Functional Studies on Blockade by Neosurugatoxin of Nicotinic Receptors in Nitroxidergic and Sensory Nerve Terminals and Intramural Ganglionic Cells

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ABSTRACT—In isolated canine cerebral and cutaneous arteries and duodenum, effects of neosurugatoxin (NSTX) on the response to nicotine were compared. Nicotine-induced relaxations are mediated by nitric oxide (NO) from vasodilator nerves in cerebral arteries and by calcitonin gene-related peptide (CGRP) in cutaneous arteries treated with NO synthase inhibitors. Duodenal relaxation to nicotine is mediated by NO from inhibitory nerves. Cerebral arterial strips without endothelium responded to nicotine with relaxations that were inhibited by NSTX (3×10^{-10} to 3×10^{-9} M) concentration-dependently. Relaxations to nicotine of duodenal strips were attenuated by NSTX and abolished by tetrodotoxin, whereas those induced by K^+ and electrical stimulation were not influenced. In cutaneous arteries treated with N^G -nitro-L-arginine, nicotine-induced relaxations were attenuated by NSTX as low as 10^{-10} M, but unaffected by tetrodotoxin. The inhibitory potency of NSTX was in the order of cutaneous artery > duodenum > cerebral artery. It is concluded that NSTX selectively antagonizes actions on nicotinic receptors in nitroxidergic nerves of cerebral arteries, CGRP-mediated sensory nerves of cutaneous artery and ganglionic cells of the duodenum, but the affinity of this toxin to nicotinic receptors in sensory nerves is the highest.

Keywords: Nicotine, Neosurugatoxin, Nitric oxide-mediated nerve, Sensory nerve, Duodenal ganglion

Nicotine is recognized to be a substance that stimulates the nicotinic receptor responsible for the release of neurotransmitters in the central and peripheral nervous system. In peripheral nerves, this receptor is located not only on ganglionic nerve cells but also on nerve terminals (1, 2). Isolated arteries and veins without ganglia respond to nicotine with contraction, relaxation or release of neurotransmitters. The responses are sensitive to hexamethonium (1, 3) or other ganglionic blocking agents, but resistant to tetrodotoxin (4). The mechanical and biochemical responses are identical to those obtained with electrical nerve stimulation, except for the fact that the electrical stimulation-induced responses are mediated by nerve action potentials that are abolished by tetrodotoxin (5). Nicotine generates action potentials possibly by opening Na^+ channels when the receptor is stimulated in ganglionic nerve cells, whereas nicotine is expected to open receptor-operated Ca^{2+} channels for the release of transmitter in nerve terminals. However, the functional characteristics of the nicotinic receptors in nerve cells and neurons have not been investigated.

Isolated cerebral arteries relax in response to nicotine that stimulates vasodilator nerve terminals to liberate nitric oxide (NO) as a neurotransmitter (6–8). Tetrodotoxin does not interfere with the response to nerve stimulation by nicotine, suggesting the mediation of nicotinic receptors in nerve terminals (5). Our recent publication suggests that nicotine-induced relaxation of isolated canine cutaneous arteries is mediated not only by NO but also calcitonin gene-related peptide (CGRP) (9), which is considered to be a neurotransmitter of sensory nerves (10). Canine duodenal longitudinal muscle treated with atropine responds to nicotine and K^+ with relaxation that is abolished by NO synthase inhibitors and restored by L-arginine (11). This relaxation to nicotine seems to be due to stimulation of receptors located in intramural ganglionic cells and generation of nerve action potentials because tetrodotoxin abolishes the response (11).

The present study was undertaken to determine the effects of neosurugatoxin (NSTX), a selective nicotinic receptor antagonist (12), on relaxations induced by nicotine in isolated canine cerebral and cutaneous arteries and

duodenal muscle and to compare the functional natures of nicotinic receptors located in nitroxi-dergic nerve terminals, sensory nerve terminals and ganglionic cell bodies. We elucidated whether different subtypes of nicotinic receptors are involved in the nerve cells and fibers tested.

MATERIALS AND METHODS

The Animal Care and Use Committee at Shiga University of Medical Science approved the use of dog materials along with the experimental protocol in this study. Mongrel dogs of both sexes, weighing 7 to 14 kg, were anesthetized with intravenous injections of sodium thiopental (30 mg/kg) and then killed by bleeding from the common carotid arteries. The brain and pieces of abdominal skin together with subcutaneous tissues (9) were removed. Basilar and middle cerebral arteries (0.3- to 0.5-mm internal diameter) and cutaneous arteries (0.2–0.4 mm) were isolated. The arteries were helically cut into strips, approximately 20-mm-long. The endothelium was removed by gently rubbing the intimal surface with a cotton ball, except for some cerebral arterial strips that were carefully prepared to preserve the endothelium. Removal of the endothelium was verified by abolishment of relaxations induced by substance P (10^{-8} M) for cerebral arterial strips or by acetylcholine (10^{-6} M) for cutaneous arterial strips. On the other hand, the duodenum of 2nd to 3rd portion was also removed from the sacrificed dogs, and longitudinal muscle strips with a length of approximately 10 mm were prepared (13). The specimens were vertically fixed between hooks in a muscle bath (20-ml capacity) containing the modified Ringer-Locke solution, which was aerated with a mixture of 95% O_2 and 5% CO_2 and maintained at $37 \pm 0.3^\circ C$. The hook anchoring the upper end of the arterial and duodenal strips was connected to the lever of a force-displacement transducer (Nihon Kohden Kogyo Co., Tokyo). The resting tensions were adjusted to 1.5 g for cerebral arteries, 0.7 g for cutaneous arteries and 1.5 g for duodenum, which were optimal for inducing maximal contractions (9, 13, 14). Constituents of the solution were as follows: 120 mM NaCl, 5.4 mM KCl, 2.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 25.0 mM $NaHCO_3$ and 5.6 mM dextrose; the pH of the solution was 7.36 to 7.42. Before the start of experiments, all of the strips were equilibrated in the bathing media for 60 to 90 min, during which time the solution was replaced every 10 to 15 min.

Mechanical responses were displayed on an ink-writing oscillograph. The contractile response to 30 mM K^+ (cerebral and cutaneous arteries) or 5 mM Ba^{2+} (duodenum) was first obtained, and then the strips were washed three times with fresh media and equilibrated for 30 to 40 min. To examine relaxant responses to agonists, the ar-

terial strips were partially contracted with prostaglandin (PG) $F_{2\alpha}$ (10^{-7} to 10^{-6} M), and duodenal strips were contracted with bradykinin (5×10^{-9} to 2×10^{-8} M), the contractions being in a range between 30% and 45% of the K^+ (30 mM)-induced contraction for arteries and between 45% and 60% of the Ba^{2+} (5 mM)-induced contraction for duodenal strips. Effects of single concentrations of nicotine (10^{-5} or 10^{-4} M), K^+ (10 mM) for duodenal muscle and NO (10^{-7} M for cerebral artery, 10^{-6} M for cutaneous artery and 10^{-4} M for duodenal muscle; acidified $NaNO_2$ solution) were determined by direct application to the bathing media. Reasons for the use of different concentrations of nicotine (10^{-4} M for arteries and 10^{-5} M for duodenum) were that these concentrations produced submaximal and comparable relaxations which were reproduced by repeated applications (1, 9, 11), and the duodenal response to the higher concentrations of nicotine was not abolished by hexamethonium. To exclude the possible effects of acetylcholine released by nicotine and K^+ in the duodenum and those of PGs and norepinephrine by nicotine in the arteries, the strips were treated with 10^{-7} M atropine (for duodenal strips) or 10^{-6} M indomethacin and/or 10^{-5} M prazosin (for arterial strips). In some experiments, cerebral arterial and duodenal strips were placed between stimulating electrodes to electrically stimulate nerve terminals. Square pulses of 0.2-msec duration with supramaximal intensity at 5 Hz for 40 sec (cerebral artery) or 10 Hz for 20 sec (duodenum), which have been determined to be optimal for consistent relaxant responses (4, 15), were delivered by an electronic stimulator (Nihon Kohden Kogyo Co.). At the end of each series of experiments, papaverine (10^{-4} M) was applied to attain the maximal relaxation. Relaxant responses to agonists and transmural electrical stimulation were expressed as relative values to the papaverine-induced relaxation.

The results shown in the text and figures are expressed as mean values $\pm$ S.E. Statistical analyses were made with the Student's unpaired *t*-test for two groups and the Tukey's test after one-way analysis of variance for three or more groups. Drugs used were nicotine (base) (Kanto, Tokyo); indomethacin and α -bungarotoxin (Sigma, St. Louis, MO, USA); N^G -nitro-L-arginine (L-NA); bradykinin, substance P, calcitonin gene-related peptide (CGRP) (Peptide Institute, Minoh); atropine sulfate (Tanabe, Osaka); prazosin hydrochloride (Wako, Osaka); acetylcholine chloride (Daiichi Pharmaceutical, Tokyo); tetrodotoxin (Sankyo, Tokyo); hexamethonium bromide (Nacalai Tesque, Kyoto); prostaglandin (PG) $F_{2\alpha}$ (Pharmacia Upjohn, Tokyo); and papaverine hydrochloride (Dainippon, Osaka). Neosurugatoxin (NSTX) was prepared by the method described in the previous report (16). Responses to NO were obtained by adding $NaNO_2$ solu-

tion adjusted to pH 2 (17), and the concentrations in the bathing media were expressed as those of the NaNO_2 solution.

RESULTS

Cerebral artery

Cerebral arterial strips without the endothelium were partially contracted with $\text{PGF}_{2\alpha}$ and treated with 10^{-6} M indomethacin. The addition of nicotine in a concentration of 10^{-4} M was sufficient to produce submaximal and reproducible relaxation (1) or transmural electrical stimulation at 5 Hz was sufficient to produce relaxations (4). The nicotine-induced relaxation was abolished by 10^{-5} M hexamethonium, and the response to electrical stimulation was abolished by tetrodotoxin (3×10^{-7} M), suggesting the involvement of vasodilator nerve stimulation. Treatment with NSTX in concentrations of 3×10^{-10} to 3×10^{-9} M inhibited the response to nicotine in a concentration-dependent manner (Fig. 1). The toxin in a concentration of 10^{-10} M did not attenuate the response ($n=5$). Similar results were also obtained in endothelium-intact strips ($n=3$), in which NSTX (3×10^{-9} M) abolished the nicotine-induced relaxation but did not affect the endothelium-dependent relaxation by 10^{-8} M substance P. Typical tracings of the response are illustrated in Fig. 2. Repeated washing of the strips with drug-free media completely reversed the effect of NSTX on nicotine-induced relaxations (Figs. 1 and 2). On the other hand, the relaxation induced by NO (10^{-7} M) was not influenced by high concentrations of NSTX, as shown in Table 1. Relaxations induced by electrical stimulation

Fig. 1. Effect of NSTX (neosurugatoxin) on the relaxant response to nicotine (10^{-4} M) in dog cerebral arterial strips treated with indomethacin (IM, 10^{-6} M) and precontracted with $\text{PGF}_{2\alpha}$. Relaxations induced by 10^{-4} M papaverine were taken as 100%. Numbers in parentheses indicate the number of strips from separate dogs. Significantly different from the control (C), $^aP < 0.01$; significantly different from the value with 3×10^{-10} M NSTX, $^bP < 0.05$ and $^cP < 0.01$, respectively; significantly different from the value with 10^{-9} M NSTX, $^dP < 0.01$, and from those with 3×10^{-9} M NSTX, $^eP < 0.01$ (Tukey's method). Vertical bars, S.E. 'W' denotes the responses after washout of NSTX.

Fig. 2. Typical tracings of the responses to nicotine (N, 10^{-4} M), substance P (SP, 10^{-8} M) and NO (10^{-7} M) of a dog middle cerebral arterial strip with the intact endothelium treated with indomethacin (IM, 10^{-6} M) before and after treatment with neosurugatoxin (NSTX, 3×10^{-10} and 3×10^{-9} M) and after washout of NSTX. Papaverine (PA, 10^{-4} M) was used to attain the maximal relaxation. The strip was precontracted with 10^{-7} M $\text{PGF}_{2\alpha}$.

($10.9 \pm 2.1\%$) were not affected by treatment with 10^{-8} M NSTX ($11.6 \pm 2.6\%$, $n=5$, $P>0.05$, unpaired t -test). Treatment with α -bungarotoxin (10^{-7} M) did not significantly change the response to nicotine (Table 1).

Duodenum

In isolated duodenal strips contracted with bradykinin and treated with 10^{-7} M atropine, nicotine (10^{-5} M) (Table 1), K^+ (10 mM) ($n=4$) and transmural electrical stimulation (5 Hz) ($n=6$) produced transient relaxations, which were abolished by treatment with 3×10^{-7} M tetrodotoxin. The response to nicotine was also abolished by hexamethonium (10^{-5} M). NSTX (3×10^{-10} to 3×10^{-9} M) concentration-dependently inhibited the relaxation elicited by nicotine (Fig. 3), but did not alter the relaxations caused by K^+ (Table 1). NSTX at 10^{-10} M was without effect ($n=3$). NO-induced relaxations were also unaffected by NSTX (Table 1). Typical results are shown in Fig. 4. Electrical stimulation-induced relaxations ($40.8 \pm 5.4\%$, $n=6$) were not significantly influenced by 10^{-8} M NSTX ($41.4 \pm 5.4\%$, $P>0.05$, unpaired t -test). The nicotine-induced relaxation was not inhibited by 10^{-7} M α -bungarotoxin (Table 1).

Cutaneous artery

Our previous study indicates that nicotine-induced relaxations are mediated not only by NO but also CGRP derived from vasodilator nerves (9). To determine the

Fig. 3. Effect of NSTX (neosurugatoxin) on the relaxant response to nicotine (10^{-4} M) in dog duodenal muscle strips treated with atropine (10^{-7} M) and precontracted with bradykinin. Relaxations induced by 10^{-4} M papaverine were taken as 100%. Numbers in parentheses indicate the number of strips from separate dogs. Significantly different from the control (C), ^a $P<0.01$; significantly different from the value with 3×10^{-10} M NSTX, ^b $P<0.05$ and ^c $P<0.01$, respectively; significantly different from the value with 10^{-9} M NSTX, ^d $P<0.05$, and from those with 3×10^{-9} M NSTX, ^e $P<0.01$ (Tukey's method). Vertical bars, S.E. 'W' denotes the responses after washout of NSTX.

Table 1. Modifications by antagonists of relaxation induced by agonists in cerebral arteries, duodenal strips and cutaneous arteries

Materials	Agonist/Antagonist	n	†Relaxation (%)	
			Control	Experimental
cerebral artery	nicotine/ α BTX	7	29.7 ± 4.5	31.2 ± 5.5
	NO/NSTX	7	45.4 ± 5.1	40.4 ± 5.8
duodenum	nicotine/ α BTX	4	59.8 ± 12.6	65.0 ± 7.7
	nicotine/TTX	4	59.8 ± 12.6	$0.0 \pm 0.0^*$
	NO/NSTX	6	54.9 ± 6.8	58.9 ± 5.7
	K^+ /NSTX	6	71.2 ± 7.6	76.2 ± 9.2
cutaneous artery	nicotine/ α BTX	3	59.5 ± 22.1	54.2 ± 20.7
	nicotine/ C_6	6	42.5 ± 11.9	$0.0 \pm 0.0^*$
	NO/NSTX	5	75.8 ± 3.6	71.9 ± 2.7
	CGRP/NSTX	5	35.5 ± 5.2	34.5 ± 4.2

Nicotine: 10^{-4} M nicotine for cerebral and cutaneous arteries and 10^{-5} M nicotine for duodenum. NO: 10^{-7} M for cerebral artery, 10^{-4} M for duodenum and 10^{-6} M for cutaneous artery. K^+ : 10 mM K^+ . α BTX: 10^{-7} M α -bungarotoxin. NSTX: 3×10^{-9} M neosurugatoxin. C_6 , 10^{-5} M hexamethonium. CGRP: 3×10^{-10} M calcitonin gene-related peptide. *Significantly different from the control ($P<0.001$, unpaired t -test). Cerebral artery, treated with 10^{-6} M indomethacin; duodenum, treated with 10^{-7} M atropine; cutaneous artery, treated with 10^{-6} M indomethacin, 10^{-5} M prazosin and 10^{-4} M N^G -nitro-L-arginine. †Relaxation induced by 10^{-4} M papaverine was taken as 100%.

effect of NSTX on CGRP-mediated nerve functions, the cutaneous arterial strips were treated with high concentrations of L-NA (10^{-4} M), prazosin (10^{-5} M) and indomethacin (10^{-6} M). In the endothelium-denuded strips thus treated and contracted with $\text{PGF}_{2\alpha}$, nicotine (10^{-4} M) produced a moderate relaxation that was abolished by hexamethonium (Table 1), but not influenced by tetrodotoxin (3×10^{-7} M) (Fig. 5), as was the response of cerebral arterial strips. Treatment with NSTX (10^{-10} to 3×10^{-9} M) reduced the response in a concentration-dependent manner (Fig. 5). The NO (10^{-6} M)-induced

relaxation was not altered by NSTX (Table 1). The relaxations induced by exogenously-applied CGRP (3×10^{-10} M) were also unaffected by 3×10^{-9} M NSTX (Table 1). The nicotine-induced relaxation was not influenced by α -bungarotoxin (10^{-7} M) (Table 1).

Comparison of inhibitory effect of NSTX in various tissues

Mean values of the relaxation induced by nicotine in the cerebral and cutaneous arteries and duodenal strips were $32.3 \pm 3.4\%$ ($n=6$), $47.4 \pm 6.6\%$ ($n=7$) and

DOG DUODENUM (Atropine-treated)

Fig. 4. Typical tracings of the responses to nicotine (N, 10^{-5} M), K^+ (10 mM) and NO (10^{-4} M) of a dog duodenal muscle strip treated with atropine (10^{-7} M) before and after treatment with neosurugatoxin (NSTX, 3×10^{-10} and 10^{-9} M). Papaverine (PA, 10^{-4} M) was used to attain the maximal relaxation. The strip was precontracted with 10^{-8} M bradykinin.

DOG CUTANEOUS ARTERY (IM + PZ + L-NA-treated)

Fig. 5. Effects of NSTX (neosurugatoxin) and TTX (tetrodotoxin, 3×10^{-7} M) on the relaxant response to nicotine (10^{-4} M) in dog cutaneous arterial strips treated with indomethacin (IM, 10^{-6} M), prazosin (PZ, 10^{-5} M) and N^{G} -nitro-L-arginine (L-NA, 10^{-4} M) and precontracted with $\text{PGF}_{2\alpha}$. Relaxations induced by 10^{-4} M papaverine were taken as 100%. Numbers in parentheses indicate the number of strips from separate dogs. Significantly different from the control (C), $^aP < 0.01$; significantly different from the value with 10^{-10} M NSTX, $^bP < 0.05$ and $^cP < 0.01$, respectively (Tukey's method). Vertical bars, S.E.

Fig. 6. Concentration-inhibition curves of NSTX in the relaxation caused by nicotine (10^{-4} M for arteries and 10^{-5} M for duodenum) in the cerebral artery (●), duodenum (○) and cutaneous artery (□). Numbers in parentheses indicate the number of strips from separate dogs. Vertical bars, S.E.

$30.5 \pm 4.1\%$ ($n=7$), respectively, the differences being statistically insignificant.

Concentration-inhibition curves of NSTX in the cerebral artery, duodenum and cutaneous artery are compared in Fig. 6. IC_{50} values of NSTX in these tissues were $[7.8 \pm 0.8]$ ($n=6$), $[5.6 \pm 0.5]$ ($n=7$) and $[2.4 \pm 0.3] \times 10^{-10}$ M ($n=7$), respectively. Differences in the values are statistically significant in the cutaneous artery vs duodenum and cerebral artery ($P < 0.01$, Tukey's method).

DISCUSSION

The present study revealed that NSTX, a selective blocker of neuronal nicotinic receptor (12), concentration-dependently inhibited the relaxant response to nicotine acting on nitroxidergic nerve terminals innervating the canine cerebral artery, sensory nerve terminals present in the cutaneous arterial wall and intramural ganglion cells in duodenal longitudinal muscle. However, the relaxations induced by transmural electrical stimulation (cerebral and duodenum), NO (all three tissues) and K^+ (duodenum) were unaffected by the nicotinic receptor blocking toxin. On the other hand, α -bungarotoxin in a high concentration (10^{-7} M) sufficient to completely block the nicotinic receptor in chick skeletal muscle (18) did not inhibit the inhibitory response to nicotine in the tissues used. α -Bungarotoxin was first found as a selective inhibitor of muscle-type nicotinic receptors (19, 20).

However, recent studies have demonstrated that this toxin has the ability to bind to nicotinic receptors in brain neurons and motoneurons (21). Possible inhibition by α -bungarotoxin of Ca^{2+} entry into motoneurons is suggested (21). Zamudio et al. (18) have postulated the presence of muscle and central neural types of α -bungarotoxin-sensitive nicotinic receptors. As far as the concentration used is concerned, this toxin does not seem to interfere with binding of nicotine to neuronal receptors and with Ca^{2+} influx upon nerve excitation in the tissues used.

Potencies of NSTX in inhibiting the response to nicotine appreciably differ in the cerebral and cutaneous arteries and duodenum strips. Sensory nerve terminals in the cutaneous artery were most sensitive to the toxin, and the sensitivity of nitroxidergic nerve terminals in the cerebral artery was least. The concentrations of nicotine could not be matched in these tissues; the reason for the use of different concentrations of nicotine (10^{-4} M for arteries and 10^{-5} M for duodenum) was that these concentrations produced submaximal and comparable relaxations. Average relaxations to nicotine in the cutaneous and cerebral arteries and the duodenum did not significantly differ (unpaired *t*-test). Therefore, on the basis of NSTX efficacy in these tissues, different subtypes of nicotinic receptors might be postulated.

Nicotine-induced relaxations of isolated canine cutaneous arteries, resistant to NO synthase inhibition, are abolished by treatment with capsaicin or a CGRP receptor antagonist and in the strips made unresponsive to CGRP due to tachyphylaxis, indicating that CGRP would be a relaxing factor derived from nerves (9). CGRP is recognized to be one of transmitters or mediators in sensory nerves (10). In antidromic vasodilatation, like the axon reflex, and neurogenic inflammation, chemical mediators released from sensory nerve terminals, including CGRP, are involved (22, 23). Although nicotine is expected to liberate CGRP from the nerve innervating the cutaneous artery, it has not been determined whether the generation of nerve action potentials participates in the transmitter release. The present study demonstrated that tetrodotoxin did not inhibit the response to nicotine of cutaneous arteries, in contrast to duodenum, suggesting no contribution of impulse conduction along the nerve. Therefore, in sensory as well as autonomic nerve terminals, nicotine appears to stimulate nicotinic receptors responsible for opening of Ca^{2+} channels, resulting in the release of neurotransmitters. Jancso et al. (24) have reported that a wheal response, but not flare, induced by capsaicin, a chemical irritant, in human skin is not abolished by local anesthesia, although the presence of functional nerves is required, and have suggested that the response is not dependent upon neuronal conduction.

This means that the substance(s) responsible for localized increase in vascular permeability, but not widespread arteriolar dilatation, is involved in the response to capsaicin. Therefore, CGRP, a potent vasodilator, is not expected to be the substance liberated by capsaicin. Whether substances, including nicotinic receptor stimulants if any, locally liberated in association with acute inflammation possess the ability to stimulate sensory nerve endings and release different vasodilator mediators remains unanswered.

In summary, NSTX was effective in attenuating nicotine actions on nitroxidergic and sensory nerve terminals and ganglionic cells, and the inhibitory potency was greater in the sensory nerve innervating cutaneous arteries than in the other tissues used. Stimulation of nicotinic receptors in the sensory nerve does not appear to generate nerve action potentials but liberates CGRP for vasodilatation, possibly due to receptor-coupled channels responsible for the Ca^{2+} entry.

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