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Partial Alanine Scan of Mast Cell Degranulating Peptide (MCD): Importance of the Histidine- and Arginine Residues

ANGELIKI BUKU,[a]* MILTON MENDLOWITZ,[b] BARRY A. CONDIE[c] and JOSEPH A. PRICE[d]

[a] Department of Physiology and Biophysics, Mount Sinai School of Medicine, one Gustave L. Levy Place, New York, NY 10029, USA
[b] Department of Molecular Cardiology, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, USA
[c] The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA
[d] Department of Pathology, College of Osteopathic Medicine, Oklahoma State University, 1111 W. 17th Street, Tulsa, OK 74107, USA

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Abstract: The influence of the two histidine and two arginine residues of mast cell degranulating peptide (MCD) in activity and binding was studied by replacing these amino acids in the MCD sequence with L-alanine. Their histamine releasing activity was determined on rat peritoneal mast cells. Their binding affinity to the FcεRIα binding subunit of the human mast cell receptor protein, was carried out using fluorescence polarization. The histamine assay showed that replacement of His^{13} by Ala occurred without loss of activity compared with the activity of MCD. Alanine substitutions for Arg^{7} and His^{8} resulted in an approximately 40-fold increase, and for Arg^{16} in a 14-fold increase in histamine-releasing activity of MCD. The binding affinities of the analogs were tested by competitive displacement of bound fluorescent MCD peptide from the FcεRIα binding protein of the mast cell receptor by the Ala analogs using fluorescence polarization. The analogs Ala^{8} (for His) and Ala^{16} (for Arg) showed the same binding affinities as MCD, whereas analog Ala^{7} (for Arg) and analog Ala^{13} (for His) showed slightly better binding affinity than the parent compound. This study showed that the introduction of alanine residues in these positions resulted in MCD agonists of diverse potency. These findings will be useful in further MCD structure–activity studies. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Ala-scan; fluorescence polarization; histamine release; MCD; mast cell receptor

INTRODUCTION

Continued studies have been carried out on the biological properties of mast cell degranulating peptide (MCD) a bee venom peptide [1]. MCD is a 22-amino acid basic peptide with the sequence shown in Figure 1.

The sequence contains two disulfide bridges, $Cys^{3,15}$ and $Cys^{5,19}$, and a plethora of basic amino acids (Figure 1). Its biological activities that are thought to derive from these structural features include histamine release at low concentrations [2] and inhibition of mast cell degranulation at higher concentrations [3,4]. These mast cell activities relate MCD to the type I hypersensitivity reaction, i.e. allergy and to IgE (immunoglobulin E). IgE is the main allergic reagent because it binds with high affinity to mast cell receptors. Cross-linking of adjacent receptor bound IgE molecules by allergens initiates a cascade of mast cell intracellular events leading to release of histamine and other mediators of allergy [5]. It has already been shown that MCD

*Correspondence to: Dr Angeliki Buku, Department of Physiology and Biophysics, Mount Sinai School of Medicine, Box 1218, New York, NY 10029, USA; e-mail: Angeliki.Buku@mssm.edu
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H-Ile[1]-Lys-Cys[3]-Asn-Cys[5]-Lys-Arg[7]-His[8]-Val-Ile-Lys-Pro-His[13]-Ile-Cys[15]-
-Arg[16]-Lys-Ile-Cys[19]-Gly-Lys-Asn[22]-NH_2

Figure 1 Mast cell degranulating peptide.

binds to the mast cell receptors and inhibits IgE binding to these receptors. [6]. Because of the properties of IgE, the studies aimed to recognize MCD analogs with diminished histamine-releasing activity and at the same time with high binding affinity to the mast cell receptor in order to compete with and inhibit IgE binding to these receptors. In spite of its interesting biological properties no structure–activity studies of MCD have been explored previously.

In order to understand the biological actions of MCD, a preliminary evaluation of the contribution of its basic amino acids that are generally responsible for histamine release [7] was initiated. For this purpose, truncated analogs of MCD [8,9] were synthesized. In these studies it became apparent that basic amino acids in the *C*-terminal end of MCD, which was found to comprise a helical part, are the most important for histamine-releasing activity. Other studies were directed toward determining the role of the cysteine and the arginine residues in MCD activity by substituting these residues with Ala and Orn, respectively [10]. Furthermore, to study the role of the individual basic residue side chains an Ala-scan was initiated. The substitution of the naturally occurring residues with Ala eliminates the side-chain at that position while preserving the configuration of the peptide backbone. Therefore, it provides an estimation of the contribution of the side-chain to activity and receptor binding. At first, the five lysine residues of MCD were replaced with Ala and histamine assays and binding studies were performed with these analogs using the soluble FcεRIα binding subunit protein of the human mast cell receptor. Some of these analogs showed reduced histamine-releasing activity and a better binding affinity to the mast cell receptor than MCD [11].

The present study was conducted to understand the role of the remaining basic residues, namely the two arginine and the two histidine residues in MCD biological actions. An Ala-scan for these residues was also performed. The alanine analogs were tested for their histamine-releasing activity on rat peritoneal mast cells. As in the case of the Lys substitutions [11], the binding affinity of the analogs to the FcεRIα binding subunit of the human IgE mast cell receptor was determined in a homogeneous assay, i.e. in solution, using fluorescence polarization (FP).

MATERIALS AND METHODS

Peptide Synthesis

All protected L-amino acids and resins were purchased from Novabiochem (San Diego, CA). All reagents and solvents were obtained from Aldrich (Milwaukee, WI).

The peptides [Ala[7]]-MCD, [Ala[8]]-MCD, [Ala[13]]-MCD, and [Ala[16]]-MCD (Figure 1) were synthesized by stepwise solid phase synthesis on *p*-methylbenzhydrylamine (MBHA) resin with standard Boc/Bzl methodology. The synthesis, oxidations, purification and analysis of the analogs followed a well-established protocol described previously for the synthesis of other MCD analogs [8–13].

Histamine Assay

The histamine-releasing activity of the peptides was determined by a method in which histamine release and histamine measurement were done on 96-well microplates [14]. Rat peritoneal mast cells from 300–400 g Sprague-Dawley rats of mixed sexes and ages were lavaged into modified Tyrode's buffer without carbonate and bivalent cations and buffered with 15 mM HEPES (pH 7.2) containing 0.1% BSA (bovine serum albumin). They were isolated on an Accudenz gradient (Accurate Chemicals Westbery, NY) as described [15].

The cells were counted with toluidine blue staining and their viability determined by the Trypan blue exclusion test. Each well contained 25 µl of various concentrations of peptide plus 100 µl prewarmed cells ($1–2 \times 10^3$ per well), and the plates were kept at 37 °C for 20 min. The cells were washed with modified Tyrode's buffer and centrifuged in a microplate centrifuge (ICN centrifuge, Needham, MA). Cell associated histamine was released on the same plates with 100 µl of 0.1% Triton X-100 for 20 min at 37 °C followed by 100 µl of 14% trichloroacetic acid and the plates were kept in the cold over night. The plate contents were centrifuged and aliquots of the supernatants were assayed for histamine. After addition of 2N NaOH and phthalaldehyde the histamine release was measured fluorometrically [16]. Statistical analysis of the results ($n = 6$) was evaluated by nonlinear regression done with Sigma Plot®. p values less than 0.05 were considered statistically significant.

Binding Assay

Binding affinity for each alanine analog was performed using the FcεRIα soluble mast cell receptor binding protein (HESKA, Fort Collins, CO). For this purpose borosilicate tubes (6 × 50 mm) containing 7 nM FcεRIα protein, 4 nM fluorescent MCD, and various concentrations of the analogs in PBS (phosphate buffered saline) to a final volume of 100 µl were incubated at room temperature for 5 min. The fluorescence polarization was measured with a Beacon 2000 fluorescence polarization analyser (PanVera, Madison, WI) and expressed in millipolarization units (mP). The filters used were 485 nm for excitation and 535 nm for emission with a 3 nm bandwidth [17]. The mP values were the average of many consecutive readings of each sample. The background reading of the fluorescent MCD was subtracted from all samples. The IC_{50} of the peptides was analysed by non-linear regression using the Origin® program.

RESULTS AND DISCUSSION

Effect of the Ala-substitutions on Histamine Release

Histamine-releasing activity of the alanine analogs performed on peritoneal mast cells are shown in Table 1, together with the activities of previously synthesized alanine analogs replacing lysine residues for comparison. Alanine analogs **2** and **3** substituting for Arg^7 and His^8 showed an almost 40-fold increase in histamine release and analog **5** for the substituted Arg^{16} showed a 14-fold increase in this activity compared with the parent MCD. The histamine-releasing activity of these two analogs also showed the greatest increase in this activity of all the tested analogs with alanine substitutions for the basic amino acids. In contrast, analog **4** for the His^{13} substitution was almost equipotent with MCD. The high potency of the tested Ala analogs indicates that the side-chain groups of each single Arg^7, His^8, His^{13} and Arg^{16} residue are not essential for the histamine-releasing activity of MCD.

Effect of the Ala-scan on Binding to the FcεRIα Receptor Protein

The binding affinities of the analogs were determined using fluorescence polarization (FP) [18–20]. This measures in a homogeneous state the change in anisotropy of a fluorescent probe in free and bound states. An increase in anisotropy is proportional to the ligand–receptor protein complex formation. For this purpose the previously synthesized and characterized fluorescent labeled MCD peptide Flu^1 MCD [6] and human soluble mast cell receptor protein in the form of its FcεRIα binding subunit were used. The binding, after addition of various Ala peptide concentrations, was followed by changes in the fluorescence polarization expressed as millipolarization units. From the calculated IC_{50}, (Table 1) analogs **3** and **5** showed binding affinities in the same range as MCD. Analogs **2** and **4** showed a slightly better binding affinity than MCD. In general the affinity and activity of the peptides did not follow the same pattern. Each Ala substitution in positions 7, 8, 13, and 16 seemed to affect the biological activity more than the binding.

Table 1 Comparison of Histamine-Releasing Activity and Binding Affinity of Alanine Analogs of MCD peptide

Peptide	$ED_{50} \times 10^{-5}$ M[a]	Ratio[b]	IC_{50} (µM)[c]	Ratio[d]
1 MCD standard	0.42 ± 0.07	1	132 ± 41	1.0
2 [Ala^7]-MCD	0.01 ± 0.02	0.02	112 ± 62	0.8
3 Ala^8]-MCD	0.01 ± 0.01	0.02	138 ± 17	1.0
4 [Ala^{13}]-MCD	0.41 ± 0.02	1	106 ± 62	0.8
5 [Ala^{16}]-MCD	0.03 ± 0.01	0.07	135 ± 26	1.0
Ala substitution for Lys[e]				
6 MCD standard	0.16 ± 0.04	1	114 ± 30	1.0
7 [Ala^2]-MCD	1.57 ± 0.05	10	82 ± 21	0.7
8 [Ala^6]-MCD	12.54 ± 0.03	76	71 ± 14	0.6
9 [Ala^{11}]-MCD	1.07 ± 0.02	6	153 ± 21	1.3
10 [Ala^{17}]-MCD	0.77 ± 0.03	4	153 ± 54	1.3
11 [Ala^{21}]-MCD	0.78 ± 0.04	4	153 ± 54	1.3

[a] Effective concentration for half-maximal response ± SEM ($n = 6$).
[b] Ratio of histamine release by the alanine analogs to MCD peptide (1.0).
[c] Concentrations of peptides at 50% binding as determined by fluorescence polarization (millipolarization units were the average from at least 10 runs).
[d] Ratio of binding affinities of the alanine analogs to MCD peptide (1.0).
[e] Data from Ref. [11].

Rationale and Evaluation of the Ala-scan on Basic Residues of MCD

The accessibility of MCD synthetic analogs makes it feasible to probe the effect of MCD on the inhibition

of IgE binding or IgE cross-linking on the mast cell receptor. Previous attempts in this direction showed that cyclic peptides were better inhibitors than their linear counterparts [21–23]. MCD with its bicyclic structure and its mast cell receptor affinity provides a natural choice for the study of allergic reactions in order to explore the inhibition of IgE to the mast cell receptor.

To obtain leads for the design of such analogs it is necessary to study the role of specific amino acids, and in the case of MCD, basic amino acids thought to be the main cause of histamine release [7]. One way to do this is to replace these residues with Ala [24]. Alanine has a non-functional side chain, although it is a more hydrophobic and a stronger helix-promoting amino acid than the basic residues [25,26].

Following up previous studies with Lys [11], Ala analogs were synthesized by modifying the residues Arg^{7}, Arg^{16} and His^{8}, His^{13} in the MCD sequence (Figure 1). The results showed that the alanine replacement of the two Arg residues in analogs **2** and **5** increased histamine release significantly compared with MCD. Also, in previous studies replacement of the Arg residues with the less basic but more hydrophobic ornithine resulted in analogs with increased histamine release [10]. Generally, it appears that enhanced hydrophobicity in the arginine positions favors histamine release. The histamine-releasing activity of alanine analog **2** (Arg^{7}) was greater than that of alanine analog **5** (Arg^{16}) where the alanine residue is located in the *C*-terminal helical part of MCD (His^{13}-Asn^{22}) that is important for histamine release [8,9]. Probably the loss of the Arg^{16} residue inside the helix has a greater impact on the change of hydrogen bonding, charge, and electrostatic interaction and thus activity than in position 7 toward the *N*-terminal end of the molecule.

The replacement of the His residues with Ala resulted in analog **3** (His^{8}) with very high histamine-releasing activity and analog **4** (His^{13}) with the same activity as MCD. As with analog **2**, enhanced hydrophobicity may have contributed to the high activity of analog **3**. In analog **4** the substitution occurs in the vicinity of the helix. In contrast to analog **5**, the replacement of His in analog **4**, does not increase histamine release. The loss of His properties such as hydrogen bonding, electrostatic interactions, and aromaticity [27] appeared not to be compensated for by the helix-increasing propensity of alanine [25,26] as is the case with analog **5**.

To compare the binding affinities of the Ala analogs to the mast cell receptor, fluorescence polarization was used [18,19,20]. This was an alternative because of the difficulties encountered with the binding of MCD to the mast cell receptor using conventional methods [28, McDonell JM, personal communication]. Furthermore, the FcεRIα soluble receptor binding protein subunit became available [29,30] and proved to be sufficient for binding to the mast cell receptor [31]. For the binding the fluorescence polarization homogeneous assay was used with fluorescent MCD as the ligand [6]. As Table 1 shows, analogs **3** and **5** showed the same binding affinities as MCD. The analogs **2** and **4** showed a tendency toward a somehow better binding affinity than the parent compound. Apparently, there is no linear relationship between the activities and binding of the Ala analogs indicating that full receptor occupancy may not be required to trigger biological response.

CONCLUSION

Based on the results of the functional and binding assays, the Ala analogs in this study resulted in MCD agonists with the same or higher potency than the native MCD. Consequently, the high biopotency that is retained in the $Ala^{7,8,13,16}$ analogs suggests that the side chains of each one of these positions are most likely not essential for receptor binding. In view of these results it seems that the performed alanine substitutions do not disrupt the global structure of the peptides. Introduced in these positions they rather enhance the hydrophobic interactions between peptides and the mast cell receptor. In this case these interactions seem to be stronger than the ionic ones of the parent MCD. Furthermore, the Arg and His substitutions compared with the Lys substitution showed (Table 1) [11], that the Lys residues might be more relevant factors for the design of MCD as IgE binding inhibitors. Overall, the Ala-scan of the basic residues of MCD provided valuable knowledge in the sense that it indicates which basic amino acid positions can be excluded or which can be considered for further design of MCD analogs to study MCD/IgE interactions, aiming to block IgE binding to the mast cell receptor.

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