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Characterization of nicotinic acetylcholine receptor-mediated noradrenaline release from the isolated rat stomach

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Abstract

We characterized nicotinic acetylcholine receptor-mediated noradrenaline release from the isolated, vascularly perfused rat stomach. The stomach was perfused via the coeliac artery with Krebs–Ringer solution at a constant flow rate of 4 ml per minute. Endogenous noradrenaline released into the perfusate was electrochemically measured using high-performance liquid chromatography. Nicotinic receptor agonists were applied once into the perfusion medium for 2 min and nicotinic receptor antagonists were administered throughout the experiments. The (−)-nicotine (3×10^{-5} M)-induced noradrenaline release was abolished by tetrodotoxin and hexamethonium and partially blocked by dihydro-β-erythroidine (up to 10^{-5} M) (a relatively selective antagonist of α4β2 nicotinic receptors) and abolished by mecamylamine (10^{-5} M) (a relatively selective antagonist of α3β4 nicotinic receptors), but not influenced by α-bungarotoxin (3×10^{-7} M) or α-conotoxin ImI (10^{-6} M) (antagonists of α7 nicotinic receptors). (±)-Epibatidine (3×10^{-7} M) (a very potent, but non-selective agonist) and (−)-cytisine (3×10^{-4} M) (an agonist of β4 nicotinic receptors) effectively evoked the release of noradrenaline, while (*E*)-*N*-methyl-4-(3-pyridinyl)-3-butene-1-amine (RJR-2403) (up to 10^{-4} M) (an agonist of α4β2 nicotinic receptors) had no effect. The potency of these agonists was as followed; (±)-epibatidine ≫ (−)-nicotine > (−)-cytisine > > > RJR-2403. These results are compatible with the published view that α3β4 nicotinic receptors are predominant in other parts of the autonomic nervous system. These receptors (probably located on the gastric sympathetic ganglia) are involved in the release of noradrenaline from the rat stomach.

Keywords: Coeliac ganglion; Nicotine; Noradrenaline release; Stomach, rat; Nicotinic receptor

1. Introduction

In the past decade, it has become evident that there is a great diversity of neuronal nicotinic receptors (Sargent, 1993; McGehee and Role, 1995; McGehee, 1999; Lukas et al., 1999). Molecular biological techniques reveal the presence of at least eight types of α subunit (α2–α9) and three types of β subunit (β2–β4). Three α subunits (α2, α3, and α4) have been shown to form functional, heteromeric receptors in combination with a β subunit (β2 or β4) in a recombinant expression system (Boulter et al., 1987; Wada et al., 1988; Cooper et al., 1991). Three other α subunits (α7, α8, and α9) form functional, homomeric receptors (Schoepfer et al., 1990; Séguéla et al., 1993). By recombinant expression study with specific combinations of these subunits, the relative efficacy and potency of available nicotinic agonists and antagonists have been defined (Brioni et al., 1997; Holladay et al., 1997; Lloyd and Williams, 2000). Based on these observations, the current study attempted to pharmacologically identify the subtypes of native nicotinic receptors involved in physiological and pathophysiological responses.

We had reported previously that intravenously administered (−)-nicotine inhibits vagally stimulated gastric acid output in anesthetized rats by activation of gastric sympathetic nerves and adrenal medulla, since this antisecretory effect was abolished by phentolamine (an antagonist of α-adrenoceptors) or by combined treatment with bilateral adrenalectomy and chemical sympathectomy with reserpine (Yokotani et al., 1986). Released catecholamines activate presynaptic α-adrenoceptors located on the vagus nerve terminals and reduce the release of acetylcholine, thereby inhibiting vagally stimulated gastric acid output (Yokotani et al., 1984, 1993). Recently, we tried to detect

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the (−)-nicotine-induced release of noradrenaline from gastric sympathetic nerves using isolated, vascularly perfused rat stomach (Wang et al., 2000).

In the present study, therefore, we attempted to characterize the nicotinic receptors involved in (−)-nicotine-induced release of noradrenaline, using several kinds of agonists and antagonists of nicotinic receptors.

2. Materials and methods

2.1. Perfusion experiments

Male Wistar rats (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) weighing about 350 g were housed for at least 2 weeks in an air-conditioned room and fasted overnight before the experiments were performed. Details of the experimental procedures were as described elsewhere (Yokotani et al., 1992). Briefly, under urethane anesthesia, the abdomen was opened with a midline incision. After ligation of the abdominal aorta just above where the coeliac artery branches, a cannula was inserted into the coeliac artery via an incision placed on the opposite site of the aorta and modified Krebs–Ringer solution (pH 7.4) bubbled with a mixture of 95% O_2–5% CO_2 maintained at 37°C was perfused at a constant flow rate of 4 ml per minute. Modified Krebs–Ringer solution was composed of 117.5 mM NaCl, 4.7 mM KCl, 2.4 mM $CaCl_2$, 1.1 mM $MgCl_2$, 1.1 mM NaH_2PO_4, 25 mM $NaHCO_3$, 11.1 mM glucose, 0.1% of bovine serum albumin, 10 μM pargyline and 1 μM phentolamine. The tube was inserted into the lumen of the stomach via a pylorus ring to drain the contents of the stomach throughout the experiment. The esophagus, duodenum, spleen and pancreas were dissected after ligation of the vessels, and the vascularly perfused stomach was kept in a chamber prewarmed at 37°C. In this preparation, the coeliac ganglia (the gastric sympathetic ganglia) remain intact (Yokotani et al., 1992). Each 2-min effluent from the portal vein was collected in chilled tubes containing 0.5 ml of 4 N perchloric acid, 2 ng of 3,4-dihydroxybenzylamine as an internal standard, and one drop of 2% sodium pyrosulfite solution.

After an equilibration period of 60 min, the following experiments were done: (1) (−)-nicotine was applied for 2 min in the perfusion medium in the presence or absence of nicotinic receptor antagonists such as hexamethonium, α-bungarotoxin, α-conotoxin ImI, dihydro-β-erythroidine or mecamylamine; (2) nicotinic receptor agonists such as (−)-nicotine, (±)-epibatidine, (*E*)-*N*-methyl-4-(3-pyridinyl)-3-butene-1-amine (RJR-2403) or (−)-cytisine were applied for 2 min in the perfusion medium. Each nicotinic receptor agonist was applied once to each preparation to avoid the appearance of tachyphylaxis after repeated administration.

All experiments were conducted in compliance with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society and the Guidelines for Animal Experiments of the Kochi Medical School.

2.2. Noradrenaline assay in the medium and the stomach

At the end of each experiment, the stomach was homogenized in 20 ml of 0.4 N perchloric acid containing 16.8 mg of disodium EDTA, two drops of 4% sodium pyrosulfite solution and 500 ng of 3,4-dihydroxybenzylamine as an internal standard. The homogenate was centrifuged for 10 min at 14,000 × *g* at 4°C. The supernatant was analyzed to determine the tissue level of noradrenaline.

Catecholamines in the effluent and the supernatant of the tissue homogenate were extracted by the method of Anton and Sayre (1962) with a slight modification, and were assayed electrochemically using high-performance liquid chromatography (Yokotani et al., 1992). Specifically, to each 3 ml of acidified sample or an aliquot (0.1 ml) of supernatant, was added 30 mg of activated alumina. The pH was then adjusted to 8.6 with 3 ml of 1.5 M Tris HCl (pH 8.6) containing 0.1 M disodium EDTA, and then samples were shaken for 10 min. The supernatant was discarded and the alumina was washed three times with double-deionized water, and catecholamines were eluted with 300 μl of 4% of acetic acid containing 0.1 mM disodium EDTA.

The high-performance liquid chromatography-electrochemical detection system consisted of a solvent delivery system (Model 880-PU; Japan Spectroscopic, Tokyo, Japan), a sample processor (Model 851-AS; Japan Spectro-

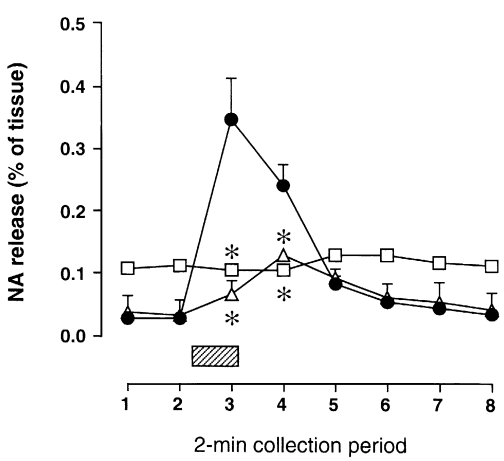

Fig. 1. Effects of hexamethonium and tetrodotoxin on (−)-nicotine-induced release of noradrenaline from the stomach. Hexamethonium (10^{-4} M) or tetrodotoxin (3×10^{-7} M) was administered 10 min before the start of experiment and continued throughout the experiment. (−)-Nicotine (3×10^{-5} M) was applied in the perfusion medium for 2 min (shown as shaded bar). The actual release of noradrenaline is expressed as percent of its tissue content per 2 min. Values are the means ± S.E.M. * Significantly different ($P < 0.05$) from the values of the control treated with (−)-nicotine alone. ●, nicotine alone ($n = 6$); △, hexamethonium plus nicotine ($n = 5$); □, tetrodotoxin plus nicotine ($n = 3$).

scopic), an ODS column (Cosmosil 5C18; Nacalai Tesque, Kyoto, Japan) and an electrochemical detector (Model CB-100; Eicom, Kyoto, Japan) equipped with a graphite electrode. The solvent system consisted of 100 mM KH_2PO_4, 0.02 mM disodium EDTA, 4.5 mM sodium octane sulfonate and 15% methanol. This assay could measure 2 pg of noradrenaline accurately.

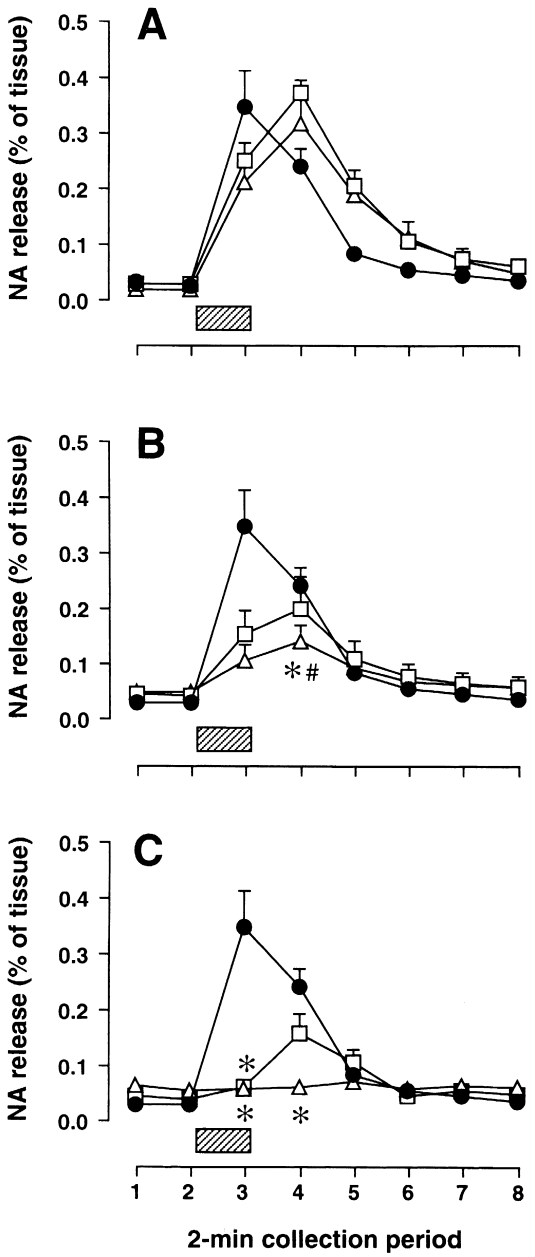

Fig. 2. Effects of α-bungarotoxin, α-conotoxin ImI, dihydro-β-erythroidine and mecamylamine on (−)-nicotine-induced release of noradrenaline from the stomach. These antagonists of nicotinic receptors were administered 10 min before the start of the experiment and continued throughout the experiment. (−)-Nicotine (3×10^{-5} M) was applied in the perfusion medium for 2 min (shown as shaded bar). (A) ●, nicotine alone (cited from Fig. 1); □, 3×10^{-7} M α-bungarotoxin ($n=6$); △, 10^{-6} M α-conotoxin ImI ($n=5$). (B) ●, nicotine alone (cited from Fig. 1); □, 10^{-6} M dihydro-β-erythroidine ($n=4$); △, 10^{-5} M dihydro-β-erythroidine ($n=4$). (C) ●, nicotine alone (cited from Fig. 1); □, 10^{-6} M mecamylamine ($n=4$); △, 10^{-5} M mecamylamine ($n=4$). * Significantly different ($P<0.05$) from the values of the control treated with (−)-nicotine alone. # Significantly different ($P<0.05$) from the values before administration of (−)-nicotine (B). Other conditions were the same as those for Fig. 1.

2.3. Evaluation and statistical analyses

The amount of noradrenaline in each sample was calculated using the peak height ratio relative to that for 3,4-dihydroxybenzylamine, an internal standard. Spontaneous and evoked release of noradrenaline is expressed as a percentage of its tissue content per 2 min. All values are expressed as the means ± S.E.M.

All data were analyzed by repeated-measure analysis of variance (ANOVA), followed by post-hoc analysis with the Bonferroni method for comparing the group treated with (−)-nicotine alone to the group treated with (−)-nicotine plus other reagent in Figs. 1 and 2. Student's *t*-test was used for evaluating the significance of differences between two values [before and after administration of (−)-nicotine] in Fig. 2B. *P* values less than 0.05 were taken to indicate significance.

2.4. Compounds

The following drugs were used: (−)-cytisine, 3,4-dihydroxybenzylamine hydrobromide, hexamethonium chloride, mecamylamine hydrochloride, (−)-nicotine hydrogen tartrate, pargyline hydrochloride, phentolamine hydrochloride (Sigma, St. Louis, MO, USA); α-bungarotoxin, dihydro-β-erythroidine hydrobromide, (±)-epibatidine dihydrochloride, tetrodotoxin (Research Biochemicals International, Natick, MA, USA); RJR-2403 (Tocris Cookson, Ballwin, MO, USA), α-conotoxin ImI (Peptide Institude, Osaka, Japan). All other reagents were of the highest grade available (Nacalai Tesque).

3. Results

3.1. Effects of hexamethonium and tetrodotoxin on (−)-nicotine-induced release of noradrenaline from the isolated stomach

The amount of noradrenaline remaining in the stomach was 752 ± 12 ng ($n=62$). Spontaneous release of noradrenaline was about 0.03% of its tissue content per 2 min. We recently reported that (−)-nicotine (10^{-6}–10^{-4} M) concentration dependently evoked the release of noradrenaline from the isolated rat stomach (Wang et al., 2000). In the present experiments, the maximal release of noradrenaline evoked by 3×10^{-5} M (−)-nicotine was 0.35 ± 0.07% of its tissue content per 2 min ($n=6$) (Fig. 1). After application of this alkaloid, the evoked levels of noradrenaline quickly declined to their basal levels.

Hexamethonium (10^{-4} M) had no effect on the basal release of noradrenaline, but tetrodotoxin (3×10^{-7} M) slightly increased the basal release (Fig. 1). Hexamethonium and tetrodotoxin abolished the release of noradrenaline evoked by (−)-nicotine (3×10^{-5} M).

3.2. Effects of nicotinic receptor antagonists on (−)-nicotine-induced release of noradrenaline from the isolated stomach

The effect of (−)-nicotine was examined in the presence of α-bungarotoxin (3×10^{-7} M) or α-conotoxin ImI (10^{-6} M) (Fig. 2A). The basal release of noradrenaline was not affected by these antagonists of nicotinic receptors. The release of noradrenaline evoked by (−)-nicotine (3×10^{-5} M) was not influenced by these toxins. The maximal release of noradrenaline evoked by this alkaloid was $0.38 \pm 0.02\%$ per 2 min in the presence of α-bungarotoxin ($n = 6$) and $0.32 \pm 0.05\%$ per 2 min in the presence of α-conotoxin ImI ($n = 5$). These values were not significantly different from that of the control group treated with nicotine alone ($0.35 \pm 0.07\%$ per 2 min, $n = 6$).

In the next experiment, the effect of (−)-nicotine (3×10^{-5} M) was examined in the presence of dihydro-β-erythroidine (10^{-6} and 10^{-5} M) (Fig. 2B). The basal release of noradrenaline was not affected by these concentrations of dihydro-β-erythroidine. The release of noradrenaline evoked by (−)-nicotine was reduced, but not blocked by this antagonist at concentrations up to 10^{-5} M. The maximal release of noradrenaline evoked by (−)-nicotine in the presence of 10^{-6} and 10^{-5} M dihydro-β-erythroidine was $0.20 \pm 0.06\%$ ($n = 4$) and $0.14 \pm 0.03\%$ ($n = 4$) per 2 min, respectively. However, these values were still significantly higher than the basal values before the administration of (−)-nicotine.

The effect of (−)-nicotine was also examined in the presence of mecamylamine (10^{-6} and 10^{-5} M) (Fig. 2C). The basal release of noradrenaline was not affected by these concentrations of mecamylamine. The release of noradrenaline evoked by (−)-nicotine (3×10^{-5} M) was effectively reduced by this reagent (10^{-6} and 10^{-5} M). Mecamylamine, at 10^{-5} M, abolished the response evoked by (−)-nicotine. The maximal release of noradrenaline evoked by (−)-nicotine in the presence of 10^{-6} M and 10^{-5} M mecamylamine was $0.16 \pm 0.01\%$ ($n = 4$) and $0.07 \pm 0.01\%$ (n = 4) per 2 min, respectively.

3.3. Effect of nicotinic receptor agonists on the release of noradrenaline from the isolated stomach

(±)-Epibatidine (3×10^{-7} M) markedly increased the release of noradrenaline from the stomach. The maximal release of noradrenaline evoked by this alkaloid was $0.78 \pm 0.03\%$ per 2 min ($n = 4$), a value greater than that of the release evoked by (−)-nicotine (3×10^{-5} M) ($0.35 \pm 0.07\%$ per 2 min, $n = 6$) (Fig. 3A).

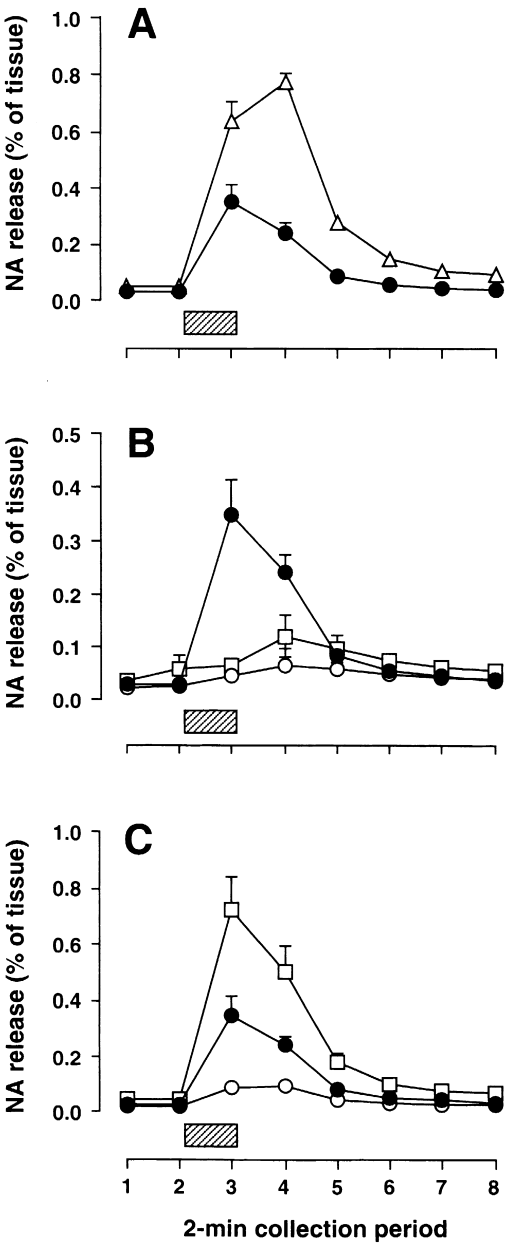

Fig. 3. Effect of (±)-epibatidine, RJR-2403, and (−)-cytisine on the release of noradrenaline from the stomach. These agonists of nicotinic receptors were applied in the perfusion medium for 2 min (shown as shaded bar). (A) ●, 3×10^{-5} M nicotine (cited from Fig. 1); △, 3×10^{-7} M (±)-epibatidine ($n = 4$). (B) ●, 3×10^{-5} M nicotine (cited from Fig. 1); ○, 3×10^{-5} M RJR-2403 ($n = 4$); □, 10^{-4} M RJR-2403 ($n = 4$). (C) ●, 3×10^{-5} M nicotine (cited from Fig. 1); ○, 3×10^{-5} M (−)-cytisine ($n = 4$); □, 3×10^{-4} M (−)-cytisine ($n = 5$). Other conditions were the same as those for Figs. 1 and 2.

RJR-2403 (3×10^{-5} M and 10^{-4} M) had little effect on the release of noradrenaline from the stomach (Fig. 3B). The maximal release of noradrenaline evoked by this reagent was $0.06 \pm 0.03\%$ (3×10^{-5} M, $n = 4$) and $0.12 \pm 0.04\%$ (10^{-4} M, $n = 4$) per 2 min, respectively. These values were markedly smaller than that for nicotine (3×10^{-5} M).

(−)-Cytisine (3×10^{-5} M) had little effect. However, at a higher concentration (3×10^{-4} M), this reagent effectively increased the release of noradrenaline from the stomach (Fig. 3C). The maximal release of noradrenaline evoked by this reagent was $0.10 \pm 0.01\%$ (3×10^{-5} M, $n = 4$) and $0.73 \pm 0.12\%$ per 2 min (3×10^{-4} M, $n = 5$), respectively.

4. Discussion

(−)-Nicotine can evoke the release of noradrenaline from sympathetic neurons by activation of nicotinic receptors located on both sympathetic nerve terminals and their cell bodies (ganglia) (Lindmar et al., 1968; Ikushima et al., 1982). Presynaptic nicotinic receptors have been shown to modulate the release of neurotransmitters in the peripheral and central nervous system (Vizi et al., 1995; Haass and Kübler, 1996). Ganglionic nicotinic receptors have also been reported to be involved in the release of neurotransmitters, because (−)-nicotine-induced release of acetylcholine from the myenteric plexus (Torocsik et al., 1991; Vizi et al., 1995) or release of dopamine from the rat striatum (Marshall et al., 1996) was abolished by tetrodotoxin, a blocker of Na^+ channels (Ritchie, 1979). In the present study, the (−)-nicotine-evoked, hexamethonium-sensitive release of noradrenaline from the isolated rat stomach was attenuated by tetrodotoxin. In this preparation, the gastric sympathetic ganglia are intact, because the release of noradrenaline evoked by electrical stimulation of the preganglionic gastric sympathetic nerve (the greater splanchnic nerve) was attenuated by hexamethonium (Yokotani et al., 1992). Therefore, it seems likely that (−)-nicotine acts on the nicotinic receptors located on the sympathetic ganglia, thereby evoking the release of noradrenaline from sympathetic nerve terminals in the stomach.

Nicotinic synaptic transmission has been demonstrated unequivocally in the peripheral autonomic ganglia such as rat superior cervical ganglia (Chiappinelli and Dryer, 1984) and the chick ciliary ganglia (Chiappinelli, 1983). Experiments carried out with these ganglia have helped define the pharmacology of the nicotinic receptors, which participate in neurotransmission. These ganglia express the same complement of nicotinic genes (α3, α5, α7, β2 and β4 subunit genes) (Corriveau and Berg, 1993; Mandelzys et al., 1995). Nicotinic receptors containing the α4 subunit were also demonstrated in the superior cervical ganglia, using polyclonal and monoclonal antibodies against synthetic peptides matching the sequence of the region (181–192) of α3, α4, α5 and α7 subunits of rat neuronal nicotinic receptors (Skok et al., 1999).

In the present experiments, (−)-nicotine-induced release of noradrenaline from the rat stomach was not influenced by either α-bungarotoxin or α-conotoxin ImI, which are antagonists of the homomeric nicotinic receptor containing α7 subunit (Couturier et al., 1990; Pereira et al., 1996). The nicotinic receptor containing α7 subunit is prominent in autonomic ganglia, however, α-bungarotoxin is unable to block functional responses in these ganglia, suggesting that the α7 subunit-containing receptor is not responsible for mediating ganglionic transmission (Chiappinelli et al., 1981). In the stomach, this subunit-containing receptor seems not to be involved in the (−)-nicotine-induced release of noradrenaline.

The (−)-nicotine-induced release of noradrenaline from the stomach was effectively attenuated by mecamylamine and dihydro-β-erythroidine. The potency of mecamylamine is greater than that of dihydro-β-erythroidine. Oocyte transfection studies indicate that mecamylamine is more effective than dihydro-β-erythroidine at α3β4 subunit-containing receptors and dihydro-β-erythroidine is most effective at α3β2 subunit-containing receptors (Luetje et al., 1991; Alkondon and Albuquerque, 1993; Cachelin and Rust, 1995). Based on these results, our observations favor the involvement of α3β4 subunit-containing receptors in the (−)-nicotine-induced release of noradrenaline from the stomach.

(±)-Epibatidine, an alkaloid isolated from the skin of the Ecuadorian frog, *E. tricoloris* (Spande et al., 1992), is a potent but non-selective agonist of nicotinic receptors. Both isomers of epibatidine have similar functional activity and are full agonists at α4β2, α3β2, α3β4, α7 and α8 subunit-containing nicotinic receptors and at ganglionic nicotinic receptors (Fisher et al., 1994; Sacaan et al., 1996). In the present study, (±)-epibatidine effectively evoked the release of noradrenaline from the stomach. The potency of (±)-epibatidine is greater than that of (−)-nicotine, indicating that (±)-epibatidine is effective against nicotinic receptors located on gastric sympathetic ganglia.

RJR-2403 is a marked selective agonist of nicotinic receptors containing α4β2 subunits, which are dominant in the brain (Bencherif et al., 1996). In the present experiments, RJR-2403 had no effect on the release of noradrenaline from the stomach. This result also suggests that the nicotinic receptor containing α4β2 subunits is not involved in (−)-nicotine-induced release of noradrenaline from the stomach.

(−)-Cytisine is a partial agonist of nicotinic receptors containing the β2 subunit, but a full agonist at nicotinic receptors containing the β4 subunit in oocytes (Luetje and Patrick, 1991; Papke and Heinemann, 1994). In the present study, (−)-cytisine effectively evoked the release of noradrenaline from the stomach, indicating that the activation of nicotinic receptors containing the β4 subunit evokes the release of noradrenaline from gastric sympathetic neurons.

In conclusion, nicotinic receptors containing α3β4 subunits are probably located on the gastric sympathetic ganglia. (−)-Nicotine seems to act on these nicotinic receptors, thereby evoking the release of noradrenaline from sympathetic nerve terminals in the rat stomach.

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