate of the mature receptor (Fig. 3 and Table 1).

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Antagonist-promoted KOR up-regulation *in vivo*

Following chronic administration of naloxone, naltrexone or buprenorphine, the KOR in brain regions was up-regulated (Morris et al., 1988; Belcheva et al., 1993; Lesscher et al., 2003). After chronic antagonists, functional supersensitivity to kappa agonist-mediated antinociception was demonstrated (Millan et al., 1988), indicating that the up-regulated KOR is functional. It is likely that the mechanisms uncovered here are applicable *in vivo*.

Non-peptide ligands act as pharmacological chaperones of other GPCRs

μ and δ opioid receptors: Non-peptide agonists and antagonists, but not peptide ligands, have been reported to act as chaperones to stabilize the ER forms and enhance cell surface levels of the wild-type and D95A mutant of the DOR and two deletion mutants and several constitutively active mutants of the MOR (Li et al., 2001a; Li et al., 2001b; Petaja-Repo et al., 2002; Chaipatikul et al., 2003).

Non-opioid receptors: Ligand-induced enhancement in cell surface receptors has been demonstrated for several other wild-type and mutant GPCRs, for example, vasopressin V_2, dopamine D_4 and β_2-adrenergic receptors (Samama et al., 1997; Morello et al., 2000; Van Craenenbroeck et al., 2005). Cell-permeable antagonists/inverse agonists and agonists up-regulated cell surface receptors. Infusion with the β_2-antagonist ICI 118,551 for 7 days resulted in a 50-fold increase in density of constitutively active mutated β_2-adrenergic receptor in the myocardium (Samama et al., 1997), indicating that pharmacological chaperone effects occur *in vivo*.

Differential effects of non-peptide and peptide ligands

In contrast to non-peptide ligands examined, the two peptide ligands, dynorphin A and B, did not have pharmacological chaperone actions. Similar differential effects of non-peptide *vs.* peptide ligands have been observed for other GPCRs, including μ and δ opioid, kinin B_1 and gonadotropin-releasing hormone receptors and a V_2 vasopressin receptor mutant (Morello et al., 2000; Petaja-Repo et al., 2002; Janovick et al., 2002; Chaipatikul et al., 2003; Fortin et al., 2006).

Membrane permeability of peptide ligands

Most peptide ligands are generally accepted to be cell impermeable, whereas some water-soluble short peptides (≤ 35 residues) that contain the so-called membrane translocation sequence are able to cross cell membrane and are classified as cell-penetrating peptides (Magzoub and Graslund, 2004). Our results that both dynorphin A and dynorphin B did not enhance the expression of the new intracellular 51-kDa species following BFA treatment indicate that the two peptides do not penetrate plasma membranes. This finding is different from the recent report by Marinova et al. (2005) that dynorphin A and big dynorphin, but not dynorphin B, were able to translocate across plasma membrane in HeLa, COS-1 and PC12 cells. However, all peptide ligands tested for many other GPCRs did not display the chaperone-like ability (see above), demonstrating that they are membrane impermeable in CHO, HEK293 and COS-7 cells. Therefore, it is possible that membrane permeability of dynorphin A and big dynorphin may be cell-specific.

Conclusion

Treatment with different KOR ligands resulted in distinct regulatory effects on the mature FLAG-hKOR. Membrane-impermeable peptide full agonists lead to receptor down-regulation to greater extents as they do not act as pharmacological chaperones. Membrane-permeable non-peptide antagonists act as pharmacological chaperones to induce receptor up-regulation without causing down-regulation. Membrane-permeable non-peptide agonists have two opposing effects on cell surface receptors: to decrease the receptor by causing activation-dependent endocytosis and degradation, and to increase the receptor by acting as pharmacological chaperones. These findings have great implications for the field of neuropeptides in general. Many non-peptide agonists or antagonists of neuropeptide receptors are being developed as pharmacological tools and therapeutic agents, primarily because of better pharmacokinetic properties and penetration through blood-brain barrier. The present study indicates that, non-peptide ligands have an added advantage over peptides in that they can act as pharmacological chaperones. Thus, non-peptide agonists will cause less down-regulation of the receptors than peptide agonists, leading to less tachyphylaxis following long-term treatment.

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Footnotes

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Legends for Figures

Figure 1. Differential regulation by prolonged treatment (4 h) with KOR ligands of the steady-state level of the mature form (doublet at ~55 kDa) of FLAG-hKOR stably expressed in CHO cells.

(A). Immunoblotting pattern of FLAG-hKOR in CHO cells. Cells were solubilized with 2× Laemmli sample buffer, and proteins were separated by SDS-PAGE followed by immunoblotting with polyclonal anti-FLAG antibody. This represents one of the three experiments performed with similar results.

(B and C). Immunoblotting and quantitation of ligand-induced change in mature receptor level. Cells were incubated with each drug for 4 h at a concentration ~1000-fold of its EC_{50} (agonists) or *Ki* (antagonists). Subsequently, 2×10^5 cells were solubilized and subjected to SDS-PAGE. FLAG-hKOR was detected with immunoblotting with anti-FLAG antibody and quantitated (mean ± S.E., n=4) using ImageGauge software. B represents one of the four experiments performed with similar results. Abbreviations used in the figures: dynorphin A (Dyn A), dynorphin B (Dyn B), U50,488H (U50), TRK-820 (TRK), bremazocine (Brema), ethylketocyclazocine (EKC), asimadoline (Asim), ICI 204,488 (ICI), etorphine (Etor), pentazocine (PTZ), naloxone (Nal), Norbinaltorphimine (Nor-BNI), naloxonazine (NalAz), naltrindole (NTI) and R(+)-SCH 23,390 (SCH)

Figure 2. Time- and concentration-dependence of ligand-mediated regulation of the steady-state level of mature FLAG-hKOR.

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Cells were treated with *(A)* naloxone (10 μM) or U50,488H (5 μM) for indicated time periods or *(B and C)* different concentrations of naloxone or U50,488H for 4 h. FLAG-hKOR was detected by immunoblotting and quantitated (mean ± S.E., n=3) by densitometry as described in Methods.

Figure 3. Effect of naloxone (10 μM) on kinetics of newly-synthesized FLAG-hKOR: Pulse-chase study.

(A). Time courses of generation and decline of the immature and mature receptors in the absence or presence of 10 μM naloxone. After 90-min incubation in Met/Cys-free DMEM depletion medium, cells were pulse-labeled with 150 μCi/ml of [^{35}S]-Met/Cys at 37°C for 30 min and then incubated with complete medium containing 5 mM Methionine (chase) for specified time periods. Cells were treated with naloxone or vehicle during the chase phase. After chase, cells were harvested and solubilized with TTSEC buffer and proteins were immunoprecipitated with anti-FLAG antibodies and PANSORBIN twice. Immunoprecipitated materials were resolved with SDS-PAGE followed by gel drying and autoradiography. The figure represents one of the four experiments performed with similar results.

(B). Quantitative autoradiography results were acquired by densitometric analysis using the OptiQuant software (mean ± S.E., n=4).

Figure 4. Naloxone enhanced the 51-kDa form in cells treated with BFA.

(A). Immunoblotting pattern of FLAG-hKOR in control and BFA-treated CHO cells. After incubation with vehicle or 5 μg/ml of BFA for 6 h, cells were solubilized for SDS-PAGE and immunoblotting as described in Methods.

(B). BFA treatment resulted in production of a new receptor intermediate (Mr 51 kDa) from the immature form (Mr 45 kDa) as demonstrated with pulse-chase study. All experiments were conducted using the same protocol as described in Fig. 3 except that cells were treated with BFA during 1.5-h depletion phase, 0.5-h pulse phase and the subsequent chase phase for indicated time periods.

(C). Naloxone promoted production of the mature (55-kDa) FLAG-hKOR in control cells, and the 51-kDa form in BFA-treated cells. Pulse-chase assay was performed as described in Fig. 3. In BFA-treated cells, BFA was added into medium during the entire depletion-pulse-chase phases lasting for 6 h. Naloxone treatment was only carried out during the 4-h chase phase. Each figure represents one of three experiments performed with similar results.

Figure 5. BFA-induced 51-kDa FLAG-hKOR is located intracellularly.

(A). BFA treatment reduced cell surface (mature) FLAG-hKOR immunofluorescence. After treated with vehicle or BFA (5 μg/ml) for 6 h, cells were incubated with monoclonal M_1 anti-FLAG antibody without membrane permeabilization and then fixed by 4% paraformaldehyde. The surface receptors were further stained with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody and visualized using a fluorescence microscope. This figure represents one of four experiments performed with similar results. Scale bar = 30 μm.

(B). BFA treatment significantly decreased cell surface FLAG-hKOR. Cells were incubated with BFA or vehicle for 6 h, and cell surface receptors were labeled by monoclonal M_1 anti-FLAG antibody and then Alexa Fluor 488-conjugated goat anti-mouse IgG antibody. Immunofluorescence intensity was determined (mean ± S.E., n=4) using fluorescence activated cell sorter (FACS).

* $P < 0.05$ compared to the control using two-tailed Student's *t*-test.

(C). BFA-induced 51-kDa form was located intracellularly. Following pulse-chase labeling as described in Fig. 4C, cell surface receptors were biotinylated with water-soluble EZ-link NHS-sulfo-SS-biotin. Cells were solubilized and incubated with immobilized streptavidin to precipitate biotinylated proteins, which were then incubated with polyclonal anti-FLAG antibody and PANSORBIN for immunoprecipitation of biotinylated receptors. Immunoprecipitated materials were resolved with SDS-PAGE and radioactivity was detected with autoradiography. This figure represents one of three experiments performed with similar results.

Figure 6. Non-peptide KOR ligands enhanced the BFA-induced intracellular 51-kDa form of FLAG-hKOR, but peptide ligands did not. Pulse-chase experiments were conducted as described in Fig. 4C. Cells were only treated with each ligand during the 4-h chase phase at the same concentration as indicated in Fig. 1. This figure represents one of three experiments performed with similar results.

Figure 7. Effects of BFA on the ligand-induced regulation of cell surface FLAG-hKOR. CHO-FLAG-hKOR cells were pre-incubated with BFA 2 h and vehicle or ligands were added for 4 h in the presence of BFA. Following these treatments, cell surface FLAG-hKOR was labeled and quantitated (mean ± S.E., n=4) as described in Fig. 5B.

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Table 1. Kinetic parameters of both newly-generated receptor species (55-kDa mature form and 45-kDa immature form) in control and naloxone-treated cells. All parameters were determined from the data in Fig. 3B as described in Methods.

		Mature Form (55-kDa)	**Immature Form (45-kDa)**
Maturation Extent ($\times 10^7$, AUC, DLU$\times$hour^{-1})	***Veh***	**3.27 ± 0.22**	-
	Nal	**4.03 ± 0.28***	-
Generation Rate Constant (k_a, hour^{-1})	***Veh***	**1.46 ± 0.20**	-
	Nal	**1.81 ± 0.09**	-
Peak Time (t_{max}, hour)	***Veh***	**1.95 ± 0.19**	-
	Nal	**1.52 ± 0.23**	-
Turnover Rate Constant (k_e, hour^{-1})	***Veh***	**0.092 ± 0.002**	**1.37 ± 0.13**
	Nal	**0.094 ± 0.004**	**1.97 ± 0.14***
Half Life ($t_{1/2}$, hour)	***Veh***	**7.57 ± 0.12**	**0.52 ± 0.04**
	Nal	**7.42 ± 0.32**	**0.36 ± 0.03***

* $P < 0.05$ compared to the control using two-tailed Student's *t*-test.

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Figure 1.

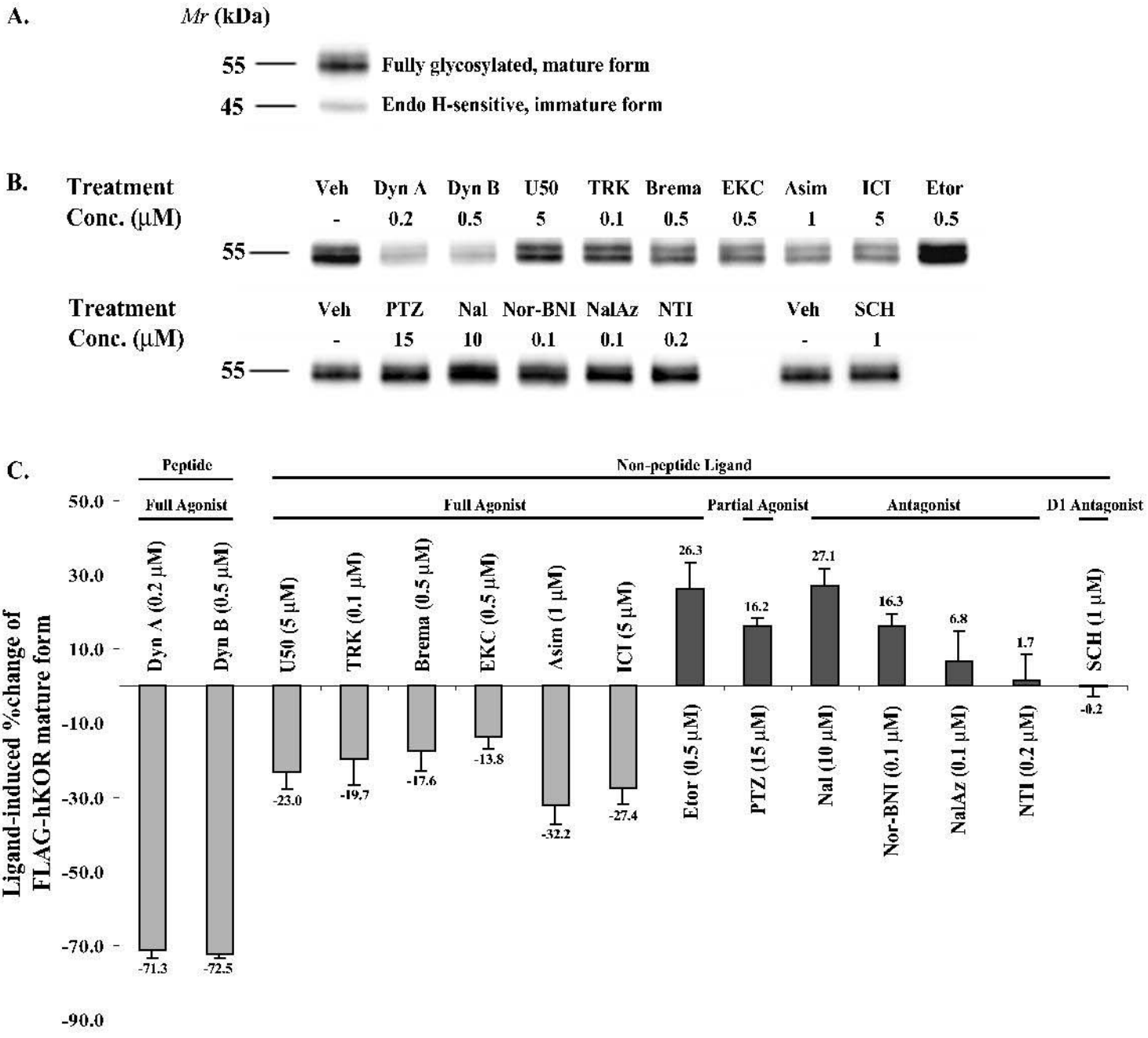

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Figure 2.

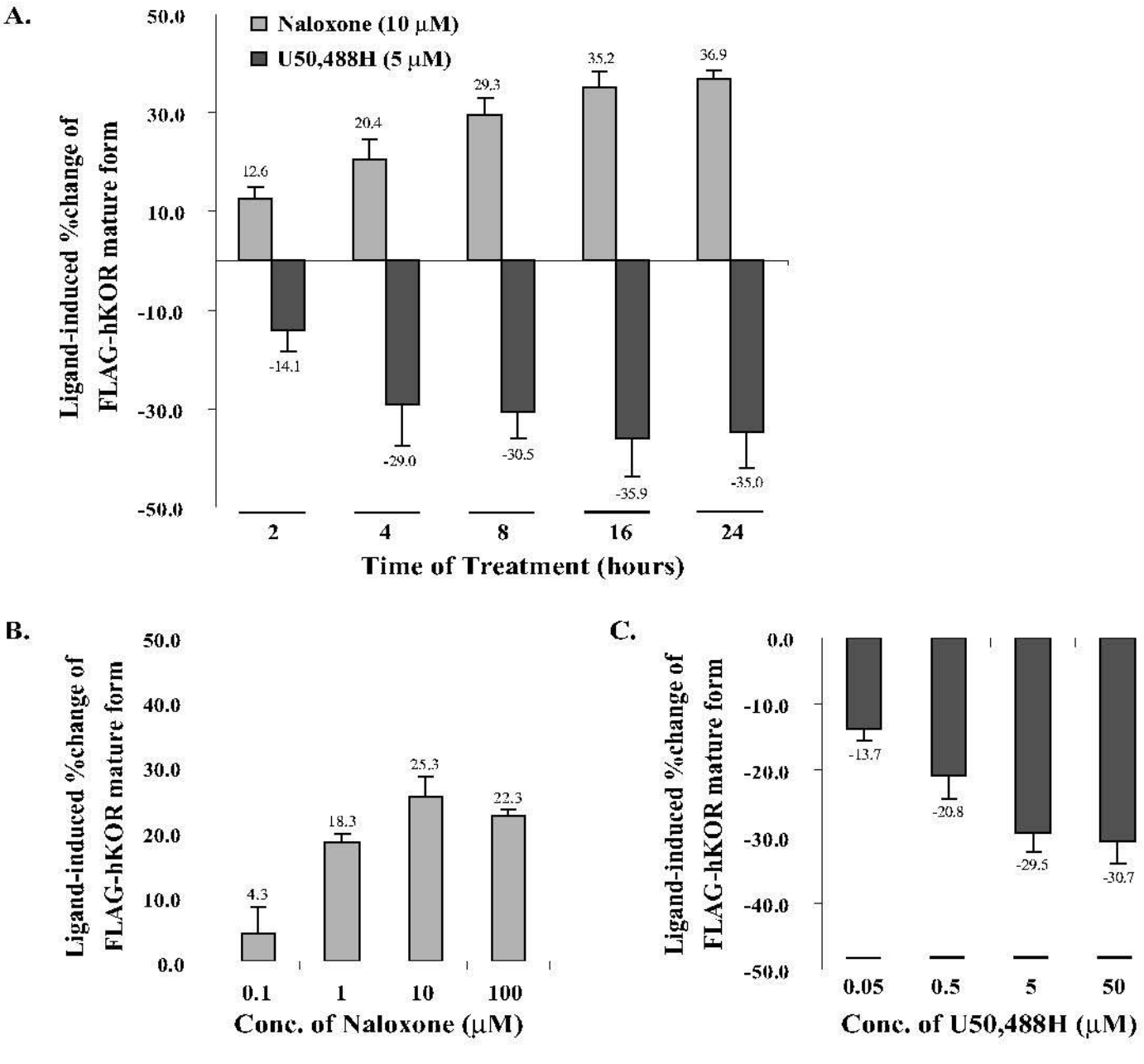

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Figure 3.

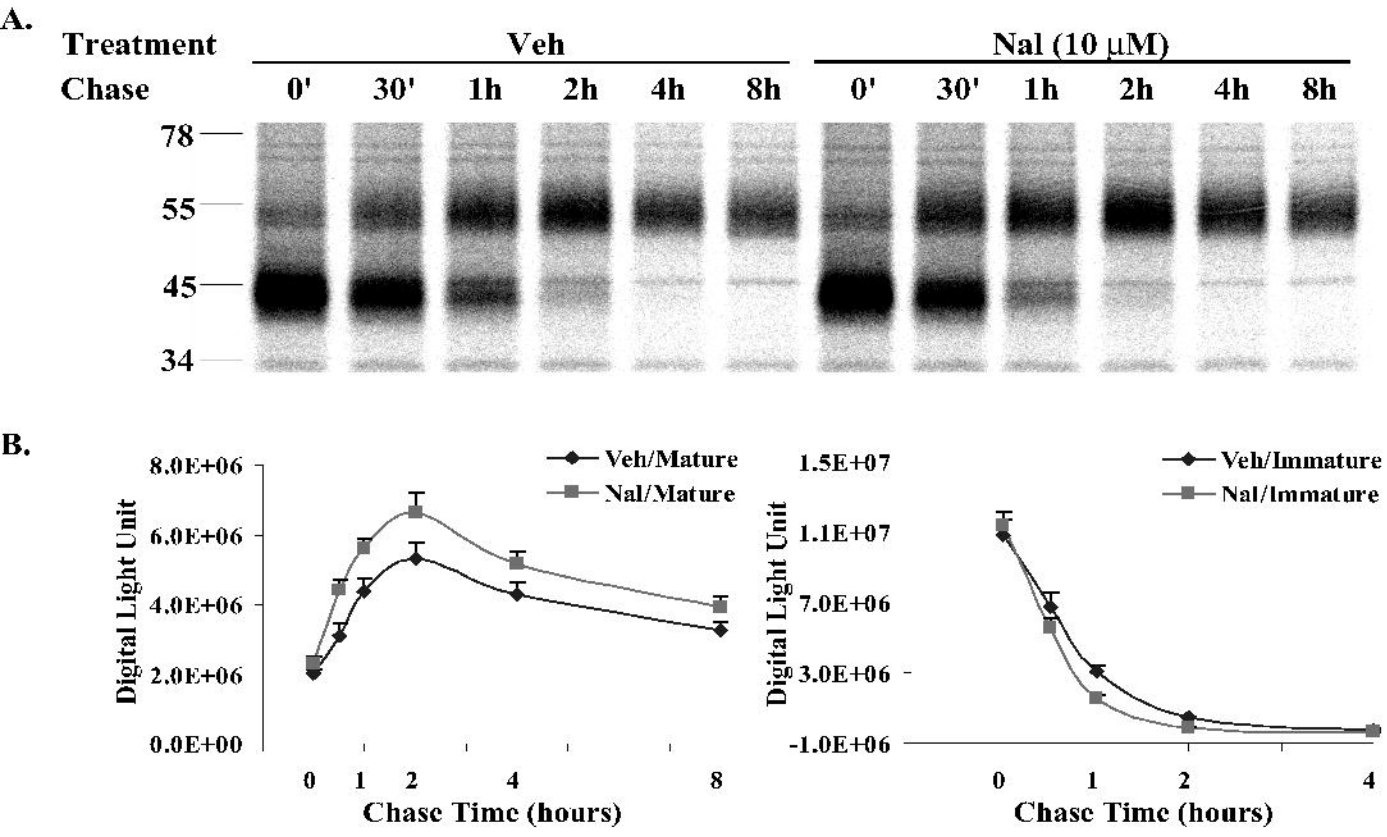

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Figure 4.

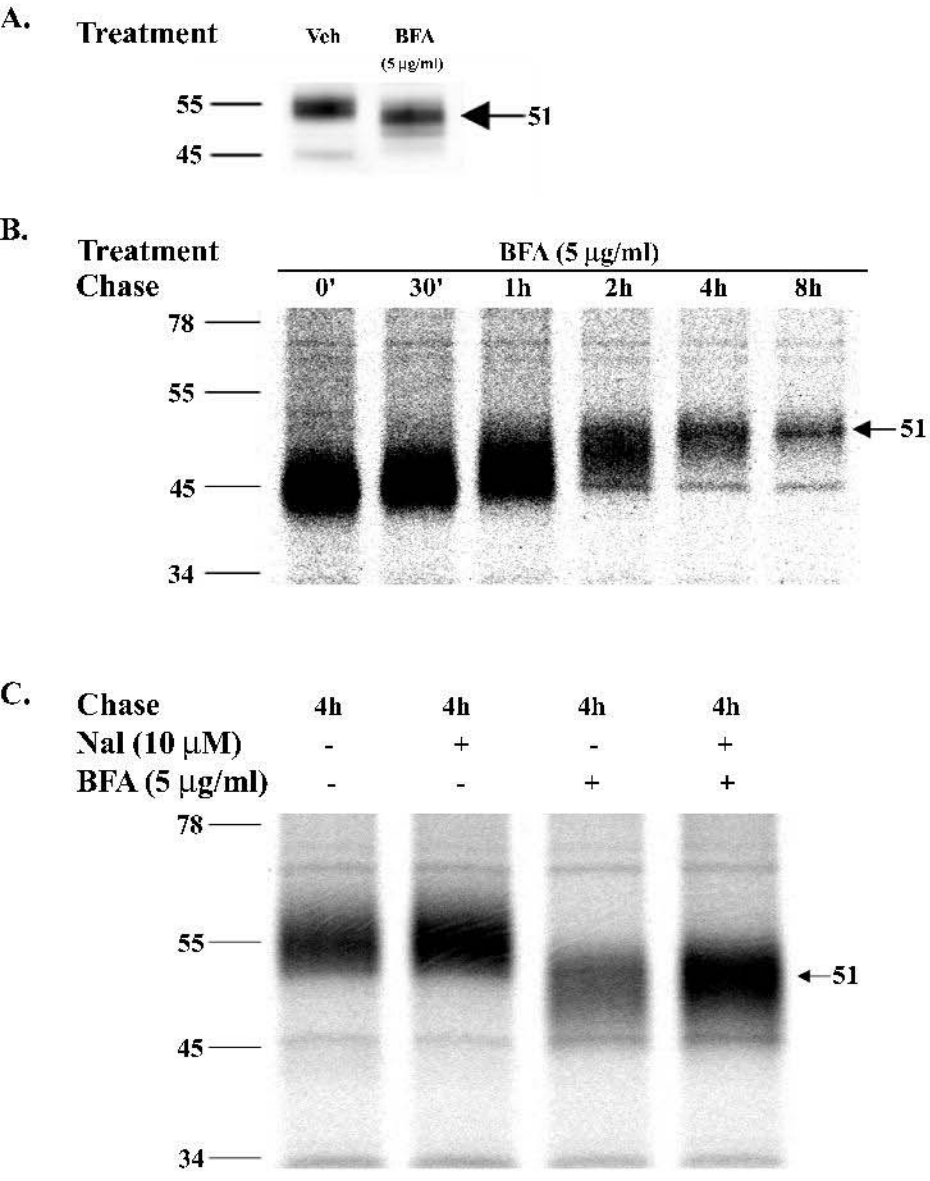

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Figure 5.

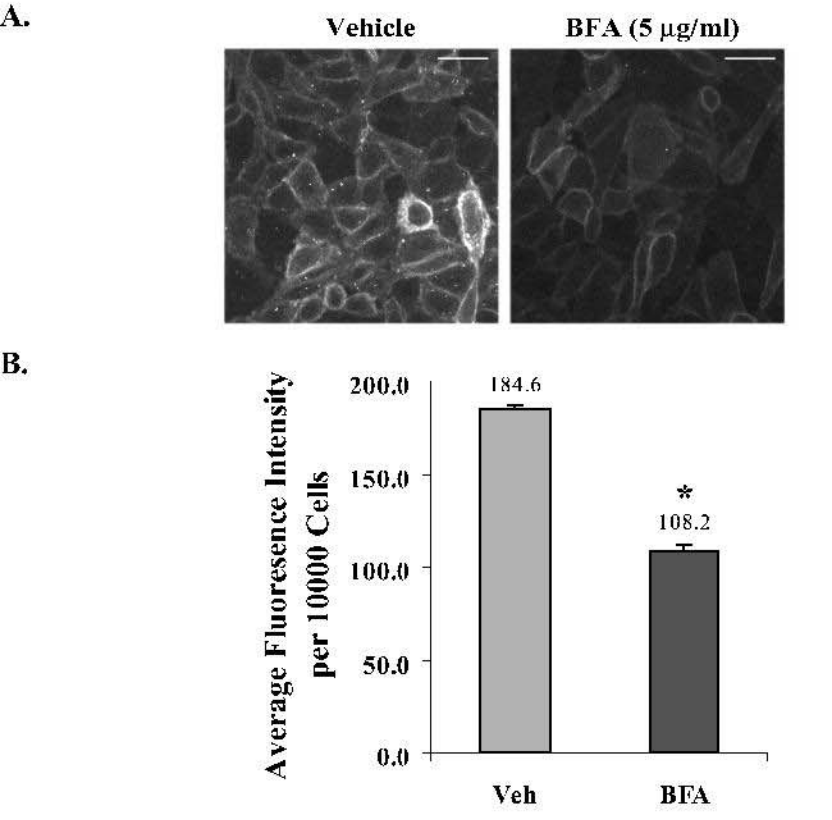

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Figure 6.

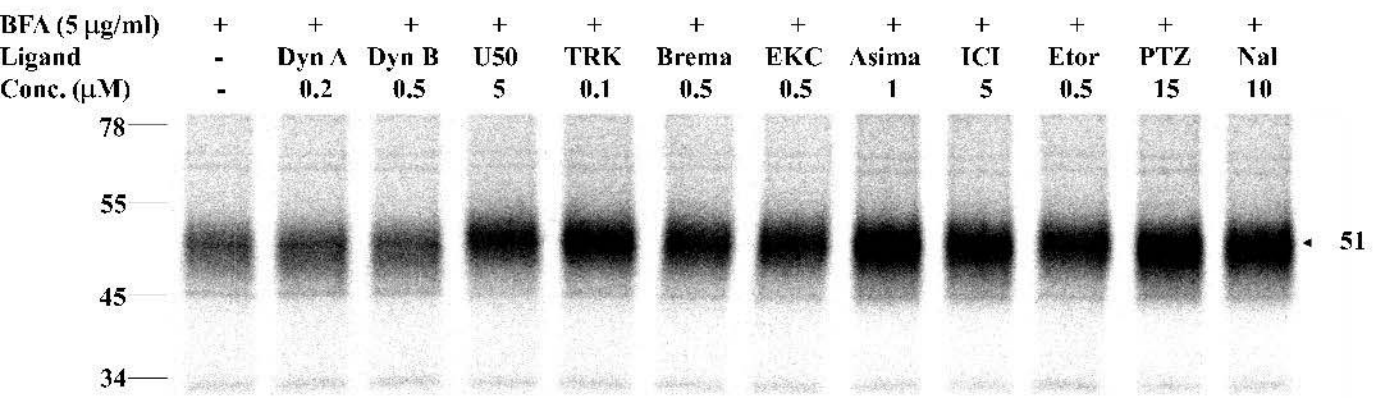

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Figure 7.