Inhibition by Tramadol of Muscarinic Receptor-Induced Responses in Cultured Adrenal Medullary Cells and in *Xenopus laevis* Oocytes Expressing Cloned M₁ Receptors

MUNEHIRO SHIRAISHI, KOICHIRO MINAMI, YASUHITO UEZONO, NOBUYUKI YANAGIHARA, and AKIO SHIGEMATSU

Department of Anesthesiology, University of Occupational and Environmental Health, School of Medicine, Kitakyushu, Japan (M.S., K.M., A.S.); Department of Second Pharmacology, Nagasaki University, School of Medicine, Nagasaki, Japan (Y.U.); and Department of Pharmacology, University of Occupational and Environmental Health, School of Medicine, Kitakyushu, Japan (N.Y.)

Received April 20, 2001; accepted June 18, 2001 This paper is available online at http://jpet.aspetjournals.org

ABSTRACT

Tramadol is a widely used, centrally acting analgesic, but its mechanisms of action are not completely understood. Muscarinic receptors are known to be involved in neuronal function in the brain and autonomic nervous system, and much attention has been paid to these receptors as targets of analgesic drugs in the central nervous system. This study investigated the effects of tramadol on muscarinic receptors by using two different systems, i.e., a *Xenopus laevis* oocyte expression system and cultured bovine adrenal medullary cells. Tramadol (10 nM–100 μM) inhibited acetylcholine-induced currents in oocytes expressing the M₁ receptor. Although GF109203X, a protein kinase C inhibitor, increased the basal current, it had little effect on the inhibition of acetylcholine-induced currents by tramadol.

On the other hand, tramadol did not inhibit the current induced by AIF₃, a direct activator of GTP-binding protein. In cultured bovine adrenal medullary cells, tramadol (100 nM–100 μM) suppressed muscarine-induced cyclic GMP accumulation. Moreover, tramadol inhibited the specific binding of [³H]quinuclidinyl benzilate (QNB). Scatchard analysis showed that tramadol increases the apparent dissociation constant (Kᵦ) value without changing the maximal binding (Bₘₐₓ), indicating competitive inhibition. These findings suggest that tramadol at clinically relevant concentrations inhibits muscarinic receptor function via QNB-binding sites. This may explain the neuronal function and anticholinergic effect of tramadol.

Pain perception is modulated by a variety of neurotransmitters, including opioids, norepinephrine, and serotonin (Yaksh, 1988). Tramadol, (1RS,2RS)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol hydrochloride, is a centrally acting analgesic that is used clinically. Tramadol has the ability to bind to μ-opioid receptors, and this is considered the mechanism of antinociception by this compound, although its binding affinity is relatively low (Hennies et al., 1988). A further mode of action of tramadol has been identified as inhibition of the reuptake of monoamines, such as norepinephrine and serotonin, released from nerve endings. This inhibitory effect may also contribute to the analgesic effects of tramadol by the inhibition of pain transmission in both the central nervous system (CNS) and the spinal cord (Raffa et al., 1992; Reimann and Hennies, 1994).

Although μ-opioid receptors and monoamine transporters are thought to be the sites of action of tramadol, there would be additional sites to explain analgesic effects.

Muscarinic receptors are involved in various neuronal functions in the CNS and autonomic nervous systems (Caulfield, 1993). Cholinergic antagonism interferes with learning behavior, whereas cholinesterase inhibitors enhance learning (Fibiger et al., 1991). Furthermore, inhibition of muscarinic receptors leads to sedation or non-rapid eye movement sleep (Durieux, 1996). The therapeutic potential of muscarinic antagonists is compromised by several effects on the autonomic nervous system, including dry mouth, tachycardia, constipation, urinary retention, and pupillary dilation (Eglen et al., 1999). Recent molecular cloning studies have revealed the existence of five subtypes of muscarinic receptors (M₁–M₅) (Wess, 1996). By using pharmacological techniques, many of the muscarinic responses in peripheral tissues have been thoroughly studied. However, relatively little is known about the functional roles of individual muscarinic subtype receptors in the CNS. Recent studies of their anatomic distribution have been used to predict their functions.

ABBREVIATIONS: CNS, central nervous system; QNB, quinuclidinyl benzilate; ACh, acetylcholine; PKC, protein kinase C; KRP, Krebs-Ringer phosphate; IBMX, 3-isobutyl-1-methylxanthine.
in the CNS. For example, cortical and hippocampal M₁ receptors are involved in memory and learning, and striatal M₄ receptors play a key role in the regulation of extrapyramidal motor function (Levey et al., 1991). Furthermore, in recent review, many anesthetics inhibit M₁ receptor function and muscarinic receptors are thought to be one of the sites of anesthetic action (Durieux, 1996). Several lines of evidence have revealed that M₁ receptors may be the site of action of general anesthetics and that they play an important role in their actions in the CNS (Minami et al., 1997a), suggesting that the inhibition by anesthetics of M₁ receptors leads amnesia and impairment of memory. However, the mechanisms by which tramadol inhibits M₁ receptors have not yet been clarified in detail.

The Xenopus laevis oocyte expression system has been widely used to study a multiplicity of brain receptors with pharmacological properties that mimic those of native brain receptors (Harris et al., 1995). Stimulation of muscarinic M₁ receptors expressed in oocytes activates Ca²⁺-activated Cl⁻ currents (Pritchett et al., 1988); stimulation of M₁ receptors leads to the Gq protein-mediated activation of phospholipase C, which causes the formation of inositol-1,4,5-trisphosphate. The latter releases Ca²⁺ from the endoplasmic reticulum and triggers the opening of endogenous Ca²⁺-activated Cl⁻ channels. This system has been well characterized and has proven useful for studying the effects of drugs acting on Gq protein-coupled receptors.

Adrenal medullary cells are derived from the neural crest and share a number of physiological and pharmacological properties with postganglionic sympathetic neurons. Adrenal medullary cells abundantly express muscarinic receptors, including M₁ receptors (Yamanaka et al., 1986), which elicit cyclic GMP accumulation in cells (Yanagihara et al., 1979). Accordingly, adrenal medullary cells provide a convenient in situ model for studying the effects of anesthetics on muscarinic receptors (Minami et al., 1994).

This study investigated whether tramadol has antimuscarinic effects. To accomplish this, we examined the effects of tramadol on the function of the M₁ muscarinic acetylcholine receptor, by using the X. laevis oocyte expression system and cultured bovine adrenal medullary cells in an in situ experiment.

**Experimental Procedures**

**Materials.** Adult female X. laevis frogs were purchased from Seac Yoshitomi (Fukuoka, Japan). Acetylcholine and atropine were purchased from Sigma (St. Louis, MO). The Escherichia coli transformation kit was from Invitrogen (San Diego, CA). A kit from QIAGEN (Valencia, CA) was used to purify plasmid cDNA. Muscarinic M₁ receptor cDNA was kindly provided by Dr. H. Lester (Caltech, Pasadena, CA) and cRNA for the M₁ receptor was synthesized in vitro with T7 polymerase (Stratagene, La Jolla, CA) from cDNA linearized with HindIII; bisindolylmaleimide I (GF190203X) was from Calbiochem (San Diego, CA); Eagle's minimum essential medium was from Nissui Pharmaceuticals (Tokyo, Japan); fetal calf serum and HEPES were from Nacalai Tesque, (Kyoto, Japan); collagenase was from Nitta Zerachin (Osaka, Japan); and tramadol hydrochloride was a kind gift from Nippon Shinyaku (Kyoto, Japan). The 125I cyclic GMP assay kit was purchased from Yamasa (Chiba, Japan). [3H]Quinuclidinyl benzilate (QNB) (48 Ci/mmol) was from Amersham Pharma Biotech UK, Ltd. (Little Chalfont, Buckinghamshire, UK).

**Whole-Cell Voltage Clamp with X. laevis Oocytes.** Isolation and microinjection of X. laevis oocytes were performed as described by Sanna et al. (1994). X. laevis oocytes were injected with 50 ng of cRNA encoding the M₁ receptor, and electrophysiological recording was performed 2 to 5 days after injection. Oocytes were placed in a 100-μl recording chamber, and perfused with modified Barth’s saline containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 10 mM HEPES, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, 0.91 mM CaCl₂, pH 7.5 adjusted with NaOH, at a rate of 1.8 ml/min at room temperature. Recording electrodes (1–5 MΩ) filled with 3 M KCl were inserted into the animal pole. A Warner oocyte-clamp OC 725-C (Warner, Hampden, CT) was used to voltage clamp each oocyte at −70 mV. We measured the peak of the transient inward currents as the acetylcholine (ACh)-induced currents, because this component is dependent on ACh concentrations and is reproducible, as performed by Minami et al. (1997a). Tramadol was preapplied for 2 min to allow for complete equilibration in the bath. The control responses were measured before and after each drug application to take into account possible shift in the control currents as recording proceeded.

To study whether the inhibitory effects of tramadol on ACh-induced currents are modulated by protein kinase C (PKC), oocytes expressing M₁ receptors were exposed to the PKC inhibitor GF109203X (200 nM) (Toullec et al., 1991) in modified Barth’s saline for 120 min. After exposure to GF109203X, oocytes were exposed to 1 μM ACh and the currents elicited were measured.

AIFc₂⁺ was used as a direct activator of G proteins, and with this system we can bypass the signal to G proteins from activated receptors. Under a two-electrode voltage clamp, we injected 30 nl of solution containing NaF and AlCl₃ into the oocytes by using a pressure injector (PM2000B; Microdata Instruments, South Plainfield, NJ). The concentrations of NaF and AlCl₃ used in this study were 10 mM and 30 μM, respectively (Bigay et al., 1987).

**Isolation and Culture of Adrenal Medullary Cells.** Adrenal medullary cells were isolated by collagenase digestion of slices of bovine adrenal medulla as described previously (Yanagihara et al., 1979). The cells were plated at a density of 4 × 10⁶/dish (35 mm; Falcon, Becton Dickinson, Lincoln Park, NJ) in Eagle’s minimal essential medium containing 10% fetal calf serum, 60 μM aminobenzylpenicillin, 100 μg/ml streptomycin, 0.3 mg/ml amphotericin B, and 3.0 μM cytosine arabinoside. The cells were cultured in 5% CO₂, 95% air in an incubator at 37°C for 4 days of culture.

**Measurement of Cyclic GMP.** Cyclic GMP in the adrenal medullary cells was measured as reported previously (Yanagihara et al., 1979). In brief, cells (4 × 10⁶/dish) were washed four times with 1 ml of 37°C Krebs-Ringer phosphate (KRP) buffer composed of 154 mM NaCl, 0.85 mM Na₂HPO₄, 2.15 mM NaH₂PO₄, 5.6 mM KCl, 2.2 mM CaCl₂, 1.1 mM MgSO₄, and 10 mM glucose, pH 7.4. Cells were preincubated at 37°C for 15 min with 0.3 mM 3-isobutyl-1-methylxanthine (IBMX), and then incubated in medium with or without muscarine (100 μM) for another 5 min in the presence or absence of varying concentrations of tramadol. The reaction medium also contained 0.3 mM IBMX. After aspirating the medium, the cells were rapidly scraped into ice-cold 7% trichloroacetic acid and centrifuged. Cyclic GMP in the supernatant was assayed with a Yamasa cyclic GMP assay kit.

[3H]QNB Binding to Adrenal Medullary Cells. Adrenal medullary cells cultured for 3 days were used for the experiments. The cells were isolated from the dish with 0.05% trypsin (Difco, Detroit, MI) and suspended in KRP buffer at a cell density of 3.0 × 10⁶ cells/100 μl. The cells were incubated for 30 min at 37°C with incubation medium (final volume 500 μl) containing [3H]QNB (0.1–2.0 nM) in the presence or absence of 10 μM tramadol. After incubation, binding was terminated by the addition of 5 ml of ice-cold KRP buffer and rapid filtration of the membrane suspension under vacuum through Whatman GFPC glass-fiber filters (Whatman, Maidstone, UK). The filters were rapidly washed twice with 1 ml of ice-cold KRP buffer and placed in counting vials containing a scintillation cocktail. The radioactivity was counted in an Aloka LSC-
The inhibitory effect of tramadol on M1 receptor function was examined in oocytes expressing cloned M1 muscarinic receptors in the presence of AlF4−. Tramadol (10 nM) inhibited ACh-induced currents in oocytes expressing M1 receptors. A, tracings were obtained from a single oocyte expressing muscarinic M1 receptors with or without tramadol treatment. B, concentration-response relationship of tramadol on ACh-induced currents. The half-maximal inhibitory concentration (IC50) of tramadol for the ACh-induced currents was 3.4 ± 2.3 μM (n = 8). It has been reported that several anesthetics, such as halothane, inhibit M1 receptor function via stimulation of PKC activity (Minami et al., 1997a). Therefore, we examined the effect of tramadol on M1 receptor-stimulated currents with oocytes that had been pretreated with the PKC inhibitor GF109203X, which has a Kd value for inhibiting protein kinase C activity of 20 nM (Toullec et al., 1991). The treatment of oocytes expressing the M1 receptor with 200 nM GF109203X for 120 min enhanced the initial currents induced by 1 μM ACh to 280 ± 44% (Fig. 2A), which was consistent with our previous report (Minami et al., 1997a). The inhibitory effects of tramadol on ACh-induced currents were still observed after pretreatment with GF109203X (Fig. 2B).

We also examined the effect of tramadol on AlF4−-induced Cl− currents in oocytes to further clarify the site of action of tramadol in signal transduction after receptor stimulation, i.e., G protein dissociation, phospholipase C activation, Ca2+ release, or Ca2+-activated Cl− channels. AlF4− binds to GDP on heterotrimeric G protein and GDP-AlF4− complex promotes the dissociation of heterotrimeric G proteins into Ga and Gβγ subunits, which subsequently leads to the activation of G protein (Gilman, 1987). The peak amplitude of AlF4−-induced currents was 350 ± 40 nA (n = 22), and tramadol did not affect the currents (340 ± 50 nA, n = 17) induced by AlF4−.

We next investigated the effects of tramadol on M1 receptor function in cultured bovine adrenal medullary cells that endogenously express M1 receptor (Yamanaka et al., 1986). Muscarine (100 μM) caused an approximately 5-fold increase in cyclic GMP accumulation, as previously reported by Yanagihara et al. (1979). Tramadol inhibited the stimulatory effects of muscarine to 54, 36, and 11% of control at 1, 10, and 100 μM, respectively (Fig. 3) (IC50 = 2.2 μM).

We further examined the effects of tramadol on the binding of [3H]QNB to adrenal medullary cells. Specific binding of [3H]QNB was saturable with increasing concentration of [3H]QNB (0.1–2.0 nM; Fig. 4A). Scatchard analysis showed a single population of binding sites, with an apparent dissociation constant (Kd) of 0.6 ± 0.1 nM and maximal binding (Bmax) of 13.2 ± 1.3 fmol/3.0 × 106 cells (Fig. 4B). The specific binding of [3H]QNB was inhibited by 10 μM tramadol, and this was reversed by increasing the concentration of [3H]QNB (Fig. 4A). From the analysis of the Scatchard plot,
tramadol significantly increased the $K_D$ value of $[^3H]$QNB binding (1.6 ± 0.3 nM) without changing $B_{\text{max}}$ (13.3 ± 2.6 fmol/3.0 × 10⁶ cells) (Fig. 4B). Tramadol concentration dependently inhibited $[^3H]$QNB binding to cells to $87 \pm 3$, $72 \pm 3$, and $32 \pm 3$% of the control value at 1, 10, and 100 μM, respectively (Fig. 5).

**Discussion**

In this study, we demonstrated that tramadol inhibited both the ACh-mediated response of M₁ receptors expressed in X. laevis oocytes and the muscarine-induced accumulation of cyclic GMP in cultured bovine adrenal medullary cells. To our knowledge, this is the first evidence demonstrating that tramadol inhibits the function of muscarinic acetylcholine receptors. According to the report by Lintz et al. (1986), the concentration of tramadol in human serum reaches approximately 600 ng/ml (about 2 μM) after intravenous injection of 100 mg of tramadol, which is the clinical dosage. In the mouse tail-flick test, the plasma concentrations of tramadol for the threshold and maximum effective doses are 0.8 and 10.8 μM, respectively (Friderichs and Becker, 1991). In the present study, tramadol inhibited the ACh-induced Cl⁻ currents with an IC₅₀ of 3.4 ± 2.3 μM. In adrenal medullary cells, tramadol suppressed the muscarine-induced cyclic GMP accumulation to 54 and 36% of control at concentrations of 1 and 10 μM, respectively. From these findings, it is likely that tramadol suppresses the function of muscarinic receptors at clinically relevant concentrations.

The role of brain muscarinic receptors in antinociception and analgesic action has been investigated. Several lines of evidence have shown that muscarinic agonists enhance antinociceptive effects that are blocked by pretreatment with either M₁, M₂, or M₃ muscarinic receptor antagonists, and that M₁ receptors may play a major role in antinociception (Bartolini et al., 1992; Naguib and Yaksh, 1997). Moreover, Ghelardini et al. (2000) reported a loss of muscarinic antinociception by antisense inhibition of M₁ receptors in mice by using the hot-plate test, suggesting that activation of the M₁ receptor subtype may be fundamental for inducing central cholinergic analgesia. These data are not consistent with our findings that a centrally acting analgesic, tramadol, inhibits M₁ muscarinic receptor function. In contrast, inhibition of the muscarinic signaling pathway induced by the reduction of acetylcholine levels, inhibiting its release or administering scopolamine in rat brains, decreases the minimal alveolar concentration of inhaled anesthetics (Zucker, 1991). Ketamine (Durieux, 1995a), halothane (Durieux, 1995b), and isoflurane (Minami et al., 1994) are well known to depress muscarinic receptor function. Thus, the actions of analgesics or anesthetics on muscarinic receptors may be more complex than currently considered (Durieux, 1996), and further studies are needed to define the relationship between antinociception and muscarinic receptor function. Recently, Gomeza et al. (1999) reported that muscarine-induced analgesia is mediated predominantly, but not exclusively, by the M₂ receptor subtype in behavioral experiments by using M₂ knockout mice. Furthermore, a recent article reported an involvement of M₃ receptors of the spinal cord in formalin-induced nociception in mice (Honda et al., 2000). To clear analgesic

**Fig. 3.** Effects of various concentrations of tramadol on muscarine-stimulated cyclic GMP formation in cultured bovine adrenal medullary cells. Cells (4 × 10⁶ cells/dish) were preincubated at 37°C for 15 min with 0.3 mM IBMX and then incubated in medium with or without muscarine (100 μM) for another 5 min in the presence or absence of tramadol (0.1–100 μM). Data represent the mean ± S.E.M. (n = 4). **P < 0.01 and ***P < 0.001.

**Fig. 4.** Saturation and Scatchard analyses of $[^3H]$QNB binding to bovine adrenal medullary cells. A, cells (3 × 10⁶ cells/dish) were incubated for 30 min at 37°C in the presence (●) or absence (○) of 10 μM tramadol with increasing concentrations of $[^3H]$QNB (0.1–2.0 nM). Data shown are the means ± S.E.M. of eight separate experiments performed in duplicate. B, Scatchard analysis of specific $[^3H]$QNB binding. The data are from Fig. 4A.

**Fig. 5.** Effects of tramadol on $[^3H]$QNB binding to bovine adrenal medullary cells. Cells were incubated with $[^3H]$QNB (0.5 nM) and various concentrations of tramadol (100 nM–1 mM) for 30 min at 37°C. The data shown are the means ± S.E.M. *P < 0.05 and ***P < 0.001.
mechanisms of tramadol, it would be interesting to study the effects of tramadol on M₂ or M₃ receptors.

There have been a number of reports that show cyclic GMP accumulation by acetylcholine or muscarine in adrenal medullary cells (Schneider et al., 1979; Yanagihara et al., 1979; Derome et al., 1981; Lemaire et al., 1981). Previously, Yamanaka et al. (1986) characterized muscarinic receptors in bovine adrenal medulla by radioligand binding assay with [³H]QNB. They showed that at least two distinct subtypes of muscarinic receptors exist in the adrenal medullary cells, and these receptors are predominantly composed of M₁ receptors. Because M₁ receptors are reported to couple with Gq type (Caulfield, 1998), in the present study muscarine may stimulate cyclic GMP accumulation via Gq protein in adrenal medulla. On the other hand, other subtypes, such as M₂ (Aguilar et al., 1992), M₃ (Aguilar et al., 1992), or M₄ (Fernando et al., 1991), have been reported to exist in adrenal medullary cells. Although the molecular mechanism of cyclic GMP accumulation by acetylcholine or muscarine has not been well understood, the inhibition by tramadol on cyclic GMP accumulation suggests the anticholinergic effects in vivo. In a clinical situation, tramadol sometimes causes anticholinergic effects such as dry mouth and constipation (Katz, 1996). Northern blot analysis (Maeda et al., 1988) and receptor-specific antibody immunoprecipitation studies (Dörje et al., 1991) demonstrate mainly the presence of M₁ and M₃ receptors in peripheral glandular tissue. These anticholinergic effects of tramadol in clinical treatment suggest that tramadol would inhibit not only M₁ but also other subtypes of muscarinic receptor functions.

This study raised the question of how tramadol inhibits M₁ receptor-mediated responses. There is considerable evidence that PKC plays an important role in the regulation of G protein-coupled receptors (Sakuta et al., 1991; Minami et al., 1997a). We recently reported that halothane, F₃ (1-chloro-1,2,2-trifluorocyclobutane), and ethanol inhibited the function of the 5-hydroxytryptamineₐ receptor (Minami et al., 1997b) as well as that of the M₁ receptor (Minami et al., 1997a) in a PKC-dependent manner. In addition, M₁ receptors are phosphorylated by PKC (Haga et al., 1996). In our experiments, however, GF109203X did not have any effect on the inhibitory effects of tramadol on muscarinic function, suggesting that PKC is not involved in the inhibitory effects of tramadol on M₁ function. Moreover, tramadol had few effects on AIFᵢ -induced currents, suggesting that tramadol does not interfere with the pathway after G protein-coupled signal transduction, such as phospholipase C activation, intracellular Ca²⁺ release, and Ca²⁺-activated Cl⁻ current. From these results, it is likely that the effect of tramadol on the ACh-induced Cl⁻ current is due to direct inhibition of M₁ receptors.

To confirm our hypothesis, we next examined the effects of tramadol on [³H]QNB binding to muscarinic receptors in cultured bovine adrenal medullary cells. Tramadol inhibited the specific binding of [³H]QNB to the cells, and this was reversed by increasing the concentration of [³H]QNB. Scatchard plot analysis of [³H]QNB binding revealed that tramadol increased the Kₐ value without altering the Bₘₐₓ, indicating competitive inhibition. These findings suggest that tramadol shares the binding sites on muscarinic receptors with QNB. Yamanaka et al. (1986) reported that the [³H]QNB binding sites also bind to adenral medulla are able to be displaced with atropine, which binds to ACh binding sites on ACh receptors. From the present findings, tramadol may inhibit M₁ receptor function by interacting with the binding sites of muscarine or ACh. It is of interest to define the region of M₁, responsible for tramadol action by using site-directed mutagenesis and such studies are currently underway in our laboratory.

In conclusion, tramadol at clinically relevant concentrations inhibits M₁ muscarinic receptor function by interfering with the QNB binding sites on the receptor. Our findings help to unveil the pharmacological basis for the better understanding of the neuronal action and anticholinergic effects of tramadol.

References:
Katz WA (1996) Pharmacology and clinical experience with tramadol in osteoarthri-
tis. Drugs 52:39–47.
type 2A receptor-induced currents by n-alcohols and anesthetics. J Pharmacol Exp Ther 281:1136–1143.


Address correspondence to: Koichi Minami, M.D., Ph.D., Department of Anesthesiology, University of Occupational and Environmental Health, School of Medicine, 1-1 Iseigaoka, Yahatanishiku, Kitakyushu 807-8555, Japan. E-mail: kminami@med.uoeh-u.ac.jp