Differential secretion of proteins by rat submandibular acini and granular ducts on graded autonomic nerve stimulations

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1. The influence of graded parasympathetic and sympathetic nerve stimulations on the secretion of protein from rat submandibular gland was studied. Peroxidase was used as a marker for the acini and rat tissue kallikrein (official nomenclature rK1) as the marker for granular ducts. Tonin (rK2) was also measured, and the ratio of rK2 : rK1 was calculated as an indication of the cellular route of secretion.

2. Continuous parasympathetic nerve stimulation caused a copious flow of saliva that had a low protein content. The secretion of peroxidase (acini) showed a gradual moderate increase as the frequency increased. However, the concentrations of rK1 and rK2 (granular ducts) showed little change throughout, and the ratio of rK2 : rK1 remained relatively constant.

3. Graded sympathetic stimulation was applied against a background of parasympathetic stimulation. Secretion of peroxidase was increased by the addition of 0·1 Hz continuous sympathetic stimulation. The amount increased thereafter up to 2 Hz, but showed no further increase if the stimulation was applied as bursts of 10 or 20 Hz. In comparison, the secretion of proteinase activity showed little change with superimposed continuous sympathetic stimulation, and the rK2 : rK1 ratio was similar to that in saliva produced by parasympathetic stimulation alone. Sympathetic stimulation applied in bursts, however, caused a large increase in the secretion of proteinase activity, and with 20 Hz burst stimulation the rK2 : rK1 ratio was indistinguishable from that of sympathetic saliva *per se*. There was an augmented secretion of both peroxidase and kallikrein when 20 Hz burst stimulation was combined with parasympathetic stimulation. The effects of sympathetic stimulation were abolished by α- and β-adrenoceptor blockade.

4. It is concluded that parasympathetic stimulation causes a moderate increase in the concentration and output of acinar peroxidase with increasing stimulation rates. On the other hand, the concentration of kallikreins secreted from the granular ducts did not change significantly. Since the rK2 : rK1 ratio remained significantly different from that in saliva produced by sympathetic stimulation alone, which is attributable to exocytotic release of secretory granules, it is likely that parasympathetic secretion of kallikreins was by vesicular (constitutive) transport. Sympathetic impulses provide a much greater drive for protein secretion by both acini and granular ducts, but increased secretion from the latter required a much higher frequency of impulse formation. These data imply that complex central integration is required to induce granule secretion from these ducts.

Several studies have assessed protein and fluid secretion from rat submandibular glands in response to sympathetic and parasympathetic nerve stimulations *in vivo* (Matthews, 1974; Abe & Dawes, 1978; Garrett, Suleiman, Anderson & Proctor, 1991), and to autonomimetic agonists *in vivo* and *in vitro* (Abe & Dawes, 1978; Bogart & Picarelli, 1978). In general terms, sympathetic electrical stimulation of nerves evokes a secretion of saliva low in volume but rich in protein, and causes an extensive degranulation of both acinar and granular duct cells. On the other hand, parasympathetic electrical stimulation of nerves induces a copious flow of saliva low in protein. Although greater flow rates are achieved than during sympathetic nerve activation, Garrett *et al.* (1991) found no morphological evidence that 10 Hz parasympathetic stimulation for 1 h caused any acinar or granular duct degranulation.

The secretion of protein from the granular ducts is generally considered to be an α-adrenergic response, whereas that from the acini involves β-adrenergic receptor activation (Abe & Dawes, 1978; Iwabuchi, Aoki & Matsuhara, 1985). Despite this seemingly orderly compartmentalization of autonomic function, several observations raise questions about the neural regulation of protein secretion in the rat submandibular gland. Rigby & Templeton (1984) found that very low frequency sympathetic nerve impulses (0·1–0·8 Hz) superimposed on a background of parasympathetic stimulation led to a significant increase in protein release. They assumed, but did not establish, that this protein originated from the acinar cells. Abe, Yoneda, Fujita, Yokota & Dawes (1980) reported that low doses of α-adrenergic agonists initially caused a 'β-type' response by the glands, as judged by the electrophoretic pattern of proteins secreted into the saliva, whereas much higher doses were required to induce the typical 'α-type' response. Jones & Wilson (1985), using more physiological methods, provided additional evidence for the apparent dose dependence of adrenergic secretory responses. A 'β-type' response to sympathetic nerve stimulation was observed at 5 Hz, but an 'α-type' response occurred when the stimulation was at 20 Hz. Finally, Shori, Proctor, Chao, Chan & Garrett (1992) observed that the specific kallikrein-related proteinases secreted from the granular ducts depend on the type of nerve stimulation applied. The ratios of different proteinases secreted from granular ducts into saliva during sympathetic stimulation (50 Hz in bursts of 1 s every 10 s) were similar to those in glandular extracts, whereas in saliva evoked by parasympathetic stimulation (10 Hz continuously) there was proportionally more tissue kallikrein (rK1; Berg *et al.* 1992) and less tonin (rK2). The kallikreins in parasympathetic saliva were thought to arise from a vesicular (constitutive) route, rather than from secretory granule release as occurs during sympathetic stimulation (Shori *et al.* 1992). Thus, the granular duct cells appear to be capable of a diverse set of responses to autonomic nerve stimulation.

These earlier findings demonstrate that a simple sympathetic *vs.* parasympathetic dichotomy is an inadequate paradigm for the neural regulation of submandibular secretion in the rat. The purpose of the present investigation has been to examine more thoroughly the protein secretory responses by each of the two main types of secretory cells in rat submandibular glands to graded stimulation of the autonomic nerve supply. Peroxidase was used as a marker of acinar cell secretion. Anderson (1986) exploited the use of peroxidase as a marker for protein secretion from submandibular acinar cells *in vitro* and found that some secretion occurred in response to the cholinergic agonist carbachol, albeit less than with β-adrenergic stimulation. Recently, Proctor & Chan (1994) showed that there is a secretion of acinar peroxidase in response to parasympathetic nerve stimulation, but that the output was far less than with sympathetic nerve stimulation. Kallikrein activity was assessed to monitor secretion from the granular ducts, and the rK2 : rK1 ratio was taken as an indicator of the cellular pathway involved (Shori *et al.* 1992). Graded sympathetic stimulations were superimposed on a steady background of parasympathetic impulses, in order to generate sufficient volumes of saliva for analysis at the lower rates of sympathetic stimulation.

METHODS

Animals

Male Wistar rats ($n = 13$) initially weighing 350–500 g were used for these studies. All animals were maintained on a 12 h light–dark cycle, and allowed free access to food and water.

Stimulation of salivary secretion

The animals were fasted overnight, and anaesthesia was induced with pentobarbitone (35 mg kg^{-1} I.P.) followed by chloralose via the femoral vein (75 mg kg^{-1} I.V.). The trachea was cannulated with a polythene tube, and the right submandibular duct was cannulated with a fine glass cannula. Body temperature was recorded by means of a rectal thermometer and maintained between 37 and 38 °C.

For graded sympathetic stimulations, the right sympathetic nerve trunk was exposed and sectioned caudal to the superior cervical ganglion. The peripheral stump was placed in a bipolar electrode for stimulation at frequencies of 0·1–2 Hz (4–6 V, 2 ms duration) continuously, or 10 and 20 Hz in bursts of 1 s every 10 s (as in Anderson, Garrett & Proctor, 1988). To ensure an adequate salivary flow, sympathetic stimulation of the submandibular gland was carried out against a background of continuous parasympathetic nerve impulses (4 Hz at 4–6 V, 2 ms duration). For both background and graded (0·5–40 Hz) parasympathetic stimulations, the lingual nerve was exposed and carefully reflected onto the cannulated submandibular duct. Both the duct and nerve were then placed on a bipolar electrode. The effects of the α- and β-adrenergic blockers, dihydroergotamine (1 mg kg^{-1} I.V.; Sandoz, East Hanover, NJ, USA) and propranolol (1 mg kg^{-1} I.V.; ICI, Wilmington, DE, USA) on sympathetic responses were tested in three animals.

Salivary flow was recorded for 5 min at each frequency, and each stimulation period was followed by a short rest period (1 min) while the collection tubes were changed. The initial drop of saliva formed at each frequency was discarded. Salivary flow was determined by observing and recording the number and rate of drops falling from the cannula, and by collection of the saliva into preweighed vials, cooled by ice, which were then reweighed (1 g of saliva was taken to be equivalent to 1 ml). To compensate for variability in submandibular gland weights, flow rates were expressed as microlitres per gram gland wet weight per minute.

Biochemical analyses

The protein content of each saliva sample was estimated by measuring absorbancy at 215 nm (Arneberg, 1971) using a human albumin/globulin standard.

Peroxidase activity was measured as described by Proctor & Chan (1994). One hundred microlitres of a stock solution of 5×10^{-5} M diacetyldichlorofluorescin in ethanol was activated to dichloro-

fluorescin by adding 900 μl of 0·01 M NaOH and leaving at room temperature for 30 min. The reaction was stopped by the addition of 1 ml of 67 mM phosphate buffer (pH 6·0) and the solution was stored on ice. Peroxidase activity was measured at 37 °C by adding 20 μl of diluted sample to 1 ml of phosphate buffer containing 1 mM potassium thiocyanate (freshly prepared). After the addition of 10 μl of hydrogen peroxide (1:400 dilution of a 30% stock solution), the assay was immediately activated by adding 100 μl of dichlorofluoroscin. After a 4 min incubation, the assay was stopped by the addition of 500 μl of NaOH and the fluorescence of dichlorofluorescein (DCF) was measured at 488 nm excitation and 530 nm emission in a luminescence spectrometer. Peroxidase activity and output were expressed as nanomoles DCF generated per minute per millilitre saliva and picomoles DCF per minute per gram gland wet weight per minute of salivary flow, respectively.

Tissue kallikrein (rK1) activity was assayed as described by Shori *et al.* (1992) using the synthetic peptide substrate D-Val-Leu-Arg-7-amino-4-trifluoromethylcoumarin (AFC) in the presence of 0·2 mg ml^{-1} soyabean trypsin inhibitor (SBTI). Under these conditions the activities of all other submandibular kallikrein-like proteinases are totally inhibited. Tonin (rK2) activity was measured in a similar manner using the substrate, z-Val-Lys-Lys-Arg-AFC in the presence of aprotinin (1 μM). For both assays, 20 μl of diluted sample were added to 1·95 ml of 20 mM Tris–HCl (pH 8·0) at 30 °C, containing 100 mg ml^{-1} bovine serum albumin. Twenty microlitres of SBTI (20 mg ml^{-1}) or aprotinin (100 μM) were added to the appropriate tubes and the mixture was incubated for 5 min. Twenty microlitres of substrate (1 mM) were then added and, after 10 min, fluorescence of the cleaved AFC was measured at 405 nm excitation and 505 nm emission in a luminescence spectrometer. Kallikrein activity was expressed as micromoles AFC (rK1) or nanomoles AFC (rK2) produced per minute per millilitre of saliva by comparison with AFC standards. The rK2 : rK1 ratio for these amounts was used to provide further information on the type of saliva secreted. Kallikrein output was expressed as nanomoles AFC (rK1) or picomoles AFC (rK2) per minute per gram gland wet weight per minute of salivary flow.

Statistical analysis

Values were expressed as means ± S.E.M., and initial statistical analyses were carried out using a repeated-measure ANOVA. Differences between individual means were then analysed for statistical significance using Student's *t* test (Bonferroni correction). Differences between means were considered to be significant at the level of $P < 0·05$.

RESULTS

Effects of graded parasympathetic stimulation

In five animals stepwise changes in parasympathetic stimulation frequency (from 1 to 10 Hz) resulted in corresponding increases in the rate of submandibular salivary flow (Fig. 1*A*). When the pattern of stimulation was reversed, i.e. from 10 to 1 Hz, flow rates were virtually identical to those observed at the same frequencies when the rate of stimulation was being increased (data not shown). Maximum salivary flow rate was achieved at 10 Hz, and no significant changes in flow rate were observed when stimulation was increased to 20 and 40 Hz.

The secretion of peroxidase (pmol DCF (g tissue)$^{-1}$ (min flow)$^{-1}$) increased with increasing stimulation frequency up to 10 Hz (Fig. 2), and the output of peroxidase at 20 and

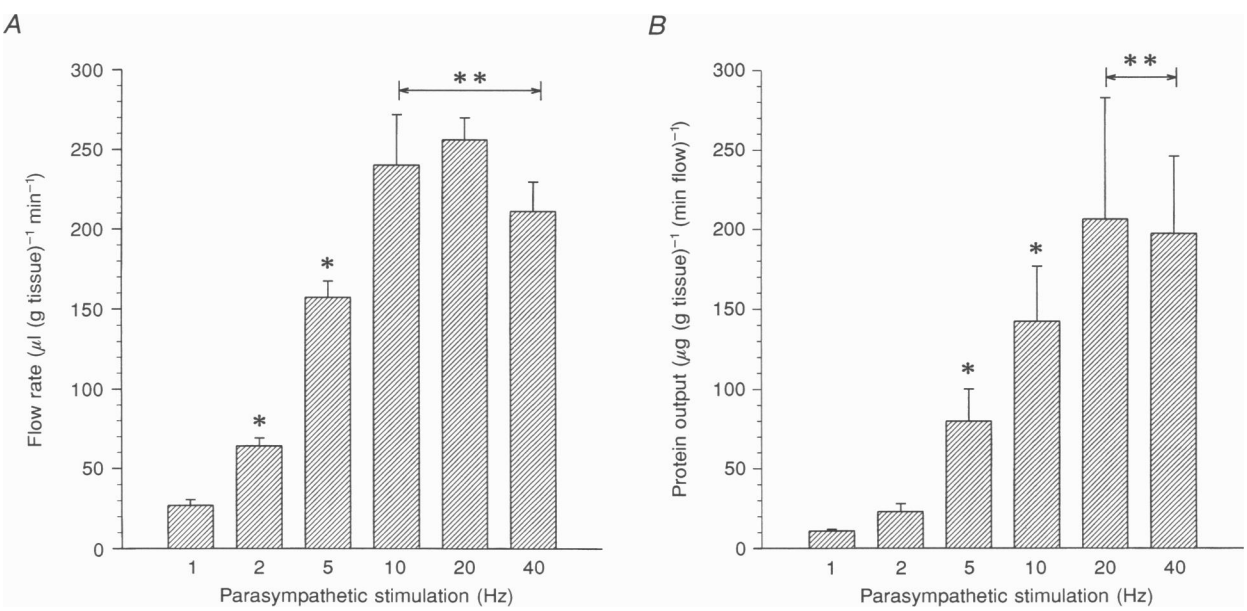

Figure 1. The effects of graded parasympathetic stimulation on salivary flow rate (*A*) and protein secretion (*B*) from the rat submandibular gland

Stimulation was graded between 1 and 40 Hz. Each bar represents the mean ± S.E.M. of at least 5 animals. For flow rate: * $P < 0·05$ compared with preceding stimulation(s); ** $P < 0·05$ compared with 1, 2 and 5 Hz, but not each other. For protein secretion: * $P < 0·05$ compared with 1 Hz and the preceding sample; ** $P < 0·05$ compared with 1, 2 and 5 Hz, but not each other.

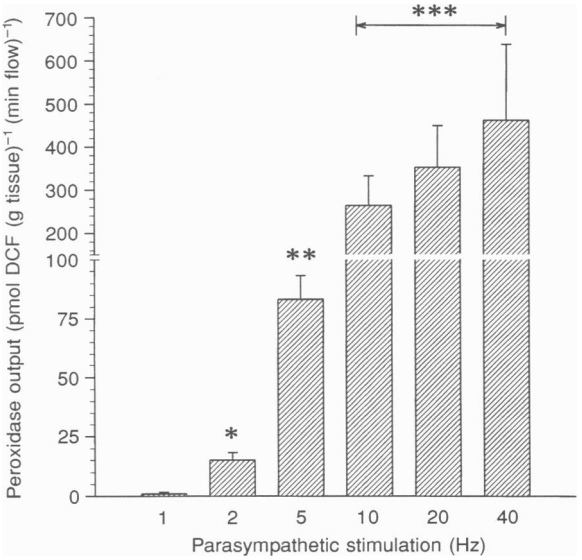

Figure 2. The effects of graded parasympathetic stimulation on peroxidase secretion from the rat submandibular gland

Stimulation was graded between 1 and 40 Hz. Each bar represents the mean ± S.E.M. of 5 animals. * $P < 0.05$ compared with 1 Hz; ** $P < 0.05$ compared with 1 and 2 Hz; *** $P < 0.05$ compared with 1, 2 and 5 Hz, but not with each other.

40 Hz was not significantly greater than at 10 Hz. The increases in peroxidase output reflected an increasing concentration of peroxidase in the saliva samples, as well as the greater salivary flow rates obtained with increasing frequency. Peroxidase concentrations at 1, 10 and 40 Hz were significantly different from each other (0·06 ± 0·02, 1·10 ± 0·15 and 1·95 ± 0·34 nmol DCF ml^{-1}, respectively, $P < 0.05$).

The patterns of rK1 (Fig. 3) and rK2 (not shown) output during parasympathetic stimulation related to changes in flow rate, as only insignificant changes in proteinase concentrations were observed over differing rates of stimulation. In addition, the ratio of rK2 (nmol AFC ml^{-1}) to rK1 (μmol AFC ml^{-1}) in parasympathetic saliva did not change significantly between 1 Hz (0·78 ± 0·04) and 40 Hz (0·42 ± 0·1).

Total protein secretion (Fig. 1*B*) at 1 Hz was less than 5 μg (g tissue)$^{-1}$ (min flow)$^{-1}$, and showed an increase with increasing stimulation frequency up to 20 Hz. As for peroxidase, the increases correlated with both flow rate and an increasing protein concentration. The concentration of protein in parasympathetic saliva was 176 ± 17 μg ml^{-1} at 1 Hz, and increased to 391 ± 91 μg ml^{-1} at 40 Hz ($P < 0.05$). Maximum protein output occurred at 20 Hz and was unchanged at 40 Hz.

Effects of graded sympathetic stimulation

In a second group of five animals, increasing rates of sympathetic stimulation were superimposed on a background of parasympathetic stimulation. Parasympathetic stimulation alone (4 Hz) evoked a rapid flow of submandibular saliva, approximately 140 μl (g tissue)$^{-1}$ min^{-1}. The addition of sympathetic impulses at very low frequencies, 0·1–0·5 Hz, had no significant effect on submandibular fluid production, but 1 and 2 Hz continuous and 10 and 20 Hz burst (1 s every 10 s) sympathetic stimulation led to a reduction in flow rate (Fig. 4).

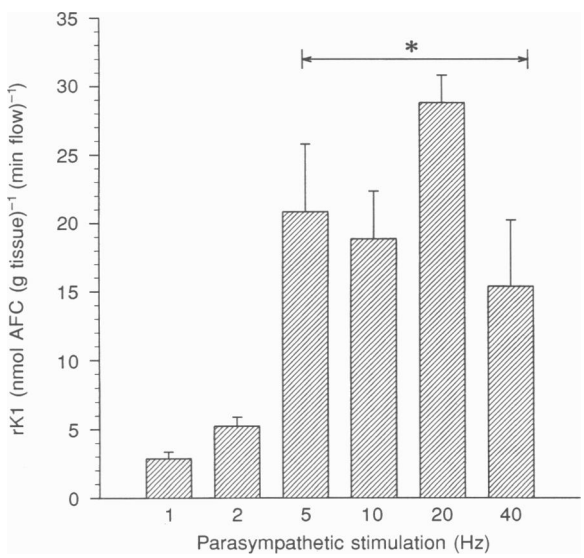

Figure 3. The effects of graded parasympathetic stimulation on tissue kallikrein (rK1) secretion from the rat submandibular gland

Stimulation was graded between 1 and 40 Hz. Each bar represents the mean ± S.E.M. of 4 animals. * $P < 0.05$ compared with 1 and 2 Hz, but not with each other.

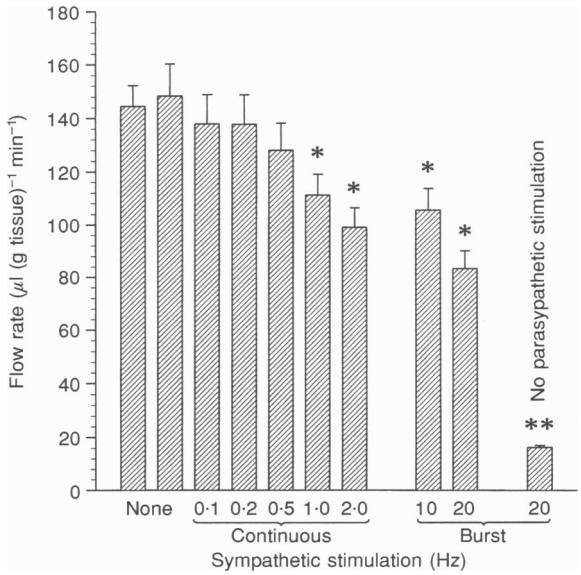

Figure 4. The effects of graded sympathetic stimulation during parasympathetic stimulation on salivary flow rate from the rat submandibular gland
Graded stimulation was applied at 0·1–2 Hz continuously and in 10 and 20 Hz bursts of 1 s every 10 s against a background parasympathetic stimulation rate of 4 Hz. Each bar represents the mean ± S.E.M. of at least 4 animals. * $P < 0·05$ compared with 4 Hz parasympathetic stimulation *per se*; ** $P < 0·05$ compared with all other flow rates.

Nonetheless, submandibular salivary flow rates under these conditions were considerably greater than those seen during 20 Hz burst sympathetic stimulation *per se* (80–110 μl (g tissue)$^{-1}$ min^{-1} compared with 16 ± 1 μl (g tissue)$^{-1}$ min^{-1}).

During parasympathetic stimulation alone, peroxidase secretion was relatively small. Sympathetic stimulation applied at increasing frequencies (0·1–2 Hz continuously) led to progressive significant increases in peroxidase output (Fig. 5). Peroxidase secretion during 10 and 20 Hz burst stimulation was similar to that observed using 1 and 2 Hz continuous sympathetic stimulation. Both peroxidase concentration (40 ± 5 nmol DCF ml^{-1}) and output (0·73 ± 0·14 nmol DCF (g tissue)$^{-1}$ (min flow)$^{-1}$) during 20 Hz burst sympathetic stimulation *per se* were significantly lower than in the presence of a parasympathetic background stimulation (68 ± 22 nmol DCF ml^{-1} and 5·04 ± 1·65 nmol DCF (g tissue)$^{-1}$ (min flow^{-1}), respectively). Peroxidase secretion returned to levels similar to those found in the saliva evoked by parasympathetic stimulation alone (0·16 ± 0·02 nmol DCF (g tissue)$^{-1}$ (min flow)$^{-1}$) after the cessation of sympathetic stimulation.

Extremely low levels of rK1 and rK2 were secreted during parasympathetic stimulation alone, and the ratio of rK2 to rK1 was 0·8 ± 0·15 (Fig. 6, inset). Addition of low frequency sympathetic impulses (0·1–2 Hz) had no apparent effect on rK1 output (Fig. 6). A small increase in rK1 release was seen during 10 Hz burst stimulation, but 20 Hz burst sympathetic stimulation resulted in an enormous output of rK1 activity. In addition, as the frequency of continuous sympathetic stimulation increased there was a trend towards higher rK2 : rK1 ratios, which was most marked at 10 and 20 Hz burst stimulation (3·09 ± 1·06 and 5·67 ± 0·51, respectively). As observed for peroxidase secretion, rK1 concentration (6·2 ± 1·1 μmol AFC ml^{-1}) and output (98·9 ± 13·8 nmol AFC (g tissue)$^{-1}$ (min flow)$^{-1}$) were

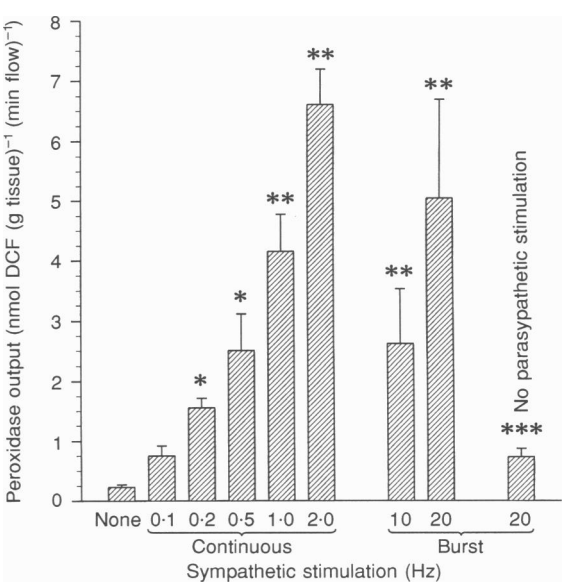

Figure 5. The effects of graded sympathetic stimulation during parasympathetic stimulation on peroxidase secretion from the rat submandibular gland
Graded stimulation was applied at 0·1–2 Hz continuously and 10 and 20 Hz bursts of 1 s every 10 s against a background parasympathetic stimulation rate of 4 Hz. Each bar represents the mean ± S.E.M. of at least 5 animals. * $P < 0·05$ compared with 4 Hz parasympathetic stimulation *per se*; ** $P < 0·05$ compared with 4 Hz parasympathetic stimulation and the previous sample; *** $P < 0·05$ compared with 20 Hz burst plus 4 Hz parasympathetic stimulation.

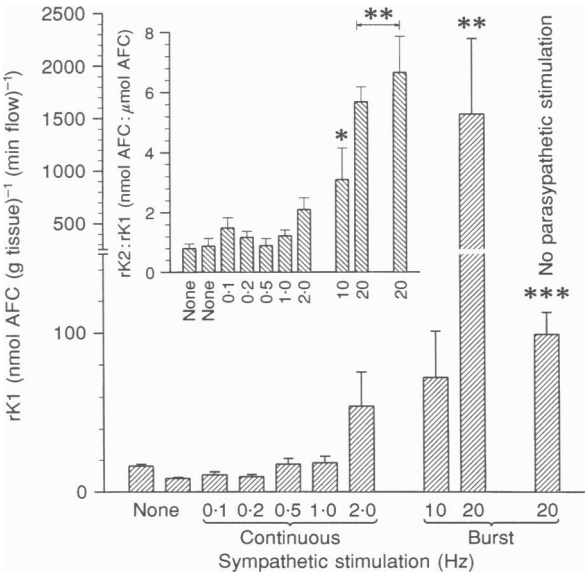

Figure 6. The effects of graded sympathetic stimulation during parasympathetic stimulation on rK1 output and on the rK2 : rK1 ratio of saliva from the rat submandibular gland

Graded stimulation was applied at 0·1–2 Hz continuously and in 10 and 20 Hz bursts of 1 s every 10 s against a background parasympathetic stimulation rate of 4 Hz. rK2 units, nmol AFC; rK1 units, μmol AFC. Each bar represents the mean ± S.E.M. of at least 4 animals. * $P < 0·05$ compared with 4 Hz parasympathetic stimulation *per se*; ** $P < 0·05$ compared with all previous samples but not with each other; *** $P < 0·05$ compared with 20 Hz burst stimulation.

significantly lower in saliva collected during 20 Hz burst sympathetic stimulation *per se* than in the presence of 4 Hz parasympathetic background stimulation (21·3 ± 6·5 μmol AFC ml^{-1} and 1535 ± 720 nmol AFC (g tissue)$^{-1}$ (min flow)$^{-1}$, respectively).

Protein output during 4 Hz parasympathetic stimulation was relatively small (30 μg (g tissue)$^{-1}$ (min flow)$^{-1}$), reflecting the low protein concentration in parasympathetic saliva (246 ± 22 μg ml^{-1}). Both protein concentration and output increased with increasing sympathetic stimulation frequency, and similar values were recorded for 1 Hz continuous and 10 Hz burst stimulation (Fig. 7). At 20 Hz burst stimulation, however, a marked increase was observed in total protein output. Consistent with the flow rate data, protein output during 20 Hz burst sympathetic stimulation *per se* was significantly reduced compared with that observed during combined parasympathetic and sympathetic nerve activation. Nonetheless, protein concentration was similar for each of the two samples (16·78 ± 6·05 and 13·30 ± 6·70 mg ml^{-1}, respectively). When sympathetic drive was removed, protein concentrations decreased dramatically (0·55 ± 0·44 mg ml^{-1}).

In three additional animals the effects of adrenoceptor blockade were tested on the sympathetic responses. Initially, in the absence of α- and β-blockers, 4 Hz parasympathetic stimulation was tested alone and with simultaneous sympathetic stimulation at 2 Hz continuously and in bursts of 20 Hz (1 s every 10 s). The results were similar to those described above. After dual blockade with the α-blocker dihydroergotamine and the β-blocker propranolol the increases in peroxidase and rK1 secretion previously seen with sympathetic stimulation were abolished.

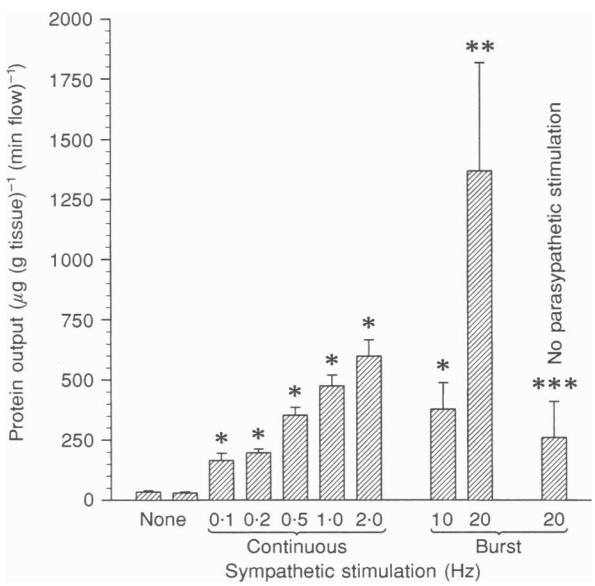

Figure 7. The effects of graded sympathetic stimulation during parasympathetic stimulation on protein secretion from the rat submandibular gland

Graded stimulation was applied at 0·1–2 Hz continuously and in 10 and 20 Hz bursts of 1 s every 10 s against a background parasympathetic stimulation rate of 4 Hz. Each bar represents the mean ± S.E.M. of at least 5 animals. * $P < 0·05$ compared with 4 Hz parasympathetic stimulation *per se*; ** $P < 0·05$ compared with all previous samples; *** $P < 0·05$ compared with 20 Hz burst stimulation.

DISCUSSION

The results of the present study confirm and extend previous findings of a functional dichotomy between the actions of sympathetic and parasympathetic nerves in the regulation of secretion in the rat submandibular gland (Abe & Dawes, 1978; Jones & Wilson, 1985; Iwabuchi *et al.* 1985; Garrett *et al.* 1991). As a result of studying the secretion of peroxidase derived from acinar cells and rK1 derived from granular duct cells the present work has also provided new information that further delineates the dual nature of sympathetic neural activation of these secretory cells in the submandibular gland of the rat. The enzymes were assessed by their enzymatic activity, which closely parallels molecular presence for rK1 (Chan *et al.* 1993). No similar study, however, has been undertaken with respect to peroxidase activity.

Parasympathetic impulses caused a copious flow of saliva that, in comparison to saliva evoked by sympathetic stimulation, was relatively low in protein. Nevertheless, although remaining relatively low, the protein concentration and output during parasympathetic stimulation *per se* did increase with the frequency of stimulation. Analysis of the saliva demonstrated that there was an increase in both concentration and output of peroxidase, particulary at frequencies greater than 2 Hz, but kallikrein concentrations remained at a relatively constant, low level. Thus, the increase in protein concentration was likely to be mainly due to progressive effects of parasympathetic impulses on acinar rather than ductal cells. Previously, morphometric evidence suggested that the secretion of protein during parasympathetic stimulation at 10 Hz for 1 h occurs in the absence of detectable acinar cell degranulation (Garrett *et al.* 1991). Nonetheless, small amounts of granule release beyond the limits of measurement may have occurred, especially if synthesis kept abreast of secretion.

It is likely that sympathetic secretory nerves do not operate in isolation in salivary glands, and that their effects normally occur in cells that are receiving simultaneous parasympathetic stimulation (Emmelin, 1987; Garrett, 1987). Thus, a dual type of stimulation may reflect natural events more closely than when stimulation of the sympathetic nerves is applied in isolation. However, in our experiments graded sympathetic impulses superimposed on a continuous background of parasympathetic stimulation (4 Hz) resulted in a frequency-dependent decrease in flow rate. The effects of sympathetic stimulation on flow rate, however, may be related to the unavoidable vaso-constriction caused by electrical stimulation of the sympathetic nerves supplying vessels, as well as those for secretomotor function (see Thulin, 1976), even when burst stimulation is used (Anderson *et al.* 1988). Despite the reduction in flow rate, there was a significant augmentation of protein secretion when sympathetic impulses were introduced in the presence of parasympathetic stimulation. When sympathetic stimulation at 20 Hz in bursts was added to parasympathetic stimulation at 4 Hz, the output of peroxidase was far greater than the sum of the activity secreted when each type of stimulation was performed separately. These observations further support the idea that parasympathetic influences are normally involved in the cellular response of the acinar cells to sympathetic stimulation. Interestingly, only peroxidase, and not kallikrein or tonin, concentration and output were increased at the lower sympathetic stimulation frequencies (0·1–2 Hz). Thus, our findings also serve to confirm Rigby & Templeton's (1984) original, but unsubstantiated, assumption that low frequency sympathetic stimulation superimposed on a parasympathetic background elicits protein secretion from the acinar cells of the rat submandibular gland.

A completely different pattern of secretion was elicited for rK1, which is synthesized and secreted by the granular ducts. A small secretion occurred during parasympathetic nerve stimulation, and although the concentrations did not increase with the frequency of stimulation, there was a gradual small increase in output. In addition, in parasympathetic saliva the rK2 : rK1 ratio remained relatively constant throughout, 0·42–0·78, which is very different from the ratio found during 20 Hz sympathetic burst stimulation alone (6·66 ± 1·2). Differences in the ratio of rK2 (nmol AFC) to rK1 (μmol AFC) give an indication of the different pools from which they are secreted, granule exocytosis or vesicular transport. Shori *et al.* (1992) found similarly low rK2 : rK1 ratios in parasympathetic saliva and high ratios in glandular extracts and sympathetic saliva, and the secretion of kallikreins in parasympathetic saliva was considered to be by the vesicular (constituitive) route (Kelly, 1985).

In contrast to the progressive effects of sympathetic impulses on the acinar cells, no similar increases in rK1 secretion occurred from the granular ducts with sympathetic stimulation up to 2 Hz continuously or at 10 Hz in bursts, and the rK2 : rK1 ratio did not differ significantly from the that of parasympathetic saliva *per se*. At 20 Hz burst stimulation, however, there was a surge of kallikrein secretion (exocytosis of secretory granules), and the rK2 : rK1 ratio (5·67 ± 0·51) became similar to that seen on sympathetic stimulation alone (6·66 ± 1·20).

When sympathetic stimulation in bursts of 20 Hz was superimposed on parasympathetic stimulation at 4 Hz, an extraordinary augmentation of kallikrein secretion occurred, compared with the outputs when stimulating each nerve separately. These data suggest that under physiological conditions, parasympathetic impulses may also be involved in the sympathetic secretory responses in the granular ducts. Nevertheless, the pattern of sympathetic impulses required for kallikrein secretion is very different from that for the secretion of peroxidase by acinar cells. Acinar cells secrete peroxidase in response to

very low rates of sympathetic stimulation, but sympathetic secretion of kallikreins from the granular ducts depends on high frequency stimulation. Thus, not only is there a dichotomy between the secretory responses of rat submandibular parenchymal cells to parasympathetic and sympathetic stimulation, there is also a dichotomy between the sympathetic responses of the two main secretory cell populations in the gland.

That there are differences in the α- and β-adrenergic involvement in protein secretion by the acinar and granular duct cells in the rat submandibular gland is well documented (Matthews, 1974; Orstavik & Gautvik, 1977; Abe & Dawes, 1978; Iwabuchi *et al.* 1985); secretory responses are predominantly β-adrenergic for the acini and α-adrenergic for the granular ducts. However, this cannot explain the requirement for different impulse frequencies in order to activate acinar and ductal cells. The pharmacological studies of Abe *et al.* (1980), however, may shed some light on this phenomenon. They demonstrated differences in the sensitivity of glandular responses to α-adrenergic agonists. Low doses of phenylephrine, for example, evoked a 'β-type' response, but higher doses were required to induce the secretion of 'α-type' proteins. By analogy, our results suggest that a small amount of neurotransmitter release from very few impulses in postganglionic sympathetic secretomotor nerves is sufficient to activate acinar cells, whereas the granular ducts require a much higher local concentration of neurotransmitter, and this greater release of neurotransmitter can only be achieved by high frequency impulse formation. The use of blocking drugs confirmed that the sympathetic effects were mediated by the activation of α- and β-adrenoceptors, rather than by neuropeptides, such as neuropeptide Y, which are released from some sympathetic nerve terminals along with noradrenaline.

Small amounts of kallikrein-like proteinases probably enter the saliva constituitively under all physiological conditions (Zhang, Garrett, Proctor, Anderson & Shori, 1994), presumably to fulfil some basic function(s). However, the reflex secretion of granules from the granular ducts that occurs only under special circumstances, so far undefined, must involve special sympathetic secretomotor drive with a very different pattern of impulse generation than for acinar cell secretion of peroxidase. This poses an intriguing question about the physiological control of the secretion of proteins in the submandibular gland. How is this differential impulse formation orchestrated? One possibility is that the two functions are controlled by separate populations of sympathetic adrenergic nerves under separate central control. It is not known whether similar differential stimulation requirements for protein secretion from different secretory cells exist in the other salivary glands of the rat, or in other species including man, but it should be noted that granular tubules, as studied here, are a special feature of rodent submandibular glands.

In conclusion, this study has shown that the two main types of secretory cells in the rat submandibular gland are very differently controlled, not only by parasympathetic and sympathetic nerve impulses, but also by the manner of delivery of or response to sympathetic nerve stimulation. Our results give experimental support to the prophetic words of Abe *et al.* (1980), based on pharmacological studies: 'it may be that in the intact animal the types of proteins secreted by the submandibular glands are dependent on the intensity of sympathetic stimulation of the glands'.

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