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Involvement of Nitric Oxide in Parasympathetic and Antidromic Vasodilatations in Cat Lower Lip

Hirotaka Suzuki, Naofumi Iwatsuki, Keishiro Karita[1] and Hiroshi Izumi[1]

Departments of Anesthesiology and [1]Orofacial Functions, Tohoku University School of Dentistry, Sendai 980-8575

Suzuki, H., Iwatsuki, N., Karita, K. and Izumi, H. *Involvement of Nitric Oxide in Parasympathetic and Antidromic Vasodilatations in Cat Lower Lip.* Tohoku J. Exp. Med., 2000, **191** (2), 59-70 —— The involvement of nitric oxide (NO) in the lower lip vasodilatations mediated via parasympathetic and antidromic mechanisms was examined in α-chloralose/urethane-anesthetized cats, with the two types of blood flow responses being recorded separately (by laser Doppler flowmeter) from the two sides of the lower lip. The central cut end of the lingual nerve (LN) or the peripheral cut end of the inferior alveolar nerve (IAN) was electrically stimulated to elicit parasympathetic or antidromic vasodilatation, respectively, in the lower lip. N^G-nitro-L-arginine methyl ester (L-NAME), but not N^G-nitro-D-arginine methyl ester (D-NAME) (each at 30 mg/kg), markedly reduced the increases in lip blood flow evoked by stimulation, the reduction being to a similar degree irrespective of whether LN or IAN was stimulated. Pretreatment with L-arginine did not prevent the L-NAME-induced attenuation of either type of vasodilatation. In conclusion, these results suggest that synthesized NO may have a common site of action in antidromic and parasympathetic vasodilator pathways to the cat lower lip. ———— parasympathetic; antidromic; vasodilatation; nitric oxide; cat

Nitric oxide synthase (NOS) inhibitors such as N^G-nitro-L-arginine methyl ester (L-NAME), have been reported to inhibit both the antidromic vasodilator response to an irritant oil in rat hindpaw skin (Lippe et al. 1993; Holzer and Jocic 1994) and vasodilator responses to parasympathetic nerve stimulation in the orofacial region (in the submandibular gland [SMG] of the pig [Modin et al. 1994] and cat [Edwards et al. 1996] and in the cat lower lip [Izumi et al. 1997a]). However, Kerezoudis et al. (1993b) found that infusion of L-NAME actually enhanced antidromic vasodilatation in the dental pulp and lip of the rat in the presence in α-adrenoceptor blockers. They suggested that nitric oxide

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Address for reprints: Hiroshi Izumi, Ph.D., Department of Orofacial Functions, Tohoku University School of Dentistry, Sendai 980-8575, Japan.

e-mail: izumi@physiol.dent.tohoku.ac.jp

(NO) may exert a prejunctional effect by inhibiting the release of vasoactive peptides, especially substance P, from afferent nerves. This lack of inhibition by L-NAME on antidromic vasodilatation may be due to absence of NOS activity, since no evidence for NOS activity has been obtained in pulp nerve fibers (Kerezoudis et al. 1993c). This may suggest that the inhibition obtained with L-NAME may have some selectively for parasympathetic vasodilator fibers contain NOS (Modin et al. 1994). However, this is not a tenable explanation for the lack of inhibition by L-NAME observed in the lip by Kerezoudis et al. (1993b), since positive staining for NOS has been reported in both parasympathetic and trigeminal nerves innervating such periodontal tissues as lip and gingiva (Ceccatelli et al. 1994; Alm et al. 1995; Blottner et al. 1995) as well as in the endothelial cells of periodontal and gingival blood vessels (Kerezoudis et al. 1993c). The above discrepancy concerning the effects of L-NAME on the lip vasodilator responses elicited by parasympathetic and antidromic mechanisms may be due to differences in methodology such as (i) the diameter of the blood vessels under study (Kurz et al. 1991; Moncada et al. 1991), (ii) the sites used for electrical stimulation of nerves and for blood flow measurements, and (iii) the anesthetic agents used. There may also be differences between the rat and cat in the role of endothelium-derived relaxing factor (now known to be NO) in the maintenance of vascular tone (Kerezoudis et al. 1993b) and/or in the role of NO in peptide release from parasympathetic and trigeminal nerves (Gustafsson et al. 1990; Modin et al. 1994).

To examine the involvement of NO in the vasodilatations mediated by activation of parasympathetic and antidromic mechanisms in the same vascular bed under the same conditions, we designed the experiments so that these two types of vasodilatation could be easily and separately evoked in one and the same cat, one on each side of the lower lip (Izumi and Karita 1990, 1992; Izumi et al. 1990; Izumi and Ito 1998; Izumi 1999a). We needed to be cautious in selecting the vascular bed used in this study since L-NAME has been reported to inhibit not only NOS, but also muscarinic receptors (Buxton et al. 1993). The vascular bed of the cat's lower lip was a suitable one for two main reasons: (i) because the parasympathetic- and antidromic-mediated vasodilatations in this bed has been reported to be exclusively mediated by non-muscarinic receptors since prior administration of antimuscarinic agents such as atropine and scopolamine had no effects on both vasodilatations (Izumi et al. 1990; Izumi and Karita 1991, 1992), and (ii) because unilateral electrical stimulation of the peripheral cut end of the inferior alveolar nerve (IAN) elicits a vasodilator response on the same side that is mediated only via an antidromic mechanism (Izumi and Karita 1990) and electrical stimulation of the central cut end of the lingual nerve (LN) elicits a parasympathetic-mediated reflex vasodilatation that is restricted to one side (the ipsilateral side) of this bed (Izumi and Ito 1998; Izumi 1999b).

Materials and Methods

Preparation of animals

Thirty adult cats, unselected as to sex and of 3.5–4.5 kg body weight, were initially sedated with ketamine hydrochloride (30 mg/kg, i.m.) and then anesthetized with a mixture of α-chloralose (50 mg/kg, i.v.) and urethane (100 mg/kg, i.v.). These anesthetics were supplemented if and when necessary throughout the experiment. The anesthetized animals were intubated, paralyzed by intravenous injection of pancuronium bromide (Mioblock; Organon, Teknika, Boxtel, The Netherlands; 0.4 mg/kg initially, supplemented with 0.2 mg/kg every hour or so after testing the level of anesthesia; see below), and artificially ventilated via the tracheal cannula with a mixture of 50% air-50% O_2. The ventilator (Model SN-480-6; Shinano, Tokyo) was set to deliver a tidal volume of 10–12 cm^3/kg at a rate of 20 breaths/minute, and the end-tidal concentration of CO_2 was determined by means of an infrared analyzer (Capnomac Ultima; Datex Co., Helsinki, Finland) as reported previously (Izumi 1999b). Blood pH, PaO_2, and $PaCO_2$ data were obtained at intervals of 90 minutes using a blood-gas analyzer (Model 148; Ciba-Corning, Medfield, MA, USA) and ventilation was adjusted to keep these parameters within normal limits. Normal (0.9%) saline solution (Otsuka Pharmaceutical Co., Tokyo) was continuously infused at a rate of approximately 8 ml/hour, and 8.4% $NaHCO_3$ solution was added if necessary (both solutions from Otsuka Pharmaceutical Co.). Rectal temperature was maintained at 37–38°C using a heating pad.

The criteria for maintenance of an adequate depth of anesthesia were the persistence of miotic pupils and the absence of a reflex elevation of heart rate and arterial blood pressure during stimulation of the central cut end of the lingual nerve. If the depth of anesthesia was considered inadequate, additional α-chloralose and urethane (i.e., intermittent doses of 5 mg/kg and 10 mg/kg i.v., respectively) was administered. Once an adequate depth of anesthesia had been attained, supplementary doses of pancuronium were given approximately every 60 minutes to maintain immobilization during periods of stimulation. We have established that when reflex stimulation is repeated at intervals of 10 minutes under the anesthetic conditions used in the present experiments, similar vascular effects can be evoked for up to 5–6 hours, indicating that highly reproducible responses can be obtained using our reflex method (Izumi et al. 1997b; Izumi and Ito 1998; Izumi 1999a). The above methodology is now well established in our laboratory for experiments on blood flow responses to nerve stimulation in cats (e.g., Izumi 1999b).

In all experiments, the vagus and superior cervical sympathetic trunks were cut bilaterally in the neck prior to any stimulation. All cats were killed, at the end of the experiment, by an overdose (about 150 mg) of sodium pentobarbital.

The experimental protocols were reviewed by the Committee on the Ethics of

Animal Experiments in Tohoku University School of Medicine, and they were carried out in accordance with both the Guidelines for Animal Experiments issued by Tohoku University School of Medicine and The Law (No. 105) and Notification (No. 6) issued by the Japanese Government. All animals were cared for and used in accordance with the recommendations in the current National Research Council guide.

Electrical stimulation of LN and IAN

The present study involved principally electrical stimulation of the central cut end of the LN (A in Fig. 1) or the peripheral cut end of the IAN (B in Fig. 1). In this study, nerves were sectioned and stimulated unilaterally under a binocular microscope. A bipolar silver electrode attached to a stimulator (Model SEN-7103, Nihon Kohden, Tokyo) was used for stimulation. The LN was stimulated for 20 seconds using a supramaximal voltage (30 V) at 10 Hz with pulses of 2 milliseconds duration. The IAN was dissected free from the mandibular canal on the side on which antidromic lip blood flow (LBF) responses were to be measured, and stimulated for 20 seconds using supramaximal voltage (30 V) at 10 Hz with pulses of 2 milliseconds duration.

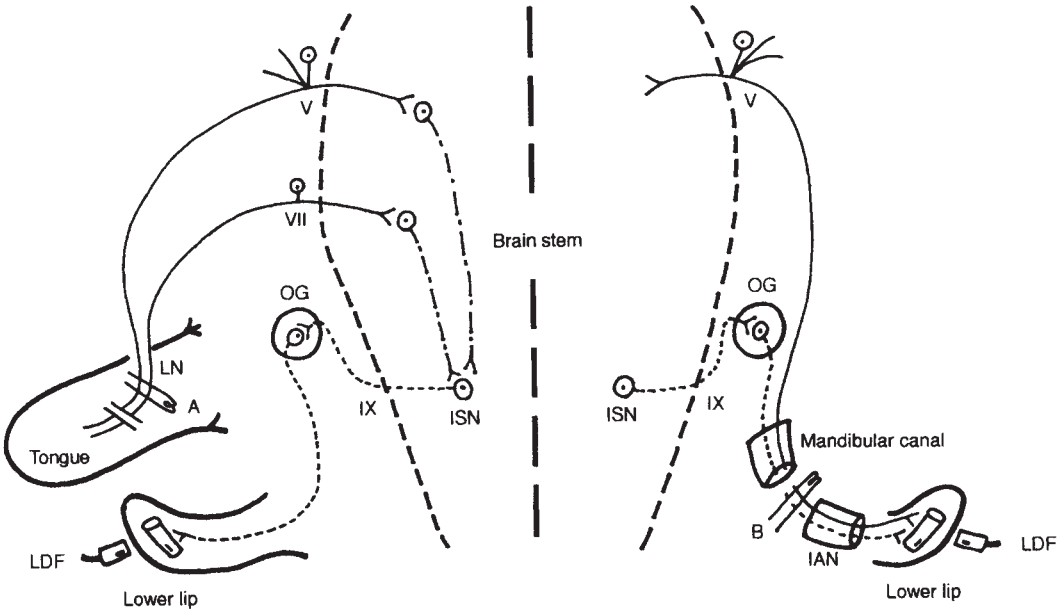

Fig. 1. Schematic representation of the sites of electrical stimulation for experiments illustrated in Figs. 2–4. Stimulation sites: The central cut end of the lingual nerve (A) and the peripheral cut end of the inferior alveolar nerve (B). The dotted lines indicate the parasympathetic vasodilator fibers from the inferior salivatory nuclei. The solid lines indicate trigeminal and facial sensory inputs to the brain stem. The dash-dotted lines indicate brain stem connections between primary afferents and parasympathetic preganglionic neurons. IAN, inferior alveolar nerve; ISN, inferior salivatory nucleus; LDF, laser-Doppler flowmeter; OG, otic ganglion; V, trigeminal nerve root; VII, facial nerve root; IX, glossopharyngeal nerve root.

Measurement of lower LBF

Blood flow was monitored in the lower lip adjacent to the canine tooth on the side ipsilateral to the stimulated side using a laser-Doppler flowmeter (LDF; ALF21R, Advance, Tokyo), as described before (Izumi and Karita 1993, 1994; Izumi et al. 1995, 1997b; Karita and Izumi 1995; Izumi and Ito 1998). The probe was placed against the lower lip without exerting any pressure on the tissue. The LDF values obtained in this way represent the blood flow in superficial vessels. Previous studies have indicated a significant correlation between blood flow recordings from oral tissues obtained by LDF and by other well-established methods (Edwall et al. 1987; Kim et al. 1990). The analog output of the equipment does not give absolute values, but shows relative changes in blood flow (for technical details and evaluation of the LDF method: see Stern et al. [1977]). Electrical calibration for zero blood flow was performed for all recordings. Several gains were selectable and the maximum output of a given gain level (defined electrically) was taken as 100%. At the settings used in this study, the ratio between the magnitude of the LBF increases and the amplitude of the baseline fluctuations ("signal to noise ratio") was 8–10 when either the IAN or LN was stimulated with a supramaximal voltage. The output from the various devices was continuously displayed on an 8-channel chart recorder (Model W5000; Graphtec, Tokyo) at a speed of 10 mm/minute. The magnitude of the blood flow changes elicited by nerve stimulation was assessed by making measurements in mm on the chart record. In the figures, flow levels are expressed in arbitrary units.

Measurements of basal LBF level and of systemic arterial blood pressure

The changes occurring in the basal LBF level (increase or decrease) after infusion of drugs were calculated as the percentage change (100% being taken as the response to LN stimulation at 10 Hz with pulses of 2 milliseconds duration for 20 seconds)

Systemic aortic blood pressure was recorded from a femoral catheter via a Statham pressure transducer. A tachograph (Model AT-610G; Nihon Kohden, Tokyo) triggered by the arterial pulse was used to monitor heart rate.

Drugs

L-NAME, D-NAME, and L-arginine (Nakalai tesque, Kyoto) were dissolved in normal saline immediately before injection. L-NAME, D-NAME, and L-arginine were each given as an intravenous bolus infusion. When L-arginine was used, it was given as pretreatment beginning 15 minutes before the start of an L-NAME infusion. L-NAME (30 mg/kg) was used to inhibit the synthesis of NO since the vasodilator response evoked by parasympathetic (chorda lingual nerve) stimulation has been reported to be largely abolished by L-NAME at this dose in the cat

submandibular gland (Edwards et al. 1996). All other chemicals were of reagent grade and were obtained from commercial sources.

Statistical analysis

All numerical data are given as the mean±S.E.M. The significance of changes in the test responses was assessed using a paired or unpaired Student's t-test, Welch's t-test, or an analysis of variance (ANOVA) followed by a contrast test. Differences were considered significant at the level $p<0.05$. Data were analyzed using a Macintosh Computer with StatView 5.0 and Super ANOVA (Abacus Concepts, Berkley, CA, USA).

Results

Effects of L-NAME and D-NAME on LBF increases

Typical recordings of the effects of L-NAME, D-NAME, and L-arginine plus L-NAME on the LBF increases evoked by electrical stimulation of the central cut end of LN and of the peripheral cut end of IAN are shown in Fig. 2, and averaged

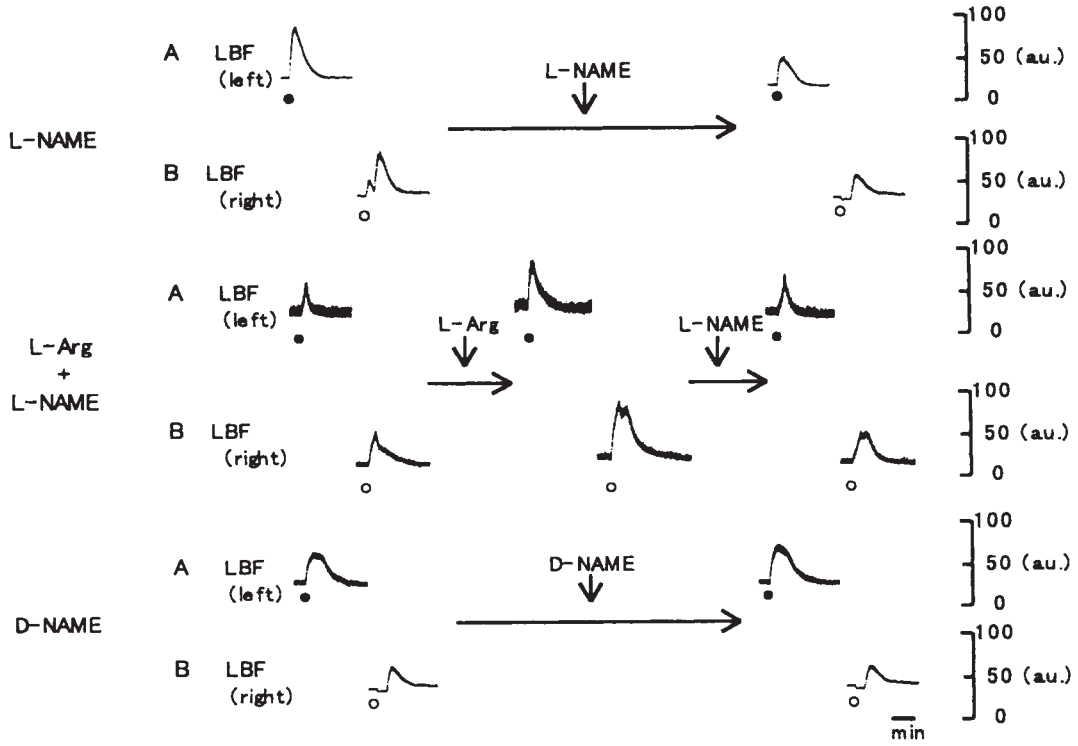

Fig. 2. Typical examples of the effects of L-NAME, D-NAME, and L-arginine plus L-NAME on vasodilator responses evoked by stimulation of the central cut end of the LN or the peripheral cut end of the IAN in vago-sympathectomized cats. The LN and IAN were stimulated where indicated by filled circles and open circles, respectively, 10–15 minutes after the end of iv infusion of L-NAME (30 mg/kg), D-NAME (30 mg/kg), or L-arginine (300 mg/kg). L-arginine was administered 15–20 minutes before L-NAME infusion. Stimulation was for 20 seconds at a supramaximal voltage (30 V) at 10 Hz with pulses of 2 milliseconds duration. Ordinates, lower lip blood flow (LBF) in arbitrary units (a.u.).

data in Fig. 3. L-NAME (30 mg/kg) reduced the LBF increases evoked by LN and IAN stimulation to 52.9±6.7% of control ($p<0.01$, $n=11$) and 47.3±6.7% ($p<0.01$, $n=10$) of control, respectively. D-NAME did not affect the LBF increases evoked by LN or IAN stimulation (94.2±8.7% of control, $n=6$, and 95.9±8.8% of control, $n=5$, respectively; both $p>0.05$).

Effects of L-arginine

Typical recordings of the effects of bolus infusions of L-arginine (300 mg/kg) alone and of L-arginine plus L-NAME (30 mg/kg) on the LN- and IAN-induced increases in LBF are shown in Fig. 2, with averaged data in Fig. 3. The LBF increases evoked by electrical stimulation of LN or IAN were both significantly greater in the presence of L-arginine than in its absence (150.6±10.0% of control response, $p<0.05$, $n=5$, and 202.8±45.0% of control response, $p<0.05$, $n=4$, respectively). The percentage reductions produced by L-NAME in the LN- and IAN-induced LBF increases were similar whether L-arginine was absent or present

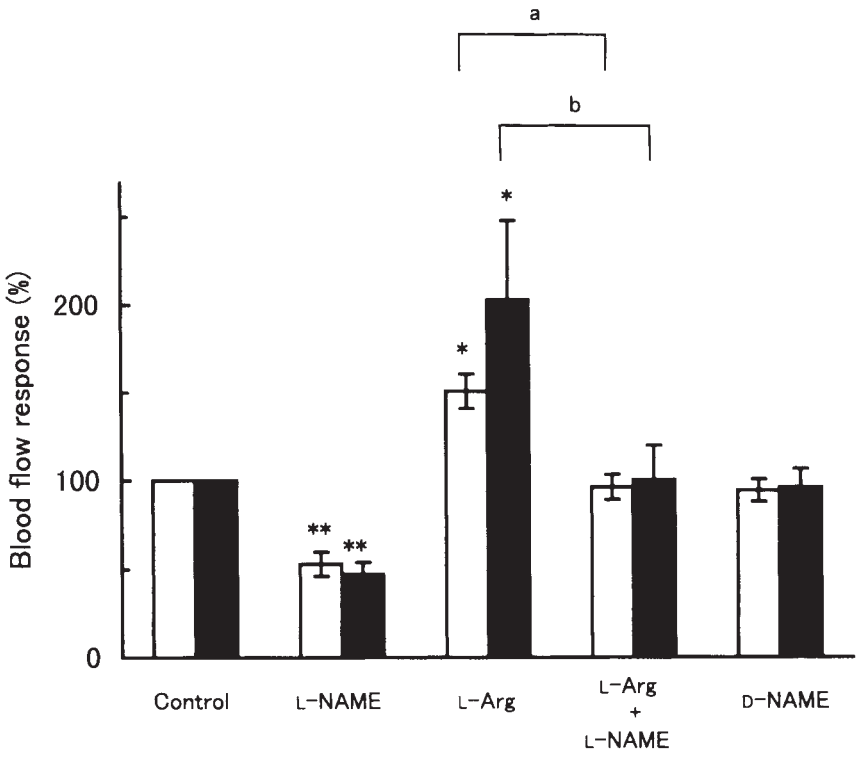

Fig. 3. Mean data (±S.E.M.) for the effects of L-NAME, D-NAME, and L-arginine (L-Arg) on vasodilator responses evoked by stimulation of the central cut end of the LN (open columns) and the peripheral cut end of the IAN (filled columns) in vago-sympathectomized cats. Experimental conditions as for Fig. 2. The ordinate shows the evoked change in LBF (expressed as a percentage of the response to LN or IAN stimulation in the absence of drug; "control"). Statistical significance from control was assessed by means of ANOVA followed by a contrast test. $*p<0.05$, $**p<0.01$, vs. control. "a" and "b" indicate significant difference between the "L-arginine" and "L-arginine+L-NAME" data (unpaired Student's t-test).

(47.1% vs. 36.1%, respectively, for LN stimulation, n.s, ANOVA) and 52.7% vs. 50.6%, respectively, for IAN stimulation (n.s, ANOVA). In nine anesthetized cats, at 20 minutes after administration of L-NAME mean aortic blood pressure had risen from 98.9 ± 2.3 to 120.5 ± 8.5 mmHg ($p < 0.05$, paired t-test, $n = 9$). A prior intravenous bolus infusion of L-arginine (300 mg/kg) evoked a decrease in mean aortic blood pressure of 13.8 ± 5.1 mmHg ($p < 0.05$, paired t-test, $n = 6$), but did not reverse the pressor effect of L-NAME (123.8 ± 4.8 mmHg, $p < 0.05$ vs. control, $n = 5$). The effects of continuous administration of L-arginine (300 mg/kg + 120 mg/kg per hour for 30 minutes) on the LBF increases were somewhat similar to those of its bolus infusion (300 mg/kg); continuous administration of L-arginine increased the LN- and IAN evoked LBF responses to 149.7 ± 10.7 and 167.4 ± 17.3%, respectively, of control. The percentage reductions produced by L-NAME in the two types of LBF response were not significantly different between tests employing bolus and continuous infusions of L-arginine.

Discussion

We have previously reported that electrical stimulation of the peripheral cut end of the IAN elicits one of three different patterns of vascular response in the cat gingiva and lower lip: vasodilatation (80%, 45/56 animals), vasoconstriction (13%, 7/56 animals), or a biphasic response (vasoconstriction followed by vasodilatation: 7%, 4/56 animals)(Izumi and Karita 1990; Izumi et al. 1990; Izumi 1995, 1999b). These vasodilator and vasoconstrictor responses or components were considered to be due to activation of sensory fibers and sympathetic α-adrenergic fibers, respectively, since the former was abolished by prior treatment with capsaicin, the pungent agent of the hot pepper, and the latter was abolished by phentolamine, an adrenergic α-receptor blocker. Thus, the IAN-induced vasodilatation is mediated exclusively via an antidromic mechanism. At present, it is unclear why parasympathetic-mediated vasodilatation does not occur in the lower lip on electrical stimulation of the peripheral cut end of the IAN even though we found evidence of parasympathetic vasodilator fibers in the IAN originating from the otic ganglion in a previous study (Izumi and Karita 1992). In the present study, animals in which a pure vasodilator response (with no preceding vasoconstriction) occurred on electrical stimulation of the IAN were used for our examination of the effects of a NOS inhibitor on the IAN-induced antidromic vasodilatation. We decided on this approach to avoid having to use α-adrenoceptor blockers such as phentolamine and phenoxybenzamine, since antidromic vasodilatation in the rat dental pulp seems to be enhanced when these agents are present (Kerezoudis et al. 1993a), and since sympathetic vasoconstriction may modulate a vasodilator response evoked by antidromic stimulation (although pretreatment with an α-adrenoceptor blocker did not enhance the IAN-induced vasodilatation) (Izumi 1999b).

To evoke parasympathetic vasodilatation, we used a "pseudo-reflex" method

(electrical stimulation of the central cut end of the LN) to activate parasympathetic efferents, as reported before (Izumi 1995, 1999b). The parasympathetic nature of this response is supported by our previous observations that prior administration of an autonomic ganglion blocking agent (hexamethonium) or section of the glossopharyngeal nerve root abolished the vasodilatation evoked in the lower lip by electrical stimulation of the central cut end of the infraorbital nerve or LN (Izumi and Karita 1992; Izumi 1999b). We chose this method because it is not possible selectively to activate parasympathetic vasodilator fibers to the lower lip when the peripheral cut ends of nerves such as IAN are electrically stimulated. Essentially, we believe that this pseudo-reflex stimulation mimics processes occurring during physiological forms of parasympathetic vasodilatation.

As shown in Figs. 2 and 3, in our cats the LBF increases elicited by electrical stimulation of either LN or IAN were reduced by pretreatment with L-NAME. These effects were stereoselective since D-NAME was ineffective in reducing either response. In magnitude, the two reductions were not significantly different. This finding is in sharp contrast to the observation of Kerezouidis et al. (1993b) that the blood flow increases (measured by LDF) in the rat lip and dental pulp following electrical stimulation of the IAN were enhanced by the NO inhibitor, L-NAME. Besides the species difference and the use of α-adrenoceptor blockade in the earlier study, we can offer no persuasive explanation for this discrepancy.

At first sight, the addition of L-arginine, regardless of whether it was given as an intravenous bolus infusion (300 mg/kg) or by continuous infusion (300 mg/kg followed by 120 mg/kg per hour for 30 minutes) seemed to prevent the attenuating effects of L-NAME on the LBF increases elicited by LN and IAN stimulation in the cat lower lip (compare "control," "L-NAME," and "L-arginine+L-NAME" in Fig. 3). This result seemed to be at variance with that of Kerezoudis et al. (1993b) who reported that L-arginine did not reverse the L-NAME-induced enhancing effect on the blood flow increases in the rat lower incisor and lip evoked by electrical stimulation of the IAN (although they did not do experiments to test the effect of L-arginine alone on these blood flow increases). However, the present data should probably be analyzed in a different way since L-arginine itself enhanced the LBF increases evoked by both LA and IAN stimulation (Fig. 3). When we use the LBF increases evoked in the presence of L-arginine as our control responses, injection of L-NAME in the presence of L-arginine reduced the LBF increases evoked by LN and IAN stimulation by 36.1% and 50.6%, respectively. These percentage decreases correspond well to those obtained when L-NAME was given in the absence of L-arginine (47.1% and 52.7%, respectively). On this basis, our data do not support the idea that L-arginine attenuates the effect of L-NAME on the LBF increases elicited by IAN and LN stimulation. The possibility that L-NAME exerts these inhibitory effects via an antimuscarinic effect had to be considered since inhibition of muscarinic receptors by L-NAME has been reported to occur in vitro (Buxton et al. 1993). However, this seems an

untenable explanation since prior administration of antimuscarinic agents such as atropine and scopolamine has no effect on either type of vasodilatation in this vascular beds (Izumi et al. 1990; Izumi and Karita 1991, 1992). Moreover, the inhibitory effect of L-NAME seen in vitro has not been observed in vivo: muscarinic activation in the mesenteric and pulmonary vascular beds of intact cats was not blocked by L-NAME (Cheng et al. 1994; Santiago et al. 1994). Further, whichever way we analyze these data one conclusion remains the same: namely, in the cat lower lip reflex parasympathetic vasodilatation and antidromic vasodilatation respond in a very similar way to L-NAME.

It has been suggested that NO is involved in the regulation of neurogenic vasodilatation in the rat lip (Kerezoudis et al. 1993b), rabbit ear (Khan et al. 1993), pig and cat SMGs (Modin et al. 1994; Edwards et al. 1996), and canine lip (Okamura et al. 1999). Modin et al. (1994) reported that in the submandibular gland of the pig, NO may act at both the prejunctional and postjunctional levels to exert its function as an intracellular regulator of the parasympathetic vascular control system. However, Edwards et al. (1996) were able to establish that the role of NO in parasympathetic vasodilatation in the cat SMG is largely or entirely postsynaptic. They did this by showing that the postsynaptic effects of NO can be substantially reduced without affecting its role in presynaptically mediating the release of vasoactive intestinal polypeptide (VIP) in the cat SMG. On the other hand, Okamura et al. (1999) have recently suggested that the relaxation of canine labial arteries induced by transmural nerve stimulation is mediated exclusively by NO synthesized from L-arginine within the nerve terminal. These results suggest that the sites of involvement of NO vary from tissue to tissue and from species to species, and whether NO is derived from the vasodilator nerve, the endothelium, or both has yet to be determined in the case of the cat lower lip. To enable us to elucidate the relative importance of neural versus endothelial NO synthase to the parasympathetic and antidromic vasodilatations in the cat lower lip, agents that are more selective than L-NAME are required.

In conclusion, the vasodilatations in the cat lower lip elicited by activation of antidromic or parasympathetic mechanisms were attenuated by the NO inhibitor, L-NAME, each to much the same extent. Regardless of how we analyzed the effect of pretreatment with L-arginine on these L-NAME-induced attenuations, the effect of L-arginine was not different between antidromic and parasympathetic vasodilatations. This may suggest that in these two vasodilator mechanisms, NO has a common target site, although the present experiments do not tell us whether NO is acting prejunctionally or postjunctionally. Further studies will be needed on this point.

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