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Nicotine-Induced Noradrenaline Release From the Isolated Rat Stomach by Activation of L- and N-type Calcium Channels

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ABSTRACT—We examined the effect of nicotine on the release of endogenous noradrenaline (NA) from the isolated, vascularly perfused rat stomach. The stomach was perfused via the coeliac artery with Krebs-Ringer solution containing 10 μM pargyline at a constant flow rate of 4 ml per minute. Nicotine was once applied in the perfusion medium for 2 min. Nicotine ($10^{-6} - 10^{-4}$ M) evoked NA release in a concentration-dependent manner. The nicotine (3×10^{-5} M)-evoked NA release was abolished by hexamethonium and tetrodotoxin. Diltiazem and isradipine [blockers of L-type voltage-activated calcium channel (VACC)] and ω-conotoxin GVIA (a blocker of N-type VACC) also abolished this nicotine-evoked NA release. Previously we reported that N-type, but not L-type, VACCs are located on the gastric postganglionic sympathetic nerve terminals, since the NA release evoked by electrical stimulation of periarterial nerves around the left gastric artery (postganglionic sympathetic nerves) was abolished by ω-conotoxin GVIA, but not by diltiazem (Yokotani et al., Jpn. J. Pharmacol. **78**, 75 – 77, 1998). From these results, it was suggested that nicotine activates nicotinic acetylcholine receptors located on the sympathetic ganglia, thereby evoking NA release by activation of L-type VACC located on the gastric sympathetic ganglia and N-type VACC probably located on the sympathetic nerve terminals in the rat stomach.

Keywords: Nicotine, Release of noradrenaline, Rat stomach, L-type voltage-activated calcium channel, N-type voltage-activated calcium channel

The effects of nicotine on the gastric acid output have been extensively studied but the results are confusing: acute administration of nicotine inhibited the gastric acid output (1, 2); chronic administration of this alkaloid produced an increase in acid output (3, 4). These mechanisms of this alkaloid remain obscure. We previously showed that intravenously administered nicotine inhibits vagally stimulated gastric acid output in anesthetized rats (5). Since this nicotine-induced antisecretory effect was abolished by phentolamine (an antagonist of alpha-adrenoceptors) or by combined treatment with bilateral adrenalectomy and chemical sympathectomy with reserpine, it seems likely that the catecholamines released by nicotine from gastric sympathetic neurons and adrenal medulla inhibit vagally stimulated gastric acid output by reducing acetylcholine release from the vagus nerve terminals by activation of presynaptic alpha-adrenoceptors (6, 7).

In the present study, therefore, we tried to observe the nicotine-induced noradrenaline release from gastric sympathetic neurons using the isolated, vascularly perfused rat stomach in vitro.

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MATERIALS AND METHODS

Perfusion experiments

Male Wistar rats (Shizuoka Laboratory Animal Center, Hamamatsu) weighing about 350 g were housed for at least 2 weeks in a conditioned room and fasted overnight before the experiments were performed. Details of the experimental procedures were as described elsewhere (8). 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 min. 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) are intact (8). 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 (DHBA), as an internal standard, and one drop of 2% sodium pyrosulfite solution.

After an equilibration period of 60 min, nicotine was once applied for 2 min in the perfusion medium to avoid the appearance of tachyphylaxis after repeated administration. Test substances such as hexamethonium, tetrodotoxin, diltiazem, isradipine and ω-conotoxin (ω-CTX) GVIA were added in the perfusion medium 14 min before application of nicotine and continued until the end of experiments.

Noradrenaline assay in the medium and 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, 2 drops of 4% sodium pyrosulfite solution and 500 ng of DHBA as an internal standard. The homogenate was centrifuged for 10 min at $14,000 \times g$ at 4°C. The supernatant was analyzed to determine the tissue level of noradrenaline.

Catecholamines in the effluent and the supernatant of tissue homogenate were extracted by the method of Anton and Sayre (9), with a slight modification, and were assayed electrochemically using high-performance liquid chromatography (10). Specifically, to each 4 ml of acidified sample or an aliquot (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 the samples were shaken for 10 min. The supernatant was discarded and alumina was washed three times with double-deionized water, and catecholamines were eluted with 500 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 Co., Ltd., Tokyo), a sample processor (Model 851-AS, Japan Spectroscopic Co., Ltd.), an ODS column (Cosmosil $_5C_{18}$; Nacalai Tesque Inc., Kyoto) and an electrochemical detector (Model CB-100; Eicom, Kyoto) 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. By this assay, 2 pg of noradrenaline could be determined accurately.

Evaluation and statistical analyses

The amount of noradrenaline in each sample was calculated by using the peak height ratio relative to that of DHBA, an internal standard. Spontaneous and evoked release of noradrenaline was expressed as a percentage of its tissue content. All values are expressed as the means ± S.E.M.

All data were analyzed by repeated-measure ANOVA, followed by *post-hoc* analysis with the Bonferroni method for comparing a control with all other means except when only two means were compared. In the latter case, Student's *t*-test was employed for significantly different variations between two groups. *P* values less than 0.05 were taken to indicate significance.

Drugs

The following drugs were used: 3,4-dihydroxybenzylamine hydrobromide, diltiazem hydrochloride, pargyline hydrochloride (Sigma Chemical Co., St. Louis, MO, USA); isradipine (Sandoz Pharma, Ltd., Basle, Switzerland); ω-CTX GVIA (Peptide Institute, Inc., Osaka). All other reagents were the highest grade available (Nacalai Tesque).

RESULTS

Effect of nicotine on noradrenaline release from the isolated stomach

The content of noradrenaline remaining in the stomach was 752 ± 21 ng (n = 38). These values were not influenced by treatments with several types of reagents as described below. Spontaneous release of noradrenaline was about 0.03% of its tissue content per 2 min.

Nicotine (10^{-6} M) had no effect, but higher concentrations of nicotine (10^{-5}, 3×10^{-5} and 10^{-4} M) concentration-dependently evoked the release of noradrenaline. The evoked release of noradrenaline 2 min after application of nicotine was 0.20 ± 0.07% of its tissue content (10^{-5} M nicotine, n = 4), 0.49 ± 0.05% (3×10^{-5} M nicotine, n = 6) and 1.13 ± 0.34% (10^{-4} M nicotine, n = 4), respectively (Fig. 1). After cessation of the nicotine infusion, the evoked levels of noradrenaline quickly declined to the basal levels. In the following experiments, 3×10^{-5} M of nicotine was applied.

Effects of hexamethonium and tetrodotoxin on the release of noradrenaline evoked by nicotine

Hexamethonium (10^{-4} M) had no effect on the basal release of noradrenaline, but tetrodotoxin (3×10^{-7} M) slightly elevated the basal release (Fig. 2). In the presence of hexamethonium or tetrodotoxin, the release of noradrenaline evoked by nicotine (3×10^{-5} M) disappeared. The evoked release of noradrenaline 2 min after applica-

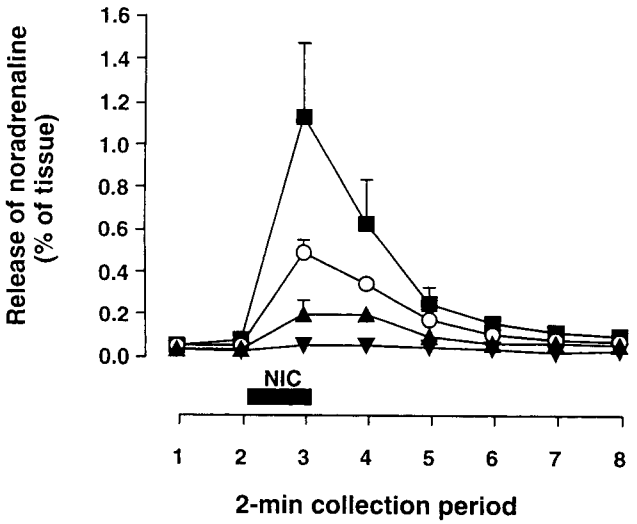

Fig. 1. Effect of nicotine on the release of noradrenaline from the isolated rat stomach. The stomach was perfused via the coeliac artery with modified Krebs-Ringer solution and effluent from the portal vein was collected every 2 min. Nicotine (NIC) (10^{-6}–10^{-4} M) was applied for 2 min in the perfusion medium. Noradrenaline release was expressed as % of its tissue content. Values are the means ± S.E.M. ▼, 10^{-6} M nicotine (n = 4); ▲, 10^{-5} M nicotine (n = 4); ○, 3×10^{-5} M nicotine (n = 6); ■, 10^{-4} M nicotine (n = 4).

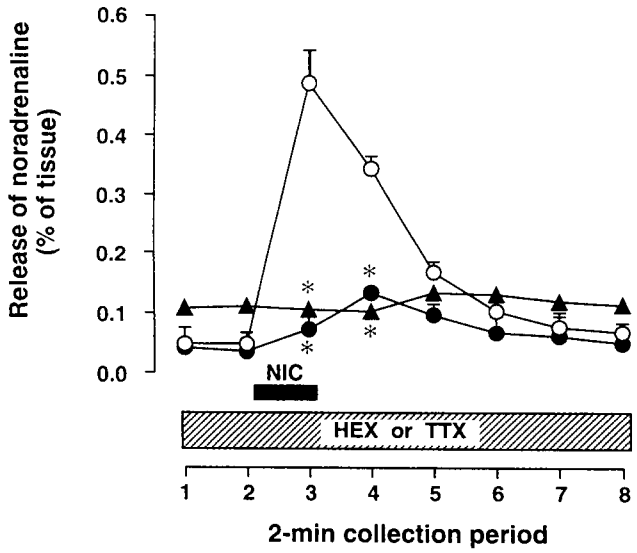

Fig. 2. Effect of hexamethonium or tetrodotoxin on the release of noradrenaline evoked by nicotine. Hexamethonium (HEX) (10^{-4} M) or tetrodotoxin (TTX) (3×10^{-7} M) was administered throughout the experiments. Nicotine (NIC) (3×10^{-5} M) was applied in the perfusion medium for 2 min. *Significantly different ($P<0.05$) from nicotine alone. Other conditions were the same as those for Fig. 1. ○, nicotine alone (cited from Fig. 1); ●, hexamethonium plus nicotine (n = 4); ▲, tetrodotoxin plus nicotine (n = 4).

tion of nicotine was 0.07 ± 0.03% of its tissue content in the hexamethonium-treated experiment (n = 4) and 0.11 ± 0.01% in the tetrodotoxin-treated experiment (n = 4), respectively (Fig. 2). These values were significantly different from that of the control experiment treated with nicotine (3×10^{-5} M) alone (0.49 ± 0.05%, n = 6).

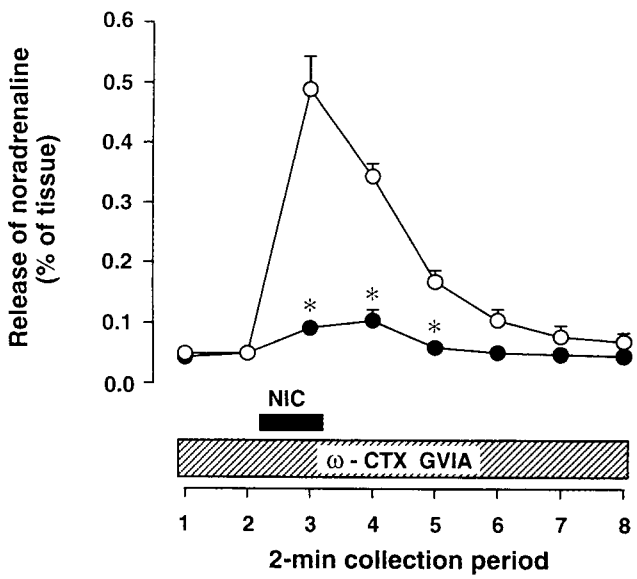

Fig. 3. Effects of ω-conotoxin (ω-CTX) GVIA on the release of noradrenaline evoked by nicotine. ω-CTX GVIA (10^{-8} M) was administered throughout the experiments. Nicotine (NIC) (3×10^{-5} M) was applied in the perfusion medium for 2 min. *Significantly different ($P<0.05$) from nicotine alone. Other conditions were the same as those for Figs. 1 and 2. ○, nicotine alone (cited from Fig. 1); ●, ω-CTX GVIA plus nicotine (n = 4).

Effects of ω-CTX GVIA on the release of noradrenaline evoked by nicotine

ω-CTX GVIA (10^{-8} M) had no effect on the basal release of noradrenaline (Fig. 3). In the presence of ω-CTX GVIA, the release of noradrenaline evoked by nicotine (3×10^{-5} M) disappeared (Fig. 3). The evoked release of noradrenaline 2 min after application of nicotine was 0.09 ± 0.01% of its tissue content in the ω-CTX GVIA-treated experiment (n = 4). This value was significantly different from that of the control experiment treated with nicotine (3×10^{-5} M) alone (0.49 ± 0.05%, n = 6).

Effects of diltiazem and isradipine on the release of noradrenaline evoked by nicotine

Diltiazem (10^{-5} M) had no effect on the basal release of noradrenaline, but isradipine (10^{-5} M) slightly elevated the basal release (Fig. 4). In the presence of diltiazem or isradipine, the evoked release of noradrenaline by nicotine (3×10^{-5} M) disappeared. The evoked release of noradrenaline 2 min after application of nicotine was 0.04 ± 0.01% of its tissue content in the diltiazem-treated experiment (n = 5) and 0.09 ± 0.01% in the isradipine-treated experiment

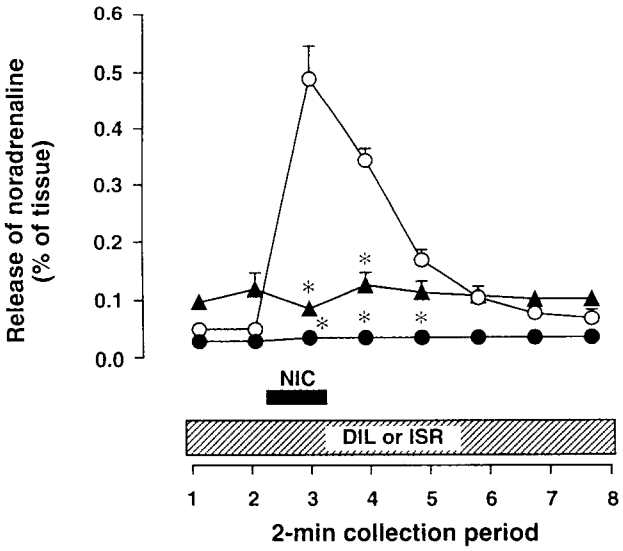

Fig. 4. Effects of diltiazem and isradipine on the release of noradrenaline evoked by nicotine. Diltiazem (DIL) (10^{-5} M) or isradipine (ISR) (10^{-5} M) was administered throughout the experiments. Nicotine (NIC) (3×10^{-5} M) was applied in the perfusion medium for 2 min. *Significantly different ($P<0.05$) from nicotine alone. Other conditions were the same as those for Figs. 1–3. ○, nicotine alone (cited from Fig. 1); ●, diltiazem plus nicotine (n=4); ▲, isradipine plus nicotine (n=4).

(n=4), respectively (Fig. 4). These values were significantly different from that of the control experiment treated with nicotine (3×10^{-5} M) alone ($0.49 \pm 0.06\%$, n=6).

DISCUSSION

In the present study, nicotine evoked the release of noradrenaline from the isolated rat stomach and the evoked release of noradrenaline was attenuated by hexamethonium. These results suggest that nicotine acts on nicotinic acetylcholine receptors (nAChRs) located on the gastric sympathetic neurons.

The nicotine-induced release of noradrenaline was also abolished by tetrodotoxin in the present study. Nicotine can evoke the release of noradrenaline from sympathetic neurons by the activation of nAChRs located on both sympathetic nerve terminals and their cell bodies (ganglia) (11–13). The presynaptic nAChRs have been shown to modulate the release of neurotransmitters in the peripheral and central nervous system (14–16). The ganglionic nAChRs have also been reported to be involved in the release of neurotransmitters because nicotine-induced release of acetylcholine from the myenteric plexus (17) or nicotine-induced release of dopamine from the rat striatum (18) was abolished by tetrodotoxin, a blocker of sodium channels (19). In the present preparation of isolated rat stomach, the gastric sympathetic ganglia are intact because the release of noradrenaline evoked by electrical stimulation of the preganglionic gastric sympathetic nerves was attenuated by hexamethonium (8). Therefore, it seems likely that nicotine acts on nAChRs located on the coeliac ganglia, thereby evoking the release of noradrenaline from the sympathetic nerve terminals.

The nicotine-induced release of noradrenaline was attenuated by ω-CTX GVIA (a blocker of N-type voltage-activated calcium channels). Voltage-activated calcium channels (VACCs) have been subdivided into L-, N-, P- and Q-type. Various polypeptide toxins have subtype-selectivities for neuronal VACCs (20). ω-CTX GVIA-sensitive N-type VACC has been shown to be involved in noradrenaline release from the rat superior cervical ganglia (21), rat isolated kidney (22), rat tail artery (23) and rat mesenteric arteries (24). Using this isolated rat stomach preparation, we also reported that N-type VACC locates on the gastric sympathetic nerve terminals, since the release of noradrenaline evoked by electrical stimulation of postganglionic sympathetic nerves (periarterial nerves around the left gastric artery) was abolished by ω-CTX GVIA (25). Therefore, it seems likely that nicotine evokes the release of noradrenaline by activation of N-type VACC probably located on the gastric sympathetic nerve terminals in the stomach.

The nicotine-induced release of noradrenaline was also abolished by diltiazem (10^{-5} M) and isradipine (10^{-5} M) (blockers of L-type VACC). These results suggest that the nicotine-induced release of noradrenaline is also mediated by the activation of L-type VACC located on the gastric sympathetic neurons. A question arises about where the L-type VACCs are located. Since the evoked release of noradrenaline by electrical stimulation of postganglionic sympathetic nerves was not influenced by diltiazem (10^{-6} and 10^{-5} M) (25), L-type VACCs seem to be located on the gastric sympathetic ganglia rather than sympathetic nerve terminals in the stomach.

Several types of VACC are colocated in sympathetic ganglia (26). In acutely dispersed coeliac-mesenteric ganglia of adult rats, the macroscopic Ca^{2+} current is composed of a small dihydropyridine-sensitive (L-type) current and a large ω-CTX GVIA-sensitive (N-type) current; however, it is not clear which type of VACC may actually be involved in excitation-secretion coupling (27). In apical dendrites of pyramidal cells of rat neocortex, subthreshold excitatory postsynaptic potentials mediated by the activation of glutamate receptors caused an increase in dendritic $[Ca^{2+}]_i$ by activation of VACCs (28). In the present study, therefore, it seems likely that binding of nicotine to ganglionic nAChRs initiates excitatory postsynaptic potentials, thereby activating ganglionic L-type VACCs. Na^+ and Ca^{2+} entries into the sympathetic ganglia through nAChRs and L-type VACCs might initiate sufficient action poten-

tial, thereby activating the release of noradrenaline from sympathetic nerve terminals in the stomach.

In conclusion, nicotine seems to act on the nAChRs located on the sympathetic ganglia. Activation of ganglionic nAChRs leads to the activation of ganglionic L-type VACC, thereby evoking the release of noradrenaline by activation of N-type VACC probably located on the sympathetic nerve terminals in the rat stomach.

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