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Analysis of the vasodilator nerve function by nicotine in isolated dog skin artery

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Abstract

Mechanisms underlying the relaxation induced by nicotine were analyzed in cutaneous arterial strips isolated from dogs and with the endothelium removed. In the strips treated with prazosin and precontracted with prostaglandin $F_{2\alpha}$, nicotine produced relaxations which were not influenced by atropine but abolished by hexamethonium. Relaxations induced by nicotine were partially inhibited by N^G-nitro-L-arginine (L-NA), a nitric oxide (NO) synthase inhibitor; the remaining relaxations were abolished by desensitization to calcitonin gene-related peptide (CGRP) or treatment with CGRP-(8–37), a CGRP receptor antagonist, or with capsaicin. Desensitization to vasoactive intestinal polypeptide (VIP) or a VIP receptor antagonist did not influence the nicotine-induced relaxation. In the strips desensitized to CGRP, the nicotine-induced relaxation was abolished by L-NA; the inhibitory effect was reversed by L-arginine. Perivascular nerves containing NADPH diaphorase and CGRP immunoreactivity were histochemically identified in the cutaneous artery. CGRP immunoreactivity was abolished by treatment with capsaicin. It is concluded that nicotine produces relaxation in dog cutaneous arterial strips, possibly mediated by NO and CGRP liberated from vasodilator nerves.

Keywords: Nitric oxide (NO); CGRP (calcitonin gene-related peptide); Nitric oxide (NO) synthase containing nerve; Vasodilation

1. Introduction

Cutaneous arterial tone, vascular resistance and blood flow can be regulated by sympathetic efferent discharges from the vasomotor center; vasodilatation can be induced by the reduction of sympathetic tonic influences. Parasympathetic nerve stimulation does not cause vasodilatation, since it is highly unlikely that neurogenic acetylcholine crosses the medial layer and reaches the endothelium to release nitric oxide (NO). However, the stimulation of parasympathetic nerves elicits arterial and venous constriction (Yoshioka et al., 1988; Kalsner and Quillan, 1989). Our recent studies have provided evidence for the presence of vasodilator nerves in dog, monkey and human peripheral arteries, in which NO acts as a neurotransmitter (Toda and Okamura, 1990, 1992; Toda, 1993). In human cutaneous arteries, nerve fibers containing peptides such as calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP) and substance P, have been histochemically demonstrated (Weihe and Hartschuh, 1988). CGRP is reported to be a major neurotransmitter when trigeminal ganglia are electrically stimulated in rat facial skin (Escott et al., 1995). In the rabbit skin, CGRP is an important vasodilator substance released from capsaicin-sensitive sensory nerves and NO is implicated in this release process (Hughes and Brain, 1994). NO contributes to the activation of afferent nerve fibers and to the release of neurotransmitters, possibly CGRP, in the rat (Holzer and Jocič, 1994). In humans, parasympathetic nerve stimulation produces cutaneous vasodilatation through the release of unknown neurotransmitter(s) by a mechanism that is not acetylcholine-dependent (Kellogg et al., 1995).

Therefore, the present study was undertaken to demonstrate functionally and histologically the presence of vasodilator nerves in the isolated dog cutaneous arterial strips denuded of the endothelium, and to analyze the mechanism of neurogenic vasodilatation in reference to NO, CGRP and VIP. The involvement of substance P was not examined, because the substance P-induced relaxation was obtained only when the endothelium was retained (unpublished data). Nicotine was used to stimulate perivascular nerves, because this agent was demonstrated to release

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neurotransmitters as does transmural electrical stimulation in blood vessels (Su, 1982; Toda, 1982; Nedergaard, 1988).

2. Materials and methods

2.1. Organ-bath studies

The Animal Care and Use Committee at Shiga University of Medical Science approved the whole experimental protocol in this study. Mongrel dogs of either sex, weighing 6–15 kg, were anesthetized with intravenous injections of sodium thiopental (30 mg/kg), and killed by bleeding from the common carotid arteries. Pieces of abdominal skin together with subcutaneous tissues were removed, and cutaneous arteries (0.2–0.4 mm internal diameter) were isolated from the subcutaneous plexus. Helically cut strips approximately 20 mm long were cut and the endothelium was removed by gentle rubbing of the intimal surface with a cotton ball. Endothelium denudation of the strips was confirmed by the abolishment of relaxations caused by acetylcholine (10^{-6} M). The specimens were fixed vertically between hooks in a muscle bath (20 ml capacity) containing modified Ringer-Locke solution (the composition of the solution (mM): NaCl 120, KCl 5.4, $CaCl_2$ 2.2, $MgCl_2$ 1.0, $NaHCO_3$ 25.0, dextrose 5.6; pH of the solution: 7.38–7.45) which was maintained at 37 ± 0.3°C and aerated with a mixture of 95% O_2 and 5% CO_2. The hooks anchoring the upper end of the strips were connected to the lever of a force-displacement transducer (Nihon-Kohden Kogyo, Tokyo, Japan). The resting tension was adjusted to 0.7 g, which was optimal for inducing maximal contraction (Toda et al., 1976). Before the start of experiments, the strips were allowed to equilibrate for 60–90 min in the bathing solution, during which time the fluid was replaced every 10–15 min.

Isometric mechanical responses were displayed on an ink-writing oscillograph. The contractile response to 30 mM KCl was first obtained, and the arterial strips were repeatedly washed with fresh bathing solution and equilibrated. The KCl-induced contraction was taken as a standard for the contraction caused by agonists (340–920 mg). The strips were contracted with prostaglandin $F_{2\alpha}$ (5×10^{-7} to 2×10^{-6} M). Effects of nicotine, NO (acidified $NaNO_2$), CGRP and VIP were determined by direct application to the bathing solution, and the strips were washed 3 or more times. After the responses to the vasodilators were found to be reproducible, the preparations were treated for approximately 30 min with blocking agents. Possible involvement of CGRP or VIP in the nicotine-induced response was tested in the strips made unresponsive to the peptides by repeated applications (3–5 times) and by the use of CGRP and VIP receptor antagonists and capsaicin. At the end of each series of experiments, papaverine (10^{-4} M) was added to attain maximal relaxation. Relaxations induced by agonists were normalized to those by papaverine.

2.2. Histochemical study

Tissue blocks containing the cutaneous arteries were fixed for 3 h in ice-cold phosphate-buffered saline (PBS) (0.2 M, pH 7.4) containing 2% paraformaldehyde, and were kept in 15% sucrose at 4°C until the next stage. The cutaneous artery was dissected out microscopically in ice-cold PBS (0.1 M). NADPH diaphorase staining of whole mount was performed by incubating the free-float arteries with PBS (0.1 M, pH 8.0), containing NADPH (1 mM) (Kohjin, Tokyo, Japan), nitro blue tetrazolium (2 mM) (Sigma, St. Louis, MO, USA) and 0.3% (v/v) Triton X-100 at 37°C under a dissecting microscope with ×8 magnification. The period of incubation was based on staining intensity. The reaction was terminated by washing the arteries in PBS (0.1 M). After several washouts with distilled water, the whole mount arteries were air-dried on gelatins/chrome-alum-coated glass and covered with a coverslip, using xylene (Entellan; Merck, Darmstadt, Germany).

For immunohistochemical staining, the dissected cutaneous arteries were incubated in a modified Ringer-Locke solution, which was aerated with a mixture of 95% O_2 and 5% CO_2 at 37°C for 20 min. During the incubation the preparations were either treated with capsaicin (10^{-6} M) or not treated. After incubation for 40 min in capsaicin-free solution, the whole mount preparations of the cutaneous arteries were fixed in phosphate buffer (0.2 M, pH 7.4) containing 2% paraformaldehyde for 3 h. The preparations were kept in PBS (0.1 M), containing 0.3% (v/v) Triton X-100 at 4°C for 2 days. The specimens were exposed to rabbit anti-human CGRPII serum (1:9000; Peninsula Laboratories, Belmont, CA, USA) in PBS with 0.3% (v/v) Triton X-100 at 4°C for 2 days. After washing, the preparations were incubated for 1 h with a diluted fluorescent second antibody (rhodamine-conjugated goat affinity-purified antibody against rabbit IgG, 1:100, Cappel Research Products, Durham, NC, USA) at 4°C. The immunostained preparations were then rinsed and covered with a coverslip with 90% glycerol in PBS for photography under a confocal laser-scanning microscope (MRC600; Bio-Rad, Tokyo, Japan). Histochemical control experiments such as exclusion of NADPH or anti-CGRP antiserum from the reaction mixture gave no positive staining.

2.3. Statistics and drugs used

The results shown in the text and figures are expressed as mean values ± S.E.M. Statistical analyses were made using Student's unpaired *t*-test and Tukey's method after one-way analysis of variance. The drugs used were N^G-nitro-L-arginine (L-NA), vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP), CGRP-(8–37) (Peptide Institute, Minoh, Japan), L-arginine, nicotine (base) (Kanto, Tokyo, Japan), capsaicin, α,β-methylene ATP, indomethacin, [D-*p*-Cl-Phe^6,Leu^{17}]VIP (Sigma),

prostaglandin $F_{2\alpha}$ (Upjohn, Tokyo, Japan), prazosin hydrochloride (Wako, Osaka, Japan), timolol maleate (Banyu, Tokyo, Japan), atropine sulfate (Tanabe, Osaka, Japan), cimetidine (Fujisawa, Osaka, Japan), hexamethonium bromide, methylene blue trihydrate (Nacalai Tesque, Kyoto, Japan), acetylcholine chloride (Daiichi Pharmaceutical, Tokyo, Japan) and papaverine hydrochloride (Dainippon, Osaka, Japan). All drugs were dissolved with distilled water except capsaicin, which was dissolved with 40% alcohol. Oxyhemoglobin was prepared by adding a 10-fold molar excess of the reducing agent, sodium hydrosulfite, to a 1 mM solution of commercial hemoglobin (Sigma) in distilled water and then was dialyzed (Martin et al., 1985). Responses to NO were obtained by adding $NaNO_2$ solution adjusted to pH 2 (Furchgott, 1988).

3. Results

3.1. Organ-bath studies

The endothelium-denuded dog cutaneous arterial strips contracted with prostaglandin $F_{2\alpha}$ responded to nicotine (10^{-4} M) with slight and transient contractions followed by relaxations. The contractions were suppressed or abolished by treatment with prazosin (10^{-5} M). When the response was not abolished (2 out of 8 strips), the strip was treated additionally with α,β-methylene ATP (10^{-6} M), which abolished the remaining contraction. The mean value of the contraction in response to nicotine in the control solution was $10.6 \pm 4.4\%$ ($n = 8$) of the contractions caused by 30 mM KCl, whereas the value of the relaxation was $61.1 \pm 8.1\%$ ($n = 8$) of the relaxations caused by papaverine (10^{-4} M). Relaxations in response to nicotine during treatment with prazosin were $49.2 \pm 7.1\%$ ($n = 8$). In order to avoid tachyphylaxis, the concentration-response curves for nicotine were tested by applying one of the concentrations (5×10^{-6} to 5×10^{-4} M) of nicotine to the arterial strips treated with prazosin. The mean values of the relaxation in response to 5×10^{-6}, 2×10^{-5}, 10^{-4} and 5×10^{-4} M nicotine were 3.6 ± 2.0, 25.9 ± 5.5, 44.6 ± 8.7 and $39.7 \pm 6.5\%$ ($n = 7$), respectively. Since 10^{-4} M nicotine produced maximal and reproducible relaxation, this concentration was used for analyses of the mechanism of action in the remainder of this study.

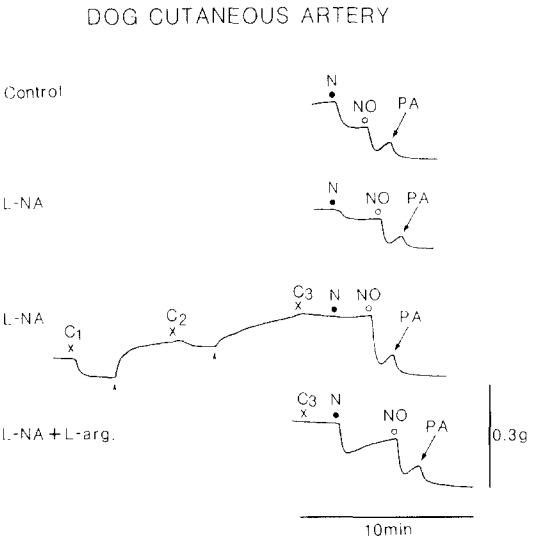

Fig. 1. Tracings of the response to nicotine and NO of a dog cutaneous arterial strip without endothelium. The strip was treated with prazosin (10^{-5} M) and was precontracted with prostaglandin $F_{2\alpha}$. 'N' and 'NO' denote nicotine (10^{-4} M) and acidified $NaNO_2$ (10^{-6} M), respectively. The tracings from top to bottom were obtained from the same strip under the conditions of (1) no treatment, (2) treatment with L-NA (10^{-5} M), (3) treatment with L-NA and repeated applications (first to third, C_1–C_3) of CGRP (10^{-8} M) or (4) treatment with L-NA, L-arginine (L-arg., 3×10^{-3} M) and repeated applications of CGRP. Desensitization to CGRP was confirmed by the abolishment of the response associated with peptide application (C_3). Upward arrowheads indicate supplemental applications of prostaglandin $F_{2\alpha}$ to raise the arterial tone. Papaverine (PA, 10^{-4} M) was used to attain maximal relaxation.

The next study on nicotine was carried out in the strips treated with prazosin. Relaxations induced by nicotine were abolished by hexamethonium (10^{-5} M) (Table 1), whereas treatment with timolol (10^{-7} M), atropine (10^{-7} M), cimetidine (10^{-5} M) or indomethacin (10^{-6} M) did not alter the degree of relaxation (Table 1). The concentrations of these blocking agents were sufficient to abolish or markedly suppress the relaxation induced by β-adrenoceptor (Scriabine et al., 1973), muscarinic (Wang et al., 1993) and histamine H_2 (Toda, 1986) receptor agonists and the endogenous vasodilator prostanoids (Miyazaki et al., 1985). The nicotine-induced relaxation was significantly inhibited but was not abolished by L-NA (10^{-5} M) alone (Figs. 1 and 2A). CGRP (10^{-8} M) caused marked relaxation in the arterial strips contracted with prostaglandin $F_{2\alpha}$. However, successive applications (3–5 times) of CGRP made the strips unresponsive to the peptide (Figs. 1 and 3). Nicotine-induced relaxation was significantly inhibited after desensitization to CGRP (Figs. 3 and 4). The relaxations caused by exogenously applied NO (acidified $NaNO_2$,

Table 1
Effect of blocking agents on the relaxant responses to nicotine (10^{-4} M) in dog cutaneous arterial strips without endothelium

Treatment	Relaxation (%) [a] induced by nicotine		
	n	Control	Experimental
Timolol (10^{-7} M)	5	41.5±4.7	39.8±4.7
Atropine (10^{-7} M)	5	43.2±2.6	43.6±5.0
Cimetidine (10^{-5} M)	5	38.4±8.5	42.1±9.2
Indomethacin (10^{-6} M)	5	48.9±5.9	42.3±9.8
Hexamethonium (10^{-5} M)	5	45.0±6.6	0 [b]

The data (means±S.E.M.) obtained before (control) and after treatment with blocking agents (experimental) were compared. The strips were treated with prazosin (10^{-5} M). n = number of strips from separate dogs. [a] Relaxations relative to those induced by papaverine (10^{-4} M). [b] $P < 0.001$ versus control (unpaired t-test).

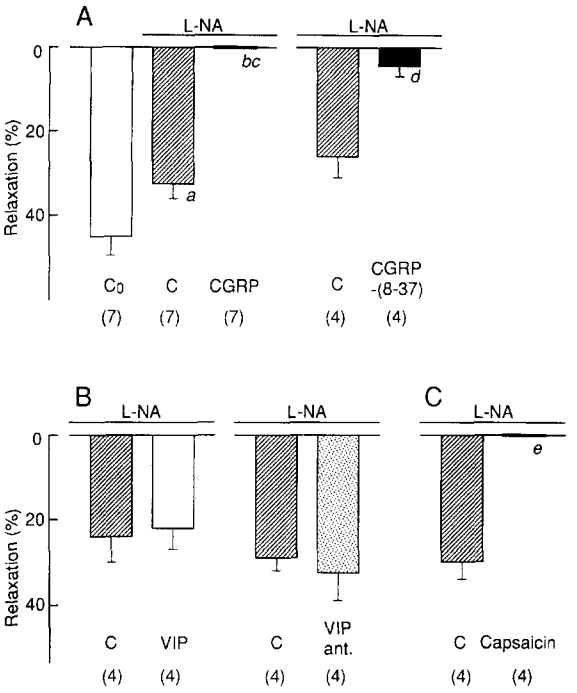

Fig. 2. Effect of L-NA in the absence and presence of desensitization to CGRP, CGRP receptor antagonist (A), desensitization to VIP, VIP receptor antagonist (B) or capsaicin (C) on the relaxant response to nicotine (10^{-4} M) in dog cutaneous arterial strips without endothelium. The strips were treated with prazosin (10^{-5} M) and contracted with prostaglandin $F_{2\alpha}$. 'CGRP' and 'VIP' in the figure denote the strips desensitized to CGRP (10^{-8} M, 3–5 times) and VIP (10^{-8} M, 3–5 times), respectively. CGRP and VIP receptor antagonists are CGRP-(8–37) (10^{-7} M) and [D-*p*-Cl-Phe^{6},Leu^{17}]VIP (VIP ant., 10^{-6} M), respectively. In capsaicin experiments, strips were exposed to capsaicin (10^{-7} M) for 20 min and repeatedly washed to remove capsaicin. Relaxations induced by papaverine (10^{-4} M) were taken as 100%. Numbers in parentheses indicate the number of strips from separate dogs. [a] $P < 0.05$ and [b] $P < 0.01$ versus control before treatment with L-NA (10^{-5} M) (C_0); [c] $P < 0.01$ versus control after treatment with L-NA (C) (panel A, Tukey's method). [d] $P < 0.01$ versus control after treatment with L-NA (C) (panel A, unpaired *t*-test). [e] $P < 0.001$ versus control after treatment with L-NA (C) (panel C, unpaired *t*-test). Vertical bars, S.E.M.

10^{-6} M) were not affected by desensitization to CGRP (from 69.0 ± 5.5 to $67.1 \pm 6.4\%$; $n = 7$). In the strips desensitized to CGRP, the remaining relaxations caused by nicotine were abolished by additional treatment with L-NA, methylene blue (10^{-5} M) and oxyhemoglobin (1.6×10^{-5} M) (Figs. 3 and 4). The inhibitory effect of L-NA was reversed by the addition of L-arginine (3×10^{-3} M) (Figs. 3 and 4). On the other hand, desensitization to CGRP abolished the relaxation in the strips treated with L-NA (Figs. 1 and 2A). In addition, CGRP-(8–37) (10^{-7} M), a specific CGRP receptor antagonist, significantly inhibited both the nicotine-induced relaxation in the strips treated with L-NA (Fig. 2A) and the relaxation in response to exogenously applied CGRP (Table 2). Other strips which responded to nicotine and CGRP (5×10^{-10} M) with moderate relaxations under control conditions were exposed to capsaicin (10^{-7} M) for 20 min and repeatedly washed to remove capsaicin. After stabilization for 40 min

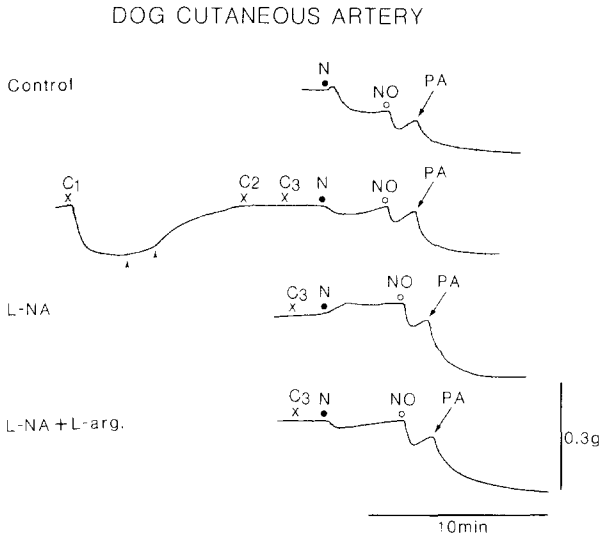

Fig. 3. Tracings of the response to nicotine and NO of a dog cutaneous arterial strip without endothelium. The strip was treated with prazosin (10^{-5} M) and was precontracted with prostaglandin $F_{2\alpha}$. 'N' and 'NO' denote nicotine (10^{-4} M) and acidified $NaNO_2$ (10^{-6} M), respectively. The tracings from top to bottom were obtained from the same strip under the conditions of (1) no treatment, (2) treatment with repeated applications (first to third, C_1–C_3) of CGRP (10^{-8} M), (3) treatment with L-NA (10^{-5} M) and repeated applications of CGRP or (4) treatment with L-NA, L-arginine (L-arg., 3×10^{-3} M) and repeated applications of CGRP. Desensitization to CGRP was confirmed by the abolishment of the response associated with peptide application (C_3). Upward arrowheads indicate supplemental applications of prostaglandin $F_{2\alpha}$ to raise the arterial tone. Papaverine (PA, 10^{-4} M) was used to attain maximal relaxation.

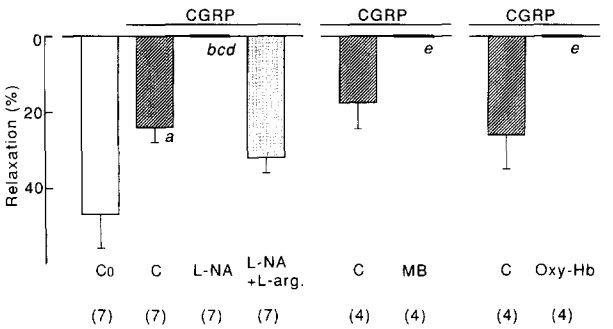

Fig. 4. Effect of desensitization to CGRP in the absence and presence of L-NA, L-NA plus L-arginine, methylene blue or oxyhemoglobin on the relaxant response to nicotine (10^{-4} M) in dog cutaneous arterial strips without endothelium. The strips were treated with prazosin (10^{-5} M) and contracted with prostaglandin $F_{2\alpha}$. 'CGRP' in the figure denotes the strips desensitized to CGRP (10^{-8} M, 3–5 times). 'MB' and 'Oxy-Hb' in the figure denote treatment with methylene blue (10^{-5} M) and oxyhemoglobin (1.6×10^{-5} M), respectively. Relaxations induced by papaverine (10^{-4} M) were taken as 100%. Numbers in parentheses indicate the number of strips from separate dogs. [a] $P < 0.05$ and [b] $P < 0.01$ versus control before desensitization to CGRP (C_0); [c] $P < 0.05$ versus control after desensitization to CGRP (C); [d] $P < 0.01$ versus desensitized to CGRP and L-NA(10^{-5} M) plus L-arginine (L-arg., 3×10^{-3} M)-treated (left panel, Tukey's method). [e] $P < 0.05$ versus control after desensitization to CGRP (C) (middle and right panels, unpaired *t*-test). Vertical bars, S.E.M.

Table 2
Effect of blocking agents on the relaxant responses to CGRP (5×10^{-10} M) or VIP (10^{-9} M) in dog cutaneous arterial strips without endothelium

Treatment	Relaxation (%) [a] induced by CGRP		
	n	Control	Experimental
CGRP-(8–37) (10^{-7} M)	4	73.3 ± 8.8	23.1 ± 8.3 [b]
Capsaicin (10^{-7} M)	4	84.2 ± 3.6	76.6 ± 7.9
		Relaxation (%) [a] induced by VIP	
[D-*p*-Cl-Phe^6,Leu^{17}]VIP (10^{-6} M)	4	64.3 ± 7.3	18.3 ± 6.7 [b]

Data (means ± S.E.M.) obtained before (control) and after treatment with blocking agents (experimental) were compared. The strips were treated with prazosin (10^{-5} M) and L-NA (10^{-5} M). *n* = number of strips from separate dogs. [a] Relaxations relative to those induced by papaverine (10^{-4} M). [b] $P < 0.01$ versus control (unpaired *t*-test).

in capsaicin-free, L-NA-containing bathing solution, the nicotine-induced relaxation was totally abolished (Fig. 2C), whereas the CGRP-induced relaxation was unaffected by capsaicin treatment (Table 2). In other experiments desensitization to VIP did not affect the response to nicotine (Fig. 2B). [D-*p*-Cl-Phe^6,Leu^{17}]VIP (10^{-6} M) at a concentration sufficient to significantly inhibit the relaxation induced by VIP (10^{-9} M) (Table 2) failed to attenuate the response to nicotine (Fig. 2B).

CGRP (10^{-10} to 3×10^{-9} M) relaxed arteries in a concentration-dependent manner (Fig. 5, right). The relaxations were slightly reduced in the second trial, but were reproducible after the third trial. Therefore, the third concentration-response curve was taken as control. The mean values for the maximal relaxation caused by CGRP in the control solution averaged 91.6 ± 1.1% ($n = 15$) and the apparent median effective concentration (EC_{50}) was (6.87 ± 0.16) × 10^{-10} M ($n = 15$). Relaxations induced by CGRP were not significantly affected by treatment with L-NA (10^{-5} M), oxyhemoglobin (1.6×10^{-5} M) or methylene blue (10^{-5} M).

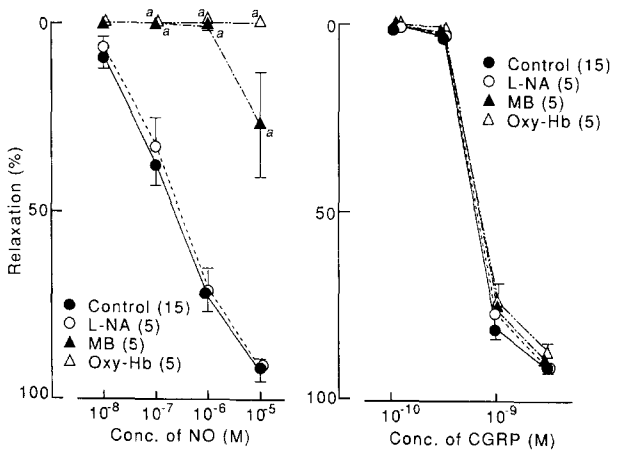

Fig. 5. Effect of arginine-NO pathway inhibitors on the relaxant responses to NO (acidified $NaNO_2$) (left panel) and CGRP (right panel) in dog cutaneous arterial strips without endothelium. Preparations were treated with prazosin (10^{-5} M) and precontracted with prostaglandin $F_{2\alpha}$. Inhibitors are L-NA (10^{-5} M), methylene blue (MB, 10^{-5} M) and oxyhemoglobin (Oxy-Hb, 1.6×10^{-5} M). Relaxations induced by papaverine (10^{-4} M) were taken as 100%. Numbers in parentheses indicate the number of strips from separate dogs. [a] $P < 0.01$ versus control (Tukey's method). Vertical bars, S.E.M.

Fig. 6. Histochemistry of NADPH diaphorase-containing nerve fibers in a whole mount preparation of the dog cutaneous artery. Scale bar, 50 μm.

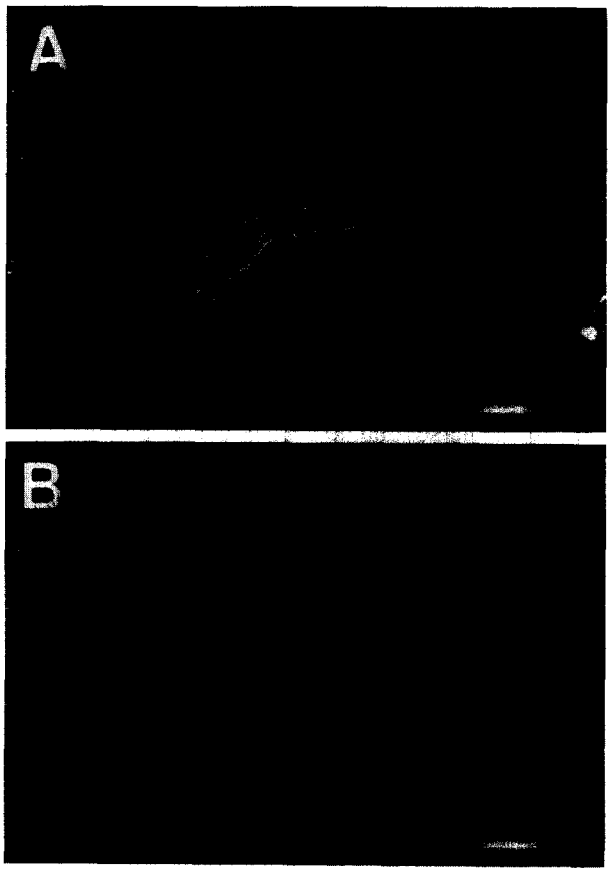

Fig. 7. Abolishment of CGRP immunoreactive nerves in a whole mount preparation of the dog cutaneous artery after exposure to capsaicin (10^{-6} M, 20 min). Control (A). Capsaicin-treated (B). Scale bars, 50 μm.

The addition of NO (acidified $NaNO_2$, 10^{-8} to 10^{-5} M) produced a transient relaxation of prostaglandin $F_{2\alpha}$-precontracted dog cutaneous arterial strips without endothelium (Fig. 5, left). The mean value of the maximal relaxation caused by NO (acidified $NaNO_2$) averaged 92.4 ± 2.4% ($n = 15$), and the apparent EC_{50} value was (5.37 ± 2.03) × 10^{-7} M ($n = 15$). The NO (acidified $NaNO_2$)-induced relaxation was not affected by treatment with L-NA (10^{-5} M) (from 72.6 ± 3.8% ($n = 15$) to 69.7 ± 4.5% ($n = 5$) at 10^{-6} M), but was abolished by oxyhemoglobin (1.6×10^{-5} M) and markedly attenuated by methylene blue (10^{-5} M).

3.2. Histochemical study

Fig. 6 shows the histologically demonstrated nerve fibers containing NADPH diaphorase in a whole mount preparation of a dog cutaneous artery. There are networks of positively stained fibers in the arterial wall. Similar findings were also obtained with arteries isolated from two additional dogs.

CGRP immunoreactivity of perivascular nerve fibers in a whole mount preparation of the artery (Fig. 7A) was abolished by treatment with capsaicin (exposure for 20 min to capsaicin (10^{-6} M) and then incubation for 40 min in capsaicin-free solution) (Fig. 7B).

4. Discussion

Contractions caused by nicotine were markedly attenuated or abolished by prazosin, suggesting the possible stimulation of α_1-adrenoceptors by neurogenic norepinephrine. In some strips in which the response was not completely abolished by prazosin, the remaining response was abolished by α,β-methylene ATP which desensitizes the excitatory response mediated by P_{2X} purinoceptors (Burnstock and Kennedy, 1985). Therefore, the contraction caused by nicotine appears to be associated with activation of adrenergic nerves and, to a lesser extent, that of purinergic nerves.

Following treatment with the α_1-adrenoceptor antagonist, dog cutaneous arterial strips responded to nicotine with relaxation. This relaxation was abolished by treatment with hexamethonium, suggesting that the response is elicited by nerve stimulation. However, the relaxation caused by nicotine was not influenced by timolol, atropine, cimetidine or indomethacin in concentrations capable of blocking β-adrenoceptor, muscarinic and histamine H_2 receptors and the synthesis of prostanoids. These findings would suggest that nicotine relaxes the dog cutaneous artery possibly via stimulation of nicotinic receptors in non-adrenergic, non-cholinergic nerve terminals, in a histamine- and prostanoid-independent manner.

The nicotine-induced relaxation of endothelium-denuded arteries was moderately attenuated by treatment with the NO synthase inhibitor, L-NA, whereas relaxations caused by the NO releasing agent, acidified $NaNO_2$, were not influenced by L-NA. These findings would suggest that NO or NO-like substance(s) appear to be involved in the relaxation caused by nicotine. However, it is possible that other substance(s) are also involved in the relaxation, because the nicotine-induced relaxation was not abolished even after treatment with L-NA at a concentration capable of inhibiting the relaxant response to nicotine in human, monkey and canine cerebral arteries (Toda, 1993; Toda and Okamura, 1991, 1992). In the strips treated with L-NA, the nicotine-induced relaxation was abolished by desensitization to CGRP and by capsaicin, as Kawasaki et al. (1988) reported, and was markedly suppressed by CGRP-(8–37), a specific CGRP receptor antagonist (Chiba et al., 1989). Capsaicin is considered to deplete neural CGRP and substance P (Saito et al., 1989), and CGRP immunoreactivity in the cutaneous artery was abolished by treatment with capsaicin. On the other hand, desensitization of the strips to VIP and treatment with [D-*p*-Cl-Phe^6,Leu^{17}]VIP, a specific VIP receptor antagonist (Pandol et al., 1986), did not influence the relaxation induced by nicotine when endogenous NO production was blocked. These findings strongly suggest that the NO-independent relaxation is mediated by liberation of CGRP but not VIP from the nerve terminals.

Further, in the arteries desensitized to CGRP, the nicotine-induced relaxation was abolished by treatment with L-NA, methylene blue, a soluble guanylate cyclase inhibitor (Martin et al., 1985), and oxyhemoglobin, a scavenger of NO (Martin et al., 1985). The inhibition by the NO synthase inhibitor was reversed by additional treatment with L-arginine. The relaxation caused by exogenously applied NO (acidified $NaNO_2$) was abolished by methylene blue or oxyhemoglobin in the dog cutaneous artery. These findings provide additional evidence for the role of NO synthesized from L-arginine. The above discussion prompts the suggestion that the relaxation caused by nicotine in dog cutaneous arteries is mediated by NO or NO-like substance(s) and CGRP. This hypothesis is supported by the histochemical demonstration of perivascular nerves containing NADPH diaphorase, reported to be identical to NO synthase (Dawson et al., 1991), together with CGRP immunoreactivity in dog cutaneous arteries. The release of neurogenic NO by CGRP is unlikely, as exogenously applied CGRP-induced relaxations were not inhibited by L-NA in dog cutaneous arteries without endothelium (present study) or in rat skin microvasculature (Ralevic et al., 1992). However, Goldsmith et al. (1996) suggested that CGRP-induced vasodilatation in human skin in vivo is mediated partially by NO.

We conclude that nicotine stimulates nicotinic receptors located in nerve terminals containing NO synthase and CGRP which subsequently release NO or NO-like substance(s) and CGRP, respectively, resulting in relaxation of the dog cutaneous artery. In human skin, nerve fibers containing CGRP have been histologically demonstrated to

exist around blood vessels (Weihe and Hartschuh, 1988) and are thought to mediate vasodilatation. NO synthase immunoreactive nerve fibers are also reported to be present in human arteries (Toda et al., 1994), and evidence suggests that NO is involved in the maintenance of resting blood flow in human skin (Goldsmith et al., 1996). In patients with diabetes mellitus and Raynaud's disease, nerve fibers containing CGRP are depleted (Levy et al., 1989; Bunker et al., 1990). Impairment of these vasodilator nerve functions might reduce cutaneous blood flow and be involved in the pathogenesis of skin diseases such as Raynaud's disease and diabetic gangrene.

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References

Bunker, C.B., G. Terenghi, D.R. Springall, J.M. Polak and P.M. Dowd, 1990, Deficiency of calcitonin gene-related peptide in Raynaud's phenomenon, Lancet 336, 1530.

Burnstock, G. and C. Kennedy, 1985, Is there a basis for distinguishing two types of P_2-purinoceptor?, Gen. Pharmacol. 16, 433.

Chiba, T., A. Yamaguchi, T. Yamatani, A. Nakamura, T. Morishita, T. Inui, M. Fukase, T. Noda and T. Fujita, 1989, Calcitonin gene-related peptide receptor antagonist human CGRP-(8–37), Am. J. Physiol. 256, E331.

Dawson, T.M., D.S. Bredt, M. Fotuhi, P.M. Hwang and S.H. Snyder, 1991, Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues, Proc. Natl. Acad. Sci. USA 88, 7797.

Escott, K.J., D.T. Beattie, H.E. Connor and S.D. Brain, 1995, Trigeminal ganglion stimulation increases facial skin blood flow in the rat: a major role for calcitonin gene-related peptide, Brain Res. 669, 93.

Furchgott, R.F., 1988, Studies on relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid-activatable inhibitory factor from bovine retractor penis is inorganic nitrite and the endothelium-derived relaxing factor is nitric oxide, in: Vasodilatation, ed. P.M. Vanhoutte (Raven Press, New York, NY) p. 401.

Goldsmith, P.C., T.A. Leslie, N.A. Hayes, N.J. Levell, P.M. Dowd and J.C. Foreman, 1996, Inhibitors of nitric oxide synthase in human skin, J. Invest. Dermatol. 106, 113.

Holzer, P. and M. Jocič, 1994, Cutaneous vasodilatation induced by nitric oxide-evoked stimulation of afferent nerves in the rat, Br. J. Pharmacol. 112, 1181.

Hughes, S.R. and S.D. Brain, 1994, Nitric oxide-dependent release of vasodilator quantities of calcitonin gene-related peptide from capsaicin-sensitive nerves in rabbit skin, Br. J. Pharmacol. 111, 425.

Kalsner, S. and M. Quillan, 1989, Cholinergic contraction to field stimulation in coronary arteries of cattle, J. Pharmacol. Exp. Ther. 249, 785.

Kawasaki, H., K. Takasaki, A. Saito and K. Goto, 1988, Calcitonin gene-related peptide acts as a novel vasodilator neurotransmitter in mesenteric resistance vessels of the rat, Nature 335, 164.

Kellogg Jr., D.L., P.E. Pérgola, K.L. Piest, W.A. Kosiba, C.G. Crandall, M. Grossmann and J.M. Johnson, 1995, Cutaneous active vasodilation in humans is mediated by cholinergic nerve cotransmission, Circ. Res. 77, 1222.

Levy, D.M., S.S. Karanth, D.R. Springall and J.M. Polak, 1989, Depletion of cutaneous nerves and neuropeptides in diabetes mellitus: an immunocytochemical study, Diabetologia 32, 427.

Martin, W., G.M. Villani, D. Jothianandan and R.F. Furchgott, 1985, Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta, J. Pharmacol. Exp. Ther. 232, 708.

Miyazaki, M., M. Yamamoto and N. Toda, 1985, Interaction between non-steroidal antiinflammatory agents and sodium salicylate in the relaxant response of dog renal arteries to angiotensin II, Arch. Int. Pharmacodyn. Ther. 274, 210.

Nedergaard, O.A., 1988, Effect of nicotine on neuroeffector transmission in blood vessels, in: The Pharmacology of Nicotine, eds. M. Rand and K. Thurau (IRL Press, Washington, DC) p. 143.

Pandol, S.J., K. Dharmsathaphorn, M.S. Schoeffield, W. Vale and J. Rivier, 1986, Vasoactive intestinal peptide receptor antagonist [4Cl-D-Phe^6,Leu^{17}]VIP, Am. J. Physiol. 250, G553.

Ralevic, V., Z. Khalil, G.J. Dusting and R.D. Helme, 1992, Nitric oxide and sensory nerves are involved in the vasodilator response to acetylcholine but not calcitonin gene-related peptide in rat skin microvasculature, Br. J. Pharmacol. 106, 650.

Saito, A., T. Masaki, Y. Uchiyama, T.J.F. Lee and K. Goto, 1989, Calcitonin gene-related peptide and vasodilator nerves in large cerebral arteries of cats, J. Pharmacol. Exp. Ther. 248, 455.

Scriabine, A., M.L. Torchiana, J.M. Stavorski, C.T. Ludden, D.H. Minsker and C.A. Stone, 1973, Some cardiovascular effects of timolol: a new β-adrenergic blocking agent, Arch. Int. Pharmacodyn. 205, 76.

Su, C., 1982, Actions of nicotine and smoking on circulation, Pharmacol. Ther. 17, 129.

Toda, N., 1982, Relaxant responses to transmural stimulation and nicotine of dog and monkey cerebral arteries, Am. J. Physiol. 243, H145.

Toda, N., 1986, Mechanisms of histamine-induced relaxation in isolated monkey and dog coronary arteries, J. Pharmacol. Exp. Ther. 239, 529.

Toda, N., 1993, Mediation by nitric oxide of neurally-induced human cerebral artery relaxation, Experientia 49, 51.

Toda, N. and T. Okamura, 1990, Mechanism underlying the response to vasodilator nerve stimulation in isolated dog and monkey cerebral arteries, Am. J. Physiol. 259, H1511.

Toda, N. and T. Okamura, 1991, Role of nitric oxide in neurally induced cerebroarterial relaxation, J. Pharmacol. Exp. Ther. 258, 1027.

Toda, N. and T. Okamura, 1992, Regulation by nitroxidergic nerve of arterial tone, News Physiol. Sci. 7, 148.

Toda, N., S. Hayashi and Y. Hatano, 1976, Length-tension relationship in isolated canine cerebral arteries, Jpn. J. Pharmacol. 26, 129.

Toda, N., T. Kimura, K. Yoshida, D.S. Bredt, S.H. Snyder, Y. Yoshida and T. Okamura, 1994, Human uterine arterial relaxation induced by nitroxidergic nerve stimulation, Am. J. Physiol. 266, H1446.

Wang, Y., T. Okamura and N. Toda, 1993, Mechanisms of acetylcholine-induced relaxation in dog external and internal ophthalmic arteries, Exp. Eye Res. 57, 275.

Weihe, E. and W. Hartschuh, 1988, Multiple peptides in cutaneous nerves: regulators under physiological conditions and a pathogenetic role in skin disease?, Semin. Dermatol. 7, 284.

Yoshioka, K., T. Furuta, A. Hayakawa, N. Ishikawa and T. Shigei, 1988, Excitatory cholinergic innervation in canine portal and mesenteric veins, Am. J. Physiol. 255, H288.