Parasympathetic reflex vasodilatation in rat submandibular gland

KEN T AO MIZUT A, KEISHI RO KARITA, AND HIROSHI IZUMI
Departments of Orofacial Functions and Pediatric Dentistry, Tohoku University School of Dentistry, Sendai 980-8575, Japan

Received 18 January 2000; accepted in final form 22 March 2000

Mizuta, Kentaro, Kei shiro Karita, and Hiroshi Izumi. Parasympathetic reflex vasodilatation in rat submandibular gland. Am J Physiol Regulatory Integrative Comp Physiol 279: R677–R683, 2000.—The present study was designed to investigate 1) whether parasympathetic reflex vasodilatation occurs in the submandibular gland (SMG) in deeply urethan-anesthetized, cervically vagotomized, and sympathectomized rats when the central cut end of the lingual nerve (LN) is electrically stimulated and 2) to what extent the neural mechanisms underlying such responses are the same as those involved in the response to direct stimulation of the chorda-LN (CLN). Stimulation of each nerve separately elicited a marked blood flow increase in SMG. Section of the chorda tympani abolished the SMG blood flow response but had no effect on the lip blood flow increase evoked by LN stimulation. Section of the CLN abolished the SMG blood flow increases evoked by stimulation of either nerve. The SMG blood flow increases (regardless of whether they were evoked by LN or CLN stimulation) were markedly reduced by the autonomic cholinergic ganglion blocker hexamethonium. The present study demonstrates that a parasympathetic reflex vasodilator mechanism is present in the rat SMG and that it can express its effects under deep general anesthesia.

autonomic reflex; autonomic ganglionic blocker; atropine; orofacial area; vasoresponse

SALIVARY SECRETION is known to occur as a reflex response to a sensory (gustatory and/or trigeminal) stimulus applied to oral or perioral regions in both humans and animals (28, 32). However, studies of the autonomic mechanisms influencing salivary secretion and blood flow in the submandibular gland (SMG) in anesthetized animals such as the rat and cat have usually been done by examining nonreflex responses [e.g., stimulating the peripheral cut end of 1) the superior cervical sympathetic trunk (CST) to produce sympathetic effects (5, 6, 10, 25, 27) or 2) the chorda-lingual nerve (CLN) or chorda tympani (CT) to produce parasympathetic effects (2–4, 7, 8, 13, 30, 31, 34, 35)]. One reason for this is that deep anesthesia has been considered to reduce reflex responses involving salivation and blood flow changes in the SMG (1).

We have reported elsewhere that in urethan-chloralose-anesthetized cats, parasympathetically mediated responses (vasodilatation in the lower lip, palate, and tongue and vasodilatation and salivation in SMG) can be evoked reflexly by electrical stimulation of afferent nerves such as the lingual nerve (LN), inferior alveolar nerve, or infraorbital nerve (14, 17–24, 26). Matsuo and Kusano (32) earlier reported that stimulation of the tongue with high concentrations of chemical solutions and/or pinching the tongue with a small clamp induced a profuse salivary secretion in the SMG (recorded unilaterally in their experiments) in chloralose-urethan-anesthetized rats. Most stimuli that induce salivary secretion also increase blood flow in the SMG (22, 23, 26). These data make it seem highly likely that parasympathetic reflex mechanisms mediating vasodilatation and salivation exist side by side in the rat SMG. However, as far as we are aware, there have been no previous reports indicating that reflex salivation and vasodilatation can be evoked by stimulation of afferent nerves in deeply anesthetized rats.

The present study was designed to investigate 1) whether parasympathetic reflex vasodilatation does indeed occur in SMG after electrical stimulation of an afferent nerve in urethan-anesthetized rats, the LN being chosen as the afferent nerve in the present experiments, and 2) if it does, whether the efferent neural mechanisms underlying the evoked increases are the same as those seen on direct stimulation of the CLN.

METHODS

Preparation of animals. Experiments were performed on male Wistar rats weighing 450–550 g. After induction with inhalation anesthetic (isoflurane), urethan (1.0 g/kg) was injected subcutaneously and then one femoral vein was cannulated to allow drug injection. The anesthetized animals were intubated, paralyzed by intravenous injection of pancuronium bromide (Mioblock; Organon, Teknika, Netherlands; 0.6 mg/kg initially, supplemented with 0.4 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% O2. The ventilator (model SN-480–7; Shinano, Tokyo, Japan) was set to deliver a tidal volume of 0.30 × body weight × respiratory rate.

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5.0–7.5 cm$^3$/kg at a rate of 70 breaths/min, and the end-tidal concentration of CO$_2$ was determined by means of an infra-red analyzer (Capnomac Ultima; Datex, Helsinki, Finland) as reported previously (17). Rectal temperature was maintained at 37–38°C with the use of a heating pad.

The criteria for the maintenance of an adequate depth of anesthesia were the absence of a reflex elevation of heart rate and systemic arterial blood pressure (SABP) during stimulation of the central cut end of the LN. If the depth of anesthesia was considered inadequate, additional urethan (i.e., intermittent doses of 100 mg/kg iv) was administered. Once an adequate depth of anesthesia had been attained, supplementary doses of pancuronium were given approximately every 60 min to maintain immobilization during periods of stimulation.

In all experiments, the cervical vagi were cut bilaterally before any stimulation. This ensured that the only parasympathetic effects involved in the present study were nonvagal. All rats were killed at the end of the experiment by an overdose of pentobarbital sodium.

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 the recommendations in the current National Research Council guide.

Electrical stimulation of the LN and the CLN. For LN stimulation, the central cut end of LN was placed on a bipolar electrode (the nerve having been sectioned at a site ~2 mm distal to the intersection of the LN and the SMG duct; site a in Fig. 1A). For CLN stimulation, the peripheral cut end of the CLN (sectioned at a site ~8–9 mm proximal to the intersection of CLN and the SMG duct) was reflected onto the SMG duct and then both the SMG duct and the CLN were placed on a bipolar electrode. Each of the above nerves was stimulated for 20 s at supramaximal intensity (20 V) and at 10 Hz with pulses of 2-ms duration using a Nihon Kohden model SEN-7103 stimulator, except as otherwise stated.

Measurement of SMG and lower lip blood flow and of SABP. Blood flow changes in the SMG and lower lip were monitored using a laser-Doppler flowmeter (LDF; model ALF21D; Advance, Tokyo, Japan). The probe was placed against the SMG or 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 (clearance) methods (9, 29). 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. (33)]. 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 LDF increases and the amplitude of the baseline fluctuations ("signal-to-noise ratio") was ~8–10 when either the LN or CLN was stimulated with a supramaximal voltage. The output from the various devices was continuously displayed on an eight-channel chart recorder (model W5000; Graphtec, Tokyo, Japan) at a speed of 5 or 10 mm/min. The blood flow changes were assessed by measuring the height of the response on the chart. In Figs. 2 and 3, flow levels are expressed in arbitrary units.

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**Fig. 1.** A: schematic representation of the site of electrical stimulation of the lingual nerve (LN; a) and sites of blood flow measurements in submandibular gland (SMG) and lower lip (by laser-Doppler flowmeter, LDF) in the rat. The dashed lines indicate parasympathetic fibers [vasodilator fibers to the lower lip from the inferior salivatory nucleus (ISN), and salivatory fibers and vasodilator fibers to SMG from the superior salivatory nucleus (SSN)]. The solid and dotted lines indicate trigeminal and facial sensory pathways to and within the brain stem. NST, nucleus of the solitary tract; OG, otic ganglion; V, trigeminal nerve root; VII, facial nerve root; IX, glosopharyngeal nerve root. Arrows indicate the sites of nerve section: b, SMG duct; c, chorda-lingual nerve (CLN) near the intersection of SMG duct and CLN; d, CLN at a site ~7–8 mm central to the intersection of SMG duct and CLN. B: possible pathways by which nerve excitation might evoke vasodilatation in SMG in response to electrical stimulation of the LN. The irregularly broken (i), regularly broken (ii), and solid (iii) lines indicate the possible pathways discussed in RESULTS.
SABP was recorded from the femoral catheter via a Statham pressure transducer. A tachograph (model AT-610G; Nihon Kohden, Tokyo, Japan) triggered by the arterial pulse was used to monitor heart rate.

Section of nerves. To examine the peripheral (afferent and/or efferent) pathway mediating the reflex vasodilator responses in the SMG, the reflex vasodilator responses evoked by electrical stimulation of the LN at site a (see Fig. 1A) were observed before and after section of the SMG duct at site b (see Fig. 1A), the CLN at sites c or d (see Fig. 1A), the CT (see Fig. 1A), or the CST.

Pharmacological agents. To examine whether the reflexly evoked vasodilator responses were mediated via activation of the autonomic nervous system, hexamethonium, an autonomic ganglion (cholinergic) blocker, was administered (1.0 and 10 mg/kg iv), and stimulation was repeated 10 min later. The responses obtained were expressed as a percentage of the response elicited by electrical stimulation of the LN or CLN before hexamethonium administration. To determine whether the vasodilator response was mediated via activation of α- or β-adrenoceptors or of muscarinic receptors, the LN or CLN was stimulated before and after administration of phentolamine (100 μg/kg iv), propranolol (100 μg/kg iv), or atropine (1, 10, and 100 μg/kg iv). The magnitude of the response obtained after each agent was expressed as a percentage of the control response recorded before its administration (means ± SE).

Statistical analysis. All numerical data are given as means ± SE. The significance of changes in the test responses was assessed using a paired or unpaired Student’s t-test or an 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 4.5 and Super ANOVA.

RESULTS

Figure 2A shows that electrical stimulation of the central cut end of the LN-evoked vasodilator responses in the SMG and lower lip of the rats (with little or no change in SABP) and also shows the effects of section of the CT (in Fig. 1A) on these responses. Section of the CT abolished the LN-evoked SMG vasodilator response (5 of 5 experiments), but had no effect on the LBF increase (4 of 4 experiments). Figure 2B shows the effects of section of the SMG duct (at site b in Fig. 1A), the CLN at site c or d (see Fig. 1A), and the ipsilateral CST on the SMG vasodilator response evoked by electrical stimulation of LN. Section of the SMG duct had no effect on this response, whereas section of the CLN at site c or d abolished it (11 of 11 experiments). Section of the CST did not alter the increase in SMG blood flow evoked by LN stimulation (6 of 6 experiments). SABP was not affected by LN stimulation whether or not the CT was sectioned.

Figure 3 shows typical examples of the effects of intravenous hexamethonium (autonomic ganglion (cholinergic) blocking agent, 1 and 10 mg/kg) and atropine (muscarinic receptor blocking agent, 1, 10, and 100 μg/kg) on the submandibular blood flow increase elicited by electrical stimulation of either LN or CLN, both at supramaximal intensity (20 V). Mean data (together with mean data for the effects of phentolamine (α-adrenoceptor blocking agent, 100 μg/kg) and propranolol (β-adrenoceptor blocking agent, 100 μg/kg) are shown in Fig. 4. Hexamethonium had a much greater effect on the increase in SMG blood flow evoked by LN stimulation than on that evoked by stimulation of CLN. In the case of LN stimulation, the SMG blood flow responses evoked after hexamethonium, expressed as a percentage of the control response, were 63.1 ± 16.0 and 7.4 ± 5.6% for doses of 1 and 10 mg/kg, respectively. When CLN was stimulated, the corresponding values were 86.0 ± 4.7 and 41.8 ± 10.5% of control. There was a statistically significant difference in the inhibitory effect of hexamethonium at a dose of...
10 mg/kg between LN (n = 5) and CLN (n = 5) stimulation (P < 0.05, ANOVA followed by contrast test).

Prior treatment with atropine at doses of 1, 10, and 100 μg/kg reduced the SMG blood flow responses elicited by stimulation of LN and of CLN, each in a dose-dependent manner (for LN stimulation, 94.0 ± 7.3, 82.6 ± 8.6, and 23.6 ± 4.3% of control, respectively; and for CLN stimulation, 102.6 ± 10.1, 97.2 ± 3.2, and 52.6 ± 10.6% of control, respectively). The inhibitory effect of atropine on the SMG vasodilator response differed in magnitude between LN stimulation and CLN stimulation [a statistically significant difference was observed in the inhibitory effect of atropine at 100 μg/kg between LN (n = 6) and CLN (n = 5) stimulation (P < 0.05, ANOVA followed by contrast test)].

The adrenoceptor blocking agents phentolamine and propranolol had no effect on the SMG blood flow increases evoked by stimulation of LN or CLN.

Figure 5 shows the effects of electrical stimulation of LN and CLN on blood flow in the ipsilateral SMG when stimulation was delivered at various intensities from 1 to 50 V (Fig. 5A) and at various frequencies from 0.5 to 100 Hz (Fig. 5B). Electrical stimulation of LN or CLN at <2 V had no significant effect, whereas increasing

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**Fig. 3.** Effects of hexamethonium (C₆) and atropine on vasodilator response in rat SMG evoked by electrical stimulation of the central cut end of the lingual nerve (LN; ●) and peripheral cut end of the CLN (●). The LN and CLN were stimulated before (control) and 10 min after intravenous administration of hexamethonium (1.0 and 10 mg/kg) or atropine (1, 10, and 100 μg/kg). Each nerve was stimulated for 20 s at a supramaximal voltage (20 V) at 10 Hz with pulses of 2-ms duration.

**Fig. 4.** Effects of hexamethonium (1.0 and 10 mg/kg), atropine (Atro; 1, 10, and 100 μg/kg), phentolamine (Phen; 100 μg/kg), and propranolol (Prop; 100 μg/kg) on the vasodilator responses in rat SMG evoked by electrical stimulation of the central cut end of the LN (open bars) and peripheral cut end of the CLN (hatched bars). Each value is expressed as a percentage of the pretreatment response and is given as means ± SE. Statistical significance of difference from control was assessed by means of ANOVA followed by a contrast test (*P < 0.05, **P < 0.01, ***P < 0.001). Brackets (P < 0.05) indicate significant difference between 2 columns. Number of animals used is shown in parentheses.

**Fig. 5.** Stimulus intensity-response (A) and frequency-response (B) relationships for changes in blood flow in the rat SMG evoked by electrical stimulation of the central cut end of the LN (●) and the peripheral cut end of the CLN (●). Stimulation was at various intensities (1–50 V) and various frequencies (0.5–100 Hz). Intensity-response curves were generated using stimulus trains at 10 Hz. Frequency-response curves were generated using stimulus trains at 20 V. Each value is given as means ± SE. *P < 0.05 indicates significant difference between the blood flow responses evoked by LN and CLN stimulation at 2 Hz (unpaired t-test). The number of animals used is shown in parentheses.
the stimulus voltage from 2 to 20 V produced successively bigger vasodilator responses, the response being saturated at 20 V. No difference in the stimulus voltage needed to elicit an SMG vasodilator response was observed between the two nerves. Electrical stimulation of LN or CLN at <1 Hz had no significant effect, and each response was saturated at 10 Hz. The optimal frequency for vasodilatation in response to LN or CLN stimulation was 10 Hz. The SMG blood flow increase evoked by CLN stimulation \((n = 5)\) was bigger than that evoked by LN stimulation \((n = 8)\) at 2 Hz \((P < 0.05; \text{ANOVA followed by contrast test})\) and tended to be bigger at 5 Hz.

**DISCUSSION**

In the years after it was found that general anesthesia decreased CT nerve activity in the rat (12) and greatly depressed the influence exerted by the salivary reflex center over three major salivary glands (1), studies of reflex effects on salivary glands were carried out by investigating the effects of unilateral sympathectomy on the morphology of the gland under conscious conditions (by comparing the acini on the sympathectomized side with those in the normally innervated control gland on the opposite side) (11). So far, reflex salivation has been reported to occur only in the zygomatic gland after pinching of the tongue in chloralose-anesthetized cats (1) and in the SMG after brushing the tongue in rats under narcotic analgesia (a combination of fentanyl citrate and fluanison) (13). A difference between the central synaptic arrangement of the reflex arc serving the zygomatic gland and those serving the three other major salivary glands could account for the lower susceptibility of the former to general anesthesia. The greater susceptibility of the other glands is indicated by the observation that the reflex salivation in the rat SMG evoked by brushing the tongue occurred only in rats under narcotic analgesia, not in rats under general anesthesia (13). Thus the salivary reflexes seem to be capable of being strongly modified by influences acting within the central nervous system (13). Nevertheless, we have recently reported the occurrence of reflex parasympathetic salivation and vasodilatation in the orofacial area in cats under general anesthesia (urethan-chloralose) [see reviews by Izumi (15, 16)]. Moreover, it has been reported that a much greater variation in secretion among individual glandular cells occurs after reflex activation (based on morphological assessment) than after electrical stimulation of the efferent nerve (11). These findings prompted us to examine whether parasympathetic responses within the SMG can be evoked reflexly in deeply anesthetized rats.

In preliminary experiments, we found that electrical stimulation of the central cut end of the LN (at a site \(-2\) mm distal to the intersection of LN and the SMG duct) elicited a blood flow increase in SMG. As it is technically difficult to stimulate the LN at sites any further from the intersection, we felt we should be cautious in our interpretation, because this blood flow increase could have been 1) an effect secondary to an SABP increase, 2) an axon reflex vasodilatation mediated via an activation of sensory fibers with collateral branches to the blood vessels of the tongue and SMG, 3) the result of a spread of stimulus current to the vasodilator fibers contained in the CLN supplying SMG (i.e., a direct parasympathetic vasodilatation), or 4) an autonomic reflex mechanism (parasympathetic or sympathetic reflex vasodilatation). However, our subsequent experiments (reported here) allow us effectively to exclude all but the last of these possibilities, as described in the following paragraphs.

The evoked blood flow changes in SMG that we observed appear not to be secondary to any changes in SABP, because the blood flow change occurred in the SMG only on the ipsilateral, not the contralateral, side and because there was no apparent increase in SABP on LN stimulation in our animals (Fig. 2A). These data suggest that the blood flow increase we elicited by stimulation of the LN is not a passive result of any evoked blood pressure change, and we thus feel justified in referring to it as “vasodilatation.” Axon reflexes were considered as a possible explanation because sensory fibers from the trigeminal ganglion may have collateral branches to the tongue and SMG and because antidromic activation of a purely sensory input could lead to peripheral vascular dilatation in the SMG via the routes shown in Fig. 1B (routes i and ii). However, the virtual abolition of the LN-evoked vasodilator response by pretreatment with the autonomic ganglion (cholinergic) blocker hexamethonium (Figs. 3 and 4) indicates that the efferent arm of the autonomic nervous system, rather than axon reflexes, is responsible for this response. This is supported by our other experiment showing that acute section of CLN at site d (Fig. 1A) abolished the LN-evoked vasodilator response in SMG (Fig. 2B), although the anatomic substrate for axon reflexes would have been intact in those circumstances. However, the above data do not rule out the possibility that parasympathetic vasodilator fibers innervating the SMG may have been either directly stimulated by the LN stimulation or activated as a result of current spread. Nevertheless, these possibilities do seem to be excluded by our data showing abolition of the LN-evoked vasodilator response in SMG by section of CLN at site c or d (Fig. 2B). In contrast, section of the SMG duct (site b in Fig. 1A) had no effect on the LN stimulation-induced SMG vasodilatation (Fig. 2B), confirming that LN stimulation did not directly activate parasympathetic preganglionic vasodilator fibers (although electrical stimulation applied to the SMG duct did elicit a vasodilatation of a magnitude similar to that evoked by CLN stimulation).

By a process of elimination, it seems reasonable to attribute the present LN-evoked increases in SMG blood flow to parasympathetic reflex activation (as indicated by pathway iii in Fig. 1B). This conclusion is supported by the following experimental observations: 1) hexamethonium (10 mg/kg) largely abolished the response (Fig. 3A), 2) atropine (100 µg/kg) diminished
the response (Fig. 3B), 3) section of CLN at site d (Fig. 1A) completely abolished the response evoked by LN stimulation (Fig. 2B), and 4) section of the CT (Fig. 1A) abolished the vasodilatation in SMG, but not that in the lip (Fig. 2A). The observation that section of the CST did not affect the SMG vasodilatation evoked by LN stimulation (Fig. 2B) effectively rules out the involvement of sympathetic fibers as the efferents mediating the present response. On the basis of our data, we suggest that the LN has to be regarded as the sensory link involved in evoking a parasympathetic reflex vasodilatation in the rat SMG, with the CT acting as the efferent link.

Our pharmacological analysis showed that the parasympathetic vasodilator response in SMG (regardless of whether it was excited directly or reflexly) was resistant to blockade by antiadrenergic agents (phentolamine and propranolol), but highly sensitive to autonomic ganglion blockade (hexamethonium) and partly sensitive to an antimuscarnic agent (atropine) (Figs. 3 and 4). This suggests that this reflex vasodilatation is mediated via final neurons that are partly cholinergic.

The effect of atropine on the blood flow increase in rat SMG evoked by CLN stimulation has been reported to depend on the frequency of stimulation (2, 34), and its effect varied from experiment to experiment. Thulin (34) reported previously that atropine at a dose of 1 mg/kg had no effect on blood flow increases evoked by CLN stimulation at 1 Hz, but slightly depressed those evoked at 10 Hz, whereas Anderson and Garrett (2) reported recently that the initial increase in blood flow in the rat SMG evoked by CLN stimulation at 2 Hz was attenuated by atropine (1 mg/kg) while the responses evoked at 5 and 10 Hz were unaffected. These results might make it seem questionable whether cholinergic vasodilator fibers truly are involved in parasympathetic vasodilatation in the rat SMG. However, the present observations show that parasympathetic vasodilatations can be elicited both by LN stimulation and by CLN stimulation and strongly suggest that cholinergic vasodilator fibers have an important role in these responses. In its dependence on cholinergic neurons, the parasympathetic vasodilatation in the rat SMG differs markedly from that previously observed in cat SMG, which is largely mediated by vasoactive intestinal peptide (30).

**Perspectives**

To our knowledge, this is the first report demonstrating that reflex vasodilatation in the SMG can be elicited via a somatoparasympathetic mechanism in the deeply anesthetized rat. When stimulation was repeated at intervals of 5–10 min under the stimulus conditions used in the present reflex stimulation experiments, similar vascular effects could be recorded for at least 4–5 h, indicating that highly reproducible responses can be obtained using our “pseudoreflex” stimulation method [as in the cat (22, 24)]. Furthermore, such reflex activation of the parasympathetic nerve fibers has several advantages over direct electrical stimulation of the peripheral cut end of the CLN. A major advantage is that it should eliminate antidromic vasodilator effects on SMG. This would allow us to stimulate the parasympathetic nerve fibers more physiologically and should enable us to investigate the precise neural mechanisms involved in the parasympathetic vasodilator and secretory inputs serving the rat SMG.

It is hoped that further studies on the properties of the afferent input, the pathways followed by the efferent nerve fibers, and the central mechanisms involved in this reflex (as well as on the interaction between salivation and vasodilatation) will provide data enabling a better understanding of the physiological role of somatoparasympathetic reflex vasodilatation in the rat SMG.

**REFERENCES**


