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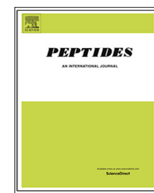

Blockade of analgesic effects following systemic administration of *N*-methyl-kyotorphin, NMYR and arginine in mice deficient of preproenkephalin or proopiomelanocortin gene

Hiroyuki Neyama[a,b], Yusuke Hamada[c], Ryoko Tsukahara[a], Minoru Narita[c], Kazuhiro Tsukamoto[b], Hiroshi Ueda[a,*]

[a] Department of Pharmacology and Therapeutic Innovation, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan
[b] Department of Pharmacotherapeutics, Nagasaki University Graduate School of Biomedical Sciences, Japan
[c] Department of Pharmacology, Hoshi University School of Pharmacy and Pharmaceutical Sciences, Japan

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ABSTRACT

Kyotorphin is a unique biologically active neuropeptide (L-tyrosine-L-arginine), which is reported to have opioid-like analgesic actions through a release of Met-enkephalin from the brain slices. *N*-methyl-L-tyrosine-L-arginine (NMYR), an enzymatically stable mimetic of kyotorphin, successfully caused potent analgesic effects in thermal and mechanical nociception tests in mice when it was given through systemic routes. NMYR analgesia was abolished in μ-opioid receptor-deficient (MOP-KO) mice, and by intracerebroventricular (i.c.v.) injection of naloxone and of *N*-methyl L-leucine-L-arginine (NMLR), a kyotorphin receptor antagonist. In the Ca^{2+}-mobilization assay using CHO cells expressing $G\alpha_{qi5}$ and hMOPr or hDOPr, however, the addition of kyotorphin neither activated MOPr-mechanisms, nor affected the concentration-dependent activation of DAMGO- or Met-Enkephalin-induced MOPr activation, and Met-enkephalin-induced DOPr activation. NMYR-analgesia was significantly attenuated in preproenkephalin (PENK)- or proopioimelanocortin (POMC)-KO mice. The systemic administration of arginine, which is reported to elevate the level of endogenous kyotorphin selectively in midbrain and medulla oblongata, pain-related brain regions, caused significant analgesia, and the analgesia was reversed by i.c.v. injection of NMLR or naloxone. In addition, PENK- and POMC-KO mice also attenuated the arginine-induced analgesia. All these findings suggest that NMYR and arginine activate brain kyotorphin receptor in direct and indirect manner, respectively and both compounds indirectly cause the opioid-like analgesia through the action of endogenous opioid peptides.

1. Introduction

Kyotorphin is an analgesic dipeptide (L-tyrosine-L-arginine), which was isolated from bovine brain by use of in vivo analgesic assay system [1]. As kyotorphin causes an in vitro release of Met-enkephalin from the striatal slices [1,2], but shows neither binding activity to opioid receptors nor inhibiting activity of enkephalin degrading enzymes [1,3,4], this dipeptide is known as an enkephalin releaser. Although details remain elusive, there are several studies showing that opioid receptor antagonist, naloxone blocked various pharmacological or physiological actions of kyotorphin [5,6,3,7]. Kyotorphin was found to bind to putative G_i-coupled receptor in brain membranes through reconstitution experiments using purified G_{i1} and membrane putative receptor, which has high-affinity to [^{3}H]-kyotorphin [8]. It should be noted that L-leucine-L-arginine (Leu-Arg) inhibits the [^{3}H]-kyotorphin binding and kyotorphin-induced GTPase activation, a sign of G-protein activity, but Leu-Arg has no agonist activity on G proteins [8], suggesting that Leu-Arg could be considered as a pure kyotorphin receptor antagonist [9,10]. Regarding the biosynthesis, we have reported that kyotorphin is synthesized from L-tyrosine and L-arginine by partially

Abbreviations: NMYR, *N*-methyl-L-tyrosine-L-arginine; PENK, preproenkephalin; POMC, proopiomelanocortin; Leu-Arg, L-leucine-L-arginine; NMLR, *N*-methyl-L-Leucine-L-arginine; WT, wild-type; KO, knockout; DAMGO, [D-Ala^2, *N*-Methyl-Phe^4, Gly^5-ol] enkephalin; MOPr, μ opioid receptor; ICS, intermittent cold stress; IPS, intermittent psychological stress; pSNL, partial sciatic nerve ligation; hMOPr, human μ opioid receptor; hDOPr, human δ opioid receptor; p.o., per os; i.c.v., intraventricular; i.t., intrathecal; AUC, area under the curve; PWL, thermal paw withdrawal latency; HBSS, Hank's balanced salt solution; L-Arg, L-arginine

* Corresponding author at: Department of Pharmacology and Therapeutic Innovation, Nagasaki University Graduate School of Biomedical Sciences, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan.

E-mail address: ueda@nagasaki-u.ac.jp (H. Ueda).

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purified synthetase from rat brains, and the distribution and subcellular localization of the synthetase [11] are similar to those of kyotorphin [12]. However, the possibility cannot be excluded that this dipeptide is generated by the enzymatic processing of precursor proteins [13]. Most recently we have reported that tyrosyl-tRNA synthetase is a potential kyotorphin synthetase and has similar biochemical characteristics to partially purified rat kyotorphin synthetase [14]. Based on the finding that Km value for substrate arginine is much higher than the plasma concentration [11,14], we examined the change in brain kyotorphin levels following the systemic administration of arginine. The elevation of kyotorphin contents was uneven throughout brain regions [14], and it was higher in the midbrain and medulla oblongata, being consistent to the brain regional distribution of partially purified kyotorphin synthetase in the rat brain [11]. Kawabata et al. [15] reported that the systemic administration with L-arginine inhibited the carrageenan-induced inflammatory hyperalgesia, being consistent to the characterization of kyotorphin synthetase.

Through various attempts to design stable and potent derivatives of kyotorphin, we found an *N*-methyl-derivative NMYR has promising potencies in a unique and very sensitive peripheral nociception test [16]. In that study, the intraplantar injection of sub-femtomoles of NMYR caused nociceptive responses, which was reversed by the co-administration of similar dose of kyotorphin antagonist *N*-methyl-Leu-Arg, NMLR. Following this study, several papers have reported that kyotorphin-amide modification and its derivatives successfully show enzymatical stability and potent analgesic activity even by systemic administration [17,18], though no attempt has been done to examine whether kyotorphin-amide analgesia is blocked by kyotorphin antagonist, Leu-Arg or NMLR. Regarding the characterization as 'opioid-like' analgesia, they used intrathecal injection (i.t.) of naloxone, an opioid receptor antagonist to demonstrate the opioid-like analgesia of kyotorphin-amide [17]. However, as this report lacks the data with a high dose (50 μg) of naloxone (i.t.) alone, it remains elusive how much the possible hyperalgesic actions of naloxone affected the analgesic action of kyotorphin-amide. In addition, the attempt to see involvement of brain opioid mechanisms through endogenous opioid peptides also remains to be determined with the analgesia of kyotorphin derivatives.

In the present study we aimed to first examine whether potent analgesic is obtained by the systemic administration of NMYR that we have previously developed [16]. Secondly, we attempted to pharmacologically characterize the analgesic effects NMYR and arginine in terms of the involvement of brain opioid peptides using mice deficient of preproenkephalin (PENK) or proopioimelanocortin (POMC) gene, as well as in vivo NMLR antagonism and in vivo and/or in vitro opioid receptor-involvements.

2. Materials and methods

2.1. Materials

Synthetic peptides, kyotorphin, NMYR and NMLR were purchased from PH Japan Co., Ltd. (Hiroshima, Japan). [D-Ala^2, *N*-Methyl-Phe^4, Gly^5-ol] enkephalin (DAMGO) and naloxone hydrochloride was purchased from Sigma Aldrich (St. Louis, MO), Met-enkephalin and L-arginine were from WAKO (Osaka, Japan). In in vivo experiments, NMYR was administered through subcutaneous (s.c.), per os (p.o.), intracerebroventricular (i.c.v.) or intrathecal (i.t.) routes, while NMLR was given by i.c.v. or i.t. injection. For the culture experiments to see opioid receptor signaling, DMEM/HAM-F12 medium, geneticine, hygromycin B were purchased from Wako, Hank's Balanced Salt Solution (HBSS) and pluronic acid were from Life Technologies (Grand Island, NY), Fluo-8 was from AAT Bioquest (Sunnyvale, CA), amaranth, probenecid, and DAMGO were from Sigma Aldrich.

2.2. Animals

Male C57BL/6 J mice (15–30 g) were purchased from TEXAM (Nagasaki, Japan) and used for most of experiments. Animals were housed in a room maintained at 22 ± 3 °C and 55 ± 5% relative humidity with a 12 h light/dark cycle (light on 8:00 A.M. to 8:00 P.M.). Food and water were available ad libitum. In some experiments, male μ opioid receptor (MOPr) gene-deficient (MOPr-KO) mice, which had been kindly supplied by Brigitte Kieffer (McGill Univ. Douglas Institute, Montreal, Canada) and backcrossed to the inbred C57BL/6J mice for at least 10 generations were used, as reported previously [19]. In some other experiments, we used male preproenkephalin-deficient (PENK-KO) mice and proopiomelanocortin-deficient (POMC-KO) mice from The Jackson Laboratory (Bar Harbor, ME) possessing C57BL/6J and 129S2/SvPas mixed genetic background, as reported previously [20]. These mice were backcrossed to the inbred C57BL/6J mice for at least 10 generations before using for behavioral experiments. All procedures were approved by the Nagasaki University Animal Care Committee (Nagasaki, Japan) and complied with the recommendations of the International Association for the Study of Pain [21]. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals [22–24].

2.3. Nociception tests

Thermal paw withdrawal, paw pressure and tail-flick tests were performed as, previously reported [25–27]. In some experiments, the analgesic activities were evaluated by use of area under the curve (AUC) as described in figure legends (Figs. 3 and 4).

2.4. Cells

The CHO cells stably co-expressing human μ opioid receptor (hMOPr) and C-terminal modified $G\alpha_q$ chimeric G protein, in which last 5 amino acids of C-terminal $G\alpha_q$ were replaced by corresponding $G\alpha_i$ amino acids to make $G\alpha_{qi5}$, and the CHO cells stably co-expressing human δ opioid receptor (hDOPr) and C-terminal modified $G\alpha_q$ chimeric G protein, in which last 5 amino acids of C-terminal $G\alpha_q$ with G66D mutation were replaced by corresponding $G\alpha_i$ amino acids to make $G\alpha_{qG66Di5}$. Both CHO cells expressing hMOPr and $G\alpha_{qi5}$ or hDOPr and $G\alpha_{qG66Di5}$ were prepared, as reported [28], and generously given by Dr. Girolamo Calo at University of Ferrara, Italy. These cells were maintained with DMEM/HAM-F12 supplemented with 10% FBS, 200 μg/mL of geneticine, 100 μg/mL of hygromycin B, 100 IU/mL penicillin and 100 IU/mL streptomycin, and incubated at 37 °C in a 5% CO_2 atmosphere.

2.5. Ca^{2+} mobilization assay

Ca^{2+} mobilization assay using the CHO cells (CHO_{hMOP}-$G\alpha_{qi5}$ and CHO_{hDOP}-$G\alpha_{qG66Di5}$) was previously described [29]. Briefly, CHO_{hMOP}-$G\alpha_{qi5}$ and CHO_{hDOP}-$G\alpha_{qG66Di5}$ cells were harvested using 0.5 mM EDTA, centrifuged, and re-suspended in culture medium described above. The cells were plated on a 384-well plate at the density of 1.0×10^4 cells/well/30 μL. Following overnight incubation, the medium was removed and the cells were loaded with 3 μM Fluo-8 dissolved in 20 mM HEPES/HBSS solution (pH7.4) containing 1 mg/mL amaranth, 2.5 mM probenecid, and 0.01% pluronic acid. After 1 h incubation, the cells were stimulated with either 10 μM kyotorphin or vehicle for 5 min. Then, Met-enkephalin or DAMGO at defined concentrations was added to the cells in the presence or absence of 10 μM kyotorphin. The fluorescence was recorded by Functional Drug Screening System/μCell (Hamamatsu Photonics K.K., Hamamatsu, Japan) and the fluorescence intensity was described as signal ratio (tested value/basal value) or fold induction. Dose-response curves were plotted as mean ± S.E.M using GraphPad prism (Graphpad Software. San Diego, CA).

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