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Rapid degradation of [^{3}H]-substance P in guinea-pig ileum and rat vas deferens *in vitro*

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1 The degradation of [^{3}H]-substance P was monitored in guinea-pig ileum and rat vas deferens. Substantial and rapid metabolism occurred in both tissues within the time course of the pharmacological responses; for example, after 1 min more than 50% of the extracted tritium from both tissues was present in the form of metabolites.

2 [^{3}H]-inulin was used to estimate the rate of equilibration of the extracellular space of guinea-pig ileum longitudinal muscle and rat vas deferens with the bath fluid; half-lives of 30 s and 5 min respectively were observed.

3 These two factors combine to give different concentrations of substance P in the biophase of the tissues. The peak concentration of substance P reached in the guinea-pig ileum longitudinal muscle was approximately five times higher than in the rat vas deferens.

4 An analogue of substance P, [^{3}H]-DiMe-C7, was found to be stable in both tissues.

5 These results are discussed in the light of the suggested existence of multiple receptors for substance P, based on agonist potency differences.

Introduction

Substance P is a potent spasmogen of a wide variety of smooth muscles, and this property has been put to use in *in vitro* bioassays to study structure-activity relationships and possible receptor heterogeneity (see review by Sandberg & Iversen, 1982). Inherent in such studies is the assumption that metabolism has a negligible influence on the potencies determined. However, recent work by Corbett, Kosterlitz & McKnight (1982) has shown this to be invalid for the enkephalins and their analogues. They were able to obtain large increases in the apparent potency of Met-enkephalin in various peripheral bioassays through the use of a mixture of peptidase inhibitors, without changing the potency of a stable analogue.

It is probable, therefore, that this also applies to substance P and analogues, and may in part explain the variations in peptide potencies and time courses of pharmacological responses on different bioassays. For example, on guinea-pig ileum the response to substance P peaks in 30 s and the EC_{50} is 1.4 nM compared with 2–3 min and 10 μM on hamster bladder (Watson, Sandberg, Hanley & Iversen, 1983). Alternatively, such differences may result from subpopulations of receptors, number of receptors, rate of equilibration of tissue extracellular space with the bath fluid, desensitization etc, or any combination of these factors.

The present study was therefore undertaken to evaluate the extent and importance of substance P degradation in *in vitro* bioassays. The two peripheral tissues chosen have been suggested to contain distinct sub-types of substance P receptors based on agonist potency differences. On guinea-pig ileum, substance P and eledoisin are equipotent and act at nanomolar concentrations, while on rat vas deferens, substance P is one hundred times less potent than eledoisin and acts in micromolar concentrations (Lee, Iversen, Hanley & Sandberg, 1982; Watson *et al.*, 1983). These putative receptor sub-types have been designated SP-P and SP-E respectively.

In addition, the stability of [pGlu^5, MePhe^8, Sar^9]-substance P (DiMe-C7) in these two bioassays was investigated. This analogue is considerably more stable than substance P to degradation by rat brain either *in vivo* or *in vitro* (Sandberg, Lee, Hanley & Iversen, 1981; Eison, Iversen, Sandberg, Watson, Hanley & Iversen, 1982) and yet has a similar potency relative to substance P (=1) on guinea-pig ileum and rat vas deferens (0.04 and 0.1 respectively; Lee *et al.* 1982) i.e. it is weakly active on SP-E systems.

Methods

In vitro *bioassays*

Tissues were set up as described by Watson *et al* (1983). Guinea-pig ileum longitudinal muscle strips were prepared by the method of Rang (1964). The EC_{50} values of substance P and its structural analogues on longitudinal muscle were similar to those found on whole ileum (unpublished observations).

The effect of peptidase inhibitors on the dose-response curve (DRC) to substance P was determined as follows. An initial DRC to substance P was obtained, the inhibitor incubated with the tissues for 30 min, and a second DRC determined with the inhibitor present throughout. Shifts were measured by comparing EC_{50} values (i.e. concentration required to produce 50% of the maximum response to substance P).

Degradation studies

Segments from the whole length of guinea-pig ileum except the duodenum were used in these experiments since the SP-P rank order of tachykinin potencies (unpublished observation) and the absolute potency of substance P is constant throughout (Holzer, Emson, Iversen & Sharman, 1981). Tissues were incubated in organ baths (2 ml) or small beakers (5 ml) containing Krebs bicarbonate (composition mM: NaCl 127, KCl 2.5, $CaCl_2$ 1.8, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 10) at 37° C for 30 min; three washes were carried out during this period. Ileal segments were then exposed to ^{3}H-peptide (1×10^{-8}M), and the tissue removed after a specific time of incubation and labelled peptides were extracted after blotting firmly on filter paper, by placing in boiling 1M acetic acid (1 ml) for 10 min, homogenizing with a Teflon/glass homogenizer and then boiling for a further 10 min. The removal of tissue from the bath to the boiling acid took 15 s. After extraction, the samples were centrifuged at 10,000 *g* for 4 min and the supernatents lyophilized. Samples were reconstituted in 10% acetic acid (100 µl) and again centrifuged at 10,000 *g* for 1 min. Portions (10 µl) were applied to LQD silica gel thin layer chromatography (t.l.c) plates.

Samples of the incubation fluid were also taken, boiled for 10 min, and applied directly to the t.l.c. plates. In addition, the appropriate peptide, its oxidised form ('O'-Met^{11}), standards of the potential labelled metabolites and control samples of ^{3}H-peptide which had been boiled for 20 min with previously homogenized and boiled tissue (100° C for 20 min) were also applied to the t.l.c. plates. The plates were then developed in ethyl acetate:pyridine: water:acetic acid (5:3:3:1). Some samples were also analysed in two other t.l.c. systems, and by high pressure liquid chromatography (h.p.l.c.): for [^{3}H]-substance P, propan-1-ol:water (7:3) and butan-1-ol:acetic acid:water (12:3:5) were used, and for [^{3}H]-DiMe-C7, propan-1-ol:water (7:3) and chloroform:methanol:ammonia (60:45:20) were used. The peptide standards were visualized using fluorescamine for substance P and fragments, while exposure to chlorine gas and then *o*-tolidine spray was used for DiMe-C7. The t.l.c. plates were then scraped at the positions of the standards and counted using liquid scintillometry. H.p.l.c. analyses of the labelled extracts were carried out with a Du Pont model 850 machine on a reverse phase µ-Bondapak C_{18} column. A gradient of 5–65% acetonitrile/50 mM H_3PO_4 over a time course of 20 min, a temperature of 35° C and a flow rate of 2 ml/min was used (Sandberg, Hanley, Watson, Brundish, Wade & Eison, 1982). Fractions were collected at 30 s intervals and analysed by liquid scintillometry.

Analysis of results of degradation studies

Over 95% of the radioactivity of ^{3}H-peptides applied directly to the t.l.c. plate co-migrated with the appropriate standard. However, this figure was reduced to approximately 80% if the labelled compounds were added to boiled tissue, presumably because of streaking over the t.l.c. plate due to the presence of interfering compounds in the tissue extracts. The recovery factors were determined individually for each t.l.c. plate, and results were corrected accordingly.

Rate of diffusion of [^{3}H]-inulin and other compounds into guinea-pig ileum and rat vas deferens

Tissue segments were incubated in small beakers with either [^{3}H]-inulin (9×10^{-7}M), [^{3}H]-phenylalanine (1×10^{-8}M), [^{3}H]-substance P (1×10^{-8}M) or [^{3}H]-DiMe-C7 (1×10^{-8}M), and were removed at various times, blotted firmly on filter paper, weighed and left in NCS tissue solubilizer at 40°C overnight. The following morning, the samples were neutralized with glacial acetic acid (50µl) and counted by liquid scintillometry. Bath samples were also taken and counted by liquid scintillometry.

Materials

[4–^{3}H–Phe^8]-substance P (specific activity, 23 Ci mmol^{-1}) was prepared and characterized as described by Allen, Brundish, Wade, Sandberg, Hanley & Iversen (1982), while [4–^{3}H–Phe^7]–DiMe–C7 (specific activity, 18.3 Ci mmol^{-1}) was prepared and

characterized as described by Sandberg *et al.* (1982). Substance P propyl ester was synthesized by Dr B.E.B. Sandberg in this laboratory (Watson *et al.*, 1983). Peptides were prepared in their oxidized forms by incubating with 0.4% H_2O_2:H_2O at room temperature for 60 min, the excess H_2O_2 being removed by lyophilization. Substance P, eledoisin and DiMe-C7 were obtained from Peninsula Lab. California. Parachloromercuribenzoate, pepstatin A, puromycin and bacitracin were purchased from Sigma Lab., Poole; Phe-Phe and Phe-Gly were purchased from Vega, Tucson, Arizona and captopril from Squibb and Sons Inc. [^{3}H]-phenylalanine (specific activity 2.48 Ci mmol^{-1}) and [^{3}H]-inulin (specific activity 33 Ci mmol^{-1}) were purchased from Amersham International Ltd., Amersham.

Results

Degradation of substance P in the bath fluid

The bath fluid in contact with segments of guinea-pig whole ileum and rat vas deferens exposed to substance P or eledoisin was sampled at intervals and bioassayed on guinea-pig ileum, and the response observed converted into the apparent concentration of substance P in the bath fluid at the time of sampling. However, the response observed might be due, in part, to the presence of bioactive fragments of substance P. The apparent rate of loss of substance P (2×10^{-9} M) from the organ bath fluid using this procedure is shown in Figure 1a (i). The semilog transformation of these data (not shown) yielded a linear slope ($P < 0.01$) and an estimated half-life of 14 min which is consistent with the removal of substance P by a 1st order process. The rate of loss of biologically active peptide from the bath fluid of the rat vas deferens exposed to eledoisin (1×10^{-8} M) or substance P (1×10^{-8} M) is shown in Figure 1a (ii), and again in semi-log transformations of these data (not shown) yielded linear slopes ($P < 0.01$ in both cases) which are not significantly different for the two peptides. The estimated half-life for substance P was 35 min. The difference in half lives between the tissues was presumably due in part to their different weights; the segments of guinea-pig whole ileum

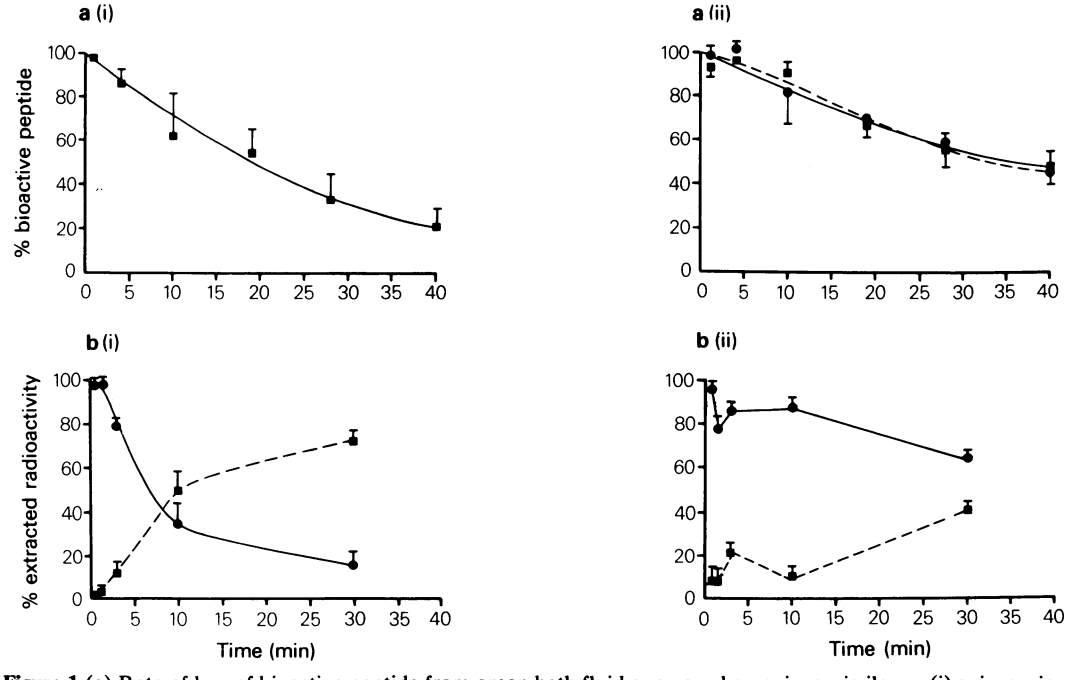

Figure 1 (**a**) Rate of loss of bioactive peptide from organ bath fluid as assayed on guinea-pig ileum: (i) guinea-pig whole ileum (approximately 10% of bath volume) (■) substance P (2×10^{-9}M); (ii) rat vas deferens (approximately 2% of bath volume) (■) substance P (1×10^{-8}M); (●) eledoisin (1×10^{-8}M). Aliquots of the bath fluid were taken and assayed on guinea-pig ileum. Each point represents the mean of 3–6 separate experiments; s.e. means shown by vertical lines. (**b**) Rate of loss of [^{3}H]-substance P (1×10^{-8}M) from bath fluid as analysed by thin layer chromatography (t.l.c.) (i) guinea-pig whole ileum approximately 10% of bath fluid; (ii) rat vas deferens approximately 2% of bath fluid. Aliquots of the bath fluid were analysed by t.l.c. (see Methods). Each point is the mean of 3 separate experiments; s.e. mean shown by vertical lines. (●) = [^{3}H]-substance P; (■) = [^{3}H]-phenylalanine. Each potential labelled intermediate accounted for less than 5% of the extracted radioactivity.

Table 1 R_F values of substance P and DiMe-C7 in different solvent systems, performed on LQD silica gel thin layer chromatography plates.

	Ethylacetate:Pyridine:Acetic acid:Water (5:5:3:1)	*Butan-1-ol:Acetic acid:Water (12:5:3)*	*Propan-1-ol:Water (7:3)*	*Chloroform:Methanol:Ammonia (60:45:20)*
Substance P	0.50	0.48	0.17	—
Oxidized substance P	0.57	0.51	0.10	—
DiMe-C7	0.93	—	0.63	1.00
Oxidized DiMe-C7	0.98	—	0.72	0.97
Phenylalanine	0.73	0.60	0.62	0.78

weighed approximately 200 mg and for the rat vas deferens approximately 40 mg.

Similar analyses were performed using [^{3}H]-substance P and the results analysed by t.l.c. The R_F values of the t.l.c. systems are shown in Table 1; all systems gave quantitatively similar results. For the rat vas deferens the rate of loss of [^{3}H]-substance P (1×10^{-8} M) from the bath fluid was not significantly different from that observed with the bioassay procedure (Figure 1b). The semi-log transformation of the data (not shown) yielded a linear slope ($P < 0.05$) and an estimated half-life of 39 min. For the guinea-pig whole ileum, the rate of loss of [^{3}H]-substance P (1×10^{-8} M) was also not significantly different from that observed with the bioassay procedure. The estimated half-life was 9.5 min although in this case there was a suggestion that the decay curve was biphasic in character (Figure 1b (i)). The slightly shorter but not significantly different, half-life estimate obtained by monitoring [^{3}H]-substance P degradation may reflect the presence of bioactive fragments which might contribute to the contraction of the guinea-pig ileum, although it must also be borne in mind that these two procedures used different concentrations of substance P. The half-life of 1×10^{-8} M substance P in the bath fluid of guinea-pig whole ileum was significantly larger ($P < 0.01$) than the half-life of loss of pharmacological responses in the tissue, which decayed with a value of 3.5 ± 1.2 min ($n = 6$, in the presence of atropine 10 μM). The reason for this difference is because of the well-documented phenomenon of substance P receptor desensitization (Lee *et al.*, 1982) as the readministration of 1×10^{-8} M substance P 5 min after the initial exposure (no washes) produced only $42 \pm 5\%$ ($n = 4$) of the original response.

The t.l.c. studies revealed that [^{3}H]-phenylalanine was the major metabolite of [^{3}H]-substance P degradation (Figure 1b) and no other metabolites were detected in individual levels greater than 5% of the total radioactivity. Thus, metabolism does take place in both tissues, but it is difficult to determine how relevant this is when evaluating potency estimates, in view of the large ratio of bath volume to tissue, and the time delay involved in diffusion between the bath fluid and the tissue.

Degradation of [^{3}H]-substance P in the biophase of guinea-pig ileum longitudinal muscle and rat vas deferens

Extensive and rapid degradation of [^{3}H]-substance P took place in the interstitial space of guinea-pig ileum longitudinal muscle and rat vas deferens within the time course of the pharmacological responses (Figure 2a; Figure 3). For example, the time to peak pharmacological response for substance P in the rat vas deferens was between 30–60 s depending on the dose, and yet after 15 s, only $53 \pm 5\%$ of the extracted radioactivity was present in the form of [^{3}H]-substance P. For the guinea-pig ileum longitudinal muscle the pharmacological response peaked after approximately 15 s and the amount of [^{3}H]-substance P recorded at this time was $54 \pm 5\%$. [^{3}H]-phenylalanine appeared to be the only metabolite formed in significant amounts; this characterization was based on the observed co-elution with the phenylalanine standard in three t.l.c. and one h.p.l.c. system (Figure 3). The large levels of phenylalanine observed are presumably due, in part, to active uptake of phenylalanine into cells (see below).

The calculated peak concentrations of [^{3}H]-substance P reached in the biophase of the guinea-pig ileum longitudinal muscle and rat vas deferens were 9 and 1.4 nM respectively (Figure 2b). This difference is presumably a function of metabolism in the interstitial space together with the rate at which the interstinial space equilibrates with the bath fluid; this latter parameter is different for the two tissues (see below). Because of these processes, half-lives of [^{3}H]-substance P in the biophase of these two tissues cannot be evaluated, and so it is not known which tissue degrades substance P more rapidly.

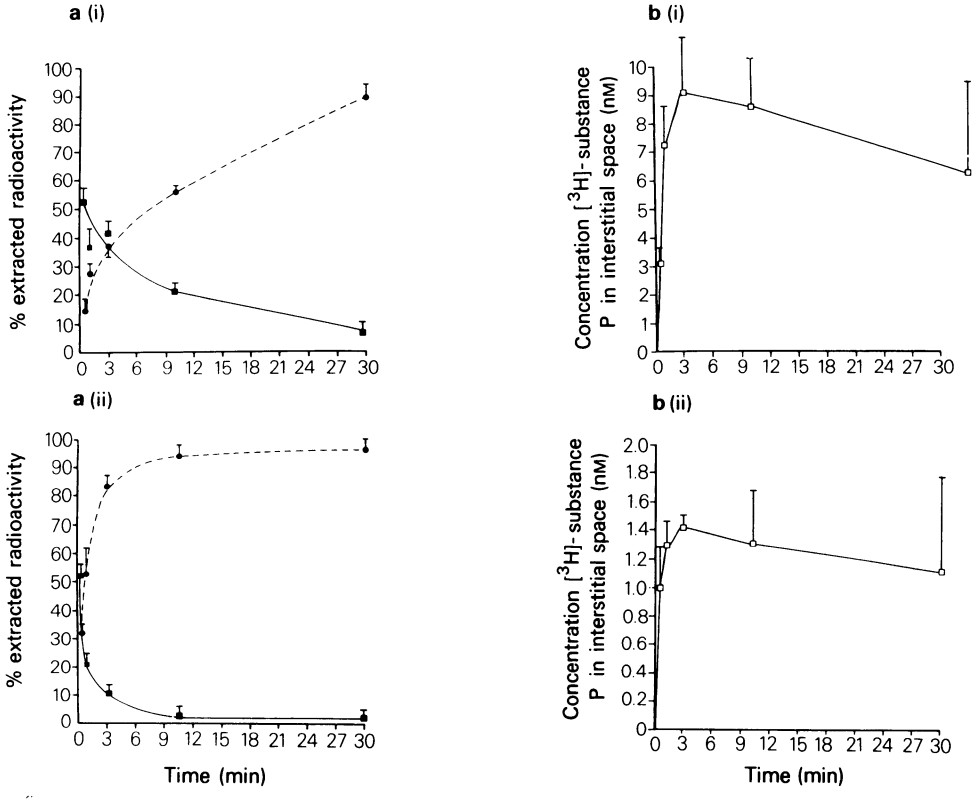

Figure 2 (**a**) Metabolism of [^{3}H]-substance P (1×10^{-8} M) by (i) guinea-pig ileum longitudinal muscle and (ii) rat vas deferens. Tissues were removed at the times indicated, blotted on filter paper, extracted for tritium and analysed by thin layer chromatography (see Methods). Each point is the mean of 3 separate experiments s.e. mean shown by vertical lines; (■) [^{3}H]-substance P; (●) [^{3}H]-phenylalanine. Each potential labelled intermediate accounted for less than 5% of the extracted radioactivity. (**b**) Concentration of [^{3}H]-substance P in the biophase of (i) guinea-pig ileum longitudinal muscle and (ii) rat vas deferens. The concentration of [^{3}H]-substance P in the tissue biophase (± s.e. mean) has been calculated as the product of,

$$[\ ] \times \frac{1}{ECV} \times U \times I$$

where [ ] = concentration of [^{3}H]-substance P (10 nM) in the bath; ECV = extracellular space volume as determined using [^{3}H]-inulin; U = tissue:medium ratio for [^{3}H]-substance P; I = amount of [^{3}H]-substance P in the extracted tritium.

Degradation of [^{3}H]-DiMe-C7 in the biophase of guinea-pig whole ileum and rat vas deferens

No detectable degradation of [^{3}H]-DiMe-C7 occurred in the biophase of segments of guinea-pig whole ileum, guinea-pig ileum longitudinal muscle (results not shown) and rat vas deferens (Figure 4a). The peak concentration of [^{3}H]-DiMe-C7 reached in the interstitial space of the rat vas deferens was approximately 7 nM (Figure 4b). This concentration is approximately 4 times greater than that for [^{3}H]-substance P and is presumably the consequence of the greater stability of [^{3}H]-DiMe-C7

Determination of extracellular space

[^{3}H]-inulin diffused into guinea-pig ileum longitudinal muscle with a half-life of approximately 30 s (Figure 5), and yielded an extracellular space estimate of 44%, which is smaller than that reported by Schnieden & Small (1971) of 60%. The semi-log transformation of these data was not linear, indicating either the inadequate removal of surface fluid or diffusion into more than one compartment. In contrast, in rat vas deferens the half-life of [^{3}H]-inulin uptake was approximately 5 min and the extracellular space estimate was 31%, which is in agreement

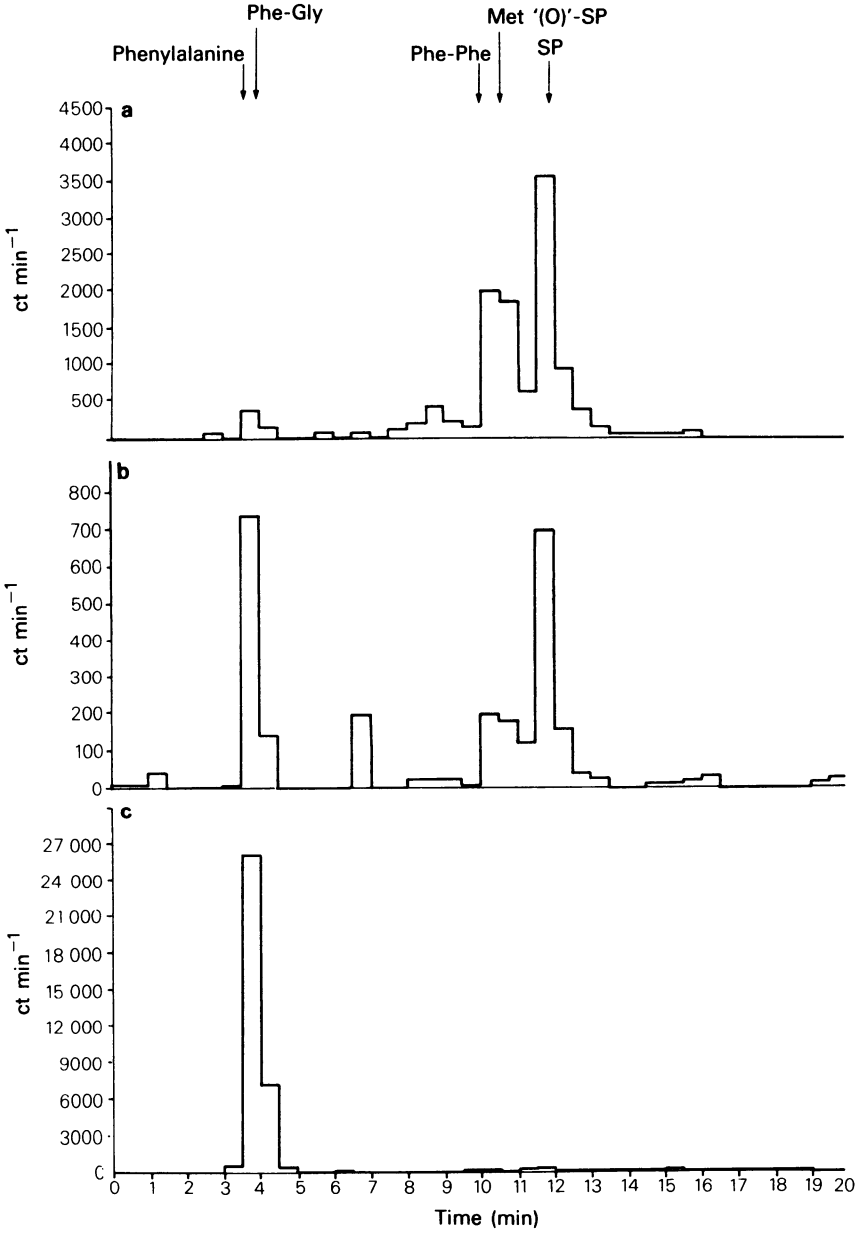

Figure 3 High pressure liquid chromatography (h.p.l.c.) analyses of [^{3}H]-substance P degradation by guinea-pig ileum longitudinal muscle. (**a**) Control: [^{3}H]-substance P was incubated with previously boiled ileum (10 min) for 20 min, extracted and analysed by h.p.l.c. (see Methods). Note the marked oxidation of [^{3}H]-substance P to 'O' Met^{11}-[^{3}H]-substance P which took place. (**b**) H.p.l.c. analysis of the extract of [^{3}H]-substance P (1×10^{-8} M) incubated with guinea-pig ileum longitudinal muscle for 1 min (see Methods). Note the presence of three main peaks which coelute with substance P, oxidized substance P and phenylalanine. Two other experiments gave similar results. (**c**) H.p.l.c. analysis of the extract of [^{3}H]-substance P (1×10^{-8} M) incubated with guinea-pig ileum longitudinal muscle for 30 min (see Methods). Note the appearance of one main peak co-eluting with phenylalanine, and the large increase in total radioactivity relative to the 1 min time point, representing the accumulation of tritium into tissue cells (see text). The absolute concentration of [^{3}H]-substance P in the tissue at 1 and 30 min is similar.

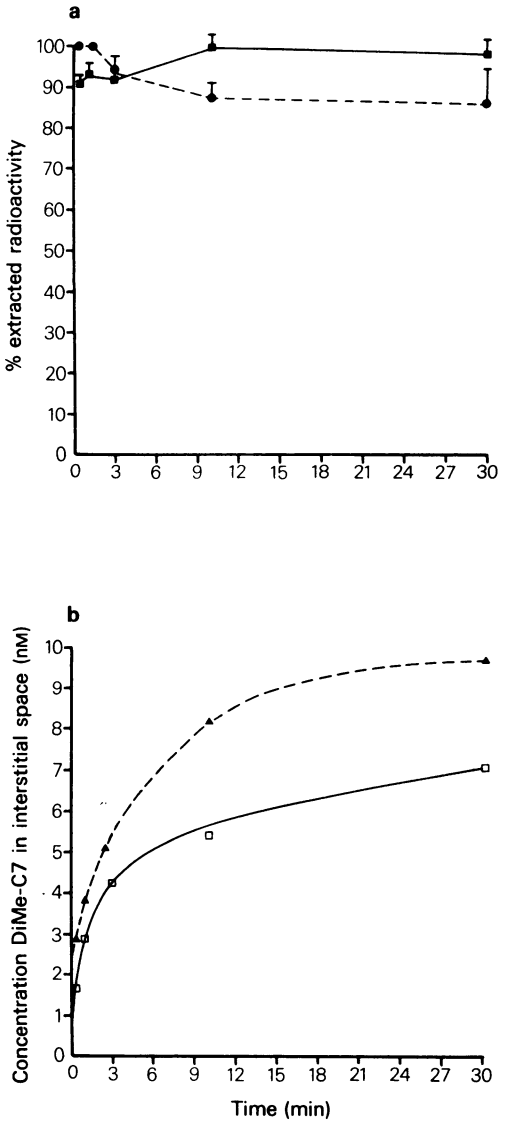

Figure 4 (**a**) Metabolism of [^{3}H]-DiMe-C7 by guinea-pig whole ileum and rat vas deferens. Tissues were removed at various times, blotted on filter paper, extracted for tritium and analysed by thin layer chromatography (see Methods). Each point is the mean of 3 separate experiments, s.e. mean shown by vertical lines. (●) Guinea-pig whole ileum; (■) rat vas deferens. (**b**) Concentration of [^{3}H]-DiMe-C7 in the interstitial space of rat vas deferens and guinea-pig whole ileum calculated as in Figure 2b. The half-life of equilibration of the biophase of guinea-pig whole ileum with the bath fluid was found to be 4.3 min (data not shown): (▲) guinea-pig whole ileum; (□) rat vas deferens.

with the value of 34% reported by Goto, Masuda & Kasuya (1976) but considerably smaller than that reported by Egan, Graham & Lewis (1976) of 80%. The semi-log transformation of these data yielded a linear slope, which is consistent with diffusion into a single compartment. Interestingly, a similar set of results were obtained with [^{3}H]-DiMe-C7 (Figure 5), suggesting that its distribution is limited to the extracellular space.

In marked contrast, [^{3}H]-phenylalanine was accumulated by both tissues to a concentration 10 times that in the medium (Figure 5). This may represent either active uptake, or the rapid metabolism of [^{3}H]-phenylalanine in the tissue so maintaining a concentration gradient. A similar picture was obtained with [^{3}H]-substance P (Figure 5), and as no uptake process for substance P has been reported, this may be the consequence of degradation to [^{3}H]-phenylalanine followed by uptake.

Effect of peptidase inhibitors on the potency of substance P

None of the peptidase inhibitors used either singly or in mixtures increased the effectiveness of substance P in guinea-pig ileum or rat vas deferens (Table 2; Figure 6).

Discussion

The present study has provided direct evidence that extensive and rapid degradation of substance P occurs in the biophase of guinea-pig ileum longitudinal muscle and rat vas deferens within the time course of the pharmacological responses. The importance of this degradation appears to be dependent on the rate at which the biophase of the tissue equilibrates with the bath fluid. In the present experiments, the half-life for [^{3}H]-inulin distribution into rat vas deferens was approximately ten times greater than that for distribution into guinea-pig ileum longitudinal muscle, and this factor combined with metabolism to produce a peak concentration of [^{3}H]-substance P in the biophase of guinea-pig ileum longitudinal muscle which was 5 times higher that that in rat vas deferens.

The implication of these observations to structure-activity work and the use of differing agonist potencies to delineate receptor sub-types is profound. If, as shown in the present study, analogues are metabolized at different rates in systems in which the agonist response is limited by metabolism, then incorrect relative as well as absolute potency estimates will be obtained. This may help to explain the inconsistency of relative potencies between peripheral bioassays (Erspamer, Erspamer & Piccinelli, 1980), and casts a good deal of doubt on their use to sub-divide recep-

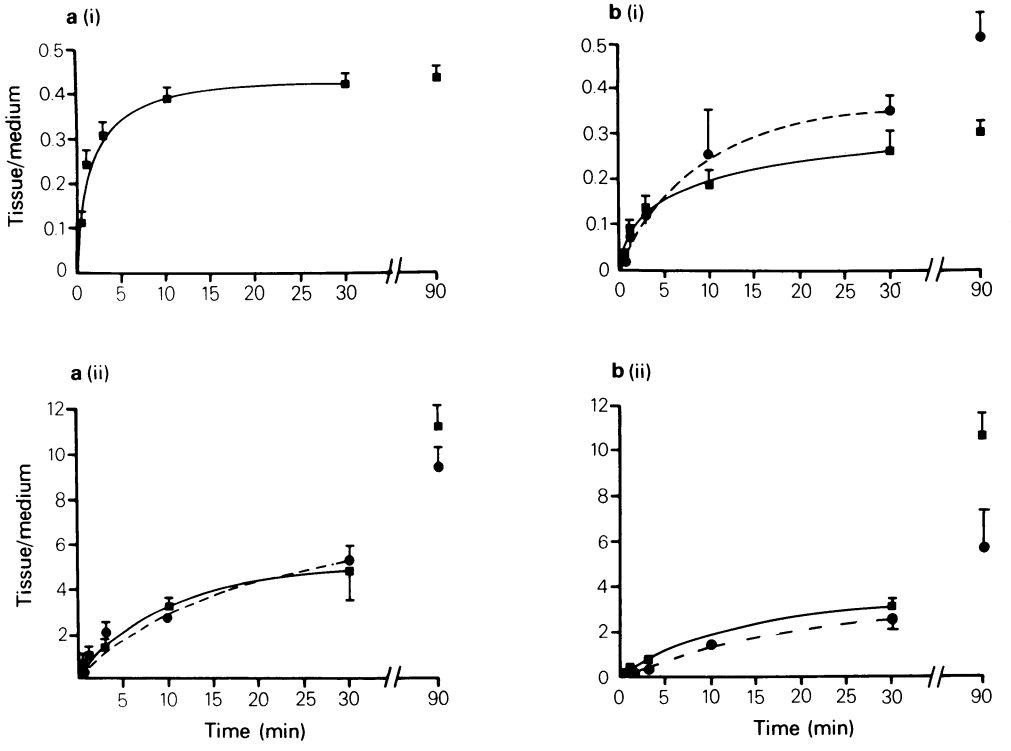

Figure 5 Accumulation of tritium into guinea-pig ileum longitudinal muscle strips and rat vas deferens. (**a**) (i) [^{3}H]-inulin uptake into guinea-pig longitudinal muscle; (ii) [^{3}H]-phenylalanine (■) and [^{3}H]-substance P (●) uptake into guinea-pig ileum longitudinal muscle. (**b**) (i) [^{3}H]-inulin (■) and [^{3}H]-DiMe-C7 (●) uptake into rat vas deferens; (ii) [^{3}H]-phenylalanine (■) and [^{3}H]-substance P (●) uptake into rat vas deferens. Tissues were incubated with the compound for various times, blotted on filter paper, weighed and left in NCS overnight (see Methods). Each point is the mean of 3–8 separate experiments; s.e. means shown by vertical lines. It is important to note that in these studies the nature of the tritium has not been identified, and so may represent possible metabolites.

Table 2 Effect of peptidase inhibitors on the responses of the guinea-pig ileum longitudinal muscle and the electrically driven rat vas deferens to substance P

Inhibitor	*Concentration*	*Inhibitory spectrum*	*Effect on the EC_{50} of substance P on guinea-pig ileum and rat vas deferens*
Bacitracin	100μM	Unknown	No apparent effect
Captopril	200μM	Angiotensin-converting enzyme	No apparent effect
Pepstatin A	14μM	Carboxyl-endopeptidases	No apparent effect
Puromycin	20μM	Amino peptidases	No apparent effect
Phe-Gly	1mM	Possible substrate inhibition	No apparent effect
Gly-Leu	1mM		
Substance P propyl ester	54μM	possible substrate inhibition	No apparent effect on rat vas deferens. This concentration elicits a maximal contraction on guinea-pig ileum (Watson *et al.*, 1983).

Dose-response curves were determined to substance P in the absence and the presence of inhibitor. Each individual experiment was performed 1–3 times.

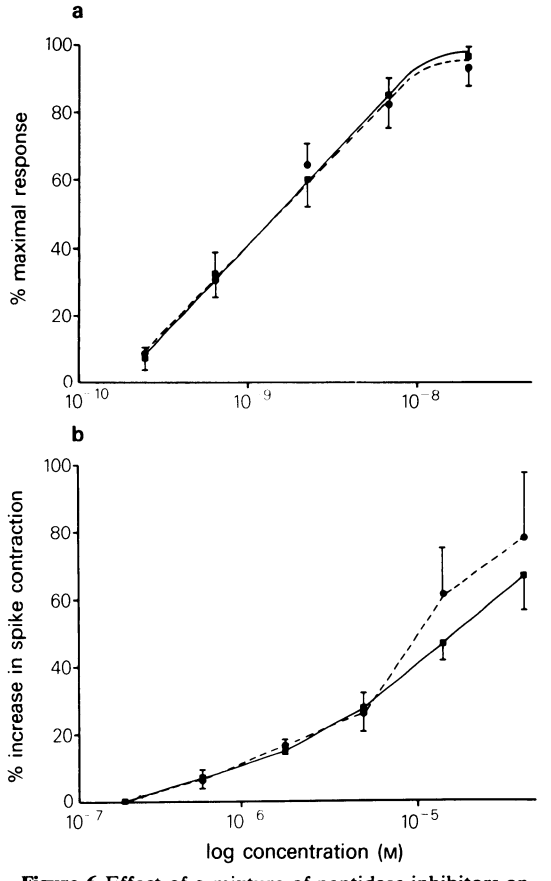

Figure 6 Effect of a mixture of peptidase inhibitors on the concentration-response curve of substance P on guinea-pig longitudinal muscle and rat vas deferens. (a) guinea-pig ileum longitudinal muscle: the inhibitors used were bacitracin (100 μM), Phe-Gly (1 mM), Phe-Phe (1 mM), puromycin (20 μM) and captopril (200 μM). Each point is the mean of 3 determinations with s.e. means shown by vertical lines. (■) Initial response curve; (●) in presence of inhibitor mixture (**b**) Rat vas deferens: the inhibitors used were bacitracin (100 μM), Phe-Gly (1 mM) Phe-Phe (1 mM), puromycin (20 μM), captopril (200 μM) and substance P propyl ester (54 μM). Each point is the mean of 3 determinations with s.e. means shown by vertical lines. (■) Initial response curve; (●) in presence of inhibitor mixture.

tors. Perhaps, therefore, this is partly the reason for the plethora of postulated substance P receptor subtypes now present in the literature (Teichberg, Cohen & Blumberg, 1981; Gater, Jordan & Owen, 1982; Lee *et al.*, 1982; Piercey, Dobry, Einspahr, Schroeder & Masiques, 1982); all these studies used as the criterion for receptor heterogeneity, differing rank orders of potency of agonists. For example, Gater *et al.*, (1982) suggested the existence of two receptors on rat parotid, based on the observation that shorter fragments of substance P are less potent relative to substance P on amylase release than ^{86}Rb efflux. However, it may be that the longer incubation periods used to monitor amylase release (5 min) than ^{86}Rb efflux (1.5 min) resulted in a greater degree of metabolism of the fragments relative to substance P, as indeed, the authors suggest.

There is obviously a need to inhibit metabolism and re-evaluate potency estimates. In the absence of knowledge of the enzymes involved, the approach adopted in the present study was to monitor the effect of potential peptidase inhibitors. No change in potency of substance P was observed, but there is no evidence that these inhibitors interfered with substance P metabolism. Further, even if inhibitors were found to block substance P degradation, it does not follow that they would be effective for related analogues; the metabolism of each analogue would have to be studied individually.

In the present study, the metabolism of substance P has been compared in two systems which have been suggested to contain different receptor sub-types, based on differences in agonist potency. The peak concentration of substance P reached in the rat vas deferens (SP-E receptors) was 20% of that reached in guinea-pig ileum longitudinal muscle (SP-P receptors), but this appears insufficient to account for the difference in EC_{50} of substance P in the two systems, 8 μM and 1.4 nM respectively (Lee *et al.*, 1982; Watson *et al.*, 1983). Further, although eledoisin is approximately 100 times more potent than substance P in rat vas deferens, and equipotent in guinea-pig ileum (Lee *et al.*, 1982), this does not appear to be due to greater metabolic stability; eledoisin and substance P were lost at similar rates from the organ bath fluid of rat vas deferens. Finally, perhaps the strongest argument that metabolism is not the cause of the agonist potency differences between these two tissues was the observation that DiMe-C7 is stable with both systems, yet has a similar pharmacological profile to substance P, i.e. it is weakly active on SP-E systems and so can be considered a selective SP-P ligand (Lee *et al.*, 1982).

In conclusion, the present study has shown that metabolism of substance P and structural analogues is a potentially important variable between different bioassays. Therefore, the sub-division of substance P receptors into SP-P and SP-E sites, must, like all receptor sub-divisions based on differing potencies of agonists, be considered as tentative until more direct evidence is obtained e.g. through the development of a selective antagonist.

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References

ALLEN, M.C., BRUNDISH, D.E., WADE, R., SANDBERG, B.E.B., HANLEY, M.R. & IVERSEN, L.L. (1982). Tritiated Peptides 13. Synthesis and biological properties of [4-^{3}H-Phe^8]-substance P. *J. med. Chem.*, **25,** 1209-1213

CORBETT, A.D., KOSTERLITZ, H.W. & McKNIGHT, A.T. (1982). Inhibition of breakdown of exogenous and endogenous enkephalins. *Br. J. Pharmac.*, **75,** 26P.

EGAN, S.M. GRAHAM, J.D.P. & LEWIS, M.J. (1976). The uptake of tritiated Δ^7-tetra-hydrocannabinol by the isolated vas deferens of the rat. *Br. J. Pharmac.*, **56,** 413-416.

EISON, A.S., IVERSEN, S.D., SANDBERG, B.E.B., WATSON, S.P., HANLEY, M.R. & IVERSEN, L.L. (1982). Substance P analog, DiMe-C7: Evidence for stability in rat brain and prolonged central actions. *Science,* **215,** 188-190.

ERSPAMER, G.F., ERSPAMER, V. & PICINELLI, P. (1980). Parallel bioassay of physalaemin and kassinin, a tachykinin dodecapeptide from the skin of the African frog *Kassina senegalensis. Naunyn-Schmiedebergs Arch. Pharmac.* **311,** 61-65.

GATER, P.R., JORDAN, C.C. & OWEN, D.G. (1982). Relative activities of substance P-related peptides in the guinea-pig ileum and rat parotid gland *in vitro. Br. J. Pharmac.*, **75,** 341-351.

GOTO, K., MASUDA, Y. & KASUYA, Y. (1976). The effect of denervation on the synchronization of contraction of the rat vas deferens. *Eur. J. Pharmac.*, **76,** 395-404.

HOLZER, P., EMSON, P.C., IVERSEN, L.L. & SHARMAN, D.F. (1981). Regional differences in the responses to substance P of the longitudinal muscle and the concentration of substance P in the digestive tract of the guinea-pig. *Neuroscience*, **6,** 1433-1441.

LEE, C.M., IVERSEN, L.L., HANLEY, M.R. & SANDBERG, B.E.B. (1982). The possible existence of multiple receptors for substance P. *Naunyn-Schmiederbergs Arch. Pharmac.*, **318,** 281-287.

PIERCEY, M.F. DOBRY, P.J.K., EINSPAHR, F.J., SCHROEDER, L.A. & MASIQUES, N. (1982). Use of substance P fragments to differentiate substance P receptors of different tissues. *Reg. Peptides,* **3,** 337-349.

RANG, H.P. (1964). Stimulant actions of volatile anaesthetics on smooth muscle. *Br. J. Pharmac. Chemother.*, **22,** 356-365.

SANDBERG, B.E.B., HANLEY, M.R., WATSON, S.P., BRUNDISH, D.E., WADE, R. & EISON, A.S. (1982). Synthesis and metabolic stability of a tritium-labelled substance P analogue. *FEBS Letts.*, **137,** 236-240.

SANDBERG, B.E.B. & IVERSEN, L.L. (1982). "Substance P" Perspective. *J. med. Chem.*, **25,** 1009-1015.

SANDBERG, B.E.B., LEE, C-M., HANLEY, M.R. & IVERSEN, L.L. (1981). Synthesis and biological properties of enzyme-resistant analogues of substance P. *Eur. J. Biochem.*, **114,** 329-337.

SCHNIEDEN, H, & SMALL, R.C. (1971). Spasmolytic effects of cadmium and zinc ions upon the guinea-pig isolated ileum preparation. *Br. J. Pharmac.*, **41,** 488-499.

TEICHBERG, V.I., COHEN, S. & BLUMBERG, S. (1981). Distinct classes of substance P receptors revealed by a comparison of the activities of substance P and some of its segments. *Reg. Peptides,* **1,** 327-333.

WATSON, S.P., SANDBERG, B.E.B., HANLEY, M.R. & IVERSEN, L.L. (1983). Tissue selectivity of substance P alkyl esters: suggesting multiple receptors. *Eur. J. Pharmac.*, **87,** 77-84.

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