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Full Length Research Paper

Morphine is an exogenous ligand for MrgX2, a G protein-coupled receptor for cortistatin

Natsuyo Akuzawa[1], Hideru Obinata[2], Takashi Izumi[2], Shigeki Takeda[1]*

[1]Department of Nano-Material Systems, Graduate School of Engineering, Gunma University, 1-5-1 Tenjin-cho, Kiryu, Gunma 376-8515, Japan.
[2]Department of Molecular Biochemistry, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan.

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MrgX2 is reported to be expressed in a specific subset of dorsal root ganglion sensory neurons, and it is activated by the endogenous peptides, cortistatin and proadrenomedullin N-terminal peptide. Here, we show that morphine but not opioid peptides stimulated a dose-dependent increase of the intracellular calcium ion level in MrgX2-expressing cells. Naloxone, an opioid receptor antagonist, did not influence these reactions. It was previously reported that the μ-opioid receptor did not show morphine dependent desensitization and internalization. In contrast, we detected morphine induced MrgX2 desensitization and internalization. Since MrgX2 gave a similar EC_{50} value for morphine with the δ- and κ- opioid receptors, we consider that MrgX2 could be the physiological morphine receptor. It was reported that some morphine effects (e.g., morphine-induced hyperalgesia) were observed even in the presence of an opioid receptor antagonist. Therefore, we propose the importance of studying MrgX2 together with the classical opioid receptors for the investigation of morphine effects.

Key words: morphine, G protein-coupled receptor, MrgX2, desensitization, internalization.

INTRODUCTION

Morphine is well known as a useful clinical agent for analgesia, although it causes serious side effects and physical dependence. Despite extensive investigations, the molecular mechanisms of morphine tolerance are not completely understood. Morphine is an exogenous opioid, and it has been revealed to act on μ-, δ-, and κ-opioid receptors, which belong to the family of G protein-coupled receptors (GPCRs) (Matthes et al., 1996; Sora et al., 1997; Narita et al., 2001). The morphine effects such as analgesia, tolerance, and addiction are mediated mainly via the μ-opioid receptor. However, the molecular effects of morphine remain to be investigated under high-dose or chronically exposed conditions. Further, the reason why only morphine and not endogenous opioid peptides (e.g., enkephalins and endorphins) shows tolerance remains to be elucidated. A possible speculation is that the classical opioid receptors are not unique receptors for morphine and that there are unknown molecular mechanisms that specifically function for morphine.

MrgX2 was originally identified as an orphan GPCR by human genome analysis. It belongs to a receptor subfamily encoded by mas-related genes (Mrgs) that include more than 30 genes of mouse (MrgAs–MrgH) (Han et al., 2002), and approximately 10 genes of humans (MrgD–MrgG and MrgX1–MrgX7) (Dong et al., 2001; Lembo et al., 2002; Choi and Lahn 2003; Grazzini et al., 2004). It was reported that the Mrg genes are expressed in the dorsal root ganglion (DRG) sensory neurons (Dong et al., 2001; Lembo et al., 2002; Robas et al., 2003). Thus, MrgXs in humans have also been termed as sensory neuron-specific receptors (SNSRs). MrgX2 is expressed not only in DRG neurons but also in a few other tissues such as neurohypophysis, blood vessels, e.t.c. (Allia et al., 2005). Some of the specific ligands for the Mrg receptors have been identified and characterized using cells stably

*Corresponding author. E-mail: stakeda@chem-bio.gunma-u.ac.jp

ABBREVIATIONS

BSA; bovine serum albumin, CHO; Chinese hamster ovary, DRG; dorsal root ganglion, FBS; fetal bovine serum, GPCR; G protein-coupled receptor, Mrg; mas-related genes, PAMP; proadrenomedullin N-terminal peptide, PCR; polymerase chain reaction.

expressing these receptors. For example, MrgA1 and MrgA4 in mouse are activated by RFamide neuropeptides (Han et al., 2002), and MrgX1 (SNSR4) and MrgX7 (SNSR3) in humans are activated by bovine adrenal medulla 22 (BAM22) peptide (Lembo et al., 2002). Recently, cortistatin (Robas et al., 2003) and proadrenomedullin N-terminal peptide (PAMP) (Kamohara et al., 2005) have been identified as endogenous ligands for MrgX2. We previously reported the screening of MrgX2 ligands by using an MrgX2-$G_i\alpha$ fusion protein expressed by Sf9 cells and found that dextrorphan and 3-methoxy morphanin, which are morphine analogs, activated MrgX2 (Takeda et al., 2003).

The aim of this paper is characterization of MrgX2 in the view of the morphine receptor. Here, we report that morphine activates MrgX2 under relatively high concentration conditions. We also present the desensitization and internalization of MrgX2 by morphine.

Our results and the fact that MrgX2 is expressed in DRG neurons (Robas et al., 2003) suggested that MrgX2 could be also candidate for physiological morphine receptor as well as the classical opioid receptors.

MATERIALS AND METHODS

Materials

Cortistatin, PAMP, α-endorphin, and leucine-enkephalin were purchased from Peptide Institute. Morphine was purchased from Shionogi. Morphine derivatives, dextrorphan, and 3-methoxy morphanin, were purchased from Sigma.

Plasmid construction

To express human MrgX2 in CHO cells, we used a pCDNA4-His/Max B expression vector (Invitrogen) that carries zeocin resistant gene for a selection marker and Xpress-tag sequence after initiation codon for antibody labeling. To assist the translocation of the expressed receptor to cell membranes, N-terminal 20 residues of bovine rhodopsin was added to MrgX2. The cDNA corresponding to the rhodopsin sequence was synthesized and inserted using *EcoR*I and *Not*I sites, resulted in formation of pCDNA4-rhod20 expression vector. The open reading frame of MrgX2 was amplified from human genomic DNA (Clonetech) as a template by polymerase chain reaction (PCR) using primers; 5'-aaagcggccgcatggatccaaccaccccg-3' as a forward primer and 5'-aaatctagactacaccagactgcttctcgacatc-3' as a reverse primer. PCR condition was 30 cycles of 95° for 5 sec, 60° for 15 sec and 68° for 30 sec by using KOD plus polymerase (TOYOBO). Resulted PCR product was cloned into pCDNA4-rhod20 vector using *Not*I and *Xba*I sites. Eventually, this pCDNA4-MrgX2 coded a fusion construct of Xpress-tag and rhodopsin N-terminal sequence upstream of human MrgX2. The DNA sequences of all constructs were confirmed by a DNA sequence ABI310 analysis system (Applied Biosystems).

Cell culture and transfection

The CHO-K1 cells were cultured in 100 mm dishes in F-12 Ham medium (Sigma) containing 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. The CHO-K1 cells were transfected with pCDNA4-MrgX2 using the LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's instructions. After selection of stable transfectants by using 400 μg/ml zeocin (Invitrogen), we screened 12 cortistatin-responsive clones by measuring their calcium mobilization as described below. The clone showing the highest potency for cortistatin was used for the subsequent experiments (CHO/MrgX2).

Measurement of calcium mobilization

Measurement of intracellular calcium mobilization was performed as described previously (Obinata et al., 2005). CHO/MrgX2 cells were seeded into black 96 well plates with clear-bottom at a density of 1 x 10^5 cells/well in 75 μl medium (F-12 Ham/10%FBS). After the cells were cultured for 24 h at 37°C in 5% CO_2, 75 μl of Hepes-tyrode buffer (25 mM Hepes/NaOH (pH 7.4), 140 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 12 mM $NaHCO_3$, 0.49 mM $MgCl_2$, 0.37 mM NaH_2PO_4, 5.6 mM D-glucose, 0.1% bovine serum albumin (BSA) containing 5 μM Fura-2 AM (Dojin), 2.5 mM probenecid (Sigma) and 0.04% F-127(Sigma) was added into each well. After incubation for 1 h at 37°C in 5% CO_2, the cells were washed with 80 μl of Hepes-tyrode buffer and then 80 μl of same buffer was added into each well. Ligands were diluted to the appropriate concentration in Hepes-tyrode buffer containing 5% BSA. Intracellular calcium concentrations were measured using FlexStation II fluorometric imaging plate reader (Molecular Device). For dose-response studies, all data points were carried out in six independent wells.

For measurement of desensitization, conventional fluorescence measurements were done using spectrofluorometer (RF-5300PC, Shimazu) in glass tubes. CHO/MrgX2 (1 x 10^7 cells) were harvested and incubated in 10 ml Hepes-tyrode buffer containing 5 μM Fura-2 AM, 1.25 mM probenecid, 0.02% F-127 for 1 h at 37°C in 5% CO_2.

After centrifugation at 1000 rpm for 5 min, cells were resuspend in 8 ml Hepes-tyrode buffer. Intracellular calcium mobilization measured using 600 μl of cell suspension and 6 μl of ligand solution were added. The second ligand was added 5 min after the first ligand stimulation and alteration of calcium mobilization were measured.

Fura-2 fluorescence was measured with excitation at 340 nm and 380 nm, and emission at 510 nm. The fluorescence intensity ratio, I_{340}/I_{380}, was utilized to determine intracellular calcium mobilization. The mock transfected CHO-K1 cells were used for control experiments. All ligands were dissolved in Hepes-tyrode buffer.

Enzyme linked immunosorbent assay (ELISA) for detection of cell surface receptors

For quantification of receptor internalization, amounts of receptors on a cell surface were evaluated by ELISA. CHO/MrgX2 (5 x 10^3 cells) were plated on 96 well plate in 100 μl F-12 Ham/10% FBS medium, and incubated for 24 h at 37°C, 5% CO_2. The medium was removed and fresh medium was added into each well. Then, an MrgX2 agonist was added to each well (final concentration of 1 μM cortistatin or 100 μM morphine) and incubated for indicated times at 37°C, 5% CO_2.

After washing with 100 μl of PBS for three times, the cells were fixed with 4% formaldehyde for 30 min. Cells were washed again with PBS, nonspecific binding was blocked with 1% BSA/PBS for 1 h at room temperature. The cells were labeled with anti-Xpress antibodies and horseradish peroxidase-conjugated anti-mouse IgG antibodies (Promega) diluted to 1:5000 with 1% BSA in PBS, and then washed with 0.1% BSA in PBS for three times. The colorimetric reaction was initiated using 100 μl of a TMB substrate solution (Promega) for peroxidase and was stopped by adding 10 μl 0.1 M HCl. The absorbance at 450 nm was measured using a microplate reader (Model 680, Bio-Rad).

Immunolocalization by fluorescence microscopy

CHO/MrgX2 cells (1 × 10^5) were grown on a 35 mm glass-based

Table 1. The EC_{50} values for the agonists of MrgX2.

Ligand	EC50
Cortistatin	78 ± 19 nM
PAMP	500 ± 130 nM
Somatostatin	350 ± 100 nM
Morphine	4.5 ± 1.7 μM
Dextrorphan	1.4 ± 1.1 μM
3-methoxy morphanin	4.7 ± 2.1 μM
Leucin-enkephalin	>100 μM
α-endorphin	>100 μM

The EC_{50} values for the agonists of MrgX2 determined by the intracellular calcium mobilization assay. All data points represent average of independent triplicate measurements. The intracellular calcium mobilization was measured in fura-2-loaded CHO/MrgX2 cells using FLEXstation II. Each EC_{50} represents the mean ± SEM of three determinations.

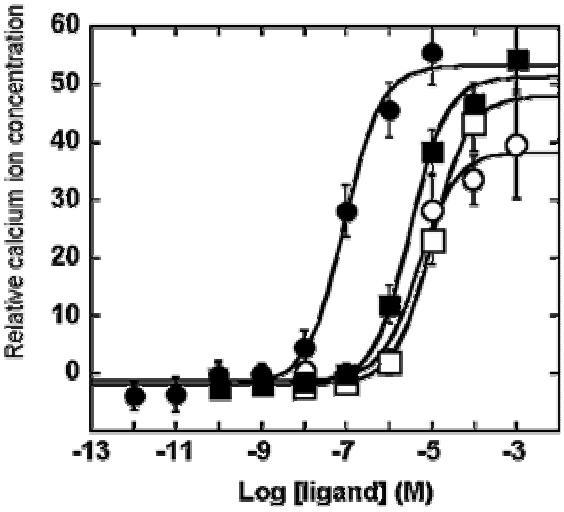
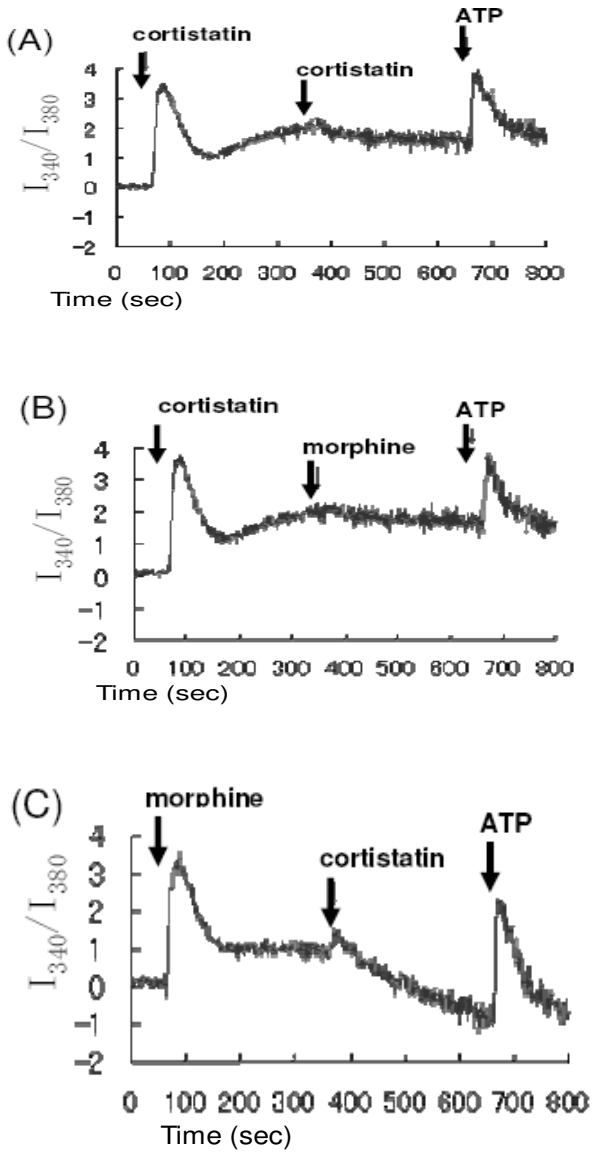


Figure 1. Representative results of intracellular calcium mobilization in CHO/MrgX2 cells induced by cortistatin (closed circles), morphine (open circles), dextrorphan (closed squares), and 3-methoxy morphanin (open squares). The Fura-2 fluorescence was measured with excitation at 340 nm and 380 nm, and emission at 510 nm. The fluorescence intensity ratio, I_{340}/I_{380}, was evaluated to be intracellular calcium mobilization. Each point represents the averate ± standard deviation of six determinations (n = 6), and is representative of at least three independent experiments. Mock-transfected cells did not respond to all ligands including Table I (data not shown).

plate at 37℃ in 5% CO_2. After 10 min stimulation with 1 μM cortistatin or 100 μM morphine, the cells were fixed with 4% formaldehyde, permeabilized with 0.1% saponin, and incubated with 1% BSA in PBS for 1 h at room temperature to block non-specific antibody binding. Antibody staining was performed by incubation with anti-Xpress antibodies (Invitrogen) and following Alexa 488-conjugated anti-mouse IgG antibodies (Molecular Probes) diluted to 1:200 with 1%



BSA in PBS. The cells were analyzed under a fluorescence microscope (Axiovert, Carl Zweiss) and by using the imaging software (Axiovision, Carl Zweiss).

RESULTS

First, we examined ligand-dependent calcium mobilization in CHO/MrgX2 cells. We observed significant calcium mobilization when the CHO/MrgX2 cells were stimulated with cortistatin, PAMP, and somatostatin with EC_{50} values of 78, 500, and 350 nM, respectively. Morphine and its derivatives showed less potent activation of MrgX2. The EC_{50}values determined for morphine, dextrorphan, and 3-methoxy morphanin were 4.5, 1.4, and 4.7 μM, respectively. Since the maximum response of morphine was approximately 80% of that of cortistatin, morphine influenced MrgX2 as a partial agonist (Figure 1). The activation of MrgX2 by morphine was not affected after 10 min pre-incubation with

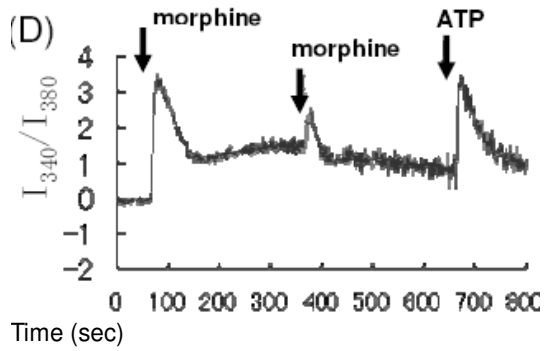


Figure 2. Cortistatin (1 μM) and morphine (100 μM) produced rapid desensitization of MrgX2. Time course of intracellular calcium mobilization in Fura-2-loaded CHO/MrgX2 cells is shown. The second ligand (A and C; cortistatin, B and D; morphine) was added 5 min after the stimulation with the first ligand (A and B; cortistatin, C and D; morphine), and alterations in calcium mobilization were measured. Adenosine 5'-triphosphate (1 μM) was also added 5 min after the administration of the second ligand to confirm that heterologous desensitization did not occurred. The Fura-2 fluorescence was measured with excitation at 340 nm and 380 nm, and emission at 510 nm. The fluorescence intensity ratio, I_{340}/I_{380}, was evaluated to be intracellular calcium mobilization.

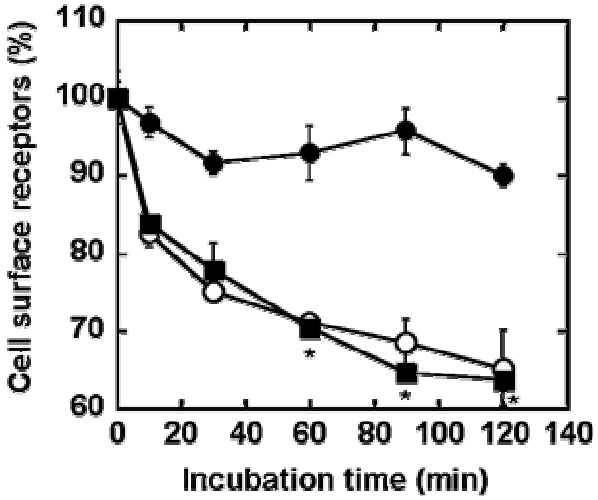


Figure 3. Representative results of MrgX2 receptor internalization induced by 1 μM cortistatin or 100 μM morphine; cortistatin (open circles), morphine (closed squares), and buffer control (closed circles). The percentage of MrgX2 receptors remaining on the cell surface after agonist stimulation were measured by ELISA analysis. Each point represents the average of two determinations (n = 2), and is representative of at least three independent experiments. *Significantly different from the control group receiving the buffer instead of morphine. Analysis of variance, Student's test, $p < 0.05$.

100 μM naloxone, a nonspecific classical opioid receptor antagonist (data not shown). In contrast, leucine-enkephalin and α-endorphin, which are endogenous opioid peptides, did not activate MrgX2 (Table 1).

Next, we examined the desensitization of MrgX2 by stimulation with saturated concentrations of cortistatin (1 μM) and morphine (100 μM). Although initial stimulation with cortistatin induced a large mobilization of calcium ions, the addition of another cortistatin after 5 min did not induce any response in the CHO/MrgX2 cells (Figure 2A). This result indicated that MrgX2 was desensitized at the first cortistatin stimulation. When a second stimulation was performed with morphine, no peak was observed (Figure 2B). These results indicated that cortistatin and morphine shared the same receptor MrgX2. We also examined the desensitization of MrgX2 following activation with morphine. Initial morphine stimulation induced calcium mobilization; a subsequent cortistatin or morphine stimulation after 5 min resulted in weak responses in CHO/MrgX2 cells (Figure 2C, 2D). Thus, both cortistatin and morphine desensitized MrgX2 expressed in the CHO cells, although the degree of desensitization by morphine was less than that by cortistatin.

The amount of MrgX2 on the cell surface was quantitatively analyzed by ELISA. Cortistatin (1 μM) induced a decrease in the amount of MrgX2 on the cell surface to 78 and 62% after 10 min and 120 min of the ligand treatment, respectively (Figure 3). The amount of MrgX2 on the cell surface was also decreased to 83 and 64% after stimulation with 100 μM morphine for 10 and 120 min, respectively. We also observed the ligand-treated CHO/MrgX2 cells under a fluorescence microscope. The plasma membranes were stained uniformly in untreated cells, suggesting that the MrgX2 receptors expressed homogeneously on the plasma membrane prior to ligand stimulation (Figure 4A). After stimulation with 100 μM morphine for 10 min, the cells showed several intracellular punctuate fluorescence and bright vesicles (Figure 4B).

This result suggested that the receptor of morphine treated cells were internalized as well as 1 μM cortistatin-treated cells (Figure 4C). Therefore, we considered that the ligand-dependent loss of MrgX2 from the cell surface resulted from receptor internalization.

DISCUSSION

CHO/MrgX2 cells were stimulated not only by cortistatin and PAMP but also by morphine and its derivatives. In contrast, leucine-enkephalin and α-endorphin, which are endogenous opioid peptides, did not activate MrgX2. The plasma concentration of morphine after clinical administration is approximately 10 nM (Collins et al.1998).

Since the EC_{50} values of morphine for the μ-opioid receptor and MrgX2 are 17 nM (Gharagozlou et al., 2003) and 4.5 μM, respectively, it is reasonable to consider that MrgX2 does not possess physiological functions for morphine under normal conditions. However, the plasma concentration of morphine is assumed to be higher than that of the normal clinical value under chronic treatment,

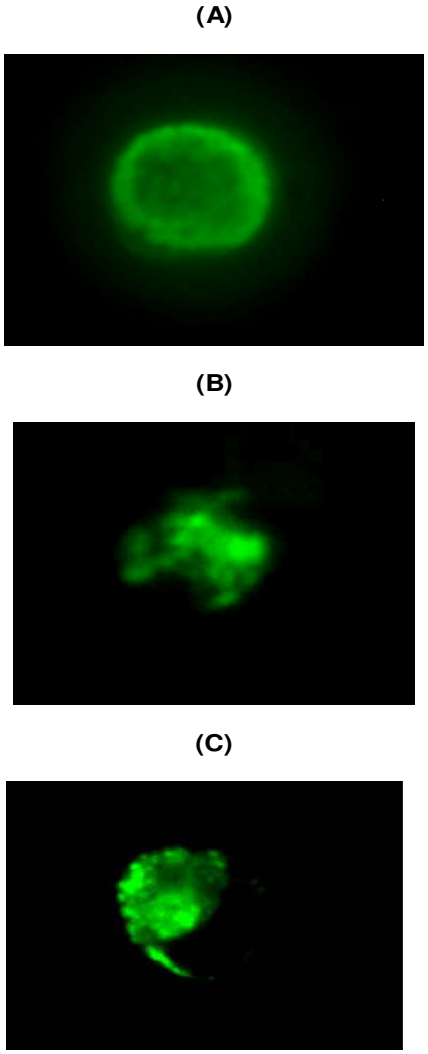


Figure 4. Fluorescence observations of the agonist-induced MrgX2 internalization. (A), no stimulation with a ligand; (B), after 10 min stimulation with 100 μM morphine; (C), after 10 min stimulation with 1 μM cortistatin.

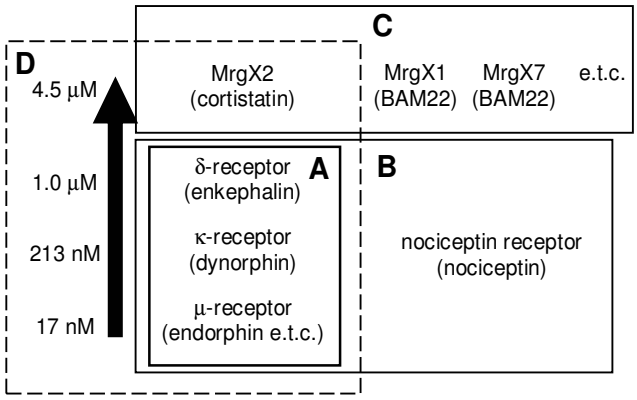


Figure 5. An expanded concept for the morphine and related receptors (A), classical opioid receptors; (B), the opioid receptor subfamily including a nociceptin receptor; (C), the Mrg subfamily; (D), a proposed new functional category for morphine receptors. We suppose that the types of morphine receptor might be able to increase depended on a morphine concentration *in vivo*. The numbers correspond to EC_{50}s for morphine. The endogenous agonist peptide for a receptor is demonstrated in a parenthesis. We notice that enkephalin and BAM22 are produced form a same precursor, a proenkephalin A.

development of tolerance, and addictive condition. To understand the functions of morphine under relatively high concentrations, several investigations have been carried out for additional morphine receptors.

One candidate for the additional morphine receptor is the δ-opioid receptor that shows an EC_{50} value of 1.0 μM for morphine (Gharagozlou et al., 2002). In addition, it is reported that the δ-opioid receptor is involved in morphine tolerance and dependence (Zhu et al., 1999; Pradhan et al., 2006; Zhang et al., 2006; Narita et al., 2001). In our experiment, the EC_{50} value of MrgX2 for morphine was 4.5 μM; this value was higher but almost equal to that of the δ-opioid receptor. Not only the δ-opioid receptor but also the κ-opioid receptor was considered to be the physiologically functional morphine receptor because a high concentration of morphine induced analgesia through the κ-opioid receptor in the μ-opioid receptor knockout mice (Yamada et al., 2006). According to these facts, we consider that MrgX2 could be physiological morphine receptor as well as the δ- and κ- opioid receptors. We are also interested in the evidence for unknown morphine receptors. Morphine-induced hyperalgesia, for example, was observed even when all the three types of opioid receptors were blocked simultaneously by using naltrexone (Wu et al., 2004; Juni et al., 2006). In the immune system, immunosuppression induced by morphine occurred via the naloxone-insensitive receptor (Roy et al., 1998).

In our experiments, MrgX2 was completely desensitized after stimulation with cortistatin (Figure 2A, 2B). On the other hand, the remaining weak response for morphine was observed even after morphine stimulation (Figure 2C, 2D).

These results were consistent with our assumption that morphine was a partial agonist for MrgX2 (Kovoor et al., 1998). The tolerance for morphine is partly explained by receptor desensitization and internalization (Bailey and Connor 2005; Narita et al., 2006). Thus, the difference in the degree of desensitization between endogenous peptides and morphine should be considered in order to understand morphine tolerance not only for opioid recaptors but also for MrgX2. Moreover, it is reported that the μ-opioid receptor dose not show morphine dependent desensitization and internalization (Keith et al., 1996; Minnis et al., 2003) in contrast with MrgX2.

Based on the above discussion, we propose the importance of studying MrgX2 together with the classical opioid receptors for the investigation of the morphine effects.

MrgX2 belongs to the Mrg subfamily. The homologies of amino acid sequences between MrgXs and opioid receptors are low. However, the facts that morphine activates MrgX2 and BAM22 (a proenkephalin A gene product) activates MrgX1 (SNSR4) and MrgX7 (SNSR3) (Lembo et al., 2002) suggest that MrgX members are functionally similar to opioid receptors (Figure 5). In addition, both MrgXs and opioid receptors are expressed in DRG neurons and could work together for modulation of nociception. Further investigations will clarify the physiological relationship between opioid receptors and MrgXs.

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