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Role of nitric oxide and β-adrenoceptors of the central nervous system on the salivary flow induced by pilocarpine injection into the lateral ventricle

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Abstract

Our studies have focused on the effect of L-*NG*-nitroarginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase (NOS), and L-arginine, the substrate of NOS, on salivary secretion induced by the administration of pilocarpine into the lateral cerebral ventricle (LV) of rats. The present study has also investigated the role of the β-adrenergic agonists and antagonist injected into LV on the salivary secretion elicited by the injection of pilocarpine into LV. Male Holtzman rats with a stainless-steel cannula implanted into the LV were used. The amount of salivary secretion was studied over a 7-min period after injection of pilocarpine, isoproterenol, propranolol, salbutamol, salmeterol, L-NAME and L-arginine. The injection of pilocarpine (10, 20, 40, 80 and 160 μg/μl) into LV produced a dose-dependent increase in salivary secretion. The injection of L-NAME (40 μg/μl) into LV alone produced an increase in salivary secretion. The injection of L-NAME into LV previous to the injection of pilocarpine produced an increase in salivary secretion. L-Arginine (30 μg/μl) injected alone into LV produced no change in salivary secretion. L-Arginine injected into LV attenuated pilocarpine-induced salivary secretion. The isoproterenol (40 nmol/μl) injected into LV increased the salivary secretion. When injected previous to pilocarpine at a dose of 20 and 40 μg/μl, isoproterenol produced an additive effect on pilocarpine-induced salivary secretion. The 40-nmol/μl dose of propranolol injected alone or previous to pilocarpine into LV attenuated the pilocarpine-induced salivary secretion. The injection of salbutamol (40 nmol/μl), a specific β-2 agonist, injected alone into LV produced no change in salivary secretion and when injected previous to pilocarpine produced an increase in salivary secretion. The 40-nmol/μl dose of salmeterol, a long-acting β-2 agonist, injected into LV alone or previous to pilocarpine produced no change in salivary secretion. The results have shown that central injections of L-NAME and L-arginine interfere with the salivary secretion, which implies that might participate in pilocarpine-induced salivary secretion. The interaction between cholinergic and β-adrenergic receptors of the central nervous system (CNS) for the control of salivary secretion can also be postulated.

Keywords: CNS; Nitric oxide; β-adrenoceptors; Pilocarpine; Salivary secretion

1. Introduction

The alkaloid pilocarpine (pye loe KAR peen) is a useful cholinergic agonist compared with acetylcholine (ACh) and its derivates but is far less potent. It is unaffected by acetylcholinesterase. Pilocarpine presents mainly muscarinic activity. Pilocarpine is able to enter the brain and stimulate sweat secretion.

This muscarinic cholinergic agonist induces copious salivation when administered systemically, simulating an activation of the parasympathetic system (Fergunson, 1993). Pilocarpine HCl stimulates labial (minor) salivary gland flow in patients with Sjögren's syndrome (Rhodus, 1997). Parasympathetic stimulation involving muscarinic cholinergic

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receptors induces the vasodilatation and the salivary secretion (Garret et al., 1991). The controlled release of pilocarpine in normal individuals has been studied (Lockhart et al., 1996).

The involvement of some areas of the central nervous system (CNS) for the control of salivary secretion in rats has been shown by several studies (Golkar et al., 2000; Flynn et al., 1981, Kanosue et al., 1990).

We have investigated the effect of cholinomimetic agonist pilocarpine injected into LV on salivary secretion of rats with anteroventral third ventricle (AV3V) electrolytic lesion. We have concluded that the CNS, particularly the AV3V region, is important for the effect of pilocarpine on salivary secretion in rats (Renzi et al., 1993). Morphological, morphometric and stereological changes of submandibular glands were observed after the lesion of the ventromedial nucleus of hypothalamus and the lesion of the AV3V region (Renzi et al., 1989, 1990).

Both the histochemical and functional results have suggested that nitric oxide (NO) plays an excitatory role in the regulation of the parasympathetic nerve, inducing salivary secretion in the submandibular gland of rats (Bodis and Haregewoin, 1993; Wang et al., 1994; Takai et al., 1999). L-*NG*-nitroarginine methyl ester (L-NAME), a NO synthase (NOS) inhibitor, increased the salivation induced by pilocarpine (Damas, 1994). Pilocarpine has been used extensively over the last century as one of the best sialogogues rather than other cholinomimetics agents (Wiserman and Faulds, 1995). NO plays an important role in the hydromineral and cardiovascular regulation (Saad et al., 1999a). Increased generation of NO upon stimulation of cells by muscarinic agonists was detected. A series of muscarinic agonists such as pilocarpine and carbachol stimulated NOS (Wang et al., 1994). Extracellular NO seems to inhibit the ability of the M(2) receptors to decrease ACh release from the parasympathetic nerves. It has been demonstrated in vitro that endogenous NO had inhibited the ability of M(2) receptors to decrease ACh release by using L-NAME and pilocarpine (Golkar et al., 2000). These studies have presented the ability of pilocarpine in affecting the NO activity. However, the role of NO found in the central structures in saliva secretion as well as the side effects of pilocarpine have not been studied before.

Two types of β-adrenergic receptors have been reported: β-1 and β-2, both present in secretory cells (Lands et al., 1967). Isoproterenol, β-1 and β-2 adrenoceptors agonist (Wheeldon et al., 1993), when administered chronically, produces a number of events in the salivary gland such as gland weight increase and acinar cell volume increase (Selye et al., 1961). Long-term treatment with selective β-2 adrenoceptors agonist, commonly used in asthma therapy, causes hypertrophy and hyperplasia of the salivary gland rats and an increase in DNA contents (Schneyer, 1962). Salmeterol is a long-acting, highly selective β-2 adrenoceptor agonist, which lasts at least 12 h. It has potent bronchodilating and possibly anti-inflammatory properties and seems to be especially effective in patients with nocturnal symptoms or exercise-induced asthma (Lötvall and Svedmyr, 1993). The central or peripheral administration of β-agonists and antagonists is considered to be involved in many physiological mechanisms (Saad et al., 1995). The β-adrenoceptors of the CNS play an important role in the water and electrolytic homeostase of the body. A series of studies have demonstrated the relation between β-adrenoceptors and pilocarpine in many salivary gland functions. Thus, pilocarpine may be have some affinity for these β-adrenoceptors (Kawaguchi et al., 1997; Iwabuchi et al., 1994; Iwabuchi and Masuhara, 1992; Takayanagi et al., 1992; Offer et al., 1991; Slomiany et al., 1991). In this study, we have investigated the effects of L-NAME and L-arginine injected into lateral cerebral ventricle (LV) on salivary secretion rate, respectively, when injected alone or in association with pilocarpine and β-adrenergic agonists and antagonist. A possible interaction between cholinergic and β-adrenergic receptors of the CNS in the regulation of salivary secretion has also been studied.

2. Material and methods

2.1. Animals

Male Holtzman rats (250–300) were housed in individual metabolic cages, with free access to food pellets and tap water.

2.2. Brain surgery

The rats were anaesthetized with urethane (1.25 g/kg body weight ip) and restrained in a stereotaxic apparatus (David Kopf model for rats). A longitudinal incision was made on the skin of the animal's head, the subcutaneous tissue was pulled back and the skull was drilled with a spherical drill. A stainless-steel cannula (14 × 0.7 mm o.d.) was introduced into the LV. The skull was positioned using bregma and lambda at the same level. The coordinates for approaching the LV were obtained from the Paxinos and Watson (1986) atlas. The cannula was fixed to the skull with screws and acrylic resin. A prophylactic dose of penicillin (Pentabiotic Fontoura Wyeth; 60,000 IU) was injected intramuscularly after brain surgery.

2.3. Drug injection

The pilocarpine, isoproterenol, propranolol, salbutamol, salmeterol, L-NAME, L-arginine and 0.15-M NaCl (as control) were injected into the LV by using a Hamilton microsyringe (5 μl) connected by a PE 10 polyethylene tubing (25 cm) to a needle (0.3 mm o.d.), which was introduced into the brain through the cannula previously fixed to the animal's head. The volume of injection was always 1 μl, injected over a period of 30–60 s. All the compounds were dissolved in 0.15-M NaCl.

2.4. Salivary secretion

Salivary flow was stimulated by pilocarpine (10, 20, 40, 80 and 160 μg/μl) injected intracerebroventricularly (icv). The animals were anaesthetized with urethane (1.25 g/kg body weight ip). Saliva was collected with preweighed small cotton wool balls inserted into the animal's mouth, a technique slightly different from that used by Schallet et al. (1978). Such technique led us to collect the whole saliva. Saliva was collected with four cotton balls weighing approximately 20 mg each, two of which were placed on either side of the oral cavity and with the other two placed under the tongue. The amount of saliva secreted was measured 7 min before the injection of pilocarpine (baseline saliva secretion) and 7 min after the injection of pilocarpine, L-NAME, L-arginine and β-agonists and antagonist. The L-NAME, L-arginine and the β-agonists and antagonist adrenoceptors were injected into LV 5 min before pilocarpine and β-agonists and antagonist.

2.5. Histology

After the experiments, the animals were anaesthetized with ether and perfused through the heart with saline and 10% formalin. The brain was removed and stored in 10% formalin for at least 1 week. The brain was then frozen and the coronal section (20–30 μm) was cut and stained with hematoxylin–eosin for examination with a light microscope. Only the results of animals whose LV were reached by the injections were used for data analyses (Fig. 1).

2.6. Statistical analysis

The results are reported as mean ± S.E.M. The ANOVA and Dunnett's *t* test were used to determine the significance. The values were considered statistically significant when $P < .05$.

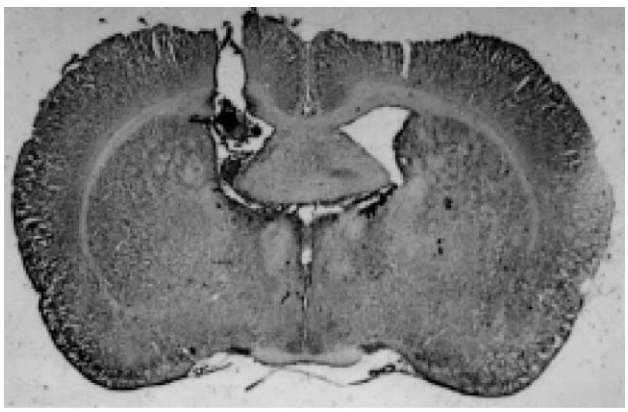

Fig. 1. Photomicrograph of a hematoxylin-stained transverse section of the rat brain showing the site of injection into the LV (arrow).

2.7. Experimental protocol

For the study of salivary flow, measurement was started 5 days after the brain surgery. Each animal was submitted to three or four experimental sessions at 3-day intervals. These parameters were obtained from different experimental sessions and from several groups of animals after the injection of the following drugs into the LV of satiated animals:

1. 0.15-M NaCl injected into the LV (control).
2. Pilocarpine (Sigma, USA; 10, 20, 40, 80 and 160 μg/μl) injected into the LV.
3. L-NAME (Sigma; 40 μg/μl) and L-arginine injected into the LV 5 min previous to pilocarpine and agonists and antagonist β-adrenoceptors intracerebroventricularly.
4. Isoproterenol, propranolol, salbutamol and salmeterol (Sigma; 40 nmol/μl) injected into the LV alone or previous to pilocarpine.

The doses used in these experiments were based on several studies of our lab as well as other labs. The results were obtained by dose–response curves previously determined.

3. Results

3.1. Histological analyses

A photomicrograph of the rat brain representing the group shows where the cannula is in the LV and it is illustrated in Fig. 1. The arrow indicates where the cannula was introduced into the LV.

3.2. The effect of treatment with isoproterenol on salivary secretion induced by intracerebroventricular injection of pilocarpine

The injection of pilocarpine (10, 20, 40, 80 and 160 μg/μl) into the LV induced a dose-dependent increase in the salivary secretion with values of 41 ± 2, 69 ± 4, 143 ± 9, 293 ± 7 and 414 ± 19 mg/7 min, respectively. ANOVA showed significant differences among all doses [$F(4,30) = 229.29$, $P < .0001$]. L-NAME (40 μg/μl) injected intracerebroventricularly previous to pilocarpine produced an increase in this sialogoguic effect of pilocarpine with values of 70 ± 5, 133 ± 7, 265 ± 15, 360 ± 21 and 556 ± 35 mg/7 min [$F(4,35) = 53.93$, $P < .0001$]. The injection of isoproterenol (40 nmol/μl) alone intracerebroventricularly produced an increase in the salivary secretion. Isoproterenol injected intracerebroventricularly previous to pilocarpine (20 and 40 μg/μl) produced an increase in the salivary secretion, showing a possible additive effect. ANOVA showed significant differences among these various treatments [$F(4,35) = 46.48$, $P < .0001$; Fig. 2].

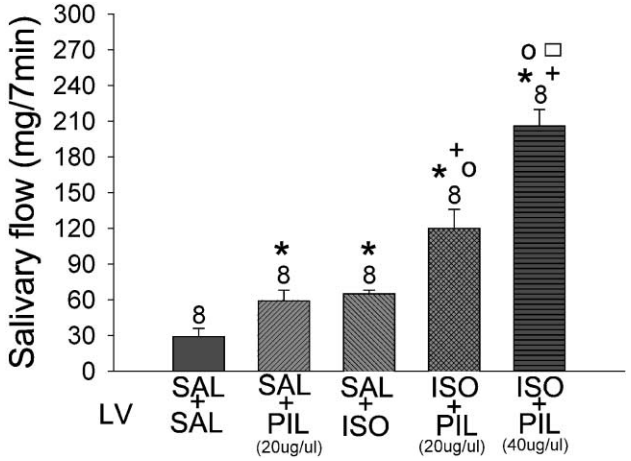

Fig. 2. Salivary flow after injection of 0.15-M saline (NaCl), saline + 20-μg/μl pilocarpine (SAL + PIL), saline + 40-nmol/μl isoproterenol (SAL + ISO), isoproterenol + 20-ug/μl pilocarpine (ISO + PIL) and isoproterenol + 40-μg/μl pilocarpine (ISO + PIL). The results are reported as means ± S.E.M. The number of animals is indicated at the top of each column. * $P < .05$ compared with saline (control). $^{+}P < .05$ compared with pilocarpine. $^{\circ}P < .05$ compared with isoproterenol. $^{\square}P < .05$ compared with ISO + PIL (20 ug/μl).

3.3. The effect of treatment with propranolol, salbutamol and salmeterol on salivary secretion induced by intracerebroventricular injection of pilocarpine

Propranolol (40 nmol/μl) injected intracerebroventricularly alone produced no change in salivary secretion and when injected previous to pilocarpine (40 μg/μl) attenuated the sialogoguic effect of pilocarpine. Salbutamol (40 nmol/μl) injected intracerebroventricularly alone produced no change in salivary secretion and when injected previous to pilocarpine (40 μg/μl) produced an increase in salivary flow. Salmeterol (40 nmol/μl) injected intracerebroventricularly alone or previous to pilocarpine (40 μg/μl) produced no change in salivary flow. ANOVA showed significant differences among these various treatments [$F(7,64) = 32.31$, $P < .0001$; Fig. 3).

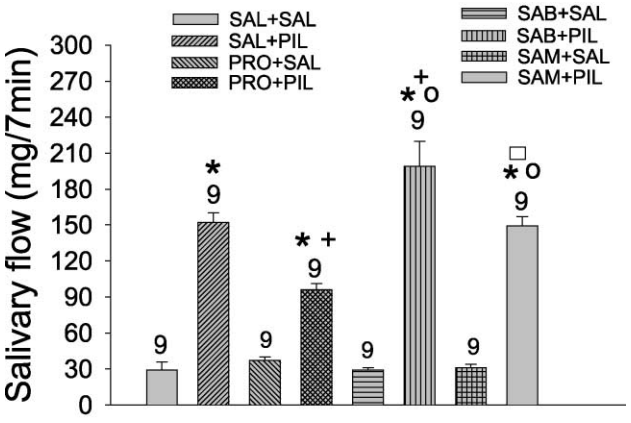

Fig. 3. Salivary flow after injection of 0.15-M saline (NaCl), saline + 40-ug/ul pilocarpine (SAL + PIL), 40-nmol/ul propranolol + 40-μg/μl pilocarpine (PRO + PIL), 40-nmol/μl salbutamol + 40-μg/μl pilocarpine (SAB + PIL) and 40-nmol salmeterol + 40-μg/μl pilocarpine (SAM + PIL). The results are reported as means ± S.E.M. The number of animals is indicated at the top of each column. * $P < .05$ compared with saline (control). $^{+}P < .05$ compared with pilocarpine. $^{\circ}P < .05$ compared with PRO + PIL. $^{\square}P < .05$ compared with 40-μg/μl SAM + PIL.

Table 1
Effect of injection of isotonic saline, L-NAME, L-arginine and isoproterenol into LV on salivary flow induced by pilocarpine injection into LV

Treatment	Number of animals	Salivary flow (mg/7 min)
Saline + saline	11	30 ± 2
Saline + pilocarpine	11	128 ± 11*
Saline + isoproterenol	11	61 ± 4*
L-NAME + saline	8	70 ± 5*
L-NAME + pilocarpine	11	182 ± 230*,+,#
L-Arginine + saline	7	31 ± 1+,#,†
L-Arginine + pilocarpine	11	33 ± 9+,#,†
L-NAME + isoproterenol + pilocarpine	11	274 ± 14*,+,#
L-Arginine + isoproterenol + pilocarpine	11	32 ± 8+,#,†

* $P < .05$ compared with saline.
+ $P < .05$ compared with pilocarpine.
# $P < .05$ compared with isoproterenol + pilocarpine.
† $P < .05$ compared with L-NAME + pilocarpine.

3.4. The effect of treatment with L-NAME, L-arginine and isoproterenol on salivary secretion induced by intracerebroventricular injection of pilocarpine

Isoproterenol (40 nmol/μl) injected intracerebroventricularly produced an increase in salivary flow. L-NAME (40 μg/μl) injected intracerebroventricularly alone produced an increase in the salivary flow. When injected previous to isoproterenol (40 nmol/μl) and pilocarpine (40 μg/μl), L-NAME enhanced the sialogoguic effect of

Table 2
Effect of injection of isotonic saline, L-NAME, L-arginine and propranolol into LV on salivary flow induced by pilocarpine injection into LV

Treatment	Number of animals	Salivary flow (mg/7 min)
Saline + saline	7	30 ± 2
Saline + pilocarpine	7	139 ± 11*
Saline + propranolol	6	43 ± 2*
Propranolol + pilocarpine	7	112 ± 9*,+
L-NAME + saline	8	79 ± 7*,+
L-NAME + pilocarpine	7	182 ± 13*,+,#
L-Arginine + saline	9	29 ± 2+,#,†
L-Arginine + pilocarpine	7	27 ± 1+,#,†
L-NAME + propranolol + pilocarpine	7	270 ± 21*,+,#
L-Arginine + propranolol + pilocarpine	7	25 ± 2+,#,†

* $P < .05$ compared with saline.
+ $P < .05$ compared with pilocarpine.
# $P < .05$ compared with propranolol + pilocarpine.
† $P < .05$ compared with L-NAME + pilocarpine.

Table 3
Effect of injection of isotonic saline, L-NAME, L-arginine and salbutamol into LV on salivary flow induced by pilocarpine injection into LV

Treatment	Number of animals	Salivary flow (mg/7 min)
Saline + saline	8	30 ± 2
Saline + pilocarpine	8	131 ± 13*
Saline + salbutamol	6	29 ± 2
Salbutamol + pilocarpine	8	231 ± 27*,+
L-NAME + saline	7	74 ± 4*,+
L-NAME + pilocarpine	8	273 ± 15*,+
L-Arginine + saline	7	31 ± 3+,#,†
L-Arginine + pilocarpine	8	33 ± 4+,#,†
L-NAME + salbutamol + pilocarpine	8	404 ± 22*,+,#
L-Arginine + salbutamol + pilocarpine	8	39 ± 7+,#,†

* $P < .05$ compared with saline.
\+ $P < .05$ compared with pilocarpine.
\# $P < .05$ compared with salbutamol + pilocarpine.
† $P < .05$ compared with L-NAME + pilocarpine.

isoproterenol and pilocarpine. L-Arginine (30 μg/μl) injected intracerebroventricularly alone produced no change in the salivary secretion. When injected previous to isoproterenol (40 nmol/μl) and pilocarpine (40 μg/μl), L-arginine produced a decrease in the salivary flow. ANOVA showed significant differences among these various treatments [$F(8,83) = 91.42$, $P < .0001$; Table 1].

3.5. The effect of treatment with L-NAME, arginine and propranolol on salivary secretion induced by intracerebroventricular injection of pilocarpine

Propranolol (40 nmol/μl) injected intracerebroventricularly alone or previous to pilocarpine (40 μg/μl) attenuated the salivary flow. L-NAME (40 μg/μl) injected intracerebroventricularly previous to propranolol (40 nmol/μl) and pilocarpine (40 μg/μl) produced an increase in salivary secretion. L-Arginine (30 μg/μl) injected intracerebroventricularly previous to propranolol (40 nmol/ul) and pilocarpine (40 μg/μl) produced an inhibitory effect in the salivary flow. ANOVA showed significant differences among these various treatments [$F(9,62) = 122.14$, $P < .0001$; Table 2).

3.6. The effect of treatment with L-NAME, L-arginine and salbutamol on salivary secretion induced by intracerebroventricular injection of pilocarpine

Salbutamol (40 nmol/μl) injected intracerebroventricularly alone produced no change in the salivary secretion and when injected previous to pilocarpine (40 μg/μl) produced an increase in the salivary flow. L-NAME (40 μg/μl) injected intracerebroventricularly previous to salbutamol (40 nmol) and pilocarpine (40 μg/μl) enhanced the sialogogue effect of pilocarpine. L-Arginine (30 μg/μl) injected intracerebroventricularly previous to salbutamol (40 nmol/μl) and pilocarpine (40 μg/μl) produced an inhibitory effect in the salivary flow. ANOVA showed significant differences among these various treatments [$F(9,66) = 97.30$, $P < .0001$; Table 3].

4. Discussion

The results of the present study have shown that the injection of pilocarpine into the LV stimulates the salivary flow in a dose-dependent manner. It has been demonstrated that pilocarpine, when injected intracerebroventricularly, produced salivary secretion at a significantly different level from that of the control. We have used the LV based on many studies to demonstrate that this structure was chosen in order to investigate the possible involvement of some areas of the CNS in physiological and pharmacological mechanisms (Hainsworth and Epstein, 1966; Emmelin, 1967; Hübschle et al., 1998). The electrolytic lesion of the AV3V produced a decrease in the salivary flow induced by pilocarpine injected intracerebroventricularly. These results have revealed that some new areas of the CNS, such as the areas surrounding the AV3V, are important for pilocarpine-induced salivary secretion (Renzi et al., 1993). The areas surrounding the AV3V are also important for the regulation of hydromineral and cardiovascular balances as well as for the control of the salivary composition and salivary flow. These results have matched with results obtained before (Hübschle et al., 1998). Xerostomy has debilitating effects in terms of both physical health and psychological well-being (Fox, 1997). Difficulty in swallowing (dysphagia) is a very ordinary upper gastrointestinal disorder caused by salivary malfunction (Valdez and Fox, 1991). Current systemic treatments with sialogogues for patients with xerostomy are limited because of little efficiency (Lockhart et al., 1996). The present results have shown that CNS is likely to participate in the control of the salivary flow, demonstrating that a treatment combining new drugs with mixed pharmacological profile may be efficient by prolonging salivary secretion and reducing side effects.

The central participation of NO and β-1 and β-2 adrenoceptors in the regulation of the salivary flow has also been demonstrated by these experiments. Previous studies demonstrated the influence L-arginine/NO in the amylase secretion and the role of NO in the salivary secretion (Lomniczi et al., 1998; Lohinai et al., 1997).

Precocious morphological development occurs in the salivary glands of neonatal rats treated with thyroid hormone, similar to that reported by chronic administration of isoproterenol (Barka and van der Noen, 1997). Saad et al. (1999a) demonstrated the effects of central injection of salbutamol and salmeterol on the salivary flow. Rats treated chronically with salmeterol, a long-acting β-2 adrenoceptor agonist, presented an impaired secretion of salivary proteins and calcium. Such effects resemble those of salbutamol (Ryberg and Johansson, 1998). The chronic use of propanolol reduced the total protein concentration in saliva,

increased caries susceptibility but did not reduce the stimulated salivary flow rate (O'Connell et al., 1993). These present results have demonstrated that isoproterenol, a β-1 and β-2 adrenoceptor agonist, when injected into the LV, produced an increased salivary flow and produced an additive effect on the sialogoguic action of pilocarpine. These data have demonstrated the participation of β-adrenoceptors of the CNS in the regulation of the salivary flow. These results have confirmed that propranolol, a β-1 and β-2 adrenoceptor antagonist, attenuated the sialogoguic effect of pilocarpine. Salbutamol, a specific β-2 agonist, enhanced the sialogoguic effect of pilocarpine, whereas β-1 decreased the salivary flow. These results demonstrated that β-2 adrenoceptors of the CNS interfere in the sialogoguic action of pilocarpine. These results have been confirmed by the experiments using isoproterenol. They imply that there is an interaction between the cholinergic and β-adrenergic receptors of the CNS in the control of the salivary flow.

The recognition of the role of NO in cell–cell communication has changed the concept of traditional neurotransmission. *N*-methyl-D-aspartate receptors mediate the dipsogenic response of c-Fos expression induced by intracerebroventricular infusion of angiotensin II (Zhu and Herbert, 1997). The effect of captopril and bradykinin on chord tympani-induced salivation in cats has been demonstrated (Stojic, 1999). NO might influence ganglionic transmission in parasympathetic pathways controlling salivary secretion. The preganglionic parasympathetic salivary neurons in the brain stem contain marks of NO synthesis (Zhu et al., 1996). The presence of NO in many structures of the CNS has been described (Bredt et al., 1991; Kadekaro et al., 1994; Saad et al., 1999b). It has been demonstrated that L-NAME increased the salivary flow induced by pilocarpine (Damas, 1994). The present results have shown that L-NAME, when injected into the LV previous to pilocarpine injection, increased the salivary flow. NO contributed to the control of vascular tone in salivary glands of rats (Edwards and Garret, 1993). We have also observed that the L-arginine injected into the LV previously to pilocarpine reduced the sialogoguic effect of pilocarpine.

These results have contributed to the studies of the mechanisms that regulate the salivary secretion by showing the importance of pilocarpine acting in the CNS. Pilocarpine hydrochloride is a parasympathomimetic cholinergic agent with powerful stimulating effect on exocrine glands. It has already been used in ophthalmologic dosage forms for the management of glaucoma. It has been used recently for the treatment of the symptoms of xerostomy caused by the hypofunction of the salivary gland due to radiotherapy in patients who have head and neck cancer. It has also been used for stimulating the salivary flow in patients with Sjögren's syndrome.

The present results show that intracerebroventricular injection of L-NAME induced salivary secretion and enhanced the sialogoguic effect of pilocarpine. L-Arginine attenuated the pilocarpine-induced salivary secretion. An interaction between central NO and β-adrenergic and cholinergic receptors in the control of the salivary secretion can be postulated. Thus, the therapeutical value of the combination of the little doses of these drugs in the control of salivary secretion and the participation of CNS can be efficient in future studies.

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