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

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 AIF₄, 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 (Bₘᵦ), 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. 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.

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, whereas cholinesterase inhibitors enhance learning (Fibiger et al., 1991). Furthermore, inhibition of muscarinic receptors leads to sedation or nonrapid eye movement sleep (Durieux, 1996). The therapeutic potential of muscarinic antagonists is complicated 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 M_1 receptors are involved in memory and learning, and striatal M_4 receptors play a key role in the regulation of extrapyramidal motor function (Levey et al., 1991). Furthermore, in recent reviews, many anesthetics inhibit M_1 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 M_1 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 M_1 receptors leads to amnesia and impairment of memory. However, the mechanisms by which tramadol inhibits M_1 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 M_1 receptors expressed in oocytes activates Ca^{2+}-activated Cl^− currents (Pritchett et al., 1988); stimulation of M_1 receptors leads to the G protein-mediated activation of phospholipase C, which causes the formation of inositol-1,4,5-trisphosphate. The latter releases Ca^{2+} from the endoplasmic reticulum and triggers the opening of endogenous Ca^{2+}-activated Cl^− channels. This system has been well characterized and has proven useful for studying the effects of drugs acting on G 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 M_1 receptors (Yamanaka et al., 1986), which elicit pharmacological properties that mimic those of native brain receptors. 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 M_1 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 M_1 receptor cDNA was kindly provided by Dr. H. Lester (Caltech, Pasadena, CA) and cRNA for the M_1 receptor was synthesized in vitro with T7 polymerase (Stratagene, La Jolla, CA) from cDNA linearized with HindIII; bisindolylmaleimide I (GF109203X) 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 Worthington (Freehold, NJ); collagenase type XI was from SIGMA (Munich, FRG); ibuprofen was from Sannat et al. (1994). _X. laevis_ oocytes were injected with 50 ng of cRNA encoding the M_1 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 NaHCO_3, 10 mM HEPES, 0.82 mM MgSO_4, 0.33 mM Ca(NO_3)_2, 0.91 mM CaCl_2, 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 M_1 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.

AlF_4^- 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 AlCl_3 into the oocytes by using a pressure injector (PM2000B; MicroData Instruments, South Plainfield, NJ). The concentrations of NaF and AlCl_3 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 × 10^6/dish (35 mm; Falcon, Becton Dickinson, Lincoln Park, NJ) in Eagle's minimal essential medium containing 10% fetal calf serum, 60 μg/ml aminobenzopyrenicillin, 100 μg/ml streptomycin, 0.3 mg/ml amphotericin B, and 3.0 μM cytosine arabinoside. The cells were cultured in 5% CO_2, 95% air in an incubator at 37°C. The concentrations of NaF and AlCl_3 used in this study were 10 mM and 30 μM, respectively.

**Measurement of Cyclic GMP.** Cyclic GMP in the adrenal medullary cells was measured as reported previously (Yanagihara et al., 1979). In brief, cells (4 × 10^6/dish) were washed four times with 1 ml of 37°C Krebs-Ringer phosphate (KRP) buffer composed of 144 mM NaCl, 0.85 mM NaH_2PO_4, 2.15 mM Na_2HPO_4, 5.6 mM KCl, 2.2 mM CaCl_2, 1.1 mM MgSO_4, 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 × 10^6 cells/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 GF/C glass-fiber filters (Whatman, Maidstone, UK). The filters were rapidly washed twice with 5 ml of ice-cold KRP buffer and placed in counting vials containing a scintillation cocktail. The radioactivity was counted in an Aloka LSC-
3500E counter (Tokyo, Japan). Specific binding of [3H]QNB was defined as the binding inhibited by 100 μM atropine.

**Statistical Analysis.** The results are expressed as percentages of control responses due to variability in the oocyte expression (Figs. 1A and 2A). The control responses were measured before and after drug application. All values are presented as the mean ± S.E.M. 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 performed using either a t test or a one-way analysis of variance.

**Results**

By using methods previously described in the report by Minami et al. (1997a), the effects of tramadol on ACh-induced currents were examined using an ACh concentration of 1 μM. In the X. laevis oocytes expressing cloned M1 muscarinic receptors, 1 μM ACh induced robust Ca2+-activated Cl− currents (1800 ± 300 nA, n = 40) (Fig. 1A). Tramadol inhibited ACh-induced Ca2+-activated Cl− currents to 94 ± 5, 76 ± 3, and 68 ± 6% of control at 10 nM, 100 nM, and 1 μM tramadol, respectively (Fig. 1B). The half-maximal inhibitory concentration (IC50) of tramadol for the 1 μM ACh-induced Cl− 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 K values 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 (Yamana 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 at 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,

**Fig. 1.** Effects of tramadol on M1 receptor-stimulated currents. A, tracings obtained from a single oocyte expressing muscarinic M1 receptors show the effect of tramadol on 1 μM ACh-induced currents. ACh was applied for 20 s with or without 2-min treatment with 10 μM tramadol. B, concentration-response relationship of tramadol on ACh-induced currents. Tramadol (10 nM–100 μM) was applied to the oocytes for 2 min and then 1 μM ACh was applied for 20 s. Data represent the mean ± S.E.M. of 40 oocytes. **P < 0.01 and ***P < 0.001 compared with the control response, by using analysis of variance.

**Fig. 2.** Effects of GF109203X on ACh-induced currents in oocytes expressing M1 receptors. A, tracings were obtained from a single oocyte showing the effect of tramadol on 1 μM ACh-induced currents in oocytes expressing muscarinic M1 receptors before and after treatment with GF109203X. Oocytes were incubated with 200 nM GF109203X for 2 h and were then stimulated by ACh in the presence of tramadol (10 μM). B, effects of tramadol (10 μM) on 1 μM ACh-induced currents with or without GF109203X (200 μM) pretreatment. Values are the mean ± S.E.M. of 15 oocytes.
tramadol significantly increased the $K_d$ value of $[^3H]$QNB binding (1.6 ± 0.3 nM) without changing $B_{max}$ (13.3 ± 2.6 fmol/3.0 × 10^6 cells) (Fig. 4B). Tramadol concentration dependently inhibited $[^3H]$QNB binding to cells at 87 ± 3, 72 ± 3, and 32 ± 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 IC$_{50}$ 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.

Tramadol significantly increased the $K_d$ value of $[^3H]$QNB binding (1.6 ± 0.3 nM) without changing $B_{max}$ (13.3 ± 2.6 fmol/3.0 × 10^6 cells) (Fig. 4B). Tramadol concentration dependently inhibited $[^3H]$QNB binding to cells at 87 ± 3, 72 ± 3, and 32 ± 3% of the control value at 1, 10, and 100 μM, respectively (Fig. 5).
mechanisms of tramadol, it would be interesting to study the effects of tramadol on M₃ or M₂ 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, 1995), 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 an-

GMP accumulation suggests the anticholinergic effects in vivo. In a clinical situation, tramadol sometimes causes an-

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 an-

cholinergic 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, F₂-1-chloro-1,2,2-trifluorocyclobutane, and ethanol inhibited the function of the 5-hydroxytryptamine[IA] 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 receptor function, suggesting that PKC is not involved in the inhibitory effects of tramadol on M₁ function. Moreover, tramadol had few effects on AIF[IA]-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 CI⁻ current. From these results, it is likely that the effect of tramadol on the ACh-induced CI⁻ 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 Kᵦ value without altering the B_max, indicating competitive inhibition. These findings suggest that tramadol shares the binding sites on muscarine receptors with QNB. Yamanaka et al. (1986) reported that the [³H]QNB binding sites are also able to be displaced with atropine, which binds to ACh binding sites on ACh receptors. From the present findings, tramad-

ol 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 under way 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


Minami K, Minami M, and Harris RA (1997b) Inhibition of 5-hydroxytryptamine

at ASPET Journals on November 4, 2017 jpet.aspetjournals.org Downloaded from
type 2A receptor-induced currents by n-alcohols and anesthetics. J Pharmacol Exp Ther 281:1136–1143.


Address correspondence to: Kouichiro 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