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Ventimiglia MS, Rodriguez MR, Morales VP, Elverdin JC, Perazzo JC, Castañeda MM, Davio CA, Vatta MS, Bianciotti LG. Endothelins participate in the central and peripheral regulation of submandibular gland secretion in the rat. *Am J Physiol Regul Integr Comp Physiol* 300: R109–R120, 2011. First published October 13, 2010; doi:10.1152/ajpregu.00041.2010.—We previously reported that endothelins (ETs) are involved in the rat central and peripheral regulation of bile secretion. In this study we sought to establish whether ET-1 and ET-3 modulated submandibular gland secretion when locally or centrally applied. Animals were prepared with gland duct cannulation to collect saliva samples and jugular cannulation to administer sialogogues. ETs were given either into the submandibular gland or brain lateral ventricle. Intraglandularly administered ETs failed to elicit salivation per se. However, ET-1, but not ET-3, potentiated both cholinergic- and adrenergic-evoked salivation through ET_A receptors. ET-1 decreased cAMP content but increased phosphoinositide hydrolysis, whereas ET-3 attenuated both intracellular pathways. The expression of ET_A and ET_B receptor mRNAs as well as that of ETs was revealed in the submandibular gland by RT-PCR. Immunohistochemical studies showed that ET_A receptor staining was localized around the interlobular ducts and acini, compatible with the myoepithelial cells' location, whereas ET_B receptor staining was restricted to small blood vessels. When applied to the brain, both ETs induced no salivation but enhanced cholinergic- and adrenergic-evoked salivary secretion through parasympathetic pathways. ET-1 response was mediated by brain ET_A receptors, whereas that of ET-3 was presumably through nonconventional ET receptors. Present findings show that ETs are involved in the brain regulation of cholinergic- and adrenergic-stimulated submandibular gland secretion through the activation of distinct brain ET receptors and parasympathetic pathways. However, when ETs were administered into the gland, only ET-1 enhanced cholinergic and adrenergic salivation likely through myopithelial cell contraction by activating ET_A receptors coupled to phospholipase C. The presence of ETs and ET receptors suggests the existence of an endothelinergic system in the submandibular gland.

salivary glands; ET_A receptors; ET_B receptors; endothelin-1; endothelin-3

ENDOTHELINS (ETs) are a family of related peptides that bind to specific receptors widely expressed in numerous tissues and cell types (1). The family comprises three isopeptides (ET-1, ET-2, and ET-3) that exert a wide variety of biological effects (5, 37). They are synthesized in various tissues and organs including the endothelium, brain, and gastrointestinal tract and function as locally released peptides. ETs play a relevant role in the regulation of blood pressure either when centrally or peripherally applied, acting synergically with other vasoactive substances like angiotensin II and catecholamines (5). ETs also regulate the synthesis and release of various hormones and neurotransmitters (9, 10).

Biological effects of ETs are mediated by two pharmacologically well-characterized G protein-coupled receptors, ET_A and ET_B, which generally have opposing actions and activate multiple signaling pathways (5, 6, 37). The ET_A receptor displays higher affinity for ET-1 and ET-2 than for ET-3, whereas the ET_B receptor is equally sensitive for the three isopeptides (5, 37). Various studies support the existence of additional receptor subtypes, termed atypical receptors, based on the observation that various ET-mediated effects, particularly in the brain, fail to be mimicked by selective agonists and/or inhibited by selective antagonists in different species including the rat (10, 15, 27, 31). Whether these receptors are subtypes of the conventional ET receptors (ET_A and ET_B) or represent new receptors, it remains to be established. Although this issue is still controversial, recent reports suggest that the atypical responses may result from the formation of homodimers and/or heterodimers of conventional ET receptors (12, 14).

We previously reported that ET-1 and ET-3 applied to the brain modulate bile secretion through different ET receptors likely located in the dorsal vagal complex. ET-1 response is mediated by vagal pathways, whereas the ET-3 effect involves nitric oxide pathways (31, 32). ETs are also involved in the central regulation of gastrointestinal motility. It was shown that ETs applied to the brain stem increase intragastric pressure and stimulate gastric motility through a vagally mediated pathway (18).

Immunoreactive ETs and ET binding have been reported in the salivary glands; however, the role of these peptides in the secretory process remains to be established (13, 19). The submandibular gland is one of the major salivary glands together with the parotid and sublingual glands. Salivary glands are composed of the acinar cells that produce the salivary fluid as well as most of the proteins (primary saliva) and ductal cells that secrete some proteins and modify the composition of primary saliva (26). Submandibular gland also possess mioepithelial cells distributed on the basal layer of the acini and intercalated ducts, which contract by autonomic nerve stimulation and assist the secretion by compressing and/or reinforcing the underlying parenchyma (23). Salivation is under the synergic control of the parasympathetic and sympathetic nervous system. Activation of the parasympathetic system releases acetylcholine that by activating muscarinic

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receptors (mainly the M3 subtype) stimulates salivary secretion with a low protein concentration. The sympathetic nervous system controls salivation through the activation of α- and β-adrenoceptors, which stimulate fluid-rich and protein-rich secretion, respectively. In addition, salivation is also influenced by various factors as well as peptides and neuropeptides locally released by nervous stimulation (33). Salivary secretion is centrally controlled by the dorsal vagal complex, which is the major brain site for the autonomic regulation of digestive secretions and gastrointestinal motility.

In the present study, we sought to establish the participation of ET-1 and ET-3 in the central and peripheral regulation of the submandibular gland secretion in the rat focusing on the underlying mechanisms and ET receptors involved. We further investigated the expression and localization of ET receptors in the gland. Our results support the modulatory role of ETs in the central and peripheral regulation of submandibular gland secretion in the rat.

MATERIALS AND METHODS

Materials and Animals

The following drugs were used: ET-1, ET-3, BQ-788, and BQ-610 (American Peptide); methacholine (MC), norepinephrine (NE), chloralose and IBMX (Sigma, St. Louis MO), [^{3}H]-cAMP, ^{125}I-GMPc, and Myo-[^{3}H]-inositol (GE Healthcare). Other reagents were of analytical or molecular biology quality and were obtained from standard sources. Sprague-Dawley rats (School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina) weighing between 250 and 300 g were used in the experiments. The rats were housed in steel cages and maintained at 22–24°C in a controlled room with 12:12-h light-dark cycle (light from 7:00 to 19:00 h) with free access to food and tap water. Rats were fasted for 14 h before experimental procedures to avoid variations in salivary secretion. All experiments were conducted following the recommendations of the National Institutes of Health guidelines for the care and use of laboratory animals (NIH Publication 1985, revised 1996). The experimental protocols were approved by the Animal Care Committee of the School of Pharmacy and Biochemistry of the University of Buenos Aires, Buenos Aires, Argentina.

In Vivo Experiments

Effect of ET-1 and ET-3 applied to the submandibular gland. Rats were prepared as previously described (3, 4, 20, 41). Briefly, animals were anesthetized with chloralose (100 mg/kg iv; ether was used as inducer) and the right femoral vein was cannulated with a polyethylene catheter (PE-40 catheter; Rivero & Cia, Argentina) to administer the sialogogic agonists MC and NE used in the study (20). The agonists were chosen based on the fact that salivation is under the physiological control of the parasympathetic and sympathetic nervous systems. Through a midline incision in the neck the trachea was intubated, and the ducts of submandibular glands were exposed and cannulated with a fine glass cannula to collect saliva samples. As expected, no basal salivation was observed since no secretion occurs when ducts are cannulated. Different doses of ET-1 and ET-3 (1, 10, and 100 nM) were injected into the parenchyma of the gland to determine whether these peptides were able to induce salivation. Experiments were then conducted to evaluate the effect of ETs on stimulated-salivation. Salivary secretion was stimulated by MC (1, 3, and 10 μg/kg) and NE (3, 10, and 30 μg/kg) (20, 41). Dose response curves to the sialogogic agonists were performed in the absence (rats received an equal volume of saline) or the presence of ET-1 or ET-3. The secretory response to the agonists subsided in < 3 min after the injection, and three additional min were allowed until the administration of the next dose. Following the dose response curve to MC or NE and after a resting period of 30 min, 1 nM ET-1 or ET-3 was administered into the submandibular gland, and the dose response curve to the agonist was repeated. We have previously tested that the dose of ET-1 and ET-3 administered into the gland induced no modifications in blood pressure (data not shown). Salivary samples were collected for 3 min in preweighed vessels, and the quantity of secretion was determined by weighing. Results were expressed as microliters per 3 min, assuming saliva density as 1.0 g/ml. To identify the ET receptor involved in ET-1 response, animals were pretreated with 100 mM BQ-610 (ET_A antagonist) or 100 mM BQ-788 (ET_B antagonist) administered into the gland 10 min before the dose response curve to MC or NE in the presence or absence of ETs.

Effect of ET-1 and ET-3 applied to the brain. Animals were prepared with a guide cannula in the left lateral ventricle 1 wk before secretory experiments as previously reported (31, 32, 34). Briefly, animals under anesthesia were mounted in a stereotaxic apparatus, and a small hole was drilled through the skull. An intracranial cannula (21-gauge stainless steel, 1.5 cm in length) was placed into the left lateral ventricle by using appropriate stereotaxic coordinates (1.3 mm posterior to the bregma, 2.0 mm lateral to the midline, and 4.0 mm ventral to the skull surface) (29). The cannula was secured by two screws inserted into the surface of the bone using cyanoacrylate. Rats were placed in individual cages with free access to food and water, and allowed to recover from surgery. After being fasted overnight, rats were anesthetized and cannulated in the submandibular salivary ducts as detailed above. The effect of centrally applied ETs was evaluated on spontaneous or stimulated salivary secretion. Different doses of ET-1 and ET-3 (total volume, 1 μl) were first applied to the brain in the absence of sialogogic agonists (MC or NE) to evaluate whether the peptides were able to induce salivation. In another set of animals, dose-response curves to MC (1, 3, and 10 μg/kg) and NE (3, 10, and 30 μg/kg) were preformed in the presence or absence of centrally applied ET-1 and ET-3 (5 ng/μl, total volume 1 μl). Rats not treated with ETs received an equal volume of artificial cerebrospinal fluid. The dose of centrally applied ETs induced no changes in blood pressure as previously reported (31, 32). Salivary samples were collected as indicated above. The identification of ET receptors in the brain were performed by centrally applied 1 mM BQ-610 and 1 mM BQ-788 10 min before dose response curves to MC or NE in the presence or presence of intracerebroventricular ET-1 or ET-3 (31, 32).

Experiments with ET-1 and ET-3 were also performed in rats with acute parasympathetic decentralization achieved by right chorda timpani nerve section as in previous studies (11). Dose response curves to MC and NE were performed before and following chorda timpani section to evaluate the effect of parasympathetic blockade on salivary secretion. In another set of rats with parasympathetic decentralization, dose response curves to the agonists were performed in the presence or absence of centrally applied ETs.

As in previous studies, the accuracy of intracerebroventricular injections was assessed at the end of each secretory experiment by the administration of 1 μl methylene blue icv (31, 32, 34). Animals were then killed, and through the opening of the skull the brain was removed and the presence of methylene blue in the lateral ventricle and its absence in surrounding regions were verified.

In Vitro Experiments

Determination of cAMP and cGMP in the submandibular gland. Studies were performed in submandibular gland slices as previously described (20, 41). Animals were killed by cervical dislocation, and the submandibular glands were removed and detached from free connective and fat tissue. One-millimeter thick-sliced submandibular gland were preincubated for 15 min in a modified Krebs-Henseleit bicarbonate buffer of the following composition (in mM): 118 NaCl, 4.7 KCl, 1 $NaHPO_4$, 1.1 $MgCl_2$, 2.5 $CaCl_2$, 25 $NaHCO_3$, and 2.5 mg/ml D-glucose supplemented with minimum essential amino acid

solution and basal medium Eagle vitamin solution (Biomedicals). The medium was equilibrated with 95% O_2-5% CO_2 and adjusted to pH 7.40. The accumulation of cAMP was assessed as previously described (7, 35). Tissues were incubated for 3 min in Krebs solution containing 1 mM IBMX and further exposed for 12 min to ET-1 (1, 10, and 100 nM) or ET-3 (1, 10, and 100 nM). To identify the ET receptor subtype involved in the response, tissues were pretreated for 10 min with 100 nM BQ-610 or BQ-788 before the addition of ET-1 or ET-3. In all experiments reaction was stopped by homogenization in ice-cold absolute ethanol and centrifuged for 15 min at 1,200 *g*. The supernatant was dried, and the remaining residue was suspended for cAMP determination that was assessed by competition of [^{3}H]-cAMP for PKA (7). Studies were also performed in the presence of MC and NE alone or combined with ETs. Results were expressed as picomoles per milligram protein. Proteins were measured as described by Lowry et al. (22).

The content of cGMP in submandibular gland slices was determined in the presence of ET-1 (1, 10, and 100 nM) or ET-3 (1, 10, and 100 nM) by radioimmunoassay as previously detailed (39). In other experiments, tissues were pretreated with 100 nM BQ-610 or BQ-788 before the addition of ETs. Accumulation of cGMP was expressed as picomoles per milligram protein.

Phosphoinositide hydrolysis assessment in the submandibular gland. Phosphoinositide turnover was determined as previously detailed (2, 41). Submandibular gland slices were incubated in Krebs-Henseleit bicarbonate buffer (KHB) containing 4 μCi/ml myo-[^{3}H]-inositol for 120 min. Tissues were washed and further incubated with 10 nM LiCl followed by exposure to 10 nM ET-1 or ET-3 for 30 min. Tissues were then washed twice with fresh cold KHB solution and homogenized with chloroform-methanol (1:2 vol/vol). In other experiments, tissues were pretreated with 10 μM U73122 (selective PLC inhibitor) or with 100 nM BQ-610 or BQ-788 before the addition of ETs. Phases were separated by the addition of chloroform and water followed by centrifugation at 2,000 *g* for 15 min. The upper phase was applied to an anion exchange column (Bio-Rad X8 resin, 100–200 mesh, formate form) followed by the addition of 5 nM unlabeled myoinositol. Columns were washed and eluted with 1 M ammonium formate and 0.1 M formic acid. The eluted fraction containing inositol 1,4,5-triphosphate, inositol 1,3,4-phosphate, and inositol 1,2,3,4-tetraphosphate represents PLC activity because inositol 1,4,5-triphosphate, the immediate product of PLC activity, is the precursor from which the other forms are synthesized. Experiments were also performed in the presence of MC and NA alone or in combination with ETs. Tritium activity was determined by usual scintillation counting methods, and results are expressed as a percentage of control ± SE.

Detection of ET-1 and ET-3 as well as ET receptors mRNAs in the rat submandibular gland. The mRNA expression of ET-1 and ET-3 as well as that of ET_A and ET_B receptors in the submandibular gland was assessed by RT-PCR. The following primers were used: rat ET-1: 5′-GCTCCTGCTCCTCCTTGATG-3′ (sense), 5′-CTCGCTCTATG-TAAGTCAT GG-3′ (antisense); rat ET-3: 5′-GACTGTCCAACCACA-GAGGA-3′ (sense), 5′-GACCTCCAGTCTCCTGCTTC-3′ (antisense); rat ET_A: 5′-GTTTCCTCCAGCCGA GACTG-3′ (sense), 5′-CACAC-CTTTCCTTCCCCTTAGA-3′ (antisense); rat ET_B: 5′-CAAAGACTG-GTGGCTGTTCAGTT-3′ (sense), 5′- TCAAGGCAATCTGCATACC ACTT- 3′ (antisense) (Genosys, Sigma). Total RNA was isolated from the submandibular gland and the lung by using MasterPure RNA Purification Kit (Epicenter Biotechnologies, Madison, WI). To eliminate genomic DNA, RNA samples were treated with DNAase RQI (Invitrogen). RNA quality and quantity was assessed by 1% agarose gel electrophoresis and UV spectrometry, respectively. Reverse transcription was carried out by the addition of 200U M-MuLV reverse transcriptase (Fermentas Life Sciences, Glen Burnie, MD) and oligo(dT)15 followed by incubation for 60 min at 42°C in M-MuLV buffer. Reverse transcription was terminated by heat-inactivating the reverse transcriptase for 10 min at 70°C. The PCR was carried out in the same reaction mixture containing forward and reverse primers and Go Taq Green master mix (Promega, WI). PCR consisted in denaturation at 95°C for 30 s, annealing at 58°C for 50 s, and extension at 72°C for 50 s, for 50 cycles. A negative control was run in the absence of reverse transcriptase to check specificity of the amplification and a positive control using lung samples. The products were then submitted to electrophoresis on 1% ethidium bromide-stained agarose gel.

Immunohistochemistry studies. Anesthetized rats were perfused through the heart with buffered saline (PBS, pH 7.4) followed by perfusion with 4% paraformaldeyde and 0.2% picric acid solution in 0.1 phosphate buffer, pH 7.4. Submandibular glands were removed and cryopreserved in graded sucrose solutions and frozen in N2-cooled acetone. Cryosections (12 μm) were processed for immunohistochemistry. Rabbit polyclonal anti-ET_A antibodies mapping the COOH terminus or the NH_2 terminus of the ET_A receptor (Alomone and Assay Designs, respectively) and rabbit polycolonal anti-ET_B antibody (Alomone) were used (working dilution 1:200). Biotinylated secondary antibodies (goat anti-rabbit IgG, 1:100) followed by avidin-biotinylated peroxidase complex (Vector Labs, Burlingame, CA) were used for immunoenzymatic detection. Incubation without primary antibodies served as control. A color reaction was obtained using nickel-enhanced diaminobencidine staining (40).

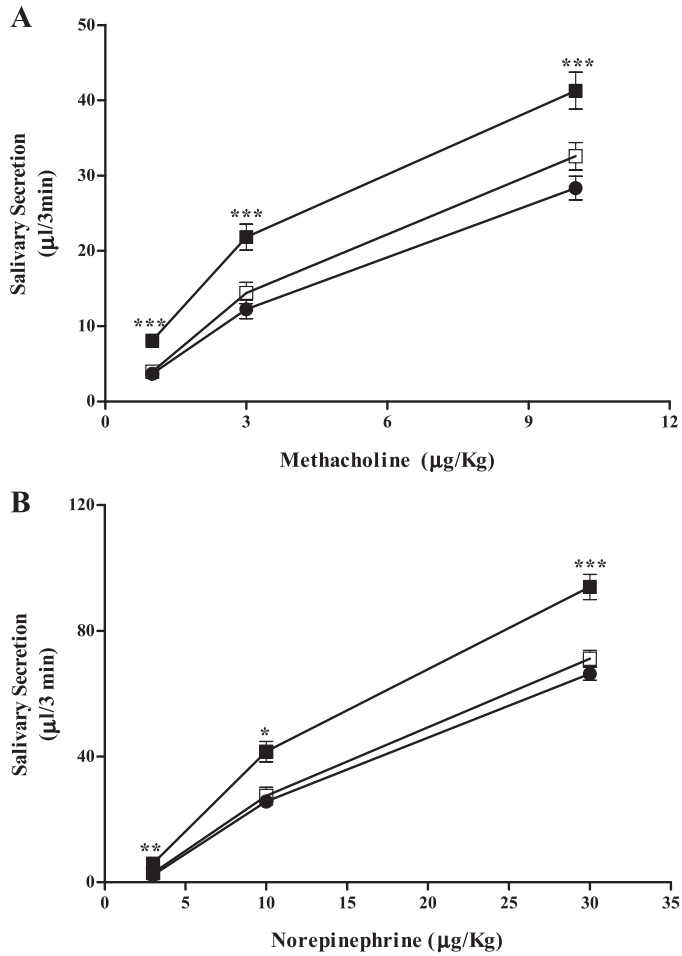

Fig. 1. Effect of endothelin (ET)-1 and ET-3 on cholinergic- and adrenergic-stimulated secretion in the submandibular gland (SMG). Dose-response curves to methacholine (MC; 1, 3, and 10 μg/kg, □; *A*) and norepinephrine (NE; 3, 10 and 30 μg/kg, □; *B*) were performed in the absence or presence of intraglandularly administered ET-1 (■) or ET-3 (●). Saliva was collected as detailed in MATERIALS AND METHODS. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ vs. MC (*A*) or NE (*B*), respectively. Number of experiments: 6–9.

Statistical Analysis

Results are expressed as the means ± SE. The statistical analysis was performed by ANOVA followed by the Student Newman Keuls test. A P of 0.05 or less was considered statistically significant.

RESULTS

Regulation of Submandibular Gland Secretion by Intraglandularly Applied ETs

We first evaluated whether ETs applied to the submandibular gland were able to induce salivation. It was observed that neither ET-1 nor ET-3 evoked secretion per se supporting that these peptides, at least in the tested doses, are not sialogogic agonists. It should be noted that no saliva flows through the cannula when salivary ducts are cannulated, unless salivation is stimulated.

We next conducted experiments to determine whether ETs were able to modify cholinergic- or adrenergic-evoked salivation. Stimulation with MC or NE dose-dependently increased salivary flow as expected. The intraglandular administration of ET-1 enhanced both cholinergic- and adrenergic-evoked salivation (Fig. 1, *A* and *B*). In the presence of ET-1 salivation evoked by 1, 3, and 10 μg/kg MC was increased by 105, 52, and 30%, respectively, whereas that stimulated by 3, 10, and 30 μg/kg NE was enhanced by almost similar percentages (98, 52, and 30%, respectively). The intraglandular administration of ET-3 failed to modify stimulated salivation (Fig. 1, *A* and *B*).

Selective antagonists of conventional ET receptors applied to the gland were used to identify the ET receptor, which mediated ET-1 response. BQ-610 and BQ-788 failed to elicit salivation per se or to modify cholinergic- or adrenergic-evoked secretion (Fig. 2). Blockade of ET_B receptors by BQ-788 (Fig. 2, *C* and *D*) did not modify ET-1 response, but blockade of ET_A receptors by BQ-610 abolished ET-1 stimulatory effect on MC and NE-induced salivation, supporting that ET-1 response was mediated by ET_A receptor activation (Fig. 2, *A* and *B*).

Effect of ETs on cyclic nucleotides content and phosphoinositide hydrolysis in the submandibular gland. We next addressed the intracellular signaling pathways activated by ETs in the submandibular gland. ET-1 and ET-3 decreased basal cAMP levels (Fig. 3*A*); pretreatment with BQ-788 did not modify ETs' response, but blockade of ET_A receptors by BQ-610 abolished ETs' inhibitory effect on basal cAMP intracellular content (Fig. 3*B*). MC modified neither basal cAMP nor ET-1-induced cAMP reduction (Fig. 3*B*). On the

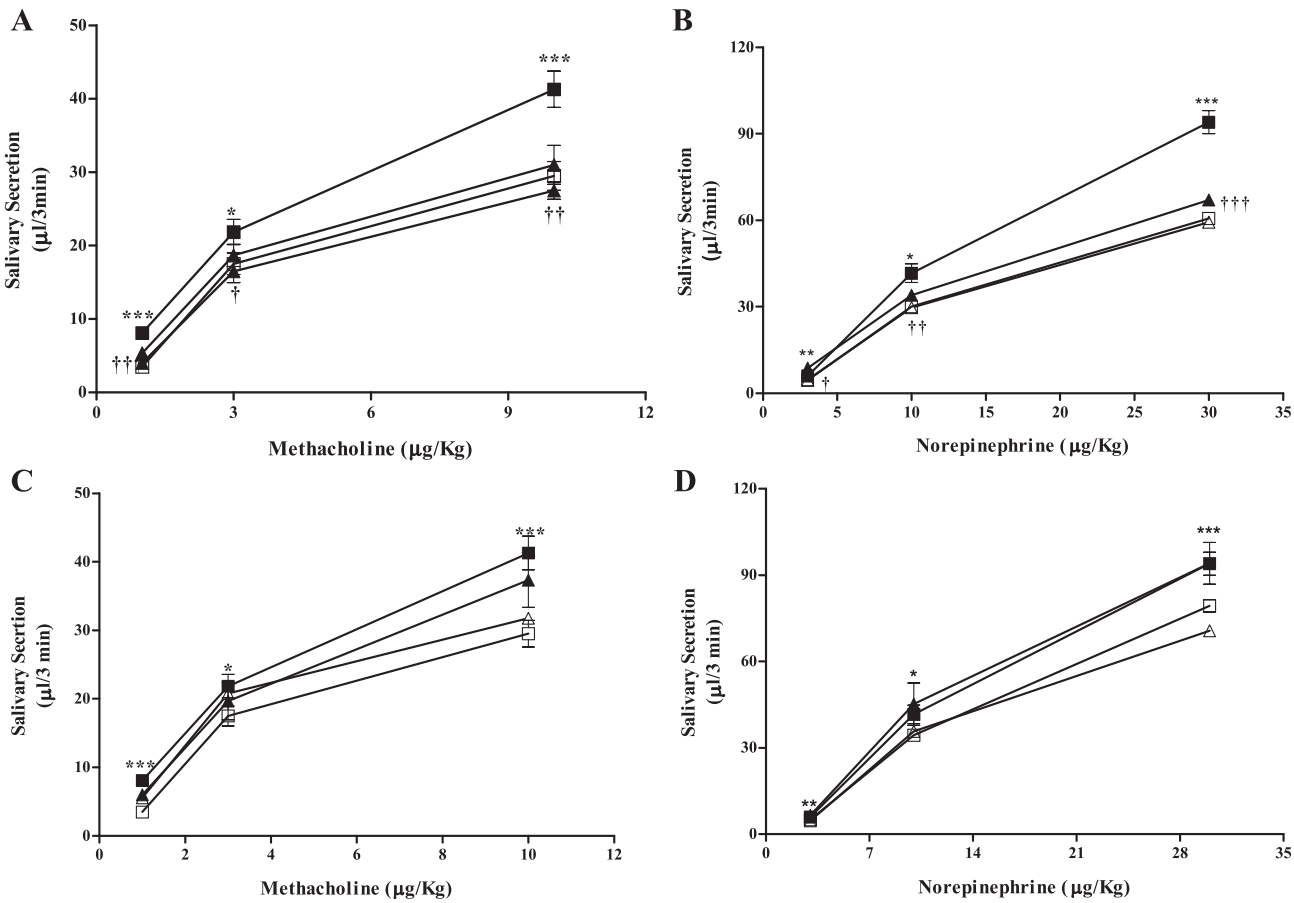

Fig. 2. Effect of ETs on cholinergic- and adrenergic-stimulated secretion following ET_A (*A* and *B*) and ET_B (*C* and *D*) receptor blockade. ET_A and ET_B receptors where inhibited by the selective antagonists BQ-610 and BQ-788, respectively, administered into the gland 10 min before dose response curves to MC (*A* and *C*) or NE (*B* and *D*). *A* and *B*: MC (□) or NE (■); MC or NE + ET-1; BQ-610 (△); BQ-610 + MC or NE + ET-1 (▲). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. MC (*A*) or NE (*B*); †$P < 0.05$, ††$P < 0.01$, and †††$P < 0.001$ vs. MC + ET-1 (*A*) or NE + ET-1 (*B*). Number of experiments: 6–8. *C* and *D*: MC or NE (□); MC or NE + ET-1 (■); BQ-788 (△); BQ-788 + MC or NE + ET-1 (▲). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. MC (*A*) or NE (*B*). Number of experiments: 6–8.

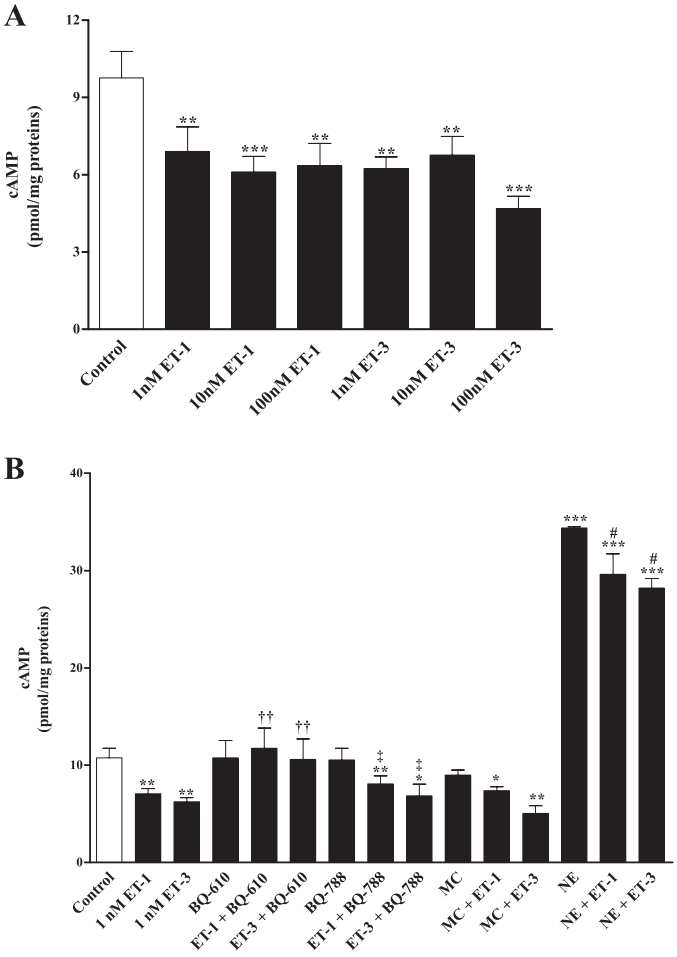

Fig. 3. Effect of ETs on cAMP content in the SMG. *A*: tissues were exposed to 1, 10, and 100 nM ET-1 or ET-3, and cAMP was assessed as detailed in MATERIALS AND METHODS. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control. Number of experiments: 6–8. *B*: effect of ETs on cAMP content in the SMG following ET_A and ET_B blockade or coincubation with MC and NE. SMG slices were incubated with BQ-610 (ET_A selective antagonist) or BQ-788 (ET_B selective antagonist) following exposure to 1 nM ET-1 or ET-3. The content of cAMP was determined as detailed in MATERIALS AND METHODS. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. control; $\dagger\dagger P < 0.01$ vs. ET-1 or ET-3; $\ddagger P < 0.01$ vs. MC; $\#P < 0.05$ vs. NE.

other hand, NE increased the cyclic nucleotide content, but NE response was slightly attenuated in the presence of ETs (Fig. 3*B*).

When the cGMP pathway was investigated it was found that 1, 10, and 100 nM ET-3 increased cGMP through the activation of ET_A receptors (Fig. 4, *A* and *B*). Conversely, ET-1 at 100 nM enhanced cGMP content through the same receptor subtype but failed to modify it at lower concentrations (1 and 10 nM) (Fig. 4, *A* and *B*).

ETs had opposing actions on the phospholipase C pathway. ET-1 enhanced phosphoinositide turnover by 100%, whereas ET-3 decreased it by 25% (Fig. 5*A*). The response was mediated by ET_A receptor activation since it was abolished by BQ-610 (Fig. 5*A*). Both MC and NE increased phosphoinositide turnover as expected. In the presence of MC or NE, ET-1 response was further enhanced, whereas that of ET-3 was not observed (Fig. 5*B*).

Detection of ETs and ET receptors mRNAs in the submandibular gland. RT-PCR analysis showed that ET_A and ET_B receptors were expressed in the submandibular gland (Fig. 6*A*). ET_A and ET_B primers yielded products of the expected size (133 and 119 bp, respectively). In addition, ET-1 and ET-3 mRNAs were also expressed in the submandibular gland, suggesting local production of both peptides (Fig. 6*B*). ET-1 and ET-3 primers also yielded products of the expected size (500 and 207 bp, respectively).

Localization of ET receptors in the submandibular gland. Immunohistochemistry studies revealed that ET_A receptor staining had intense periductular localization, particularly around interlobular ducts (ET_A antibody mapping the NH_2 terminus of the ET_A receptor) (Fig. 7, *A–C*). Furthermore, studies carried out with the ET_A antibody mapping the COOH terminus of the ETA receptor showed that ET_A immunoreactivity was not only observed around interlobular ducts (Fig. 7*E*) but also around acini (Fig. 7*F*). These findings suggest that

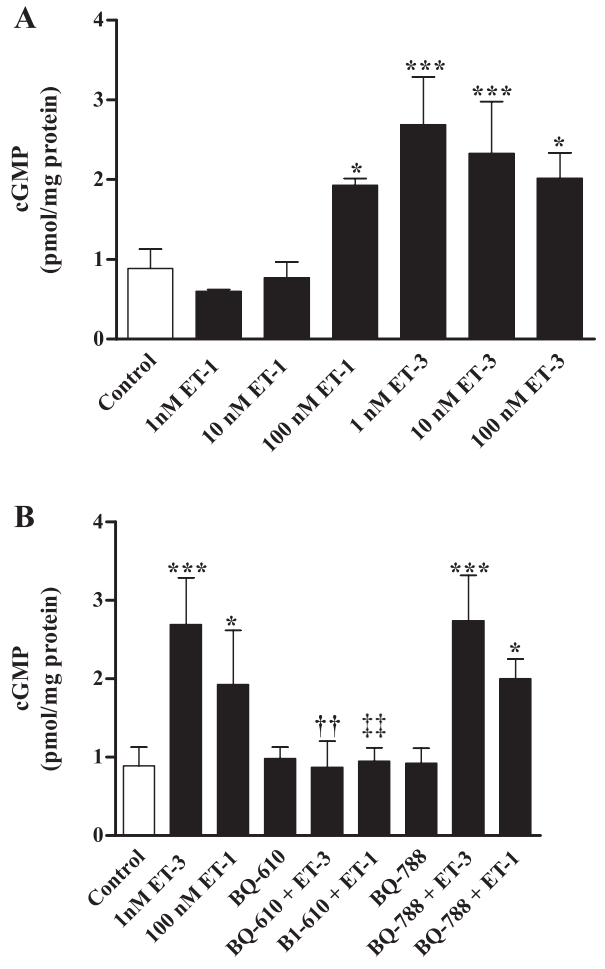

Fig. 4. Effect of ETs on cGMP content in the SMG. *A*: tissues were incubated with 1, 10, and 100 nM ET-1 or ET-3, and cGMP was determined as detailed in MATERIALS AND METHODS. $^{*}P < 0.05$, and $^{***}P < 0.001$ vs. control. Number of experiments: 6–8. *B*: effect of ETs on cGMP content in the SMG following ET_A and ET_B blockade. SMG slices were incubated with BQ-610 (ET_A selective antagonist) or BQ-788 (ET_B selective antagonist) following exposure to 1 nM ET-3 or 100 nM ET-1. The content of cGMP was determined as detailed in MATERIALS AND METHODS. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. control; $\dagger\dagger P < 0.01$ vs. 1 nM ET-3; $\ddagger\ddagger P < 0.01$ vs. 100 nM ET-1. Number of experiments: 4–6.

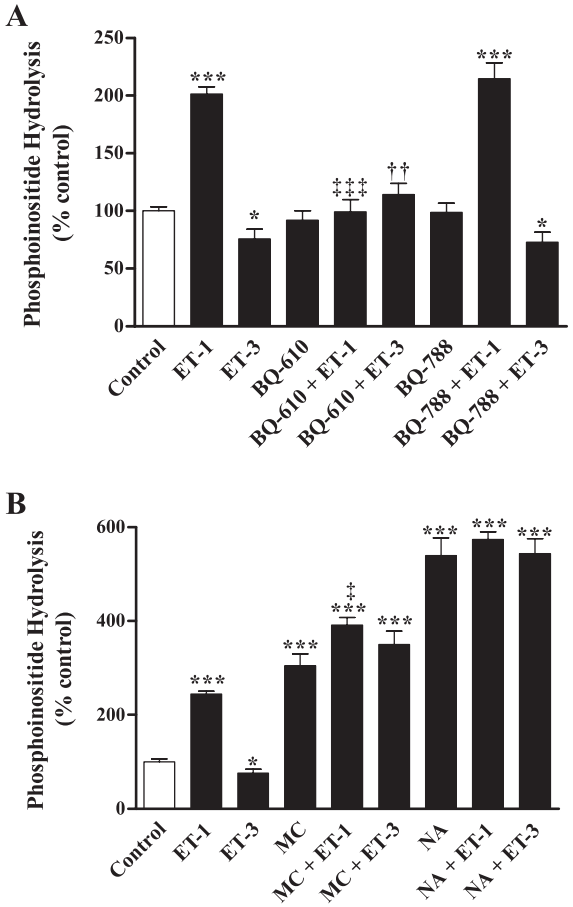

Fig. 5. effect of ET-1 and ET-3 on phosphoinositide hydrolysis in the SMG. *A*: SMG slices were incubated with ET-1 or ET-3 in the presence or absence of BQ-610 (ET_A selective antagonist) or BQ-788 (ET_B selective antagonist) pretreatment. Phosphoinositide hydrolysis was determined as detailed in MATERIALS AND METHODS. $*P < 0.05$ and $***P < 0.001$ vs. control; $‡‡‡P < 0.001$; $††P < 0.01$ vs. 1 nM ET-3. *B*: tissues were incubated with ET-1 and ET-3 alone or combined with MC and NE. $***P < 0.001$ vs. control; $†*P < 0.05$ vs. MC; $‡P < 0.05$ vs. MC. Number of experiments: 5–7.

the ET_A receptor is presumably located in myopithelial cells. On the other hand, ETB receptor staining was restricted to blood vessels (Fig. 7*D*). Individual acinar cells showed no immunoreactivity to ET_A or ET_B receptors.

Regulation of Submandibular Gland Secretion by Centrally Applied ETs

We first addressed whether centrally applied ETs induced submandibular gland secretion. ET-1 and ET-3 induced no salivation, suggesting that ETs do not behave as sialogogic agonists when applied to the brain, at least in the tested doses. However, both peptides potentiated cholinergic- and adrenergic-evoked salivary secretion when intracerebroventricularly administered (Fig. 8). ET-3 induced a more pronounced stimulatory effect than ET-1. In the presence of ET-1, 1, 3, and 10 μg/kg MC-evoked salivation was increased by 50, 50, and 30%, respectively, whereas that of 3, 10, and 30 μg/kg NE was by 20, 30, and 50%, respectively (Fig. 8, *A* and *B*). On the other hand, in the presence of ET-3 cholinergic-evoked salivation (1, 3, and 10 μg/kg MC) was increased by 96, 80, and 65%, whereas adrenergic induced salivation (3, 10, and 30 μg /kg NE) by 78, 75, and 95%, respectively (Fig. 8, *A* and *B*). Acute parasympathetic decentralization by chorda timpani nerve section did not affect MC- or NA-evoked salivation, but it prevented the increase induced by ETs applied to the brain suggesting that parasympathetic activation mediated central ETs' response (Fig. 9).

To identify the brain ET receptors involved in the secretory response, rats were pretreated with intracerebroventricularly administered BQ-610 or BQ-788 (Fig. 10). Centrally applied ET_A and ET_B antagonists did not elicit salivation by the submandibular gland and did not modify cholinergic- or adrenergic-induced secretion. However, ET-1 response was blocked by BQ-610 supporting activation of central ET_A receptors (Fig. 10). Both BQ-610 and BQ-788 individually abolished ET-3-induced stimulation of MC- and NE-evoked salivation, suggesting that the effect could be mediated by nonconventional ET receptors (Fig. 11).

DISCUSSION

The major findings of the present study were that ETs participated in the central and peripheral regulation of submandibular salivary secretion, and further, that both ETs were expressed in the gland, suggesting that they are locally released

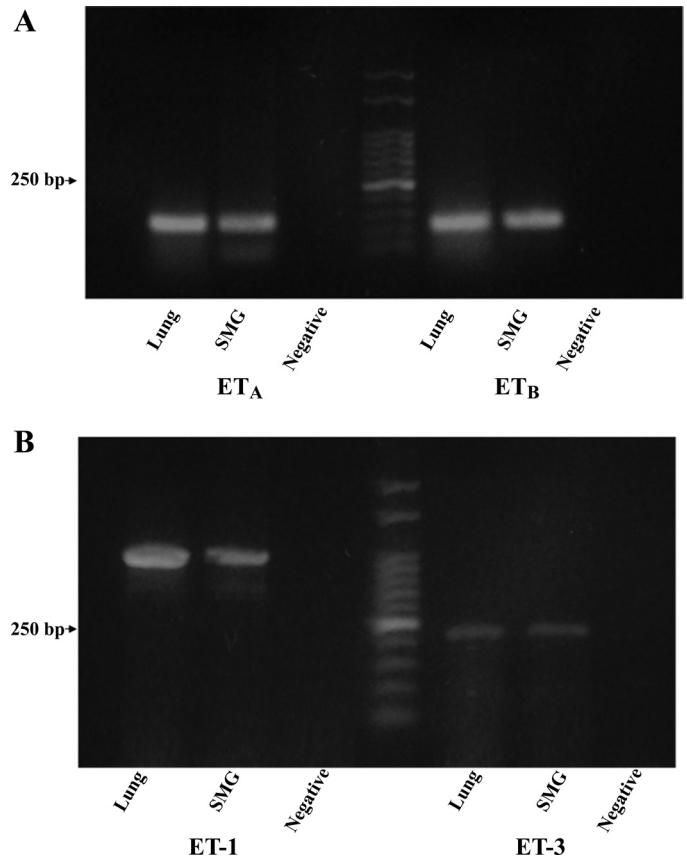

Fig. 6. Expression of ET receptors and ETs in the SMG. *A*: expression of the conventional ET receptors, ET_A and ET_B, were assessed in the SMG and in the lung (positive control) by RT-PCR as detailed in MATERIALS AND METHODS. ET_A and ET_B primers yielded products of the expected size (133 and 119 bp, respectively). *B*: expression of ETs in the SMG. Expressions of ET-1 and ET-3 was assessed in the SMG and in the lung (positive control) by RT-PCR as detailed in MATERIALS AND METHODS. ET-1 and ET-3 primers yielded products of the expected size (500 and 207 bp, respectively).

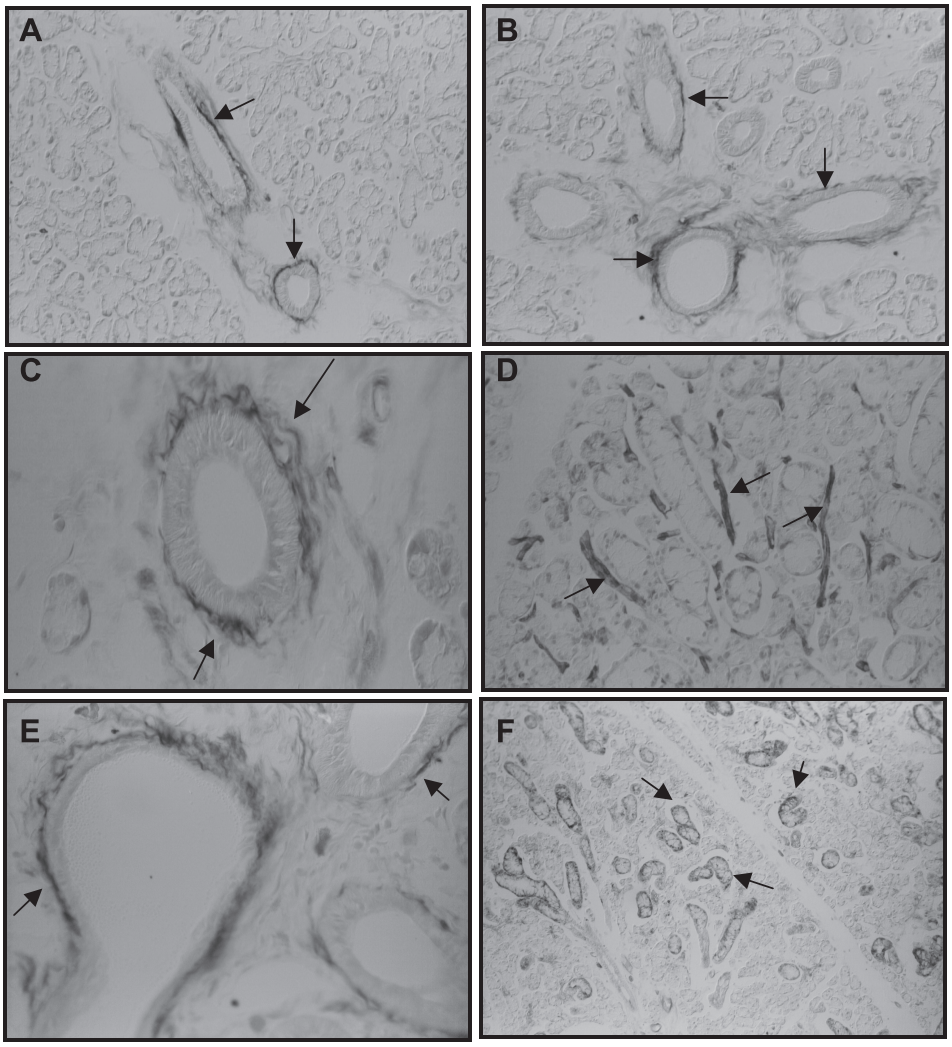

Fig. 7. Immunohistochemical localization of ET receptors in the SMG. ET_A (*A–C* and *E–F*) and ET_B (*D*) receptors were detected by immunohistochemistry as detailed in MATERIALS AND METHODS. *A* and *B*: periductular localization of the ET_A receptor (magnification, ×100). A strong staining was detected surrounding interlobular ducts (arrows) by using a polyclonal antibody against the COOH terminus of the rat ET_A receptor. *C*: an interlobular duct shown at a larger magnification (×400) where the immunostaining for ET_A receptor is seen in periductular cells coincident with the localization of myopithelial cells (arrows). *D*: ET_B receptor (magnification, ×200) showed a vascular localization restricted to capillaries (arrows). Periductular (*E*; arrows; magnification, ×300) and periacinar (*F*; arrows; magnification, ×100) localization of ET_A immunoreactivity using a polyclonal antibody against the NH_2 terminus of the rat ET_A receptor.

to act as paracrine and/or autacrine factors. The expression of ET receptors further suggests the existence of an endothelinergic system in the submandibular gland, which would contribute to control its functionality.

Salivary secretion is mainly under the synergic control of the parasympathetic and sympathetic nervous system, but various peptides, neuropeptides, and other factors locally released contribute to modulate salivation (26). Parasympathetic synaptic vesicles contain acetylcholine but also neuropeptides like vasoactive intestinal peptide and substance P, which are released in response to plasma membrane depolarization and have direct secretomotor effects in the submandibular gland (26). Other peptides like calcitonin gene-related peptide and atrial natriuretic factor, also produced by the glands, do not elicit salivation per se, but they enhance cholinergic- and adrenergic-evoked salivation (17, 26). In this regard, we previously reported that atrial natriuretic factor potentiates cholinergic-, adrenergic-, and substance P-stimulated salivary secretion through the activation of NPR-C receptors coupled to the inhibition of adenylyl cyclase and stimulation of PLC (3, 4, 41). The exogenous administration of substance P elicits copious salivation, whereas calcitonin gene-related peptide is not a sialogogic agonist, although it modulates parasympathetic-evoked secretion (16). In addition, other mediators like purinergic agonists and nitric oxide also have been shown to contribute to the stimulation of salivary glands (8, 21). Nitric oxide synthase is expressed in nerve fibers surrounding the acini as well as in acinar cells, supporting the dual source of nitric oxide in salivary glands (21). Thus, the normal secretory activity in the salivary glands results from the complex and simultaneous activation of an entire repertoire of receptors and associated intracellular pathways, not only in the acinar and ductal cells, but also in myoepithelial cells. Present findings support that ETs are locally produced and contribute to the regulation of the submandibular gland secretion.

We first sought to establish whether ETs and their receptors were expressed in the submandibular gland. Immunoreactive ETs and ET binding in the salivary glands of different species have been previously reported (13, 19, 28). Furthermore, pre-pro ET-3 as well as pre-pro ET-1 was shown to be expressed in various tissues including rat submandibular glands of different species (36, 38). In the present study, RT-PCR analysis confirmed that ET-1 and ET-3 mRNAs were expressed in the rat submandibular gland, suggesting that these peptides are locally synthesized. Furthermore, ET_A and ET_B receptor mRNAs were also expressed in the tissue. The ET_B receptor was localized in capillaries, whereas ET_A receptors had periductal and periacinar localization. Intense ET_A immunostaining was observed surrounding the inter-

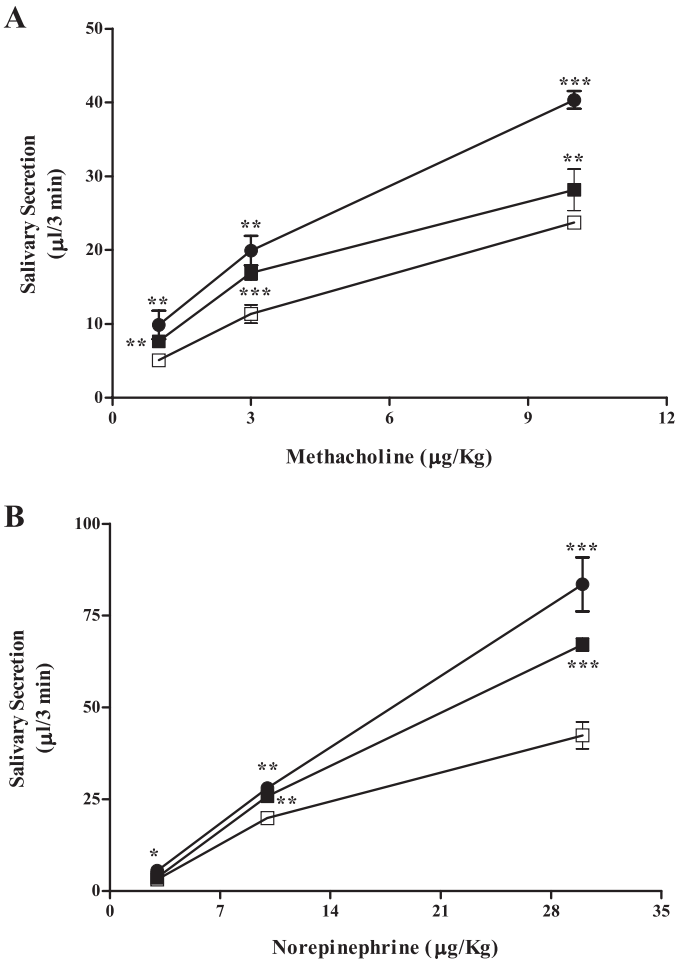

Fig. 8. Effect of centrally applied ET-1 and ET-3 on cholinergic- and adrenergic-evoked SMG secretion. Dose response curves to MC (*A*) or NE (*B*) were performed in the absence (□) or presence of intracerebroventricularly applied ET-1(■) or ET-3 (●). Saliva was collected as detailed in MATERIALS AND METHODS. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ vs. MC (*A*) or NE (*B*). Number of experiments: 6–8.

lobular ducts, and around the acini coincident with the localization of myopithelial cells. The presence of ET_B receptors in endothelial cells has been previously reported, whereas the localization of ET_A receptors in myopithelial cells has been shown in lacrimal glands (25). The authors showed that ET_A receptors were expressed in myoepithelial cells, and although no functional studies were performed, it was suggested that ETs may have a role in lacrimal secretion. Given the contractile property of ETs and the localization of ET_A receptors, presumably in myoepithelial cells, it is possible to assume that ETs may target this cell type.

Local administration of ET-1 in the gland failed to elicit salivation per se, but it potentiated cholinergic- and adrenergic-induced secretion. Blockade of ET_A receptors in the gland by the selective antagonist BQ-610 abolished ET-1 response, supporting that the effect was mediated by ET_A activation. Conversely, ET-3 failed to evoke salivation or to modify stimulated-secretion. ET-1 is a potent vasoconstrictor peptide that acts directly on vascular smooth muscle cells increasing the intracellular calcium concentration and hence causing their contraction. Therefore, it could have been expected that the potent vasoconstrictor properties of ET-1 could have reduced salivary blood flow and eventually the rate of salivary secretion. However, the contribution of local hemodynamic changes to ET-1 secretory response seems rather unlikely. Previous studies showed that the production of saliva is compromised when blood flow is substantially reduced for 5 min, but it is virtually immune to deprivation over shorter periods (23). It was proposed that this may be due in part to the ability of acinar cells to withstand a fluid debt (23). In the present study, saliva was collected for 3 min so if hemodynamic changes occurred, they were not reflected on ET-1 secretory response. Furthermore, the presence of ET_A receptors likely in the myoepithelial cells of the submandibular gland suggests that ET-1 may target this cell type. Myoepithelial cells are found surrounding the acini and the proximal ductal system (interlobular ducts) and contract upon stimulation to constrict the underlying parenchyma, thus aiding in the expulsion of saliva. Present findings suggest that ET-1 is produced in the gland, thereby serving an autocrine/paracrine function within the salivary gland. Contraction of myoepithelial cells by ET-1 binding to ET_A receptors is likely the underlying mechanism of ET-1-enhanced cholinergic and adrenergic-evoked salivation in the rat.

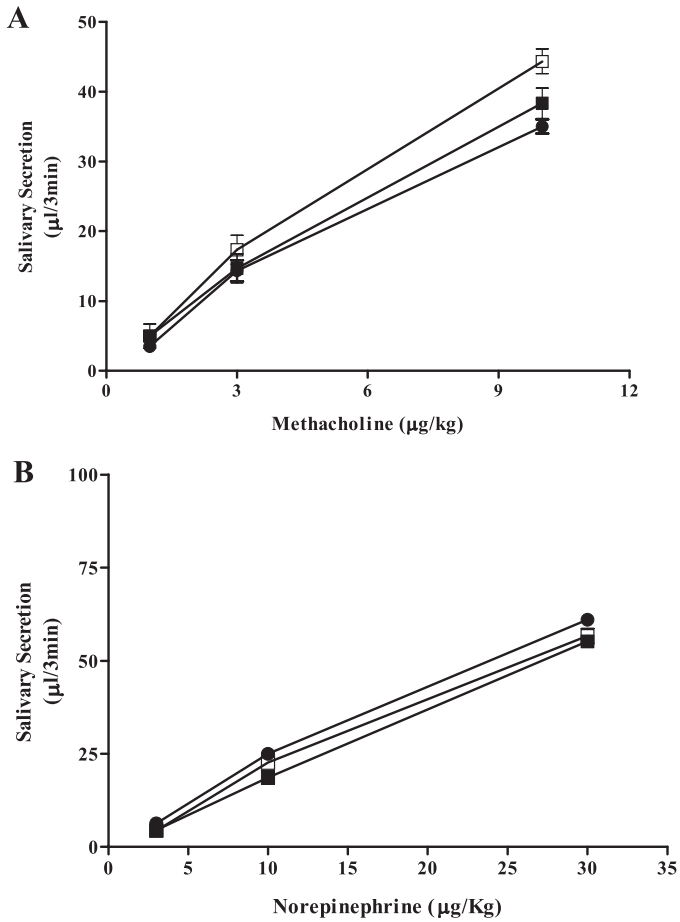

Fig. 9. Effect of centrally applied ETs on cholinergic- and adrenergic-evoked salivation in rats with acute parasympathetic decentralization. Dose response curves to MC (*A*) or NE (*B*) were performed in the absence (□) or presence of intracerebroventricularly applied ET-1 (■) or ET-3 (●) in rats with chorda timpani section (acute parasympathetic decentralization). Saliva was collected as detailed in MATERIALS AND METHODS. Number of experiments: 4–6.

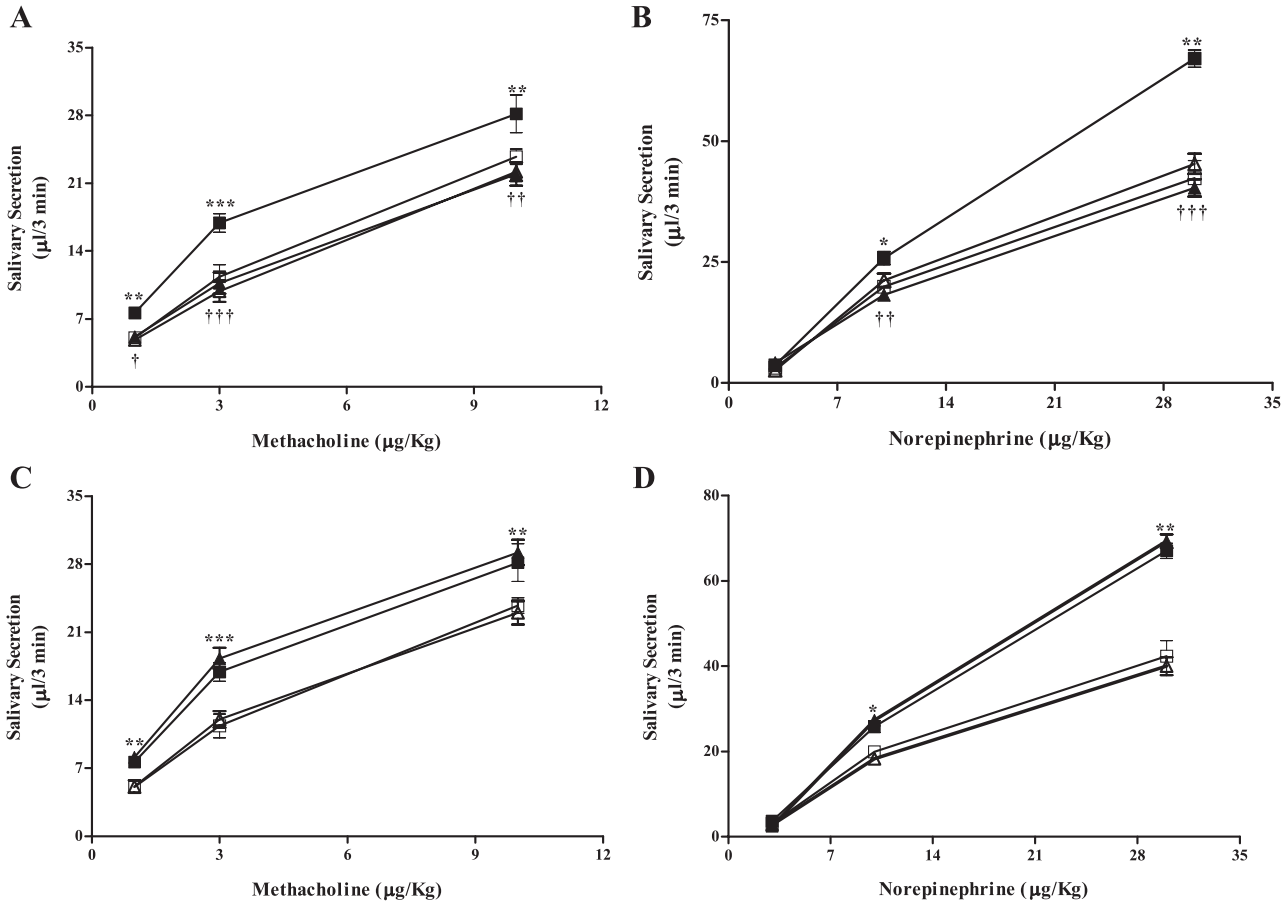

Fig. 10. Effect of centrally applied ET-1 on cholinergic- and adrenergic-evoked SMG secretion following brain ET_A (*A* and *B*) and ET_B (*C* and *D*) receptor blockade. Brain ET_A receptors were inhibited by the intracerebroventricular administration of BQ-610 and dose response curves to MC (*A*) and NE (*B*) were performed in the presence of centrally applied ET-1 (5 ng/µl). Saliva samples were collected as detailed in MATERIALS AND METHODS. *A*: MC (□); MC+ ET-1 (■); BQ-610 + MC (△); BQ-610 + MC + ET-1 (▲). *B*: NE (□); NE+ ET-1 (■); BQ-610 + NE (△); BQ-610 + NE + ET-1 (▲).$^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. MC (*A*) or NE (*B*); $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$ vs. ET-1. Number of experiments: 5–7. Brain ET_B receptors were inhibited by the intracerebroventricular administration of BQ-788 and dose response curves to MC (*C*) and NE (*D*) were performed in the presence of centrally applied ET-1 (5 ng/µl). Saliva samples were collected as detailed in MATERIALS AND METHODS. *C*: MC (□); MC+ ET-1 (■); BQ-788 + MC (△); BQ-788 + MC + ET-1 (▲). *D*: NE (□); NE+ ET-1 (■); BQ-788 + NE (△); BQ-788 + NE + ET-1 (▲). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. MC (*C*) or NE (*D*). Number of experiments: 5–7.

Although ET-3 may bind ET_A receptors, this peptide failed to modify cholinergic- or adrenergic-evoked salivation. ET-3 may presumably regulate functions others than the secretory process. ETs promote cell proliferation in various tissues including the salivary glands. It was recently reported that ET-3 through ET_A receptors directs extension of axons of a subset of sympathetic neurons from the superior cervical ganglion to a preferred intermediate target, the external carotid artery, which serves as the gateway to select targets including the salivary glands (24).

We next attempted to address the intracellular signaling triggered by ET-1 that mediated its response in the submandibular gland. ET receptors are able to stimulate or inhibit multiple signaling pathways in diverse tissues and cell types (37). Salivation is linked to both calcium-mobilizing and cAMP-generating signaling pathways (26). ET-1 through ET_A receptors reduced basal cAMP levels but enhanced phosphatidylinositol hydrolysis in the submandibular gland. These findings suggest that ET-1 enhanced cholinergic- and adrenergic-evoked salivation through ET_A receptors coupled to PLC activation. However, as shown in the present study, ET_A receptors were not localized in acinar cells but presumably in myopithelial cells. Therefore ET-1 activation of ET_A receptors coupled to PLC activation would be the intracellular signaling underlying the contraction of myopithelial cells, thus resulting in enhanced cholinergic and adrenergic salivation in the presence of the peptide. On the other hand, ET-3 reduced both cAMP content and phosphoinositide turnover. The reduction in phosphoinositide hydrolysis may account for the lack of effect of ET-3 on cholinergic- and adrenergic-evoked salivation.

As ET receptors may activate multiple signaling pathways we also evaluated the effect of ETs on the guanylyl cyclase pathway. Both ET-1 and ET-3 increased cGMP content in the submandibular gland through the activation of ET_A receptors. cGMP is not involved in the stimulus-secretion coupling mechanism in salivary glands. However, in secretory cells like pancreatic acinar cells, it has been shown that agonists that stimulate PLC, and in turn cause intracellular calcium elevation, also increase cGMP. Diverse studies have implicated this messenger in the stimulation of capacitative or store-operated calcium entry, which is activated following the emptying of agonist-sensitive intracellular calcium stores in different tis-

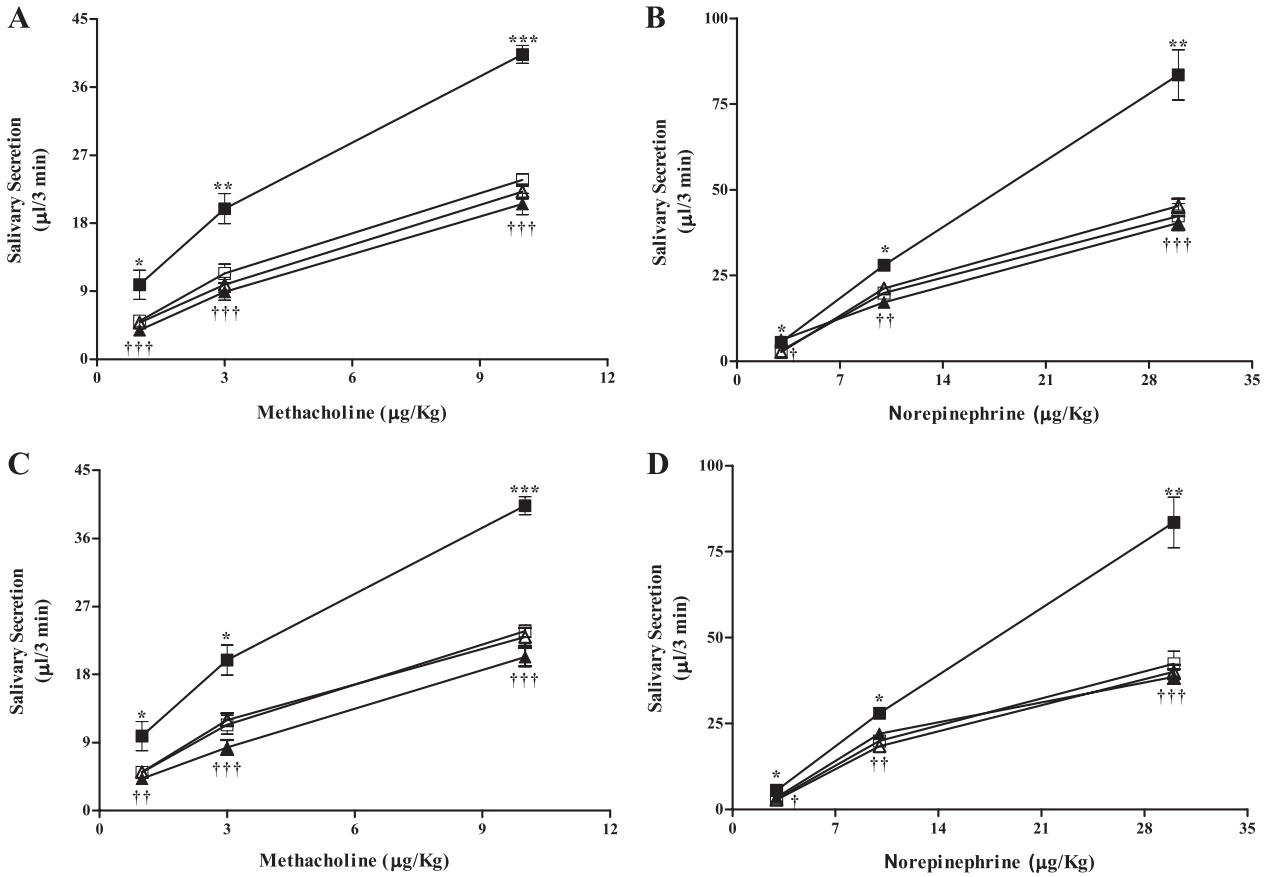

Fig. 11. Effect of centrally applied ET-3 on cholinergic- and adrenergic-evoked SMG secretion following brain ET_A (*A* and *B*) and ET_B (*C* and *D*) receptor blockade. Brain ET_A receptors were inhibited by the intracerebroventricular administration of BQ-610, and dose response curves to MC (*A*) and NE (*B*) were performed in the presence of centrally applied ET-3 (5 ng/μl). Saliva samples were collected as detailed in MATERIALS AND METHODS. *A*: MC (□); MC+ ET-3 (■); BQ-610 + MC (△); BQ-610 + MC + ET-3 (▲). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. MC (*A*) or NE (*B*); †$P < 0.05$, ††:$P < 0.01$, and †††$P < 0.001$ vs. ET-3. Number of experiments: 6–8. Brain ET_B receptors were inhibited by the intracerebroventricular administration of BQ-788, and dose response curves to MC (*C*) and NE (*D*) were performed in the presence of centrally applied ET-3 (5 ng/μl). *C*: MC (□); MC+ ET-3 (■); BQ-788 + MC (△); BQ-788 + MC + ET-3 (▲). Saliva samples were collected as detailed in MATERIALS AND METHODS. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. MC (*C*) or NE (*D*); †$P < 0.05$, ††$P < 0.01$, and †††$P < 0.001$ vs. ET-3. Number of experiments: 6–8.

sues and cell types. However, the regulation of the activity of the store-operated calcium channels in salivary glands remains poorly understood.

The lack of effect of ET_A and ET_B blockade on cholinergic- and adrenergic-evoked salivation argues against the existence of an endothelinergic tone in the gland to control salivation. ETs are produced in various tissues but the stimuli, which promote their release are not fully understood. Hypoxia and various cytokines have been shown to promote ETs' release in diverse tissues. In addition, ETs and ET receptors are overexpressed in diverse cardiovascular disorders like hypertension. Thus it can be speculated that ET-1 may play a role in pathophysiological situations where salivation may be impaired or compromised.

We previously reported that ET-1 and ET-3 applied to the brain modify bile secretion in the rat (31, 32). Furthermore, ETs act in the lower brain stem to increase intragastric pressure and gastric smooth muscle contractile activity via a vagally mediated pathway (18). Therefore we sought to establish whether ETs applied to the brain-modulated submandibular gland secretion. The sites and situations of synthesis and/or release of ETs under physiological conditions in the central nervous system are scarcely understood. Various studies show that immunoreactive ET converting enzyme is detectable predominantly in neuronal cells with a pattern similar to ET-like immunoreactivity, whereas nonneuronal cells are generally not immunoreactive, supporting that ETs are released mainly from neuronal cells. ET-1 and ET-3 in the brain also participate in the regulation of brain neurotransmission by affecting the release and uptake of neurotransmitters like NE, dopamine, and GABA (9, 10, 30). ET receptors are widely expressed in the central nervous system including the dorsal vagal complex, which is the major brain site involved in the control of the digestive function. In the present study, ETs applied to brain did not elicit salivation per se but they enhanced cholinergic- and adrenergic-evoked salivary secretion through parasympathetic pathways. Acute parasympathetic decentralization did not affect MC- or NE-evoked salivation, but it prevented the response of centrally applied ETs. These findings support that central regulation of submandibular secretion by ETs is mediated by parasympathetic pathways.

ET-1 response was mediated by the activation of central ET_A receptors, whereas ET-3 effects appeared to be mediated by nonconventional ET receptors. The observation that ET-3 response was inhibited by both ET_A and ET_B selective antagonists is an atypical response, which suggests the involvement

of atypical ET receptors. Current evidence shows that several ET-3 mediated effects in the brain involve nonconventional ET receptors. In this regard, we reported that ET-3 applied to the brain through non-ET_A and non-ET_B receptors modulate bile secretion in the rat (31). Furthermore, ET-3 increases tyrosine hydroxylase activity and expression in the posterior hypothalamus through nonconventional ET receptors (30). Recent studies suggest that atypical responses may result from dimerization of the conventional ET_A and ET_B receptors (12, 14). However, further studies are necessary to prove this assumption. Nevertheless, it can also be possible that drug application by the intracerebroventricular pathway may affect circuits including both ET_A and ET_B receptors, not necessarily present in the same cell.

In conclusion, present findings show that ETs do not induce salivation per se but they regulate submandibular gland secretion in the rat. When intraglandularly applied, ET-1 by activating ET_A receptors coupled to the PLC pathway enhanced cholinergic- and adrenergic-evoked salivation presumably by the contraction of myoepithelial cells since ET_A receptors appear to be localized in that cell type. However, both peptides increased stimulated secretion when applied to the brain through distinct ET receptors through the activation of parasympathetic pathways. The detection of ET-1 and ET-3 mRNAs in the gland support that ETs are locally released to modulate the function of the gland in a paracrine and/or autacrine manner. This study shows that ETs are involved in the peripheral and central regulation of salivary secretion in the rat.

Perspectives and Significance

The importance of saliva is evident when secretion is reduced or absent. Saliva in the oral cavity is relevant in mastication, digestion, swallowing, speech, taste, and denture holding. It has anticaries activity, antihalitosis activity, and antiinfective activity. Xereostomia impairs swallowing of food leading to malnutrition and also results in ulceration of the oral mucosa. Current therapeutic tools for this pathophysiological situation are limited because only a few pharmacological agents are available to promote salivary secretion. Therefore studies conducted to characterize new ways of stimulating salivary gland function might eventually lead to the discovery of new sialogogues. We here report that ETs enhanced salivation but whether these peptides represent potential new therapeutic targets to stimulate salivary secretion remains to be fully elucidated.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

1. **Barton M, Yanagisawa M.** Endothelin: 20 years from discovery to therapy. *Can J Physiol Pharmacol* 86: 485–498, 2008.
2. **Berridge MJ, Downes CP, Hanley MR.** Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochem J* 206: 587–595, 1982.
3. **Bianciotti LG, Elverdin JC, Vatta MS, Fernandez BE.** Atrial natriuretic factor modifies the composition of induced salivary secretion in the rat. *Regul Pept* 65: 139–143, 1996.
4. **Bianciotti LG, Elverdin JC, Vatta Colatrella C, Fernandez BE.** Atrial natriuretic factor enhances induced salivary secretion in the rat. *Regul Pept* 49: 195–202, 1994.
5. **Davenport AP, Maguire JJ.** Endothelin. In: *Handbook of Experimental Pharmacology, The Vascular Endothelium I.* Berlin, Germany: Springer Verlag, 2006, vol. 176/I, p. 295–329.
6. **Davenport AP.** International Union of Pharmacology XXIX: Update on endothelin receptor nomenclature. *Pharmacol Rev* 54: 219–226, 2002.
7. **Davio CA, Cricco GP, Bergoc RM, Rivera E.** H1 and H2 histamine receptors in *N*-nitroso-*N*-methylurea (NMU)-induced carcinomas with atypical coupling to signal transducers. *Biochem Pharmacol* 50: 91–96, 1995.
8. **Dehaye JP, Moran A, Marino A.** Purines, a new class of agonists in salivary glands? *Arch Oral Biol* 44: S39–S43, 1999.
9. **di Nunzio AS, Jaureguiberry MS, Rodano V, Bianciotti LG, Vatta MS.** Endothelin 1 and 3 diminish neuronal NE release through a NO mechanism in the rat anterior hypothalamus. *Am J Physiol Regul Integr Comp Physiol* 283: R615–R622, 2002.
10. **di Nunzio AS, Legaz G, Rodano V, Bianciotti LG, Vatta MS.** Modulatory effect of endohtelin 1 and 3 on neuronal norepinephrine release in rat posterior hypothalamus. *Regul Pept* 118: 51–59, 2004.
11. **Fernández Solari J, Prestifilippo JP, Vissio O, Enhart Bornstein M, Bornstein SR, Rettori V, Elverdin JC.** Anandamide injected into the lateral ventricle of the brain inhibits submandibular salivary secretion by attenuating parasympathetic neurotransmission. *Braz J Med Biol Res* 42: 537–544, 2009.
12. **Gregan B, Jurgensen J, Papsdorf G.** Ligand dependent differences in the internalization of endothelin A and endothelin B receptor heterodimers. *J Biol Chem* 279: 27679–27687, 2004.
13. **Gresik EW, Hosoi K, Kurihara K, Maruyama S, Ueha T.** The rodent convoluted tubule cell–an update. *Eur J Morphol* 34: 221–224, 1996.
14. **Harada N, Himeno A, Shigematsu K.** Endothelin binding to endothelin receptors in the anterior pituitary gland: possible formation of and ET_A-ET_B receptor heterodimer. *Cell Mol Neurobiol* 22: 207–226, 2002.
15. **Henry PJ, King SH.** Typical endothelin ETA receptors mediate atypical endothelin 1-induced contractions in sheep isolated tracheal smooth muscle. *J Pharmacol Exp Ther* 289: 1385–1390, 1999.
16. **Iwabuchi Y, Kimura T.** Effect of calcitonin and calcitonin-gene related peptide on the substance P-mediated secretion of fluid from the rat submandibular gland. *Gen Pharmacol* 31: 137–141, 1998.
17. **Janowski M, Tremblay J, Gutkowska J.** Natriuretic peptide system in the rat submaxillary gland. *Regul Pept* 62: 53–61, 1996.
18. **Krowicki Z, Nathan N, Hornby P.** Excitatory gastric motor and cardiovascular effects of endothelins in the dorsal vagal complex are mediated through ET_A receptors. *J Pharmacol Exp Ther* 282: 535–542, 1997.
19. **Lam HC, Takahashi k, Ghatei MA, Suda K, Kanse SM, Bloom SR.** Presence of immunoreactive endothelin in human saliva and rat parotid gland. *Peptides* 12: 883–885, 1991.
20. **Lomniczi A, Mohn C, Falletti A, Franchi A, McCann SM, Rettori V, Elverdin JC.** Inhibition of salivary secretion by lipopolysaccharide: possible role of prostaglandins. *Am J Physiol Endocrinol Metab* 281: E405–E411, 2001.
21. **Looms D, Tritsaris K, Pedersen AM, Nauntofte B, Dissing S.** Nitric oxide signalling in salivary glands. *J Oral Pathol Med* 31: 569–584, 2002.
22. **Lowry OH, Rosenbrough NJ, Farr AI, Randall RJ.** Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265–275, 1951.
23. **Lung MA.** Autonomic nervous control of myoepithelial cells and secretion in submandibular gland of anaesthetized dogs. *J Physiol* 546: 837–850, 2003.
24. **Makita T, Sucov HM, Gariepy CE, Yanagisawa M, Ginty DD.** Endothelins are vascular derived axonal guidance cues for developing sympathetic neurons. *Nature* 452: 759–763, 2008.
25. **Matsumoto Y, Ishibashi T, Niiya A, Yamada H, Kurokawa K, Kinoshita S.** Distribution of endothelin and endothelin A receptor in the lacrimal glands of the monkey (*Macaca fuscata*). *Exp Eye Res* 64: 127–132, 1997.
26. **Melvin JE, Shuthleworth T, Begenisich T.** Regulation of fluid and electrolyte composition in salivary gland acinar cells. *Annu Rev Physiol* 27: 445–469, 2005.
27. **Motte S, McEntee K, Naeije R.** Endothelin receptor antagonists. *Pharmacol Rev* 110: 386–414, 2006.

28. **Nakamizo M, Pawankar R, Ohkubo K.** Presence of endothelin 1 in human salivary glands and tumors. *Nippon Ika Daigaku Zasshi* 65: 471–477, 1998.
29. **Paxinos E, Watson J.** *The Rat Brain Stereotaxic Coordinates* (2nd Ed.), New York: Academic, 1986.
30. **Perfume G, Nabhen SL, Riquelme Barrera K, Otero MG, Bianciotti LG, Vatta MS.** Long-term modulation of tyrosine hydroxylase activity and expression by endothelin 1 and 3 in the rat anterior and posterior hypothalamus. *Am J Physiol Regul Integr Comp Physiol* 294: R905–R914, 2008.
31. **Rodríguez MR, Sabbatini ME, Santella G, Dabas P, Villagra A, Vatta MS, Bianciotti LG.** Endothelin 3 applied to the brain evokes opposite effects on bile secretion mediated by a central nitric oxide pathway. *Peptides* 26: 1219–1227, 2005.
32. **Rodriguez MR, Sabbatini ME, Santella G, Vescina C, Vatta MS, Bianciotti LG.** Vagally mediated cholestatic and choleretic effects of centrally applied endothelin-1 through ETA receptors. *Regul Pept* 135: 54–62, 2006.
33. **Rougeot C, Rosinski-Chupin I, Mathison R, Rougeon F.** Rodent submandibulargland peptide, hormones and other biologically active peptides. *Peptides* 21: 443–455, 2000.
34. **Sabbatini ME, Rodriguez MR, Corbo NS, Vatta MS, Bianciotti LG.** C-type natriuretic peptide applied to the brain enhances exocrine pancreatic secretion through a vagal pathway. *Eur J Pharmacol* 524: 67–74, 2005.
35. **Sabbatini ME, Vatta MS, Davio CA, Bianciotti LG.** Atrial natriuretic factor negatively modulates secretin intracellular signalling in the rat pancreas. *Am J Physiol Gastrointest Liver Physiol* 292: G349–G357, 2007.
36. **Sakurai T, Yanagisawa M, Inoue A, Ryan US, Kimura S, Mitsui Y, Goto K, Masaki T.** cDNA cloning sequence analysis and tissue distribution of rat preprotendothelin 1 mRNA. *Biochem Biophys Res Commun* 175: 44–47, 1991.
37. **Schneider MO, Boesen EI, Pollock DM.** Contrasting actions of endothelin ET_A and ET_B receptors in cardiovascular disease. *Annu Rev Pharmacol Toxicol* 47: 731–759, 2007.
38. **Shiba R, Sakurai T, Yamada G, Morimoto H, Saito A, Masaki T, Goto K.** Cloning and expression of rat preproendothelin-3 cDNA. *Biochem Biophys Res Commun* 186: 588–594, 1992.
39. **Steiner AL, Parker C, Kipnis DM.** Radioimmunoassay for cyclic nucleotides. *J Biol Chem* 247: 1106–1113, 1972.
40. **Suburo AM, Wheatley SC, Horn DA, Gibson SJ, Jahn R, Fisher-Colbrie R, Wood JN, Latchman DS, Polak JM.** Intracellular distribution of neuropeptides and secretory proteins during differentiation of neuronal cell lines. *Neuroscience* 46: 881–889, 1992.
41. **Ventimiglia MS, Rodriguez MR, Elverdin JC, Davio CA, Vatta MS, Bianciotti LG.** Atrial natriuretic factor intracellular signalling in the rat submandibular gland. *Regul Pept* 150: 43–49, 2008.