Antibacterial activity of secretolytin, a chromogranin B-derived peptide (614–626), is correlated with peptide structure

Jean-Marc Strub, Pierre Hubert, Gérard Nullans, Dominique Aunis, Marie-Hélène Metz-Boutigue*

Institut National de la Santé et de la Recherche Médicale, Unité 338 de Biologie de la Communication Cellulaire, 5 rue Blaise Pascal, 67084 Strasbourg, France

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Abstract Amongst the chromogranin B (CGB) derived fragments naturally generated in bovine chromaffin granules and detected in the extracellular space, we recently identified a major peptide corresponding to the 614–626 sequence of CGB. This peptide, named secretolytin, shared an interesting sequence homology with the lytic domain of cecropins and displayed a potent antibacterial activity. The aim of the present study was to determine the structural features of secretolytin necessary for this biological activity. Our results suggest that an α-helical amphipathic structure common to secretolytin, cecropins and pig myeloid antibacterial peptide may account for the antibacterial activity.

Key words: Bovine adrenal medulla; Chromaffin granule; Chromogranin; Secretogranin; Cecropin; Antibacterial peptide

1. Introduction

Chromogranins are proteins stored in secretory granules of many endocrine cells, neuroendocrine cells and neurons [1]. The widespread occurrence of chromogranins in mammals as well as in primitive living organisms suggests that important biological roles remain to be defined. An intracellular function as a helper protein in the packaging of peptide hormones and neuropeptides and as modulators of the processing of these components has been suggested [2]. Within chromaffin granules, chromogranin A, B and C (CGA, CGB/SgI, CGC/SgII) represent more than 80% of the soluble proteins [3,4] and they are actively processed [5–7]. Because of the occurrence of numerous chromogranin-derived peptides, an extracellular role as a precursor to biologically active peptides has also been proposed [1].

A major short 13-residue peptide corresponding to the 614–626 C-terminal region of CGB has recently been identified amongst the CGB-fragments naturally released into the extracellular medium from depolarized bovine cultured chromaffin cells [7]. In this study, we reported a strong relationship between the sequence of secretolytin and the N-terminal lytic helix of cecropins. Cecropins are a family of strongly basic peptides with very potent antibacterial activity, that were first isolated from the hemolymph of *Drosophila* [8], then from the giant silk moth, *Hyalophora cecropia* [9,10], and subsequently from a wide range of other insects [11]. Later, an antibacterial peptide with 33% homology to insect cecropins was isolated from porcine small intestine [12]. More recently, a new antibacterial peptide was isolated from pig myeloid cells (PMAP-37): its sequence also shows a high degree of similarity with the N-terminal helix of cecropin domain [13]. The isolation of cecropin from mammalian sources and from cuticular matrix [14] suggests that cecropins are widely distributed in the animal kingdom and are active mediators of innate immunity [15]. Recent efforts have been directed towards understanding the mechanism of action of the cecropins and their derivatives. Studies using chemically synthesized selective analogues have established that the active peptides do not function by chiral interactions with either receptors, enzymes or lipids. However, the mechanism leading to the death of a bacterium is not yet understood.

The aim of the present study was to identify the structural features of secretolytin that are necessary for its antibacterial activity. Synthetic related peptides were prepared taking into consideration modifications related to the length of secretolytin and the distribution of polar and hydrophobic residues. Computer design models of secretolytin and derived peptides were constructed and are discussed in relation to their antibacterial activity.

2. Materials and methods

2.1. Materials

Chemicals (analytical grade) were from Sigma or Prolabo except those employed for the protein microsequencer and the peptide synthesizer which were obtained from Applied Biosystems (Perkin Elmer). *Micrococcus luteus* (strain A 270) and *Bacillus megaterium* (strain M A) were purchased from the Pasteur Institute.

2.2. Peptide synthesis

Peptides were synthesized in our laboratory on an Applied Biosystems 432 A peptide synthesizer, SYNERGY, using the stepwise solid-phase synthetic approach and 9-fluorenylmethoxycarbonyl (Fmoc) chemistry [16]. All residues were incorporated with a double coupling. Peptides were further purified by reverse-phase HPLC on a Brownlee Aquapore OD-300 (7 mm × 250 mm) and lyophilized.

2.3. Sequence analysis

The N-terminal sequence of purified synthetic peptides was determined by automatic Edman degradation on an Applied Biosystems 473 A microsequencer. Samples purified by HPLC were loaded onto polybrene-treated and precycled glass-fibre filters [7]. Phenylthiohydantoin amino acids were identified by chromatography on a PTH C-18 column (2.1 mm × 200 mm).

*Corresponding author. Fax: (33) (88) 60-08-06.

Abbreviations: CGA, chromogranin A; CGB, chromogranin B/secretogranin I; CGC, chromogranin C/secretogranin II; PMAP-37, pig myeloid antibacterial peptide.

Secretolytin sequence has been deposited in the EMBL sequence data bank and is available under accession number P23389.

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2.4. Mass spectrometry

Mass analyses were performed using electrospray mass spectrometry technique (ESMS) on a VG Bio-Q quadrupole with a mass range of 4000 Da (Bio-Tech, Manchester, UK) in the positive mode [17]. The peptide (50 pmol) was dissolved in 10 μl of acetic acid/water/acetonitrile (1/49/50; v/v/v) and introduced into the ion source at a flow rate of 4 μl/min. Scanning was usually performed from m/z = 500 to m/z = 1500 in 10 s and the resolution was adjusted so that the peak at m/z = 998 from horse heart myoglobin was 1.5–2 wide on the foot. Calibration was performed using the multiply-charged ions produced by a separate introduction of horse heart myoglobin (169550.4 Da).

2.5. Antibacterial activity

Bacteria were grown aerobically at 37°C in yeast extract-free Luria-Bertani medium (1% Bactotryptone, 0.5% NaCl (m/v), pH 7.5). Antimicrobial activity was established from the growth inhibition of *Micrococcus luteus* (strain A 270) and/or *Bacillus megaterium* (strain M A) (in Luria-Bertani medium) according to the method previously described [18]. Aliquots of peptide extract (200 pmol; 10 μl) were incubated in microtiter plates with 100 μl of a midlogarithmic phase culture of bacteria with a starting absorbance at 620 nm of 0.001. Microbial growth was assessed by the increase of A_{620} nm after 16 h incubation at 37°C (*Micrococcus luteus*) or 20°C (*Bacillus megaterium*). The A_{620} nm value of control cultures growing in the absence of peptide (10 μl of water in place of peptide solution) was taken as 100%.

2.6. Sequence comparisons

Sequence alignment of secretolytin (bovine CGB 614–626) with corresponding fragments of human, rat and mouse CGB, of cecropins from different species and of PMAP-37 was performed using the Clustal V multiple sequence alignment program [19] using default parameters. Chromogranin, cecropin and PMAP-37 sequences were retrieved from the Swiss-Prot database.

2.7. Computer predicted models

The models of secretolytin, its related peptides and PMAP-37 were built taking into account the sequence similarity of secretolytin and cecropin (Antpe) from *Antheraea Pernyi* (chinese oak silk worm; protein Data Bank entry P01509) and using the program Pro-Explore (Oxford molecular, Oxford, UK).

3. Results and discussion

The purpose of this work was to examine the relationship between the structure and the antibacterial activity of secretolytin, the 614–626 fragment of bovine CGB and to establish whether the amphipathic α-helix model previously proposed for cecropins [20,21] might also account for the interaction of secretolytin with bacterial membranes.

3.1. Characterization of the residues necessary for the antibacterial activity of secretolytin

In order to characterize the residues important for the antibacterial activity of secretolytin, this peptide (1) and several related peptides numbered 2 to 10, were synthesized (Fig. 1A). During storage of secretolytin, we observed the natural formation of a pyrolidone glutamic acid at the N-terminal end, giving a molecular mass of 1460 Da. This peptide was isolated after HPLC purification (peptide 2). The synthetic peptide 3 (molecular mass of 1292 Da) has two deleted residues (G9, T10) and R11 changed into Q11 and corresponds to the C-terminal CGB peptide of several species (human, rat and mouse). Peptide 4 (molecular mass of 1052 Da) corresponds to a shorter form of secretolytin. In peptides 5 and 6 (molecular mass 1421 Da), the

A

peptide number	sequence													molecular mass Da
				1	2	3	4	5	6	7	8	9	10	11	12	13	
1				Q	K	I	A	E	K	F	S	G	T	R	R	G	1478
2				Q*	K	I	A	E	K	F	S	G	T	R	R	G	1460
3				Q	K	I	A	E	K	F	S			Q	R	G	1292
4								A	E	K	F	S	G	T	R	R	1052
5				Q	K	I	A	E	A	F	S	G	T	R	R	G	1421
6				Q	A	I	A	E	K	F	S	G	T	R	R	G	1421
7				Q	K	A	A	E	K	A	S	G	T	R	R	G	1360
8		D	L	E	L	Q	K	I	A	E	K	F	S	G	T	R	R	G	1949
9	A	M	D	L	E	L	Q	K	I	A	E	K	F	S	G	T	R	R	G	2151
10	A	W	D	L	E	L	Q	K	I	A	E	K	F	S	G	T	R	R	G	2206
11							Q	K	+	I	A	E	K	+	F	S	G	T	R	+	R	+	G	N.D.

B

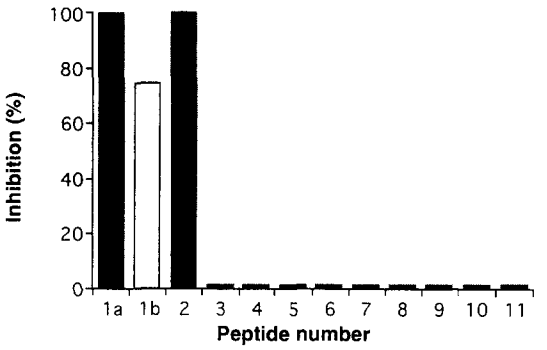

Fig. 1. Antibacterial activity of synthetic secretolytin-derived peptides. (A), Amino acid sequence of the different synthetic peptides (peptide 1–10) was characterized after complete automatic Edman degradation, and molecular mass was obtained using electrospray mass spectrometry technique (ESMS). Q*, corresponds to the natural formation of pyrolidone glutamic acid. The mixture of peptides resulting of the tryptic digest of secretolytin was numbered 11. (B), Peptides 1–11 were tested against *Micrococcus luteus* (strain A 270) by incubation with 2 μM of peptide in yeast-extract-free Luria-Bertani medium (100 μl). The microbial growth was assessed by measuring the increase at 620 nm after incubation for 16 h at 37°C (grey columns 1a and 2–11). Peptide 1 (*secretolytin*) was also tested against *Bacillus megaterium* (strain M A; white column 1b). The microbial growth was assessed by measuring the increase at 620 nm after incubation for 16 h at 20°C. Values found with control cultures grown in the absence of peptide was taken as 0%. Data are typical of several experiments and are given ±10%.

two basic K residues in positions 6 and 2 were alternatively changed into A. In peptide 7 (molecular mass 1360 Da), two A residues were included instead of the two hydrophobic residues I3 and F7. In addition, a mixture of peptides resulting from a tryptic digest of secretolytin was used as a control (line 11, Fig. 1A).

The antibacterial activity against *Micrococcus luteus* was measured for each peptide (1–10 in Fig. 1A). Secretolytin and its pyrolidone glutamic derivative displayed antibacterial activity against *Micrococcus luteus*, reaching 100% activity around 2 μM (1a and 2 in Fig. 1B). In addition, secretolytin was active in the micromolar concentration range towards another bacteria gram-positive, *Bacillus megaterium* (1b in Fig. 1B). None of

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Fig. 2. Computer models of antibacterial peptides. (A), Helical models of (a) Antpe-cecropin B (AC P01509), (b) secretolytin and (c) PMAP-37. Polar and hydrophobic residues are indicated. (B), Computer models for secretolytin (1) together with inactive variants (peptides 3–7 in Table 1A): Residues which have been modified are depicted in solid representation.

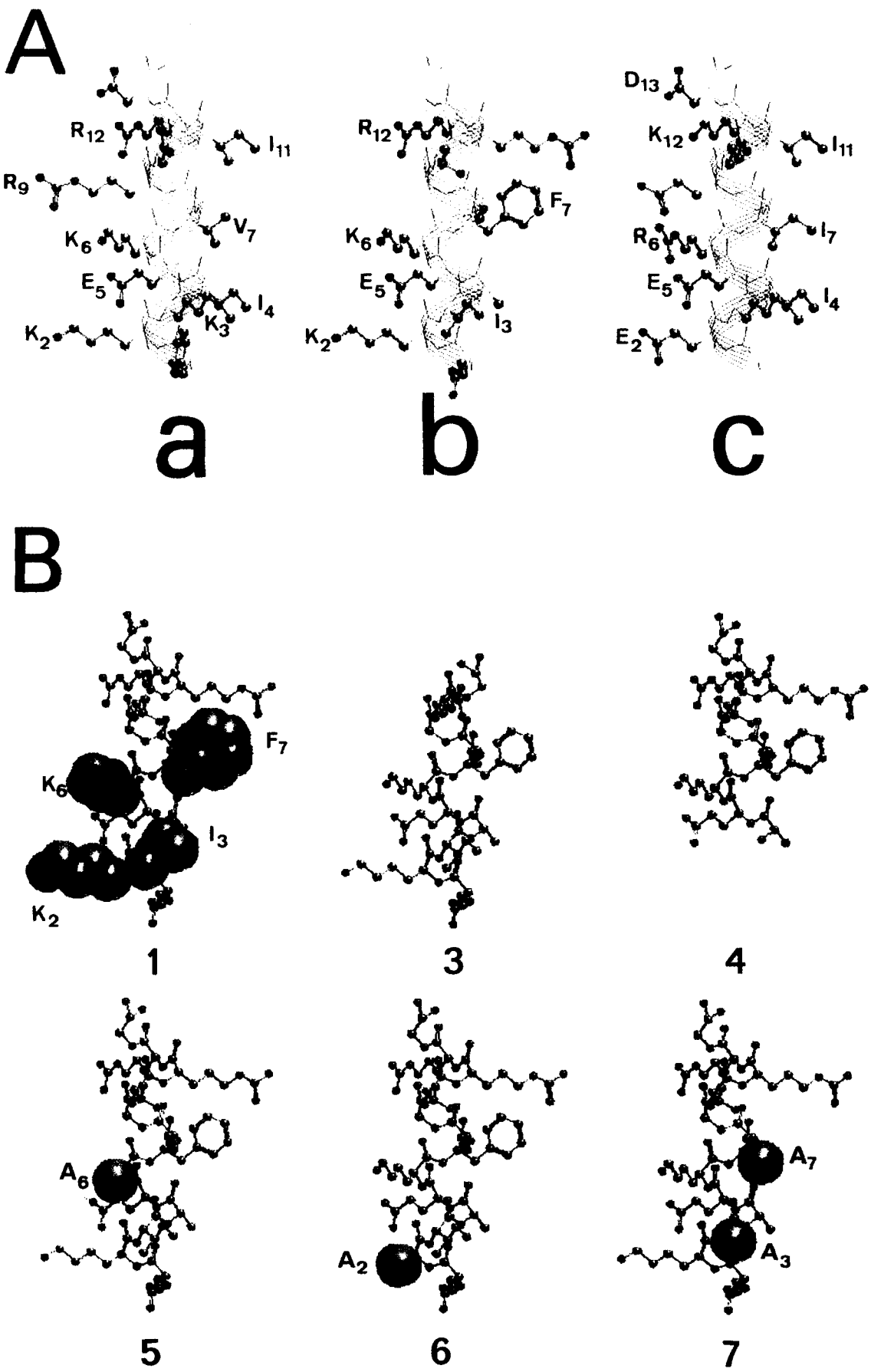
A
R12
I11
R9
V7
K6
E5
I4
K3
K2
a
R12
F7
K6
E5
I3
K2
b
D13
K12
I11
I7
R6
E5
I4
E2
c
B
F7
K6
I3
K2
1
3
4
A6
5
A2
6
A7
A3
7

Table 1
Sequence comparison of the antibacterial secretolytin (CGBb) with the homologous chromogranin-derived peptides (CGBh, CGBm and CGBr), PMAP-37, and cecropins from different species Sequence alignment was performed using the Clustal V multiple sequence alignment program [19].

1	CGB-b	Q		K	I	A	E	K	F	S	G	T	R	R	G
2	CGB-h	Q		K	I	A	E	K	F	S			Q	R	G
3	CGB-m	Q		K	I	A	E	K	F	S			Q	R	G
4	CGB-r	Q		K	I	A	E	K	F	S			Q	R	G
5	PMAP-37	G	E	K	I		E	R	I	G	Q	K	I	K	D
6	Cec1_Cerca	G	K	K	I		E	R	V	G	Q	H	T	R	D
7	Cec1_Pig	A	K	K	L		E	N	S	A	K		K	R	
8	Cec2_Manse	F	K	E	L		E	R	A	G	Q	R	V	R	D
9	Cec4_Bommo	F	K	K	I		E	K	V	G	Q	N	I	R	D
10	CecA_Bommo	F	K	K	I		E	K	M	G	R	N	I	R	D
11	CecA_Hyace	F	K	K	I		E	K	V	G	Q	N	I	R	D
12	CecB_Antpe	F	K	K	I		E	K	V	G	R	N	I	R	N
13	CecB_Drome	G	K	K	I		E	R	I	G	Q	H	T	R	D
14	CecB_Hyace	F	K	K	I		E	K	M	G	R	N	I	R	N
15	CecC_Drome	G	K	R	I		E	R	I	G	Q	H	T	R	D
16	CecD_Hyace	F	K	E	L		E	K	V	G	Q	R	V	R	D
17	Sr1C_Sarpe	G	K	K	I		E	R	V	G	Q	H	T	R	D
18	Sr1D_Sarpe	G	K	R	I		E	R	V	G	Q	H	T	R	D
Homology (%)				89	100		100	94	89	100				100	
Consensus				K/R	I/L		E	K/R	X_1	X_2				R/K	

X_1 = F, V, I, M.
X_2 = G, A, S.

the modified peptides numbered 3–7 nor tryptic digest numbered 11 had any effect on the growth of *Micrococcus luteus* suggesting the importance of: (i) the length of the peptide to interact with the microbial membrane, (ii) the presence of basic residues K2, K6 and R11 and (iii) the presence of hydrophobic residues I3 and F7. In addition, these data show that the antibacterial activity seems to be specific to bovine CGB (614–626).

3.2. *Antibacterial activity of secretolytin is related with the extracellular processing of chromogranin B /secretogranin I (CGB)*

The study of the processing of CGB in bovine chromaffin granules allowed us to characterize the different cleavage sites along the protein chain [7]. Secretolytin, the major CGB-derived fragment (614–626) was identified in the extracellular medium from directly depolarized bovine cultured chromaffin cells. In secretory granules, its immediate precursor is the 610–626 fragment [7]. This fragment was synthesized (peptide 8 in Fig. IA) and found to be inactive against bacteria growth. The natural peptide extracted from the soluble granule core was also inactive. In order to compare the antibacterial activity of secretolytin with the larger CGB fragment, allowing its alignment with cecropin 1–15, the corresponding peptide 9 (Fig. 1A) was synthesized. This peptide was inactive towards the growth of the bacteria gram-positive *Micrococcus luteus* (Fig. 1B). Since the aromatic residue in position 2 (W) of the lytic helix of cecropin has been described to be important for antibacterial activity [22], the residue M2 was changed into W2 (peptide 10): no antibacterial activity was found (Fig. 1B). These results suggest that any additional N-terminal residues modify the helical conformation of secretolytin and thus abolish the antibacterial activity.

We conclude that only secretolytin, the natural matured peptide released from cells and recovered in the extracellular medium, and its corresponding synthetic peptide possess the length and the residues necessary for the antibacterial activity against bacteria gram-positive.

3.3. *Sequence comparison of secretolytin with the corresponding sequences of CGB from several species, cecropins and PMAP-37*

The alignment of secretolytin with homologous (inactive) CGB fragments from human (sequence 2), mouse (sequence 3) and rat (sequence 4) and with cecropins from different species (sequences 6–18) is shown in Table 1. Recently, a novel antibacterial peptide composed of 37 residues has been extracted and purified from myeloid tissue and named PMAP-37 (Porcine Myeloid Antibacterial Peptide) [13]. In the sequence of PMAP-37 a central fragment (sequence 5) shows a high degree of similarity with the active domain of cecropin B. Sequence alignment confirms the consensus sequence confering the antibacterial activity: X-X-K/R-I/L-X-E-K/R-F/V/M/I/-G/A/S-X-X-X-R/K-X (Table 1). By comparison with the consensus pattern for cecropins previously proposed : W-X(0,2)-K/N-X-X-K-K/E-L/I-E-R/K/N [23; PS00268], the alignment reported in Table 1 suggests that the C-terminal moiety is crucial to antibacterial activity.

3.4. *Structural properties of secretolytin*

From the alignment data (Table 1), a comparative prediction of the secondary structure of cecropin (Antpe; sequence 12) from *Antheraea Pernyl* and bovine secretolytin (sequence 1) was performed according to the Homologue method provided in Pro-Explore (Fig. 2A). Studying the antibacterial activity of 6 analogues to the 37-residue cecropin A, Andreu et al. [22] demonstrated that the α-helical 5–21 region of cecropin A was clearly involved in the interaction against *Micrococcus luteus, Bacillus megaterium* and *Pseudomonas aeruginosa*. All modifications causing conformational changes in this region had a marked effect on the activity of the corresponding peptide indicating a strong correlation between the loss of helicity and absence of antibacterial activity. As shown in Fig. 2A, the 2 peptides cecropin-Antpe (model a) and secretolytin (model b) have a similar arrangement concerning polar and hydrophobic residues (K2, E5, K6, R12, I3/4 and F/V7). The charged and polar groups are grouped together suggesting that the basic amino acids could interact with the negatively charged phospholipids of the bacterial membranes. Both models predict a hydrophobic domain covering approximatively one third of the molecule which is likely to orientate the peptide towards the hydrophobic core of membranes [24]. Furthermore, several possibilities of internal interactions between charged groups exist. The most significant of them is that the δ carboxylate group of E5 is so close to the ε amino group of K2 and K6 that a hydrogen bond or salt link may be formed, contributing to the stabilization of the helix. For PMAP-37 (model c ; Fig. 2A), the predicted model shows a similar arrangement of charged residues (E2, E5, R6, K12 and Di3) defining a hydrophobic domain comprising the 3 residues I4, I7 and I11.

Previously, we determined the circular dichroism spectra of the synthetic secretolytin at various pHs and in the presence or absence of trifluoroethanol [7]. The results showed that increasing amounts of trifluoroethanol induced a higher α-helical con-

tent. In 100% trifluroethanol, the circular dichroïsm spectra became clearly helical as characterized by two negative bands at 208 and 220 nm. More recently, experiments were performed by use of a combination of two-dimensional NMR techniques (NOESY, two-dimensional nuclear Overhauser effect spectroscopy and HOHAHA, homonuclear Hartmann-Hahn spectroscopy). Results obtained at 25°C and 40°C show an equilibrium between two conformations of the sequence Q1 to G9, including respectively loops located at K2-I3 and A4-E5 (25°C) and loops located at A4-E5 and F7-S8 (40°C). In addition, a unique non-structured conformation was assigned for the C-terminal sequence T10-G13. In these conditions, the stabilization of the helical structure by the interaction between E5 and K2/K6 seems possible in peptide conformations obtained at 40°C and including loops A4/E5 and F7/S8.

These results show that in aqueous solution secretolytin presents a conformational flexibility. Two parameters are important to define the flexibility of this peptide. The first concerns the rate at which conformational changes occur, and another is the energetics of the various conformations. In addition, amino acid side chains of peptides present into membrane are much more restricted in their flexibility than are those of peptides in aqueous solutions. Taking into account this last point, it seems now important to further define the interaction of secretolytin with phospholipid vesicles.

In 1989, the first mammalian cecropin was isolated from pig small intestine [12]. This peptide, porcine cecropin P1, presents extensive homology with insect cecropins except that it is not amidated on the C-terminus. In addition, cecropin P1 has a more narrow antibacterial spectrum. The structures of cecropin A and a hybrid of cecropin A and mellitin in solution have been determined by Holak et al. [20] and Sipos et al. [25]. More recently, the conformation of the mammalian cecropin P1 has been studied by two-dimensional NOE spectroscopy (NOESY) [21]. The conclusion from these studies is that a central region of P1 composed of 13 or 14 residues forms a well identified α-helix. Adjacent to this region, there is another, rather well-characterized α-helix structure which bends away from the first α-helix axis. The first 2–4 residues in the N-terminus and the last 3–5 residues in the C-terminus are not structurally well determined and are likely to be flexible.

It is important to point out that the structure of porcine cecropin P1 in solution is remarkably different from the structures of insect cecropins [20], mellitin [26] or a hybrid between these last two [25]. Whereas porcine cecropin shows a continuous amphipathic α-helix over nearly the whole length of the peptide, the structure of insect cecropins consists of two helices connected by a flexible hinge. The N-terminal helix is amphipathic while the other internal α-helix is essentially hydrophobic.

Using the Homologue method in Pro-Explore, secondary structure predictions for secretolytin (peptide 1) and for different related peptides (peptides 3 to 7 in Fig. 1A) were compared (Fig. 2B). Residues K2, K6, I3 and F7 in the prediction model 1 and residue A which replaces these residues in models 5, 6 and 7 have been represented as compact models. These models show clearly the structural features critical for the antibacterial activity as previously discussed (see above).

3.5. Antibacterial mechanisms

Antibacterial peptides are produced by numerous species from different classes including mammals, amphibians and insects. These peptides appear to play an important role in the innate immune response to invasion by pathogenic microorganisms [27]. Nevertheless, the precise mode of action of these antibacterial peptides is not yet fully understood. It has been suggested that the antibacterial activity appears to be due to the permeation of membranes via peptide–lipid interaction, rather than to receptor-mediated recognition [28,29]. To understand the structural requirements for this antibacterial activity, the enianto, retro, and retroenianto isomers of hybrid analogs of cecropin were synthesized [30]. They concluded that chirality of peptide was not a critical feature, and full activity could be achieved with peptides containing either all L- or D-amino acids in their respective right- or left-handed helical conformations.

Recently, studies on the interaction of pig cecropin with phospholipid vesicles have been reported [14]. By using a variety of biophysical methods (e.g. fluorescence spectroscopy, NMR), a non-pore mechanism was suggested as a possible mode of action of magainins [31], dermaseptin [24] and insect cecropins [14]. Further support for non-pore mechanism as the mode of action comes from the finding that cecropin-mellitin hybrids as short as 15 amino acids [32], or the 12 amino acid N-terminal analogue of dermaseptin [33] had antibacterial activity similar to that displayed by their intact parent molecules. The antibacterial characterization of secretolytin and PMAP-37 provides further examples arguing for the non-pore mechanism. The lengths of these α-helices are too short to span the 30 Å thick phospholipid bilayers membrane and form a conducting pore.

Antibacterial peptides have to be positively charged in order to bind to bacterial surfaces, which are negatively charged. In the non-pore mechanism suggested for the antibacterial peptide dermaseptin [24], four steps were possibly required. One of the steps include the laying of amphipathic α-helical monomers on the surface of the membrane such that the positive charges of the basic amino acids interact with the negatively charged phospholipid headgroups or water molecules. Secretolytin presents a similar basic feature with a net charge of +3. The different unactive peptides reported on Fig. 1A have a net charge of +2 that may be too low to allow interaction of the peptide with the bacterial membrane. The inability of peptide 7 (net charge of +3) to inhibit bacterial growth may be related to the presence of two residues A3 and A7 in place of the hydrophobic residues I3 and F7 which prevent the building of an amphipathic helix.

3.6. Conclusions

During the past few years, much has been learned about the biochemistry and cell biology of chromogranins, but the function of these proteins and their derived peptides is still unclear. However, the use of these proteins as neuroendocrine tumor markers has been clearly defined [34] and in Alzheimer's disease, CGA is colocalized with beta-amyloid precursor protein in senile plaques [35]. With the antibacterial properties displayed by secretolytin we have determined a new cryptic biological activity associated with endogenous chromogranin-derived peptides. The interesting structural homology of secretolytin with cecropins and PMAP-37 suggests that a common α-helical amphipathic domain may underlie this antibacterial activity.

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References

[1] Simon, J.P. and Aunis, D. (1989) Biochem. J. 262, 1–13.
[2] Huttner, W.B., Gerdes, H.H. and Rosa, P. (1991) Trends Biochem. Sci. 16, 27–30.
[3] Helle, K. (1966), Mol. Pharmacol. 2, 298–310.
[4] Blaschko, H., Comline, R., Schneider, F., Silver, M. and Smith, A. (1967) Nature 215, 58–59.
[5] Winkler, H. (1976) Neuroscience 1, 65–80.
[6] Metz-Boutigue, M.H., Garcia-Sablone, P., Hogue-Angeletti, R. and Aunis, D. (1993) Eur. J. Biochem. 217, 247–257.
[7] Strub, J.M., Garcia-Sablone, P., Lonning, K., Taupenot, L., Hubert, P., Van Dorsselaer, A., Aunis, D. and Metz-Boutigue, M.H. (1995) Eur. J. Biochem. 229, 356–368.
[8] Boman, H.G., Nilsson, I. and Rasmuson, B. (1972) Nature 237, 232–235.
[9] Hultmark, D., Steiner, H., Rasmusson, T. and Boman, H.G. (1980) Eur. J. Biochem. 106, 7–16.
[10] Steiner, H., Hultmark, D., Engström, A., Bennich, H. and Boman, H.G. (1981) Nature 292, 246–248.
[11] Boman, H.G. (1994) in: Phylogenetic perspectives in immunity: the insect host defense (Hoffmann, J.A., Janeway, C.A., Natori, S. eds.) pp. 3–17, R.G. Landes Company, Austin.
[12] Lee, J.Y., Boman, A., Chuanxin, S., Andersson, M., Jörnvall, H., Mutt, V. and Boman, H.G. (1989) Proc. Natl. Acad. Sci. USA 86, 9159–9162.
[13] Tossi, A., Scocchi, M., Zanetti, M., Storici, P. and Gennaro, R. (1995) Eur. J. Biochem. 228, 941–946.
[14] Brey, P.T., Lee, W.J., Yamakawa, M., Koizumi, Y., Perrot, S., François, M. and Ashida, M. (1993) Proc. Natl. Acad. Sci. USA 90, 6275–6279.
[15] Gazit, E., Boman, A., Boman, H.G. and Shai, Y. (1995) Biochemistry 34, 11479–11488.
[16] Merrifield, R.B. (1963) J. Am. Chem. Soc. 85, 2149–2154.
[17] Jaquinod, M., Potier, N., Klarskov, K., Reymann, J.M., Sorokine, O., Kieffer, S., Barth, P., Andriantomanga, V., Biellmann, J.F. and Van Dorsselaer, A. (1993) Eur. J. Biochem. 218, 893–903.
[18] Bulet, P., Dimarck, J.L., Hetru, C., Lagueux, M., Charlet, M., Hegy G., Van Dorsselaer, A. and Hoffman, J. (1993) J. Biol. Chem. 268, 14893–14897.
[19] Higgins, D.G., Bleasby, A.J. and Fuchs, R. (1992) CABIOS 8, 189–191.
[20] Holak, T.A., Engström, A, Kraulis, P.J., Lindeberg, G., Bennich, H., Jones, T.A., Gronenborn, A.M. and Clore, G.M. (1988) Biochemistry 27, 7620–7629.
[21] Sipos, D., Andersson, M. and Ehrenberg, A. (1992) Eur. J. Biochem. 209, 163–169.
[22] Andreu, D., Merrifield, R.B., Steiner, H. and Boman, H. G. (1985) Biochemistry 24, 1683–1688.
[23] Bairoch, A. (1991) Nucleic Acids Res. 19, 2241–2245.
[24] Pouny, Y., Rapaport, D., Mor, A., Nicolas, P. and Shai, Y. (1992) Biochemistry 31, 12416–12423.
[25] Sipos, D., Chandrasekhar, K., Arvidsson, K., Engstrom, A. and Ehrenberg, A. (1991) Eur. J. Biochem. 199, 285–291.
[26] Bazzo, R., Tappin, M.J., Pastore, A., Harvey, S., Carver, J.A. and Campbell, I.D. (1988) Eur. J. Biochem. 173, 139–146.
[27] Zasloff, M. (1992) Curr. Opin. Immun. 4, 3–7.
[28] Wade, D., Boman, A., Wahlin, C.M., Drain, D., Andreu, D., Boman, H.G. and Merrifield, R.B. (1990) Proc. Natl. Acad. Sci. USA 87, 4761–4765.
[29] Bessalle, R., Kapitovsky, A., Gorea, A., Shalit, I. and Fridkin, M. (1990) FEBS Lett. 274, 151–155.
[30] Merrifield, R.B., Juvvadi, P., Andreu, D., Ubach, J., Boman, A. and Boman, H.G. (1995) Proc. Natl. Acad. Sci. USA 92, 3449–3453.
[31] Matsuzaki, K., Murase, O., Tokuda, H., Funakoshi, S., Fujii, N. and Miyajima, K. (1994) Biochemistry 33, 3342–3349.
[32] Andreu, D., Ubach, J., Boman, A., Wahlin, B., Wade, D., Merrifield, R.B. and Boman, H.G. (1992) FEBS Lett. 296, 190–194.
[33] Mor, A., Hani, K. and Nicolas P. (1994) J. Biol. Chem. 269, 31635–31645.
[34] Deftos, L.J. (1991) Endocr. Rev. 12, 181–187.
[35] Wang, D. and Munoz, D.G. (1995) J. Neuropathol. Exp. Neurol. 54, 548–556.