Histamine-Releasing Activity and Binding to the FcεRIα Human Mast Cell Receptor Subunit of Mast Cell Degranulating Peptide Analogues with Alanine Substitutions

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We have investigated the effects on mast cell binding and the histamine-releasing activity of L-alanine substitutions for the five lysine residues and the proline residue in the MCD peptide (**1**) sequence. All synthesized analogues Ala^{2} (**2**), Ala^{6} (**3**), Ala^{11} (**4**), Ala^{12} (**5**), Ala^{17} (**6**), and Ala^{21} (**7**) showed a loss of histamine release compared to the parent MCD peptide **1**. The order of decreased potency was **1** > **6** > **7** > **4** > **2** > **3** > **5**. The alanine-substituted analogues showed a 5- to 6-fold decrease in histamine release for analogues **6**, **7**, and **4** and a 10-fold decrease for analogue **2**. A more significant loss was observed in analogue **3** with a 75-fold loss of activity. The greatest loss of activity was observed with alanine substituting for proline in position 12. This analogue **5** showed a 130-fold loss of histamine release compared to the parent peptide **1**. The ability of each analogue to interact with the FcεRIα subunit of the human mast cell receptor was analyzed by competitive binding of the fluorescent peptide **1** and the alanine analogues using fluorescence polarization. The binding affinities of analogues **4**, **6**, and **7** for the mast cell receptor were less than the affinity of the native peptide **1**. Analogues **2**, **3**, and **5** showed an increase in binding affinity, with analogue **5** showing the highest increase compared to the native peptide **1**. The order of increased affinity was **5** > **3** > **2** > **1** > **4**, **6**, **7**. On the basis of these results, the possibility that analogue **5** inhibits peptide **1**-stimulated histamine release was examined. We found that peptide **5** did not inhibit histamine release by peptide **1**. The analogues **2**, **3**, and especially analogue **5** may be useful leads toward study of agents that prevent binding of IgE to mast cell receptors.

Introduction

Bee venom consists of a rich and complex mixture of peptides with many biological and pharmacological properties.[1] One of these peptides is mast cell degranulating (MCD) peptide **1**. Unlike other peptides in the venom such as the hemolytic agent melittin and the neurotoxic agent apamin, peptide **1** is a concentration-dependent histamine modulator. Although it releases histamine from mast cells at low concentrations, peptide **1** also inhibits histamine release in the presence of IgE at concentrations higher than those in which it releases histamine.[2] Besides these properties, peptide **1** has been found to bind to RBL mast cell lines by receptor-mediated endocytosis which is responsible for the functioning and recycling of the MCD peptide receptor on the mast cell surface.[3] Therefore, peptide **1** has been found to inhibit IgE binding to its high affinity mast cell receptor.[4] IgE is the main globulin in allergic reactions. Allergic reactions are initiated with high affinity IgE binding to its mast cell receptors. Subsequent cross-linking of IgE molecules occupying these receptors by allergens releases histamine and other inflammatory mediators.[5] Peptide **1**, therefore, is a natural choice for the study of IgE actions.

Isolation of peptide **1** from venom is impractical since it is only present in small amounts and is similar in structure to other peptides in the mixture.[6] We therefore synthesized peptide **1**.[7] Peptide **1** is a 22-amino acid, bicyclic peptide with two disulfide bridges at cysteines in positions 3, 13 and 5, 19 (Table 1). The amino acids in this sequence, with few exceptions, are either basic or hydrophobic. This peptide pattern and especially the basic amino acids are thought to be important for the ability of peptide **1** to release histamine.[8] At first, we investigated the effects on histamine release of truncated analogues of peptide **1** by removing clusters of basic amino acids. These results showed that histamine-releasing activity was lost when positive charges were removed from the α-helical part of the C-terminal end of peptide **1**.[9−11] Other studies were directed at defining the role of the two disulfide bridges and the two arginine residues in the peptide **1** molecule.[12]

These structure−activity relationships provided a background for the study of IgE/MCD peptide interactions. Because of the properties of IgE,[5] this process requires analogues of peptide **1** with high binding affinity to the mast cell receptor but without histamine-releasing activity. To this end, our first approach was a partial alanine scan of peptide **1**. The objectives of this

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Table 1. Histamine Release and Binding Affinities of MCD Peptide Analogues to the FcεRIα Human Mast Cell Receptor I K C^3 N C^5 K R H V I K P H I C^{15} R K I C^{19}G K N-NH_2[a]

	peptide [Ala^x]MCD	histamine release ED_{50} (10^{-5} M)[b]	ratio[c]	binding IC_{50} (μM)[d]	ratio[e]
1	MCD standard	0.16 ± 0.04	1	114 ± 30	1.0
2	[Ala^2]MCD	1.57 ± 0.05	10	82 ± 21	0.7
3	[Ala^6]MCD	12.54 ± 0.03	76	71 ± 14	0.6
4	[Ala^{11}]MCD	1.07 ± 0.02	6	153 ± 21	1.3
5	[Ala^{12}]MCD	21.24 ± 2.19	129	25 ± 10	0.2
6	[Ala^{17}]MCD	0.77 ± 0.03	4	153 ± 54	1.3
7	[Ala^{21}]MCD	0.78 ± 0.04	4	153 ± 54	1.3

[a] Primary structure of MCD peptide. Numbers mark the positions of the disulfide bonds connected at $C^{3,15}$ and $C^{5,19}$. [b] Concentration of peptide at 50% maximal histamine release [±SEM] (n = 6). [c] Ratio of histamine release of each analogue to MCD peptide. [d] Concentration of unlabeled peptide displacing 50% of receptor bound fluorescent MCD peptide in the fluorescence polarization assay. The results are the average of many consecutive readings for each sample. [e] Ratio of the binding affinities of each analogue to MCD peptide.

study were (a) to replace each of the five lysines and the proline residues in the MCD peptide sequence (Table 1) with L-alanine and (b) to develop a throughput binding assay for comparing the relative affinity of analogues of peptide **1** to the mast cell receptor.

Amino acid replacement by L-Ala is a common strategy for testing the role of the substituted amino acids side chain to peptide activity and receptor binding. L-Ala is used because it has a nonfunctional hydrophobic side chain and generally preserves the backbone conformation of the peptides.[13,14] On the other hand, a throughput binding assay is desirable because peptide **1** is not amenable to standard binding methods such as radiolabeling or surface plasmon resonance.[15,16] For this purpose we used the fluorescent-labeled peptide **1**[4] and the FcεRIα binding subunit protein of the human IgE mast cell receptor expressed in insect cells[17] to test the binding affinity of the alanine analogues to this receptor. We followed the binding of the Ala analogues in the homogeneous solution by means of a fluorescence polarization assay (FP).[18] Here, we report the binding affinities of these analogues and their histamine-releasing activity tested on peritoneal mast cells.

Results

Chemistry. The synthesis and purification of the analogues in Table 1 were carried out according to procedures published previously for the preparation of several peptide **1** analogues.[7,9−12] These procedures are also described in the Experimental Section. Briefly, the peptides were synthesized by solid-phase synthesis using Boc/benzyl type protective chemistry. Couplings were performed as hydroxybenzotriazole esters in the presence of diisopropylethylamine. An orthogonal protection has been used for the formation of the S−S bonds. A "shared resin" protocol was applied. Synthesis of each analogue was performed on the starting resin and forwarded until the incorporation of the residue preceding the one to be substituted. The resin was shared, and an aliquot was removed for the synthesis of the analogue. Synthesis was continued on the remaining resin until the position of the next desired substitution was reached, and the same procedure was repeated for the other substitutions. The synthesis of the analogues was accomplished by introducing the required sequences in the corresponding resin aliquots. The remaining resin was used to synthesize MCD peptide **1** as a control. No synthetic or purification problems were experienced following the well-established protocol used previously for the synthesis of MCD peptide analogues.[7,9−12] HPLC analysis of the analogues showed a purity of 97% or better. The results of mass spectrometric analysis of the analogues were consistent with the expected structures (Table 2).

Table 2. Analytical Data for the Synthesized MCD Peptide Alanine Analogues[a]

	peptide [Ala^x]MCD	HPLC[a] RT min	HPLC[b] RT min	mass (calcd)	mass (found)	purity, %
1	MCD standard	11.6	13.4	2587.2	2587.4	>98
2	[Ala^2]MCD	12.6	13.7	2530.1	2529.6	>97
3	[Ala^6]MCD	12.5	13.5	2530.1	2530.8	>98
4	[Ala^{11}]MCD	12.8	13.	2530.1	2530.6	>97
5	[Ala^{12}]MCD	12.8	13.3	2561.2	2561.3	>99
6	[Ala^{17}]MCD	12.2	13.4	2530.1	2529.9	>97
7	[Ala^{21}]MCD	12.3	13.3	2530.1	2530.8	>98

[a] RT = retention time in a gradient of 10−40% B in 15 min; buffer A was 0.1% trifluoroacetic acid in water and buffer B was 0.1% trifluoroacetic acid in acetonitrile. [b]RT = retention time in a gradient of 0−50% B in 25 min; buffer A was 10 mM TEAP/5 mM butanesulfonate/5% 1-propanol, pH 3.0 and buffer B was 10 mM TEAP/5mM butanesulfonate/ 15% 1-propanol. An analytical Vydac C18 column was used with a flow rate of 1 mL/min.

Binding Assay. The receptor binding affinity of the alanine analogues to the FcεRIα receptor protein was evaluated by competition binding experiments in a homogeneous assay using fluorescence polarization (FP). With this technique, ligand binding or competitive binding assays are based on changes in polarization of light emitted by an excited fluorescent ligand as it undergoes changes of its rotational mobility in solution during binding to the receptor. This translates into changes in FP reflecting the binding process.[19,20] For the FP assay we used the previously synthesized fluorescent ligand of peptide **1** (Flu 1) which is fully functional in the histamine-releasing assay compared with the unlabeled peptide **1**.[4] The Flu 1 peptide bound to the receptor protein was competitively displaced with increasing concentrations of unlabeled alanine analogues, and the change in fluorescence polarization was measured in millipolarization units (mP). Table 1 summarizes the binding affinities of the alanine analogues compared to peptide **1** after binding to the FcεRIα subunit of the mast cell receptor protein. On the basis of the relative IC_{50}, values, the analogues **4**, **6**, and **7** showed a similarly reduced binding affinity, whereas analogues **2** and **3** showed an increase in binding affinity. A more pronounced increase in binding affinity occurred with analogue **5**. This analogue showed a 5-fold increase in affinity compared to peptide **1**.

Histamine Assay. The histamine-releasing activity of the alanine analogues **2**−**7** from rat peritoneal mast cells was tested and compared to peptide **1**[21] (Table 1). The ED_{50} values obtained in this assay showed that all the alanine substitutions yielded analogues with decreased histamine-releasing activity, compared to peptide **1**. The substitutions in positions 17 in analogue **6** and 21 in analogue **7** of two Lys residues from the carboxyl terminus of MCD peptide had a moderate loss of potency (>4-fold), whereas analogue **4** with the lysine

substitution in the middle part of the molecule at position 11 showed a greater loss of potency (>6-fold). Analogue **2** (Ala^2) and analogue **3** (Ala^6) with alanine substitutions in the amino end of MCD peptide showed a 10-fold and 80-fold decrease in histamine releasing-activity, respectively. The largest loss of activity was caused by analogue **5** with the proline substitution in the middle of the sequence at position 12. This analogue showed a more than 125-fold loss in activity.

Analogue **5** was therefore tested as a competitive antagonist of the histamine-releasing activity of peptide **1**. At a concentration up to 2 μM, peptide **5** did not inhibit peptide **1**-stimulated histamine release.

Discussion

In the search for MCD peptide analogues as inhibitors for IgE we initiated an L-Ala scan and used fluorescence polarization to test the affinity of the alanine analogues to the mast cell receptor. For this purpose we used the soluble FcεRIα subunit of the IgE receptor. It has been shown that human FcεRIα protein is sufficient for high affinity IgE binding.[22] The structure of the FcεRIα subunit free and bound to IgE has been revealed by X-ray crystallography.[17,23] Various designed peptides derived from FcεRIα sequences have been found to inhibit IgE binding to the mast cell receptor. In these studies it was also found that cyclic peptides have stronger inhibitory activity than linear ones when competing with IgE binding.[24−27] In this respect, peptide **1** has an inherent cyclic disulfide structure and inhibits IgE binding.[4] In contrast to some designed peptides, peptide **1** acts through and activates the mast cell receptor by releasing histamine[2] and by elevating Ca^{2+} in the cell.[28] Therefore, such functional studies and the possible identification of receptor binding sites by fluorescent-labeled MCD peptides may facilitate the design of analogues to inhibit IgE binding. This is of importance in view of the suggestion that two regions of FcεRIα interact with IgE.[17,23]

In the L-Ala scan, the substitution of the Lys residues with L-Ala changed an important property of peptide **1**, namely its overall basicity. All the l-Ala analogues showed a loss of histamine-releasing activity compared to the parent peptide **1**. The loss of histamine releasing-activity increased significantly as the L-Ala substitutions proceeded from the carboxyl to the amino terminus of MCD peptide. Analogues **6** and **7** showed a moderate loss of histamine release. In these two analogues, the L-Ala substitutions are included in the α-helical part of peptide **1** between His^{13}-Asn^{22}, which is important for this activity.[8−10] Alanine, a stronger helix-promoting amino acid than lysine,[29] apparently reinforces the helix and thus diminishes the loss of histamine-releasing activity. Analogue **4** with the L-Ala substitution located just outside the boundaries of the helix showed a greater loss in activity, whereas analogues **2** and **3**, with the alanine substitutions remote from the helix, showed the greatest loss in histamine-releasing activity. Previous studies showed that a change in the charge distribution of the Ile^1-Lys^2 amino end of peptide **1** does not affect histamine activity significantly.[10] However, analogue **2**, with alanine in position 2, showed relatively low histamine releasing-activity. Therefore, the decrease in activity of analogue **2** and of all the other L-Ala analogues seem to be due to the increase in hydrophobicity after the removal of positive charge.

The binding affinities of the alanine analogues to the FcεRIα receptor protein were determined in homogeneous medium via FP in a competitive binding assay. The FP assay showed that the affinity of the analogues **4**, **6**, and **7** changed in the same direction as their activity, i.e., decreased potency and decreased activity compared to the parent peptide **1**. These analogues behaved as weak agonists of peptide **1**. The Lys residues in these analogues were replaced by L-Ala in positions 11, 17, and 21 which are located in and around the MCD peptide's α-helical part, known to regulate biological activity.[8−10] Judging from our binding studies, these positions also seem to be important for receptor binding.

Analogues **2**, **3**, and **5** showed increased binding affinity and decreased histamine release compared to peptide **1**, i.e., an opposite effect between activity and binding affinity. These analogues were partial agonists of peptide **1**. The binding affinity of analogues **2** and **3** indicated that the Lys residues in positions 2 and 6, located in the N-terminal end of peptide **1**, seem to be more important for activity than for binding.

Of special interest was analogue **5** with L-Ala substituting for the Pro residue in position 12. This analogue showed the greatest loss in activity and the highest binding affinity compared to all analogues tested (Table 1). The restricted torsion angle between Pro and its preceding residue (x-Pro) imposes structural constraints on the secondary structure of proteins and peptides and allows only a limited number of conformations. One conformation often associated with proline is a cis−trans isomerization with the isomers being convertible to each other.[30] Chromatographic observations[7,14,31] supported by NMR studies[31] with peptide **1** indicated that the conformation of the molecule includes a cis−trans isomerization of the proline bond. The L-Ala side chain replacing Pro lacks such unusual backbone dihedral angle preferences.[30] Thus the change in the dihedral angle induced changes in the overall conformation and in the structural integrity of the molecule. These changes, in turn, seem to play a decisive role in the biological profile of analogue **5**. Despite the apparent conformational and functional differences between peptide **1** and **5**, analogue **5** did not inhibit the functional activity of peptide **1**.

Conclusion

All the synthesized monosubstituted L-Ala analogues in this study have been found to decrease histamine-releasing activity compared to the parent peptide **1**. The differences in the binding affinities of these analogues to the FcεRIα mast cell receptor protein, as seen by fluorescence polarization, did not parallel those of their histamine-releasing activity. Consequently, the action of these analogues varied from weak to partial agonists of peptide **1**. Analogue **5** in this series showed the most intriguing properties concerning activity and binding.

As an interesting beginning, the alanine analogues in this study provide a basis for further search of MCD peptide antagonists. When combined in the same molecule, some of the alanine substitutions may result in competitive or noncompetitive antagonists of peptide **1**

and consequently can be used as inhibitors of IgE/MCD peptide interaction on the mast cell receptor.

Experimental Section

Peptide Synthesis and Purification. All Boc-protected amino acids and resins were purchased from Novabiochem (San Diego, CA). Coupling reagents and solvents were purchased from Aldrich (Milwaukee, WI). They were all used as received. The protected amino acids used were Boc-Lys(2-Cl-Z), Boc-His(Bom), and Boc-Arg(Tos). The cysteine[3,15] pair was protected with the Mbzl and the cysteine[5,19] pair with the Acm group.

The peptides were synthesized on a 1% cross-linked MBHA resin (100−200 mesh, 0.54 mmol/g) on a 430 Applied Biosystems peptide synthesizer and manually. 50% TFA in DCM was used for the removal of the Boc groups followed by several washings with DCM and neutralization with 10% DIPEA in DCM. The resin was washed thoroughly with NMP before coupling. Three-fold excess protected amino acid was added as preformed HOBt ester in NMP, neutralized with DIPEA, and allowed to react for 90−120 min. The completion of the reaction was checked by the Kaiser test[32] and repeated couplings and acetylations (excess acetic anhydride in DMF and HOBt for 15 min) were performed if necessary. After completion of the peptide chain the N-terminal Boc group was removed as usual and the resin neutralized, washed thoroughly with DCM and dried prior to HF cleavage. One gram of fully protected MCD peptide resin was deprotected and cleaved from the resin by the low/high hydrogen fluoride method.[33] The HF was evaporated, the resin was washed with ethyl acetate, and the peptides were extracted from the resin with 100 mL of 0.2 N AcOH. This solution was diluted to 1.5 L with water and adjusted to pH 8.0 with ammomium hydroxide, and the Cys[3,15] disulfide bond was formed by addition of potassium ferricyanide until a permanent yellow color persisted. After 30 min, the pH was adjusted to pH 3.0, and the solution was stirred with Bio-Rad AG3-X4A (chloride form, 200−400 mesh) ion exchanger for another 30 min. The solution was filtered and evaporated in vacuo. This residue was successively chromatographed on Sephadex G25 in 0.2 N AcOH, carboxymethyl cellulose with a linear gradient of ammonium acetate (0.1 M to 0.75 M), and on Sephadex G15 with 0.2 N acetic acid. The structure of the [Ala^{x}Cys(Acm)^{5,19}]MCD analogues was assured by amino acid analysis and mass spectrometry.[7] The removal of the remaining two Acm cysteine protecting groups and the formation of the second disulfide bond was performed with I_2. For this oxidation, 50 mg of monocyclic MCD peptide analogue in 8 mL of HOAc and 2 mL of water was added dropwise at 0 °C in a solution of 18 mg I_2 in 48 mL of HOAc, 42 mL of water and 0.2 mL of 1 N HCl. The reaction mixture was stirred for 15 min at 0 °C and 15 min at room temperature and quenched with ascorbic acid. The solution was reduced in vacuo and chromatographed on Sephadex G 15 with 0.2 N AcOH.[7]

The bicyclic peptides were further purified by RP-HPLC on a semipreparative Vydac C18 column (218TP510, 1 × 25 cm) on an Altex-Beckman chromatographic system.[7] A standard linear gradient of 10−40% of 0.1% trifluoroacetic acid and 0.1% trifluoroacetic acid in acetonitrile was used. The yields of the final products ranged from 30 to 60%. Generally, it is difficult to retain MCD peptides on reversed-phase HPLC columns, due to their hydrophilic and highly charged properties. A better retention time can be achieved by using ion pairing agents in a linear gradient of 10 mM triethylammonium phosphate (TEAP)/5 mM butanesulfonate/5%1-propanol, pH 3.0; 10 mM TEAP/5 mM butanesulfonate/15% 1-propanol.[34] When using the latter gradient in a preparative scale the peptides must afterward be desalted on Sephadex G15 in 0.2 N AcOH. The final products were analyzed in the two gradient systems and by electrospray mass spectrometry (Micromass Q-Tof spectrometer). Analytical data are shown in Table 2.

Binding Assay. The competitive binding of the alanine analogues to the FcεRIα receptor protein were measured by FP. Fixed amounts of 7 nM FcεRIα receptor protein, 4 nM of peptide Flu 1 in PBS (pH 7.4), and increasing concentrations (7−500 μM) of peptides **1**−**7** were incubated at 25 °C for 5 min in borosilicate tubes (6 × 50 mm) in a final volume of 100 μL. The fluorescence polarization was measured on a Beacon 2000 fluorescence polarization analyzer (PanVera, Madison, WI) and expressed in millipolarization units (mP). The filters used were 485 nm for excitation and 535 nm for emission with 3 nm bandwidth.[35] The background reading of the labeled peptide alone was 47 mP and was subtracted from all samples. The mP values were the average of many consecutive readings of each sample. Preliminary experiments using different lots of peptides and of the protein gave similar results. The IC_{50} of the peptides was analyzed by nonlinear regression with the computer curve-fitting program Origin.

Histamine Assay. Histamine-releasing activity of the peptides was tested using a recently developed microplate assay.[21] Rat peritoneal mast cells from Sprague−Dawley rats of different sexes and ages were lavaged into Tyrode's buffer without carbonate and bivalent cations and buffered with 15 mM Hepes (pH 7.2) and 0.1% bovine serum albumin. They were isolated on an Accudenz gradient (Accurate Chemicals, Westbury, NY).[36] Prior to use, the cells were counted by toluidine blue staining, and their viability was determined using Trypan blue exclusion. Each well of the microplates contained 25 μL of various concentrations of peptide and 100 μL of prewarmed mast cells ((1−2) × 10^3 per well), and the plates were kept at 37 °C for 20 min. The cells were washed by centrifugation with Tyrode buffer using a microplate centrifuge. Cell-associated histamine was released on the plates with 0.1% Triton X-100 (37 °C for 20 min) followed by 14% trifluoroacetic acid. The plates were kept in the cold overnight. The plate contents were centrifuged, and aliquots of the supernatants were assayed for histamine fluorometrically after addition of 2 N NaOH and phthalaldehyde.[37] Samples were replicated (n = 6). Statistical analysis of the data was evaluated by nonlinear regression done with Sigma Plot. P values <0.05 were considered statistically significant. P values of all analogues were between 0.0003 and 0.04.

For competitive inhibition of the histamine releasing activity of peptide **1** by peptide **5**, a fixed amount of peptide **1** (0.5 μg/mL) and increase concentrations of peptide **5** (0.05−50 μg/mL) were mixed, and peritoneal mast cells were added. After incubation for 35 min at 37 °C, histamine release was extracted and measured as above. The histamine-releasing values of peptide **1** controls and of the mixture of peptide **1** with peptide **5** did not show statistical differences.

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Abbreviations

The abbreviations of all the L-amino acids used are in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9−37). Additional abbreviations: AcOH, acetic acid; Acm, acetamidothetyl; Bom, benzyloxymethyl; CL-Z, 2-chlorobenzyloxycarbonyl; DCM, dichloromethane; DIPEA, *N*,*N*-diisopropylethylamine; HOBT, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; IgE, immunoglobulin E; Mbzl, 4-methylbenzyl; NMP, *N*-methylpyrrolidone; TFA, trifluoroacetic acid; Tos, 4-toluenesulfonyl.

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