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Amino Acid Sequence of Bumblebee MCD Peptide: A New Mast Cell Degranulating Peptide From the Venom of the Bumblebee *Megabombus pennsylvanicus*

ANTONIO ARGIOLAS,* PATRICIA HERRING AND JOHN J. PISANO

*Institute of Pharmacology, University of Cagliari, Via Porcell 4, 09100 Cagliari, Italy
and Section of Physiological Chemistry, Laboratory of Chemistry
National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20205*

ARGIOLAS, A., P. HERRING AND J. J. PISANO. *Amino acid sequence of bumblebee MCD peptide: A new mast cell degranulating peptide from the venom of the bumblebee* Megabombus pennsylvanicus. PEPTIDES 6: Suppl. 3, 431–436, 1985.—A new 28 amino acid peptide, we recently isolated from the venom of the bumblebee *Megabombus pennsylvanicus*, has been characterized. The peptide, Met-Cys-Ile-Cys-Lys-Asn-Gly-Lys-Pro-Leu-Pro-Gly-Phe-Ile-Gly-Lys-Ile-Cys-Arg-Lys-Ile-Cys-Met-Met-Gln-Gln-Thr-His(NH_2), has been named bumblebee mast cell degranulating (MCD) peptide due to its ability to degranulate rat peritoneal mast cells and its resemblance to the bee venom MCD peptide. Bumblebee MCD peptide, unlike bombolitins, the other mast cell degranulating heptadecapeptides of bumblebee venom, is not lytic and releases histamine at a dose as low as 0.05 μg/ml (1.6×10^{-8} M).

Amino acid sequence Bumblebee MCD peptide Bumblebee venom

RECENTLY we reported the isolation and characterization of bombolitins, a new class of lytic mast cell degranulating heptadecapeptides from the venom of the bumblebee *Megabombus pennsylvanicus* [3]. In addition to bombolitins, we found that bumblebee venom contains other active peptides. Although less abundant than bombolitins, one of these peptides stimulated histamine release from rat peritoneal mast cells at a dose as low as 20 nM. We report here the complete sequence of this specific bumblebee mast cell degranulating (MCD) peptide.

METHOD

The following were purchased: venom sacs and pure dry venom of *Megabombus pennsylvanicus* (Vespa Laboratories, Spring Mills, PA); trypsin-TPCK treated, thermolysin, subtilisin and carboxypeptidase A (Boehringer Mannheim Biochemicals). Reagents and chemicals for sequencing were sequanal grade from Pierce Laboratories. All other reagents were of the highest available purity.

Peptide Isolation

Bumblebee MCD peptide was isolated from pure venom or venom sac extracts as previously described [3]. Briefly, aliquots equivalent to 0.16 mg of pure venom or to 0.5 sac were fractionated at room temperature on a C_{18} μBondapak column (0.39×30 cm, Waters Assoc.) mounted in a Varian 5000 high performance liquid chromatograph equipped with a variable wavelength UV detector. The linear gradient (20 to 100% B in 60 min, flow 1 ml/min) was made with A=0.05% trifluoroacetic acid and 0.025% triethylamine in water, and B=the same reagents in 80:20 acetonitrile:water. Bumblebee MCD peptide eluted as a very sharp peak at 19 min (Fig. 1).

Bioassay

Hemolysis of guinea pig erythrocytes and histamine release from purified rat peritoneal mast cells were performed as previously described [2].

Amino Acid and Sequence Analysis

Samples were hydrolyzed in evacuated sealed tubes with 6 N HCl for 24 hr at 110°C and amino acids were determined with a Beckman 6300 amino acid analyzer essentially by the method of Spackman, Moore and Stein [11]. Amino acid sequences were determined manually by dansyl [5] and dimethylamino-azobenzene-isothiocyanate (DABITC)-phenylisothiocyanate (PITC) methods [4]. Dansyl- and dimethylamino-azobenzene-thiohydantoin (DABTH) amino acids were identified by thin layer chromatography using 5×5 cm polyamide plates (Schleicher & Schuell). Automated Edman degradation was performed with a Caltech gas phase

TABLE 1

AMINO ACID ANALYSIS OF BUMBLEBEE MCD PEPTIDE AND ITS FRAGMENTS

Amino acid	BB MCD P	BB MCD T1	BB MCD T2	BB MCD T1a	BB MCD T1b	BB MCD T1c	BB MCD T1SS
CmCys				0.8(1)	1.7(2)	0.9(1)	
Asx	1.2(1)		1.1(1)				
Thr	0.8(1)	0.8(1)				0.8(1)	0.8(1)
Glx	2.1(2)	2.2(2)				2.0(2)	2.1(2)
Pro	1.9(2)		2.0(2)				
Gly	3.3(3)		3.2(3)				
½Cys	3.8(4)	4.1(4)					1.8(2)
Met	2.5(3)	2.8(3)			0.9(1)	1.8(2)	1.7(2)
Ile	3.8(4)	3.0(3)	1.0(1)	1.0(1)	1.0(1)	1.0(1)	0.8(1)
Leu	1.0(1)		1.2(1)				
Phe	0.8(1)		0.9(1)				
His	1.2(1)	1.0(1)				1.0(1)	1.0(1)
Lys	3.9(4)	1.9(2)	1.8(2)		0.8(1)	0.9(1)	0.8(1)
Arg	0.9(1)	0.9(1)		0.9(1)			
Residues	28	17	11	3	5	9	10

The peptide did not show any absorbance peaks near 280 nm indicating the absence of Trp.

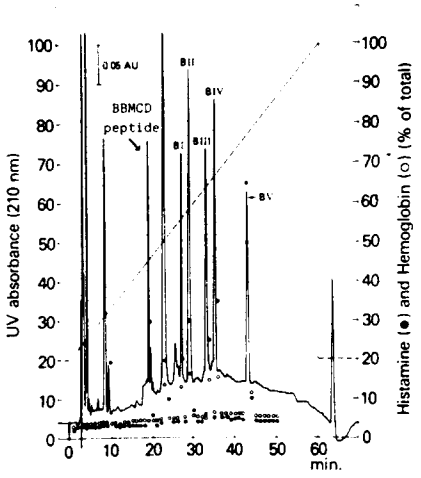

FIG. 1. High performance liquid chromatography fractionation of peptides in *Megabombus pennsylvanicus* venom. Sample: 0.16 mg dry weight venom in 50 μl of 0.05% trifluoroacetic acid. Column: C_{18} μBONDAPAK 0.39×30 cm; temp. ambient. Solvent A: 0.05% trifluoroacetic acid and 0.025% triethyl amine in water; Solvent B: the same reagents in 80:20 acetonitrile:water. Linear gradient: from 20 to 100% B in 60 min. Flow: 1 ml/min. One ml fractions were collected, concentrated in a Speed Vac (Savant), dissolved in 200 μl water and assayed for histamine release and erythrocytes hemolysis [2].

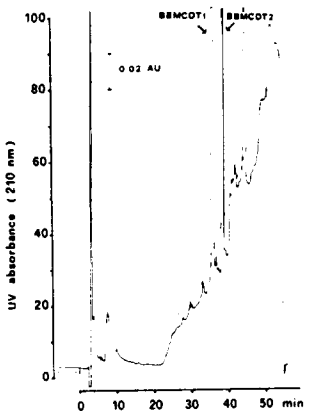

FIG. 2. High performance liquid chromatography fractionation of tryptic peptides of the bumblebee MCD peptide. Two nmol of intact peptide were incubated at 37° for 1 hr with 0.5 μg trypsin-TPCK in 50 μl of 50 mM Tris-HCl pH 8.0. Sample: the entire reaction mixture. Linear gradient: 2 to 20% B in 30 min and to 80% B in the next 20 min. The other conditions were as in Fig. 1, except that triethylamine was ommitted from Solvent A and B.

sequenator. Phenyl-thiohydantoin (PTH) amino acids were identified at 269 nm on a Hewlett Packard high performance liquid chromatograph equipped with a 0.4×25 cm Zorbax ODS column (Du Pont) as previously described [10].

RESULTS

From amino acid analysis, we calculated that from 9 mg of dry venom 8 nmol of bumblebee MCD peptide were isolated, and from one sac 0.2–0.3 nmol.

Amino Acid and Sequence Analysis

Amino acid analysis revealed that bumblebee MCD peptide is a highly basic 28 amino acid peptide (Table 1). It contains 4 Lys, 1 Arg and 1 His together with 4½ Cys and no

TABLE 2

MANUAL DETERMINATION OF THE SEQUENCE OF BUMBLEBEE MCD PEPTIDE AND ITS FRAGMENTS

	1	5	10	15	20	25	28
BB MCD peptide	Met-Cys-Ile-Cys-Lys-Asn-Gly-Lys-Pro-Leu-Pro-Gly-Phe-Ile-Gly-Lys- Ile-Cys-Arg-Lys-Ile-Cys-Met-Met-Gln-Gln-Thr-His(NH_2)						
BB MCD T1a*					Ile-Cys-Arg		
BB MCD T1b*	Met-Cys-Ile-Cys-Lys						
BB MCD T1c*						Lys-Ile-Cys-Met-Met-Gln-Gln(Thr,His)	
BB MCD T2			Asn-Gly-Lys-Pro-Leu-Pro-Gly-Phe-Ile-Gly-Lys				

The arrow (→) indicates residues determined by the DABITC-PITC Edmand degradation performed on 1 nmol of the peptides. DABTH-amino acids were identified by two dimensional chromatography on 5×5 cm polyamide sheets. Solvent 1: water, acetic acid (10:5); solvent 2: toluene, n-hexane, acetic acid (10:5:2.5). The arrow (-) indicates residues determined by the dansyl procedure [5]. The arrow (|) indicates the trypsin cleaved peptide bounds. *Cys indicates Carboxymethyl-cysteine.

TABLE 3

AUTOMATED EDMAN DEGRADATION OF BUMBLEBEE MCD PEPTIDE

Step	Amino acid	Yield (%)
1	Met	78
2	—	—
3	Ile	80
4	—	—
5	Lys	63
6	Asn	81
7	Gly	71
8	Lys	54
9	Pro	72
10	Leu	51
11	Pro	65
12	Gly	46
13	Phe	53
14	Ile	48
15	Gly	36
16	Lys	25
17	Ile	10
18	—	—
19	Arg	1
20	Lys	8
21	Ile	5
22	—	—
23	Met	4
24	Met	3
25	Gln	2
26	Gln	2
27	Thr	1
28	His	1

The intact peptide (1.3 nmol) was sequenced. PHT-amino acids were identified by high performance liquid chromatography [10]. No PTH amino acid was identified at cycles 2, 4, 18 and 22 (Cys).

free sulfidryl group. The amino terminal amino acid Met was determined by both the dansyl and DABITC methods. Digestion with trypsin gave only two peptides, BB MCD T1 and BB MCD T2 (Fig. 2 and Table 1) with a 75% yield, although 6 peptides were theoretically expected. N-terminal amino acid analysis revealed Met, Ile and Lys at the N-terminal of BB MCD T1 and Asn at the N-terminal of BB MCD T2. Reduction with dithiothreitol and carboxymethylation with iodoacetic acid of BB MCD T1 produced 3 peptides which were separated by high pressure liquid chromatography, BB MCD T1a, BB MCD T1b and BB MCD T1c, with Ile, Met and Lys at their N-terminal, respectively (Fig. 3 and Table 1). BB MCD T1a was covered by a huge peak of UV absorbing material (Fig. 3). In order to localize BB MCD T1a, one ml fractions were collected during the elution of the UV absorbing material, concentrated with a Sped-Vac, subjected to acid hydrolysis and analyzed for the presence of amino acids. As the UV absorbing material did not interfere with amino acid analysis, BB MCD T1a was found 7 min after the injection. Manual sequencing of BB MCD T1a, BB MCD T1b, BB MCD T1c and BB MCD T2 was straightforward, except for the last 2 amino acids of BB MCD T1c (Thr

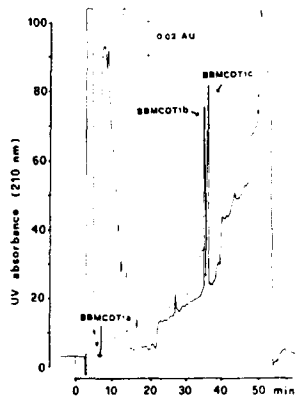

FIG. 3. High performance liquid chromatography fractionation of BB MCD T1 after reduction and carboxymethylation. BB MCD T1 (Fig. 1) (1.4 nmol) was incubated 1 hr at 37° with 0.12 mM dithiothreitol in 50 μl of 0.2 M N-methyl-morpholine-HCl pH 8.5. Iodoacetic acid was added to the reaction mixture in 20 μl of the above buffer to a final concentration of 0.24 mM. The incubation was continued for 1 hr at 37° and stopped by injecting the entire reaction mixture in the column. The peptides were fractionated by using the same conditions reported in Fig. 2. In order to localize BB MCD T1a one ml fractions were collected and concentrated with a Speed Vac. After acid hydrolysis at 110° in sealed evacuated tubes, the amino acid composition revealed the presence of the peptide. No interference was found due to the huge peak of UV absorbing material.

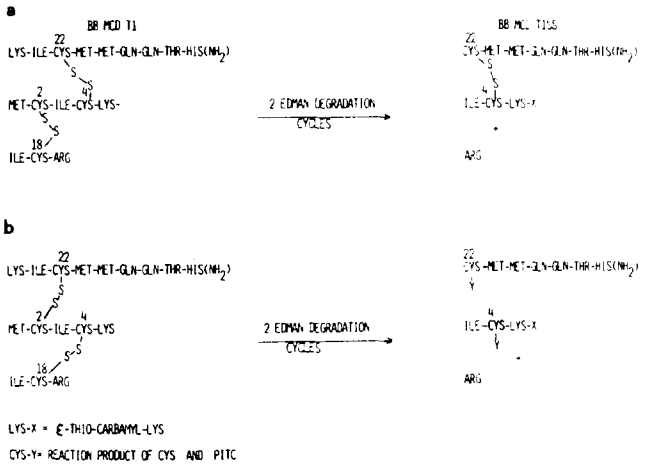

FIG. 4. Scheme of the reaction for the assignment of the disulfide bridges in BB MCD T1. (a) Disulfide bridges between Cys^{2}–Cys^{18} and Cys^{4}–Cys^{22}; (b) Disulfide bridges between Cys^{2}–Cys^{22} and Cys^{4}–Cys^{18}. See the Results section for details.

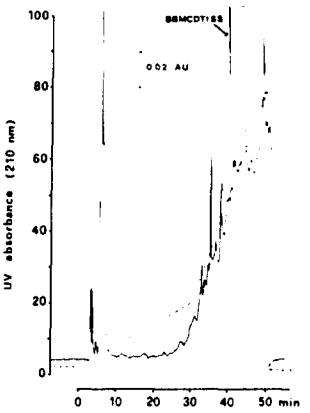

FIG. 5. High performance liquid chromatography of BB MCD T1 after 2 Edman degradation cycles. One nmol of BB MCD T1 was subjected to 2 manual Edman degradation cycles. The shortened peptide was redissolved in 50 μl Solvent A, injected and fractionated as described in Fig. 2.

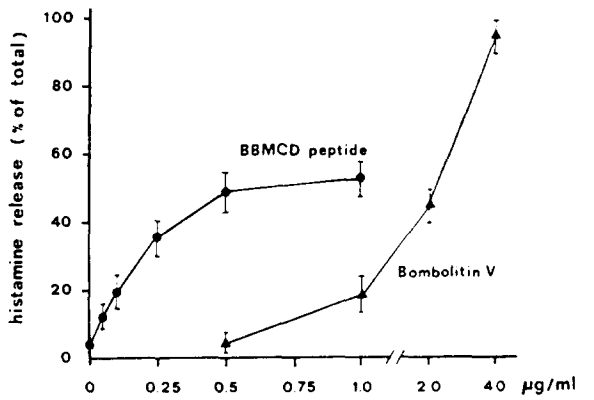

FIG. 6. Histamine release from rat peritoneal mast cells induced by bumblebee MCD peptide and bombolitin V. The peptides were incubated with 10^{5} cell/sample at 37°C for 5 min without shaking in 200 μl of medium [12]. The reaction was stopped by cooling the samples with ice cold water and centrifugating at 1500×g for 10 min. Histamine was determined in the supernatant spectrophotofluorometrically [1]. Total histamine was determined by sonication of the cells.

TABLE 4

STRUCTURE OF BUMBLEBEE MCD PEPTIDE, BEE MCD PEPTIDE, APAMINE AND BOMBOLITIN V

	1	5	10	15	20	25	28
BB MCD peptide	Met-Cys-Ile-Cys-Lys-Asn-Gly-Lys-Pro-Leu-Pro-Gly-Phe-Ile-Gly-Lys-Ile-Cys-Arg-Lys-Ile-Cys-Met-Met-Gln-Gln-Thr-His(NH_2)						
Bee MCD peptide	Ile-Lys-Cys-Asn-Cys-Lys-Arg-His-Val-Ile-Lys-Pro-His-Ile-Cys-Arg-Lys-Ile-Cys-Gly-Lys-AsnNH_2						
Apamine	Cys-Asn-Cys-Lys-Ala-Pro-Glu-Thr-Ala-Leu-Cys-Ala-Arg-Arg-Cys-Gln-Gln-HisNH_2						
Bombolitin V	Ile-Asn-Val-Leu-Gly-Ile-Leu-Leu-Gly-Lys-Ala-Leu-Ser-His-LeuNH_2						

and His) which could not be assigned by the manual methods. The knowledge of the N-terminal amino acid, the partial sequence of the intact peptide and trypsin cleavages permitted the alignment of the tryptic fragments (Table 2). Automatic Edman degradation of 1.3 nmol of the intact peptide confirmed the sequence obtained manually and allowed the assignment of Thr^{27}–His^{28}, although no amino acid was identified at cycles 2, 4, 18 and 22, occupied by Cys (Table 3). Incubation of the intact peptide with carboxypeptidase A (peptide:enzyme 1:20, 2 hr at 37°C) did not release any His or other amino acids, indicating that the C-terminal is blocked, probably amidated. However, attempts to release $HisNH_2$ with thermolysisn and subtilisin [13] were unsuccessful, although many amino acids were released.

Assignment of Disulfide Bridges

Digestion by trypsin of the intact peptide ruled out the presence of disulfide bridges between Cys^2–Cys^4 and Cys^{18}–Cys^{22} leaving only 2 possibilities: Cys^2–Cys^{18} and Cys^4–Cys^{22} or Cys^2–Cys^{22} and Cys^4–Cys^{18}. Since both bridges were present in BB MCD T1, 2 manual Edman degradation cycles were performed on this peptide. The scheme of the reaction is shown in Fig. 4. According to the first possibility, free Arg and only one peptide containing the intact disulfide bridge Cys^4–Cys^{22} would be obtained, while free Arg and 2 peptides would be produced if the second possibility were correct. High pressure liquid chromatography of BB MCD T1 after 2 manual Edman degradation cycles produced free Arg which eluted in the void volume and only one peptide with the expected amino acid composition and an intact disulfide bridge (BB MCD T1SS, Fig. 5 and Table 1), indicating that the first possibility is the correct one. Determination of the N-terminal amino acid of BB MCD T1SS gave only Ile, since the other expected amino acid, Cys, does not give any measurable product.

Biological Activity

Bumblebee MCD peptide was tested in 2 bioassays: hemolysis of guinea pig erythrocytes and histamine release from rat peritoneal mast cells. The peptide was without effect up to a dose of 1 μg/ml on guinea pig erythrocytes (results not shown). On the other hand, the peptide induced a significant release of histamine at a dose as low as 0.05 μg/ml (1.6×10^{-8} M) (Fig. 6). The maximal release (55% of total histamine) was obtained with 0.5 μg/ml. On the contrary, bombolitin V, the most potent bombolitin in the histamine release bioassay [3], was without effect at 0.5 μg ml (3×10^{-7} M) and induced only a 20% release at 1 μg/ml. Due to its lytic effect, bombolitin V released the total histamine content at doses higher than 4 μg/ml (Fig. 6).

DISCUSSION

Bumblebee MCD peptide represents less than 1% of the dry weight of the venom. On the contrary, bombolitins constitute 25–30% of the dry venom. The low amount of peptide caused some problems in the manual determination of the sequence of bumblebee MCD peptide. However, digestion with trypsin, reduction and carboxymethylation produced shorter peptides in amounts sufficient for the amino acid and sequence analysis. The presence of a Lys-Pro bound, resistent to trypsin, did not cause any problem, and only the last 2 amino acids could not be assigned by manual methods. On the other hand, the automatic Edman degradation of the in-

tact peptide was straightforward, probably because of the high hydrophilic structure of the peptide, although the yield dropped to values lower than 10% in the last cycles. The isolation of a peptide that contains one of the 2 disulfide bridges intact allowed the assignment of both disulfide bridges. The only point that needs more investigation is the determination of the C-terminal blocking group. Although $HisNH_2$ was not released by thermolysin or subtilisin, the peptide is probably amidated, as are the majority of the peptides isolated from hymenopteran venoms.

Bumblebee MCD peptide is not related to bombolitins and its sequence represents a new structure that has to be added to the class of the specific, not lytic, mast cell degranulating peptides. Although a new sequence, bumblebee MCD peptide is somewhat related to the bee MCD peptide and apamine (Table 4). In fact, all 3 peptides contain 2 disulfide bridges, are rich in basic amino acids charged at physiological pH and lack the surfactant character of melittin, mastoparans and bombolitins. This is probably due to the disulfide bridges, which restrain the peptides in a particular configuration. The similarity is greater with bee MCD peptide than with apamine. The fragment 17–22 of bumblebee MCD peptide is identical to the fragment 14–19 of the bee MCD peptide [8,15] and the 2 peptides show the same potency in releasing histamine from rat peritoneal mast cells [6,15]. On the contrary, the similarity with apamine is restricted to the C-terminal, since apamine does not release histamine [6,15]. Apamine is thought to be a neurotoxin [6] apparently for its ability to bind with high affinity to calcium-dependent potassium channels [9]. It is unknown whether this property is common also to bee and bumblebee MCD peptides. Recently a neurotoxic action of bee MCD peptide has been reported [7]. When injected in mice at a dose of 4 nmol/kg intracerebroventricularly, but not endovenously, the peptide causes convulsions and hyperactivity followed by death. In addition high affinity binding sites for bee MCD peptide have been reported in brain synaptic membranes [14]. In view of the structural similarity of bee and bumble bee MCD peptides, it would be not surprising if bumblebee MCD peptide also exerts a neurotoxic action and binds to brain membranes.

Although MCD peptides are present in the venom in very low concentration, their potent pharmacological action certainly justifies more investigation. The availability of synthetic MCD peptides will greatly facilitate the study of their mechanism of action and perhaps will permit the discovery of some yet unknown function of these peptides.

ACKNOWLEDGEMENT

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REFERENCES

1. Anton, A. H. and D. F. Sayre. A modified fluorometric procedure for tissue histamine and its distribution in various animals. *J Pharmacol Exp Ther* **166**: 285–292, 1969.
2. Argiolas, A. and J. J. Pisano. Facilitation of phospholipase A_2 activity by mastoparans, a new class of mast cell degranulating peptides from wasp venom. *J Biol Chem* **258**: 13697–13702, 1983.
3. Argiolas, A. and J. J. Pisano. Bombolitins, a new class of mast cell degranulating peptides from the venom of the bumblebee *Megabombus pennsylvanicus*. *J Biol Chem* **260**: 1437–1444, 1985.
4. Chang, J. Y., D. Brauer and B. Wittman-Liebold. Microsequence analysis of peptides and proteins using 4-N,N-dimethylamino - azobenzene - 4' - isothiocyanate/phenylisothiocyanate double coupling method. *FEBS Lett* **93**: 205–214, 1978.
5. Gray, W. R. Sequence analysis with dansyl chloride. In: *Methods in Enzymology*, Vol XXV, edited by C. H. W. Hirs and S. N. Timasheff. New York: Academic Press, 1972, pp. 333–344.
6. Haberman, E. Bee and wasp venoms. *Science* **117**: 314–322, 1972.
7. Haberman, E. Neurotoxicity of apamine and MCD peptide upon central administration. *Naunyn Schmiedebergs Arch Pharmacol* **300**: 188–191, 1977.
8. Haux, P. Die aminosaürense von MCD-peptid, einem spezifich mastzellendegranulierenden peptid aus bienengift. *Z Physiol Chem* **350**: 536–546, 1969.
9. Hugues, M., G. Romey, D. Duval, J. P. Vincent and M. Lazdunski. Apamine as a selective blocker of the calcium dependent potassium channel in neuroblastoma cells: voltage clamp and biochemical characterization of the toxin receptor. *Proc Natl Acad Sci USA* **79**: 1308–1312, 1982.
10. Parker, D. S., J. P. Raufman, T. L. Donohue, M. Bledsoe, H. Yoshida and J. J. Pisano. Amino acid sequences of helospectins, new members of the glucagon superfamily, found in *Gila monster* venom. *J Biol Chem* **259**: 11751–11755, 1984.
11. Spackman, D. H., W. H. Stein and S. Moore. Automated recording apparatus for use in the chromatography of amino acids. *Anal Chem* **30**: 1190–1206, 1958.
12. Sullivan, T. J., K. L. Parker, W. Stenson and E. W. Parker. Modulation of cyclic AMP in purified rat mast cells. I. Responses to pharmacologic, metabolic and physical stimuli. *J Immunol* **114**: 1473–1479, 1975.
13. Tatemoto, K. and V. Mutt. Chemical determination of polypeptide hormones. *Proc Natl Acad Sci USA* **75**: 4115–4119, 1978.
14. Taylor, J. W., J. N. Bidard and M. Lazdunski. The characterization of high affinity binding sites in rat brain for the mast cell degranulating peptide from bee venom using the purified monoiodinated peptide. *J Biol Chem* **259**: 13957–13967, 1984.
15. Vernon, C. A., J. M. Hanson and R. W. Brimblecombe. Peptides British Patent No. 1324823.