Short Communication

The Tramadol Metabolite *O*-Desmethyl Tramadol Inhibits Substance P–Receptor Functions Expressed in *Xenopus* OocytesKouichiro Minami^{1,2,*}, Toru Yokoyama^{1,2}, Junichi Ogata¹, and Yasuhito Uezono²¹Department of Anesthesiology and Critical Care Medicine, Jichi Medical University, Tochigi 329-0498, Japan²Division of Cancer Pathophysiology, National Cancer Center Research Institute, Tokyo 104-0045, Japan

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Abstract. Tramadol has been widely used as analgesic. *O*-Desmethyl tramadol (ODT) is one of the main metabolites of tramadol, having much greater analgesic potency than tramadol itself. Substance P receptors (SPR) are well known to modulate nociceptive transmission within the spinal cord. In this study, we investigated the effects of ODT on SPR expressed in *Xenopus* oocytes by examining SP-induced Ca^{2+} -activated Cl^- currents. ODT inhibited the SPR-induced Cl^- currents at pharmacologically relevant concentrations. The protein kinase C (PKC) inhibitor bisindolylmaleimide I did not abolish the inhibitory effects of ODT on SP-induced Ca^{2+} -activated Cl^- currents. The results suggest that the tramadol metabolite ODT inhibits the SPR functions, which may be independent of activation of PKC-mediated pathways.

Keywords: *O*-desmethyl tramadol (ODT), tramadol, substance P

Substance P (SP) acts as a neurotransmitter released from C fibers located within nociceptive primary afferent neurons into the spinal cord and mediates a part of the excitatory synaptic input to nociceptive neurons at this level (1). SP and its receptors (SPR) are widely distributed in the central and peripheral nervous systems (2). Several studies showed that pain sensitivity is altered in mice lacking the gene encoding SPR; a reduction in nociceptive responses to certain somatic and visceral noxious stimuli occurs in SPR knockout mice (3).

SPR belongs to the family of Gq protein-coupled receptors that activate the protein kinase C (PKC) and Ca^{2+} -mobilization by stimulation of phospholipase C. Our recent reports have shown that the function of SPR is inhibited by volatile anesthetics and intravenous anesthetics. Halothane, isoflurane, enflurane, diethyl ether, and ethanol inhibit the function of SPR (4). Moreover, ketamine and pentobarbital inhibited the SPR-induced currents at pharmacologically relevant concentrations, whereas propofol had little effect on the currents in *Xenopus* oocytes expressing SPR (5). These results suggest that SPR is one of the targets of some anesthetics.

O-Desmethyl tramadol (ODT) is one of the metabolites

of analgesic, tramadol. Only ODT among these metabolites has been shown to have analgesic activity in mice and rats, as assessed by the tail-flick responses. Analgesic potency of ODT is 2 – 4-times higher than that of tramadol (1, 3). In addition, ODT has more affinity for the μ -opioid receptor than does tramadol in biochemical receptor binding studies, although its chemical structure is quite similar to tramadol (1). There have been several reports suggesting that ODT, at pharmacologically relevant concentrations, inhibited 5-HT-evoked Ca^{2+} -activated Cl^- currents in oocytes expressing 5-HT_{2C}R, and inhibited the functions of NMDA receptors, but not those of glycine and GABA_A receptors (6). We have previously reported in *Xenopus* oocytes expressing SPR that tramadol had little effect on the SP-induced Ca^{2+} -activated Cl^- currents (5). However, a recent report has shown that tramadol, given intraperitoneally or intravenously, produced significant inhibition of the biting behavior induced by intrathecal injection of SP (7). We have previously reported the different effects on the Gq-coupled muscarinic M₃ receptors (M₃R) between ODT and tramadol: tramadol inhibited acetylcholine (ACh)-induced currents in oocytes expressing M₃R, whereas ODT did not. In the report we suggest that ODT does not affect the M₃R-mediated signaling in spite of having only a small difference in its structure compared with that of tramadol (8). Collectively these data suggest that inhibitory effects of

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tramadol on SP-induced biting behavior could be due to ODT, although the effects of ODT on SPR functions have not been studied in detail.

The *Xenopus* oocyte expression system has been used to study a multiplicity of receptors including Gq-coupled receptors (5). Stimulation of SPR results in activation of phospholipase C-mediated Ca^{2+} -activated Cl^- currents in *Xenopus* oocytes (4, 5). In the present study we examined the effects of the ODT on the SP-induced Ca^{2+} -activated Cl^- currents in SPR-expressing *Xenopus* oocytes.

Adult *Xenopus laevis* female frogs were purchased from Seac Yoshitomi (Yoshitomi, Fukuoka). SP was from Sigma (St. Louis, MO, USA). ODT hydrochloride was a kind gift from Nippon Shinyaku (Kyoto). Bisindolylmaleimide I (GF109203X) was from Calbiochem (La Jolla, CA, USA). The Ultracomp *E. coli* Transformation Kit was from Invitrogen (San Diego, CA, USA). A Qiagen (Chatsworth, CA, USA) Kit was used to purify plasmid cDNA. Rat SPR cDNA was kindly provided by Dr. J.E. Krause (Washington University School of Medicine, St. Louis, MO, USA). The cDNA for the SPR was inserted into the pBlueScriptIISK(-) vector and linearized with *Xba*I. The SPR synthetic RNA was prepared by using a mCAP mRNA Capping Kit and transcribed with a T7 RNA Polymerase in vitro Transcription Kit (Stratagene, La Jolla, CA, USA).

Isolation and microinjection of *Xenopus* oocytes were performed as described by Sanna et al. (9). Briefly, *Xenopus* oocytes were injected with 50 ng of synthetic RNA encoding SPR and incubated for 2 days. Oocytes were placed in a 100- μl recording chamber and perfused with modified Barth's saline (MBS) containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO_3 , 10 mM HEPES, 0.82 mM MgSO_4 , 0.33 mM $\text{Ca}(\text{NO}_3)_2$, and 0.91 mM CaCl_2 (pH 7.5) at a rate of 1.8 ml/min at room temperature. Recording and clamping electrodes (1–5 M Ω) were pulled from 1.2-mm outside diameter capillary tubing and filled with 3 M KCl. A recording electrode was imbedded in the animal's pole, 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 (Hampden, CT, USA) was used to voltage-clamp each oocyte at -70 mV. We analyzed the peak of the transient inward current component of the SPR-induced currents because this component is dependent on SP concentration and is quite reproducible, as described by Minami et al. (4, 5). The ODT were pre-applied for 2 min to allow for complete equilibration in the bath. The solutions of ODT were freshly prepared immediately before use. The concentrations in the figures represent the bath concentrations.

To determine whether activation of PKC plays a role

in ODT modulation of SPR-mediated events, oocytes were exposed to a PKC inhibitor, bisindolylmaleimide I (GF109203X) (200 nM) (10), in MBS for 120 min. We then compared the effects of anesthetics on SP-induced Ca^{2+} -activated Cl^- currents in *Xenopus* oocytes expressing SPR between before and after the exposure to GF109203X.

Results were expressed as a percentage of control responses, due to the variable SPR expression rate in oocytes. The control responses were measured before and after application of each test compound to take into account possible shifts in the control currents as recording preceded. The "n" values refer to the number of oocytes studied. Each experiment was performed with oocytes from at least two different frogs. Statistical analyses were performed using either a *t*-test or a one-way ANOVA (analysis of variance).

The tramadol metabolite ODT inhibited the action of

Fig. 1. Effects of *O*-desmethyl tramadol (ODT) on substance P (SP)-stimulated currents in *Xenopus* oocytes expressing SP receptors (SPR). A) Chemical structures of tramadol and *O*-desmethyl tramadol (ODT). B) ODT suppresses the SP-induced Ca^{2+} -activated Cl^- currents in *Xenopus* oocytes expressing SPR. Tracings obtained from a single oocyte expressing SPR show the effect of ODT on currents induced by 100 nM SP. SP was applied for 20 s with or without 2-min ODT treatment.

100 nM SP to $71.0 \pm 12.3\%$, $73.6 \pm 9.2\%$, and $56.7 \pm 8.6\%$ of the control at concentrations of 0.1, 1, and 10 μM , respectively (Figs. 1 and 2). After washout of the ODT, the size of SPR-induced currents was reversed to almost the same as the control levels.

We previously reported that treatment with the PKC inhibitor GF109203X (200 nM), which has a K_i value of 20 nM for the inhibition of PKC activity (10), produced the enhancement of the initial Cl^- currents activated by 100 nM SP (4, 5). The control currents before ODT treatment was 35.1 ± 27.6 nA. GF109203X enhanced the currents to $398 \pm 86\%$ of the control currents (119 ± 79.6 nA), which was similar to our previous report. The inhibitory effects of ODT on SP-induced currents were observed in the oocytes pretreated with GF109203X (Fig. 3). ODT (10 μM) inhibited the action of 100 nM SP to $52.0 \pm 9.7\%$, while treatment of GF109203X resulted in the action of ODT (10 μM) to $45.9 \pm 14.6\%$ of control (Fig. 3), although the effect was not significantly different.

Tramadol undergoes biotransformation in the liver by two metabolic pathways to form five *N*- or *O*-desmethylated metabolites. ODT is one of the five main metabolites of tramadol; and the others are mono-*N*-desmethyl tramadol, di-*N*-desmethyl tramadol, tri-*N,O*-desmethyl tramadol, and di-*N,O*-desmethyl tramadol. We have previously reported that tramadol had little effect on SPR function (5). In another paper, we reported that a low

Fig. 2. Concentration-response relationship of *O*-desmethyl tramadol (ODT) on substance P (SP)-induced currents. ODT (10 μM – 10 nM) was applied to the oocytes for 2 min, and then 100 nM of SP was applied for 20 s. Data represent the mean $\pm$ S.E.M. of 40 oocytes. * $P < 0.05$ and ** $P < 0.01$, compared with the control response using analysis of variance.

concentration (under 0.1 μM) of ODT did not suppress SP-induced currents in oocytes expressing the SPR (11). Grond et al. (12) reported the mean ODT concentrations after a 200 mg bolus IV infusion of tramadol and those after patient controlled analgesia with demand doses of 20 mg for 24 h in 92 patients. In our study, the mean concentration of ODT was 84.0 ± 34 ng/mL (approximately 0.3 μM). Sindrup et al. (13) also reported mean ODT concentrations of 5.0 – 122 ng/mL (maximally 0.4 μM) in patients who received 200 – 400 mg of tramadol. In the present study, 0.1 μM and higher concentrations of ODT actually inhibited SP-induced Ca^{2+} -activated Cl^- currents. From the present results, ODT at higher levels,

Fig. 3. The effects of GF109203X (protein kinase C inhibitor: PKCI) on inhibition by *O*-desmethyl tramadol (ODT) on substance P (SP)-stimulated currents in *Xenopus* oocytes expressing SP receptors (SPR). A) Tracings obtained from a single oocyte expressing SPR show the effect of ODT on SP (100 nM)-induced currents before and after treatment of PKCI. SP was applied for 20 s with or without 2-min ODT treatment. PKCI was treated for 120 min. B) Comparison of the effects of PKCI on the inhibitory effects of ODT. Oocytes were incubated with 200 nM GF109203X (PKCI) for 120 min. ODT (10 μM) shown was preapplied for 2 min before being co-applied with SP (100 nM) for 20 s. “Before treatment” indicates the effects of ODT before application of bisindolylmaleimide. Data represent the mean $\pm$ S.E.M. for 10 separate determinations. A paired Student’s *t*-test was used for the statistical analysis.

although within the clinically relevant concentrations, would inhibit SPR functions clinically.

The present study raises the question of how ODT inhibits SPR-mediated responses. We have reported that ODT had little effect on the function of muscarinic M₃R, which share the same downstream signaling steps as the SPR following Gq protein activation, expressed in *Xenopus* oocytes. These findings suggest that the inhibitory effect of ODT on the SP-induced Cl⁻ current is likely due to the inhibition of the SPR before activation of Gq proteins. There is considerable evidence that PKC plays an important role in regulating the function of GPCRs (14) and the functions of some GPCRs are inhibited by PKC activation. We reported that the inhibitory effects of halothane, isoflurane, enflurane, diethyl ether, and ethanol on SP-induced currents were suppressed in oocytes treated with the PKC inhibitor, suggesting that these anesthetics and ethanol inhibit SPR function via activation of PKC. However, in our present experiments, GF109203X did not alter the inhibitory effects of ODT on SPR function, suggesting that PKC may not be involved in the cases of the inhibitory effects of ODT on SPR.

Although much attention has been paid to the μ -opioid receptor and monoamine uptake in the central nervous system as targets for tramadol and ODT, several studies have shown that some GPCRs and ligand-gated ion channels are also targets for tramadol (15). In our present results, the inhibitory effects of ODT seem to be weaker than that on μ -opioid receptors and transporters. Nonetheless, SPR might also be one of the targets for ODT. The inhibitory effects of ODT on SPR might also contribute to the side effects of tramadol. More information about SPR may help to elucidate the role of SPR in the mechanisms of tramadol activity.

In conclusion, we demonstrated that the tramadol metabolite ODT inhibited SPR function. Our findings might help to elucidate the pharmacological basis of ODT and provide a better understanding of its neuronal action and the antinociceptive effects of tramadol. More definitive studies, such as the use of the SPR knockout mouse model, would be required.

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