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SUBSTANCE P IS A FUNCTIONAL NEUROTRANSMITTER IN THE RAT PAROTID GLAND

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SUMMARY

1. The technique of electrical field stimulation was employed to stimulate the intrinsic nerves of isolated rat parotid gland fragments. Responses to field stimulation were recorded as changes in enzyme secretion (amylase release), radiolabelled ion fluxes (^{86}Rb efflux) and electrophysiological effects (changes in acinar cell membrane potential and input resistance). All effects of field stimulation were abolished by the neurotoxin, tetrodotoxin (TTX).

2. Selective use of pharmacological antagonists revealed that both the sympathetic and parasympathetic nerves to this tissue were being excited by field stimulation. Importantly a significant component of the response to field stimulation persisted in the presence of combined autonomic receptor blockade by atropine, phentolamine and propranolol, i.e. due to release of a non-cholinergic, non-adrenergic neurotransmitter.

3. The non-cholinergic, non-adrenergic neurotransmitter evoked amylase release, ^{86}Rb efflux and electrophysiological effects seen as changes in acinar cell membrane potential and conductance, i.e. stimulus–permeability coupled.

4. Two biologically active peptides, substance P (SP) and vasoactive intestinal polypeptide (VIP) were shown to evoke amylase release in the presence of combined autonomic blockade. VIP however did not evoke any increase in ^{86}Rb efflux, i.e. not stimulus–permeability coupled. All the effects of the non-cholinergic, non-adrenergic transmitter were mimicked by substance P which evokes ^{86}Rb efflux and electrophysiological effects in addition to amylase release.

5. The non-cholinergic, non-adrenergic field stimulus effects on amylase release and ^{86}Rb efflux were abolished or markedly attenuated in tissues which had been desensitized by prior exposure to exogenous substance P. In the presence of VIP, however, the non-cholinergic, non-adrenergic effects persisted and were apparently potentiated.

6. Acute application of the neurotoxin capsaicin first stimulated a transient release of amylase and subsequently abolished the non-cholinergic, non-adrenergic field stimulus-evoked enzyme release.

7. The putative substance P antagonist, D-Pro^2, D-Trp^{7,9} substance P, reversibly blocked the response to both non-cholinergic, non-adrenergic nerve stimulation and exogenous substance P. It was demonstrated however that prolonged exposure to this

antagonist is associated with non-reversible and, importantly, non-specific neurotoxic effects.

8. It is concluded that substance P or a closely related peptide is a functional neurotransmitter in the rat parotid gland.

INTRODUCTION

Secretion from salivary glands is initiated and regulated by activity in the sympathetic and parasympathetic nerves which supply the glands, However in the characterization of the mode of action of the autonomic neurotransmitters it is the *in vitro* salivary gland preparation which has been most extensively utilized. Stimulation of these isolated salivary glands could only be achieved by application of exogenous agonists, i.e. pharmacological stimulation. These studies have revealed that the autonomic receptors regulate two separate stimulus-secretion pathways in these tissues (see Peterson, 1980; Butcher & Putney, 1981). Cholinergic (muscarinic) and α-adrenergic receptors regulate a common, calcium-dependent pathway which is associated with changes in the permeability of, and ion fluxes across, the basolateral membrane, i.e. stimulus–permeability coupled. The β-adrenergic receptors regulate a different pathway, one which is associated with activation of the adenylate cyclase/cyclic AMP system, a pathway which does not give rise to the stimulus–permeability effects described above. Such *in vitro* experiments have also revealed that in addition to the autonomic agonists there is a family of closely related peptides, the tachykinins, which are potent stimulators of salivary secretion (see Erspamer, 1981). Of this group of peptides only one, the undecapeptide substance P (SP) is of mammalian origin and it is the effects of this peptide that have been most extensively investigated. Early studies revealed that the effects of this peptide were mediated via interaction with a specific peptide receptor on the salivary acinar cells (Leeman & Hammerschlag, 1967; Rudich & Butcher 1976) and there is now considerable evidence that these peptidergic receptors regulate the same stimulus–permeability pathway as the cholineric and α-adrenergic receptors (Putney, 1977; Gallacher & Petersen 1980*a*). These peptide receptors have been characterized by investigating the responses evoked by application of exogenous substance P and to date no functional role has been reported for the receptors. Recently however the techniques of radioimmunoassay and immunohistochemistry have demonstrated substance P-like immunoreactivity localized within the varicosities of nerves in salivary glands including the rat parotid gland (Hokfelt, Johansson, Kellerth, Ljungdahl, Nilsson, Nygards & Pernow, 1977; Robinson, Schwartz & Costa, 1980; Brodin & Nilsson, 1981). Additionally, Nishiyama, Katoh, Saitoh & Wakui (1980) and Gallacher & Petersen (1980*b*) have demonstrated that the technique of electrical field stimulation is an effective means of stimulating the nerves of the isolated salivary gland preparation. In this study the technique of field stimulation was employed to excite the intrinsic nerves of the isolated rat parotid gland to investigate the role of the intrinsic nerves in activation of acinar cells, in particular to investigate the possibility that a functional non-cholinergic, non-adrenergic innervation exists in this tissue. A preliminary report of this work has been published as an abstract (Gallacher, 1982*a*).

METHODS

Experiments were carried out on 231 isolated parotid glands from 118 adult male rats of 200–250 g in weight. The glands were divided into segments and superfused with a physiological salt solution warmed to 37 °C and gassed with a mixture of 95% O_2 and 5% CO_2. The standard solution had the following composition (mM): NaCl, 103; KCl, 4·7; $CaCl_2$, 2·56; $MgCl_2$, 1·13; $NaHCO_3$, 25; $NaHPO_4$, 1·15; D-glucose, 2·8; Na pyruvate, 4·9; Na glutamate, 4·9, Na fumarate, 2·7.

For electrophysiological recording, segments of the glands were secured to a Perspex platform in a tissue bath (volume, 7 ml.) through which physiological saline flowed at about 15 ml. min^{-1}. Measurements of cell membrane potential and input resistance were carried out using one intracellular micro-electrode for recording and current injection, as previously described (Nishiyama & Petersen, 1974). All micro-electrodes were filled with 3 M-KCl + 10 M-K citrate and had tip resistances of 10–30 MΩ. Electrical field stimulation was achieved via a pair of fine platinum electrodes brought into light contact with the tissue as previously described (Gallacher & Petersen, 1980*b*).

The rate of ^{86}Rb efflux (after Putney, 1976) was monitored from segments of parotid gland, loaded by pre-incubation with ^{86}Rb, 3–5 μCi in 1 ml. physiological saline, for 30 min. After loading the tissue was transferred to a flow chamber (volume 1 ml., flow rate 6 ml. min^{-1}) and after 10 min of superfusion the effluent was collected at 1 min intervals and analysed in a scintillation counter for ^{86}Rb activity. The radioactivity remaining in the tissue at end of each experiment was determined and the activity in the minute samples converted to rate coefficients by computer, (Putney, 1976; Gallacher, 1982*b*).

For measurement of amylase release the parotid segments were placed in a flow chamber identical to that described above but superfused at 1 ml. min^{-1}. The effluent from the flow chamber passed directly to an automated on-line fluorometric assay for continuous measurement of amylase output, (Matthews, Petersen & Williams, 1974). Amylase release is expressed as units min^{-1} 100 mg $tissue^{-1}$ ± S.E. of mean.

Nerve stimulation during the ion flux or amylase release experiments was achieved via a pair of silver electrodes constructed into the flow chamber.

When pharmacological antagonists were used they were included in the superfusing media. Atropine sulphate (Sigma) was used at 10^{-5} M phentolamine mesylate (Ciba) at 10^{-5} M and propanolol hydrochloride (I.C.I.) at 5×10^{-6} M. The neurotoxins tetrodotoxin and capsaicin (Sigma) were used at 10^{-6} M and 10^{-5} M respectively. The substance P analogue D-Pro^2,D-$Trp^{7,9}$ substance P (two sources: Peninsula Laboratories, U.S.A. and Cambridge Research Biomedicals, England) was superfused at 10^{-5} M.

RESULTS

Effects of electrical field stimulation

Field stimulation evoked amylase release from superfused parotid segments at frequencies above 3 Hz. Fig. 1*A* shows the increase in amylase release evoked by three successive stimulations, each at 10 Hz. The amplitude of the field stimulus-evoked response diminished with successive stimulations as did the responses to successive applications of exogenous agonists. Fig. 1*B* demonstrates field stimulus-induced ^{86}Rb efflux from pre-loaded segments of parotid gland. Fig. 2*A* shows the electrophysiological response of the parotid acinar cells to short-duration field stimulation, demonstrating changes in both membrane potential and input resistance (i.e. conductance). The field stimulus-evoked electrophysiological responses are identical to those evoked in this tissue by ionophoretic application of any of the stimulus–permeability agonists (see Fig. 2*B* and Gallacher & Petersen, 1980*a*).

In this as in previous studies (Gallacher & Petersen, 1980*b*) all effects of field stimulation were abolished by the neurotoxin, TTX, at 10^{-6} M; TTX blockade of field stimulus responses was demonstrated for amylase release in eight experiments, ^{86}Rb

efflux in two experiments, and electrophysiological effects in two experiments. TTX blockade was specific for the field stimulus-evoked effects and did not block the tissue response to exogenous agonists as previously reported (Gallacher & Peterson, 1980*b*).

The selective use of pharmacological blockers of autonomic receptors at concentrations which will completely inhibit the effects of saturating concentrations of the

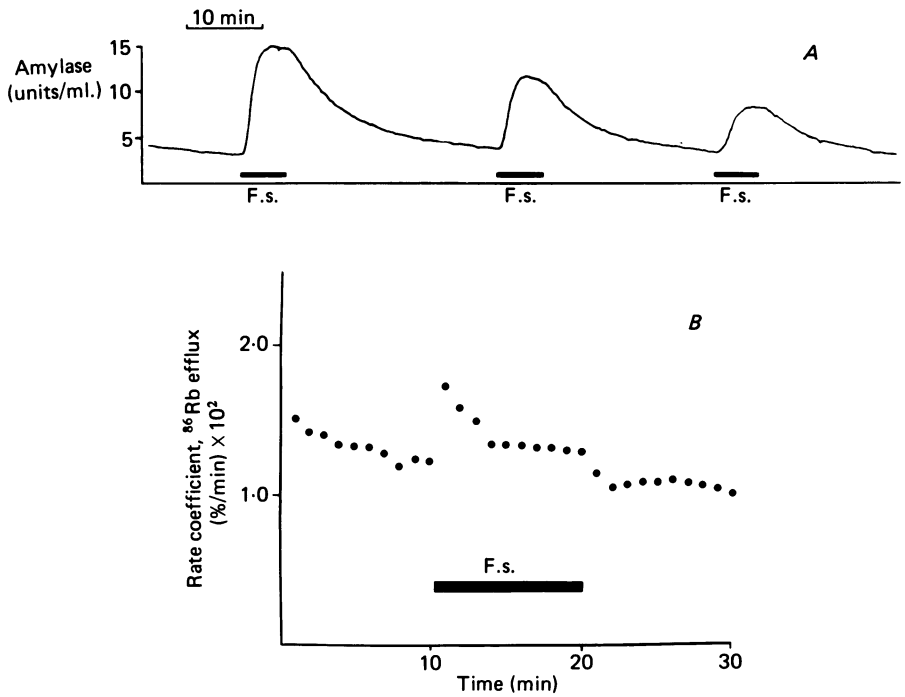

Fig. 1. Effects of electrical field stimulation (F.s.) on amylase release and ^{86}Rb efflux from isolated superfused segments of rat parotid glands. *A*, amylase release evoked by field stimulation. The amylase content of the effluent from the tissue chamber (volume 1 ml.; flow rate 1 ml. min^{-1}) was continually assayed by an automated on-line fluormetric method. The Figure shows three successive field stimulus responses evoked in normal physiological saline. Duration of field stimulation is shown by horizontal bars under the record. The parameters of field stimulation were 10 Hz, 1 msec pulses, 50 V. TTX (10^{-6} M) totally abolished the field stimulus responses in each of eight experiments. *B*, rate of ^{86}Rb efflux from segments of rat parotid gland. The Figure shows the mean ^{86}Rb efflux evoked by field stimulation in normal saline solution ($n = 6$). The horizontal bar indicates the period of field stimulation at 10 Hz, 1 msec, 50 V. (S.E. of mean throughout these experiments never exceeds 10%). In two experiments it was shown that TTX (10^{-6} M) totally abolished the field stimulus responses.

respective agonists in this tissue (Rudich & Butcher, 1976; Gallacher & Petersen, 1980*b*) revealed that both cholinergic and adrenergic mechanisms were involved in the field stimulus-evoked amylase release (Fig. 3). Amylase release in response to field stimulus in control media (i.e. no blockers) was $3{\cdot}69 \pm 0{\cdot}5$ units of amylase min^{-1} 100 mg tissue^{-1} (mean of the first responses evoked in eight experiments). In

the presence of the β-adrenergic blocker propranolol (5×10^{-6} M) the field stimulus-evoked amylase release was reduced to $2{\cdot}01 \pm 0{\cdot}26$ units min^{-1} 100 mg^{-1} ($n = 14$). Importantly, when atropine (10^{-5} M), phentolamine (10^{-5} M) and propranolol (5×10^{-6} M) were superfused in combination, field stimulation still evoked an amylase release of $1{\cdot}72 \pm 0{\cdot}71$ units min^{-1} 100 mg^{-1} ($n = 24$). This field stimulus-evoked

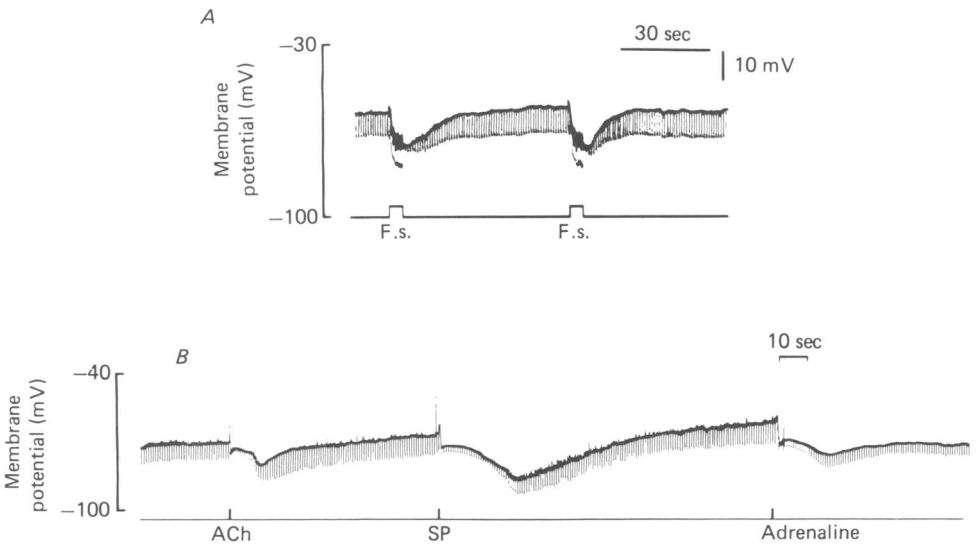

Fig. 2. Recordings of membrane potential and input resistance from acinar cells of isolated rat parotid glands. The electrotonic potential changes superimposed on the recordings of membrane potential are due to the repetitive injection of hyperpolarizing current pulses of constant amplitude through the recording electrode (2 nA, 100 msec, 1 Hz). The amplitude of the evoked electrotonic potentials corresponds to the input resistance of the acinar unit. *A*, the field stimulus response evoked in control media (20 Hz, 1 msec, 30 V). A stimulus artifact is present during field stimulation but it is seen that upon cessation of field stimulation there is a hyperpolarization of the membrane potential associated with a marked reduction in input resistance. Such responses are characteristic of parotid acinar cell activation. *B*, the electrophysiological responses evoked by ionophoresis of the stimulus–permeability coupled agonists acetylcholine (ACh; 30 nA retaining current, 100 nA eject, 1 sec), adrenaline (80 nA retaining current, 500 nA eject, 1 sec) and substance P (SP; 0 retaining current, 100 nA eject, 1 sec).

amylase release in the presence of complete autonomic receptor blockade (Fig. 4*A*) was observed in each of forty-five experiments carried out and indicates that field stimulus was releasing a non-cholinergic, non-adrenergic neurotransmitter. Fig. 4 demonstrates that the field stimulus-evoked ^{86}Rb efflux (Fig. 4*B*) (seen in each of eight experiments) and electrophysiological effects (Fig. 4*C*) (seen in a total of seven cells from three preparations) also persist in the presence of all three autonomic blockers. These experiments demonstrate that the non-cholinergic, non-adrenergic neurotransmitter released by field stimulation activates the stimulus–permeability pathway.

Comparison of non-cholinergic, non-adrenergic field stimulus effects and effects evoked by application of substance P and VIP

The effects of the non-cholinergic, non-adrenergic neurotransmitter were compared with those evoked by application of two biologically active peptides, substance P and VIP. Both substance P and VIP evoked amylase release from the isolated parotid segments in the presence of combined autonomic blockers (Fig. 5). In Fig. 5 (upper

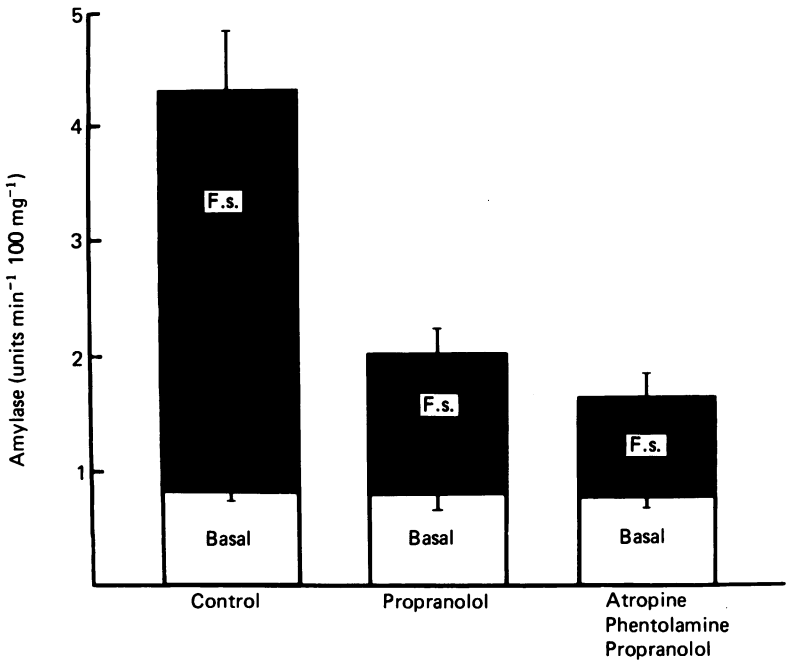

Fig. 3. Comparison of field stimulus-evoked amylase release over basal, (±S.E. of mean); in control media (i.e. no pharmacological antagonists), $n = 8$; in the presence of the β-blocker propranolol (5×10^{-6}), $n = 14$; and in the presence of combined autonomic blockade by atropine (10^{-5} M), phentolamine (10^{-5} M) and propranolol (5×10^{-6} M), $n = 24$. The field stimulus parameters were the same as in Fig. 1 *A*, i.e. 10 Hz, 1 msec, 50 V for 6 min.

record) it is demonstrated that the non-cholinergic, non-adrenergic field stimulus-evoked amylase release could be readily superimposed on the sustained secretion evoked by continued superfusion of 10^{-7} M-VIP ($n = 4$). Superfusion of substance P (5×10^{-9} M) evoked amylase release but in contrast to the VIP-induced secretion the response was characterized by rapid desensitization in the continued presence of the agonist. The non-cholinergic, non-adrenergic field stimulus response was attenuated if field stimulation was applied during the period of desensitization to substance P (Fig. 5, lower record). This record also shows that the tissue response to VIP was not inhibited by prior desensitization to substance P.

VIP failed to evoke ^{86}Rb efflux (two experiments) and the non-cholinergic, non-adrenergic field stimulus-evoked ^{86}Rb efflux persisted in the presence of 10^{-7} M-VIP. Substance P in this study as in others (Rudich & Butcher, 1976) evoked ^{86}Rb efflux from parotid segments and, as observed for amylase release, the response showed a rapid desensitization. If field stimulation was applied during this period

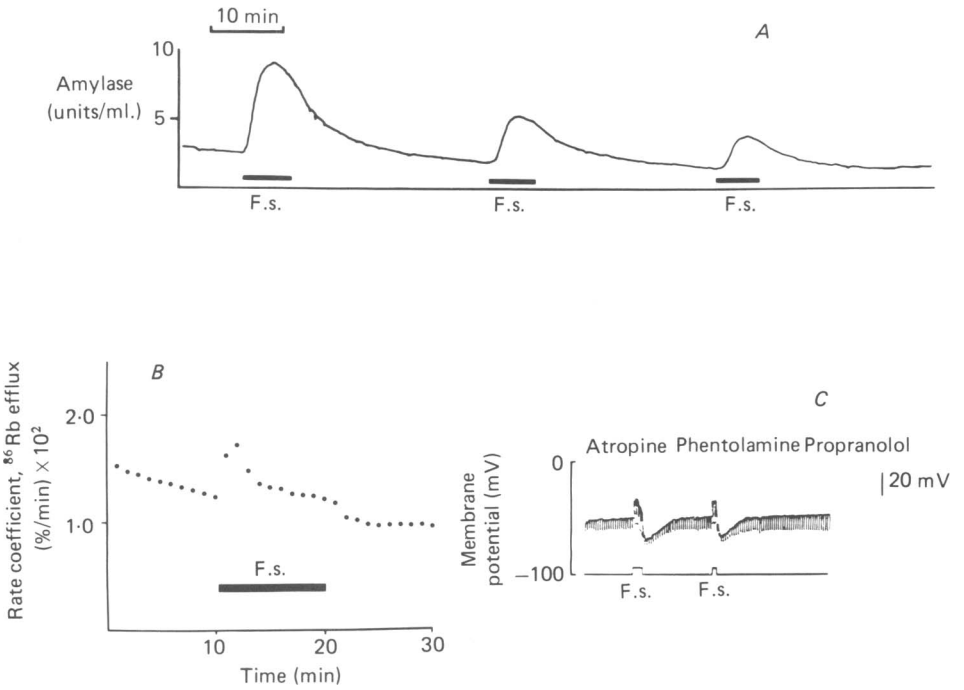

Fig. 4. Non-cholinergic, non-adrenergic field stimulus effects. *A*, amylase release; *B*, ^{86}Rb efflux and *C*, electrophysiological effects. *A*, field stimulus-evoked amylase release in the presence of combined autonomic blockade by atropine (10^{-5} M), phentolamine (10^{-5} M) and propranolol (5×10^{-6} M). The horizontal bars indicate the periods of field stimulation (f.s.), the parameters of field stimulation are identical to those in Fig. 1*A* i.e. 10 Hz, 1 msec, 50 V for 6 min. *B*, field stimulus-evoked ^{86}Rb efflux from rat parotid segments superfused with media containing atropine (10^{-5} M), phentolamine (10^{-5} M) and propranolol (5×10^{-6} M), $n = 7$. S.E. of mean was never greater than 10%. *C*, recording from rat parotid acinus showing the changes in membrane potential and input resistance evoked by field stimulation in the presence of combined autonomic blockade by atropine (10^{-5} M), phentolamine (10^{-5} M) and propranolol (5×10^{-6} M) (20 Hz, 1 msec, 30 V). All the field stimulus responses demonstrated were blocked by application of TTX (10^{-6} M).

of desensitization to substance P there was no increase in ^{86}Rb efflux ($n = 3$). There was then a marked interaction between the non-cholinergic, non-adrenergic field stimulus responses and the substance P-evoked effects. In contrast the VIP-evoked amylase release was apparently mediated by a different mechanism.

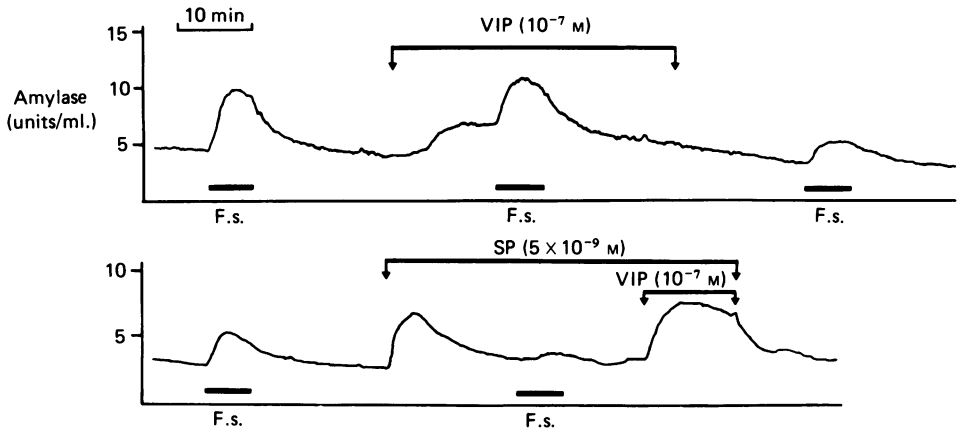

Fig. 5. Interaction between the non-cholinergic, non-adrenergic field stimulus-evoked amylase release and secretory responses to biologically active peptides, vasoactive intestinal polypeptide (VIP) and substance P (SP). The autonomic blockers atropine (10^{-5} M), phentolamine (10^{-5} M) and propranolol (5×10^{-6} M) were present throughout. The parameters of field stimulation were 10 Hz, 1 msec, 50 V, duration indicated by the horizontal bars. In the upper record it is seen that VIP can evoke amylase secretion but the field stimulus (f.s.) response can be superimposed upon this secretion. This record is representative of four such experiments. In the lower record it is seen that substance P (5×10^{-9} M) evokes amylase secretion, but in the continued presence of the agonist desensitization develops and field stimulation during this period evokes only a small response. VIP applied during this refractory period evokes a marked secretion of amylase. This record is one of two such experiments.

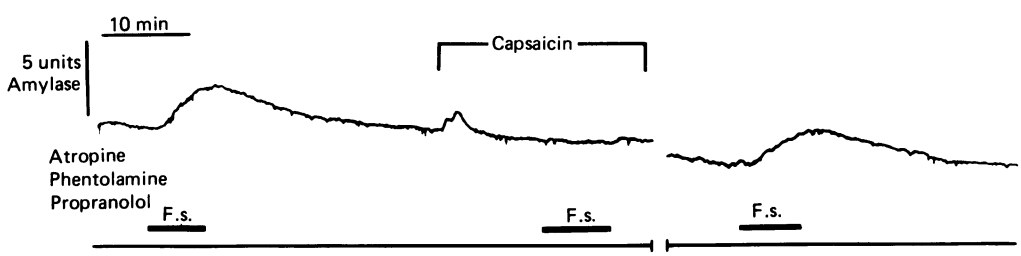

Fig. 6. Field stimulus-evoked amylase release in the presence of combined autonomic blockade by atropine (10^{-5} M), phentolamine (10^{-5} M) and propranolol (5×10^{-6} M). Field stimulation (10 Hz, 1 msec, 50 V) is indicated by the horizontal bars. Capsaicin was superfused (10^{-5} M) for the period indicated. Capsaicin evokes a small transient release of amylase and subsequently blocks the field stimulus response. The effects of capsaicin are reversible and a field stimulus response is evoked 20 min after removal of capsaicin.

Effects of capsaicin and a substance P antagonist on the non-cholinergic, non-adrenergic field stimulus responses

The experiments detailed above demonstrate that the non-cholinergic, non-adrenergic field stimulus effects are mimicked by application of the peptide substance P. Capsaicin, an extract of red peppers, is a neurotoxin that has been shown to release and deplete populations of small afferent neurones of substance P (Jessel, Iversen &

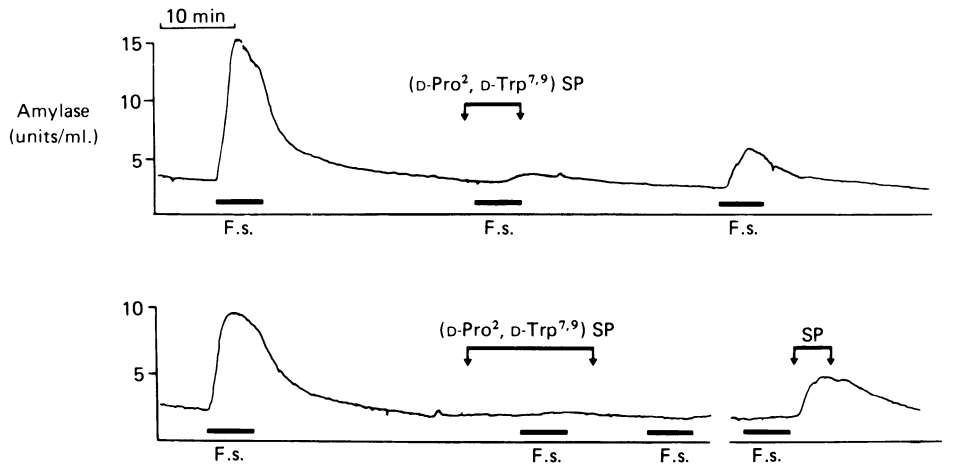

Fig. 7. Two records showing the effect of the substance P antagonist, D-Pro²,D-Trp⁷,⁹ SP (10^{-5} M) on the non-cholinergic, non-adrenergic field stimulus response. The autonomic blockers atropine (10^{-5} M), phentolamine (10^{-5} M) and propranolol (5×10^{-6} M) are present throughout. The upper record shows that a short exposure (6–8 min) produces a reversible blockade of the non-cholinergic, non-adrenergic field stimulus response. The record is representative of seven such experiments. The lower record reveals that a prolonged exposure to the substance P antagonist produces a blockade that is not reversible. The gap in this record represents a 16 min wash period. This non-reversible blockade is not due to the persistence of receptor antagonism because application of exogenous substance P (SP) (5×10^{-9} M) evokes a response at a time when the response to field stimulation is still abolished. (Representative of four such experiments).

Cuello, 1978; Gamse, Holzer & Lembeck, 1980). In this study capsaicin was applied by inclusion in the superfusing media at 10^{-5} M. In each of seven experiments application of capsaicin was associated with a small transient increase in amylase release. Following this the non-cholinergic, non-adrenergic field stimulus response was abolished if superfusion of the neurotoxin was maintained. In a number of experiments the non-cholinergic, non-adrenergic field stimulus response could be recovered, but only after prolonged wash-out of capsaicin (Fig. 6).

Recently Folkers and his colleagues have described a series of substance P analogues which have been found to block peripheral effects of substance P in a specific and reversible manner (Folkers, Horig, Rosell & Bjorkroth, 1981; Engberg, Svensson, Rosell & Folkers, 1981; Leander, Hakanson, Rosell, Folkers, Sundlers & Torqvist, 1981). In this study one of these analogues, D-Pro²,D-Trp⁷,⁹ substance P

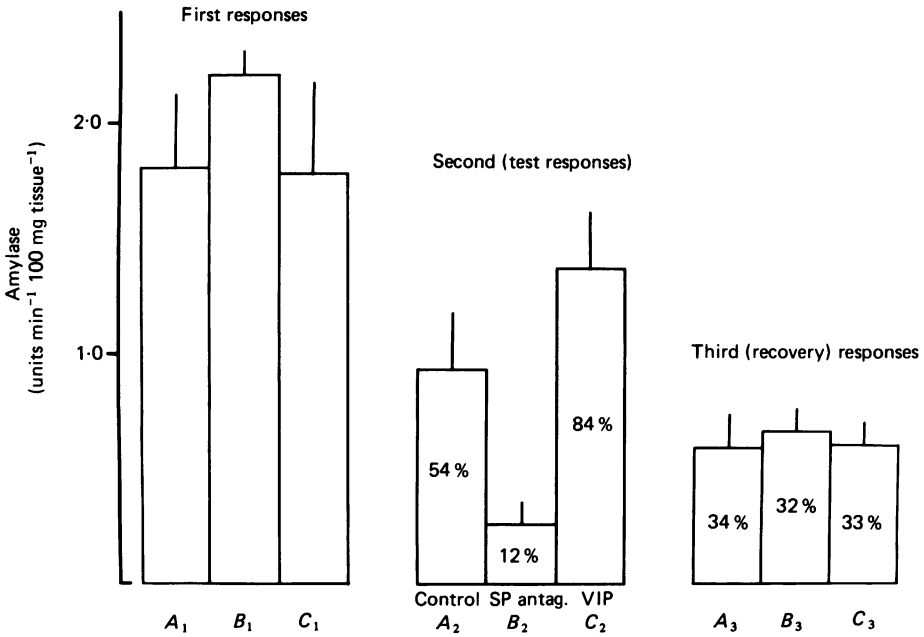

Fig. 8. Field stimulus-induced amylase release. The autonomic blockers are present throughout, i.e. non-cholinergic, non-adrenergic nerve stimulation. This Figure presents the quantitative data (±S.E. of means) from three series of experiments of which Figs. 4*A* and 5 are representative. The protocols are as shown in the previous Figures. In series *A* ($n = 5$) three successive field stimulus responses are evoked (A_1, A_2 and A_3) as controls. In series *B* ($n = 7$) the substance P analogue (D-Pro^2,D-Trp^{7,9} SP) is superfused (10^{-5} M) during the second field stimulus response (B_2); the third field stimulus response (B_3) after removal of the antagonist indicates by comparison with A_3 the degree of reversibility of the blockade. In series *C* ($n = 4$) VIP (10^{-7} M) was superfused during the second field stimulus period (C_2); a third response was evoked after removal of the VIP (C_3). The parameters of field stimulation were identical in each case: 10 Hz, 1 msec pulses, 50 V for 6 min. The percentages shown in the bars express the second and third responses as a percentage of the first responses in that series of experiments. There is no significant difference between the first responses (A_1, B_1, C_1) in each series, or between the third response (A_3, B_3, C_3). The reduction in amylase release in the presence of the substance P antagonist (B_2) is statistically significant when compared to the control second responses (A_2) (Student's *t* test) either in quantitative terms ($P < 0{\cdot}05$) or expressed as percentages of the respective first responses ($P < 0{\cdot}001$). Comparison of A_2 and C_2 shows that superfusion of VIP during field stimulation potentiates the non-cholinergic, non-adrenergic field stimulus response.

was tested on the non-cholinergic, non-adrenergic field stimulus response. The antagonist at 10^{-5} M reversibly abolished the response to application of exogenous substance P at concentrations up to 10^{-8} M, the highest concentration tested. It did not interfere with the responses to application of either VIP or acetylcholine, indicating that it does not act by a general blockade of secretory mechanisms. Fig. 7 (upper record) shows the effect of the substance P antagonist on the non-cholinergic,

non-adrenergic field stimulus response. A short exposure to the antagonist (6–8 min) inhibited the non-cholinergic, non-adrenergic field stimulus-evoked amylase release in a manner that was readily reversible (seven experiments). It became apparent however that more prolonged exposure to the antagonist resulted in a blockade of the non-cholinergic, non-adrenergic field stimulus responses that was not, or only poorly, reversible (four experiments). This non-reversibility was not due to sustained blockade of the peptide receptor because it persisted at a time when superfusion of 5×10^{-9} M-substance P evoked amylase release (Fig. 7, lower record). Importantly this blockade was not specific for the non-cholinergic, non-adrenergic field stimulus effects, since prolonged exposure to the antagonist (12–20 min) markedly reduced, in a non-reversible manner, the field stimulus response when the only autonomic blocker present was propranolol ($0{\cdot}56 \pm 0{\cdot}1$ units amylase min^{-1} 100 mg^{-1} in the presence of the substance P antagonist, compared with $2{\cdot}01 \pm 0{\cdot}26$ units amylase min^{-1} 100 mg $tissue^{-1}$ in controls). In the light of these non-specific effects it was necessary to adopt a protocol of short exposure to the antagonist and it was superfused only for the duration of field stimulation (6–8 min). The field stimulus responses were not totally abolished in most of these experiments but Fig. 8 presents the quantitative data from seven such experiments, comparing them with control experiments; these demonstrated that the D-Pro^2,D-Trp^{7,9} substance P analogue used in such a protocol achieved an inhibition of the non-cholinergic, non-adrenergic field stimulus-evoked amylase release that was statistically significant and that was totally reversible as compared to control experiments. The non-cholinergic, non-adrenergic field stimulus response in the presence of the substance P antagonist (B_2 in Fig. 8) was $0{\cdot}25 \pm 0{\cdot}09$ units amylase min^{-1} 100 mg^{-1}. In an identical protocol but with propranolol the only autonomic blocker the field stimulus response in the presence of the substance P antagonist was $1{\cdot}1 \pm 0{\cdot}14$ units 100 mg ($n = 4$, $P < 0{\cdot}01$). This indicates that not only were the effects of brief exposure to the antagonist reversible, but specific for the non-cholinergic, non-adrenergic field stimulus responses.

DISCUSSION

Nishiyama *et al.* (1980) and Gallacher & Petersen (1980*b*) reported that the technique of electrical field stimulation was an effective means of stimulating the intrinsic nerves of the isolated salivary glands of the mouse. Both these studies described only electrophysiological responses evoked in salivary acinar cells in response to field stimulation. Gallacher & Petersen (1980*b*) reported that the electrophysiological responses in the mouse parotid gland were completely abolished by atropine, i.e. mediated by acetylcholine. In the present study we have utilized the technique of field stimulation, but extended the range of experimentation to investigate the effects of nerve stimulation in the isolated rat parotid gland on amylase release, and ^{86}Rb efflux in addition to electrophysiological effects. Figs. 1 and 2*A* demonstrate that field stimulation evokes amylase release, ^{86}Rb efflux, and electrophysiological responses. Such responses have formerly only been demonstrated in the isolated rat parotid gland following application of exogenous agonists. Additionally the sequential inclusion of pharmacological antagonists of autonomic

receptors in the superfusion media revealed that a component of the field stimulus response was susceptible to blockade by propranolol, i.e. mediated via β-adreno-receptor mechanisms. This demonstrates for the first time that field stimulation excites both the parasympathetic and sympathetic components of the autonomic nerves. Importantly the study revealed that a component of the field stimulus response was resistant to combined blockade of cholinergic, α-adrenergic and β-adrenergic receptors, i.e. due to release of a non-cholinergic, non-adrenergic neuro-transmitter. The non-cholinergic, non-adrenergic transmitter released by field stimulation is shown to evoke amylase release and ^{86}Rb efflux and is associated with changes in membrane potential and conductance, i.e. stimulus–permeability coupled.

Recent immunohistochemical studies have identified two biologically active peptides that are located in nerve terminals within salivary glands. These are substance P (Hokfelt *et al.* 1977; Robinson & Schwartz, 1980), and VIP (Lundberg, Anggard, Fahrenkrug, Hokfelt & Mutt, 1980). Specific binding of ^{125}I-labelled substance P has been demonstrated in rat parotid acinar cells (Liang & Cascieri, 1980) and these substance P receptors have been characterized *in vitro* by application of exogenous substance P. Activation of substance P receptors on parotid acinar cells is characteristically associated with ion fluxes, amylase release (Rudich & Butcher, 1976) and membrane conductance changes (Gallacher & Petersen, 1980*a*), i.e. stimulus–permeability coupled. While VIP has been shown to be released on stimulation of parasympathetic nerves to the cat submandibular gland it has been reported that VIP does not cause secretion from acinar cells, but is most probably associated with the atropine-resistant vasodilation observed in this tissue (Lundberg *et al.* 1980). However this present study has demonstrated that in rat parotid gland VIP does evoke secretion of amylase, most probably via a specific receptor on parotid acinar cells (Fig. 4). Both substance P or VIP could then be the transmitter mediating the non-cholinergic, non-adrenergic field stimulus-evoked release of amylase.

During preparation of this paper a report has appeared (Inoue & Kanno, 1982) which also describes VIP-evoked amylase release from the rat parotid gland. These authors report that 10^{-7} M was the saturating concentration for VIP-evoked amylase release. In the present study VIP (10^{-7} M) failed to evoke ^{86}Rb efflux and did not inhibit the non-cholinergic, non-adrenergic field stimulus-induced ^{86}Rb efflux. Also the non-cholinergic, non-adrenergic field stimulus-evoked amylase release could be superimposed upon the VIP-evoked secretion even at 10^{-7} M-VIP. However substance P at 5×10^{-9} M (saturating concentrations of this peptide for amylase release in rat parotid are reported as 10^{-7}–10^{-6} M: Brown & Hanley, 1981) did mimic all the effects of non-cholinergic, non-adrenergic field stimulation, including the ^{86}Rb efflux, and there was a notable interaction between the non-cholinergic, non-adrenergic field stimulus responses and the response to exogenous substance P. Superfusion of substance P resulted in a marked secretion of amylase and ^{86}Rb efflux, but in the continued presence of the agonist these effects rapidly returned to basal levels i.e. desensitization. Field stimulation during this period of desensitization was associated with a much attenuated response. The VIP-evoked amylase release was unaffected by this refractoriness to substance P (Fig. 2).

While this study has demonstrated a direct VIP-induced enzyme secretion this peptide cannot mimic the membrane permeability increase evoked by the non-

cholinergic, non-adrenergic neurotransmitter released by field stimulation. The observations reported above suggest that VIP activates a secretory mechanism different from that associated with permeability effects. Such a system, the adenylate cyclase/cyclic AMP system, has been demonstrated in this tissue but was formerly considered to be regulated only by β-adrenoreceptors (see Butcher & Putney, 1980). It has recently been reported (Fredholm & Lundberg, 1982) that VIP can cause an increase in cycle AMP levels in the cat submandibular gland. As demonstrated in Fig. 8 there is an apparent potentiation of the non-cholinergic, non-adrenergic field stimulus response in the presence of VIP, and a similar potentiating effect of VIP on cholinergic activation has been reported (Lundberg, Hedlund & Bartfai, 1982). Substance P and acetylcholine activate the same intracellular (calcium-dependent) secretory pathway and it is most likely that the potentiating effect of VIP for both these agonists is exerted at this intracellular level. It is clear that VIP is not the non-cholinergic, non-adrenergic neurotransmitter released by field stimulation.

The study has then demonstrated that field stimulation of the rat parotid gland is associated with release of a non-cholinergic, non-adrenergic neurotransmitter in addition to the autonomic neurotransmitters acetylcholine and noradrenaline. This non-cholinergic, non-adrenergic transmitter activates the stimulus–permeability pathway in parotid acinar cells. These effects of the non-cholinergic, non-adrenergic transmitter are mimicked by the peptide substance P but not by VIP. Since it has been demonstrated that substance P is contained within the varicosities of nerves in salivary glands, and since the presence of a substance P receptor on parotid acinar cells has been demonstrated, it seems reasonable to assume that substance P could be the non-cholinergic, non-adrenergic transmitter released by field stimulation. The marked interaction between the non-cholinergic, non-adrenergic field stimulus responses and those evoked by exogenous substance P is indirect evidence that substance P is the non-cholinergic, non-adrenergic transmitter. However Davis, Oleander, Maury & McDaniels (1980) have reported that sustained activation of any one of the stimulus–permeability coupled receptors, i.e. cholinergic, α-adrenergic and peptidergic in this tissue, can induce a general refractoriness in the secretory mechanism to all stimulus–permeability coupled agonists. The interaction between substance P and the non-cholinergic, non-adrenergic field stimulus response could then be interpreted as demonstrating only that the non-cholinergic, non-adrenergic transmitter activates the same stimulus–permeability pathway as substance P and could in fact be a different transmitter. There are no other non-cholinergic, non-adrenergic stimulus–permeability coupled agonists known to be effective in this tissue. While it has been reported that ATP is a stimulus–permeability coupled agonist in the mouse parotide gland, acting via a P_2 purinergic receptor the nucleotide is ineffective in the rat parotid gland (Gallacher, 1982*b*). In the present study the effects of histamine were tested and histamine (10^{-4} M) was without effect on either amylase release or ^{86}Rb efflux.

At the present time the pharmacological tools available for the identification of non-cholinergic, non-adrenergic neurotransmitters are limited. In a number of tissues where substance P is considered to be a putative neurotransmitter the chemical capsaicin has been employed in the identification of the peptide (Ueda, Maramatsu, Sakakibara & Fujiwara, 1981). Capsaicin is an extract of red peppers which has been

shown to release and deplete substance P from populations of small afferent neurones (Jessel *et al.* 1978; Gamse *et al.* 1980) and to inhibit axoplasmic transport of the peptide (Gamse, Petsche, Lembeck & Jansco, 1982). In this study capsaicin was applied by inclusion in the superfusing media at 10^{-5} M in the presence of the three autonomic blockers. In each case application of capsaicin was associated with what was, by comparison to the non-cholinergic, non-adrenergic field stimulus response (Fig. 6), a small transient increase in amylase release. Following this the responses to non-cholinergic, non-adrenergic field stimulation were totally abolished if superfusion of capsaicin was maintained. The effects of capsaicin were often reversible but only after prolonged wash-out of the drug (Fig. 6). Capsaicin did not abolish control field stimulus responses. It is now recognized that the effects of capsaicin are not specific for substance P-containing neurones (Gamse, Leeman, Holzer & Freedman, 1981) and the unequivocal identification of substance P as the neurotransmitter mediating non-cholinergic, non-adrenergic responses in a tissue requires the demonstration of blockade of these effects by a specific antagonist of substance P receptors.

Recently Folkers and his co-workers (Folkers *et al.* 1981; Engberg *et al.* 1981; Leander *et al.* 1981) have described various synthetic analogues of substance P which are reported to block the effects of substance P in peripheral tissues specifically and reversibly. In this present study one of the most effective of these analogues, D-Pro^2,D-Trp^{7,9} substance P, was tested on the non-cholinergic, non-adrenergic field stimulus responses. This analogue reversibly abolished the responses to application of exogenous substance P at concentrations up to 10^{-8} M, the highest concentration tested. It did not interfere with the tissue responses to either acetylcholine or VIP, indicating that it does not act by general, non-specific blockade of secretory mechanisms. In the protocol that was adopted throughout this study (see upper record in Fig. 7) it was possible to demonstrate that this analogue specifically and reversibly antagonized (Fig. 8) the non-cholinergic, non-adrenergic field stimulus effects in a manner that was statistically significant as compared with control experiments. It became apparent however that prolonged exposure to this antagonist (10–20 min) resulted in blockade of the non-cholinergic, non-adrenergic field stimulus responses that was not, or only poorly, reversible (see lower record in Fig. 7). The non-reversibility was not due to sustained blockade of the peptide receptor, because the field stimulus response was still abolished at a time when substance P applied by superfusion evoked a marked amylase release. This effect of the peptide must then be due to some presynaptic blockade of nerve stimulation, perhaps due to some neurotoxic effect similar to that of capsaicin. Importantly this neurotoxic effect of the substance P analogue, unlike the capsaicin blockade, was not specific for non-cholinergic, non-adrenergic field stimulus effects, as it markedly inhibited the field stimulus response when the only other antagonist present was propranolol. Such rapidly developing non-specific effects have not been reported previously in peripheral nerves, but a recent study (Hokfelt, Vincent, Hellsten, Rosell, Folkers, Markey, Goldstein & Cuello, 1981) reports an apparent neurotoxic effect of this analogue in the brain 24 hr after intraventricular injection. When this analogue of substance P is employed in the identification of substance P as a transmitter released by stimulation of nerves it is then necessary to carry out control experiments to demonstrate the specificity and reversibility of the antagonist. Such control experi-

ments were carried out in this study and it was demonstrated that by adopting a protocol of short exposure to the antagonist its effects were specific and fully reversible as compared to the control experiments.

The data presented above demonstrated that there is a non-cholinergic, non-adrenergic innervation to the rat parotid gland. The non-cholinergic, non-adrenergic neurotransmitter activates amylase secretion and evokes a marked increase in acinar cell membrane permeability to potassium. While it is not possible to measure fluid secretion in this preparation it is of interest to note that Thulin (1976) reported that the secretory response to stimulation of the parasympathetic nerves to rat parotid gland *in vivo* often persisted after intraperitoneal injection of atropine. It is possible that what Thulin reported was fluid secretion due to non-cholinergic, non-adrenergic neurotransmitter release. It is shown that VIP can evoke amylase release but it cannot mimic the non-cholinergic, non-adrenergic neurotransmitter in its ability to activate the membrane permeability mechanism. The effects of the non-cholinergic, non-adrenergic transmitter are however mimicked in all respects by the peptide substance P. The drugs available for the identification of substance P as a neurotransmitter are not ideal; they have however been utilized in this study in conjunction with a series of control experiments and it is shown that the D-Pro^{7},D-Trp^{7,9} analogue of substance P (a putative substance P receptor antagonist) reversibly antagonizes the non-cholinergic, non-adrenergic field stimulus effects. On the basis of the several different lines of evidence presented in this study it is concluded that substance P, or a closely related peptide, is a functional neurotransmitter in the rat parotid gland.

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