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Effect of Substance P and Receptor Antagonists on Secretion of Lingual Lipase and Amylase from Rat von Ebner's Gland

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FIELD, R. B., S. J. CHIRTEL AND R. S. REDMAN. *Effect of substance P and receptor antagonists on secretion of lingual lipase and amylase from rat von Ebner's gland.* PEPTIDES **18**(2) 277–285, 1997.— Substance P (SP, 1 μM) when incubated with minced von Ebner's glands for 15, 30, and 60 min, stimulated secretion of lingual lipase (12.14% ±0.90) and amylase (8.30% ±0.42). Only 10 μM of the SP receptor antagonist CP-96,345 significantly inhibited SP-evoked secretion. D-Pro^2-D-Phe^7-D-Trp^9-SP (Ia), D-Pro^2-D-Trp^{7,9}-SP (Ib), D-Arg^1-D-Trp^{7,9}-D-Leu^{11}-SP (Ic), or 1 μM CP-96,345 were not effective, suggesting that the SP receptor of von Ebner's gland might be an isoform. Propranolol and timolol, β_1/β_2-adrenergic receptor antagonists were not effective and the cholinergic receptor antagonist, atropine, was effective in only slightly reducing amylase secretion but not lingual lipase. Differential secretion of the two enzymes was observed for basal and stimulated secretion. Thus, exocytosis may not be the only pathway involved in SP-evoked protein secretion. Published by Elsevier Science Inc.

Substance P   Substance P receptors   von Ebner's glands   Lingual lipase   Amylase   Protein secretion
Substance P analogs   Substance P receptor antagonists β-Adrenergic receptor antagonists
Cholinergic receptor antagonist   CP-96,345   Exocytosis   Basal secretion   Stimulated secretion

SUBSTANCE P (SP), a undecapeptide, is a member of the tachykinin family of peptides that share a common carboxyl-terminal sequence, Phe-X-Gly-Leu-Met (30). It was originally isolated from brain and tissue extracts by von Euler and Gaddam (55) and characterized by Chang and Leeman (8). SP modulates a variety of biological responses including vasodilation, hypotension, contraction of nonvascular smooth muscle, and secretion from exocrine glands (30,34). SP stimulates salivary flow from the rat parotid (29,56) and submandibular glands (56). SP also evokes amylase release from isolated parotid cells (29), parotid slices (36,40), guinea pig pancreas (43), and in vivo from rat parotid gland (56). In addition, SP stimulates phosphoinositol turnover in the parotid (24,39), submandibular (24,31), and sublingual glands (24). Thus there are considerable data on the effects of SP on the pancreas and major salivary glands. However, no studies of the effects of SP on protein secretion from minor salivary glands have been reported.

Von Ebner's glands are minor exocrine salivary glands that are embedded in muscle fiber beneath and anterior to the circumvallate papilla and medial to the foliate furrows in the posterior and pharyngeal portion of the tongue (22,50). They contain serous acinar cells that are morphologically similar to those of the parotid gland and exocrine pancreas (9,22,28,50) and are thus a good model system for studying secretion from exocrine glands. These serous glands of the tongue are the sole source of an important digestive enzyme, lingual lipase (21), as well as amylase (11–13,19). Lingual lipase, which hydrolyzes triacylglycerols to fatty acids and partial acylglycerols in the stomach at an acid pH (18,20,21), is the enzyme responsible for the first step in fat digestion. The optimal pH for enzyme activity by lingual lipase is a broad range from 4.0 to 6.5 (14). The activity of lingual lipase was not decreased after incubations with pepsin at pH 2-6 for up to 1 h (37). In addition, the secretions of von Ebner's glands bathe the taste buds that are found in the troughs of the circumvallate and foliate papillae, and thus they play a role in the perception of taste (48). Recently, a putative tastant carrier, MWr=18,000, was found in rat von Ebner's glands and in human von Ebner's gland saliva (3,27,42,49).
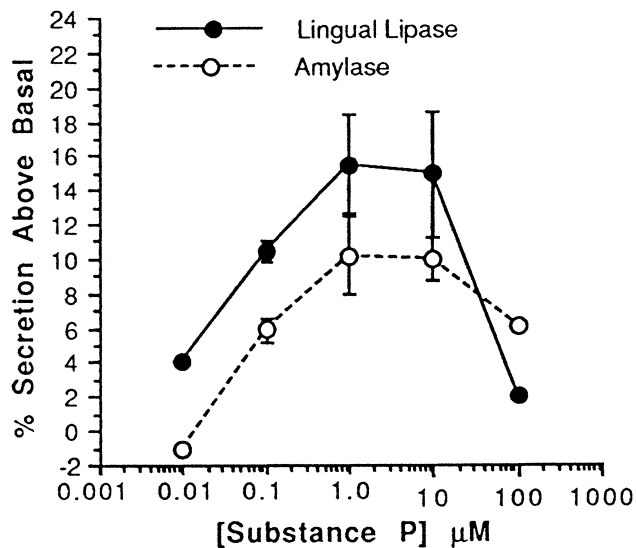

FIG. 1. Percentage secretion above basal of lingual lipase and amylase activity, showing dose responses when minced von Ebner's glands were incubated for 60 min with 0.01 ($n = 1$), 0.1 ($n = 3$), 1.0 ($n = 3$), 10 ($n = 3$), or 100 ($n = 1$) μM SP. Data points are the mean ± standard error, and n is the number of experiments, each using the pooled glands of 8 rats according to the analytical and statistical methods described.
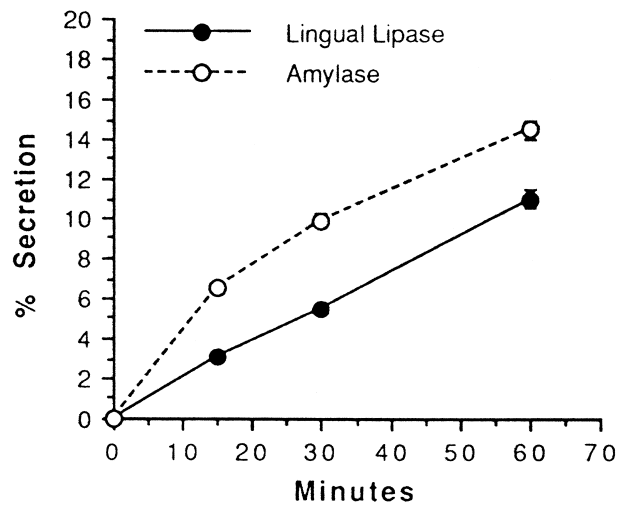

FIG. 2. Percentage basal secretion of lingual lipase and amylase activity after 15, 30, and 60 min incubations of minced von Ebner's glands in DMEM, $n = 43$. Data points are the mean ± standard error, and n is the number of experiments, each using the pooled glands of 8 rats according to the analytical and statistical methods described. Secretion of both enzymes increased with time, $p = 0.0001$, secretion of amylase was greater than lingual lipase, $p = 0.0001$, and the time courses of the enzymes and the curvature of the curves differed, $p = 0.0102$ and $p = 0.0001$, respectively.

The β-adrenergic and cholinergic regulation of protein secretion from von Ebner's glands is particularly interesting because it is much more like that of the pancreas than that of the of the major salivary glands. Protein secretion from the parotid gland is mediated mainly by the β-adrenergic receptor and fluid secretion is mediated by the cholinergic receptor (1). However, lingual lipase and amylase are secreted from von Ebner's gland mainly in response to cholinergic stimulation (12). To a lesser extent protein secretion from von Ebner's gland is evoked by β-adrenergic stimulation but not by a-adrenergic stimulation (12). Histamine also stimulates protein secretion from von Ebner's gland (10).

The present study was undertaken to determine whether SP is a secretagogue for the secretion of lingual lipase and amylase from minced rat von Ebner's glands. Antagonists of the SP receptor (analogs of SP and a nonpeptide compound) and β-adrenergic and cholinergic receptor antagonists were evaluated for their effects on secretion evoked by SP.

METHODS

Chemicals

SP and the SP analogs (SP receptor inhibitors), D-Pro^2-D-Phe^7-D-Trp^9-SP (Ia), D-Pro^2-D-Trp^{7,9}-SP (Ib), and D-Arg^1-D-Trp^{7,9}-D-Leu^{11}-SP (Spantide, Ic), the β-adrenergic receptor antagonists, timolol maleate and propranolol, and the cholinergic receptor antagonist, atropine, were purchased from Sigma, St. Louis, MO. A nonpeptide inhibitor of SP receptors, CP-96,345 {(2S,3S)-cis-2-(diphenylmethyl)-N-[(2-methoxyphenyl) methyl]-1-azabicyclo [2.2.2] octan-3-amine}, was kindly provided by Dr. R. Michael Snider of Pfizer, Inc., Groton, CT. Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco Laboratories, Grand Island, NY. The radioisotopes used in the lingual lipase assay, tri [9,10-^{3}H]

FIG. 3. Percentage secretion of lingual lipase and amylase activity above basal after stimulation of minced von Ebner's glands with 1.0 μM SP for incubations of 15, 30, and 60 min, $n = 39$. Data points are the mean ± standard error, n is the number of experiments, each using the pooled glands of 8 rats according to the analytical and statistical methods described. The secretions of lingual lipase and amylase were different from zero, $p = 0.0001$, the curvature of the lingual lipase and amylase curves differed, $p = 0.0001$, and * signifies the difference between the enzymes, $p = 0.0001$, seen only at 60 min, by paired Student t-test.
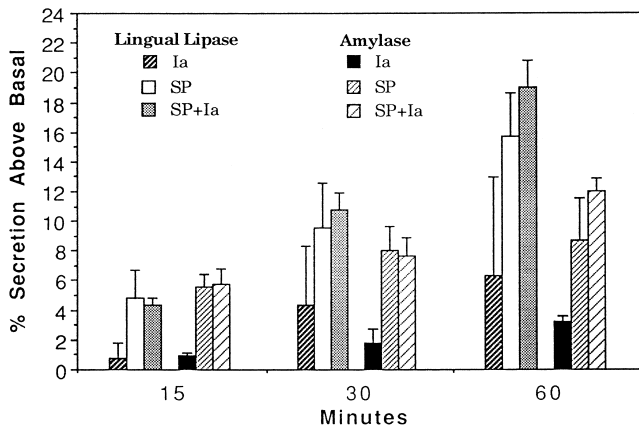
FIG. 4. Effect of 1.0 μM SP receptor antagonist Ia (D-Pro[2]-D-Phe[7]-D-Trp[9]-SP) on the percentage secretion of lingual lipase and amylase activity evoked by 1.0 μM SP. Minced von Ebner's glands were incubated with SP, $n = 3$, or Ia, $n = 2$, alone or preincubated with Ia for 20 min and then incubated with 1.0 μM SP combined with 1.0 μM Ia, $n = 3$, for 15, 30, and 60 min. Data points are the mean $\pm$ standard error, and n is the number of experiments, each using the pooled glands of 8 rats according to the analytical and statistical methods described. Ia did not elicit secretion nor did it have any effect on SP-elicited secretion.

oleoylglycerol and [9,10-^{3}H] oleic acid, were from Dupont NEN Research Products, Boston, MA. Chemicals needed for lingual lipase and amylase assays, L-α-Phosphatidyl choline, type III-E, taurodeoxycholine, trioleoylglycerol (99%), and starch were also from Sigma. All other chemicals were reagent grade.

Solutions of Substance P and Receptor Antagonists

DMEM that had been previously gassed with 95% O2, 5% CO2 was used to prepare stock solutions and dilutions except for preparations of SP, SP analogs, and CP-96,345.

Stock solutions of SP, Ia, Ib, and Ic were prepared in 0.05 M acetic acid and aliquots were stored under nitrogen at $-70°C$. Dilutions were made with DMEM that had been gassed with nitrogen. DMEM buffered the acid pH of the stock solutions so that the pH of the final solutions was the same as DMEM. CP-96,345 was prepared in and diluted with DMEM previously gassed with nitrogen. Stock timolol was prepared in saline.

Tissue Preparation

These experiments were carried out as previously described, with some modifications (10,12). Sprague–Dawley male rats, 200 $\pm$ 30 g, were obtained either from Charles River Laboratories, Raleigh, NC, certified free of sialodacryoadenitis and rat corona viruses or from their progeny in the breeding colony at the DVA Medical Center. They were housed under controlled lighting conditions (12 h off and 12 h on) with unlimited access to water and a commercial pelleted diet. Rats were fasted overnight, anesthetized with nembutal (50 mg/kg, IP), and exsanguinated prior to removal of the tongues. Von Ebner's glands were dissected from the tongues with the aid of a dissecting microscope. Care was taken not to include any mucous glands that are located posterior and lateral to von Ebner's glands. For most experiments, the glands of 8 rats were dissected, minced, and

pooled in 1.33 ml DMEM, previously gassed with 95% O2, 5% CO$_2$. The glands were gassed after each addition of tissue and gently shaken in a water bath at 37°C. When the dissection was completed, DMEM was added, and the tissue was randomly divided into eight 50 ml round-bottom, screw-capped, polycarbonate tubes.

Incubation of Tissue with Substance P and Receptor Antagonists

Incubations were done as described previously (10,12) with some changes. The tubes were centrifuged to $240 \times g$ and the media aspirated and discarded. One ml DMEM or 1 ml of a DMEM solution of a receptor antagonist was added to the tubes for preincubation. The tubes were gassed and incubated for 20 min at 37°C in a water bath shaker at 90 cycles/min. The media were aspirated and discarded and 1 ml of DMEM alone (basal secretion) or DMEM solutions of the agonist, antagonist, or a combination of agonist and antagonist solutions were added to the tissue. The incubation was carried out on duplicate samples with gassing every 15 min. Each experiment included untreated samples (controls), to determine basal secretion. Two 0.1 ml aliquots were removed from each sample at 15 and 30 min as indicated, and the volume was immediately replaced with the appropriate solution. These aliquots were stored in cryogenic tubes in liquid nitrogen. At 60 min, the final time interval, all the media were aspirated and stored in 2 cryogenic tubes/sample in liquid nitrogen. The tissue was washed with DMEM, frozen on dry ice, and homogenized with a Polytron (Brinkmann Instruments) for 30 sec in Tyrode's solution (glucose free). A total volume of 4 ml Tyrode's solution was used to homogenize the tissue and rinse the Polytron generator. The homogenates were centrifuged for 15 min at $850 \times g$, and the supernatants were measured and then stored in two cryogenic tubes/sample in liquid nitrogen. Using concentrations of 0.01,
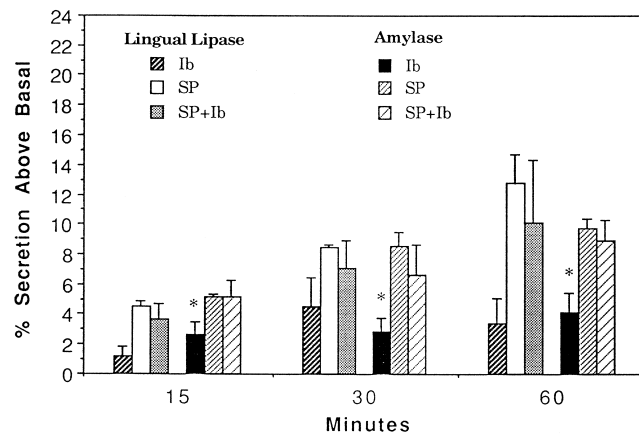

FIG. 5. Effect of 1.0 μM SP receptor antagonist Ib (D-Pro[2]-D-Trp[7,9]-SP) on the percentage secretion of lingual lipase and amylase activity evoked by 1.0 μM SP. Minced von Ebner's glands were incubated with SP or Ib alone or preincubated with Ib for 20 min and then incubated with 1.0 μM SP combined with 1.0 μM Ib for 15, 30, and 60 min, $n = 5$. Data points are the mean $\pm$ standard error, and n is the number of experiments, each using the pooled glands of 8 rats according to the analytical and statistical methods described. * signifies the difference between secretion above basal of amylase elicited by Ib alone and basal, $p < 0.05$, by paired Student t-test. Ib did not elicit secretion of lingual lipase and had no effect on SP-elicited secretion.
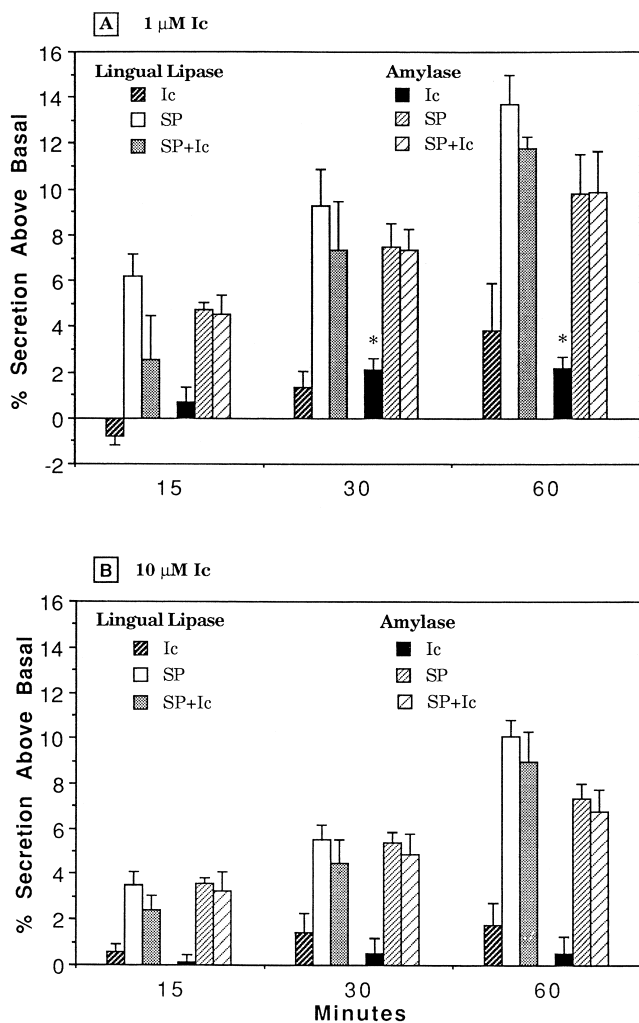
feld (2) and for lingual lipase activity the following day by the method of Field and Scow (14). A unit of amylase activity is defined as a milligram equivalent of maltose formed in 3 min at 30°C. A unit of lingual lipase activity is defined as a
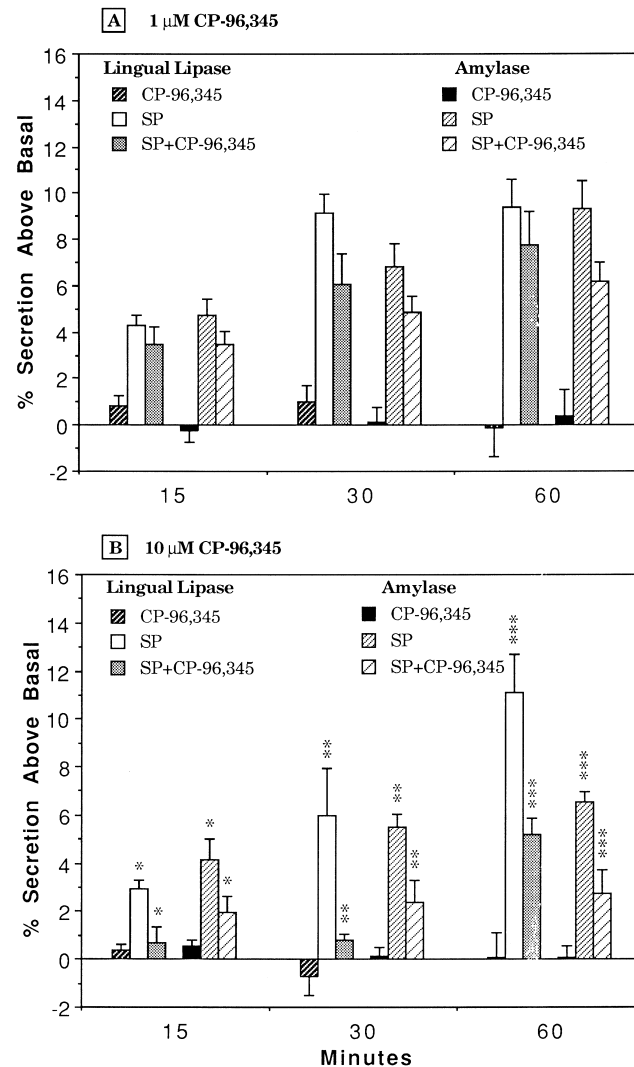

FIG. 6. Effect of 1.0 μM (A) or 10.0 μM (B) SP receptor antagonist Ic (D-Arg[1]-D-Trp[7,9]-D-Leu[11]-SP, Spantide) on the percentage secretion of lingual lipase and amylase activity evoked by 1.0 μM SP. Minced von Ebner's glands were incubated with SP or Ic alone or preincubated with Ic for 20 min and then incubated with 1.0 μM SP combined with 1.0 μM (A) or 10.0 μM (B) Ic for 15, 30, and 60 min, $n = 4$ (A), $n = 6$ (B). Data points are the mean ± standard error, and n is the number of experiments, each using the pooled glands of 8 rats according to the analytical and statistical methods described. * signifies the difference between secretion above basal of amylase elicited by 1 μM Ic alone and basal, $p < 0.05$, by Student t-test. Neither concentration of Ic elicited secretion of lingual lipase or had an effect on SP-elicited secretion.

0.1, 1.0, 10.0, and 100 μM SP, the optimal dose for SP was determined to be 1.0 μM. For the other experiments, the concentration used for Ia and Ib, was 1.0 μM, for Ic and CP-96,345 the concentrations were 1.0 and 10 μM and the concentration for atropine, propranolol, and timolol was 10.0 μM. Preliminary experiments (data not shown) were done with 0.1, 5.0, and 10 μM Ia and 5 μM Ib, but no differences were seen in the results when compared to 1 μM.

Enzyme Assays

All media and supernatants of tissue homogenates were assayed for amylase activity the next day by the method of Bern-

FIG. 7. Effect of 1.0 μM (A) or 10 μM (B) of a nonpeptide inhibitor of SP receptors, CP-96,345, {(2S,3S)-*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl) methyl]-1-azabicyclo [2.2.2] octan-3-amine}, on the percentage secretion of lingual lipase and amylase activity evoked by 1.0 μM SP. Minced von Ebner's glands were incubated with SP or CP-96,345 alone or preincubated with CP-96,345 for 20 min and then incubated with 1.0 μM SP combined with 1.0 μM (A) or 10 μM (B) CP-96,345 for 15, 30, and 60, $n = 7$ (A), $n = 5$ (B). Data points are the mean ± standard error and n is the number of experiments, each using the pooled glands of 8 rats according to the analytical and statistical methods described. (A) 1 μM CP-96,345 did not elicit secretion of either enzyme, nor did it have an effect on CP-96,345-elicited secretion. (B) 10 μM CP-96,345 did not elicit secretion of either enzyme, but was very effective in inhibiting secretion elicited by CP-96,345. *, ⁑, ⁂ represent differences between secretion evoked by SP alone and SP+10 μM CP-96,345 at 15, 30, and 60 min, respectively. For lingual lipase, $p = 0.0116$, 0.0001, and 0.0189 at 15, 30, and 60 min, respectively. For amylase, $p = 0.0337$, 0.0135, and 0.0050 at 15, 30, and 60 min, respectively, by Student t-test.
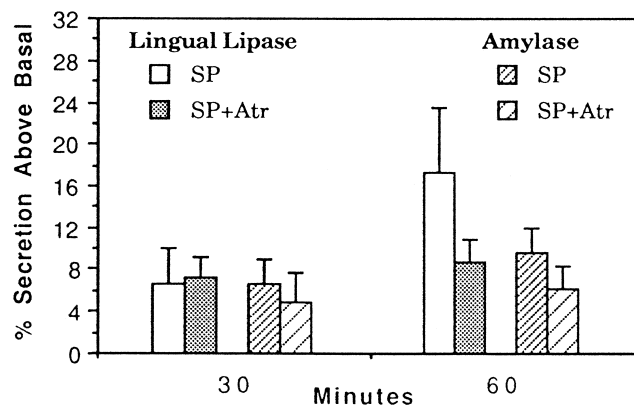
FIG. 8. Effect of 10.0 μM of a cholinergic receptor antagonist, atropine (Atr), on the percentage secretion of lingual lipase and amylase activity evoked by 1.0 μM SP. Minced von Ebner's glands were incubated with SP alone or preincubated with Atr for 20 min and then incubated with 1.0 μM SP combined with 10.0 μM Atr for 30 and 60 min, $n = 4$. Data points are the mean $\pm$ standard error, and n is the number of experiments each, using the pooled glands of 8 rats according to the analytical and statistical methods described. SP-evoked lingual lipase secretion was not inhibited by atropine. For SP-evoked amylase secretion, although atropine did not cause significant differences at the individual time points, there was a significant overall combined effect (sum of 30 and 60 min) of atropine on SP-evoked amylase as determined by repeated measures ANOVA, $p = 0.0298$.

micromole of fatty acid produced per minute at 37°C. Since samples were stored in two cryogenic tubes/sample, each assay could be performed on samples that had been thawed only once.

Calculations

The data were expressed as percentage secretion, which was calculated by dividing the enzyme activity secreted at each time interval by total activity in the sample. The units of enzyme activity were calculated for media samples at each time interval and homogenate samples at the conclusion of the experiment. Appropriate corrections were made for the enzyme activity in the volume of sample removed at each time interval. The total enzyme activity was the sum of the units in the media and in the tissue homogenates at the final time interval. Secretion that resulted from the treatment alone was expressed as percentage secretion above basal and was determined by subtracting the percentage secretion of the controls (basal secretion) from the total percentage secretion.

Statistics

All the analyses were performed on percentage secretion above basal data. The data were examined to ensure that the assumptions of normality and homoscedasticity were justified. Normality was checked by analyzing the residuals from the ANOVA with the Wilk-Shapiro statistic. The group variances were examined visually with residuals plots. These assumptions were violated in one group of experiments involving SP and propranolol. Nonparametric Wilcoxon two-sample tests were done separately for 30, 60 min values for this group of experiments. The other data were analyzed using the repeated measures procedure in the SAS Proc GLM statistical software package (SAS Institute, Cary, NC). Main effects and interactions were tested
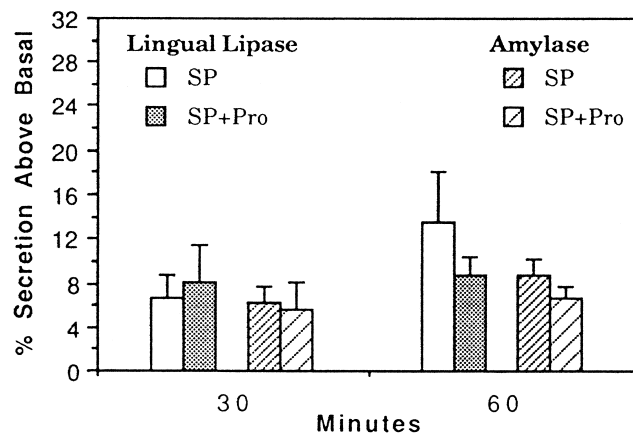

FIG. 9. Effect of 10.0 μM of a β_1/β_2-adrenergic antagonist, propranolol (Pro), on the percentage secretion of lingual lipase and amylase activity evoked by 1.0 μM SP. Minced von Ebner's glands were incubated with SP alone or preincubated with Pro for 20 min and then incubated with 1.0 μM SP combined with 10.0 μM Pro for 30 and 60 min, $n = 6$. Data points are the mean $\pm$ standard error, and n is the number of experiments, each using the pooled glands of 8 rats according to the analytical and statistical methods described. Pro alone had no effect on secretion of either enzyme or on SP-elicited secretion.

using the Geisser-Greenhouse corrected F-values. The nature of the time course differences was examined using the orthogonal polynomial contrast option in PROC GLM (23,41). Differences were considered statistically significant when $p < 0.05$. Statistical analyses, using Student's t-tests, were done on the data resulting from incubating the tissues with receptor antagonists alone to determine whether secretions elicited by these compounds were different from zero.
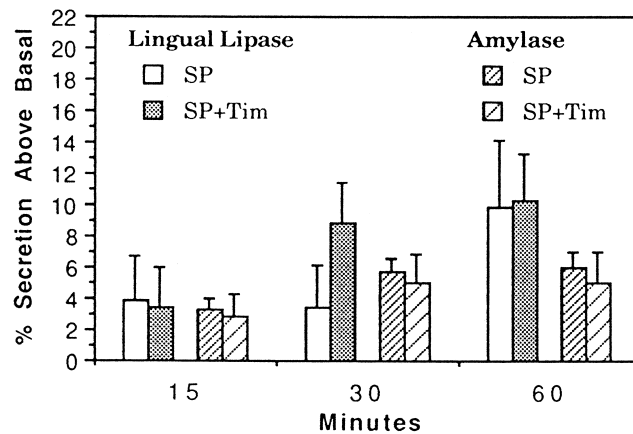
FIG. 10. Effect of 10.0 μM of a β_1/β_2-adrenergic antagonist, timolol (Tim), on the percentage secretion of lingual lipase and amylase activity evoked by 1.0 μM SP. Minced von Ebner's glands were incubated with SP alone or preincubated with Tim for 20 min and then incubated with 1.0 μM SP combined with 10.0 μM Tim for 15, 30, and 60 min, $n = 3$. Values are the mean $\pm$ standard error, and n is the number of experiments, each using the pooled glands of 8 rats according to the analytical and statistical methods described. Tim alone had no effect on secretion of either enzyme or on SP-elicited secretion.

RESULTS

To determine the optimal dose of SP, preliminary experiments were conducted in which minced von Ebner's glands were incubated with 0.01 ($n = 1$), 0.1 ($n = 3$), 1.0 ($n = 3$), 10 ($n = 3$), and 100 ($n = 1$) μM SP. The percentage secretion was determined at 15, 30, and 60 min. Figure 1 shows the dose response curve at 60 min expressed as percentage secretion above basal. Very little or no secretion was evoked by 0.01 μM, somewhat more by 0.1 μM, and the maximal secretion by 1 μM SP. The percentage secretion evoked by 10 μM was almost the same as by 1 μM, and 100 μM evoked very little secretion. The curves in Fig. 1 show that the percentage secretion above basal for lingual lipase tends to be greater than for amylase, except for the 100 μM dose. Statistical analyses were not performed on these preliminary data.

Figure 2 shows the basal secretion of lingual lipase and amylase that occurred when minced tissue was incubated in DMEM for 15, 30, and 60 min. Repeated measures ANOVA revealed that the percentage basal secretion of lingual lipase was significantly lower than the percentage basal secretion of amylase, $p = 0.0001$, $n = 43$. The time courses of lingual lipase and amylase basal secretion were significantly different from each other, $p = 0.0102$. Secretion of both enzymes significantly increased with time, $p = 0.0001$. The nature of the difference between the time courses was analyzed using orthogonal polynomial contrasts. The two enzymes did not differ in the linear component of their time course, $p = 0.91131$. There was a significant difference in the quadratic component or curvature, $p = 0.0001$. An examination of the graph shows that by 15 min basal amylase secretion was much greater than basal lipase secretion. The slope of the curve for amylase secretion decreased between 30 and 60 min, but the slope of the lipase curve remained constant.

SP-elicited secretion of lingual lipase and amylase is shown in Fig. 3. The percentage secretion of both enzymes increased with time. The slopes of both curves decreased with time, but the decrease in the slope of the amylase curve was greater. This observation was confirmed by statistical evaluation. The quadratic components of the time courses of the two enzymes were statistically different, $p = 0.0001$, while the linear components were not. The differences between the two enzymes were significant only at 60 min, $p = 0.003$. The 15, 30, and 60 min values for percentage secretion of lingual lipase and amylase were all statistically different from zero, $p = 0.0001$. The maximum percentage secretion above basal of lingual lipase was 12.14 ± 0.90 and for amylase, 8.30 ± 0.42, $n = 39$.

The mechanism of stimulation of secretion by SP was investigated by preincubating the tissue with the following SP receptor antagonists: the SP analogs, D-Pro^2-D-Phe^7-D-Trp^9-SP (Ia), D-Pro^2-D-Trp^{7,9}-SP (Ib), and D-Arg^1-D-Trp^{7,9}-D-Leu^{11}-SP (Spantide, Ic), and a nonpeptide inhibitor of SP receptors (kindly provided by Dr. R. Michael Snider of Pfizer, Inc.), CP-96,345, {(2S,3S)-cis -2-(diphenylmethyl)-N-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine}.

Figures 4–6 show the results of treating the tissue with the SP analogs that are known to be SP receptor antagonists (26). One μM Ia (Fig. 4) had no significant effect on enzyme secretion when incubated with the tissue alone. When combined with SP, 1 μM Ia did not have any effect on SP-evoked enzyme secretion. Neither 1 μM Ib (Fig. 5) nor 1 or 10 μM Ic (Fig. 6) had statistically significant effects on secretion evoked by SP. However, 1 μM Ib, when incubated with the

tissue (Fig. 5) stimulated secretion of amylase at all time intervals, $p < 0.05$. One μM Ic alone stimulated secretion of amylase at 30 and 60 min, $p < 0.05$, but 10 μM Ic alone did not significantly stimulate secretion of either enzyme. In preliminary experiments, inhibition of SP-evoked stimulation was not observed using 0.1, 5.0, and 10 μM Ia and 5 μM Ib (data not shown).

CP-96,345 is an inhibitor of SP receptors that is not a peptide or an analog of SP (45). Figure 7A shows the results of seven experiments in which 1 μM CP-96,345 was preincubated for 20 min with minced tissue and was then incubated with 1 μM SP and CP-96,345 for 60 min. Although, when combined with SP, a trend was seen towards inhibition of SP-evoked secretion, there were no statistically significant changes in the time courses of SP-evoked secretion of lingual lipase or amylase in the presence of CP-96,345 ($p = 0.0587$ and 0.0671 for lingual lipase and amylase, respectively). CP-96,345 (1 μM) alone did not significantly stimulate secretion of either enzyme at this concentration. However, with 10 μM CP-96,345 (Fig. 7B), there was inhibition of SP-evoked secretion. The percentage reduction in lingual lipase secretion was 79.5 ± 18.0, 84.9 ± 4.8, and 47.0 ± 12.3 at 15, 30, and 60 min, respectively and for amylase the percentage reduction in secretion was 48.3 ± 15.2, 56.3 ± 13.3, and 58.5 ± 10.5 at 15, 30, and 60 min, respectively. The differences between SP and SP+CP were statistically significant with p values for lingual lipase of 0.0116, 0.0001, and 0.0189 at 15, 30, and 60 min, respectively and for amylase the p values were 0.0337, 0.0135, and 0.0050 at 15, 30, and 60 min, respectively. Secretion of neither enzyme was statistically significantly different from basal secretion when the tissue was incubated with 10 μM CP-96,345 alone.

Since SP-analog receptor antagonists did not inhibit SP-evoked secretion of lingual lipase or amylase, the effects of antagonists to other receptors were investigated. The effects of SP on major salivary glands involved the phosphoinositol pathway mediated by cholinergic stimulation (24). Cholinergic stimulation is the major effector of protein secretion from von Ebner's gland (12). Therefore, the cholinergic receptor antagonist, atropine, was used to inhibit any cholinergic responses that could possibly be due to SP. Figure 8 shows the effects on secretion of lingual lipase and amylase elicited by SP after preincubation of minced von Ebner's gland tissue with atropine followed by incubation with SP and atropine. Statistical analyses did not demonstrate a significant effect of atropine on the SP-evoked secretion of lingual lipase. There was no significant difference between atropine and atropine + SP on amylase secretion at the individual time points, however, statistical analysis by repeated measured ANOVA revealed an overall effect (sum of 30 and 60 min) of the atropine treatment, $p = 0.0298$. Thus, there was some evidence of cholinergic mediation of SP-evoked amylase secretion, but not lingual lipase secretion. Two β-adrenergic receptor antagonists, propranolol (Fig. 9) and timolol (Fig. 10), having both β_1 and β_2 activities had no significant effect on SP-evoked secretion, nor did they evoke secretion when incubated alone (data not shown).

DISCUSSION

Our experiments showed that SP stimulated secretion of both lingual lipase and amylase from von Ebner's gland in a dose- and time-dependent manner. In addition, recent histochemical studies of rat von Ebner's glands revealed degranulation of acinar cells in response to SP (53) and the presence of SP immunoreactivity in selective cells (38) and neuronal elements (33).

SP-evoked responses are mediated by NK-1 receptors (6,45). Receptors for SP have been implicated in the secretory responses of the submandibular (4,15,24,56), parotid (17,24,32,56), and sublingual glands (24), and of the exocrine pancreas (26,47). Although most tissues have distinct types of receptors for a variety of tachykinin peptides, there is a lack of specificity among the tachykinin receptors, making it difficult to find specific antagonists (5,6,30).

In order to characterize the SP receptor in von Ebner's glands, three SP analogs (Ia, Ib, and Ic) known to antagonize SP receptors were tested. None of these analogs significantly inhibited secretion evoked by SP at concentrations of 1, 5, or 10 μM. However, two SP-analogs stimulated secretion of amylase, 1 μM D-Pro^2-D-Trp^{7,9}-SP (Ib) alone stimulated amylase secretion at all time intervals and 1 μM D-Arg^1-D-Trp^{7,9}-D-Leu^{11}-SP (Spantide, Ic) alone stimulated secretion of amylase at 30 and 60 min, but none of the SP-analog inhibitors alone evoked secretion of lingual lipase. However, in other exocrine glands, investigators found that these compounds could inhibit SP-evoked secretion. In superfused segments of isolated parotid glands, Ib (10 μM) reversibly inhibited SP-induced amylase secretion (17) and Ia, Ib, and Ic also reversibly inhibited amylase release from dispersed pancreatic acinar cells (26).

CP-96,345, an SP receptor antagonist that is not a peptide, also was used to characterize the SP-induced secretion The evidence is very strong that CP-96,345 is an antagonist of the SP receptor, NK-1, in exocrine glands. In vivo, CP-96,345 has inhibited salivation elicted by SP in rats (46) and has been shown to be an antagonist of SP receptors in pancreatic acinar cells of the guinea pig (44). The current experiments did not reveal any statistically significant effects of 1 μM CP-96,345 on the secretion evoked by SP, nor did 1 μM CP-96,345 evoke any secretion alone. However, 10 μM CP-96,345 did inhibit SP-evoked secretion as shown in Fig. 7B. While a concentration of CP-96,345 that is equimolar with SP did not interfere with the effects of SP on the SP receptor, a 10-fold concentration of CP-96,345 was effective in reducing SP-evoked secretion.

One possible explanation for these differences can be drawn from reports that the SP receptor has species and organ specificity. Thus, the SP receptor in von Ebner's gland could be an isoform of the receptor found in other organs and this receptor does not bind the above mentioned SP analogs as strongly as it binds SP. Species specificity of the NK-1 receptor was revealed when CP-96,345 was found to be a more potent inhibitor of the human than the rat NK-1 receptor (16). A minor change in the structure of the receptor could increase the binding of one class of antagonists while decreasing another (16). A species difference was also observed in the affinity of the nonpeptide NK-1 antagonist, CP-96,345, between rat and guinea pig brains (35). In addition, in contrast to the rat, SP had no effects on parotid or submandibular secretion in the mouse, but the responses to carbachol and isoproterenol in the mouse were similar to those in the rat (51). An example of organ specificity was found between rat submandibular glands and the pancreas (52). Inhibitory effects of SP on [^{3}H]myoinositol transport were mediated through NK-1 receptors in rat submandibular glands, but not in rat pancreas that also have NK-1 receptors.

The effects of cholinergic and β-adrenergic receptor antagonists were investigated to determine whether the SP effects could possibly be cholinergic or β-adrenergic mediated. The lack of effect of propranolol or timolol, β-adrenergic receptor antagonists, on SP-stimulated secretion, indicated that the effects of SP were not mediated by β-adrenergic receptors. However, although atropine, a cholinergic receptor antagonist, had no statistically significant effect on the SP-stimulated secretion of lingual lipase, it did show an overall effect on SP-stimulated secretion of amylase (Fig. 8). Thus indicating the possibility of a cholinergic response that resulted from SP stimulation of protein secretion that was differential between the two enzymes. Using the same methods, propanolol and atropine were shown to inhibit secretion of both enzymes when the tissue was stimulated by isoproterenol and carbachol, respectively (12).

Another interesting finding was that there was differential secretion of lingual lipase and amylase in both basal and SP-stimulated secretion. During basal secretion more amylase than lingual lipase was secreted in the first 15 min. Significant differences were found between the curvatures of the lingual lipase and amylase secretion time courses (Fig. 2). The slope of the amylase time course decreased after 30 min, whereas the slope of the lingual lipase curve remained the same. If exocytosis was the only pathway of secretion, these slopes should have been identical. Similar differences were also seen when the secretion was stimulated by carbachol (12), isoproterenol (12), and histamine (10). The only explanation for these findings is that factors other than exocytosis may be involved in basal or stimulated secretion of these enzymes. These factors include unstimulated or constitutive secretion (7), and the maturative secretory pathway. This pathway is postulated to exist between the trans-Golgi network and the mature secretory granule and to provide a continuous delivery of protein to the cell surface (54). It has been suggested that this pathway accounts for differences observed between the patterns of proteins secreted from the exocrine pancreas of conscious hogs when secretion is blocked by atropine as compared to regulated (cholinergic) secretion (54). It is possible that the constitutive, maturative, or another as yet unknown pathway that operates in addition to exocytosis is responsible for the differential secretion of lingual lipase and amylase. In a recent review article (25), it was suggested that processes, in addition to exocytosis, for the secretion of digestive enzymes would be important to allow for modulation of the amounts of different enzymes secreted depending upon the content of the food ingested. Whereas, exocytosis allows for the secretion of stored enzymes that have been previously synthesized, other means of secretion could modulate the synthesis and independent, immediate secretion of enzymes needed to digest a meal that required more of a particular enzyme.

REFERENCES

1. Baum, B. J. Regulation of salivary secretion. In: Sreebny, L. M. ed. The salivary system. Boca Raton, Florida: CRC Press Inc.; 1987:123–134.

2. Bernfield, P. Amylases α and β. In: Colowick, S. P.; Kaplan, N. O., eds. Methods in enzymology, vol. 1. New York: Academic Press; 1955:149–158.

3. Bläker, M.; Kock, K.; Ahlers, C.; Buck, F.; Schmale, H. Molecular cloning of human von Ebner's gland protein, a member of the lipocalin superfamily highly expressed in lingual salivary glands. Biochim. Biophys. Acta 1172:131–137; 1993.

4. Buck, S. H.; Burcher, E. The rat submaxillary gland contains predominantly P-type tackykinin binding sites. Peptides 6:1079–1084; 1985.

5. Buck, S. H.; Fanger, B. O.; van Giersbergen, P. L. M. Pharmacological and biochemical evidence for multiple types of tachykinin NK2 receptors. Ann. N.Y. Acad. Sci. 632:112–115; 1991.

6. Burcher, E. The study of tachykinin receptors. Clin. Exp. Pharmacol. Physiol. 16:539–543; 1989.

7. Burges, T. L.; Kelley, R. B. Constitutive and regulated secretion of proteins. Ann. Rev. Cell Biol. 3:243–293; 1987.

8. Chang, M. M.; Leeman, S. E. Isolation of a sialogic peptide from bovine hypothalmus and its characterization as substance P. J. Biol. Chem. 245:4784–4790; 1970.

9. Dixon, J. S. Histology: Ultrastructure. In: Howat, H. T.; Sarles. H. eds. The exocrine pancreas. London: W. B. Saunders Co. Ltd.; 1979:31–49.

10. Field, R. B.; Chirtel, S. J. The effects of histamine, pyrilamine, cimetidine, and ranitidine on secretion of lingual lipase and amylase from rat von Ebner's glands. Agents Actions 37:210–219; 1992.

11. Field, R. B.; Dromy, R.; Hand, A. R. Regulation of secretion of enzymes from von Ebner's gland of rat tongue. J. Dental Res. 66:586–587; 1986.

12. Field, R. B.; Hand, A. R. Secretion of lingual lipase and amylase from rat lingual serous glands. Am. J. Physiol. 253:G217–G225; 1987.

13. Field, R. B.; Spielman, A. I.; Hand, A. R. Purification of lingual amylase from serous glands of rat tongue and characterization of rat lingual amylase and lingual lipase. J. Dent. Res. 68:139–145; 1989.

14. Field, R.B.; Scow, R.O. Purification and characterization of rat lingual lipase. J. Biol. Chem. 258:14563–14569; 1983.

15. Fleming, N.; Bilan, P. T.; Graham, K. W. Effect of substance P on exocrine secretion by rat submandibular gland cells J. Dent. Res. 63:1022–1027; 1984.

16. Fong, T. M.; Yu, H.; Strader, C. D. Molecular basis for the species selectivity of the neurokinin-1 receptor antagonists CP-96,345 and RP67580. J. Biol. Chem. 267:25668–25671; 1992.

17. Gallacher, D. V. Substance P is a functional neurotransmitter in the rat parotid gland. J. Physiol. 342:483–498; 1983.

18. Hamosh, M.; Burns, W. A. Lipolytic activity of human lingual glands (Ebner). Lab. Invest. 37:603–608; 1977.

19. Hamosh, M.; Hand, A. R. Development of secretory activity in serous cells of the rat tongue. Develop. Biol. 65:100–113; 1978.

20. Hamosh, M.; Klaeveman, H. L.; Wolf, R. O.; Scow, R. O. Pharyngeal lipase and digestion of dietary triglyceride in man. J. Clin. Invest. 55:908–913; 1975.

21. Hamosh, M.; Scow, R. O. Lingual lipase and its role in the digestion of dietary lipid. J. Clin. Invest. 52:88–95; 1973.

22. Hand, A. R. The fine structure of von Ebner's gland of the rat. J. Cell Biol. 44:340–353; 1970.

23. Hand, D. J.; Taylor, C. C. Multivariate analysis of varience and repeated measures. London: Chapman and Hall; 1987.

24. Hanley, M. R.; Lee, C. M.; Jones, L. M.; Michell, R. H. Similar effects of substance P and related peptides on salivation and phosphatidylinositol turnover in rat salivary glands. Mol. Pharmacol. 18:78–83; 1980.

25. Isenman, L.; Liebow, C.; Rothman, S. Transport of proteins across membranes-a paradigm in transition. Biochim. Biophys. Acta 1241:341–370; 1995.

26. Jensen, R. T.; Jones, S. W.; Lu, Y.-A.; Xu, J.-C.; Folkers, K.; and Gardner, J. D. Interaction of substance P antagonists with substance P receptors on dispersed pancreatic acini. Biochim. Biophys. Acta 804:181–191; 1984.

27. Kock, K.; Bläker, M.; Schmale, H. Postnatal development of von Ebner's glands: Accumulation of a protein of the lipocalin superfamily in taste papillae of rat tongue. Cell Tissue Res. 267:313–320; 1990.

28. Leslie, B. A.; Putney, J. W. Ionic mechanisms in secretagogue-induced morphological changes in rat parotid gland. J. Cell Biol. 97:1119–1130; 1983.

29. Liang, T.; Casccieri, M. A. Substance P stimulation of amylase release by isolated parotid cells and inhibition of substance P induction of salivation by vasoactive peptides. Mol. Cell. Endocrinol. 15:151–162; 1979.

30. Maggio, J. E. Tachykinins. Ann. Rev. Neurosci. 11:13–28; 1988.

31. Martinez, J. R.; Martinez, A. M. Stimulatory and inhibitory effects of substance P on rat submandibular secretion. J. Dent. Res. 60:1031–1038; 1981.

32. Merritt, J. E.; Rink, T. J. The effects of substance P and carbachol on inositol tris- and tetrakisphosphate formation and cytosolic free calcium in rat parotid acinar cells. J. Biol. Chem. 262:14912–14916; 1987.

33. Oomori, Y.; Satoh, Y.; Ishikawa, K.; Ono, K. Substance P immunoreactivity in rat von Ebner's gland. Histochem. J. 27:395–400; 1995.

34. Pernow, B. Substance P. Pharmacol. Rev. 325:85–141; 1983.

35. Petitet, F.; Beaujouan, J.-C.; Saffroy, M.; Torrens, Y.; Fardin, V.; Glowinski, J. NK-1 tachykinin receptor in rat and guinea pig brains: Pharmacological and autoradiographic evidence for a species difference. Peptides 14:551–559; 1993.

36. Poat, J. A.; Cripps, H. E.; Cowburn, R.; Iversen, L. L. Synergistic interactions between forskolin, isoprenaline and substance P as secretagogues in rat parotid glands. Eur. J. Pharmacol. 144:317–326; 1987.

37. Roberts, I. M. Rat lingual lipase: Effect of proteases, bile, and pH on enzyme stability. Am. J. Physiol. 249:G496–G500; 1985.

38. Roberts, I. M.; Solomon, S. E.; Brusco, O. A.; Goldberg, W.; Bernstein, J. J. Neuromodulators of the lingual von Ebner gland: An immunocytochemical study. Histochemistry 96:153–156; 1991.

39. Rollandy, I.; Dreux, C.; Imhoff, V.; Rossignol, B. Importance of the presence of the N-terminal tripeptide of substance P for the stimulation of phosphatidylinositol metabolism in rat parotid gland: A possible activation of phospholipases C and D. Neuropeptides 13:175–185; 1989.

40. Rudich, L.; Butcher, F. R. Effect of substance P and eledoisin on K+ efflux, amylase release and cyclic nucleotide levels in slices of rat parotid gland. Biochim. Biophys. Acta 444:704–711; 1976.

41. SAS/STAL Users Guide, Release 6.03 Cary, NC: SAS Institute Inc.; 1988.

42. Schmale, H.; Holtgreve–Grez, H.; Christiansen, H. Possible role for salivary gland protein in taste reception indicated by homology to lipophilic-ligand carrier proteins. Nature 343:366–369; 1990.

43. Sjödin, L.; Conlon, T. P.; Gustavson, C.; Uddholm, K. Interaction of substance P with dispered pancreatic acinar cells from the guinea pig. Stimulation of calcium outflux, accumulation of cyclic GMP and amylase release. Acta Physiol. Scand. 109:107–110; 1980.

44. Sjödin, L.; Gylfe, E. A selective and potent antagonist of substance P receptors on pancreatic acinar cells. Biochem. Int. 27:145–153; 1992.

45. Snider, R. M.; Constantine, J. W.; Lowe, J. A., III; Longo, K. P.; Lebel, W. S.; Woody, H. A.; Drozda, S. E.; Desai, M. C.; Vinik, F. J.; Spencer, R. W.; Hess, H.-J. A potent nonpeptide antagonist of the substance P (NK1) receptor. Science 251:435–437; 1991.

46. Snider, R. M.; Longo, K. P.; Drozda, S. E.; Lowe, J. A., III; Leeman. S. E. Effect of CP-96,345, a nonpeptide substance P receptor antagonist, on salivation in rats. Proc. Natl. Acad. Sci. USA; 88:10042–10044; 1991.

47. Song, S.-Y.; Iwahita, S.; Noguchi, K.; Konishi, S. Inositol trisphosphate-linked calcium mobilization couples substance P receptors to conductance increase in a rat pancreatic acinar cell line. Neurosci. Lett. 95:143–148; 1988.

48. Spielman, A. I. Interaction of saliva and taste. J. Dent. Res. 69:838–843; 1990.

49. Spielman, A. I.; D'Abundo, S.; Field, R. B.; Schmale, H. Protein analysis of human von Ebner saliva and a method for its collection from the foliate papillae. J. Dent. Res. 72:1331–1335; 1993.

50. Testa Riva, F.; Cossu, M.; Lantini, M. S.; Riva, A. Fine structure of deep posterior lingual glands J. Anat. 142:103–115; 1985.

51. Tojyo, Y.; Tanimura, A.; Matsumoto, Y. Evidence that substance-P receptors do not exist in mouse parotid and submandibular acinar cells. Arch. oral Biol. 38:269–271; 1993.

52. Torrens, Y.; Dietl, M. M.; Beaujouan, J-C.; Glowinski, J. Myoinositol uptake in rat parotid gland. Ann. N.Y. Acad. Sci. 632:145–153; 1991.

53. Ueba, H.; Uchihashi, K. Degranulation of acinar cells in von Ebner's gland of the rat. J. Osaka Dent. Univ. 25:35–49;1991.

54. Viau, G.; Lainé, J.; Levenez, F.; Gueugneau, A.-M.; Corring, T.; Morisset, J.; Lebel, D. Evidence for an apical, nonregulated protein secretion in pig exocrine pancreas. Am. J. Physiol. 30:G764–G771; 1994.

55. von Euler, U. S.; Gaddam, J. H. An unidentified depressor substance in certain tissue extracts. J. Physiol. (Lond.) 72:76–87; 1931.

56. Yu, J.-H.; Burns, S. M.; Schneyer, C. A. Salivary secretion induced by substance P. Proc. Soc. Exp. Biol. Med. 173:467–470; 1983.