Further Studies on the Structural Requirements for Polypeptide-Mediated Histamine Release from Rat Mast Cells

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Structure–activity studies have been performed on a series of naturally occurring and ‘tailor-made’ polypeptides, by measurement of ability to induce selective histamine release from normal rat peritoneal mast cells *in vitro*. Compounds investigated include corticotropin and melittin derivatives, mast-cell-degranulating peptide from bee venom, polymyxin B, bradykinin and various synthetic poly(amino acids) and short-chain peptides. It was confirmed that a cluster of four basic residues (lysine or arginine) was optimal for histamine release by corticotropin and melittin polypeptides, provided that the C-terminal carboxyl group was substituted (by, for instance, amidation). In contrast, the presence of a free C-terminal carboxyl group or nearby dicarboxylic acid residues led to a considerable diminution in histamine-releasing activity. Likewise, polypeptides comprised essentially of acidic amino acids were inactive. On the basis of these observations it has been possible to predict that synthetic peptides comprising a particular sequence within the Fc region of human immunoglobulin E, the immunoglobulin class particularly involved in mediation of allergic reactions of the immediate type, would possess potent histamine-releasing activity when similarly made to react with normal rat mast cells. The further study of such a structure should throw new light on the molecular basis of allergen–antibody triggering of mast cells.

In a previous publication (Jasani *et al.*, 1973) we described preliminary results on the structure–activity relationships of certain natural and synthetic polypeptides capable of releasing histamine from rat peritoneal mast cells *in vitro*. The main conclusion to be drawn from these early data was that histamine-releasing activity increased with increasing structural complexity and size of the polypeptide liberator, and that although the presence of certain basic amino acid residues appeared to be a prerequisite, more remotely sited non-basic residues probably provided some contributions to the overall activity of the molecule.

In order to substantiate these general trends observed in the structure–activity relationships of peptides largely derived from corticotropin (‘ACTH’; adrenocorticotrophic hormone) and melittin (a major bee-venom peptide), we have since carried out

Abbreviations used: ACTH and MEL (in synthetic-modification nomenclature), corticotropin and melittin respectively; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; -OMe, methyl ester.

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similar studies with further naturally occurring and synthetic ‘tailor-made’ polypeptides. A detailed quantitative analysis of the previous and the newly obtained data presented here not only confirms our previous observations, but in addition, provides further insight into the mode of action of these polypeptide liberators on the rat peritoneal mast cells.

Materials and Methods

Polypeptides

Each polypeptide used was obtained in a freeze-dried form, which was dissolved in the cell-suspending medium (see below), divided into appropriately small portions and stored frozen at -20°C until used. The primary structures of some of the polypeptides tested are shown in Table 1.

Synthetic human corticotropin [ACTH-(1–39)-peptide]. This peptide and its subpeptides ACTH-(1–24), ACTH-(1–16), ACTH-(11–24), ACTH-(25–39), ACTH-(L-Ser¹, Lys¹⁷, Lys¹⁸, B1–18) amide[ACTH-(L-1–18)], ACTH-(D-Ser¹, Lys¹⁸, B1–18)-tetradecylamide [ACTH-(D-1–18)-tetradecylamide] and ACTH-(D-1–18) were all obtained through the generosity of CIBA-GEIGY Ltd., Horsham, Sussex, U.K. The purity and the structures of these have been described in detail by Jaques & Brugger (1969) and Desaulles *et al.* (1969).

Table 1. *Primary sequences of certain polypeptide histamine liberators*

1. Corticotropin-(1-39)-nonacontapeptide [ACTH-(1-39)-peptide]

¹ Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly¹⁴
¹⁵ -Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro²⁴
²⁵ -Asp-Gly-Ala-Glu-Asp-Gln-Leu-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe³⁹

2. Melittin-(1-26)-hexacosapeptide [MEL-(1-26)-peptide]

¹ Gly-Ile-Gly-Ala-Val-Leu-Lys⁷
⁸ -Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-¹⁹
²⁰ -Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH₂²⁶

3. Ceylonese-bee melittin

¹ Gly-Ile-Gly-Ala-Ile-Leu-Lys⁷
⁸ -Val-Leu-Ala-Thr-Gly-Leu-Pro-Thr-Leu-Ile-Ser-Trp¹⁹
 (-Thr-Leu-Ile-Ser-Trp)

4. Mast-cell-degranulating peptide

¹ Ile-Lys-Cys-Asn-Cys-Lys-Arg-His-Val-Ile-Lys-Pro-His-Ile-¹⁴
¹⁵ -Cys-Arg-Lys-Ile-Cys-Gly-Lys-Asn-NH₂²²

5. Polymyxin B and Colistin (DABA = $\alpha\gamma$ -diaminobutyric acid; in Colistin the D-phenylalanine residue is replaced by D-leucine)

DABA-DABA-DABA-D-Phe
¹ |
 L-Thr-DABA-L-Leu-DABA
¹ |
 6-methyloctanoic acid

6. Bradykinin

¹ Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg⁹

7. Protamine

¹ Pro-Arg-Arg-Arg-Arg-Ser-Ser-Ser-Arg-Pro-Val-Arg-¹²
¹³ -Arg-Arg-Arg-Arg-Pro-Arg-Val-Ser-Arg-Arg-Arg-Arg-²⁵
²⁶ -Arg-Gly-Gly-Arg-Arg-Arg-Arg³²

Synthetic corticotropin subpeptides ACTH-(5-24) and ACTH-(7-24) were generously given by Professor R. Schwyzler, Institute of Molecular Biology and Biophysics, Eidgenössisch Technische Hochschule, Zürich-Hönggerberg, Switzerland.

European-bee (Apis mellifera)-venom melittin [MEL-(1-26)-peptide]. This was purified from freeze-dried bee venom (Firma Mack, Illertissen, Germany), and its subpeptides MEL-(17-26), MEL-(18-26), MEL-(20-26) and MEL-(20-26*) [lacking one amide group at its C-terminus compared with MEL-(20-26)-peptide] were prepared and purified as described by Kreil & Kreil-Kiss (1967).

Ceylonese-bee (Apis florea)-venom melittin. This was prepared and purified as described by Kreil (1973).

Mast-cell degranulating peptide from bee venom. This was generously given by Professor C. A. Vernon, Department of Chemistry, University College London, the purity and the structure of which has been previously described by Brithaupt & Habermann (1968) and Hanson & Vernon (1969) respectively.

Polymyxin B sulphate (B.P., sterile) and colistin sulphomethate sodium (B.P.). These were purchased from Burroughs Wellcome and Co., London N.W.1, U.K., and Pharmax Ltd., Crayford, Kent, U.K., respectively.

Synthetic bradykinin triacetate trihydrate (chromatographically pure; batch no. 56104). This was obtained commercially from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Protamine sulphate from salmon (essentially histone-free; grade 1) was purchased from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.

Dilysine, lysylglycine, isoleucylarginine, trilycine, triornithine, triarginine and tetralysine. These were obtained commercially from Miles Laboratories, Elkhart, IN 46514, U.S.A., and Baceem A.G., Liestal, Switzerland, respectively.

Dilysine methyl ester and isoleucylarginine amide. These were purchased from Fox Chemical Company, Los Angeles, CA, U.S.A.

Triornithine methyl ester and tetralysine methyl ester. These were prepared and purified as follows. The basic peptide (triornithine or tetralysine) was dissolved in methanol by the addition of thionyl chloride. The resulting ester was purified by paper electrophoresis, the content of the free peptide being less than 1% in the final preparation.

Dansyl-triarginine and dansyl-isoleucyltriarginine. These were prepared by using a standard procedure (Gray, 1967). The prepared compounds were dried, and dissolved in acetic acid (pH 3) and extracted with butan-1-ol in order to remove any dansyl amide. Finally, the aqueous phase was passed over a small column of Dowex-1 (acetate form) to remove any free sulphonic acid. The final product was shown to be more than 90% pure, with a minor basic compound being present as the only impurity.

Tuftsin. This was generously provided by Dr. J. Morley, ICI, Macclesfield, Cheshire, U.K.

Poly(lysyl-dialanine), poly(glutamyl-dialanine) and poly(arginyl-dialanine). These were all generously given by Dr. J. Jones, Dyson Perrins Laboratories, University of Oxford, Oxford, U.K.

Cell-suspending medium. This was prepared by adding 0.1 ml portions of mineral salts solution (Dulbecco B solution; code no. SR39, Oxoid Ltd., London E.C.4, U.K.) and glucose solution (200 mg/ml) to 20 ml of sterile phosphate-buffered saline (Dulbecco A solution; code no. BR14a, Oxoid).

Rat peritoneal mast cells. These were prepared by using a standard procedure described in detail by Jasani (1973). Ice-cold sterile phosphate-buffered saline (Dulbecco A) was introduced into the peritoneum of the rat (Lister black-hooded strain) by using a plastic perforated transport tube. The abdomen was then gently massaged for 90 s in order to release the peritoneal cells, including the mast cells (2-7% of the total), into the washing fluid, but ensuring at the same time avoidance of any undue intraperitoneal haemorrhage. The peritoneal washings were collected in plastic test tubes (kept in an ice/water mixture) in which they were then centrifuged at 770g and 4°C for 1 min. The resulting cell pellets were resuspended in the cell-suspending medium and pooled, giving on average 1×10^6 - 2×10^6 mast cells/ml. The contamination of these cells with erythrocytes, due to any unavoidable haemorrhage, was found to be as low as 10% of the total number of cells harvested in most experiments.

Release of histamine from mast cells

Five concentrations of each liberator, shown through preliminary tests to give a dose-response curve extending over a range of below 15 to above 75% histamine release, were each measured into 5 ml plastic tubes to give a final fixed volume of 0.9 ml. These, together with the cell suspension, were pre-incubated for 5 min at 37°C. By using a 100 μ l Eppendorf pipette, 0.1 ml portions of the cells were then transferred to each of the liberator solutions, the resulting constituents being quickly but gently mixed and left at 37°C for a further 5 min. The tubes were then transferred to the ice/water mixture and allowed to cool to 4°C. These were then centrifuged at 770g and 4°C for 5 min, a procedure that ensured firm fixation of the cell pellets to the bottom of the tubes. The supernatants were immediately decanted into 1 ml portions of 10% trichloroacetic acid, 2 ml aliquots of 5% trichloroacetic acid being added at the same time to cell pellets. These trichloroacetic acid solutions of cell sediment and supernatant samples were finally assayed for histamine by using the spectrofluorimetric procedure of Shore *et al.* (1959).

Results

Analysis of histamine-release data

The histamine released from mast cells by a given concentration of liberator was expressed throughout as a percentage of the total available histamine (i.e. the sum of the amounts of histamine found in supernatant and the corresponding cell sediment).

A typical set of dose-response curves plotted from data on a series of peptides tested on the same cell preparation is shown in Fig. 1. Most of the curves are seen to be of sigmoidal shape. Hence, by using probit analysis (Finney, 1947) it was possible to obtain a quantitative estimate of the median effective dose, ED_{50} , which represents the dose of a liberator giving 50% histamine release.

Repetition of analyses similar to the above with a different rat mast-cell preparation gave dose-response

Fig. 1. Dose-response curves of certain polypeptide histamine liberators obtained by using portions of the same mast-cell preparation

A, mast-cell-degranulating peptide; B, protamine; C, MEL-(1-26)-peptide; D, polymyxin B; E, Synacthen; F, Colistin; G, bradykinin; and H, Tuftsin.

Fig. 2. Dose-response curves of (a) ACTH-(1-24)- and (b) ACTH-(1-18)-peptides, showing the variation in response from one mast-cell population to another

The curves were obtained by using four different cell preparations harvested on different days. The arrows indicate the points of 50% release on each curve.

curves that although retaining their overall shape and positions relative to each other, had shifted either upwards or downwards *in toto* with respect to the original family of dose-response curves. As Fig. 2 shows, the magnitude of such a shift in each of the dose-response curves was proportionately the same, irrespective of the nature of the liberator. Thus, on changing from one cell preparation to another, the ED_{50} values of the tested liberators either increased or decreased by proportionately the same amount. Hence the shift appeared to be due to some change in the property of cells from one preparation to another and not due to any change in the liberators themselves. As Fig. 3 shows, the variation in the histamine-release values at five different liberator concentrations comprising a typical dose-response curve caused by such shifts was much greater than that observed when repetitive analyses were carried out with portions of the same cell preparation. In addition, the extent of this variation was of the same order irrespective of the liberator used.

In order to determine a reliable direct measure of the average ED_{50} of a given liberator it was necessary, therefore, to obtain dose-response curves with several different cell preparations. However, because of the paucity of the available test material (in the case of most of the polypeptides investigated) it became imperative to adopt an indirect, yet reliable, method of determining their average histamine-releasing capacities. This was achieved in the following manner. Since the magnitude and the direction of the shift in a dose-response curve were the same in the

Fig. 3. Comparison of between-cell population* [Δ , curve (a)] and within-cell population† [$\square$, curve (b)] variation in histamine release induced by ACTH-(1-24)-peptide $\circ$ and $\bullet$ [Curve (a)], between-cell population variations in histamine release induced by MEL-(1-26)-peptide and mast-cell-degranulating peptide respectively. Δ [Curve (c)], estimated sampling error involved in the use of Eppendorf micropipettes in measuring out 5–100 μ l of various liberator solutions.

* Variation in histamine release resulting from the use of different cell populations in each experiment.

† Variation in histamine release encountered in repeated use of the same cell population.

case of each of the liberators tested jointly on two or more occasions, it was possible to correct for the variation in the ED_{50} value due to such a shift by inclusion, in each of the experiments, of a suitable standard polypeptide liberator. The ratio of ED_{50} of a liberator to the ED_{50} of the standard liberator obtained by using the same cell preparation (i.e. the relative potency of the liberator with respect to the standard) should, in theory, provide an index of the liberator's activity essentially free of the variation due to the unavoidable usage of different mast-cell preparations. This was indeed found to be the case, as can be seen from Table 2, which depicts the relative potencies of ACTH-(D-1-18)-peptide and MEL-(1-26)-peptide with respect to ACTH-(1-24)-peptide (included as a standard liberator) obtained using four different rat mast-cell preparations. The relative potencies of each of the three liberators are approximately constant, i.e.:

$$\frac{ED_{50} \text{ of liberator}}{ED_{50} \text{ of standard}} \approx \text{constant} \quad (1)$$

Hence:

$$\frac{ED_{50} \text{ of liberator}}{ED_{50} \text{ of standard}} \approx \frac{\overline{ED}_{50} \text{ of the liberator}}{\overline{ED}_{50} \text{ of the standard}} \quad (2)$$

where ED_{50} refers to the median effective dose obtained by using a single batch of mast cells and $\overline{ED}_{50}$ represents the mean of a fixed but relatively large number of individual ED_{50} values each obtained by using a different mast-cell preparation.

It follows from eqn. (2) that:

$$\overline{ED}_{50} \text{ of liberator} \approx \text{relative potency of the liberator} \times \overline{ED}_{50} \text{ of the standard} \quad (3)$$

Hence, if $\overline{ED}_{50}$ of the standard liberator is known, it is only necessary (at the least) to determine a single relative potency value of a given liberator in order to

obtain an approximate estimate of the highly reliable index of the liberator's histamine-releasing capacity, namely its $\overline{ED}_{50}$ value. It is also evident that if the mean of more than one separately obtained relative potency value of a liberator is used instead of any particular value, in eqn. (3), the resulting estimate of $\overline{ED}_{50}$ is likely to approximate even better to the actual $\overline{ED}_{50}$ of the liberator.

In the light of the above, ACTH-(1-24)-peptide (Synacthen) was chosen as the standard liberator throughout the present study, since, not only was it readily available, but its high purity and the strict quality control exercised by CIBA-GEIGY in its manufacture permits its use as a standard in any laboratory.

In our use of it as a standard liberator in the present study, a total of 17 separate dose-response curves of ACTH-(1-24)-peptide were derived with 17 different mast-cell preparations. By applying probit analysis to these, the $\overline{ED}_{50}$ of ACTH-(1-24)-peptide was established to be $3.1 \pm 0.8 \mu\text{M}$. The coefficients of variation (i.e. the ratios of s.e.m. values to their respective means) relating to the observed $\overline{ED}_{50}$ values of ACTH-(1-24)-peptide and MEL-(1-24)-peptide obtained from 17 and 21 separate experiments respectively, were found to have approximately the same magnitude, their individual values being 26 and 24% respectively. Hence, as with the nature of variation in histamine release observed at a given concentration (see Fig. 2), the variation in $\overline{ED}_{50}$ appeared to be of approximately the same order irrespective of the type of liberator tested. Exploiting this finding, the expected s.e.m. associated with the estimate of $\overline{ED}_{50}$ of a given liberator was calculated for each case to have a value of $\pm 25\%$ of the estimated $\overline{ED}_{50}$ value.

By using the values $\overline{ED}_{50}$ derived in the manner described above, the histamine-releasing capacities of the various polypeptide liberators examined were compared (see below).

Table 2. *Variation in relative potency of a histamine liberator from one cell population to another*
The experimental details are described in the Materials and Methods section. The potency of each histamine liberator relative to ACTH-(1-24)-peptide was derived as described in Results section under 'Analysis of histamine-release data'. Abbreviation used: MCD, mast-cell-degranulating peptide.

Cell preparation	Potency [relative to ACTH-(1-24)-peptide]		
	ACTH-(D-1-18)-peptide	MCD	MEL-(1-26)-peptide
1	2.0	0.0076	0.022
2	2.7	0.0085	0.023
3	2.1	0.0070	0.018
4	1.7	0.0067	0.022
Mean $\pm$ s.d.	2.1 $\pm$ 0.4	0.0075 $\pm$ 0.0008	0.21 $\pm$ 0.002
Coefficient of variation (%)	19	11	10

Effect of omission of N-terminal residues from ACTH-(1-24)-peptide

From Table 3 it is apparent that the omission of the first four *N*-terminal amino acid residues from ACTH-(1-24)-peptide increases the molecule's histamine-releasing activity by as much as 3-fold. On the other hand, further omission of the fifth and sixth residues causes a substantial decrease in the activity, the activity of the resulting subpeptide, ACTH-(7-24), being as much as five times less than its parent molecule, ACTH-(1-24)-peptide. However, the activity of this subpeptide is not significantly altered on omission from it of residues 7-10, since the activity of ACTH-(11-24)-peptide is approximately the same as that of ACTH-(7-24)-peptide. This is in contrast with the effect of omission of further residues, i.e. 11-16, as the resulting subpeptide [ACTH-(17-24)-peptide] was found to be completely inactive at concentrations ten times higher than the ED_{50} of ACTH-(11-24)-peptide. The fact that ACTH-(20-24)-peptide was also inactive, at even higher

concentrations, further substantiated this finding.

Effect of omission of C-terminal residues from ACTH-(1-24)-peptide

Table 4 shows that the omission of *C*-terminal residues 19-24 from ACTH-(1-24)-peptide caused a fall in activity of just over 2-fold. However, this relatively small difference in the activities of ACTH-(1-24)- and ACTH-(1-18)-peptides was amplified 4-fold when a further two residues, namely residues 17 and 18, were omitted, since the resulting subpeptide, ACTH-(1-16), was nearly eight times less active than ACTH-(1-24)-peptide.

Effect of omission of N-terminal residues from MEL-(1-26)-peptide

Table 5 shows that the removal of *N*-terminal residues of MEL-(1-26)-peptide caused a drop in the molecule's histamine-releasing capacity in a manner similar to that described above for ACTH-(1-24)-peptide. Thus omission of residues 1-16 from the structure of MEL-(1-24)-peptide caused a 7-fold decrease in activity. The resulting subpeptide, MEL-

Table 3. *Comparison of the histamine-releasing capacities of the corticotropin group of peptides: the effect of sequential omission of amino acid residues from the N-terminal end of ACTH-(1-24)-peptide*

Full experimental details are described in the Materials and Methods section. For definition and the method of derivation of $\overline{ED}_{50}$ and relative potency of a histamine liberator see under 'Analysis of histamine-release data' in the Results section. In all cases where greater than one measurement was made, the values of mean $\overline{ED}_{50} \pm 1$ s.d. are compared.

Peptide	No. of times tested	$10^8 \times$ Estimate of $\overline{ED}_{50}$ (M)	1/Relative potency
ACTH-(1-24)	17	310 ± 78	1.00
ACTH-(5-24)	1	100	3.13
ACTH-(7-24)	1	1550	0.19
ACTH-(11-24)	2	2060 ± 71	0.16
ACTH-(17-24)	2	Inactive	—
ACTH-(20-24)	2	Inactive	—

Table 4. *Comparison of the histamine-releasing capacity of the corticotropin group of peptides: the effect of sequential omission of amino acid residues from the C-terminal end of ACTH-(1-24)-peptide*

For further details, see the text and the legend to Table 3.

Peptide	No. of times tested	$10^8 \times$ Estimate of $\overline{ED}_{50}$ (M)	1/relative potency
ACTH-(1-24)	17	310 ± 78	1.00
ACTH-(1-18)	1	740	0.44
ACTH-(1-16)	1	2300	0.13

Table 5. *Comparison of the histamine-releasing capacities of the melittin group of peptides: the effect of sequential omission of amino acid residues from the N-terminal end of MEL-(1-26)-peptide*

For further details, see the text, and the legend to Table 3.

Peptide	No. of times tested	$10^8 \times$ Estimate of $\overline{ED}_{50}$ (M)	1/Relative potency
MEL-(1-26)	21	71 ± 18	1.00
MEL-(17-26)	1	560	0.12
MEL-(18-26)	3	1020 ± 250	0.06
MEL-(20-26)	3	4800 ± 1025	0.01

Table 6. *Effect of N-terminal residue modification on the histamine-releasing activities of certain polypeptide liberators*

Full experimental details are described in the Materials and Methods section. For the definition and the method of derivation of $\overline{ED}_{50}$ see the Results section under 'Analysis of histamine-release data'. In all cases where greater than one measurement was made, the values of mean $\overline{ED}_{50} \pm 1$ s.d. are compared.

Peptide	No. of times tested	$10 \times$ Estimate of $\overline{ED}_{50}$ (M)
ACTH-(1-18)	1	740
ACTH-(1-18)	4	650 ± 125
Triarginine	2	2100 ± 141
Dansyl-triarginine	2	2700 ± 35
Dansyl-isoleucyltriarginine	1	4600

(17–26), showed further ‘loss’ in activity in the event of omission of residues 18 and 19 sequentially, as was revealed by the activities of MEL-(18–26)- and MEL-(20–26)-peptides, which were found to be approx. 2–10-fold less active respectively as compared with MEL-(1–26)-peptide.

Effect of modification of the N-terminal residues of certain polypeptide liberators

The results given in Table 6 demonstrate that alteration of the *N*-terminal serine residues from the L-form to the D-form in the subpeptide ACTH-(1–18) failed to cause any significant change in the peptide's histamine-releasing activity. Similarly, the *N*-terminal attachment of one or two bulky hydrophobic residues to triarginine did not cause any significant alteration in the overall activity.

Table 7. *Effect of C-terminal residue modification on the histamine-releasing activities of certain polypeptide histamine liberators*

For further details, see the text and the legend to Table 6.

Peptide	No. of times tested	$10^8 \times$ Estimate of ED_{50} (M)
MEL-(20–26)	3	4800 ± 1025
MEL-(20–26*)	1	48 300
Dilysine	1	800000 ± 320000
Dilysine methyl ester	1	200 000
Isoleucylarginine	2	Inactive
Isoleucylarginine amide	1	797 000
ACTH-(D-1–18)	4	650 ± 125
ACTH-(D-1–18)-tetradecylamide	1	3
ACTH-(1–24)	17	310 ± 78
ACTH-(1–39)	1	14 400

* Lacking one amide group at its C-terminus compared with MEL-(20–26)-peptide.

Effect of modification of the C-terminal residues of certain polypeptide liberators

Table 7 shows that, unlike at the *N*-terminal end, even the slightest modification at the C-terminal end leads to a substantial change in a peptide's histamine-liberating activity. For example, removal of a C-terminal amide group from MEL-(20–26)-peptide caused a 10-fold change in the peptide's activity, the resulting peptide, MEL-(20–26*), being 10-fold less active. Alternatively, blockage of the C-terminal carboxy group with either an amide or an ester group caused a significant increase in activity, as evidenced by the comparison of the activities of dilysine with dilysine methyl ester, isoleucylarginine with isoleucylarginine amide, and ACTH-(D-1–18)-peptide with its analogue, ACTH-(D-1–18)-tetradecylamide; the biggest change in activity (nearly 200-fold) being observed between the latter two liberators. In contrast, addition of extra dicarboxylic acid residues at the C-terminal end, as in the case of ACTH-(1–39)-peptide compared with ACTH-(1–24)-peptide, led to an almost 15-fold decrease in the activity. This finding was consistent with the above-mentioned decrease in the activity of MEL-(20–26)-peptide on creation of an extra carboxy group by hydrolysis of one of its C-terminal amide groups, as in MEL-(20–26*).

Relationship between the length of a lysine- or an arginine-rich peptide and histamine-releasing capacity

The data in Table 8 clearly demonstrate that increasing the length of lysine- or arginine-rich peptide by addition of extra lysine or arginine residues respectively leads to an almost exponential increase in histamine-releasing capacity, the arginine peptides on the whole being more active than the corresponding lysine peptides. In addition, the smallest peptide capable of eliciting histamine release in the system used *in vitro* was found to comprise two adjacent basic residues (as in dilysine). A single basic residue, either as a sole lysine or arginine molecule,

Table 8. *Relationship between the chain length of a predominantly basic peptide liberator and its histamine-releasing capacity*
For further details see the text and the legend to Table 7.

Peptide	No. of basic residues	No. of times tested	$10^8 \times$ Estimate of ED_{50} (M)
Lysine	1	2	Inactive
Dilysine	2	3	800000 ± 320000
Trilysine	3	2	35100 ± 14100
Tetralysine	4	2	5500 ± 0
Poly(Lys-Ala-Ala)	30	1	470
Arginine	1	2	Inactive
Triarginine	3	2	2100 ± 141
Poly(Arg-Ala-Ala)	15	1	35
Protamine	21	1	31
Lysylglycine	1	2	Inactive
Isoleucylarginine	1	2	Inactive

Table 9. Comparison of the histamine-releasing capacities of a miscellaneous group of liberators

Liberator	No. of times tested	$10^8 \times$ Estimate of ED_{50} (M)
Poly(glutamyl-dialanine)	2	Inactive
ACTH-(25-39)-peptide	2	Inactive
Bradykinin	1	5300
Colistin	1	4060
Ceylonese-bee melittin	1	85
Polymyxin B	3	81 ± 32
Compound 48/80	1	25
Mast-cell-degranulating peptide	7	2 ± 0.6

or in conjunction with another non-basic residue (as in lysylglycine and isoleucylarginine) at concentrations as high as 10mM, consistently failed to release histamine. Any further increase in the concentration led to marked interference with the spectrophotofluorimetric histamine-assay system used. This observation was found to apply to lysine, arginine, lysylglycine and isoleucylarginine, indicating that by means of the present assay system it was impossible to assess the histamine-releasing activity of any molecular species for which the ED_{50} was above 10mM. But in any case, values in this range would probably not be of any physiological significance.

Histamine-releasing capacities of a miscellaneous group of compounds

Data presented in Table 9 reveal several important features about the structural requirements for polypeptide-mediated histamine release. For example, polypeptides consisting predominantly of acidic residues [e.g. poly(glutamyl-dialanine) and ACTH-(25-39)-peptide], despite their relatively large size, were incapable of releasing histamine even at very high concentrations. Secondly, the relatively high activities of the cyclic peptides Colistin, polymyxin B and mast-cell-degranulating peptide, despite their possession of relatively few basic residues, suggests that the constraint imposed on a basic peptide by intrachain bonding somehow enhances its histamine-releasing capacity. In this light, it is noteworthy, from the Table 9, that one such peptide, mast-cell-degranulating peptide, was found to be over ten times more active than the widely used artificial histamine liberator, Compound 48/80.

Discussion

The data obtained from our studies of the effect of sequential omission of *N*-terminal residues on the histamine-releasing activity of ACTH-(1-24)-peptide

show that not all residues within the *N*-terminal region (most of which are non-basic in character) are essential for the overall activity of the liberator. For example, residues 1-4 seemed to hinder the expression of the maximal activity of ACTH-(1-24)-peptide. On the other hand, residues 5 and 6 appeared to be crucial for the histamine-releasing action of the whole polypeptide. It is noteworthy, in the light of this finding, that the same two amino acid residues are implicated in the steroidogenic action *in vitro* of ACTH-(1-24)-peptide on adrenal-cortex cells (Fujino *et al.*, 1971). In fact, the observation of Schwyzer *et al.* (1971) that ACTH-(5-10)-peptide exhibits weak, but nevertheless significant, steroidogenic action on the isolated adrenal-cortex-cell system suggests that amino acid residues of the sequence His⁶-Phe⁷-Arg⁸-Trp⁹ are actually involved in some form of membrane receptor-activation process. Whether these amino acid residues play a similar role in the release of histamine from peritoneal mast cells by ACTH-(1-24)-peptide is difficult to ascertain from the data available at present. However, a survey of the existing structure-activity data on the steroidogenic action of corticotropin on isolated rat adrenal cells [see the review by Seelig *et al.* (1975)], and their comparison with our data, strongly suggests that the mode of interaction of ACTH-(1-24)-peptide with rat peritoneal mast cells and with adrenal-cortex cells is based on a very similar structural principle. For example, in both systems the following structure-activity relationships hold true: the 24-amino acid fragment of corticotropin, ACTH-(1-24)-peptide, is more potent than the parent 39-amino acid molecule; the C-terminal sequence, starting with amino acid residue 25, is totally devoid of activity; further shortening of the peptide from ACTH-(1-24)- to ACTH-(1-18)-peptide is associated with a decrease, but by no means a drastic loss, in the biological potency; and finally, when one or more of the dibasic amino acid residues at positions 15-18 (inclusive) are removed, a dramatic decrease of activity follows. The latter grouping of four dibasic amino acid residues is believed to be one of the most important affinity sites of the corticotropin molecule with respect to its action on the adrenal-cell system. Moreover, it is clear from our data that these four basic amino acid residues, and basic residues in general, play a crucial role in the histamine-release process. Thus there exists a real possibility that the same regions of the corticotropin polypeptide chain may be involved in ligand-receptor interactions in the two rat cell systems.

As we reported previously (Jasani *et al.*, 1973), melittin appeared to have a dual releasing action on mast cells, for, although the whole molecule caused histamine release by a direct lytic effect, part of the structure, depleted of its predominantly hydrophobic sequence, was capable of effecting the selective

release of histamine. For example, the lytic action of whole melittin was completely abolished on removal of residues 1–16, whereas the residual peptide, comprising residues 17–26, demonstrated only selective histamine release. The activity of the latter peptide was, however, dramatically decreased by removal of two residues (i.e. 18 and 19) from its *N*-terminal end. It is quite possible, therefore that residues 18 and 19 play some important role in the triggering of the histamine release by MEL-(18–26)-peptide analogous to that played by residues 5 and 6 in corticotropin. It is noteworthy that at least one of these pairs of apparently crucial amino acid residues has an aromatic character (e.g. in corticotropin, residue 6 is phenylalanine, whereas residue 19 of melittin is tryptophan). This feature, taken in conjunction with our finding that the replacement of the aromatic residue *D*-phenylalanine in polymyxin B by the non-aromatic residue *D*-leucine (as in Colistin) causes the liberator to lose its histamine-releasing activity by as much as 70-fold, suggests that an appropriately situated aromatic amino acid residue in the sequence of a polypeptide histamine liberator plays a certain critical role in optimal stimulation of the histamine-release process.

With regard to the influence of other structural features on the histamine-releasing capacity of polypeptide, it is noteworthy that the selective mast-cell-triggering action of basic amino acid residues is enhanced by neighbouring hydrophobic residues and, conversely, decreased by neighbouring acidic ones (Jasani, 1973; Jasani *et al.*, 1973). In the former connection it is also of significance that incorporation of a prolyl-alkyl chain within an established corticotropin polypeptide liberating structure enhances activity, presumably by increasing its membrane-penetrating capacity (Rüegg & Jaques, 1977). But, like whole melittin, such compounds are lytic in their action, in contrast with the polypeptides derivatives investigated by us in the present study.

The interpretation of the data relating to the effect of the omission of *C*-terminal residues from ACTH-(1–24)-peptide on its capacity to release histamine from rat mast cells amply confirms our previous findings (Jasani *et al.*, 1973), which indicated that the integrity of the four adjacent basic residues (i.e. residues 15–18) is crucial to the overall activity of the liberator. The latter observation applies also to MEL-(1–24)-peptide, which possesses a similar sequence of basic residues, namely residues 21–24. The significance of these basic sequences is enhanced by the data shown in Table 8, in particular those relating to the lysine-rich peptides, which confirm that a structure consisting of four adjacent basic residues is optimal for histamine release. For, as will be noted, the inclusion of further basic residues causes significantly less increase in the histamine-releasing activity as compared with that observed

when one, or two or three basic residues are attached to the sole lysine or arginine molecule.

Thus it is quite possible that this striking similarity between the nature of the optimal structure just described and the proposed active sites of ACTH-(1–24)- and MEL-(1–26)-peptides in some way reflects the probable character of the complementary active site, on or within the rat mast cell, which is associated with the triggering of the histamine-release process. Moreover, subsequent studies in our laboratory (Stanworth *et al.*, 1979) have demonstrated that a synthetic nonapeptide representative of the human immunoglobulin E ϵ -chain sequence (i.e. Arg-Lys-Thr-Lys-Gly-Ser-Gly-Phe-Phe-OMe) that incorporates this essential structure possesses rat mast-cell histamine-releasing activity *in vitro* at doses as low as 1 μ M.

The cell-activating role of such basic residues is by no means restricted to the release of histamine from rat peritoneal mast cells. On the contrary, it seems to be quite widespread in other biological systems. For example, Melrose *et al.* (1970) have proposed the active site of bradykinin, a potent effector of vascular permeability, to reside in its two terminal residues (i.e. the *C*- and the *N*-terminal residues respectively) brought together by the distortion in its backbone induced by contiguous proline residues. Similarly, some of the effects of glucagon (Hsu Chen & Feingold, 1973; Schneider & Edelhoch, 1972*a,b*), polymyxin B (Nakajima, 1967; Teuber, 1970) and melittin (Sessa *et al.*, 1969; Williams & Bell, 1972; Schroder *et al.*, 1971; Lubke *et al.*, 1971) on both artificial (i.e. liposomes) and biological membranes, appear to be mostly (if not wholly) attributable to the basic residues occurring in the structures of these biologically active molecules.

From the above discussion it seems that a combination of basic residues in one part of the molecule and certain non-basic residues in another, somehow act in unison to produce important biological effects at the cell-membrane level. In fact, it has been suggested by Rodbell and co-workers (Rodbell *et al.*, 1970, 1971; Pohl *et al.*, 1971) that the action of polypeptide hormone on cell membranes may occur through such bifunctional interactions, since, according to them, the hormone sensitivity of a cell is completely abolished on selective treatment of its surface with proteolytic and phospholipolytic enzymes. Hence it is perhaps possible to equate histamine release by polypeptides with hormone-induced activation of various cellular processes. Such an analogy is further justified by the fact that some of the highly active selective polypeptide liberators, including ACTH-(1–24)-peptide and mast-cell-degranulated peptide, were found to release histamine from mast cells over the same concentration range (viz. 100–1 nM) over which some of the highly active polypeptide hormones [e.g. glucagon and cortico-

tropin (see Greengard & Costa, 1970)] act on their respective target cells.

With regard to the physico-chemical property of the basic residues ultimately responsible for the induction of histamine release from rat mast cells, the data on the effect of modification of the C-terminal residues of several liberators (see Table 7) are particularly revealing. It was consistently observed that either formation of a C-terminal carboxy group [as in MEL-(20-26*)-peptide], or actual attachment of a polypeptide chain comprising several free carboxy groups, as in the side chains of glutamic or aspartic acid residues [such as occurs in ACTH-(1-39)-peptide], led to a considerable loss in the overall histamine-releasing activity of the liberator. In addition, polypeptides consisting primarily of acidic amino acid residues [e.g. ACTH-(25-39)-peptide and poly(glutamyl-dialanine)] were found to be totally inactive. Hence it seems that the effective positive charge more than any other property is likely to be responsible for the cell-triggering capacity of the basic residues. In view of this, it is perhaps not surprising that arginine-rich peptides are generally more active than corresponding lysine-rich peptides (see Table 8), since over the optimal pH 7.0-7.5 for polypeptide-mediated histamine release from normal rat peritoneal mast cells (Jasani, 1973) the arginine-residue side chain is more likely to be protonated than the corresponding lysine-residue side chain, as the respective pK values of the arginine guanidino and the lysine ϵ -amino groups are 12.48 and 10.69 respectively.

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