Further studies on histamine release from rat mast cells *in vitro* induced by peptides

Characteristics of a synthetic intermediate with potent releasing activity

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Previous studies on histamine release by corticotropin peptides and melittin peptides were extended, leading to the identification of a synthetic peptide intermediate, Lys(Z)-Arg(NO₂)-Arg(NO₂)OMe, (I) as an active non-cytolytic histamine releaser from rat mast cells. However, significant differences in the releasing capacity of optical isomers of this compound, and of Lys-Lys-Arg-ArgOMe [methyl ester of corticotropin-(15–18)-tetrapeptide; ‘basic core’] were observed, with the L-forms being markedly more active. A study of various analogues of the tripeptide compound (I) indicated that the structural basis for mast-cell triggering by such peptidic agents was highly specific. The relevance of these observations to the immunologically induced histamine-release processes is discussed.

We have reported previously (Jasani & Stanworth, 1973; Johnson & Erdős, 1973; Stanworth *et al.*, 1979; Jasani *et al.*, 1979) investigations performed with a number of histamine-liberating peptide sequences, including corticotropin-, mellittin- and immunoglobulin E-derived peptides, which defined similarities between peptide-induced and immunologically induced release processes, and established general structural requirements for non-cytolytic histamine-releasing activity. For example, peptide-releasing activity was found to be related primarily to the presence of the basic amino acid residues lysine and arginine. Moreover, the activity of these basic sequences was observed to be enhanced further by the presence of adjacent hydrophobic residues, but diminished by acidic functions.

Recently we have studied the histamine-releasing characteristics of synthetic peptides representing the ‘basic core’ of amino acid residues present in corticotropin [Lys-Lys-Arg-Arg; corticotropin-(15–18)-tetrapeptide] and in melittin [Lys-Arg-Lys-Arg; melittin-(21–24)-tetrapeptide] with particular reference to the implications of differential activities of enantiomers (physicochemically identical). During this work a synthetic intermediate, Lys(Z)-

Abbreviation used: Z, benzyloxycarbonyl.

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Arg(NO₂)-Arg(NO₂)OMe was found unexpectedly to be equipotent to Compound 48/80 (Read *et al.*, 1973), a well-established artificial histamine liberator. This compound was subjected to a structure–activity-relationship study and was found to display interesting stereochemical dependencies. The data in the present communication collate these observations, thereby extending information on structural requirements associated with the histamine-release trigger process by peptidic releasing agents.

Materials and methods

Materials

The peptides under study refer to the all-L-form unless otherwise stated. The tetrapeptides Lys-Lys-Arg-ArgOMe (enantiomers) and Lys-Arg-Lys-ArgOMe, the tripeptide intermediate Lys(Z)-Arg(NO₂)-Arg(NO₂)OMe (enantiomers) and structural analogues were prepared by solution synthesis by employing standard methods in peptide chemistry (Schröder & Lubke, 1965). Purification was by crystallization and column chromatography on Sephadex LH20 in water and was monitored by t.l.c. Identification of products was made by amino acid analysis and p.m.r. spectroscopy, where results obtained for the peptides studied were consistent

with expected structure. Optical-rotation measurements were made in a Perkin-Elmer 141 automatic polarimeter, providing a check on enantiomers for equal and opposite rotations.

The positive controls for histamine release used in these studies were Compound 48/80 (Wellcome Laboratories, Beckenham, Kent, U.K.), and synthetic corticotropin-(1-24)-tetraacosapeptide, marketed under the name Synacthen by CIBA/Geigy, Horsham, Sussex, U.K. The latter peptide promoted 50% release of histamine from normal rat peritoneal mast-cell preparations *in vitro* at an average concentration of $5\ \mu\text{M}$ (Jasani *et al.*, 1979).

Disodium cromoglycate (Intal) was obtained from Fisons Pharmaceutical Laboratories, Loughborough, Leics., U.K.; it was administered simultaneously with the peptide challenge in inhibition experiments.

Cell preparations and assay procedures

Mast cells were obtained from the peritoneal washings of male outbred Wistar rats (body wt. 250–300 g). In a typical experiment, duplicate samples of cell suspensions were incubated with peptide at 37°C for 5 min, and after centrifugation (at 150 g for 5 min at 4°C) the supernatants were assayed for histamine by an automated spectrofluorimetric method (Evans *et al.*, 1973). Specific histamine release was calculated as a percentage of total histamine available in each test by taking into account that remaining in the cell pellet.

The histamine-releasing activity of a given liberator is expressed throughout as the molar concentration needed to effect a 50% release of histamine from a standard rat mast-cell suspension.

Cromoglycate inhibition and temperature experiments in the rat mast-cell system were performed as described by Kusner *et al.* (1973) and Johnson & Moran (1971) respectively.

Comparison of ^{51}Cr -release and lactate dehydrogenase-release assays for cell lysis

For these studies, a more sensitive isotope procedure (^{51}Cr -release assay) was developed and used as a routine monitor of cytolysis in place of the commonly employed lactate dehydrogenase-release assay (Burch *et al.*, 1963). There was good overall correlation found between the lactate dehydrogenase-release and ^{51}Cr -release methods for assaying cell lysis, as described in the following comparative study.

Mast cells derived from the peritoneal washings of six rats were pooled and purified by the method of Cooper & Stanworth (1975), and histamine, lactate dehydrogenase-release and ^{51}Cr -release values were determined.

Purified cells were washed twice in Dulbecco's incomplete buffer (free from mineral salts) and then

resuspended in Dulbecco's buffer (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.) to the required volume. The equivalent of mast cells from four rats was used for the lactate dehydrogenase-release assay, one rat for the ^{51}Cr -release assay and one rat for the histamine-release assay.

(i) *^{51}Cr -release assay.* A solution of purified mast cells in 1.0 ml of Dulbecco's medium was incubated with 0.1 ml of $\text{Na}_2^{51}\text{CrO}_4$ (1 mCi/ml; The Radiochemical Centre, Amersham, Bucks., U.K.) at ambient temperature (20°C) for 30 min. The labelled cells were washed three times with Dulbecco's medium to remove extraneous chromate. The cell pellet was resuspended in the same buffer to the required volume, and 0.1 ml portions were added to the wells of a flat-bottomed microtitre plate (Titertek; Flow Laboratories). The plate was warmed at 37°C for 5 min, and each 0.1 ml cell suspension was treated with 0.1 ml of similarly prewarmed challenge solutions. Incubation was continued at 37°C for 15 min, and the plate was spun at 1200 rev./min for 5 min. The supernatants were separated from cells by absorption into filter pads by using a supernatant harvester system (Flow Laboratories). The filter pads were transferred into counting tubes, and radioactivity present in the recovered supernatants was measured by using a Tracerlab Spectromatic counter. The amount of ^{51}Cr released was assessed in relation to the values obtained from the positive and negative controls, i.e. Triton X-100 and buffer, respectively.

Control experiments, with unlabelled Na_2CrO_4 at concentrations equivalent to those employed when labelling was used, showed that the histamine-releasing characteristics of mast cells were not affected by the procedure.

(ii) *Lactate dehydrogenase-release assay.* Lactate dehydrogenase activity was determined by a modification of the fluorimetric method of Burch *et al.* (1963). Samples (0.50 ml) of cell suspension in Dulbecco's medium were treated with equal volumes of challenge solutions, incubated for 15 min at 37°C , and separated by centrifugation at 150 g for 5 min at 4°C . Lactate dehydrogenase activity was then measured directly in the supernatant solutions. To 0.50 ml of supernatant was added 0.50 ml of NAD^+ (1 mM in 0.2 M-Tris buffer, pH 8.5). Then 0.50 ml of this solution was treated with $50\ \mu\text{l}$ of lactic acid (50 mM in 0.2 M-Tris buffer, pH 8.5); as a control, $50\ \mu\text{l}$ of 0.2 M-Tris buffer, pH 8.5, was added to a second sample (0.50 ml) of the NAD^+ solution. The solutions were incubated at ambient temperature for 20 min, and the fluorescence emission was then measured. The excitation and emission wavelengths used were 340 and 460 nm respectively. All measurements were made with a Baird Atomic automatic spectrofluorimeter (Fluoripoint). The lactate-dehydrogenase activity was assessed in terms of the

increases of fluorescence over control due to NADH formation after the addition of lactate. The lactate dehydrogenase released was assessed in relation to the fluorescence intensity obtained in the positive control challenge solution supernatants (i.e. Triton X-100 challenge).

(iii) *Histamine-release assay.* To 0.50 ml duplicate samples of challenge solution, prepared in Dulbecco's medium and prewarmed to 37°C, was added 0.50 ml of cell suspension. After incubation for 15 min at 37°C and centrifugation for 3 min at 1000 rev./min (150 g), supernatants were separated, treated with 0.1 ml of 80% (w/v) trichloroacetic acid to precipitate protein and re-centrifuged, and the supernatant solutions were set aside for histamine analysis. The original cell residues were treated with 1 ml of 8% trichloroacetic acid and left for approx. 30 min at ambient temperature, and then treated in a manner similar to that used for the original supernatants. Histamine was measured by the method described by Evans *et al.* (1973), with a Technicon AutoAnalyzer. Histamine release was calculated as a percentage of total histamine available in each challenge solution.

Skin test method

Skin tests were carried out in the shaved backs of animals (rats and baboons) immediately after intravenous injection of Pontamine Sky Blue (5%) in aq. 0.9% NaCl at a dose of 0.1 ml/kg body wt. for the rats and 5 ml/animal for the baboons.

A series of peptide concentrations in aqueous 0.9% NaCl solution, or saline control, were injected intradermally in 0.05 ml or 0.10 ml volumes. Skin reactions were read 20 min after intradermal challenge.

The molar concentration of peptide to give an end point in intradermal titrations in rat or baboon skin was determined from at least two experiments.

Results and discussion

Table 1 lists typical histamine-release values expressed in terms of the concentration required to effect 50% release obtained from measurements made with mixed rat peritoneal mast cells after the peptide challenge.

Activity of the sequence Lys-Lys-Arg-ArgOMe, representative of the 'basic core' of corticotropin, was comparable with that of corticotropin-(1-24)-tetraacosapeptide (Synacthen), the most active releaser previously reported in the series (Jasani & Stanworth, 1973). This result further emphasizes the important role played by the basic region of corticotropin in the histamine-releasing action of the parent molecule [by contrast, the sequence Lys-Lys-Arg-Arg possesses no intrinsic steroidogenic activity (Ramachandran, 1973)]. The corre-

Table 1. *Histamine-release values in the rat mast-cell system for sequences derived from corticotropin and melittin*

Amino acid residues refer to the L-configuration unless otherwise shown. For experimental details see the text.

Synthetic peptide	Activity [concn. (M) releasing 50% histamine]
Lys-Lys-Arg-ArgOMe	3.1×10^{-6}
all-D-Lys-Lys-Arg-ArgOMe	4.0×10^{-4}
Lys-Arg-Lys-ArgOMe	2.4×10^{-5}
Lys(Z)-Arg(NO ₂)-Arg(D)OMe	5.0×10^{-7}
all-D-Lys(Z)-Arg(NO ₂)-Arg(NO ₂)OMe	4.2×10^{-5}
Compound 48/80*	9.0×10^{-7}
Corticotropin-(1-24)-tetraecosapeptide	2.5×10^{-6}

* Mol.wt. taken as 520 (trimer).

sponding optical antipode, all-D-Lys-Lys-Arg-ArgOMe, of equal basicity, was significantly less active by approx. 100-fold, suggesting that a stereospecific component is associated with the mast-cell complementary site for the peptide. The pairing of basic residues (in Lys-Lys-Arg-ArgOMe) leads to greater activity than an alternating arrangement of the two types of basic residues (as in Lys-Arg-Lys-ArgOMe, representative of the 'basic core' of melittin), though the net basic charge of each isomer is the same. When basicity is lowered by substitution of lysine and arginine residues, there is unexpected enhancement of histamine-releasing activity, an effect particularly noticeable in the synthetic peptide intermediate Lys(Z)-Arg(NO₂)-Arg(NO₂)OMe. This compound displayed potent releasing ability of the same order as that of the well-known liberator compound 48/80, a condensation product of *p*-methoxy-*N*-methylphenylamine with formaldehyde (Table 1). Stereospecificity of action of Lys(Z)-Arg(NO₂)-Arg(NO₂)OMe was evident from the significantly lower histamine-releasing activity of the corresponding all-D-enantiomer. The varied activities of a number of other analogues of the tripeptide intermediate discussed in detail below (Table 3) demonstrate that a high degree of structural specificity is associated with this releasing agent.

The mechanism of histamine release induced by the peptides under study was examined. The measurement of cytoplasmic markers and in particular the enzyme lactate dehydrogenase have been commonly employed to detect cell lysis (Becker & Henson, 1973). We developed the use of ⁵¹Cr release from prelabelled mast cells as a monitor of cytolysis in place of the lactate dehydrogenase-release assay. The ⁵¹Cr-release procedure offers advantages of greater sensitivity (only one-quarter of

the number of cells used in the lactate dehydrogenase-release assay were required) and of avoiding possible interference in the enzyme assay by components of the reaction mixture. Results validating the ^{51}Cr -release assay for cytolysis are presented in Table 2.

The unusually high activity shown by Lys(Z)-Arg(NO₂)-Arg(NO₂)-OMe was noteworthy, since, apart from its potency, the mechanism whereby it induced histamine release from mast cells resembled closely the immunological release process inasmuch as it was non-cytolytic (^{51}Cr retention; Table 3), was partially inhibited by cromoglycate (Kusner *et al.*, 1973) and showed dependence on temperature in a manner similar to that exhibited by antigen-induced release (Johnson & Moran, 1971). By contrast, lytic release by Triton X-100 could not be inhibited by cromoglycate, and was not affected by the process of cooling and rewarming cells.

A structure-activity study on the tripeptide was therefore undertaken, the results obtained from the rat mast-cell system *in vitro* for a number of analogues being shown in Table 3. In general, structural modification led to decrease in activity, except for analogue (2), in which isosteric replacement of the benzyloxycarbonyl group (Ph-CH₂-O-CO-) by the phenylpropionyl group (Ph-CH₂-CH₂-CO-) was introduced, and resulted

in increased activity. The corresponding des-dinitro derivative (3) of the latter isostere was 50-fold less active, indicating an important contribution towards activity by the nitro functions. The removal of all side-chain protecting groups to give the more basic free tripeptide ester (4) resulted in an approx. 60-fold decrease in activity, whereas omission of the benzyloxycarbonyl group alone from the parent structure provided an analogue (5) that retained appreciable activity. However, transfer of the benzyloxycarbonyl group from the ϵ -amino to the α -amino position of lysine to give the isomeric analogue (6) essentially destroyed activity. The substitution of methyl ester by ethyl ester to give analogue (7) decreased activity by 50%, and the effect of removing the ester function to give analogue (8) resulted in a somewhat greater decline in activity to approx. 17%.

The structure-activity data on the tripeptide Lys(Z)-Arg(NO₂)-Arg(NO₂)-OMe overall (Tables 1 and 3) clearly indicate that histamine release from rat mast cells by this agent is a structural and stereospecific phenomenon. Moreover, it is evident from the corresponding ^{51}Cr -release data for the analogues shown in Table 3 that histamine release by this series of compounds is essentially non-cytotoxic, there being retention of a major portion of ^{51}Cr after peptide challenge at 0.1 mM concentration.

Table 2. Comparison of the release of histamine, lactate dehydrogenase and ^{51}Cr from rat mast cells after treatment with histamine-releasing agents

For experimental details see the text. Results are means $\pm$ range for two experiments.

Challenge	Histamine release (%)	^{51}Cr release (c.p.m.)	Lactate dehydrogenase release [change in fluorescence (arbitrary units)]
Buffer	1 $\pm$ 0	84.5 $\pm$ 1	0
Triton X-100 (0.05%, w/v)	90 $\pm$ 0	1517 $\pm$ 21	53.5 $\pm$ 1.5
Compound 48/80 (1 mg/ml)	83.5 $\pm$ 1.5	1565 $\pm$ 0	31.0 $\pm$ 1.0
Compound 48/80 (1 μg /ml)	78 $\pm$ 0	176 $\pm$ 13	0
Corticotropin-(1-24)-tetraeicosapeptide (5 μM)	86.5 $\pm$ 1.5	250 $\pm$ 53	1.0 $\pm$ 1.0

Table 3. Histamine release from rat mast cells by analogues of Lys(Z)-Arg(NO₂)-Arg(NO₂)-OMe
Amino acids are in the L-form. For experimental details see the text. Abbreviation used: Php, phenylpropionyl.

Analogue no.	Synthetic peptide	Activity [concn. (M) releasing 50% histamine]	^{51}Cr release (%)
(1)	Lys(Z)-Arg(NO ₂)-Arg(NO ₂)-OMe	5.0×10^{-7}	7.0
(2)	Lys(Php)-Arg(NO ₂)-Arg(NO ₂)-OMe	1.0×10^{-7}	7.0
(3)	Lys(Php)-Arg-Arg-OMe	5.0×10^{-6}	4.0
(4)	Lys-Arg-Arg-OMe	2.9×10^{-5}	0
(5)	Lys-Arg-(NO ₂)-Arg(NO ₂)-OMe	3.6×10^{-6}	5.0
(6)	Z-Lys-Arg(NO ₂)-Arg(NO ₂)-OMe	$> 10^{-4}$	4.0
(7)	Lys(Z)-Arg(NO ₂)-Arg(NO ₂)-OEt	1.0×10^{-6}	5.0
(8)	Lys(Z)-Arg(NO ₂)-Arg(NO ₂)	3.0×10^{-6}	5.0

Interestingly, measurement of the histamine-releasing capacity in rat and baboon skin *in vivo* showed marked quantitative differences of activity in the two species. For example, whereas the tripeptide Lys(Z)-Arg(NO₂)-Arg(NO₂)OMe maintained the previously observed activity *in vitro* when tested in rat skin, it was found to be considerably less active (about 100-fold on a molar basis) in the corresponding primate system.

In conclusion, this study on structurally related peptide sequences capable of imitating the antigenically induced histamine-release process has identified new parameters associated with histamine-releasing activity. In particular, the observations of stereoselectivity of action indicate that the L-configuration of amino acid is a relatively important requirement, and is suggestive of the involvement of a natural receptor in triggering rather than a physicochemical membrane-perturbation phenomenon. Analogue studies have demonstrated that activity is not proportional simply to the degree of basicity of the peptide sequence, but rather depends on an appropriate hydrophilic/lipophilic balance; nevertheless it is also evident that the structural basis of triggering of mast cells by such peptides is highly specific.

These additional observations on model histamine-releasing compounds provide further insight into the probable structural characteristics of the mast-cell-triggering effector site, which is postulated (Stanworth, 1973) to be situated within the Fc region of immunoglobulin E and to be

mobilized when allergen cross-links cell-bound antibody molecules of this class.

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