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THE EFFECTS OF SUBSTANCE P AND RELATED PEPTIDES ON α-AMYLASE RELEASE FROM RAT PAROTID GLAND SLICES

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1 The effects of substance P and related peptides on amylase release from rat parotid gland slices have been investigated.

2 Supramaximal concentrations (1 μM) of substance P caused enhancement of amylase release over the basal level within 1 min; this lasted for at least 40 min at 30°C.

3 Substance P-stimulated amylase release was partially dependent on extracellular calcium and could be inhibited by 50% upon removal of extracellular calcium.

4 Substance P stimulated amylase release in a dose-dependent manner with an ED_{50} of 18 nM.

5 All C-terminal fragments of substance P were less potent than substance P in stimulating amylase release. The C-terminal hexapeptide of substance P was the minimum structure for potent activity in this system, having 1/3 to 1/8 the potency of substance P. There was a dramatic drop in potency for the C-terminal pentapeptide of substance P or substance P free acid. Physalaemin was more potent than substance P (ED_{50} = 7 nM), eledoisin was about equipotent with substance P (ED_{50} = 17 nM), and kassinin less potent than substance P (ED_{50} = 150 nM).

6 The structure-activity profile observed is very similar to that for stimulation of salivation *in vivo*, indicating that the same receptors are involved in mediating these responses.

7 All the fragments of substance P tested were capable of eliciting a full amylase release response. This indicates that the apparent partial agonist action of the C-terminal nonapeptide fragment on *in vivo* salivation is not explicable at the receptor level.

Introduction

The undecapeptide substance P was originally identified in extracts of horse intestine and was found to cause hypotension and powerful contractions of intestinal smooth muscle (Von Euler & Gaddum, 1931). Substance P was subsequently isolated from the hypothalamus, sequenced and synthesized (Chang & Leeman, 1970; Chang, Leeman & Niall, 1971).

One of the most potent actions of substance P in peripheral tissues is the stimulation of salivation (Leeman & Hammerschlag, 1967). Upon systemic injection it can cause up to a 50 fold increase in salivary flow with a 4 fold increase in amylase release per unit volume of saliva (Liang & Cascieri, 1979). This appears to be a direct action of substance P on the glands themselves as it is not blocked *in vivo* by atropine, propranolol or phenoxybenzamine (Leeman & Hammerschlag, 1967). Moreover, *in vitro* studies show several substance P effects on salivary gland preparations (Rudich & Butcher, 1976; Friedman & Selinger, 1978; Jones & Michell, 1978; Gallacher & Petersen, 1980), and point to the existence of separate α-adrenoceptor, substance P and muscarinic receptors acting via a common ion channel mechanism (Putney, 1977; Gallacher & Petersen, 1980).

In peripheral tissue bioassays, the potency rank order of synthetic C-terminal fragments of substance P (Bury & Mashford, 1976; Blumberg & Teichberg, 1979) or of tachykinins (naturally-occurring substance P-like peptides; Erspamer, Erspamer & Piccinelli, 1980), has been used as a tool to study the substance P receptor. Experiments on *in vivo* salivation have shown that the minimum sequence for activity is the C-terminal pentapeptide, and all C-terminal fragments of substance P have lower agonist potency than substance P (Hanley, Lee, Jones & Michell, 1980). However, these data also showed an interesting anomaly; namely that the C-terminal substance P nonapeptide behaved as a partial agonist and did not elicit a full response, unlike the other C-terminal fragments.

The investigation described here was undertaken to determine the structural requirements of the substance P receptor of rat salivary glands *in vitro*, using the release of α-amylase from parotid slices. In so doing, we found that the apparent partial agonist activity of the C-terminal nonapeptide is not a true

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receptor phenomenon but is rather a complication of *in vivo* sialogogue experiments. We also describe the characteristics of α-amylase release from parotid slices to support the use of this preparation as a model of substance P receptor function.

Methods

Preparation of the parotid slices

A gassed (95% O_2:5% CO_2) Krebs-Ringer bicarbonate buffer containing 10 mM inosine, 0.4 mM adenine and 5 mM 3-hydroxybutyrate was used throughout (Batzri & Selinger, 1973).

For a typical experiment involving 36 incubations, the parotid salivary glands were removed from three male Sprague-Dawley rats, which had been anaesthetized with chloroform and killed by exsanguination. After removal, the glands were kept in the medium at room temperature.

The glands were dissected free of lymph nodes, adipose and connective tissues and cut into small pieces about 3 mm^2 which were then cross chopped at 60° with a spacing of 0.15 mm on a McIlwain tissue chopper. The resulting slices were placed in medium (5 ml per pair of parotid glands) containing 0.5 mg/ml of collagenase, which was gently gassed with 95% O_2:5% CO_2, sealed, and incubated at 30°C for 30 min with shaking. The purpose of this treatment was to remove the collagen fibres which tend to clump the tissue slices. At the end of this incubation, the collagenase solution was poured off and the treated slices rinsed three times with medium at 30°C.

Preparation of tissue for calcium-free incubation

A calcium-free medium was prepared by omitting $CaCl_2$ from the normal medium and replacing it on a molar basis with $MgCl_2$ and adding EGTA to a final concentration of 1 mM. The slices were prepared as described above and were then dispersed with a 50 times larger volume of calcium-free medium. The slices were pipetted from the large volume of medium when they had settled, rinsed again in calcium-free medium and pipetted into incubation vials in calcium-free medium containing the various drug additions.

Incubation of tissue slices

Volumes of 20 μl of the suspension of parotid slices (0.2 mg wet weight) were pipetted into flat-bottomed polypropylene vials containing 470 μl of the medium and 10 μl of drug solution. Triplicate experiments were performed for each drug concentration. Tubes containing no drugs (basal) and tubes containing 1 μM substance P (supramaximal) were included as internal standards for each batch of tissue. The vials were gassed with 95% O_2:5% CO_2, sealed and incubated at 30°C with shaking for 30 min. Preliminary studies showed that stimulation of release was greater at 30°C than at 20°C. At the end of the 30 min incubation period, the contents of the vials were transferred into 'Microfuge' tubes which were spun for 1 min at 10,000 *g* in a Beckman 'Microfuge'. The supernatant was taken for α-amylase assay.

Amylase assay

The supernatant was diluted × 4 and 10 μl was incubated with 0.99 ml of starch solution (0.5 ml of starch solution in 0.1 M phosphate buffer (pH 7) diluted with 0.49 ml distilled water) at room temperature for 5 min. The reaction was stopped by addition of 1 ml of an alkaline solution of 3,5-dinitrosalicylic acid. The mixture was heated in a boiling water bath for 10 min. After cooling, the contents were diluted with 10 ml of water and the absorbance (1 cm light path) at wavelength 546 nm was measured against a blank to which 10 μl of incubation medium had been added in place of diluted supernatant. For each experiment, the amylase activity was expressed as maltose equivalents by comparison with a maltose standard curve. One unit of amylase is defined as the amount of enzyme that causes the formation of 1 mg of maltose in 5 min at 25°C. Owing to between-experiment variation in the magnitude of the response, data were in some instances expressed as a percentage of the stimulation by a supramaximal concentration of substance P (1 μM) over the basal α-amylase release.

In vivo *salivation*

The increase in salivary flow provoked by substance P and other peptides was determined in rats as described previously (Hanley *et al.*, 1980).

Drugs

Synthetic substance P, C-terminal fragments of substance P, substance P free acid, eledoisin, physalaemin and kassinin were obtained from Peninsula Laboratories. Collagenase was obtained from Worthington Biochemicals.

Results

Characteristics of response

Substance P stimulation of amylase release was apparent within 1 min and lasted for at least 40 min (Figure 1). There was an initial steep rise in basal amylase release and of amylase release stimulated by a supramaximal concentration of substance P (1 μM)

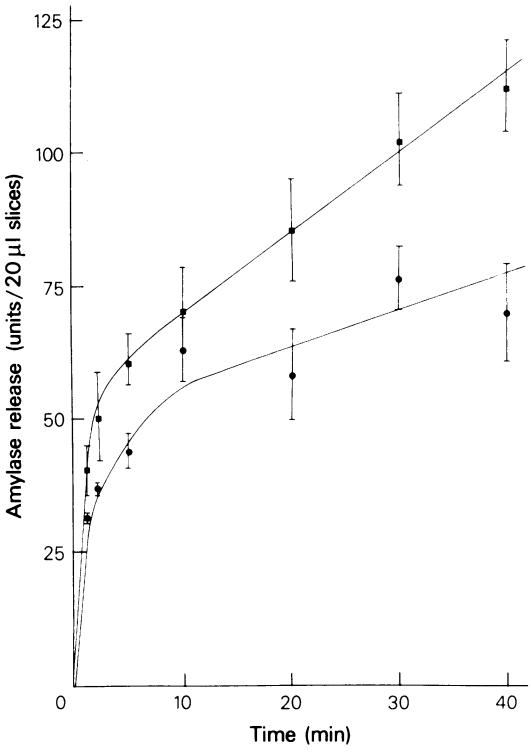

Figure 1 Time course of amylase release by parotid gland slices. (●) Basal release; (■) release in presence of 1 µM substance P. Slices (20 µl) were incubated in the medium (total volume 500 µl) at 30°C with shaking. Incubations were ended after various times by transferring to microfuge tubes and spinning down. Results are mean for triplicate determinations; vertical lines show s.e.mean.

which slowed after about 2 min. From 10 to 40 min there was a constant rate of amylase release which was greater in the presence of 1 µM substance P than in the absence of drug. These time courses indicate that 30 min is a suitable incubation time to choose for the study of stimulation of amylase release by substance P and related peptides.

When carbachol, noradrenaline and substance P were used in supramaximal concentrations, no combination of agonists produced a summated amylase release response (Table 1). The stimulation of amylase release by a supramaximal concentration of carbachol (500 µM) was not significantly different from that elicited by 1 µM substance P. However, supramaximal noradrenaline (500 µM) produced a significantly greater stimulation of amylase release than did either carbachol or substance P.

Basal amylase release and amylase release stimulated by substance P, eledoisin, carbachol and noradrenaline were reduced, but not abolished, in the absence of external Ca^{2+} (Table 2). Removing external Ca^{2+} reduced noradrenaline-stimulated amylase release less than release stimulated by other agonists.

Putative calcium antagonists also appeared to inhibit substance P-stimulated amylase release. Lanthanum chloride (1 mM) had no effect on basal release but produced a 69% inhibition of substance P-stimulated amylase release, and D600 (methoxyverapamil, 0.1 mM) inhibited substance P-stimulated release by 50%; however D600 also gave an elevated basal release.

The addition of the local anaesthetic tetracaine (1 mM) to the medium had no significant effect on the amylase release stimulated by supramaximal concentrations of substance P, carbachol or noradrenaline (Table 3).

Substance P C-terminal fragment series and tachykinins

Maximum stimulation of amylase release by substance P occurred at about 100–200 nM with half-maximum stimulation at about 18 nM (Figure 2).

Table 1 Comparison of responses to stimulation of α-adrenoceptors, muscarinic and substance P receptors

Drug additions	*Amylase release** (units/20 µl slices)
Substance P (1 µM)	64.5 ± 3.2
Carbachol (500 µM)	56.2 ± 4.5
Noradrenaline (500 µM)	77.3 ± 2.4**
Carbachol (500 µM) + substance P (1 µM)	77.3 ± 8.5
Noradrenaline (500 µM) + substance P (1 µM)	79.2 ± 17.3
Carbachol (500 µM) + noradrenaline (500 µM)	74.7 ± 8.9

* All values have had basal levels subtracted and are means ± s.e.mean ($n = 3$).

** Significantly different from 1 µM substance P ($p < 0.05$) and from 500 µM carbachol ($P < 0.01$) using Student's t test.

Table 2 Calcium-dependence of basal and stimulated amylase release

Drug	*Amylase release** (units/20 μl slices) *In absence of* Ca^{2+}	*In presence of* Ca^{2+}	*%** inhibition of stimulation by omission of* Ca^{2+}
Basal	64.8± 0.9	93.6± 3.8	(30.8)
Substance P (1 μM)	105.1± 1.5	175.0± 3.2	50.5
Eledoisin (1 μM)	97.9±10.9	201.6±23.3	69.4
Carbachol (500 μM)	105.1±26.4	192.6± 9.3	59.3
Noradrenaline (500 μM)	144.0±14.2	193.0± 0.9	20.3

* Values are means ± s.e.mean ($n = 3$).

** Determined by the ratio of $1 - \frac{\text{stimulation } (-Ca^{2+})}{\text{stimulation } (+Ca^{2+})} \times 100$

The C-terminal hexapeptide sequence of substance P and the C-terminal amide group are critical for potent biological activity in this system. All substance P fragments retaining this structure had potencies, as assessed by the ED_{50}, one third to one eighth of that of substance P (Table 4). The C-terminal pentapeptide and the substance P free acid showed a great drop in potency being about 300 times weaker than substance P (Table 4).

The tachykinin peptide family was also capable of potently stimulating amylase release from parotid slices. Physalaemin was three times more potent than substance P, and eledoisin about equipotent with substance P. The rank order of potency was physalaemin > eledoisin > kassinin.

All fragments of substance P, including the C-terminal nonapeptide, which was a partial agonist on the *in vivo* salivation response, and the weakly active C-terminal pentapeptide and substance P free acid, were capable of eliciting a full amylase release response equal to that produced by a maximal concentration of substance P. All the tachykinins were also full agonists.

Effects of other peptides

A single high concentration of some other putative neurotransmitter peptides was used to screen for ability to stimulate or inhibit amylase release from parotid slices. Table 5 lists a variety of peptides which had no effect on amylase release.

Discussion

In previous work (Hanley *et al.*, 1980), we reported the feasibility of using rat parotid gland slices to measure substance P-receptor linked events. In this paper, we have extended these observations to a physiologically relevant secretory event, the release of amylase. Upon addition of substance P, stimulated amylase release lasted for at least 40 min. The onset and persistence of this response were similar to the time course of amylase release in dispersed acinar cells (Liang & Cascieri, 1979). The amylase-release response to substance P was not additive to those of noradrenaline and carbachol, supporting the idea

Table 3 Insensitivity of α-adrenoceptor, muscarinic receptor and substance P-stimulated amylase release to tetracaine

Drug	*Amylase release** (units/20 μl slices) *Control*	*Tetracaine 1 mM*
Substance P (1 μM)	79.9±10.7	87.1±6.9
Carbachol (500 μM)	76.3± 9.1	74.2±7.1
Noradrenaline (500 μM)	82.1± 7.6	76.3±5.4

* All values have had basal levels subtracted and are means ± s.e.mean ($n = 3$).

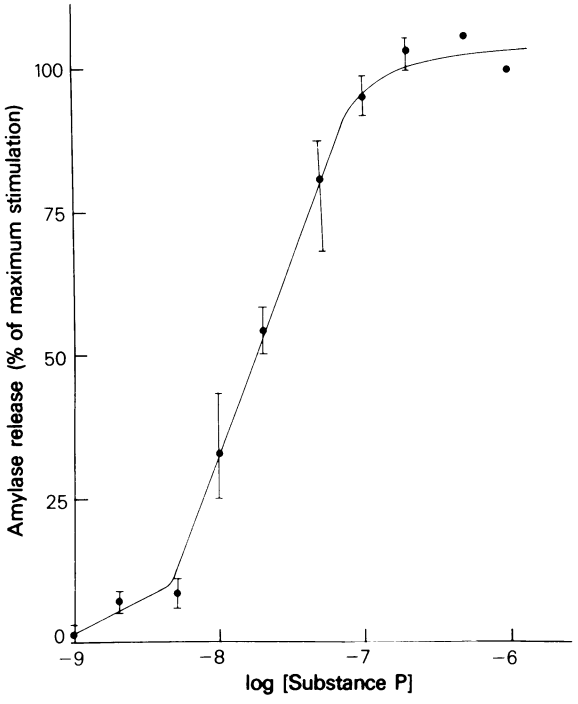

Figure 2 Effect of various concentrations of substance P on the release of amylase from parotid slices expressed as a percentage of the stimulation by 1 μM substance P. Slices (20 μl) were incubated in the medium with various concentrations of substance P in a final volume of 500 μl. Incubations were for 30 min in a shaking water bath at 30°C. Triplicate tubes were used for each concentration. The results are calculated from three separate experiments (except 20 nM: two experiments and 500 nM: one experiment). The error bars indicate the extreme values of the means of the triplicates. Maximum stimulation of amylase release over basal by substance P varied from 63.3–101.6 units/20 μl slices.

that there is a common effector mechanism for α-adrenoceptors, muscarinic cholinoceptors and substance P receptors (Marier, Putney & Van de Walle, 1978; Gallacher & Petersen, 1980). α-Adrenoceptors, muscarinic cholinoceptors and substance P receptors in the parotid have been shown to be coupled to a pool of calcium channels (Putney, 1977), and amylase release from parotid gland slices has been shown previously to be calcium-dependent (Rudich & Butcher, 1976). Butcher (1975) demonstrated that in the presence of external Ca^{2+}, the divalent cationophore, A 23187 can increase α-amylase release from slices of rat parotid gland. However isoprenaline and dibutyryl cyclic adenosine 3′,5′-monophosphate stimulation of amylase release is independent of Ca^{2+}, suggesting that β-adrenoceptors mediate their response through a calcium-independent, adenylate cyclase-coupled mechanism (Kanagasuntheram & Randle, 1976; Leslie, Putney & Sherman, 1976). Substance P has been shown to have no effect on cyclic nucleotide levels in the parotid gland (Rudich & Butcher, 1976).

The results described here show that noradrenaline-stimulated amylase release is inhibited less than substance P or carbachol-stimulated amylase release by the absence of Ca^{2+}, and this is possibly explained by the β-receptor-mediated component in the noradrenaline response.

Eledoisin-stimulated amylase release was also partially calcium-dependent; this does not support the idea of a separate receptor-coupling mechanism for eledoisin, suggested by the calcium independence of eledoisin-stimulated K^+ efflux in the submandibular gland in contrast to the calcium-dependence of physalaemin-stimulated K^+ efflux. (Spearman & Pritchard, 1977).

K^+ efflux resulting from the activation of α- and β-adrenoceptors, substance P and muscarinic receptors in parotid gland slices also shows a partial dependence on external Ca^{2+}, with an initial transient 'calcium-independent' phase, possibly mediated by intracellularly bound Ca^{2+} and a sustained calcium-dependent phase (Putney, 1977; Marier *et al.*, 1978; Friedman & Selinger, 1978). The partial calcium-dependence of amylase release observed here may be explained by calcium-independent and calcium-dependent phases, so that only the latter component of the response is affected by extracellular Ca^{2+} removal. In contrast to results obtained on amylase release from mouse parotid (Watson, Friedman & Siegel, 1980), we have found no effect of tetracaine on basal or secretogogue-stimulated amylase release. The lack of a tetracaine effect may be due to the high concentrations of secretogogues used.

The structural requirements for potency of substance P fragments and related peptides in stimulating phosphatidylinositol turnover in parotid gland slices (Hanley *et al.*, 1980) and amylase release in parotid gland slices observed in these experiments and in evoking salivation *in vivo* (Hanley *et al.*, 1980) are the same, indicating that the same receptors are involved in mediating all these responses. The concentrations of substance P and related peptides required for half-maximal stimulation of amylase release and phosphatidylinositol turnover are virtually identical, indicating that there are no spare receptors for the amylase release response relative to phosphatidylinositol turnover.

As had been previously reported for the phosphatidylinositol turnover response (Hanley *et al.*, 1980), the C-terminal nonapeptide is capable of eliciting full amylase release, indicating that it is a full agonist at the substance P receptor and that its inability to stimulate a full *in vivo* salivation response must be related to an additional interaction at some other site, possibly blood vessels. Substance P is known to affect blood pressure and circulation (Hanley & Iver-

Table 4 Potencies of substance P and related peptides in stimulating amylase release from rat parotid gland slices and comparison with *in vivo* salivation

Peptide	ED_{50}*	Relative potencies: Amylase release in vitro	Relative potencies: Salivation in vivo**
Substance P	18 nM	1.0	1.0
Substance P free acid	5.5 μM	0.0033	0.0003
C-terminal fragments of substance P			
Decapeptide	68 nM	0.26	0.26
Nonapeptide	59 nM	0.30	0.16††
Octapeptide	66 nM	0.27	0.37
Heptapeptide	132 nM	0.14	0.28
†Hexapeptide	138 nM	0.13	0.40
Pentapeptide	6 μM	0.003	0.003
Tachykinins			
Eledoisin	17 nM	1.1	1.3
Kassinin	150 nM	0.12	0.10
Physalaemin	7 nM	2.6	2.7

* ED_{50}s determined from log-dose response curves; ** Hanley *et al.*, 1980.
† Pyroglutamyl N-terminal form; †† Relative potency in evoking half of its maximum response, but this maximum was less than that evoked by the other peptides.

sen, 1980) and it seems likely that substance P-related peptides may influence blood flow through the salivary glands; particularly because it has been documented that vasoactive peptides (neurotensin, angiotensin II and vasopressin) can produce a dose-dependent decrease in substance P-stimulated salivation in the whole animal but not in isolated parotid cells (Liang & Cascieri, 1979).

Relative potencies of C-terminal fragments and tachykinins: comparison of salivary glands with other tissues

The structure-activity profile for C-terminal substance P fragments in salivary gland responses differs from those for intestinal smooth muscle and hypotension. Certain shorter substance P fragments show enhanced activity compared with substance P in causing hypotension and contraction of intestinal smooth muscle (Bury & Mashford, 1976; Blumberg & Teichberg, 1979), whereas in the salivary gland responses, activity is reduced with progressive shortening of the peptide. The relative potencies of tachykinins have been proposed as a means of discriminating between types of substance P receptor (Erspamer *et al.*, 1980; Lee, 1981). For example, whilst eledoisin is equipotent with substance P in eliciting amylase release from parotid slices, it is about 1/50th as potent as substance P in producing the same response from pancreatic acinar cells (Jensen & Gardner, 1979). It may be that the amylase response to substance P-related peptides is composed of more than one type

Table 5 Peptides that fail to stimulate amylase release in parotid slices

Peptide	Concentration	Amylase release* (units/20 μl slices): Peptide	Basal	
Angiotensin II	10 μM	87.4 ± 2.9	85.2 ± 2.7	NS
Bombesin	10 μM	77.9 ± 2.5	79.6 ± 2.2	NS
Bradykinin	19 μM	119.7 ± 2.7	118.8 ± 2.4	NS
Cholecystokinin-8	10 μM	89.6 ± 2.9	90.1 ± 2.1	NS
Gastric inhibitory polypeptide	9 μM	98.1 ± 2.6	95.4 ± 3.2	NS
β-Lipotropin	1.8 μM	138.6 ± 2.0	135.0 ± 3.5	NS
Luteinising hormone-releasing hormone	17 μM	102.6 ± 0.7	95.4 ± 3.2	NS
Neurotensin	10 μM	77.1 ± 3.0	75ı2 ± 2.5	NS
Somatostatin	10 μM	72.0 ± 3.2	78.7 ± 2.7	NS
Vasopressin	10 μM	66.3 ± 2.4	62.9 ± 2.1	NS

* Means ± s.e.mean ($n = 3$).

of pharmacological receptor. In this regard, Sjodin, Brodin, Nilsson & Conlon (1980) found two populations of ^{125}I-tyr^{8}-substance P binding sites on pancreatic acinar cells.

In summary, the substance P stimulation of amylase release may be a useful *in vitro* system for the study of structural requirements and receptor-linked events for peptide receptors.

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