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Mechanisms underlying constrictor and dilator responses to perivascular nerve stimulation in canine lingual arteries

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Abstract

In isolated canine lingual arteries denuded of the endothelium, transmural electrical stimulation (2–20 Hz) produced a frequency-related contraction which was not significantly influenced by prazosin but which was reversed to a relaxation by α,β-methylene ATP. The responses were abolished by tetrodotoxin. The stimulation-induced relaxation was abolished by treatment with N^G-nitro-L-arginine (L-NA, 10^{-6} M) and restored by the addition of L-arginine. Neurogenic relaxation resistant to L-NA was not observed after electrical stimulation, even though the pulse width and stimulus intensity were raised. Under treatment with prazosin, α,β-methylene ATP and indomethacin, the arterial strips responded to nicotine (10^{-4} M) with a marked relaxation that was abolished by hexamethonium. The relaxation was significantly inhibited but not abolished by L-NA (10^{-5} M), and raising the concentration of the inhibitor to 10^{-4} M, did not produce additional inhibition. In the strips treated with L-NA, the nicotine-induced relaxation was abolished or markedly reduced under desensitization with vasoactive intestinal peptide (VIP) or calcitonin gene-related peptide (CGRP) and by treatment with high concentrations of beraprost, a stable analog of prostaglandin I_2, but was unaffected by CGRP or VIP receptor antagonists. Relaxant responses to a low concentration of nicotine (5×10^{-6} M) were abolished by L-NA and restored by L-arginine. Histochemical study demonstrated many nerve fibers and bundles containing NADPH diaphorase in the adventitia of the arteries. It is concluded that the neurogenic arterial contraction is induced mainly by ATP via stimulation of P_2X purinoceptors, and that the relaxation induced by electrical stimulation or a low concentration of nicotine is mediated by nitric oxide (NO) released from perivascular nerves. In high concentrations, nicotine elicits marked relaxations possibly due to the liberation of NO from the nerve and also vasodilator substances that increase the content of cyclic AMP in the tissue; CGRP and VIP are unlikely to be involved.

Keywords: Lingual artery; Nitric oxide (NO); ATP; Nitroxidergic nerve; Nicotine

1. Introduction

The tongue is a special tissue with abundant vascular networks for blood supply and autonomic, motor and sensory nerve fibers for taste sensation and food swallowing. Therefore, the neural and vascular controls of characteristic functions of the tongue are expected to differ from those of other tissues and organs. However, little is known about the roles of autonomic nerves in the lingual vasculature in large mammals (Nagai and Pleschka, 1981; Bevan et al., 1982; Toda et al., 1997). Vasomotor control in the tongue is important for taste and temperature sensations and for fine movement of muscles. Sympathetic vasoconstrictor innervation is widely recognized, and there is some evidence suggesting the functional role of vasodilator nerves in the lingual vasculature (Pleschka, 1984; Faraci et al., 1986). However, whether this is the case in canine lingual arteries and what mechanism underlies the vascular response have not yet been determined.

There are numerous literature suggesting that vasoconstriction is associated with the activation of sympathetic nerves liberating norepinephrine, ATP (White, 1991) and neuropeptide Y (Granstam and Nilsson, 1991) as neurotransmitters or neuromodulators. Vasodilatation is mediated by nitric oxide (NO) from efferent nerves (called 'nitroxidergic') (Toda and Okamura, 1992a) or calcitonin gene-related peptide (CGRP) possibly from sensory nerves (Ayajiki et al., 1994; Uchiyama et al., 1997) and also by endogenous acetylcholine, which may cause the release of NO from the endothelium in resistance vessels (McMahon

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et al., 1992; Loke et al., 1994) and vasodilatation by acting as an inhibitor at prejunctional sites on adrenergic vasoconstrictor nerves (Zhang et al., 1996). Electrical stimulation with square pulses and nicotine are thought to stimulate perivascular nerves innervating the vascular wall and to share the same mechanisms of action (Nedergaard, 1988; Toda, 1995). However, there are some differences in the responses induced by these stimuli, such as different Ca^{2+} channels involved (N-type vs. non-L- and non-N-type) (Toda and Okamura, 1992b; Toda et al., 1995), release of prostanoids only by nicotine (Okamura et al., 1993), etc.

The aims of the present study were to determine vasoconstriction and dilator responses to transmural electrical stimulation and nicotine in canine lingual arteries, to clarify the mechanisms underlying the responses and to compare the responses to those elicited by electrical stimulation and nicotine.

2. Materials and methods

The study review board at our University approved the use of canine blood vessels in this study.

2.1. Tension recording

Mongrel dogs of either sex, weighing 7 to 11 kg, were killed by bleeding from the common carotid arteries under anesthesia indicated by intravenous injections of sodium pentobarbital (30 mg/kg). The tongue was rapidly removed, and the distal portion of the deep lingual artery (0.3 to 0.4 mm outside diameter) was isolated. In some experiments, proximal arteries (0.5 to 0.8 mm) were isolated for comparison of the response to that obtained with distal arteries. The arteries were cleaned off the surrounding tissues and cut into helical strips of approximately 20 mm long. The endothelium was removed by gently rubbing the intimal surface with a ball of cotton wool. Endothelium denudation was verified by abolishment of the relaxant response to 10^{-6} M acetylcholine. The specimen was vertically fixed between hooks in a muscle bath (20 ml capacity) containing modified Ringer–Locke solution which was maintained at 37 ± 0.3°C and aerated with a mixture of 95% O_2 and 5% CO_2. The hook anchoring the upper end of the strips was connected to the lever of a force-displacement transducer (Nihon-Kohden Kogyo, Tokyo, Japan). Most of the strips were placed between stimulating electrodes, and electrical pulses of 0.2 ms at frequencies of 2, 5 and 20 Hz for periods of 100, 40 and 10 s, respectively, were transmurally applied to stimulate intramural nerve terminals. Resting tensions were adjusted to 1 g for the distal arterial strips and 1.5 g for the proximal arteries, which were optimal for inducing the maximal contraction. Constituents of the solution were as follows (mM): NaCl 120, KCl 5.4, $CaCl_2$ 2.2, $MgCl_2$ 1.0, $NaHCO_3$ 25.0, and dextrose 5.6. The pH of the solution was 7.38 to 7.43. Before the start of experiments, all of the strips were allowed to equilibrate for 60 to 90 min in the bathing media, during which time the fluid was replaced every 10 to 15 min.

Isometric mechanical responses were displayed on an ink-writing oscillograph. The contractile response to 3×10^{-2} M K^+ was first obtained, and then the arterial strips were repeatedly washed with fresh media and equilibrated. The strips were partially contracted with prostaglandin $F_{2\alpha}$ (4×10^{-7} to 45×10^{-7} M), the contraction being in a range between 20 and 37% of the contraction induced by 3×10^{-2} M K^+. Transmural electrical stimulation was applied every 10 min to the strips, until reproducible responses were obtained. Nicotine and NO (acidified $NaNO_2$ solution) in single concentrations were successively applied to the bathing media which contained 10^{-6} M indomethacin to avoid the influence of cyclooxygenase products. At the end of each series of experiments, papaverine (10^{-4} M) was added to attain the maximal relaxation, which was taken as 100% for the relaxation induced by electrical stimulation or agonists. Contractions induced by 3×10^{-2} M K^+ were taken as 100% for the contractions associated with transmural stimulation. The arterial strips were treated for 10 min or longer with blocking agents before the effect of electrical nerve stimulation or agonists was elicited. Some strips treated with a NO synthase inhibitor were desensitized to CGRP (10^{-8} M) or vasoactive intestinal peptide (VIP) (10^{-8} M) by repeated application (four to five times) of the peptide, and the responses to nicotine were compared before and after desensitization.

2.2. Histochemical study

The lingual artery was fixed in ice-cold 0.1 M phosphate-buffered saline (PBS, pH 7.4) containing 0.3% glutaraldehyde and 4% paraformaldehyde and then post-fixed overnight in 0.1 M PBS with 4% paraformaldehyde, followed by cryoprotection in 15% sucrose. The fixed blocks were cut into sections (20 μm thick) in a cryostat (Cryotom, Nakagawa Seisakusho, Tokyo) and mounted onto gelatin/chrome-alum-coated glass slides. The sections were then rinsed in 0.1 M PBS. NADPH diaphorase staining was performed by incubating glass-mounted sections with 0.1 M PBS, pH 8.0, containing 1 mM NADPH (Kojin, Tokyo), 2 mM nitro blue tetrazolium (Sigma, St. Louis, MO), and 0.3% Triton X-100 at 37°C. The duration of incubation (30–60 min) was determined from the staining intensity and was kept constant in sections from individual animals. The reaction was terminated by washing the sections in 0.1 M PBS. After several washes with distilled water, the sections were air-dried and coverslipped with Entellan (Merck, Tokyo). Almost serial sections were doubly stained for NADPH diaphorase and eosin. A histochemical control experiment, in which

NADPH was excluded from the reaction mixture, gave no positive staining. The sections were then examined and photographed under a light microscope.

2.3. Statistics and drugs used

The results shown in the text, table and figures are expressed as mean values ± S.E. Statistical analyses were made by using Student's paired and unpaired *t*-tests for two groups and Tukey's test after one-way analysis of variance for more than three groups. Drugs used were N^G-nitro-L-arginine (L-NA), N^G-nitro-D-arginine (D-NA), VIP, CGRP, CGRP-(8–37), [D-Pro^4,D-Trp^{7,9}]substance P-(4–11) (Peptide Institute, Minoh, Japan), L- and D-Arginine, nicotine (base), methylene blue trihydrate, hexamethonium bromide (Nacalai Tesque, Kyoto, Japan), atropine sulfate (Tanabe, Osaka, Japan), timolol hydrochloride (Banyu, Tokyo), prazosin hydrochloride (Pfizer-Taito, Tokyo), indomethacin, α,β-methylene ATP, aminophylline, [D-*p*-Cl-Phe^6,Leu^{17}]VIP (Sigma), prostaglandin $F_{2\alpha}$ (Pharmacia-Upjohn, Tokyo), beraprost sodium (Toray Industries, Tokyo), acetylcholine chloride (Daiichi Pharmaceutical, Tokyo), tetrodotoxin (Sankyo, Tokyo) and papaverine hydrochloride (Dainippon, Osaka). Responses to NO were obtained by adding $NaNO_2$ solution adjusted to pH 2 (Furchgott, 1988), and the concentrations of $NaNO_2$ in the bathing media are expressed as those of NO. Oxyhemoglobin was prepared according to the method of Martin et al. (1985).

3. Results

3.1. Response to transmural electrical stimulation

In canine lingual arterial strips contracted with prostaglandin $F_{2\alpha}$, transmural electrical stimulation at frequencies of 2, 5 and 20 Hz produced a frequency-dependent contraction followed by a relaxation. The contraction was not or only slightly reduced by treatment with 10^{-5} M prazosin but was abolished by combined treatment with α,β-methylene ATP (10^{-6} M) (Fig. 1). The relaxation was potentiated by the combined treatment. α,β-Methylene ATP alone abolished the contraction and potentiated the relaxation; relaxations relative to papaverine at 5 Hz in the control and α,β-methylene ATP-treated groups were 7.4 ± 2.8% and 19.7 ± 4.3%, respectively ($n = 6$, $P < 0.05$, unpaired *t*-test). Additional treatment with prazosin did not potentiate the relaxation (20.1 ± 5.7%, $n = 6$). The contractile and relaxant responses were abolished by 3×10^{-7} M tetrodotoxin. In the remainder of this report, the mechanisms of the relaxant response were analyzed after treatment with prazosin and α,β-methylene ATP.

Relaxations induced by nerve stimulation at 2, 5 and 20 Hz were abolished by treatment with L-NA (10^{-6} M), and the response was restored by L-arginine (3×10^{-4} M) but

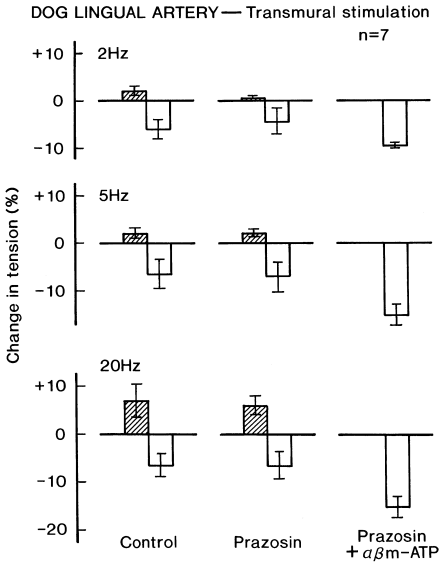

Fig. 1. Modification by prazosin (10^{-5} M) and α,β-methylene ATP (α,βm-ATP, 10^{-6} M) of the response to transmural electrical stimulation (2–20 Hz) in lingual arterial strips partially contracted with prostaglandin $F_{2\alpha}$ (1 to 3×10^{-6} M). Contractions induced by K^+ (3×10^{-2} M) were taken as 100% (plus on the ordinate, hatched column), and relaxations induced by papaverine (10^{-4} M) were taken as 100% (minus, open column). The data were obtained from strips isolated from different dogs ($n = 7$). Vertical bars represent S.E.

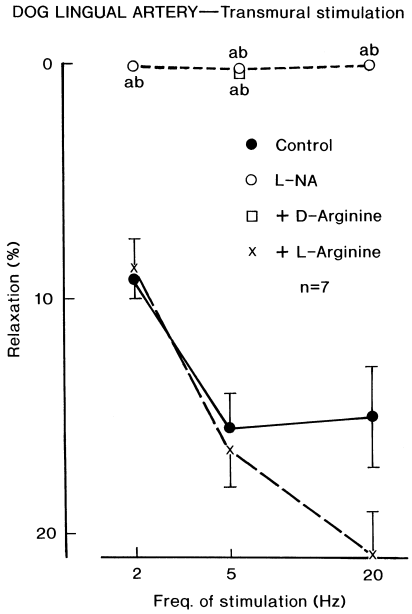

Fig. 2. Frequency-relaxant response curves for transmural electrical stimulation (2–20 Hz) in the presence of N^G-nitro-L-arginine (L-NA, 10^{-6} M), L-NA + D-arginine (3×10^{-4} M) and L-NA + L-arginine (3×10^{-4} M) in lingual arterial strips treated with prazosin (10^{-5} M) and α,β-methylene ATP (10^{-6} M) and partially contracted with prostaglandin $F_{2\alpha}$ (1 to 3×10^{-6} M). Relaxations induced by 10^{-4} M papaverine were taken as 100%. Significantly different from control, $^aP < 0.01$; significantly different from the value with L-NA + L-arginine, $^bP < 0.01$ (Tukey's test). Parameter *n* denotes the number of strips from different dogs. Vertical bars represent S.E.

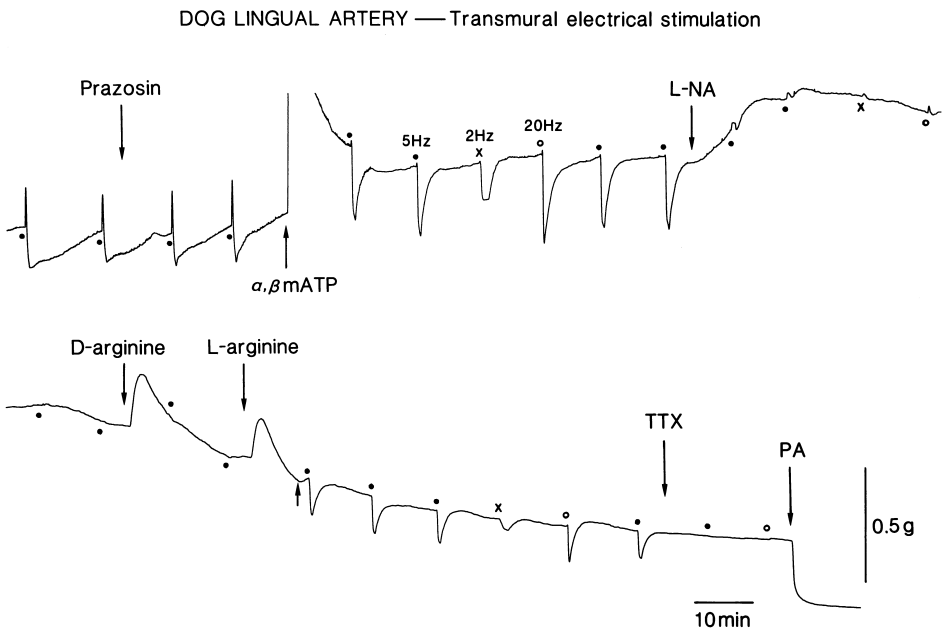

Fig. 3. Typical tracings of the response to transmural electrical stimulation at 2 (cross), 5 (solid circle) and 20 Hz (open circle) of a lingual arterial strip before and after treatment with prazosin (10^{-5} M), α,β-methylene ATP (α,βmATP, 10^{-6} M), N^G-nitro-L-arginine (L-NA, 10^{-6} M), D-arginine (3×10^{-4} M), L-arginine (3×10^{-4} M) and tetrodotoxin (TTX, 3×10^{-7} M). The recording continues from upper right to lower left. The strip was partially contracted with 10^{-6} M prostaglandin $F_{2\alpha}$. PA represents 10^{-4} M papaverine, which produced the maximal relaxation. The upward arrow indicates the addition of prostaglandin $F_{2\alpha}$ (0.5 to 1×10^{-6} M) to restore arterial tone.

not by D-arginine (3×10^{-4} M) (Fig. 2). Typical tracings of the response are illustrated in Fig. 3. The stimulation-induced relaxation was not influenced by timolol (10^{-7} M), atropine (10^{-7} M) or D-NA (10^{-6} M) but was abolished by L-NA (10^{-6} M) in seven strips from separate dogs,

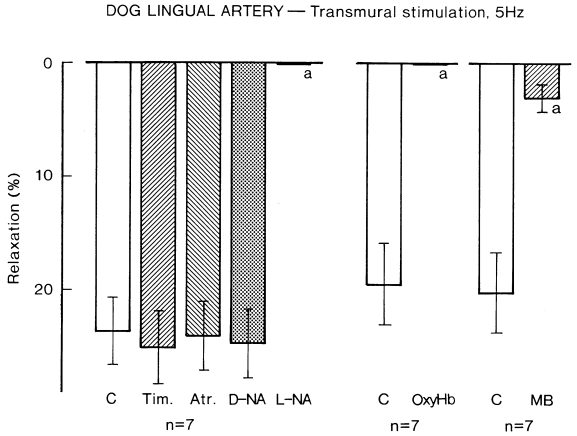

Fig. 4. Modification by timolol (Tim., 10^{-7} M), atropine (Atr., 10^{-7} M), N^G-nitro-D-arginine (D-NA, 10^{-6} M) and N^G-nitro-L-arginine (L-NA, 10^{-6} M) (for left panel), oxyhemoglobin (OxyHb, 1.6×10^{-5} M) (middle panel) and methylene blue (MB, 10^{-5} M) (right panel) of the relaxant response to transmural electrical stimulation (5 Hz) in lingual arterial strips treated with prazosin (10^{-5} M) and α,β-methylene ATP (10^{-6} M), and partially contracted with prostaglandin $F_{2\alpha}$ (1 to 3×10^{-6} M). Relaxations induced by 10^{-4} M papaverine were taken as 100%. Parameter n denotes the number of strips from different dogs. Significantly different from control (C), $^aP < 0.01$ (Tukey's test for the left panel, and unpaired t-test for the middle and right panels). Vertical bars represent S.E.

when these antagonists were applied successively. In different series of experiments, the neurogenic response was abolished or markedly suppressed by oxyhemoglobin (1.6×10^{-5} M) or methylene blue (10^{-5} M). The results are summarized in Fig. 4. Relaxation induced by nerve stimulation was not affected by aminophylline (20.8 ± 5.1 vs. $19.6 \pm 4.9\%$, $n = 6$), a P_1 receptor antagonist, in a concentration (2×10^{-5} M) sufficient to markedly inhibit the relaxation induced by ATP in the presence of α,β-methylene ATP. Mean values of the response to ATP before and after treatment with aminophylline were 16.2 ± 2.8 and 0% ($n = 7$, $P < 0.001$, unpaired t-test), respectively, at 10^{-6} M, and 52.3 ± 5.4 and $23.1 \pm 4.5\%$ ($n = 7$, $P < 0.01$, unpaired t-test), respectively, at 10^{-5} M. In the strips in which relaxations induced by electrical stimulation were abolished by L-NA, increasing the pulse width to 0.5 or 1.0 ms or the stimulation intensity up to four times as high as that usually used at frequencies of 5 or 20 Hz did not produce an additional relaxation that was abolished by tetrodotoxin (3×10^{-7} M) ($n = 6$).

3.2. Response to nicotine

In the strips treated with prazosin and α,β-methylene ATP and partially contracted with prostaglandin $F_{2\alpha}$, nicotine (10^{-4} M) produced a profound relaxation amounting to 50 to 80% of that induced by 10^{-4} M papaverine, which was abolished by hexamethonium (10^{-5} M). The relaxation rapidly developed to reach the maximal level, then declined gradually to stabilize at a level lower than

that prior to the addition of nicotine. The nicotine-induced relaxations were not influenced by treatment with aminophylline (2×10^{-5} M) (48.4 ± 6.3 vs. $50.8 \pm 8.9\%$, $n = 4$). Treatment with L-NA (10^{-5} M) attenuated the magnitude of relaxation, in addition to slowing the development of the response (cf. the tracings of upper two of the right column in Fig. 5). Raising the concentration of L-NA to 10^{-4} M did not produce additional inhibition ($n = 6$). The relaxation induced by a low concentration (5×10^{-6} M) of nicotine was abolished by L-NA (10^{-5} M) and restored by L-arginine (10^{-3} M) (Fig. 5, left column). The effects of L-arginine on the nicotine-induced relaxation are quantified in Fig. 6. Relaxations induced by exogenous NO (10^{-6} M), as acidified $NaNO_2$, were not influenced by L-NA (56.5 ± 3.9 vs. $61.8 \pm 3.6\%$, $n = 11$).

In order to determine whether involvement of NO and other factors in the response to the high concentration of nicotine was associated with the size of arteries, we determined the inhibitory effect of L-NA on the induced relaxation in artery strips taken proximal to the strips used in the present study, unless otherwise mentioned (Fig. 6, left panel). Although proximal arteries tended to respond to nicotine with a smaller relaxation than distal arteries, L-NA only partially inhibited the response in the proximal arterial strips.

The mechanisms underlying the nicotine-induced relaxation that was resistant to L-NA were analyzed in distal lingual arteries treated with 10^{-4} M L-NA. The relaxation was abolished in the strips made unresponsive to CGRP

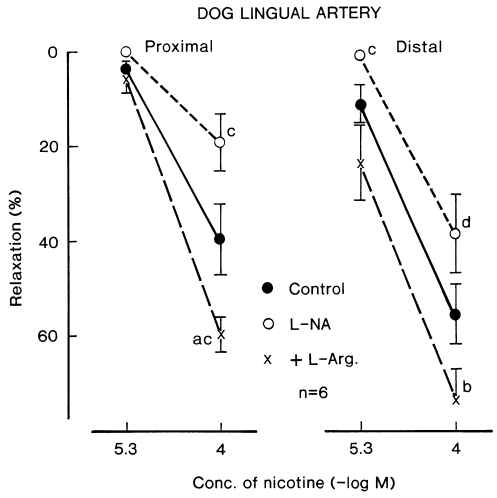

Fig. 6. Modification by N^G-nitro-L-arginine (L-NA, 10^{-5} M) and L-NA + L-arginine (L-Arg., 10^{-3} M) of the relaxant response to nicotine in low (5×10^{-6} M) and high (10^{-4} M) concentrations of proximal (left figure) and distal (right) lingual arterial strips treated with prazosin (10^{-5} M) and α,β-methylene ATP (10^{-6} M) and partially contracted with prostaglandin $F_{2\alpha}$ (0.5 to 3×10^{-6} M). Relaxations induced by 10^{-4} M papaverine were taken as 100%. Significantly different from the value with L-NA, $^{a}P < 0.01$, $^{b}P < 0.05$ (Tukey's test); significantly different from control, $^{c}P < 0.01$, $^{d}P < 0.05$ (paired *t*-test). Parameter *n* denotes the number of strips from different dogs. The proximal and distal arteries were obtained from the same dogs. Vertical bars represent S.E.

(10^{-8} M) by repeated application of the peptide, as indicated in the third tracing of the right column in Fig. 5. Quantitative data are included in Fig. 7. However, the

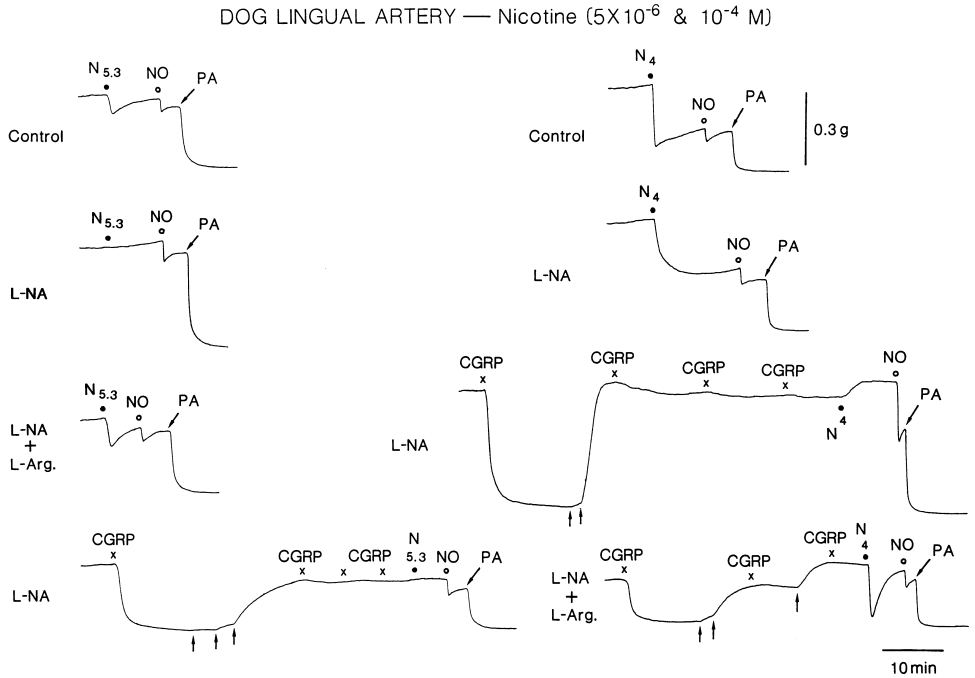

Fig. 5. Recordings of the response to nicotine (N) in low (5×10^{-6} M, left column) and high (10^{-4} M, right) concentrations and NO (10^{-6} M) of lingual arterial strips treated with prazosin (10^{-5} M) and α,β-methylene ATP (10^{-6} M) before and after treatment with N^G-nitro-L-arginine (L-NA, 10^{-5} M), L-NA + CGRP (10^{-8} M, three to four times) and L-NA + L-arginine (L-Arg., 10^{-3} M). Strips obtained from the same dog were partially contracted with prostaglandin $F_{2\alpha}$ (0.5 to 3×10^{-6} M). Upward arrows indicate the addition of prostaglandin $F_{2\alpha}$ (0.5 to 1.5×10^{-6} M) to raise arterial tone. PA represents 10^{-4} M papaverine, which produced the maximal relaxation.

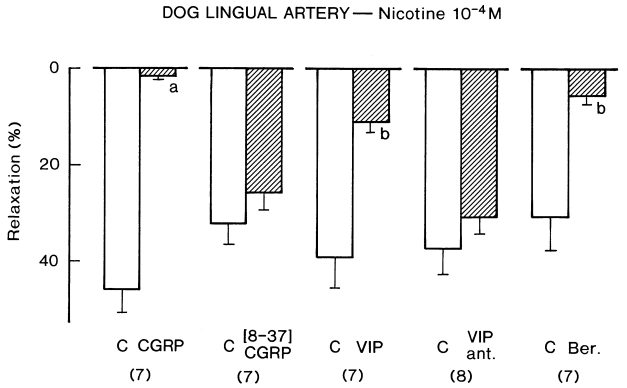

Fig. 7. Modification by CGRP tachyphylaxis (CGRP, made unresponsive to the peptide by applications of 10^{-8} M three or four times), CGRP-(8–37) (3×10^{-7} M), VIP tachyphylaxis (VIP, 10^{-8} M three or four times), [D-*p*-Cl-Phe^{6},Leu^{17}]VIP (VIP ant., 5×10^{-6} M) and beraprost (Ber., 10^{-6} M) of the response to nicotine (10^{-4} M) of lingual arterial strips treated with prazosin (10^{-5} M), α,β-methylene ATP (10^{-6} M) and N^{G}-nitro-L-arginine (10^{-6} M) and partially contracted with prostaglandin $F_{2\alpha}$ (0.5 to 3×10^{-6} M). Relaxations induced by 10^{-4} M papaverine were taken as 100%. Significantly different from control (C), $^{a}P < 0.001$, $^{b}P < 0.01$ (unpaired *t*-test). Numbers in parentheses indicate the number of strips from different dogs. Vertical bars represent S.E.

response was not significantly inhibited by CGRP-(8–37), a CGRP receptor antagonist, in a concentration (3×10^{-7} M) sufficient to markedly inhibit the relaxation induced by CGRP. Mean values of the response to 3×10^{-9} M CGRP before and after treatment with the CGRP antagonist were 77.8 ± 5.2 and $38.7\pm7.4\%$ ($n=12$, $P<0.001$, unpaired *t*-test), respectively. In the strips made tachyphylactic to VIP (10^{-8} M), the nicotine-induced relaxation was markedly inhibited, whereas [D-*p*-Cl-Phe^{6},Leu^{17}]VIP (5×10^{-6} M), a VIP receptor antagonist, was without effect. This antagonist significantly inhibited the relaxation induced by VIP (3×10^{-9} M) (53.5 ± 5.9 vs. $34.7\pm5.2\%$, $n=13$, $P<0.05$, unpaired *t*-test). CGRP and VIP produced marked relaxations, possibly associated with an increase in intracellular cyclic AMP. Therefore, beraprost, a stable analog of prostaglandin I_2 that increases cyclic AMP in vascular tissue, was used to test whether treatment with this agent, by causing a marked relaxation and increasing cyclic AMP, impaired the response to nicotine. The response was depressed by beraprost treatment (Fig. 7). Mean values of the relaxation elicited by the first application of CGRP (10^{-8} M), VIP (10^{-8} M) and beraprost (10^{-6} M) were $92.1\pm3.7\%$ ($n=5$), $85.7\pm6.2\%$ ($n=5$) and $74.9\pm6.1\%$ ($n=5$), respectively. The nicotine-induced relaxation under L-NA treatment was not influenced by timolol (10^{-7} M); mean values of the response before and after treatment were 38.6 ± 8.0 and $38.2\pm9.0\%$ ($n=7$), respectively. The substance P receptor antagonist, [D-Pro^{4},D-Trp^{7,9}]substance P-(4–11), at a concentration (10^{-7} M) sufficient to abolish the relaxation elicited by 10^{-9} M substance P, was also ineffective against the relaxation that was resistant to L-NA (41.5 ± 5.1 vs. $40.8\pm4.8\%$, $n=5$).

3.3. Histological study

There were abundant nerve fibers containing NADPH diaphorase in the adventitio-medial border and the adventitia of an oblique section of the dog lingual artery (Fig. 8). Similar data were obtained for two other dog arteries.

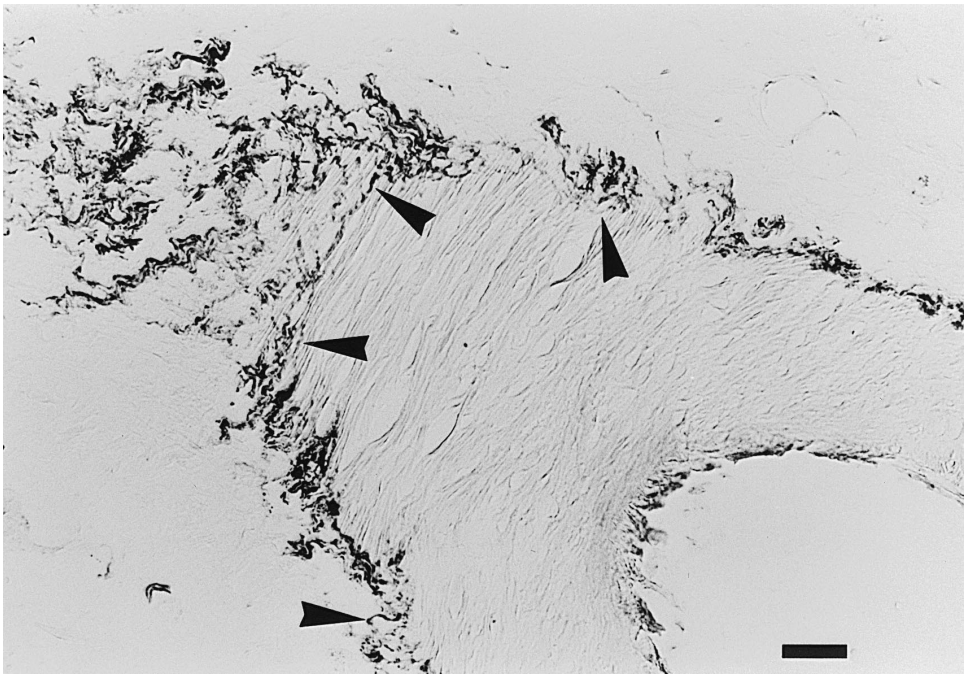

Fig. 8. Histochemistry of NADPH diaphorase in a cryostat section of the dog lingual artery with intact endothelium. The positively stained nerve fibers and bundles are seen in the adventitio-medial border (arrows) and the adventitia.

4. Discussion

Transmural electrical stimulation produced a frequency-dependent contraction in prostaglandin $F_{2\alpha}$-contracted canine lingual arterial strips denuded of the endothelium. The response was not significantly influenced by prazosin but was reversed to a relaxation by treatment with α,β-methylene ATP, which desensitizes the vascular contraction mediated via P_2X purinoceptors (Burnstock and Kennedy, 1985). Similar P_2X purinoceptor-mediated vasoconstriction has been observed in rabbit saphenous and rat renal arteries (Burnstock and Warland, 1987; Schwartz and Malik, 1989) and canine saphenous veins (Flavahan and Vanhoutte, 1986). The neurogenic relaxation in the strips treated with prazosin, α,β-methylene ATP and indomethacin was not influenced by timolol, aminophylline, atropine and D-NA but was abolished by treatment with L-NA, oxyhemoglobin, methylene blue and tetrodotoxin. The L-NA-induced inhibition was reversed by L-, but not D-arginine. Histochemical study demonstrated perivascular nerves containing NO synthase immunoreactivity, demonstrated by the NADPH diaphorase method, in the adventitia. These findings indicate that NO liberated from perivascular nerves acts as a neurotransmitter and activates soluble guanylate cyclase in smooth muscle for cyclic GMP production, resulting in arterial relaxation, as already postulated in canine and monkey arteries (Toda and Okamura, 1996). It is unlikely that ATP acts as a vasodilator transmitter by stimulating P_1 receptors. As far as the results obtained with electrical nerve stimulation are concerned, no evidence of involvement of other vasodilator substances was obtained.

The addition of nicotine (10^{-4} M) elicited profound, prolonged relaxations in endothelium-denuded, indomethacin-treated lingual arteries. The response was abolished by hexamethonium, indicating that neural activation was involved. The nicotine-induced relaxation was not inhibited by aminophylline, indicating again that ATP and P_2Y receptors were not involved in the neurogenic relaxation. Treatment with high concentrations of L-NA (10^{-5} M) partially attenuated the nicotine-induced relaxation, and L-arginine reversed the inhibition, suggesting the involvement of neurogenic NO. Additional treatment with 10^{-4} M L-NA failed to further inhibit the response. Therefore, relaxing factors other than NO appear to participate in the relaxation induced by nerve stimulation. The L-NA-resistant relaxation elicited by nicotine was not reduced by timolol in a concentration sufficient to depress the arterial relaxation mediated by β-adrenoceptor stimulation (Shiraishi et al., 1997) or by [D-Pro^4,D-Trp^{7,9}]substance P-(4–11), a substance P receptor antagonist (Caranikas et al., 1982). In the strips made unresponsive to CGRP or VIP by repeated application of the peptides, the response was markedly inhibited; however, treatment with CGRP-(8–37), a $CGRP_1$ receptor antagonist (Chiba et al., 1989), or [D-*p*-Cl-Phe^6,Leu^{17}]VIP, a VIP receptor antagonist (Pandol et al., 1986), did not significantly alter the action of nicotine. Therefore, CGRP and VIP are unlikely to contribute to the action, but the same mechanisms underlying the relaxation induced by CGRP and VIP may be involved. These peptides dilate a variety of blood vessels through the mediation of cyclic AMP (Itoh et al., 1985; Ganz et al., 1986; Poyner, 1992), and treatment with substances that increase cellular cyclic AMP concentrations inhibit the relaxant response to agonists in which cyclic AMP is an important intracellular messenger (Matsumoto et al., 1993; Okamura et al., 1997). In order to support the hypothesis that the relaxation produced by nicotine in the L-NA-treated strips is mediated by substances that stimulate adenylate cyclase in smooth muscle, another compound, beraprost, a stable analog of prostaglandin I_2 (Toda, 1988) which increases cyclic AMP concentrations, was used. Treatment with high concentrations of this compound suppressed the nicotine-induced relaxation, as did high concentrations of CGRP and VIP. These findings indicate that β-adrenoceptor agonists, cyclooxygenase products, substance P, CGRP and VIP, are excluded as factors that are possibly liberated from vasodilator nerves to elicit arterial relaxation in association with increased production of cyclic AMP.

Nitric oxide and the other relaxing factors from nerves participate in the nicotine (high concentration)-induced relaxation in proximal and distal lingual arteries, whereas NO seems to be the sole compound involved in producing relaxation induced by nicotine (low concentration) and electrical nerve stimulation, since the responses to the latter were abolished only by treatment with the NO synthase inhibitor. The results with nicotine suggest that weak stimulation liberates NO and strong stimulation causes the release of NO and other factors. However, this was not true in the case of electrical stimulation of nerves, since even though the frequency, intensity and pulse width of stimuli were raised, tetrodotoxin-sensitive, L-NA-resistant relaxations were not obtained. The mechanisms underlying the distinct ability of nicotine and electrical nerve stimulation to stimulate the nerves innervating the arterial wall have not been elucidated. Of the various canine and monkey arteries tested so far (Toda and Okamura, 1990a,b, 1992c; Okamura et al., 1993; Okamura and Toda, 1994), the canine lingual artery is the only preparation in which different mechanisms for the neurogenic relaxation have been observed. Huckabee et al. (1993) have reported that snuff applied to the buccal space increases plasma nicotine concentrations and blood flow in cheek mucosa and tongue, although the mechanism of the vasodilator action is not known.

The present functional study revealed that the canine lingual artery has vasoconstrictor and vasodilator innervation. Under the experimental conditions used, the neurogenic vasoconstriction is likely to be mediated mainly by ATP, which activates P_2X purinoceptors, and the vasodilatation appears to be associated with the release of NO.

Additional vasodilator nerves may also be stimulated by high concentrations of nicotine, which liberates substances that increase the content of cyclic AMP in smooth muscle.

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