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SHORT COMMUNICATION

μ-Opioid receptor activation by tramadol and *O*-desmethyltramadol (M1)

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Abstract Tramadol has been used as an analgesic for several decades. μ-Opioid receptors (μORs) are the major receptors that mediate the analgesic effects of opioids. Although μORs have been thought to be one of the sites of action of tramadol, there has been no report that directly proves whether tramadol is an agonist of μOR or not. In this study, we examined the effects of tramadol and its main active metabolite *O*-desmethyltramadol (M1), on the function of μORs using *Xenopus* oocytes expressing cloned human μORs. The effects of tramadol and M1 were evaluated using the Ca^{2+}-activated Cl^- current assay method for $G_{i/o}$-protein-coupled receptors by using a μOR fused to G_{qi5} (μOR-G_{qi5}) in *Xenopus* oocytes. DAMGO [(D-Ala^2, *N*-MePhe^4, Gly^5-ol)-enkephalin] evoked Cl^- currents in oocytes expressing μOR-G_{qi5} in a concentration-dependent manner. Tramadol and M1 also evoked Cl^- currents in the oocytes expressing μOR-G_{qi5}; however, relatively higher concentrations (compared to DMAGO) were necessary to induce such currents. Tramadol and M1 had a direct effect on μORs expressed in *Xenopus* oocytes. Although the monoamine uptake system and several types of ligand-gated ion channels are thought to be one of the targets for tramadol, tramadol-induced antinociception may be mediated at least in part, by the direct activation of μORs.

Keywords Tramadol · *O*-Desmethyltramadol · μ opioid receptor · *Xenopus* oocytes

Introduction

Tramadol [(1R,2R) and (1S,2S)-2-dimethylamino-methyl-1-(3-methoxyphenyl)-cyclohexanol hydrochloride] has been used as an analgesic for several decades and several lines of mechanisms of antinociception have been proposed [1]. It has been reported that blocking the serotonin (5-HT) transporter may contribute to its analgesic actions [2]. There has also been evidence that shows the inhibition of norepinephrine (NE) transporter [3–5].

Recently, we and other investigators reported that G protein-coupled receptors (GPCRs) may be one of the targets of tramadol [1]. Overall, opioid receptors (ORs) have been thought to be the site of action of tramadol. ORs belong to the $G_{i/o}$-coupled receptors in the GPCR family and three types of receptors (μ, δ and κ) have been identified by molecular cloning [6]. Within these three subtypes, μORs are the major receptors that mediate the analgesic effects of opioids [6]. Tramadol elicited dose-related antinociception in mouse abdominal constriction hot-plate and tail-flick tests and in rat air-induced abdominal constriction and hot-plate tests [7]. The antinociception of tramadol obtained with the mouse tail-flick test was completely antagonized by naloxone, suggesting an opioid mechanism of action. Although μORs have been thought to be one of the sites of

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action of tramadol, there have been few reports that prove whether tramadol is an agonist of μOR or not.

The *Xenopus* oocyte expression system has widely been employed to study the functions of a number of GPCRs [8, 9]. In the case of G_q-coupled receptors, receptor stimulation results in activation of Ca^{2+}-activated Cl^- channels in *Xenopus* oocytes by G_q-mediated phospholipase C activation and subsequent formation of IP_3 and diacylglycerol [9]. IP_3 formation causes release of Ca^{2+} from the endoplasmic reticulum which, in turn, triggers the opening of Ca^{2+}-activated Cl^- channels endogenously expressed in the oocytes [9]. However, in the case of $G_{i/o}$-coupled receptors, analysis has been difficult due to a lack of appropriate analytical output in oocytes. We have established an assay method for $G_{i/o}$-coupled GPCRs by using a μOR fused to G_{qi5} (μOR-G_{qi5}) in *Xenopus* oocytes [10].

In this study, we examined whether tramadol and its main active metabolite, *O*-desmethyltramadol (M1), activate the function of μORs or not, using this assay system directly.

Materials and methods

Materials

Adult *Xenopus laevis* female frogs were purchased from Kato Kagaku (Tokyo, Japan). The Ultracomp™ *Escherichia coli* transformation kit was from Invitrogen (San Diego, CA, USA). Purification of cDNAs was performed with a Qiagen purification kit (Qiagen, Chatsworth, CA, USA). Gentamicin, sodium pyruvate, [D-Ala^2, *N*-Me-Phe^4,Gly^5-ol] enkephalin (DAMGO) and other chemicals were from Nacalai Tesque (Kyoto, Japan). Rat μOR was provided by Dr. N. Dascal (Tel Aviv University, Ramat Aviv, Israel). The chimeric $G\alpha_{qi5}$ was a gift from Dr. B.R. Conklin (UCSF, CA, USA). Each of the cRNAs was prepared using a mCAP mRNA capping kit (Stratagene, La Jolla, CA, USA), and transcribed with a T7 RNA polymerase in vitro transcription kit (Stratagene, La Jolla, CA, USA). Tramadol and M1 were supplied by Nippon Shinyaku Co., Ltd (Kyoto, Japan).

Preparation of chimeric μOR-Gqi5

The tandem cDNAs of chimeric μOR-Gqi5 were created by ligating the receptor cDNA sequences into the NheI site of G_{qi5} cDNAs. The sequences of all PCR products were confirmed by sequencing with ABI3100 (Applied BioSystems, Tokyo, Japan). 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 [11], ensuring a high level of protein expression in the oocytes. Each of the cRNAs were synthesized using the mCAP mRNA capping kit, with a T7 RNA polymerase in vitro transcription kit (Ambion, Austin, TX, USA) from the respective linearized cDNAs.

Recording and data analysis

Isolation and microinjection of *Xenopus* oocytes were performed as previously described [10, 12–14]. *Xenopus* oocytes were injected with appropriate amounts of cRNAs (50 ng; μOR-G_{qi5}) and incubated with a composition of modified Barth's saline (MBS) [NaCl 88 mmol/l, KCl 1 mmol/l, $NaHCO_3$ 2.4 mmol/l, Hepes 10 mmol/l, $MgSO_4$ 0.82 mmol/l, $Ca(NO_3)_2$ 0.33 mmol/l, and $CaCl_2$ 0.91 mmol/l (pH 7.4 adjusted with NaOH)] supplemented with 2.5 mmol/l sodium pyruvate and 50 μg/ml gentamicin for 3–7 days until recording. Oocytes were placed in a 100-μl recording chamber and perfused with the MBS as composed above (pH 7.4 adjusted with NaOH) at a rate of 1.8 ml/min at room temperature. Recording and clamping electrodes (1–2 MΩ) were pulled from 1.2-mm outside diameter capillary tubing and filled with 3 mol/l KCl. A recording electrode was imbedded in the animal's pole of oocytes, and once the resting membrane potential stabilized, a clamping electrode was inserted and the resting membrane potential was allowed to restabilize. A Warner OC 725-B oocyte clamp (Hamden, CT, USA) was used to voltage-clamp each oocyte at −70 mV. DAMGO, tramadol and M1 were applied for 20 s. We analyzed the peak component of the transient inward currents induced by receptor agonists; this component is dependent on the concentrations of the receptor agonist applied and is reproducible, as described by Minami et al. [12].

Statistical analysis

Results are expressed as currents (nA). The '*n*' values refer to the number of oocytes studied. Each experiment was carried out with oocytes from at least two different frogs. Statistical analyses were carried out by one-way ANOVA followed by Dunnett's correction using GraphPad Prism 4 (GraphPad Softwear. Inc. La Jolla, CA, USA) (Fig. 2a). A p value <0.05 indicated statistically significant differences compared to currents at 10^{-9} M. To compare the agonistic effects of tramadol and M1 at 10^{-4} M, we carried out Student's t test (Fig. 2b). A p value <0.05 indicated statistically significant differences.

Results

We examined the effects of DAMGO, tramadol, and M1 on the μOR function in *Xenopus* oocytes expressing μOR-G_{qi5} by using the assay method we previously used for function

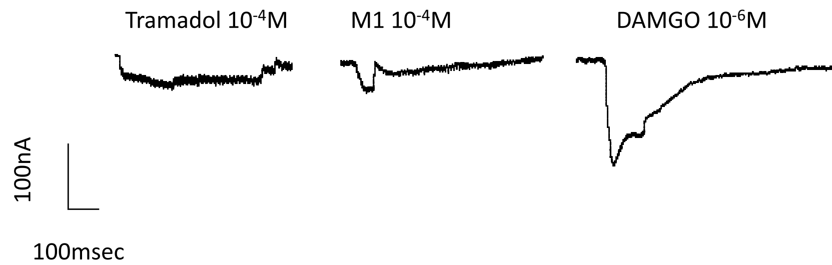

Fig. 1 Typical currents due to stimulation of DAMGO [(D-Ala[2], *N*-MePhe[4], Gly[5]-ol-enkephalin)], tramadol and *O*-desmethyltramadol (M1). Oocytes expressing μOR-G_{qi5} were voltage-clamped at −70 mV. DAMGO (1 μM), tramadol (100 μM) and M1 (100 μM) was bath-applied to the oocytes for 20 s and the peak Ca^{2+}-activated Cl^- current was measured

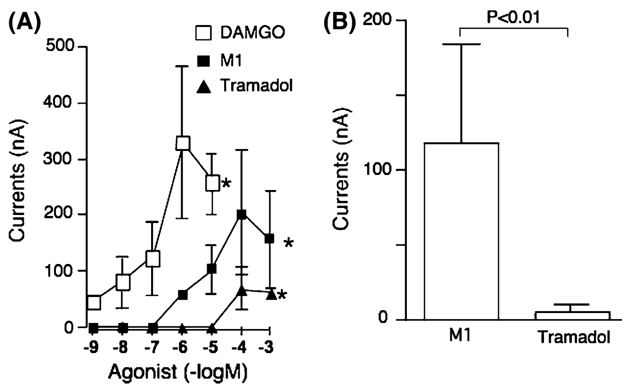

Fig. 2 **a** The concentration–response curve for DAMGO ([D-Ala[2], *N*-MePhe[4], Gly[5]-ol]-enkephalin), tramadol and *O*-desmethyltramadol (M1) activation of Ca^{2+}-activated Cl^- current in *Xenopus* oocytes expressing μOR fused to G_{qi5} (μOR-G_{qi5}). Oocytes expressing μOR-G_{qi5} were voltage-clamped at −70 mV, and a representative recording trace from a single oocyte is shown. Oocytes were treated (*open square*) with DAMGO, tramadol (*filled triangle*) and M1 (*filled square*) for 20 s. The concentration−response curve of the inhibition of DAMGO (10^{-9} to 10^{-5} M), tramadol (10^{-9} to 10^{-3} M), and M1 (10^{-9} to 10^{-3} M)-induced Cl^- currents. Data are the mean of the currents ± SEM from 8 oocytes at each tramadol concentration. Statistical analyses were carried out by one-way ANOVA followed by Dunnett's correction. *$p < 0.05$ compared with the currents at 10^{-9} M. **b** Comparison between *O*-desmethyltramadol (M1)-induced currents and tramadol-induced currents in *Xenopus* oocytes expressing μORs fused to G_{qi5} (μOR-G_{qi5}). Oocytes expressing μOR-G_{qi5} were voltage-clamped at −70 mV, and a representative recording trace from a single oocyte is shown. Oocytes were treated with tramadol (10^{-4} M) and M1 (10^{-4} M) for 20 s. Data are the mean of the currents ± SEM from 8 oocytes at each tramadol concentration. Statistics were analyzed using Student's *t* test. A *p* value <0.05 indicated a statistically significant difference

analysis of $G_{i/o}$-coupled receptors. We previously reported the effects of the μOR agonist DAMGO on the Ca^{2+}-activated Cl^- currents in *Xenopus* oocytes expressing μOR-G_{qi5} [10, 13, 14]. In the present study, DAMGO, tramadol, and M1 did not elicit any currents in oocytes without expression of μOR-G_{qi5}. DAMGO evoked Cl^- currents in oocytes expressing μOR-G_{qi5} in a concentration-dependent manner (Figs. 1, 2), which is consistent with our previous results [10, 14]. Tramadol and M1 also evoked Cl^- currents in oocytes expressing μOR-G_{qi5} (Figs. 1, 2); however, higher concentrations (than for DAMGO) were necessary to induce Cl^- currents. The values of DAMGO-induced currents were 332 ± 134 nA and 260 ± 51 nA at 1 and 10 μM, respectively ($n = 8$ each) (Fig. 2), whereas those of tramadol-induced currents were 67.9 ± 37.2 nA and 61.5 ± 9.7 nA at 100 μM and 1 mM, respectively ($n = 8$ each) (Fig. 2). M1-induced currents were 59.1 ± 0.1 nA at 1 μM and 102 ± 43 at 10 μM, respectively ($n = 8$ each) (Fig. 2a).

We next compared M1-induced currents and tramadol-induced currents at the same concentration in *Xenopus* oocytes expressing μOR-G_{qi5}. DAMGO (10^{-5} M)-induced currents were 210.3 ± 48.6 nA ($n = 7$) and M1 (10^{-5} M)-induced currents were 95.3 ± 42.1 nA ($n = 9$); however, tramadol (10^{-5} M) did not evoke any currents. We compared the agonistic effects of tramadol and M1 at 10^{-4} M. M1 (10^{-4} M)-induced currents were 118.2 ± 66.1 nA ($n = 8$) and tramadol (10^{-4} M)-induced currents were 5.1 ± 5.1 nA ($n = 8$). M1-induced currents were approximately 20 times those of tramadol.

Discussion

We have shown that tramadol and M1 had a direct effect on μOR expressed in *Xenopus* oocytes; however, higher concentrations (than for DAMGO) were necessary to induce Cl^- currents. This is the first report that shows the agonistic effects of tramadol and M1 on cells expressing μORs. Raffa and Friderichs (1992) reported the antinociceptive activity of tramadol possibly via an opioid mechanism of action. Tramadol, however, has a weak affinity for μORs [7]. Tramadol bound with modest affinity to μORs and with weak affinity to δORs and κORs, with K_i values of 2.1, 57.6 and 42.7 μM, respectively [7]. The affinity of tramadol for μORs is approximately ten-fold less than that

of codeine, and 6,000-fold less than that of morphine, an affinity that by itself does not seem to be sufficient to contribute to the analgesic action of tramadol [15, 16]. Stoops et al. [17] evaluated the effect of μOR blockade with naltrexone on the pharmacodynamic action of tramadol in humans. Naltrexone only partially attenuated tramadol's positive subjective effects, which suggest that both opioid and non-opioid actions may play a role in tramadol's subjective profile of action [17].

The tramadol metabolite M1 binds to μOR with an approximately 300-fold higher affinity than the parent compound tramadol, but with a much lower affinity than morphine [18, 19]. In in vitro receptor binding and synaptosomal uptake experiments, the (+)-forms of tramadol are specific for the μOR site. M1 shows a pronounced μ-selectivity [18, 19]. The pain thresholds induced by tramadol are only partially blocked by the opioid antagonist naloxone [20]. Our present results are consistent with these reports.

The action of tramadol has been considered as the inhibition of the reuptake of monoamines, such as NE and 5-HT, which are released from nerve endings. These inhibitory effects may also contribute to the analgesic effect of tramadol by inhibiting pain transmission in the central nervous system (CNS) [2–5]. Although μORs and monoamine transporters are thought to be the sites of tramadol activity, additional sites probably exist, based on the additional clinical and analgesic effects of tramadol [1]. Several previous studies have shown that some GPCRs and ligand-gated ion channels are also targets for tramadol [1]. Tramadol has an inhibitory effect on muscarinic receptors (M1 and M3) [21, 22], $5HT_{2C}$ receptors, and nicotinic acetylcholine ion channels [23]. Our present results and the clinical experience of a wide separation between analgesia and typical opioid side-effects suggest that tramadol-induced antinociception could be mediated by both μORs and nonopioid mechanisms.

In conclusion, tramadol and M1 had a direct effect on μORs expressed in *Xenopus* oocytes. Although the monoamine transporters in the CNS and GPCRs are thought to be one of the targets for tramadol, the inhibition of some ligand-gated ion channels have also been shown to be a possible mechanism of action of tramadol. Taken together, tramadol-induced antinociception may be mediated by opioid and some non-opioid mechanisms.

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