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RELATIVE ACTIVITIES OF SUBSTANCE P-RELATED PEPTIDES IN THE GUINEA-PIG ILEUM AND RAT PAROTID GLAND, *in vitro*

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1 The relative potencies of a series of substance P analogues have been determined for spasmogenic activity in the guinea-pig ileum *in vitro* and for the release of ^{86}Rb and α-amylase activity from rat parotid gland slices *in vitro*. Equipotent molar ratios (EMR), relative to substance P, were determined for all the compounds.

2 In the rat parotid gland, EC_{50} values for amylase release were, on average, 35.5 times greater than those for ^{86}Rb release. Analysis of Hill plots suggests that spare receptors exist for ^{86}Rb release but not for amylase release and it is suggested that the stimulus-response coupling for amylase release may be less efficient than that for ^{86}Rb release.

3 In the parotid gland, the octapeptide and [<Glu^6]-hexapeptide C-terminal fragments of substance P were less active than substance P itself, whereas in the ileum, the octapeptide was as active as substance P.

4 Substitutions at the Phe^7 or Phe^8 positions in general reduced activity relative to substance P. This effect was particularly apparent in C-terminal hexapeptide analogues.

5 Substitutions at the Phe^7 and Phe^8 positions in C-terminal hexapeptide analogues produced a greater reduction in activity in the parotid gland than in the ileum. The most marked difference was observed with eledoisin-related peptide for which the ratio of EMRs for ileum and ^{86}Rb release was 18.1. The unsubstituted C-terminal octapeptide fragment similarly showed a discrepancy between the two assay systems (EMR ratio, ileum: ^{86}Rb release = 7.75).

6 It is suggested that the results may indicate the presence of sub-populations of 'substance P receptors' which are represented at least in different proportions in the two tissues studied, although alternative explanations such as differences in metabolism of agonists are possible.

Introduction

The classical approach to classification of receptors has involved the use of specific receptor antagonists and measurements of potency ratios for series of agonists. A suitable antagonist for the study of receptor/s which mediate responses to substance P (sP) is not widely available, and so classification must primarily be derived from the latter approach. *In vitro* systems, by virtue of their simplicity and the degree to which they may be controlled, are preferable for the determination of structure-activity relationships. To date, the most sensitive and reliable preparation for bioassay of sP has been the isolated ileum of the guinea-pig and, accordingly, this has been widely used (Bury & Mashford, 1976; Rosell, Bjorkroth, Chang, Yamaguchi, Wan, Rackur, Fisher & Folkers, 1977; Yanaihara, Yanaihara, Horihashi, Sato, Iizuka, Hashimoto & Sakagami, 1977; Chipkin, Stewart, Sweeney, Harris & Williams, 1979; Couture, Fournier, Magnan, St.-Pierre & Regoli, 1979). Recently, Teichberg, Cohen & Blumberg (1981) have found that smooth muscle obtained from different sources exhibits different sensitivities to sP and they suggest that this reflects differences in receptor sub-populations.

Isolated nervous tissue preparations have also been used to investigate structural requirements of sP-like peptides (e.g. Otsuka & Konishi, 1977) and the ability of sP to stimulate [^{3}H]-inositol incorporation into rat parotid gland cells has similarly been compared with that of a number of structural analogues (Hanley, Lee, Jones & Mitchell, 1980). Whilst two groups have examined the relative activities of sP fragments and analogues in stimulating salivary secretion *in vivo* (Leeman, Mroz & Carraway, 1977; Hanley *et al.*, 1980), sP-evoked amylase secretion (Rudich & Butcher, 1976) and K^+ loss (Putney, 1977; Friedman & Selinger, 1978) from rat parotid gland have not, so far, been studied in the context of structure-activity relationships. This tissue provides a convenient system with which to examine

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a number of 'sP-receptor'-mediated phenomena, and has the potential advantage that factors such as receptor accessibility and metabolism of agonists may be common features. The use of isolated salivary glands also circumvents possible complicating factors such as changes in blood flow encountered with studies *in vivo*, and permits simple differentiation of electrolyte movement and enzyme secretion.

In the rat parotid gland, sub-populations of adrenoceptors independently mediate amylase and K^+ release (Batzri, Selinger, Schramm & Robinovitch, 1973; Leslie, Putney & Sherman, 1976; Putney, 1976). The α-adrenoceptors are primarily concerned with K^+ permeability changes (Batzri *et al.*, 1973; Putney, 1976), whereas the noradrenaline-evoked amylase release is largely β_1-adrenoceptor mediated (Leslie *et al.*, 1976; Carlsoo, Danielsson, Henriksson & Idahl, 1981). Thus, it was tempting to speculate that two distinct types of sP-receptor may govern these two phenomena, by analogy with the adrenoceptor system.

The following study compared the potencies of a range of sP-analogues in their ability to cause amylase release and modify ^{86}Rb efflux from rat parotid slices *in vitro*. ^{86}Rb was used in this context as a convenient marker of potassium movements (Putney, 1976). The results are compared with data obtained for the spasmogenic activities of sP-analogues in guinea-pig isolated ileum preparations.

Methods

All comparative assays were performed using substance P as a standard.

Parotid gland assays

Preparation of parotid gland slices Male hooded rats (120–180 g) were fasted for a period of 15–20 h before they were killed under ether. The parotid glands were quickly excised and placed in a modified Krebs-Hensleit solution (KRH) which was continuously bubbled with O_2 and CO_2 (95:5%), maintained at 37°C. The glands were cleaned of fat and connective tissue and sliced into strips, 1 mm thick, with a McIlwain tissue chopper. Samples of slices were then pre-incubated in KRH for 60 and 90 min before experiments measuring amylase and ^{86}Rb efflux, respectively. The composition of the Krebs-Hensleit solution was as follows, (mM): NaCl 118, KCl 5.6, $MgSO_4$ 1.16, KH_2PO_4 1.18, $CaCl_2$ 2.5, $NaHCO_3$ 25, sodium DL-β-hydroxybutyrate or sodium pyruvate 5.0, pH, 7.4. In experiments to study the efflux of ^{86}Rb, RbCl (1.0 mM) was included in the bathing medium in order to reduce non-specific binding of ^{86}Rb. In this case KCl was reduced to 4.6 mM.

^{86}Rb-efflux Slices were incubated for 90 min in KRH at 37°C, bubbled continuously with O_2:CO_2 (95:5%) in the presence of $^{86}RbCl$ (sp.act., 1–12 mCi.mgRb^{-1}) to a final concentration of 0.2 μCi/ml. Accumulated tissue radioactivity after this period was linearly related to the amount of tissue present. In each experiment, glands from four rats were used. Following 'loading' with ^{86}Rb, slices from each rat were divided into four aliquots, each to receive one of four treatments; for example, four agonist concentrations. Thus, tissue from each rat formed a 'block' of treatments. This block was repeated for the remaining rats. Efflux of ^{86}Rb was measured by continuously perfusing tissue samples contained within 1 ml polythene chambers. Four channels were perfused simultaneously by means of a Gilson multichannel peristaltic pump. The perfusion rate was 15 ml/min, a high rate being used in order to encourage rapid equilibration of tissue with drug and efficient washout. Temperature was maintained at 37°C by means of glass heat exchange coils adjacent to the tissue chambers. Each chamber could be allocated a particular bathing medium via 3-way plastic taps. Perfusates were collected into plastic scintillation counting vials and ^{86}Rb activity was determined by measuring Cerenkov light emission using a Packard Tri-Carb liquid scintillation spectrophotometer.

Drugs were freshly dissolved prior to each perfusion cycle which consisted of the following steps. After an initial wash-out period of 1 min, four 1 min collections were made to establish a basal efflux rate. sP analogues were applied for a period of 90 s during which three 30 s collections were made. Responses to sP agonists normally peaked by 60 s and the short collection periods provided good resolution of the response. Drug periods were followed by a 1 min washout. Glands were then removed from the perfusion system and incubated in 5 ml 0.1 M HNO_3 at approximately 100°C until completely digested. Aliquots of the digest were then removed for estimation of the total content of ^{86}Rb for each sample of tissue.

Amylase release The procedure and conditions used to measure release of amylase in response to agonists were as previously described (Owen & Jordan, 1981). The procedure consisted of the transfer of tissue samples through a series of incubation media at regular intervals of time. At the end of the experiment each sample of tissue was homogenized in KRH with a Polytron homogenizer (setting No.6 for 30 s) and stored with the collected incubation media overnight at −20°C before assay for amylase activity. The amylase assay is described in detail elsewhere (Owen & Jordan, 1981) but in essence used a fluorescent starch substrate, amylopectin anthranilate. Reaction products were separated from the substrate and measured by fluorescence spectrometry. Enzyme ac-

tivity was calculated by reference to standard amylase solutions (Sigma Type IIa).

^{86}Rb efflux and amylase release in response to sP were unaffected by the inclusion of atropine (5 μM), phentolamine (1 μM) and propranolol (1 μM) in the bathing media.

Isolated guinea-pig ileum assay

Procedures for the measurement of the contractile effect of peptides in the guinea-pig isolated ileum were as described previously (Jordan, 1980). A dose cycle of 10 min was adopted to minimize interaction between doses, the tissue being exposed to each dose for 20 s. Serial dose-response curves were obtained and suitable doses selected for use in a formal 3 + 3 assay. A balanced randomized block design was adopted (Schild, 1942; Colquhoun, 1971). Results of the assays were analysed by analysis of variance.

Treatment of data

(1) *Parotid gland*

^{86}Rb efflux: After the initial washout of extracellular ^{86}Rb the efflux of ^{86}Rb was satisfactorily described by the exponential function:-

$$Rb_t = Rb_0 e^{-kt} \qquad \text{Eq.1}$$

where Rt_0 is the ^{86}Rb content of the tissue at t = 0; Rb_t, the ^{86}Rb remaining after a period, t (min) and k is the efflux rate coefficient. The rate coefficient (k) was calculated from:-

$$k_{Rb} = \Delta Rb/\overline{Rb}.\ \Delta t \qquad \text{Eq.2}$$

where ΔRb is the amount of ^{86}Rb lost over a period t (min) with an average tissue ^{86}Rb content over that period of $\overline{Rb}$. Figure 1 illustrates a typical efflux profile in terms of the ^{86}Rb coefficient. The response (Δk), to drugs was defined as the difference between the peak drug-evoked efflux rate coefficient and the basal efflux rate coefficient (see Figure 1).

Amylase release: Since the total amylase content of each tissue sample was known, release of amylase into bathing media could be calculated as for ^{86}Rb efflux but the rate coefficient derived was expressed as % min^{-1}. Again the response tc an agonist was defined as the difference between the evoked and basal rate coefficients (see Figure 2).

Dose-response relationships: Responses to peptides, for ^{86}Rb and amylase release, were dose-related, and all analogues were full agonists compared to sP. Responses for amylase release were thus expressed in terms of the response evoked by a supramaximal dose of physalaemin (1 μM) in each assay. ^{86}Rb efflux was related to maximal responses evoked either by supramaximal concentrations of sP, or the test analogue concerned. All peptides were independently tested relative to sP for full agonist activity. Data were computer fitted directly by the method of least squares to the logistic function:-

$$y = \frac{y_{max} \cdot x^n}{K^n + x^n} \qquad \text{Eq.3}$$

where (x) is the concentration of peptide; (K) represents the EC_{50} of the peptide; (y) is the response to peptide at concentration (x), and (y_{max}) is the maximal response obtainable. (n) is equivalent to the slope of a Hill plot of the data (log [$y/y_{max} - y$)] vs. log x).

When responses were expressed as a percentage of maximum, variances became homoscedastic and dose-response curves for the different agonists could be compared. In curve fitting, logistic functions for both amylase and ^{86}Rb efflux data were initially constrained to a (y_{max}) of 100. Values of (n) were then found for the ^{86}Rb efflux and amylase release dose-response curves for each analogue. There were no significant differences between individual (n) values for either ^{86}Rb or amylase release and so a mean value of (n) was used in each case to constrain the logistic function fit. The data could then be re-fitted to obtain an estimate of (K). Essentially, therefore, data were fitted to a 'mean' logistic curve, the log dose-response curves being assumed to be parallel.

Values of (K) derived in this way were used to calculate equipotent molar ratios (EMRs) in relation to sP where EMR = EC_{50} (sP)/EC_{50} (analogue). Confidence limits for EMRs were calculated using Fieller's Theorem where the estimate of the variance for each EC_{50} value was obtained from the least squares fit to the logistic curve.

(2) *Guinea-pig ileum*

With one exception, only regressions derived from the (3 + 3) formal assays which showed no significant departure from parallelism were used to calculate EMRs. In the case of [*p*-amino-Phe^7]sP$_{1-11}$, several assays were performed but all yielded non-parallel regressions ($P < 0.05$). Values for each of the peptides tested, and their 95% confidence limits, were calculated by the analysis of variance.

Materials

Substance P, physalaemin and sP$_{4-11}$ were obtained from Beckman, Geneva. [<Glu^6] [*p*-amino-Phe^7]sP$_{6-11}$; [*p*-amino-Phe^7]sP$_{1-11}$ and [<Glu^6]sP$_{6-11}$ were prepared by Dr N.N. Petter, Peptide Group, Pharmaceutical Division, ICI Ltd., Alderley Park, Macclesfield, England. Eledoisin-related peptide

Table 1 Structures of substance P-like peptides

Peptide	1	2	3	4	5	6	7	8	9	10	11
Substance P (sP_{1-11})	Arg	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met-NH_2
Physalaemin (Phys)	<Glu	Ala	Asp	Pro	Asn	Lys	Phe	Tyr	Gly	Leu	Met-NH_2
[*p*-Amino-Phe^7]sP_{1-11}	Arg	Pro	Lys	Pro	Gln	Gln	*p*-amino-Phe	Phe	Gly	Leu	Met-NH_2
Octapeptide (sP_{4-11})				Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met-NH_2
Eledoisin-related peptide (Erp)						Lys	Phe	Ile	Gly	Leu	Met-NH_2
[<Glu^6]sP_{6-11}						<Glu	Phe	Phe	Gly	Leu	Met-NH_2
[<Glu^6] [*p*-amino-Phe^7]sP_{6-11}						<Glu	*p*-amino-Phe	Phe	Gly	Leu	Met-NH_2
[Tyr^8]sP_{1-11}	Arg	Pro	Lys	Pro	Gln	Gln	Phe	Tyr	Gly	Leu	Met-NH_2

was obtained from Sigma Chemical Co., Poole. Amylopectin anthranilate was kindly donated by Mr G.T.B. Frost, Sigma Chemical Co., Poole. Rubidium chloride was from BDH, Poole. ^{86}RbCl (1–12 mCi $mgRb^{-1}$), was obtained from the Radiochemical Centre, Amersham. Sodium DL-β-hydroxybutyrate; sodium pyruvate and amylase (Type IIa) were obtained from Sigma Chemical Co. Poole. Atropine sulphate monohydrate was from (Aldrich Chemical Co. Inc., USA); propranolol hydrochloride from ICI, Macclesfield and phentolamine-mesylate from CIBA, Horsham.

All peptides excepting [<Glu^6] [*p*-amino-Phe^7]sP_{6-11} and [<Glu^6]sP_{6-11} were dissolved in 1% acetic acid, divided into small samples and deep frozen (−20°C) until required. The exceptions above were dissolved in 50% and 74% ethanol respectively. Under these conditions, no significant deterioration of peptides, as measured by bioassay on isolated guinea-pig ileum, occurred over several months. No effects of peptide vehicles, at appropriate concentrations, were found on any of the assay systems used.

The structures of the peptides used are given in Table 1.

Results

A typical ^{86}Rb efflux response, in this case evoked by physalaemin (10 nM), is illustrated in Figure 1, and is expressed in terms of the ^{86}Rb efflux coefficient. The response (Δk_{Rb}) is defined as the difference be-

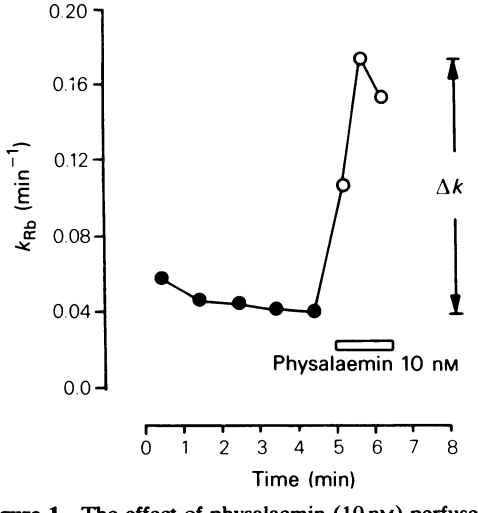

Figure 1 The effect of physalaemin (10 nM) perfused over parotid slices (90 s) on the ^{86}Rb efflux rate coefficient (k_{Rb}) calculated from Eq. 2. The response (Δk), measured as the difference between the evoked rate coefficient and the basal rate coefficient, reaches a maximum within 90 s.

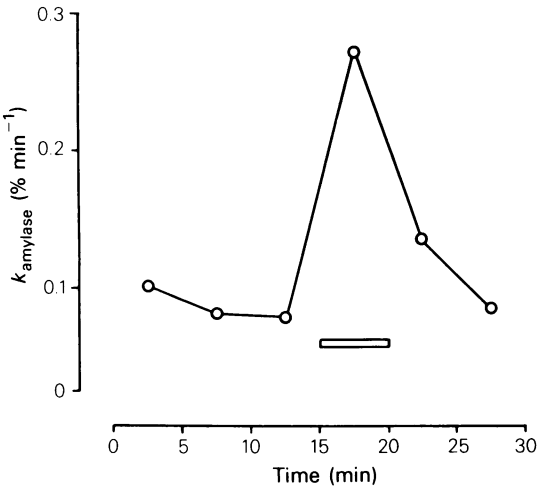

Figure 2 Amylase release from parotid slices transferred through incubation tubes at 5 min intervals. The release is expressed as a percentage of the total tissue amylase released per min (averaged over each incubation period). Substance P (1 μM) was applied during the period indicated by the bar. The response was measured as the difference between the peak evoked $k_{amylase}$ and the basal release coefficient. Points represent single incubations showing a typical basal release and response profile.

tween the peak evoked efflux rate coefficient and the basal efflux rate coefficient (Figure 1). Evoked amylase release was treated in a similar way, as shown for a typical response to sP in Figure 2.

Responses, expressed as a percentage of the maximum (i.e. % max Δk), were fitted directly to logistic dose-response functions and the corresponding log dose-response curves for ^{86}Rb efflux and amylase release are given in Figures 3 and 4, respectively. The average values of (n) used to constrain the logistic functions (see Methods) were 1.46 (s.e.mean 0.15; $n=7$) and 1.01 (s.e.mean 0.08; $n=7$) for evoked ^{86}Rb efflux and amylase release respectively. It was from the fit of data to these parameters, with a (y_{max}) of 100, that estimates of EC_{50} (and their variances) and hence, EMR's were obtained (see Methods).

Comparison of tissue equipotent molar ratios

The EMR values for the series of peptides in the three assay systems summarized in Table 2 are presented graphically in Figures 5, 6 and 7. In each figure, the EMR of an analogue in a particular system is plotted against its EMR in one of the other systems examined. An exact coincidence for a particular analogue corresponds to the theoretical line of unity slope (Figures 5, 6 and 7).

The relative change in EMR for an analogue be-

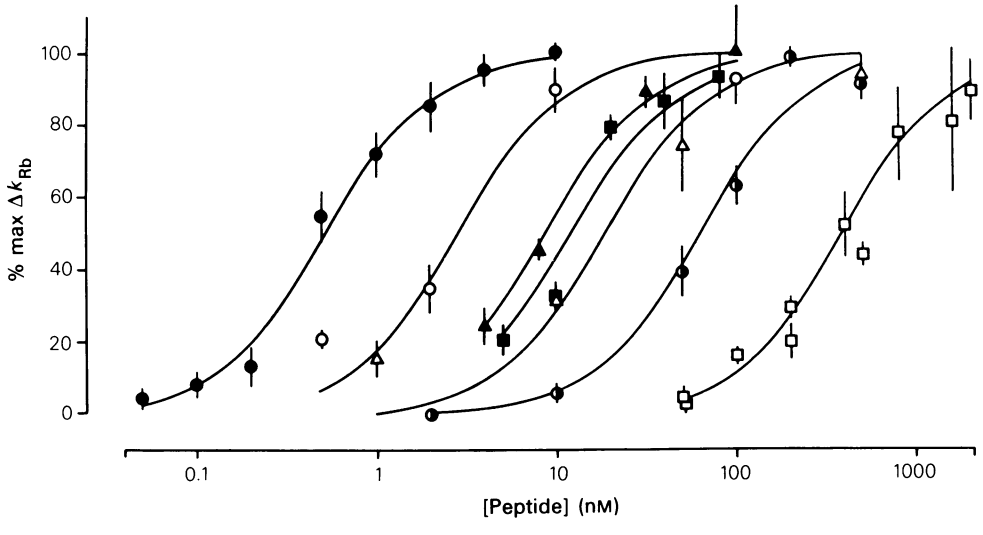

Figure 3 Summary of dose-response relationships for substance P (sP)-like peptides tested for their ability to evoke ^{86}Rb efflux from perfused parotid slices. Δk values obtained as previously described, are related to the maximal Δk obtained for each agonist by a supramaximal sP concentration. (●) Physalaemin; (○) sP; (▲) [*p*-amino-Phe^7]sP_{1-11}; (■) [<Glu^6]sP_{1-11}; (△) sP_{4-11}; (◑) eledoisin related peptide; (□) [*p*-amino-Phe^7]-sP_{6-11}. Each point is the mean of 4–8 determinations and the vertical lines represent the s.e.mean. The curves are fitted by the method of least squares to a logistic function where y_{max} is constrained to 100 and (n) to a mean value of 1.46 (see text). Weighting of points was not considered to be warranted in view of the number of determinations made.

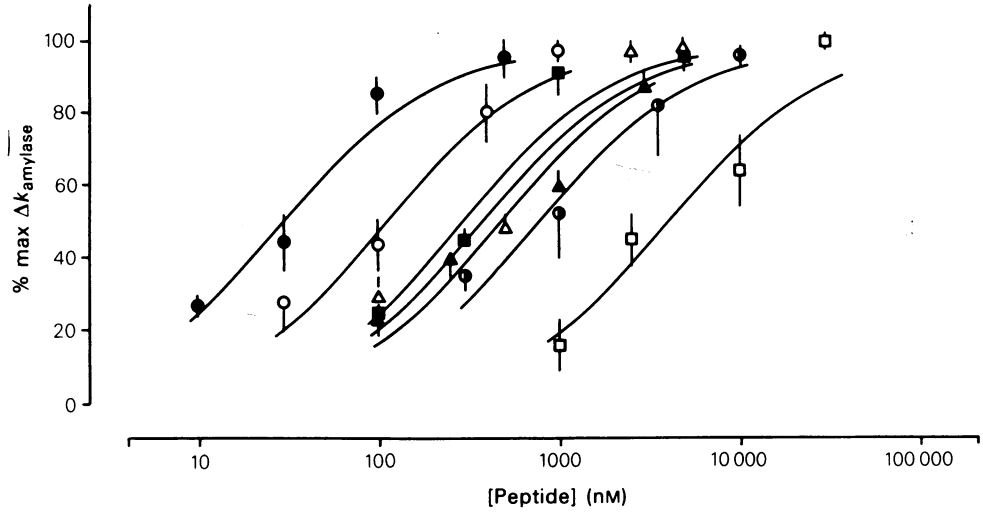

Figure 4 Dose-response curves for amylase release from parotid slices, evoked by substance P (sP)-like peptides. Δk values are expressed as a percentage of the response to a supramaximal concentration of physalaemin (1 μM). Each point is the mean of 4–6 determinations and the s.e.mean is represented by vertical lines. (●) Physalaemin; (○) sP; (▲) [*p*-amino-Phe^7]sP$_{1-11}$; (■) [<Glu^6]sP$_{6-11}$; (△) sP$_{4-11}$; (◑) eledoisin-related peptide; (□) [<Glu^6] [*p*-amino-Phe^7]sP$_{6-11}$. Curves are fitted to a logistic function where $y_{max} = 100$ and $n = 1.01$ (see text). Points were not weighted.

tween any two systems is shown in Table 3, where attention is drawn to peptides with significant differences in EMR between the two systems compared. Considerable differences between the EMRs for ileum and ^{86}Rb efflux models are evident for three peptides, sP$_{4-11}$, eledoisin-related peptide (Erp) and [<Glu^6] [*p*-amino-Phe^7]sP$_{6-11}$ (Table 3 and Figure 5). Particularly striking is the change in EMR for Erp (by a factor of 18.1). The changes in EMR for sP$_{4-11}$ and Erp effectively alter their rank orders of potencies (Table 3).

Significant deviations are seen for sP$_{4-11}$ and Erp when their EMR_{ileum} and $EMR_{amylase}$ values are contrasted, although the factors by which they change are smaller than for the ileum/^{86}Rb comparison (Figure 6).

Interestingly a contrast of ^{86}Rb and amylase EMRs also reveals small, but significant, deviations for Erp and [<Glu^6] [*p*-amino-Phe^7]sP$_{6-11}$ (Figure 7).

With the exception of sP$_{4-11}$ on the ileum, all sP fragments had a lower potency than sP itself in all three systems studied. sP$_{4-11}$ was at least equipotent with sP on the ileum, whereas physalemin was significantly more active than sP in all three systems. In fact the EMR contrast, ileum/Rb, for physalaemin showed a significant deviation from unity although the change in EMR was fairly small (a factor of 0.4).

Table 2 Equipotent molar ratios (EMRs) for substance P-like peptides for contraction of the guinea-pig ileum and efflux of ^{86}Rb and amylase from rat parotid gland

	Ileum		*Parotid*			
			^{86}Rb		Amylase	
Peptide	EMR	95% limits	EMR	95% limits	EMR	95% limits
Physalaemin	2.28	1.86–2.81	5.31	3.31 –7.63	3.66	2.15 –6.03
Substance P	1.0		1.0		1.0	
[*p*-Amino-Phe^7]sP$_{1-11}$	0.39$^+$	0.34–0.44	0.312	0.195–0.445	0.227	0.135–0.361
[<Glu^6]sP$_{6-11}$	0.26	0.23–0.29	0.227	0.141–0.333	0.378	0.217–0.661
(sP$_{4-11}$)	1.10	1.01–1.20	0.142	0.080–0.276	0.291	0.169–0.496
Erp	0.78	0.68–0.89	0.043	0.026–0.064	0.140	0.079–0.263
[<Glu^6] [*p*-amino-Phe^7]sP$_{6-11}$	0.042	0.035–0.053	0.0067	0.0042–0.0099	0.027	0.015–0.049
[Tyr^8]sP$_{1-11}$	0.99	0.90–1.14				

$^+$Log dose-response curves deviated from parallelism.

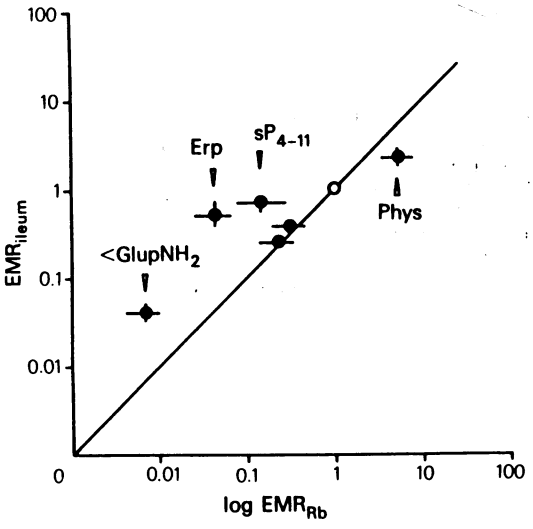

Figure 5 A double log plot of EMR_{ileum} against EMR_{Rb} for each peptide. The line represents a coincidence of EMRs for the two preparations. The open circle represents substance P (sP) and the labelled points represent peptides which deviate significantly from the line. [<GlupNH$_2$) represents [<Glu^6] [*p*-amino-Phe^7]sP$_{6-11}$. The bars represent the 95% confidence limits for the EMR estimates. EMRs for ^{86}Rb efflux were found from the ratio of each peptide EC_{50} to that of sP. The EMR_{ileum} was found from analysis of formal (3 + 3) randomized block design bioassays. Confidence limits were found by the analysis of variance and application of Fieller's theorem.

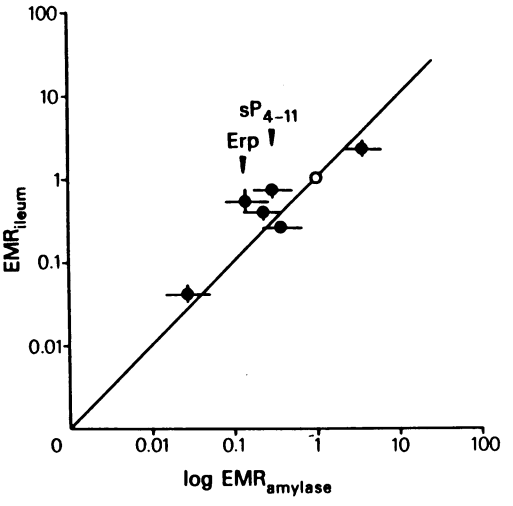

Figure 6 Double log plot of the EMR_{ileum} against the $EMR_{amylase}$ for each peptide. Details are otherwise as for Figure 5. Values for eledoisin-related peptide (Erp) and sP$_{4-11}$ deviate significantly from the line. The bars are the 95% confidence limits of each EMR estimate.

EC_{50} values for ^{86}Rb efflux and amylase release

If EC_{50} values, obtained for each analogue, for evoked ^{86}Rb efflux and amylase release are compared using a double log plot, as in Figure 8, then if there were no changes in the relative activities of these compounds between the two systems, the points would be expected to lie on a regression of unity slope. In fact a least squares regression for all the data points (Figure 8; solid line) has a slope which deviates from unity and it is clear that there is a tendency for the EC_{50} (amylase) to exceed the respective EC_{50} (Rb) for any particular analogue.

In order to obtain an estimate of the general shift in the apparent potencies of analogues the data points for Erp and [<Glu^6] [*p*-amino-Phe^7]sP$_{6-11}$ (which have been shown to deviate significantly in the comparison of EMR values for the two systems, Table 3 and Figure 7), have been omitted and the second regression fitted to the remaining points with a slope of unity (Figure 8, dotted line). The intercept of the line of unity slope corresponds to a potency ratio of 35.5 and the implications of this observation are

Table 3 Comparison of relative potencies of substance P-like peptides on guinea-pig ileum and rat parotid gland *in vitro*

Peptide	(a) *EMR contrast* Ileum/Rb	Ileum/amylase	Rb/amylase	(b) *Rank order of EMR* Ileum	^{86}Rb	amylase
Physalaemin	*0.43	0.62	1.45	1	1	1
Substance P (sP$_{1-11}$)	1.0	1.0	1.0	3	2	2
[*p*-Amino-Phe^7]sP$_{1-11}$	1.25	1.72	1.37	5	3	5
[<Glu^6]sP$_{6-11}$	1.15	0.69	0.60	6	4	3
sP$_{4-11}$	*7.75	*3.78	0.49	2	5	4
Erp	*18.14	*5.57	*0.31	4	6	6
[<Glu^6] [*p*-amino-Phe^7]sP$_{6-11}$	*6.27	1.56	*0.25	7	7	7

(a) Summary of EMR contrasts. The figures are the ratios of EMRs for the systems contrasted. e.g. sP$_{4-11}$: EMR_{ileum}/EMR_{Rb} = 7.75. * Indicates a significant difference between the two EMRs contrasted ($P < 0.05$).
(b) The rank order of EMRs for each system is based on a scale of decreasing potency, from 1–7.

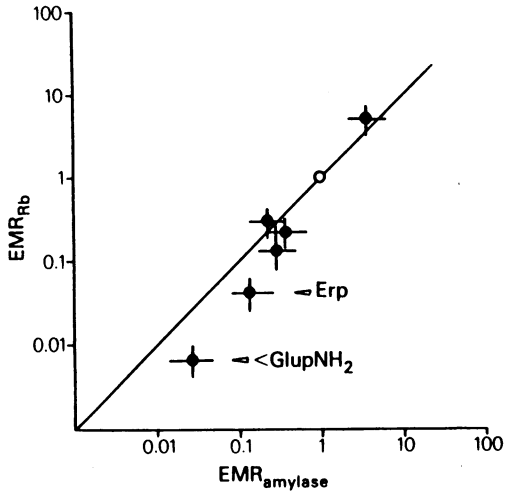

Figure 7 Double log plot of EMR_{Rb} against the $EMR_{amylase}$ for each peptide. Details are as for Figure 5. Eledoisin-related peptide (Erp) and [< Glu^6] [*p*-amino-Phe^7]sP_{6-11} (< $GlupNH_2$) deviate significantly from the line. The bars represent the 95% confidence limits of each EMR estimate.

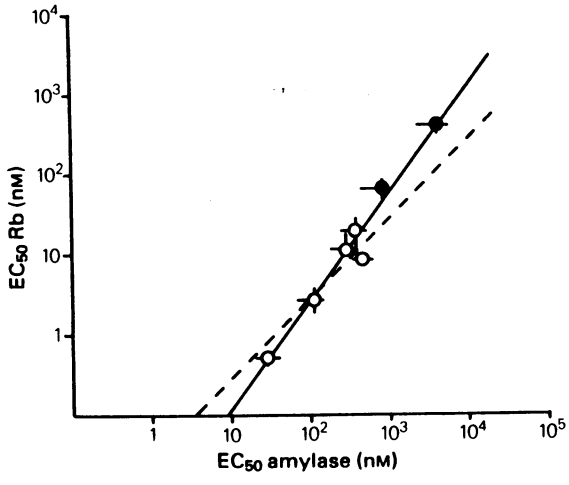

Figure 8 Double log plot of EC_{50}Rb against EC_{50} amylase for each peptide. The solid line represents the regression obtained by least squares fit to all the points. The broken line represents the fit to data excluding the peptides labelled and is constrained to a slope of 1. The intercept of the latter corresponds to a potency ratio of 35.5. The bars represent the 95% confidence limits for the EC_{50} values for each peptide.

discussed below. It may be significant that the value of (n) derived from the logistic fit of amylase data was approximately 1.0 whereas that found for the ^{86}Rb fit was 1.46. In other words, the slope of the linear portion of the log dose-response curve is shallower for the amylase release, and the dose-response relationship approaches a simple hyperbola.

p-*Amino substitution of Phe*^{7}

Substitution of Phe^7 by *p*-amino-Phe in the whole sP molecule reduced EMR_{ileum} to 0.39, EMR_{Rb} to 0.31 and $EMR_{amylase}$ to 0.23. There was no significant difference between any of these estimates. Replacement of Phe^7 in [<Glu]sP_{6-11} by *p*-amino-Phe resulted in a dramatic reduction in activity. The hexapeptide, [<Glu]sP_{6-11} gave EMRs of the same order in the three systems (Table 2) but the Phe^7 substitution, to give [<Glu] [*p*-amino-Phe^7]sP_{6-11}, resulted in substantial reduction in activity in all three systems, with a significantly greater fall in EMR_{Rb}. The factors by which EMR_{ileum}, EMR_{Rb} and $EMR_{amylase}$, were reduced with this Phe^7 substitution were 6.2, 33.9 and 14.0 respectively.

Discussion

Initiation of ^{86}Rb and amylase movements in the rat parotid gland and contraction of the guinea-pig ileum constitute distinct actions of sP. Pharmacologically it is of particular interest to know whether the receptors mediating these actions are the same, or whether it is possible to distinguish subpopulations of 'sP-receptors'. If such differentiation of sP-receptors exists the intriguing possibility of pharmacological manipulation of specific 'sP-mechanisms' suggests itself. This is of considerable interest in relation to the putative primary afferent transmitter role of sP (Henry, 1977; Hökfelt, Ljungdahl, Terenius, Elde & Nilsson, 1977; Otsuka & Konishi, 1977), since an antagonist might, for example, prove to have analgesic activity (Cutting & Jordan, 1975; 1980) and agonists might be predicted to have important behavioural effects as well (Hanley & Iversen, 1980).

In general, evidence for receptor sub-types may be obtained from a comparison of relative activities of a number of agonist analogues in producing each of the responses studied. This was the approach adopted in the present study and equipotent molar ratios have been derived by relating activities of analogues to that of sP. Studies of this kind are, of course, vulnerable to complicating factors such as (1) differences in the metabolism of agonists between tissues, and (2) receptor accessibility. There is evidence to suggest that sP-like peptides are metabolized when incubated with rat parotid gland slices (Hanley *et al.*, 1980) and this possibility is now under investigation. An advantage of comparing activities in a single tissue system, as in the ^{86}Rb-efflux and amylase release studies, is that these problems become less important. One of the most striking observations,

therefore, was the difference in the dose range for peptides required to evoke ^{86}Rb efflux and amylase release. There is an average ratio of 35.5 when the EC_{50} (^{86}Rb) is compared to EC_{50} (amylase) for a particular analogue (Figure 8). This figure was derived by excluding those points which, on the basis of the EMR comparison (Figure 7), appeared to deviate from the overall trend (i.e. those corresponding to Erp and [<Glu^6] [*p*-amino-Phe^7]sP$_{6-11}$). Although there was a change in the rank order of EMRs between the two systems, the changes in EMR lie well within their 95% confidence limits and thus, with the exception of Erp and [<Glu^6] [*p*-amino-Phe^7]sP$_{6-11}$, there seems to be simply a shift of the dose-response curves to higher agonist concentrations. This would suggest that there may be a difference in the efficiency of the receptor-response coupling for amylase secretion and ^{86}Rb efflux. Both phenomena require an external Ca^{2+} pool (Putney, 1976; 1977; Rudich & Butcher, 1976). However, measurements of ^{86}Rb efflux made in this study correspond to the transient phase described by Putney (1976) which is not dependent on extracellular Ca^{2+}, but seems to utilize an intracellular or membrane-bound Ca^{2+} pool. Such a pool may be able to provide a rapid rise in Ca^{2+} locally, which transiently reaches a level sufficient for K^+ channel activation. Enzyme secretion may require a higher Ca^{2+} level at more remote sites and thus may require a greater proportion of receptors to be occupied to achieve a more extensive Ca^{2+} influx.

The apparent discrepancy in the activities of Erp and [<Glu^6] [*p*-amino-Phe^7]sP$_{6-11}$ in evoking ^{86}Rb efflux and amylase release (Figure 7) may mean that there are different receptors governing the two processes, either of which may be preferred by certain agonist analogues. If this is so, then for agonists that cannot distinguish between the two types of receptor, the difference in EC_{50}s and Hill coefficients for ^{86}Rb efflux and amylase release would simply reflect the relative efficiencies of the stimulus-response coupling process in each case.

Alternatively, a single receptor type may be envisaged whose interaction with agonist may lead to the activation of a different number of effector molecules or a different type of effector molecule, depending upon the agonist concerned; for example via conformational changes in the receptor-agonist complex on binding. Such a model could account for discrepancies in the EMRs of certain analogues for ^{86}Rb efflux and amylase release and also accommodate the concept of an inherent difference in the stimulus-response coupling for the two processes.

Interestingly, the slope of the Hill plot for the dose-response relationship for amylase release is shallower than that for ^{86}Rb efflux ($n = 1.01$ and 1.46 respectively). Such a trend is characteristic of a relative absence of spare receptors, and assuming one receptor type, would suggest that there are more spare receptors for the ^{86}Rb efflux system. This would be in keeping with the lower concentrations of the peptides needed to elicit ^{86}Rb efflux. However, the suggestion that spare receptors are present is a different view from that arrived at on the basis of a corrrelation of ^{125}I-physalaemin displacement by sP-analogues with their ability to stimulate ^{42}K efflux, (Putney, Van der Walle & Wheeler, 1980). It may be that the iodinated analogue has different binding characteristics to physalaemin itself.

On the basis of current evidence, therefore, it is not possible to conclude whether different types of sP-receptor mediate ^{86}Rb efflux and amylase release (cf. α- and β-adrenoceptors) or whether, just as cholinoceptor agonists stimulate both processes via muscarinic receptors (Leslie *et al.*, 1976; Putney, 1977), a single class of sP-receptor may mediate the two responses.

Whether amylase release evoked by sP *per se* is a physiologically significant phenomenon is open to doubt in view of the relatively limited extent of maximal secretion rates compared to that evoked by β-adrenoceptor agonists (Owen & Jordan, 1981). There is a suggestion, however, that sP may modulate the actions of endogenous secretagogues in a synergistic manner (Owen and Jordan, unpublished observations), as has been reported for cholinoceptor agonists with β-adrenoceptor agonists (Templeton, 1980).

The finding that the octapeptide and [<Glu]-hexapeptide C-terminal fragments are less active than sP itself in the two measurements made on the parotid is in agreement with the observations of Leeman *et al.* (1977) and Hanley *et al.* (1980) on sP-induced salivary secretion *in vivo* and of Hanley *et al.* (1980) on [^3H]-inositol incorporation into parotid slices *in vitro* (PI effect). It would appear, therefore, that the reduced activity of sP fragments *in vivo* reflects a true potency difference (relative to sP) rather than the consequence of pharmacokinetic factors or the changes in blood pressure produced by these compounds.

Whilst in the parotid gland sP$_{4-11}$ is less active than sP itself, the two peptides are equiactive in the guinea-pig ileum. Discrepancies in the activity of sP$_{4-11}$ have been observed in other systems also. For example, Hanley, Sandberg, Lee, Iversen, Brundish & Wade (1980) have shown that the IC_{50} of sP$_{4-11}$ for inhibition of [^3H]-sP binding in rat brain membranes is approximately 20 times higher than that for sP, whereas sP$_{4-11}$ is approximately equiactive with sP when tested for its depolarizing effects on the rat spinal cord *in vitro*.

The effects of *p*-amino-Phe^7 substitution, in the full sP-sequence (i.e. reduced activity in all three

systems), lends support to the view that the Phe^7-Phe^8 pair of residues form a crucial interaction at the receptor. Previously documented effects of substitution at this point suggest that there are stringent requirements for biological activity. Replacement of Phe^7 with L-carboranylalanine in sP_{4-11} almost completely abolished activity in the guinea-pig ileum (Couture, Drouin, Leukart & Regoli, 1979). [Ala^7]sP_{1-11} has negligible activity on this preparation and [Ala^8]sP_{1-11} also has very much reduced potency (Couture *et al.*, 1979). Similarly [Ile^7]sP_{1-11} has very little activity on the guinea-pig ileum [EMR 0.002], (Leban, Rackur, Yamaguchi, Folkers, Bjorkroth, Rosell, Yanaihara & Yanaihara, 1979). However, [Tyr^7]sP_{1-11} (Chipkin *et al.*, 1979) and [Tyr^8]sP_{1-11} (Table 2) retain full activity on the same preparation. Furthermore, physalaemin, which has a tyrosine residue at position 8 is more active than substance P itself (Table 2). The sensitivity to manipulations of Phe^7 is further emphasized here, by the effect of *p*-amino-Phe^7 substitution in [$<$Glu^6]sP_{6-11}; that is, a very marked reduction of potency measured in all three assays (Table 2). What is particularly interesting, is the apparent differentiation by this analogue between the ileum and ^{86}Rb efflux systems. The potency in evoking ^{86}Rb efflux decreased by a factor of 34 with the substitution, whereas its spasmogenic activity only fell by a factor of about 6.

Even more striking is the departure in the EMR_{Rb} from EMR_{ileum} for eledoisin-related peptide (Erp), another hexapeptide; in this case with an isoleucine substitution for Phe^8. The EMR_{Rb} for Erp is about 18 times lower than its EMR_{ileum}. The octapeptide fragment sP_{4-11} shows a similar, though less marked tendency. It is also apparent that both Erp and [$<$Glu^6] [*p*-amino-Phe^7]sP_{6-11} differentiate between the ^{86}Rb efflux and amylase release 'systems'.

Thus, there is a suggestion, on the basis of the results of this study, that the 'sP-receptors' mediating the contraction of guinea-pig ileum and the stimulation of ^{86}Rb efflux from rat parotid slices in particular, may differ in their structural requirements for binding and/or activation. The Phe^7 and Phe^8 residue may form a particularly sensitive region of the sP molecule and further modifications in this position may assist in the future characterization of 'sP-receptors'.

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