BACKGROUND: Histamine is released from mast cells by immunologic and non-immunologic stimuli during salivary gland inflammation, regulating salivary secretion. The receptor-secretory mechanism has not been studied in detail.

Aims: The studies reported were directed toward elucidating signal transduction/second messenger pathways within the rat submandibular gland associated with 2-thiazolylethylamine (ThEA)-induced H₁-receptor responses.

Materials and methods: To assess the H₁ receptor subtype expression in the rat submandibular gland, a radioligand binding assay was performed. The study also included inositol phosphates and cyclic GMP accumulation, protein kinase C and nitric oxide synthase activities, and amylase release.

Results: The histamine H₁ receptor subtype is expressed on the rat submandibular gland with high-affinity binding sites. The ThEA effect was associated with activation of phosphoinositide-specific phospholipase C, translocation of protein kinase C, stimulation of nitric oxide synthase activity and increased production of cyclic GMP. ThEA stimulation of nitric oxide synthase and cyclic GMP was blunted by agents able to interfere with calcium mobilization, while a protein kinase C inhibitor was able to stimulate ThEA action. On the other hand, ThEA stimulation evoked amylase release via the H₁ receptor but was not followed by the L-arginine/nitric oxide pathway activation.

Conclusions: These results suggest that, apart from the effect of ThEA on amylase release, it also appears to be a vasoactive chemical mediator that triggers vasodilatation, modulating the course of inflammation.

Key words: Submandibular gland, 2-Thiazolylethylamine, H₁ binding assay, Amylase release, Phosphoinositide hydrolysis, Nitric oxide synthase activity, Cyclic GMP, Protein kinase C

Introduction
Histamine, one of the autacoids, is primarily stored and released by mast cells in mammalian tissues and produces its effect via interactions with histamine receptor subtypes, the H₁, H₂, H₃ and H₄ receptors. In general, the interaction of histamine with H₁ receptors causes the contraction of smooth muscle, and the interactions with H₂ receptors causes the secretion of gastric acid from parietal cells in the stomach and of saliva from acinar cells in the salivary glands. H₃ receptors are localized as pre-synaptic receptors present on histaminergic nerve terminals in the central nervous system and a wide variety of tissues; they regulate histamine synthesis and release by a feedback mechanism. Moreover, as histamine is the most abundant agent released during degranulation, we suspected that it might also mediate the effects of mast cells during the bone resorption process.

Histamine receptor subtypes belong to the family of receptors linked to different members of the superfamily of G-protein-coupled receptors. Since in recent years it is has been found that effector enzymes, such as adenylyl cyclase and phospholipase C (PLC), are characterized by a large molecular heterogeneity, the interaction of histamine receptor subtypes with the intracellular biochemical pathways is clearly not very straightforward. However, a role for both calcium from intracellular stores and protein kinase C (PKC) has been suggested.

It has proposed that histamine is released from mast cells by immunologic and non-immunologic stimuli during salivary gland inflammation. As a
mediator of inflammatory reactions, binding and acting on H₁ and/or H₂ receptors on the surface of exocrine cells, histamine regulates enzyme secretion. Emmelin reported that histamine acted on the submandibular gland of dogs and cats as a main regulator of salivary secretion. Furthermore, amylase release from rat parotid slices was stimulated by the H₁ histamine receptor, as observed also in guinea pig parotid glands. However, the receptor-secretory mechanism, especially the intracellular signal transduction, coupled to the H₁ receptor in the rat submandibular gland has not been studied in detail.

On the other hand, it is described that the nerve cell bodies of the ganglion located at the hilum of the rat submandibular gland are strongly positive for nitric oxide synthase (NOS) enzyme, and nerve fibres around acini and ducts are also NOS positive. So, we want to know whether the H₁ receptor stimulation could trigger NOS activation.

The studies reported in the present paper were directed toward elucidating signal transduction/second messenger pathways within rat submandibular glands associated with 2-thiazolylethylamine (ThEA)-induced responses through the H₁ receptor subtype. ThEA has been associated with activation of PLC and PKC, production of nitric oxide (NO) and cyclic GMP (cGMP), as well as amylase release in rat submandibular glands.

Materials and methods

Histaminergic binding studies

Submandibular microsomal membranes were prepared from rats as previously reported. Briefly, the glands were excised and immediately homogenized at 4°C in six to eight volumes of 50 mM sodium/potassium phosphate buffer (pH 7.5), 0.25 M sucrose and supplemented with protease inhibitors as described elsewhere. The homogenate was centrifuged at 900 × g twice, and the supernatants collected and spun down at 10,000 × g for 10 min and 40,000 × g for 60 min. The resulting pellet was resuspended in 50 mM phosphate (pH 7.5), 5 mM MgCl₂ and the same protease inhibitors to give a final protein concentration of 3–5 mg/ml, and was stored at −80°C until used. Radioligand binding assays with the H₁ histaminergic antagonist pyrilamine, [³H]-5-pyridinyl ([³H]-Pyrilamine; Dupont New England Nuclear, Boston, MA, USA), were carried essentially as described elsewhere. For the dissociation constant (Kᵰ) and the number of binding sites (Bₘₐₓ) calculations, several concentrations of [³H]-Pyrilamine (0.25–10 nM) were assessed using saturation isotherms. Membranes (0.3–0.4 mg of protein) were incubated in duplicate with various concentrations of [³H]-Pyrilamine. Non-specific binding was determined in the presence of 10 μM pyrilamine. Results were analysed with the computer-assisted curve fitting program LIGAND. All the experiments on animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publications number 80–23).

Measurement of total labelled inositol phosphates

Submandibular glands were incubated for 120 min in 0.5 ml of Krebs Ringer bicarbonate (KRB) gassed with CO₂ in oxygen with 37 kBq of [³H]-myo-inositol (specific activity 553 kBq/mmol) from Dupont New England Nuclear. LiCl (10 mM) was added for determination of inositol monophosphate accumulation according to the technique previously reported. Agonist was added 30 min before the end of the incubation period and the blockers 30 min before the addition of agonist. Water-soluble inositol phosphates (IPs) were extracted after 120 min incubation following the method previously reported. Submandibular gland slices were quickly washed with KRB and homogenized in 0.3 ml of KRB with 10 mM LiCl and 2 ml of chloroform/methanol (1:2, v/v) to stop the reaction. Then chloroform (0.62 ml) and water (1 ml) were added. Samples were centrifuged at 3000 × g for 10 min, and the aqueous phase of the supernatant (1–2 ml) was applied to a 0.7 ml column of Bio-Rad AG (Formate Form) 1 × 8 anion exchange resin (100–200 mesh) suspended in 0.1 M formic acid that had been previously washed with 10 mM Tris–formic (pH 7.4). The resin was then washed with 20 volumes of 5 mM myo-inositol followed by six volumes of water, and IPs were eluted with 1 M ammonium formate in 0.1 M formic acid. One millilitre fractions were recovered and radioactivity was determined by scintillation counting. Peak areas were determined by triangulation. Results corresponding to the second peak were expressed as absolute values of area units under the curve per milligram of tissue wet weight following the criteria of Simpson’s equation. To confirm the absence of [³H]-myo-inositol in the eluted peaks of IPs, chromatography on silica gel 60 F254 sheets (Merk, Buenos Aires, Argentina) was performed using propan-2-ol/6 N NH₄OH (14:5) as the developing solvent following the procedure of Hokin-Neaverson and Sadeghian.

PKC activity assay

PKC activity was assayed by measuring the incorporation of ³²P from ³²P-adenosine triphosphate into histone H₁ as previously described. Briefly, incubations were conducted for 30 min at 30°C in a final volume of 85 μl. In the final concentrations, the assay mixture contained 25 μmol adenosine triphosphate (14.8 kBq), 10 mM magnesium acetate, 5 mM β-mercaptoethanol, 50 μg of histone H₁, 20 mM HEPES (pH 7.4) and, unless otherwise indicated, 0.2 mM CaCl₂.
and 10 μg/ml of phosphatidylserine vesicles. The incorporation of $^{32}$P phosphate into the histone H1 was linear for at least 30 min. The reaction was stopped by the addition of 2 ml of ice-cold 5% trichloroacetic acid, 10 mM H$_3$PO$_4$. The radioactivity retained on GF/c glass-fibre filters after filtration was determined by counting the filters in 2 ml of scintillation fluid. PKC activity was determined after subtracting the incorporation in the absence of calcium and phospholipids. The data were expressed in picomoles of phosphate incorporated into the substrate per minute per milligram of protein.

**NOS activity assay**

NOS activity was measured in submandibular glands of male Wistar rats using L-[U-14C]-arginine as a substrate. Briefly, submandibular glands were carefully dissected and incubated in KRB solution containing 18.5 kBq of L-[U-14C]-arginine for 20 min before the addition of agonist. When inhibitors were used they were added from the beginning of the incubation at the final concentrations indicated in the text. The incubations were carried out under a 50% CO$_2$ in oxygen atmosphere at 37°C and stopped by homogenization of the glands in 1 ml of 20 mM HEPES (pH 7.4), 0.5 mM EGTA, 0.5 mM EDTA and 1 mM dithiothreitol at 4°C. The supernatants were passed through 2 ml of Dowex AG-50 WX-8 columns, and L-[U-14C]-citrulline was eluted with water and quantified as described previously. NOS activity was inhibited more than 90% by 0.5 mM N$^G$-monomethyl-L-arginine (L-NMMA). In the experiments with NOS activation by the agonist, we chose lower concentrations of this arginine analogue to inhibit the effects in order not to modify the basal NOS activity.

**cGMP assay**

Glands were incubated in 1 ml of KRB containing 0.1 mM isobutyl methyl xantine for 30 min under a constant current of 5% CO$_2$ in oxygen. The agonist was added in the last 10 min while inhibitors were included in the incubation volume from the beginning. Reactions were stopped by homogenization as previously stated, and samples were assayed by radioimmunoassay using [125I]-cGMP from Dupont New England Nuclear (81400 kBq/nmol) and anti-cGMP antiserum from Sigma Chemical Co. (St Louis, MO, USA).

**Determination of amylase activity**

Submandibular gland slices (5.1 ± 0.5 mg) were used throughout. Free connective tissue, fat and lymph nodes were gently remove under a magnifying glass, slices were placed in tubes containing 500 μl of KRB solution containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO$_4$, 1.8 mM CaCl$_2$, 0.96 mM NaH$_2$PO$_4$, 25 mM NaHCO$_3$ and 5 mM β-hydroxybutyric acid (pH 7.4), and were then bubbled with 95% O$_2$ and 5% CO$_2$ at 37°C. After an equilibrium period of 10 min, vehicle (basal values) or drugs to be evaluated (one concentration in each tube) were added and allowed to stay for 20 min, after which the total amylase content or that released into the medium was determined by the method described by Bernfeld using starch suspension as the substrate.

**Statistical analysis**

Student’s t-test for unpaired values was used to determine the levels of significance. When multiple comparison were necessary, after analysis of variance, the Student–Newman–Keuls test was applied. Differences between means were considered significant if $p < 0.05$.

**Results**

To assess the expression of histamine H$_1$ receptor subtype in the rat submandibular gland, a radioligand binding assay was performed. [3H]-Pyrilamine revealed a single class of high-affinity binding sites demonstrable by Scatchard analysis of the saturation curve (Table 1).

**Table 1. Specific [3H]-Pyrilamine binding to submandibular gland membranes**

| Radioligand       | $B_{max}$ (fmol/mg protein) | $K_d$ (nM) |
|------------------|----------------------------|-----------|
| [3H]-Pyrilamine   | 96.5 ± 8.1                 | 3.66 ± 0.41 |

The membranes from rat submandibular glands were prepared for binding assays as stated in Materials and methods. The number of binding sites ($B_{max}$) and the equilibrium dissociation constant ($K_d$) were analysed using weighted non-linear regression, computerized least-squares curve-fitting programs (LIGAND). Values presented as the mean ± SEM of at least four experiments performed in duplicate.

**Drugs**

ThEA (H$_1$ receptor agonist), L-NMMA (NOS inhibitor), L-arginine (natural substrate of NOS), verapamil (calcium blocker), staurosporine (PKC inhibitor), trifluoperazine (TFP) (calcium/calmodulin inhibitor), pyrilamine maleate (H$_1$ receptor antagonist), cimetidine (H$_2$ receptor antagonist) (all Sigma Chemical Co.), 1-[6(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl] amino hexyl-1H-pyrrole-2,5 dione (U-73122) (PLC inhibitor) (ICN Pharmaceuticals Inc., Costa Mesa, CA, USA) and [1H]-1,2,4-oxadiazola-(4,3–2)-quinoxaline-1-one (ODQ) (soluble guanylate cyclase inhibitor) (Tocris Cookson Inc., Ellisville, MO, USA) stock solutions were freshly prepared in the corresponding buffers. The drugs were diluted in the bath to achieve the final concentrations stated in the text.
To verify whether the H₁ receptor subtype was coupled to phosphoinositide turnover in the rat submandibular gland, the effect of ThEA on IP accumulation, in the presence or absence of histaminergic antagonists, was investigated. As can be seen in Fig. 1, ThEA (1 × 10⁻⁷ M) increased IP formation, pyrilamine (1 × 10⁻⁶ M) but not cimetidine (1 × 10⁻⁶ M) significantly inhibited the stimulatory action of ThEA, pointing to the participation of H₁ receptor activation by ThEA upon IP accumulation.

To determine the participation of PLC in the effect of ThEA, the action of U-73122 was explored. As can be seen in Fig. 1, U-73122 (5 × 10⁻⁶ M) blunted the effect of ThEA. As can be seen in Fig. 2, ThEA was also able to translocate PKC from the glandular cytosol to the glandular membrane; this effect was also abrogated by the specific H₁ antagonist.

To know whether the endogenous NO signalling system was turned on in the ThEA-activated H₁ receptor on the rat submandibular gland, NOS activity in the presence of ThEA was explored. As can be seen in Fig. 3, there was a concentration-dependent increase in NOS activity; the maximal effect being at 1 × 10⁻⁷ M, a concentration that was active on both IP accumulation and PKC translocation. The increment of NOS activity was blunted by L-NMMA (5 × 10⁻⁵ M), a reversible NOS inhibitor, while the natural substrate L-arginine (5 × 10⁻⁴ M) reversed this inhibition (Fig. 3).

To study the nature of the mechanism by which the activation of H₁ receptor increased NOS activity and cGMP production, rat submandibular glands were incubated with several inhibitors. Table 2 shows that pyrilamine inhibited the stimulatory action of ThEA on both NOS activity and cGMP production. Also, the inhibition of the calcium influx by verapamil (5 × 10⁻⁶ M), of calcium/calmodulin by TFP (5 × 10⁻⁶ M) and of NOS by L-NMMA (5 × 10⁻⁵ M) attenuated the H₁ receptor-dependent activation of NOS activity and cGMP accumulation. Moreover, the inhibitory action of L-NMMA on the ThEA effect upon NOS and cGMP was reversed by L-arginine (5 × 10⁻⁴ M) (Table 2). A selective inhibitor of NO-sensitive guanylate cyclase, ODQ was able to blunt the stimulatory action of ThEA upon cGMP accumulation. Additionally, the inhibition of PKC by staurosporine (1 × 10⁻⁹ M) increased both, NOS activity and cGMP production. None of the inhibitors had any effect per se on basal values of NOS activity and cGMP levels (data not shown) with the
exception of \( \text{L-NMMA} \), which at the concentration used decreased in 75% the NOS activity and cGMP production. In addition, the generation of amylase was stimulated by ThEA (Fig. 4). The effect of ThEA on amylase release was inhibited by pyrilamine but not by cimetidine. \( \text{L-NMMA} \) did not have an effect on ThEA-amylase release (Fig. 4). These results suggest that the stimulatory effect of ThEA on amylase release is mediated directly by the \( \text{H}_1 \) receptor and independently of NOS activation.

### Discussion

These data provide important new insights into the pathways by which the histaminergic system induces rapid activation of NOS with subsequent accumulation of cGMP. These intracellular signals were not directly associated with the capacity of the system to release amylase. However, both effects (amylase and NO releases) were triggered by the activation of the \( \text{H}_1 \) receptor subtype as ThEA (a specific \( \text{H}_1 \) agonist) and pyrilamine (a specific \( \text{H}_1 \) antagonist) exerted and blocked these effects, respectively.

### Table 2. Effect of ThEA upon NOS activity and cyclic GMP accumulation of rat submandibular gland slices: influences of different inhibitory agents

| Additions                  | NOS activity (pmol/g tissue wet weight) | cGMP (pmol/g tissue wet weight) |
|----------------------------|----------------------------------------|---------------------------------|
| None                      | 450 ± 26                               | 18 ± 1                          |
| ThEA                      | 732 ± 32*                              | 41 ± 4*                         |
| pyrilamine + ThEA         | 470 ± 28                               | 22 ± 2                          |
| Verapamil + ThEA          | 490 ± 30                               | 20 ± 3                          |
| TFP + ThEA                | 380 ± 21                               | 17 ± 2                          |
| Stauroporine + ThEA       | 920 ± 37*                              | 58 ± 2*                         |
| \( \text{L-NMMA} \) + ThEA| 280 ± 12                               | 11 ± 1                          |
| \( \text{L-NMMA} \) + ThEA + \( \text{L-arginine} \) | 686 ± 29*                                | 43 ± 3*                         |
| ODQ + ThEA                | –                                      | 21 ± 2                          |

NOS activity and cGMP accumulation were measured by incubating rat submandibular gland slices with or without inhibitory agents for 20 min and then for an additional 10 min with the \( \text{H}_1 \) receptor agonist \( (1 \times 10^{-7} \text{ M}) \) ThEA. Results presented as the mean ± SEM of seven experiments performed in duplicate in each group. The final concentrations of the inhibitors were: pyrilamine (\( \text{H}_1 \) receptor antagonist), \( 1 \times 10^{-6} \text{ M} \); verapamil (calcium blocker) and TFP (calcium/calmodulin blocker), \( 5 \times 10^{-6} \text{ M} \); stauroporine (PKC inhibitor), \( 1 \times 10^{-6} \text{ M} \); \( \text{L-NMMA} \) (NOS inhibitor), \( 5 \times 10^{-6} \text{ M} \); \( \text{L-arginine} \) (natural NOS substrate), \( 5 \times 10^{-4} \text{ M} \); and ODQ (soluble guanylate cyclase inhibitor), \( 5 \times 10^{-6} \text{ M} \). * Significantly different from basal values (none) with \( p < 0.001 \).
Our results support the assumption that ThEA-promoted increases in NO and cGMP are mediated by H₁ receptors: first, because this subtype is expressed in submandibular gland membranes, as was demonstrated by the binding assay; and, second, because pyrilamine was able to inhibit both NO production and cGMP accumulation. It is important to note that specific pyrilamine binding to H₁ receptors in submandibular gland membranes was saturable and best described by interaction of the radioligand with a single class of high-affinity binding sites.

The participation of constitutive NOS was demonstrated by the ability of the stereospecific NOS inhibitor L-NMMA to inhibit the ThEA-induced NOS activity and cGMP accumulation in the rat submandibular gland. These was further confirmed by the reversibility of the action of L-NMMA by the natural substrate L-arginine. A relationship between histamine and NO has been reported, but little is known about the mechanism by which histamine control NOS activity in the submandibular gland. Among the mechanisms involved in the H₁-receptor-dependent activation of NOS is the stimulation of soluble guanylate cyclase to increase the accumulation of cGMP. The fact that a selective inhibitor of NO-sensitive guanylate cyclase was able to prevent the ThEA H₁-receptor activation of cGMP production confirms this assumption. This is similar to the effect of other agonists, suggesting that some of the physiological effects of NO may be mediated by the activation of soluble guanylate cyclase. NO and cGMP have been suggested to act in salivary vasoregulation by several investigators. The vasculature of submandibular and parotid glands exhibit a NO-dependent basal vasodilator tone. Therefore, the vasculature of the submandibular gland is highly responsive to exogenous NO. cGMP appears to regulate amylase release of the mouse parotid gland via a cGMP-dependent kinase, but did not affect amylase release by the rat parotid gland linked to a soluble guanylate cyclase.

ThEA increased accumulation of IPs and this action was inhibited by pyrilamine, suggesting the involvement of an H₁ receptor, while the H₁ receptor antagonist, cimetidine, was without effect. This ThEA action was linked to PLC as the enzyme inhibitor U-73122 abrogated this effect. It is known that PLC hydrolysis of submandibular gland membrane phosphoinositides generates the intracellular second messengers diacylglycerol and inositol triphosphate, which activate PKC and calcium/calmodulin, respectively. Our results, however, indicate that intermediates of phosphoinositide hydrolysis oppose ThEA-induced NOS activation. Thus, rapid activation of the enzyme is related to calcium mobilization, while inhibition of NOS requires extensive translocation of PKC. This is based on the observed potentiation by staurosporine and the inhibition by TFP and verapamil on the effect of ThEA on glandular NOS activity and cGMP production. H₁ receptors are classically linked to activation of PKC, and the regulatory role for protein phosphorylation of NO by PKC and calcium/calmodulin-dependent kinase has been demonstrated.

Moreover, ThEA caused translocation of PKC from the cytosol to cell membrane in the rat submandibular gland as it does in airway epithelium and in neuroblastoma NCB-20 cells. We did not look specifically at the PKC wing of PLC activation in this work, but it is possible that activation of cell-H₁-receptor-specific isoforms of PKC may also occur in rat submandibular glands in response to ThEA-induced activation of PLC.

The receptor-secretory mechanism of ThEA-induced amylase release in the rat submandibular gland is mediated directly and selectively by the H₁ receptor. However, with present results there is no evidence supporting such a role of the l-arginine/NO pathway in regulating amylase secretion.

The vasculature of the submandibular gland exhibits a NO-dependent basal vasodilator tone. Also, the vasculature of these tissues is highly responsive to exogenous NO. Therefore, the release of endogenous NO by histamine during the first step of inflammation may be triggered by vasodilation modulating the course of inflammation.

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Histaminergic systems activation in submandibular gland

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