J. Physiol. (1982), **330**, *pp.* 393–411
With 8 text-figures
Printed in Great Britain

THE EFFECTS OF SUBSTANCE P ON HISTAMINE AND 5-HYDROXYTRYPTAMINE RELEASE IN THE RAT

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(*Received 13 January 1982*)

SUMMARY

1. Substance P (SP) induces histamine release from isolated rat peritoneal mast cells at concentrations of 0·1–10 μM.

2. Inhibitors of glycolysis and oxidative phosphorylation prevent the release of histamine induced by SP.

3. Cells heated to 47 °C for 20 min release histamine when treated with an agent causing cell lysis but fail to release histamine in response to SP.

4. SP does not release histamine by interacting with cell-bound IgE.

5. Histamine release by SP is rapid, with more than 90 % of the response occurring within 1 min of the addition of the peptide to mast cells at 37 °C.

6. Substance P, unlike antigen–antibody or compound 48/80, does not show enhanced release of histamine when calcium (0·1–1 mM) is present in the extracellular medium but calcium increases the response to SP when the ion is added after the peptide. Extracellular calcium (0·1–1 mM), magnesium (1–10 mM) and cobalt (0·01–0·1 mM) all inhibit SP-induced histamine release when added before the peptide. Pre-treatment of the cells with EDTA (10 mM) and washing in calcium-free medium inhibits the histamine release induced by SP.

7. Histamine release induced by SP was optimum at an extracellular pH of 7·2.

8. A number of peptides structurally related to SP were examined for histamine-releasing activity. At the concentrations tested, the *N*-terminal dipeptides Lys-Pro and Arg-Pro, tuftsin, physalaemin, eledoisin, SP_{3-11}, SP_{4-11} and [*p*-Glu^6, *p*-amino Phe^7]-SP_{6-11} were all found to be inactive. The relative activities of the other peptides were:

[D-Phe^7]SP_{1-11}	>			[D-Pro^2, D-Phe^7, D-Trp^9]SP_{1-11}			>	SP_{1-11}	>		
1·8	:			1·3			:	1·0	:		
SP_{1-9}	=	SP_{1-8}	⩾	ERP	>	SP_{1-7}	>	SP_{1-6}	=	SP_{1-4}	
0·3	:	0·3	:	0·2	:	0·04	:	0·006	:	0·006	

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9. Rat basophilic leukaemia cells (RBL-2H3) fail to respond to SP at concentrations which activate rat mast cells. Release of 5-hydroxytryptamine by immunological activation of RBL cells is not changed by the presence of SP.

10. The mechanism of action of SP on mast cells and the nature of the SP receptor on mast cells is discussed in relation to SP receptors in other cell types.

INTRODUCTION

Johnson & Erdös (1973) showed that substance P (SP) released histamine from rat peritoneal mast cells and this observation has subsequently been confirmed by others (Kitada, Ashida, Maki, Fujimo, Hirai, Yasuhara, Nakajima, Takeyama, Koyama & Yajima, 1980; Erjavec, Lembeck, Florjanc-Irman, Skofitsch, Donnerer, Saria & Holzer, 1981). Substance P is present in skin (Hökfelt, Johansson, Kellerth, Ljungdahl, Nilsson, Nygårds & Pernow, 1977) and Cuello, Del Fiacco & Paxinos (1978) have shown that substance P is in sensory nerves and it has been suggested that antidromic stimulation of these nerves could bring about the release of SP in the dermis at a site distinct from the point of stimulation (Lembeck & Holzer, 1979). The SP released could have direct effects on blood flow in the skin and it could also have indirect effects on microcirculation and on cells associated with inflammatory mediators from mast cells. It has already been shown that intradermal injection of SP into human skin produces wheal and flare reactions (Hägermark, Hökfelt & Pernow, 1978; Coutts, Jorizzo, Greaves & Burnstock, 1981; Foreman & Jordan, 1981). Of particular interest is the sensitivity of the flare response induced by SP to antagonists of histamine selective for H_1 receptors (Foreman & Jordan, 1981). Reduction of tissue histamine content with compound 48/80 also reduces the vascular changes in response to SP (Lembeck & Holzer, 1979). Thus, some of the vascular changes occurring as a result of injection of SP into human skin are indirectly brought about by the release of histamine in the skin.

In this paper we seek to examine the nature of the SP receptor on mast cells and to determine some characteristics of the mechanism of histamine release in response to SP. A series of peptides structurally related to SP have been compared for their histamine-releasing activity.

A preliminary communication of part of this work has been made (Foreman & Jordan, 1981) and a related study in human skin is in progress.

METHODS

Experiments were performed on mast cells obtained from Lister hooded rats of both sexes weighing 100–200 g. All experiments except those indicated were carried out on a mixed population of peritoneal cells containing 1–5% mast cells. The method used for obtaining these cells and the stimulation of histamine release by ovalbumin as antigen has been described in detail in a previous paper (Foreman, Hallett & Mongar, 1977). Cells were divided by volume into equal aliquots, each containing about 1 μg histamine in total, in 10 ml. polypropylene conical centrifuge tubes. The cells were pre-warmed to 37 °C for 10 min before addition of SP or other reagents according to the particular experimental protocol. After initiating the histamine release process with SP or other agent the cells were allowed to remain at 37 °C for 10 min to ensure completion of the release process. 3 ml. cold (4 °C) Tyrode solution was then added to stop any further reaction. Cells and supernatants were separated by centrifugation at 1000 g for 5 min. The pellets were resuspended in 4 ml. Tyrode solution and heated to boiling for 5 min to release residual histamine.

In some experiments mast cells were separated from other peritoneal cells either by density gradient centrifugation over human serum albumin as previously described (Foreman, Hallett & Mongar, 1977) or by separation over metrizamide. In the latter method, 2 ml. peritoneal washings were placed onto 5 ml. of a solution of metrizamide (22·5 % in Tyrode solution). Centrifugation for 15 min at 700 *g* yielded a pellet containing mast cells with a purity of better than 90 % and a Trypan Blue exclusion of 96 %. The supernatant was discarded and the pellet washed once with 45 ml. Tyrode containing fetal calf serum (20 %). The cells were finally resuspended in the Tyrode solution appropriate to the experiment.

Histamine assays were performed by the fluorimetric method of Shore, Burkhalter & Cohn (1959) without extraction of histamine. Histamine release was expressed as a percentage which was calculated as described previously (Foreman, Hallett & Mongar, 1977) and histamine release occurring in the absence of SP or any other stimulus which is referred to as spontaneous histamine release, has, unless otherwise stated, been subtracted from those releases observed in the presence of SP or other histamine-releasing agent as previously described.

The source of rat basophilic leukaemia cells (RBL-2H3) and the method of measuring the release of [^{3}H]5-hydroxytryptamine from them has been described in detail in previous publications (Taurog, Mendoza, Hook, Siraganian & Metzger, 1977; Siraganian, Kulczycki, Mendoza & Metzger, 1975). For these experiments RBL cells were passively sensitized by incubation of 6×10^7 cells in a volume of 5–15 ml. with mouse anti-dinitrophenol (DNP) IgE (3 μg/ml.) for 1·25 hr. After washing twice in Tyrode solution the cells were resuspended to give $2{\cdot}0 \times 10^6$ cells/ml. for use in the experiments. The agent used to trigger these cells was DNP coupled to bovine gamma globulin ($DNP_{15}BGG$) with 15 hapten groups per mole protein.

Viability of cells was assessed by Trypan Blue exclusion. A mast cell suspension of 0·1 ml. and 0·9 ml. 4 % Trypan Blue were incubated together for 5 min and the number of cells excluding the dye was counted under 100 × magnification.

The medium used for incubating the cells was based on Tyrode solution and had the following composition: NaCl, 137 mM; KCl, 2·7 mM; Na H_2PO_4, 0·4 mM; Hepes (4-(2 hydroxyethyl)-1-piperazine ethane sulphonic acid), 20 mM; glucose, 5·6 mM. Unless otherwise stated, the pH was 7·6. Where necessary, $CaCl_2$, $MgCl_2$ or $CoCl_2$ was added to give the desired concentration.

Substance P and related peptides were dissolved in 0·1 % or 1 % acetic acid to give concentrated stocks of about 1 mM. The stocks were stored at −25 °C in aliquots sufficient for one experiment. For the experiment, the aliquot was thawed, neutralized with NaOH and diluted in the modified Tyrode solution used for the experiment. The small quantities of sodium acetate did not affect the mast cell responses to antigen–antibody stimulation.

A23187 and nigericin were dissolved in ethanol to give stock solutions about 6 mM. Dilutions from these stocks into Tyrode solution were made; the final alcohol concentration did not affect mast cell response to antigen–antibody stimulation.

Sources of peptides. SP_{1-11}, SP_{4-11}, physalaemin, [Tyr^8] SP_{1-11} and eledoisin-related peptide were obtained from Beckman, Geneva. Eledoisin, [D-Pro^2, D-Phe^7, D-Trp^9]SP_{1-11} and SP_{3-11} were obtained from Peninsula Laboratories. Tuftsin was obtained from Sigma, U.K. [D-Phe^7]SP_{1-11}, [*p*-Glu^6, *p*-amino Phe^7]SP_{6-11}, and SP_{1-9} were synthesized by Mr N. N. Petter, Imperial Chemical Industries, Pharmaceutical Division, Alderley Edge. SP_{1-4}, Arg-Pro and Lys-Pro were synthesized starting from the protected Boc-Arg (NO_2)-Pro-Lys(Boc)-Pro-OH peptide. Protecting groups were removed by liquid HF and purification was achieved by ion exchange chromatography on carboxymethylcellulose as previously described (Bienert, Köller, Wohlfeil, Mehlis, Bergmann & Niedrich, 1979). SP_{1-6}, SP_{1-7}, SP_{1-8} were synthesized according to the method described by Chipkin, Stewart, Sweeney, Harris & Williams (1979). SP_{1-6}, SP_{1-7} and SP_{1-8} were synthesized as the free acids whereas SP_{1-11} and SP_{1-9} were the amidated forms.

In the text, EDTA refers to the sodium salt of ethylenediaminetetraacetic acid and EGTA to the sodium salt of ethylene glycol bis(β-aminoethylether)*N*,*N*′-tetraacetic acid.

Human serum albumin and metrizamide were obtained from Sigma. Purified rat IgE was obtained from a myeloma kindly supplied by Dr Henry Metzger, National Institutes of Health, U.S.A.

All other chemicals and reagents were of the Analar grade or the highest quality available.

RESULTS

Histamine release by substance P

Substance P releases histamine and 5-hydroxytryptamine from rat peritoneal mast cells in a concentration-dependent manner with a threshold concentration of about 0·1 μM (Figs. 1 and 7). Concentrations greater than 16 μM have not been tested and this concentration still appeared to produce a submaximal response. At these concentrations SP released histamine from either mixed peritoneal cells or from cell preparations purified on either albumin or metrizamide containing more than 90%

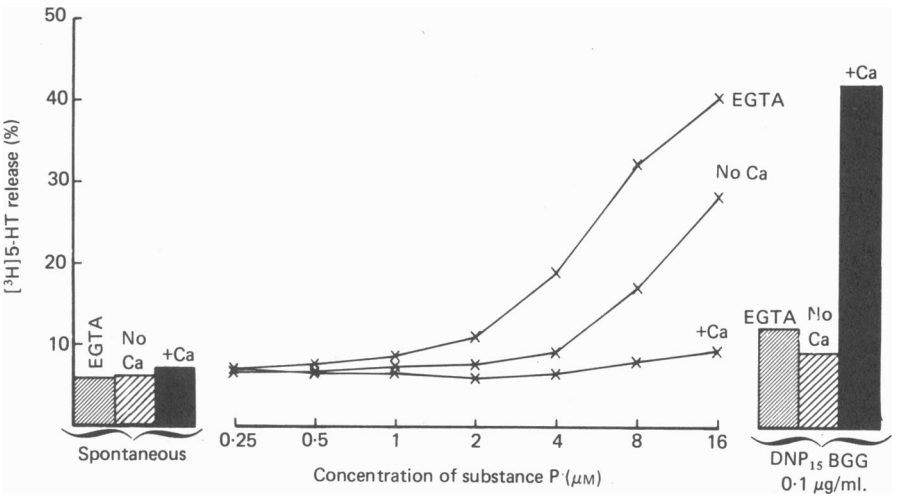

Fig. 1. Concentration–response relationship for substance P and the release of [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT) from rat mast cells purified on a metrizamide solution and passively sensitized with mouse (anti-dinitrophenol) IgE. Release of [^{3}H]5-HT was measured in the presence of EGTA (0·1 mM) in a nominally calcium-free medium or in the presence of calcium (1·8 mM). Release of [^{3}H]5-HT induced by $(dinitrophenol)_{15}$ bovine gamma globulin, a polyvalent hapten, is also shown for comparison. Each point or column is the mean of duplicate determinations in a single experiment.

mast cells. Substance P did not affect the viability of the mast cells as tested by Trypan Blue exclusion. In the absence of SP, 95·2% of the population of purified mast cells excluded Trypan Blue, and cells stimulated with SP (5 μM), which released 18·3% of their histamine, 96·7% of the population excluded the dye.

Inhibition of glycolysis and oxidative phosphorylation

Fig. 2 shows that removal of glucose from the medium produced a small inhibition of the response of the cells to SP. However, in the absence of glucose, inhibition of oxidative metabolism with either cyanide, antimycin A or oligomycin totally abolished the response to SP. Glucose (5·6 mM) reversed the inhibitory effect of cyanide and antimycin A, and the release obtained in response to SP when glucose was added in the presence of these inhibitors was greater than that obtained in their absence. Similar results were obtained with antigen stimulation of the mast cells.

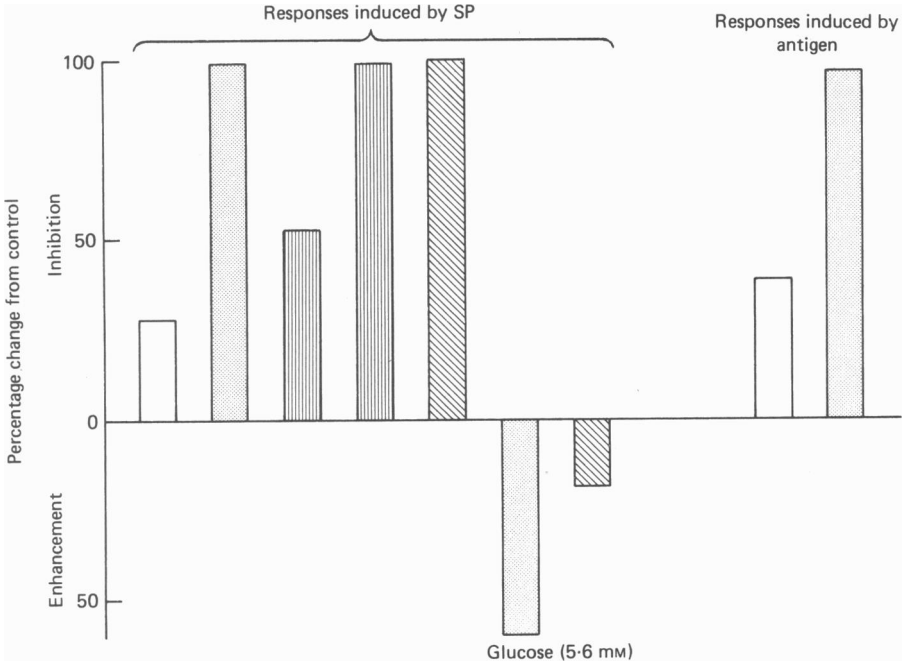

Fig. 2. Effect of inhibitors of oxidative phosphorylation on histamine release induced by SP or antigen (ovalbumin) in the presence and absence of glucose. The results are the means from a series of seven experiments. In the presence of glucose (5·6 mM), the histamine release induced by SP (2 μM) was 18 % and that induced by ovalbumin (1 g/ml.) was 45 %. Changes induced by the various compounds are expressed as percentages of these control histamine releases. □ control, no inhibitor; ▨ antimycin A (1 μM); ▥ cyanide (50 and 100 μM); ■ oligomycin (0·3 μM).

Effect of heating cells to 47 °C

Heating cells to 47 °C for 20 min denatures proteins and inactivates the cellular metabolic processes. The cells do not, however, leak histamine under these conditions. Table 1 shows that a lytic substance such as Triton X-100 or the ionophore, nigericin, release histamine from such heat-treated cells but substance P and the ionophore A23187 fail to do so.

Effect of IgE on histamine release induced by substance P

There is some evidence that SP and similar peptides may exist in fibrillar form in solutions (Perry, Oakley, Candy & Perry, 1981). It is, therefore, conceivable that SP could interact with cell-bound IgE to cross-link it in the same manner that antigens cross-link cell-bound IgE to initiate histamine secretion (see Foreman, 1981*b* for review). To test this hypothesis, mast cells were treated with either SP or anti-IgE in the presence of a large excess of soluble IgE in the incubating medium. Fig. 3 shows that the added IgE totally abolished the histamine release in response to anti-IgE but failed to affect the histamine release induced by substance P.

TABLE 1. The effect of pre-treating peritoneal cells for 20 min at 47 °C on histamine release induced by substance P

Histamine release induced by	Histamine release (%)			
	Cells pre-incubated at 37 °C		Cells pre-incubated at 47 °C	
	a	b	a	b
SP 2·5 μM	16·2	8·5	0·3	0·5
5·0 μM	28·6	23·2	1·7	0
10·0 μM	35·7	35·5	0	0
A23187 0·6 μM + Ca^{2+} 1 μM	18·2	16·2	2·4	0
Nigericin 4 μM	16·1	27·7	28·5	50·7
Triton X-100 1 mg/ml.	84·5	89·2	82·2	87·9

a and b refer to separate experiments.

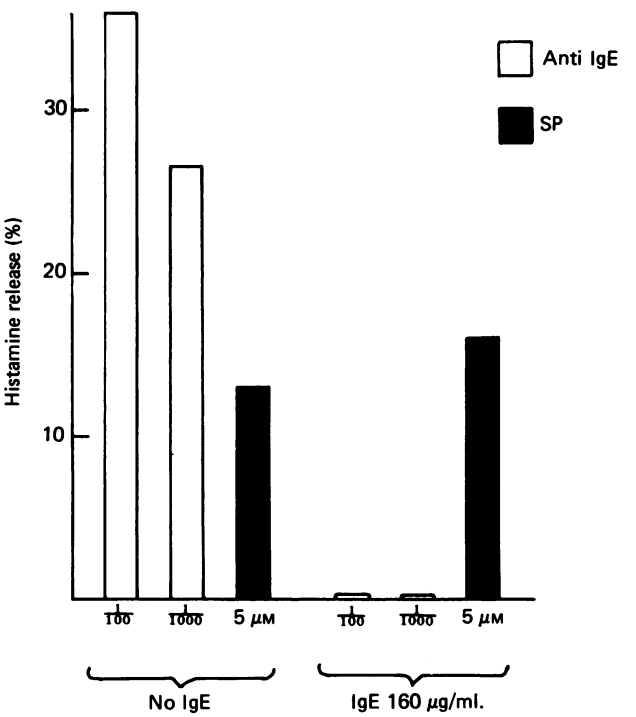

Fig. 3. The effect of excess soluble rat IgE on histamine release induced by anti-IgE serum, 1/100- or 1/1000-fold dilutions (□) or SP (5 μM) (■). Histamine releases to anti-IgE or SP are shown in the absence of IgE or in the presence of rat IgE (160 μg/ml.) added 5 min before the anti-IgE or SP. Each column represents the means of duplicate determinations in a single experiment and similar results were obtained in an identical experiment.

Time course of histamine release induced by substance P

Histamine release from mast cells induced by SP is rapid, with more than 90% of the response occurring within 15 sec of the addition of SP (Fig. 4).

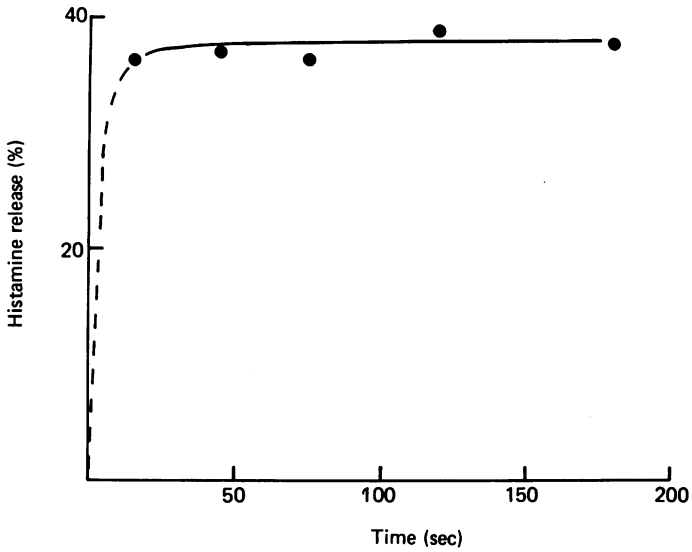

Fig. 4. Rate of histamine release induced by SP (5 μM) at 37 °C. Histamine release was stopped at the times indicated after the addition of SP by quenching the reaction with 5 ml. ice-cold Tyrode solution. Results are the means of duplicate determination in a single experiment in a series of three similar experiments.

Effect of calcium, magnesium and cobalt

Histamine release induced by SP takes place in the absence of any added extracellular calcium. Treatment of cells with EDTA (10 mM) or a nominally calcium-free medium, followed by washing, produced an increased response to substance P in the presence of calcium (1 mM) (Table 2*A*). However, treatment of cells with EDTA or a calcium-free medium, followed by restoration of the calcium concentration to 1 mM without washing, resulted in a reduced response to substance P in the presence of calcium (Table 2*B*). It can be seen that antigen- or A23187-induced histamine releases were dependent on extracellular calcium in both conditions *A* and *B* of Table 2. Compound 48/80-induced histamine release was increased in the presence of calcium after calcium-free pre-treatment and washing of the cells (Table 2*A*) but there was little or no increase in histamine release by calcium when no washing of the cells was performed (Table 2*B*). Addition of calcium to the extracellular medium before SP inhibited the response to SP over the concentration range 0·1–1 mM (Fig. 5). Magnesium (1–10 mM) and cobalt (0·01–0·1 mM) similarly inhibited SP-induced histamine release (Fig. 5). However Table 3 shows that if calcium (1 mM) was added to the cells 10 min after the addition of SP to a

TABLE 2. Effect of calcium-free medium and EDTA on histamine release induced by compound 48/80 (0·6 μg/ml.), A23187 (0·6 μM), ovalbumin (1 μg/ml.) and substance P (2 μM)

	Histamine release (%) in medium containing:			
	No Ca^{2+} (1)	Ca^{2+} (1mM) (2)	No Ca^{2+} (3)	Ca^{2+} (1 mM) (4)
(A) Cells washed once between 2 hr pre-incubation and resuspension for histamine release				
Compound 48/80	17·6±0·4	49·3±4·3	25·0±7·1	54·5±8·6
A23187	5·0±1·8	26·8±11·0	3·0±0·2	26·4±7·1
Ovalbumin	7·4±2·3	22·7±5·2	1·7±0·7	25·2±5·2
SP	5·8±2·0	15·8±7·2	4·7±1·5	18·3±2·0
(B) Cells not washed after 2 hr pre-incubation; Ca^{2+} added to achieve final required free Ca^{2+} concentration for histamine release				
Compound 48/80	31·3±6·8	37·9±13·7	46·4±5·6	40·9±13·4
A23187	7·4±1·3	73·5±5·1	4·2±2·0	69·9±7·8
Ovalbumin	7·5±1·6	26·4±3·0	9·3±1·0	30·8±7·1
SP	16·4±7·3	2·5±2·0	26·8±2·8	3·6±1·4

Each value is mean±S.E. from three experiments.

* (1) Cells pre-incubated for 2 hr 4 °C in Ca^{2+}-free medium. (2) Cells pre-incubated for 2 hr 4 °C in Ca^{2+}-free medium. (3) Cells pre-incubated for 2 hr 4 °C in Ca^{2+}-free medium + EDTA (10 mM). (4) Cells pre-incubated for 2 hr 4 °C in Ca^{2+}-free medium + EDTA (10 mM).

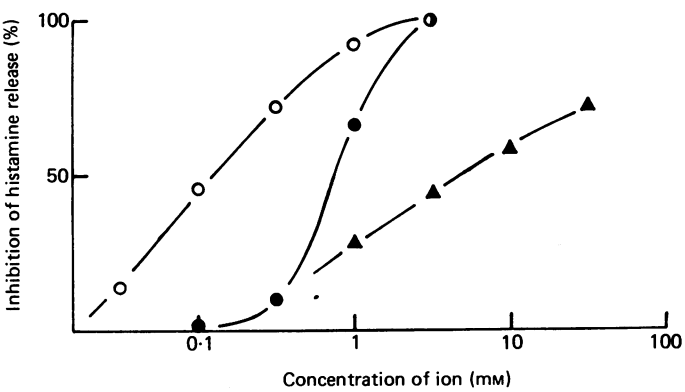

Fig. 5. Concentration-effect relationships for divalent cations and the inhibition of histamine release induced by SP (5 μM). Histamine release obtained in the absence of divalent cations was 33 %. Each point is the mean of duplicate determinations from a single experiment in a series of four similar experiments. ○–○, cobalt; ●–●, calcium; ▲–▲, magnesium.

calcium-free medium, histamine release was increased by the calcium. It should be pointed out that 10 min after the addition of SP, the initial release of histamine brought about by this peptide was over (Fig. 4).

TABLE 3. The effect of adding calcium (1 mM) before or after substance SP (5 μM)

	Histamine release (%)			
Addition	a	b	c	d
SP alone	27·1	19·8	32·6	20·1
SP–Ca^{2+}	37·5	29·5	43·8	22·9
Ca^{2+}–SP	—	—	23·3	10·5

The interval between adding either the calcium or the substance P and the second addition of either substance P or calcium was 10 min.

a, b, c and d refer to different experiments.

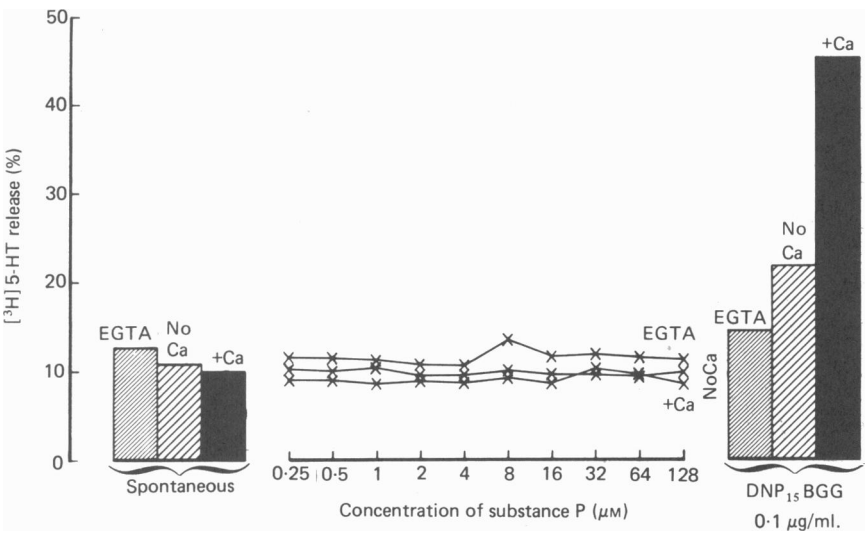

Fig. 6. Effect of substance P and polyvalent hapten $(dinitrophenol)_{15}$ bovine gamma globulin on rat basophilic leukaemia cells (RBL-2H3) passively sensitized with mouse (anti-dinitrophenol) IgE. Release of [^{3}H]5-hydroxytryptamine was measured in the presence of EGTA (0·1 mM) in a nominally calcium-free solution or in the presence of calcium (1·8 mM). Each column or point is the mean of duplicate determinations in a single experiment. Three other experiments gave similar results.

Effect of extracellular pH

The effect of changing the extracellular pH on the response to SP was investigated using HEPES as the buffer and the pH was adjusted before the experiments with sodium hydroxide or hydrochloric acid. Measurement of the pH after incubation of the cells for the release of histamine revealed no change from the pre-incubation value. In four separate experiments the optimum pH for release was found to be $7·2 \pm 0·1$ (mean $\pm$ S.E. of mean).

TABLE 4. Structure of substance P and some similar peptides

	1	2	3	4	5	6	7	8	9	10	11	
Substance P	Arg	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met	NH_2
Eledoisin	*p*-Glu	Pro	Ser	Lys	Asp	Ala	Phe	Ile	Gly	Leu	Met	NH_2
Eledoisin-related peptide	—	—	—	—	—	Lys	Phe	Ile	Gly	Leu	Met	NH_2
Physalaemin	*p*-Glu	Ala	Asp	Pro	Asn	Lys	Phe	Tyr	Gly	Leu	Met	NH_2
[*p*-Glu *p*-amino-phe7] SP_{6-11}	—	—	—	—	—	*p*-Glu	*p*-amino Phe	Phe	Gly	Leu	Met	NH_2
Tuftsin	Thr	Lys	Pro	Arg	—	—	—	—	—	—	—	—

5-Hydroxytryptamine release from RBL cells

When challenged with the polyvalent hapten, $DNP_{15}BGG$, RBL cells sensitized with mouse anti-DNP IgE release 5-hydroxytryptamine in a dose-related manner at $DNP_{15}BGG$ concentrations of 1–81 ng/ml. Optimum concentrations of $DNP_{15}BGG$ produced about 75% release of total cell 5-hydroxytryptamine content.

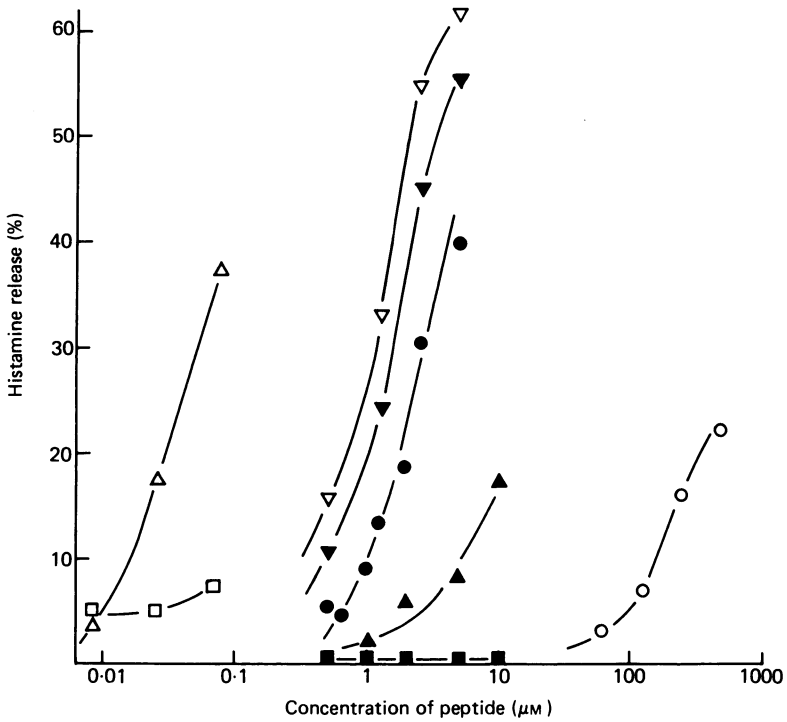

Fig. 7. Concentration–response relationships for histamine release induced by a variety of peptides. No calcium was added to the extracellular medium. Each point is the mean of two or more determinations. △–△ poly-L-lysine (molecular weight 30,000–70,000); □–□, succinylated poly-L-lysine (molecular weight 30,000–70,000); ▽–▽, [D-Phe^7]SP_{1-11}; ▼–▼, [D-Pro^2, D-Phe^7, D-Trp^9]SP_{1-11}; ●–●, SP_{1-11}; ▲–▲, eledoisin-related peptide; ■–■, eledoisin; ○–○ *N*-terminal tetrapeptide of substance P.

Substance P at concentrations from 0·33–128 μM failed to elicit 5-hydroxytryptamine release from RBL cells which did secrete in response to $DNP_{15}BGG$. Substance P (0·33–128 μM) also failed to change the dose–response curve to $DNP_{15}BGG$ (Fig. 6).

5-hydroxytryptamine release in response to $DNP_{15}BGG$ is dependent on extracellular calcium (Fig. 6), but SP failed to release 5-hydroxytryptamine from RBL cells either in the presence or absence of extracellular calcium (Fig. 6).

Substance P-related peptides

Table 4 shows the structure of SP and some peptides which are structurally related to SP. The activity of these and other peptides, relative to SP, have been compared and Fig. 7 shows the results of one such a comparison. Table 5 summarizes the relative activities of all the peptides examined. Two peptides have been found to be more active than SP (Fig. 9). (D-Phe^7)SP_{1-11} is 1·8 times more active than SP and (D-Pro^2, D-Phe^7, D-Trp^9)SP_{1-11} is 1·3 times more active than SP.

Peptides lacking the *N*-terminal Arg-Pro-Lys-Pro sequence of SP did not release histamine in the concentration ranges tested (Table 5). Thus, SP_{3-11}, SP_{4-11}, physa-

TABLE 5. The relative activity of some peptides structurally related to substance P

Peptide	Concentration range tested (μM)	Potency relative to substance P*
SP_{1-11}	0·1–10	1·0
SP_{1-9}	0·7–20	0·3 ± 0·06
SP_{1-8}	2·5–20	0·26 ± 0·003
SP_{1-7}	20–80	0·04 ± 0·005
SP_{1-6}	40–500	0·0057 ± 0·007
SP_{1-4}	62·5–500	0·0064 ± 0·0023
SP_{3-11}	0·6–10	not active†
SP_{4-11}	0·6–20	not active
Arg-Pro	6·25–50	not active
Lys-Pro	6·25–50	not active
[D-Phe^7]SP_{1-11}	0·05–10	1·83 ± 0·56
[D-Pro^2, D-Phe^7, D-Trp^9]SP_{1-11}	0·6–10	1·33 ± 0·13
Physalaemin	0·07–2	not active
Eledoisin	0·5–20	not active
Eledoisin-related peptide	0·1–10	0·18
[Tyr^8]SP_{1-11}	1–5	0·36
Tuftsin	0·1–10	not active
[*p*-Glu^6, *p*-amino Phe^7]SP_{6-11}	1·4–38·3	not active

* Potency is the ratio of the concentration of substance P to the concentration of peptide required for equal histamine releases. The average value in each experiment was calculated over the response range achieved in the experiment when the dose–response curves were not parallel. Each value is the mean of two or more experiments. Values of S.E. are given for $n > 3$.

† Not active means that a release of histamine greater than spontaneous levels was not achieved over the concentration range tested.

laemin, [*p*–Glu^6, *p*-amino Phe^7]SP_{6-11} and eledoisin were all inactive. Eledoisin-related peptide has an *N*-terminal lysine and was 0·18 times as active as SP_{1-11} (Fig. 7). It is interesting that the *N*-terminal tetrapeptide by itself was much less active than SP, having on average only 0·0064 times the activity (Fig. 7). Also, SP_{1-9} and SP_{1-8} were only 0·3 times as active as SP_{1-11}.

It is important to note that SP_{1-7} and SP_{1-6} have at least an order of magnitude less activity than SP_{1-11}, SP_{1-9}, and SP_{1-8} (Fig. 8). The activity of SP_{1-6} is not different from that of the *N*-terminal tetrapeptide SP_{1-4}. The two dipeptides Arg-Pro and Lys-Pro were inactive, as was the tetrapeptide tuftsin.

Substitution with tyrosine at position 8 reduced the activity of substance P to 0·36 of that of the parent peptide.

Poly-lysine has previously been shown to be a histamine releasing agent (Padawer, 1970; Foreman & Lichtenstein, 1980; Ennis, Pearce & Weston, 1980) and its concentration-effect curve is also shown in Fig. 7. Ablation of the basic character of the poly-lysine molecule by succinylation removes its biological activity in mast cells (Fig. 7).

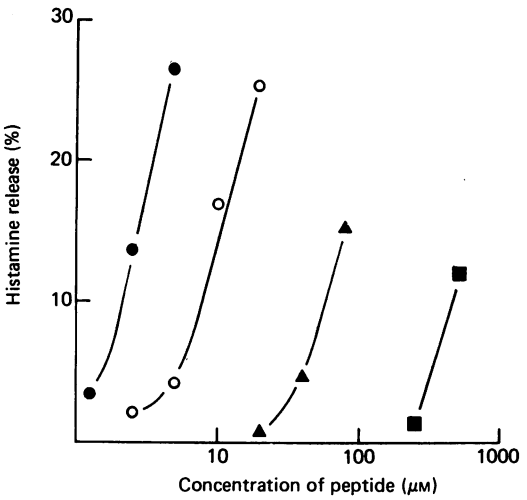

Fig. 8. Comparison of the histamine releasing activity of three fragments of SP with decreasing numbers of *C*-terminal amino acis. ●–●, SP_{1-11}; ○–○, SP_{1-8}; ▲–▲, SP_{1-7}; ■–■, SP_{1-6}.

DISCUSSION

Histamine release induced by substance P from isolated rat mast cells was first reported by Johnson & Erdös (1973) and has subsequently been confirmed by others (Kitada *et al.* 1980; Foreman & Jordan, 1981; Erjavec *et al.* 1981). In this paper we have identified some aspects of the mechanism of action of SP on isolated rat mast cells. The capacity to release histamine from mast cells has long been identified as a property of basic molecules (Paton, 1957; Jasani, Kreil, Mackler & Stanworth, 1979) and hence it could have been predicted that SP would release histamine since it is a basic peptide, having arginine and lysine residues at the 1 and 3 positions respectively (Table 4). However, substance P has an action on rat mast cells which is notably different from that of the polyamine histamine-releasing-agent, compound 48/80, and the structure–activity relationships reported in this paper show that the amino acid sequence Arg-Pro-Lys-Pro is not the only part of the molecule to influence the histamine-releasing activity of SP. Features of both the *N*-terminal and *C*-terminal sequences of the peptide are important for its histamine releasing action.

The results show a clear relationship between the magnitude of histamine secretion and the concentration of SP. A maximum effective concentration of SP has not been determined and it is important to point out that the concentrations of SP required to activate histamine release are about 1000-fold greater than those which stimulate the smooth muscle of the gastrointestinal tract or stimulate secretion from the parotid gland (Gater, Jordan & Owen, 1982).

Treatment of mast cells with SP does not reduce their viability as assessed by Trypan Blue exclusion studies. Furthermore, SP-induced histamine release requires intact metabolic processes for ATP generation within the cell, since inhibition of glycolytic and oxidative metabolism within mast cells abolished the response to SP. Additional evidence that SP cannot induce histamine release unless the biochemical processes of the cell are intact was provided by the experiments which demonstrated that heating mast cells to 47 °C for 20 min prevented the response to the ionophore A23187 and SP, whilst not inhibiting the histamine release induced by lytic agents such as the detergent Triton X-100 or the ionophore, nigericin. The requirement for metabolic processes generating ATP places the SP-induced release process in a category analagous to that initiated by an antigen–antibody reaction (Chakravarty, 1968), compound 48/80 (Peterson, 1974) or the calcium ionophore, A23187 (Foreman, Mongar & Gomperts, 1973).

Histamine release evoked by SP also has a time course which is as fast as, or faster than, that of the release process initiated by compound 48/80 or antigen (Uvnäs & Thon, 1961; Mongar & Svec, 1972). Histamine release induced by substance P is essentially complete within 15 sec of adding it to the mast cells at 37 °C.

The role of calcium in histamine secretion induced by SP differs from its involvement in the actions of compound 48/80, antigen or ionophore A23187. Addition of calcium ions (0·1–1 mM) to the incubation medium before challenge of the cells with SP inhibited the response in a concentration-dependent manner. However, it is well established that addition of these concentrations of calcium before antigen, compound 48/80 or ionophore A23187 increases the histamine release obtained with these agents (Uvnäs & Thon, 1961; Foreman & Mongar, 1972, 1973; Foreman *et al.* 1973). Magnesium and cobalt also inhibited the histamine release induced by SP, cobalt being more effective than calcium and magnesium being less effective than either of these ions.

The role of calcium in SP-induced histamine release was studied further in experiments in which the cells were pre-treated in a nominally calcium-free medium. The experiments with EDTA showed that if the mast cells were pre-treated with EDTA or a nominally calcium-free solution, washed and exposed to substance P, then the histamine release induced by SP was increased by the presence of calcium (1 mM) added to the incubating medium. Histamine release induced by antigen, compound 48/80 and A23187 behaved similarly. However, if the cells were exposed to EDTA or calcium-free medium and not washed before the extracellular calcium was raised to 1 mM then the response to SP was reduced by calcium but the responses to antigen and A23187 were still increased by calcium. Compound 48/80 responses were little changed by this experimental procedure.

One interpretation of this is that calcium ions in the cell membrane prevent SP binding. Pre-incubation of the cells in calcium-free medium or EDTA followed by washing could be sufficient to remove calcium, allow increased SP binding and hence increased secretion. However, it is also necessary to assume that SP relies upon the mobilization of internal stores of calcium to induce histamine secretion and that these stores are also depleted by incubation and washing in calcium-free medium. Thus, after such treatment, the release induced by SP appears dependent upon extracellular calcium (Table 2*A*, columns 2 and 4). If the cells are not washed after pre-treatment

in calcium-free medium, the calcium which prevents the binding of SP appears not to be removed and histamine release is limited. Further addition of calcium under these conditions produces inhibition of SP-induced histamine release (Table 2*B*, columns 2 and 4). A23187 and antigen are known not to require calcium for them to gain access to the membrane, and the release in response to these agents is always calcium-dependent (Table 2*A* and *B*). Compound 48/80 binding may be affected by calcium since it is a positively charged molecule, but it behaves differently from SP in these experiments. Such a difference may reflect difference of binding properties at the mast-cell membrane rather than differences in the mechanisms of histamine release.

The argument above is supported by the observation that SP-induced histamine release is increased by calcium when the calcium is added after the SP, even when the calcium is added after the SP has already induced histamine release. Assuming first that SP, once bound to the mast cell, releases calcium from intracellular stores and also makes the cell membrane permeable to calcium, and secondly, that calcium released intracellularly, or which enters from outside, induces histamine release, then it is reasonable to expect that the addition of calcium after SP would release more histamine. Less histamine release is induced by SP if calcium is added before SP presumably because the peptide cannot bind. Binding experiments with SP and monitoring of labelled calcium movements are in progress to test this hypothesis.

There is evidence in the literature (Perry *et al.* 1981) that in salt solutions SP may form fibrils. It is also well established that histamine release is induced by the cross-linking of membrane proteins such as IgE receptors (see Foreman, 1981*b* for review). It is possible, therefore, that SP could act by binding to and cross-linking IgE or IgE-receptors themselves. Two pieces of evidence argue against a cross-linking of IgE by SP. First, an excess of soluble IgE which absorbs anti-IgE, preventing it from inducing cross-links and hence histamine release, fails to inhibit histamine release induced by SP. Secondly, rat basophilic leukaemic cells which have about ten times more receptors for IgE than normal mast cells fail to respond to SP, whereas they do respond to hapten-antibody stimulation. Substance P in no way interfered with the hapten-antibody-induced histamine release. Substance P is unlikely, therefore, to induce histamine release by interacting with mast cell-bound IgE. The lack of response by RBL cells to SP is interesting, and more histamine-containing cells are being studied for responsiveness to the peptide.

The optimum extracellular pH for histamine release by SP was found to be 7·2. A similarly low pH optimum has been reported for compound 48/80 (Uvnäs & Thon, 1961) while the optimum for antigen-stimulated histamine secretion is a little higher, at around 7·5 (Foreman *et al.* 1977). There is evidence that extracellular acidity is associated with histamine secretion which appears to depend more on intracellular calcium stores than on extracellular calcium (Foreman, 1981*a*). This factor may be of importance in relation to SP- and compound 48/80-induced histamine release (Ennis, Pearce, Truneh & White, 1981) since both compounds appear to utilize internal calcium and have optimum action at an extracellular pH lower than 7·5.

It has already been stated that SP was predicted to have histamine-releasing action because of the basic amino acids in the *N*-terminal region, and the *N*-terminal tetrapeptide is itself active though less so than substance P. Mazurek, Pecht,

Teichberg and Blumberg (1981) also found the *N*-terminal tetrapeptide to be active. SP_{3-11}, SP_{4-11}, physalemin, [*p*-Glu^6, *p*-amino Phe^7]SP_{6-11} and eledoisin all lack the basic amino acids in the *N*-terminal region and were without histamine-releasing activity at the concentrations tested. Exposing a basic amino acid at the *N*-terminal end as in eledoisin-related peptide yields activity equivalent to almost a fifth of that of SP from a peptide, eledoisin, which is itself inactive, and confirms the importance of an *N*-terminal basic amino acid. The actual amino acid sequence Arg-Pro-Lys-Pro may, therefore, not of itself be crucial for activity provided the basic nature is retained. The tetrapeptide of the *N*-terminal sequence is about 150 times less active than SP, whilst the dipeptides Arg-Pro and Lys-Pro were inactive at concentrations up to 50 μM.

It appears, therefore, that the *C*-terminal end as well as the basic *N*-terminal end is of some importance in the histamine-releasing activity of SP. A clear demonstration of this comes from studying the relative activity of SP_{1-9}, SP_{1-8}, SP_{1-7} and SP_{1-6}. SP_{1-9} and SP_{1-8} are about one third as active as SP_{1-11}. Removal of the Phe^8 to give SP_{1-7} causes a 10-fold drop of activity, and removal of both phenylalanine residues causes almost a 100-fold fall in activity as in SP_{1-6}. The presence of phenylalanine residues at positions 7 and 8 is clearly of importance to the action of SP on mast cells possibly at the level of interaction with its receptors, and substitution with D-phenylalanine may in fact, confer increases in activity. Support for this comes from studies with [D-Phe^7]SP_{1-11} and the so-called SP antagonist [D-Pro^2, D-Phe^7, D-Trp^9]SP_{1-11} (Engberg, Svensson, Rosell & Folkers, 1981), both of which are more active than SP itself. It is important to note that [D-Pro^2, D-Phe^7, D-Trp^9]SP_{1-11} has been found to block antidromic vasodilation (Rosell, Olgart, Gazelius, Panopoulos, Folkers & Hörig, 1981) but it is more active than SP_{1-11} as a histamine releasing agent *in vitro*. Other selective SP antagonists are needed to resolve this paradox.

The SP–receptor interaction on the mast cell involves both the *N*-terminal basic tetrapeptide sequence Arg-Pro-Lys-Pro which is essential for activity, and the *C*-terminal sequence of which the Phe at positions 7 and 8 appear to be the most important part.

It is interesting to note that the dipeptide L-Phe-L-Phe-*O*-methyl has some histamine releasing action (Stanworth, Kings, Roy, Moran & Moran, 1979) and Stanworth and his colleagues have pointed out also that the segment 496–504 of the ϵ chain of IgE has the sequence:

Arg—Lys—Thr—Lys—Gly—Ser—Gly—Phe—Phe.

Although we have obtained evidence that SP does not cross-link IgE, we cannot exclude the possibility that the SP receptor is located within the IgE receptor itself, although the lack of response to SP of RBL cells argues against this because RBL cells have more IgE receptors than normal mast cells. Since the IgE-receptor has been isolated and purified, binding studies could be performed to answer this question (Rossi, Newman & Metzger, 1977).

The SP receptor on mast cells shows clear differences in structure–activity relationships compared with SP receptors elsewhere. In certain tissues such as guinea-pig ileum and rat parotid gland, physalaemin is invariably more potent than SP, whilst eledoisin and kassinin are less active (Hanley & Iversen, 1980; Brown &

Hanley, 1981; Gater *et al.* 1982). In other tissues, however, the reverse order of potencies has been observed (Erspamer, Falconieri Erspamer & Linari, 1977; Iversen, Lee, Sandberg & Watson, 1982; Bailey, Gater & Jordan, 1982) and it is possible, therefore, that different subpopulations of receptors mediate responses in these tissues. However, neither of these two apparent receptor populations has structural requirements for agonists corresponding to those found for the histamine-releasing activities of SP; the *C*-terminal sequence is of greater importance for the two receptor populations described above, with physalaemin and eledoisin both being effective agonists.

When studying structure–activity relationships for a series of compounds, apparent differences in relative potencies do not always accurately reflect differences in association with or activation of the receptor mechanism. For example, compounds may vary in their susceptibility to degradative enzymes or they may have different rates of diffusion. Whilst we have not measured the breakdown rates for substance P analogues during incubation with mast cells, certain observations argue against metabolism being a major determinant of their potencies as histamine-releasing agents. The release of histamine is complete within 15 sec exposure of the mast cells to substance P and thus metabolism would have to be extremely rapid to explain potency ratios of 100. Furthermore, when tested in the skin of the human forearm, some of the peptides such as physalaemin, which are relatively inactive as histamine releasing agents, produce negligible flare responses but marked wheal responses which are maintained for 15 min or more (Foreman & Jordan, 1981). Thus in the environment of the skin, at least, these peptides are capable of producing certain well maintained responses, despite their ineffectiveness as flare-inducing or histamine-releasing agents.

To conclude, SP exerts inflammatory effects in skin which are clearly dependent upon histamine release within the skin (Chahl, 1979; Lembeck & Holzer, 1979; Foreman & Jordan, 1981; Holzer, Saria, Skofitsch Lembeck, 1981; Lembeck & Donerer, 1981). It is clearly of interest to question the role that SP and its action on mast cells might have in the pathophysiology of inflammation in several tissues. An antagonist of SP at the mast cell receptor would be a valuable experimental tool for such purposes and may be of therapeutic significance.

We wish to thank the Medical Research Council, the Wellcome Trust and the National Institutes of Health (NS 09199) for financial support. Mr N. A. Hayes provided technical assistance.

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