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APIDAECIN-TYPE PEPTIDE ANTIBIOTICS FUNCTION THROUGH A NON-POREFORMING MECHANISM INVOLVING STEREOSPECIFICITY

Peter Casteels and Paul Tempst

Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, and Cornell University Graduate School of Medical Sciences, 1275 York Avenue, New York, NY 10021

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Insect resistance to bacterial infections is dependent on the production of specialized defense peptides. We report here that lethal acivitities of apidaecin, a small peptide from honeybees, cannot possibly be the result of a conventional 'lytic' mechanism. Evidence includes the complete lack of membrane permeabilization, at concentrations that exceed lethal doses by four orders of magnitude, and undiminished sensitivity of apidaecin-resistant mutants to 'poreforming' peptides. In addition, the D-enantiomer of apidaecin is completely devoid of antibacterial activities. We propose therefore, that the antagonistic effects of apidaecin involve stereoselective recognition of a chiral cellular target, establishing this peptide as functionally unique among insect antibacterials. Identification of the apidaecin target may provide the scientific basis for rational drug design.

During the last decade, many antibacterial peptides have been found in insects (for latest updates see Refs. 1 and 2, and references therein). We have isolated a unique peptide, 'apidaecin', from honeybees (3). Apidaecin is small (18 unmodified, L-amino acids; 33% prolines) and can easily be mass produced. It inhibits viability of many gram-negative pathogens in nanomolar doses; lethal activity is near immediate. About a 1,000-fold higher dose is required to inhibit growth of *Bacillus megaterium* and *Micrococcus lysodeikticus*; other gram positives are not affected at all (1,3,4). The almost complete lack of activity against gram positives clearly sets this peptide apart from magainins, hymenoptaecin, cecropins and defensins, that usually act quite well (1,5,6) or better (7) on gram positives. This raises intriguing questions about apidaecin mode-of-action.

Defensins, cecropins and magainins are bactericidal through a 'lytic/ionophoric' mechanism (7-9). Interaction of these peptides (Cec A, Mag B) on membranes does not involve recognition of chiral molecules as all-D enantiomers form channels in lipid bilayers and kill bacteria equally well as their natural 'all-L' counterparts (10,11). To date, few antibacterial all-L polypeptides are known to function through a 'non-lytic' mechanism. Some members of the colicin and microcin families, proteins of bacterial (*E. coli*) origin that antagonize non-identical strains, fall into the latter

Abbreviations: RP-HPLC: reversed phase high performance liquid chromatography, IPTG: isopropyl-ß-D-thiogalactopyranoside, ONPG: o-nitrophenyl-β-D-galactoside, PBS: phosphate buffered saline, LB: luria broth, BHI: brain heart infusion, LPS: lipopolysaccharide, CFU: colony forming units, MIC: minimal inhibitory concentration, OM: outer membrane, IM: inner membrane.

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category (12,13), as do insect lysozyme and attacins. Lysozyme degrades the peptidoglycan component of the bacterial cell wall; attacin reportedly inhibits synthesis of outer membrane proteins in *E. coli* through specific interference with *omp* gene transcription (14).

In this report, we present the first data on apidaecin mode-of-action and show that lethal function is not associated with a 'lytic' mechanism. We also provide evidence that the antagonistic effects of apidaecin, to kill or inhibit gram negative bacteria, involve stereoselective recognition of a chiral cellular target.

MATERIALS AND METHODS

Peptides--Peptides used in this study were: apidaecin (GNNRPVYIPQPRPPHPRL, from Ref. 3), all-D apidaecin Ib, cecropin P1 (15), magainin B (16), defensin HNP-1 (7) and polymixin B sulfate. Polymixin B sulfate and HNP-1 were purchased from Sigma (St. Louis, MO). All other peptides were chemically synthesized (MSKCC Core Facility), purified by preparative RP-HPLC and their authenticity verified by chemical sequencing and mass spectrometry (1). Quantitations were done by amino acid composition analysis.

Cytoplasmic Membrane Permeabilization --Bacterial membrane permeabilization was studied using a modified version of the method previously described (17,18). *E.coli* ML-35p was grown at 37°C in full strength LB, supplemented with 100μg/ml ampicillin (Sigma), until an A_{600} of approximately 0.5 was reached. The cells were washed three times with PBS, the A_{600} was adjusted to 0.5 and the suspension was kept on ice. The assay mixture consisted of 10% strength LB (in water) to which ampicillin and ONPG (Sigma) were added at a final concentration of 10μg/ml and 2mM, respectively; 200 μl of cells (in PBS) were then fivefold diluted in this assay mix. Ninety μl of the resulting bacterial suspension were then pipetted in microvolume 10mm pathlength cuvettes and supplemented with 10 μl of the test solution (peptide or water, as control). In selected cases, 10 μl of 1% Tween-20 was also added at this point. Hydrolysis of ONPG was recorded as A_{400}, starting immediately after addition of peptide to the cells and continued for 25 min, using an Ultrospec III spectrophotometer (Pharmacia). The reference cell contained 100 μl assay mix and cells, without peptide. Strain ML-35p was a generous gift from Dr. Robert Lehrer (UCLA, Westwood, CA).

Inhibition Zone Assay --Aliquots (25 nanomoles in 10 μl PBS) of synthetic all-L apidaecin and all-D apidaecin were applied in 3mm wells on agar plates seeded with log-phase bacteria. Microorganisms (obtained from ATCC; strain numbers listed in table 1) were grown under aerobic conditions on BHI medium from Difco (Detroit, MI), except *Agrobacterium, Erwinia, Pseudomonas syringae* and *Acinetobacter* which were grown on nutrient agar, *Rhizobium* on tryptic soy agar, *Francisella* on chocolate agar (Remel 01-300 plates) and *Haemophilus influenzae* on GC medium (Difco 0289) with 2% hemoglobin powder (BBL 11871). In general, incubation was done for 48 hours at 37°C, except *Rhizobium, Agrobacterium, Erwinia, P. syringae* which were grown at 28°C.

Growth Inhibition in Liquid Culture--Minimal inhibitory concentrations (MIC's) of peptides against four defined *E. coli* strains (see text and table 2 for details) were determined in flat-bottomed 96-well microtiter plates, essentially as described (1). The MIC values (a-b) express the highest peptide concentration at which cells were able to grow (a) and the lowest concentration at which no growth was observed (b). Strains D21 and D22 are derived from *E. coli* K-12 (discussed in Ref. 19) and were kindly provided by Dr. Hans Boman (Stockholm University, Sweden).

RESULTS AND DISCUSSION

Apidaecin does not permeabilize membranes--To investigate a possible effect of apidaecin on the inner (cytoplasmic) membrane of gram negative bacteria (e.g. *E. coli*), we used an assay that has been developed by Lehrer and coworkers (17,18) (for experimental details see "Materials and Methods"). First, apidaecin MIC to inhibit growth of *E. coli* ML-35p in 10% strength LB (assaying medium) was determined to be 0.05-0.1 μg/ml. Analysis with increasing concentrations

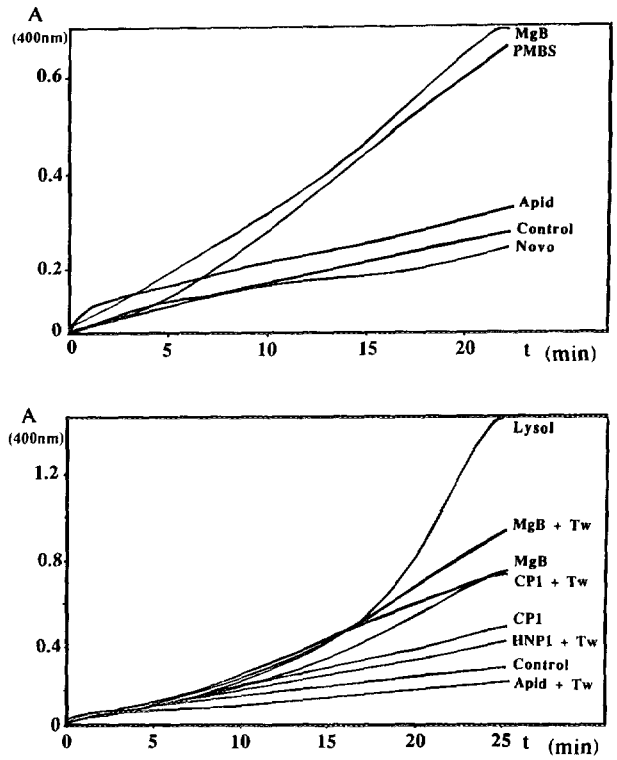

Figure 1. **Bacterial cytoplasmic membrane permeabilization.** Effects of various peptides on the permeability of inner membrane of *E. coli* strains ML-35p . Inner membrane leakage was studied measuring hydrolysis of ONPG, a β-galactosidase substrate, at 400 nm. For details see "Materials and Methods". Symbols: Lysol (0.2%); Novo, novobiocin (1 mg/ml); Apid, apidaecin (5 mg/ml); MgB, magainin B (0.2 mg/ml); CP1, cecropin 1 (0.02 mg/ml); PMBS, polymixin B sulfate (0.5 mg/ml); HNP1, defensin HNP-1 (0.005 mg/ml); symbols followed by 'Tw' indicates that 0.1% Tween-20 was also present in that experiment.

of apidaecin indicated that as much as 5 mg/ml of peptide ($5x10^4$ times the MIC) had no measurable effect in the permeabilization assay (figure 1).

Several other antibacterial peptides with established membrane-perturbing activities were then tested: magainin B (9,16), cecropin P1 (8,19), defensin HNP-1 (7,18) and polymixin B (20). Lysol, a disinfectant, was used as the definitive positive control for cell lysis (21); the 'classical' antibiotic novobiocin (DNA replication inhibitor) was chosen as negative control as it does not affect synthesis or integrity of the cell envelope (22). Not unexpectedly, the 'poreforming' peptides invariably showed permeabilizing effects, as clearly illustrated by the rapid incline of the A_{400} readings during the first 20-25 min after addition to the cells (figure 1).

All tests with peptides were then repeated in the presence of 0.1% Tween-20. Detergents of the polyoxyethylene type (e.g. Tween and Triton) solubilize cytoplasmic membranes (23). However, they are very effectively excluded by the hydrophilic shell of the gram negative OM (24). Tween-20 is therefore a suitable probe for assessment of OM sensitization or disintegration. While the detergent clearly augmented the IM permeabilization effects of magainin and cecropin, combination of 5mg/ml apidaecin and 0.1% Tween-20 did still not result in any noticable ONPG ingress and hydrolysis.

We then tested defensin HNP-1, that has been shown to permeabilize inner and outer membranes of *E. coli* at a concentration of 50 μg/ml (18) and to sensitize the cells to rifampin at 10 μg/ml (25), both at low-ionic-strength. We used the peptide at a concentration of only 5 μg/ml (plus 0.1% Tween-20); A_{400} increase over time was clearly more pronounced than during the control and the apidaecin (5 mg/ml + Tween) experiments. Conversely, the apidaecin MIC to inhibit growth of strain ML-35p is at least two orders of magnitude lower (i.e. more potent) than the defensin MIC under the same conditions (this study). Thus, overall, the data indicate a complete absence of apidaecin poreforming ability, even at concentrations that exceed lethal doses by more than four orders of magnitude.

Apidaecin resistant and hypersensitive E. coli phenotypes--In the course of assaying apidaecin activities on *E. coli* strain ATCC 11775, we isolated a spontaneous mutant (designated 11775apid^R) that could withstand about 500-fold higher concentrations of this peptide drug than those required to inhibit growth of the parental strain. Strain 11775apid^R was shown, by two-dimensional gel analysis, to have an identical protein phenotype as the strain from which it had been derived (data not shown), indicating it was not just a contaminant. Pattern comparison of total protein has long been accepted as a legitimate tool for taxonomic clustering of microorganisms (26). Analysis of both strains for sensitivity to 46 'classical' antibiotics, using the Baxter Microscan System (model autoSCAN-N/A) as commonly employed in clinical microbiological analysis, resulted in identical antibiograms.

Comparative analysis of *E. coli* strains ATCC 11775 and 11775apid^R for their susceptibility / resistance to cecropin and magainin indicated that, in contrast to apidaecin, no differences existed between the two strains (table 1). MIC values of the lytic peptides to inhibit growth of both strains were identical, with respect to strain. From these observations, we conclude that, whatever mutation caused apidaecin resistance, it had no effect on antibacterial activities of two prominent poreformers. The latter function through a mechanism of disruption / traversion of the OM and permeabilization of the IM (this study and Refs. 9,10) with concomittant dissipation of electrochemical membrane potential (for more details see Ref. 1, and references therein).

Next, we determined the MIC values of apidaecin, cecropin and magainin to inhibit growth of *E. coli* strains D21 and D22. Strain D22 is an *envA* mutant of D21 and has a barrier defect in the

Table 1. **Minimal Inhibitory Concentrations.** The MIC's of all-L apidaecin (L-Apid), all-D apidaecin (D-Apid), cecropin P1 (Cec P1) and magainin B (Mag B) to inhibit growth of four *E. coli* strains are expressed in μg/ml. In some tests, growth media were supplemented with 2mM $MgCl_2$ (+$MgCl_2$) or phosphate buffered saline (+PBS). Peptide concentrations over 40 μg/ml were not tested except for D-Apid against strain ATCC 11775, where 800 μg/ml was used. For further details see "Materials and Methods".

	Bacterial strains											
	E. coli ATCC 11775			*E. coli* 11775apid^R			*E. coli* D21			*E. coli* D22		
Peptides	-	+$MgCl_2$	+PBS	-	+$MgCl_2$	+PBS	-	+$MgCl_2$	+PBS	-	+$MgCl_2$	+PBS
	μg/ml			*μg/ml*			*μg/ml*			*μg/ml*		
L-Apid	0.5-1	5-10	1-5	>40	>40	>40	1-5	20-40	20-40	0.05-0.1	1-5	1-5
D-Apid	>800	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40
Cec P1	0.1-0.2	1-2	0.2-1	0.02-0.1	1-2	0.1-0.2	0.1-0.2	0.2-1	0.2-1	0.01-0.02	0.1-0.2	0.01-0.02
Mag B	1-5	10-20	1-5	1-5	10-20	1-5	1-5	10-20	1-5	0.1-0.5	5-10	0.5-1

outer membrane, making these cells overly sensitive to various peptide antibiotics (2,19,27). The mutant strain was sensitive to about 10-fold lower doses of cecropin and magainin than strain D21 (table 1); in the case of apidaecin, the MIC value decreased about 50-fold (table 1). Thus, while strong evidence exists that the cell envelope is not the target for apidaecin activity, a structurally impaired OM is apparently favorable for apidaecin to function as an antibacterial agent. Further evidence correlating apidaecin antibacterial potency to the ease with which it can penetrate the OM came from the observation that the addition of 2mM $MgCl_2$, known to stabilize molecular organization of the OM (28), to the culture medium resulted in a tenfold increase of MIC values; in case of hypersensitive strain D22, attenuation of apidaecin potency was by a factor of fifty.

The single major difference between apidaecin and the cecropin / magainin group of lytic peptides, in their growth inhibition of strain D22, was the strong attenuation of apidaecin activity at high ionic strength (150 mM NaCl component of PBS) (table 1). This effect cannot be simply explained by assuming decreased ionic interaction with the anionic LPS, as magainin and cecropin are also cationic peptides and their capacities to inhibit growth of strains D21 and D22 at high ionic strength were essentially undiminished (table 1). One must, therefore, assume that high salt causes interference of apidaecin interaction (ionic and/or other) with another, presently unknown, molecule. It is too early to speculate on the nature of this (these) cellular target molecule(s), but in the light of our observation that apidaecin lacks OM disorganizing properties, it may be involved in the required translocation of the peptide antibiotic inside the cell.

All-D apidaecin enantiomer does not inhibit bacterial growth--As the available data pointed towards a rather specific interaction of apidaecin with 'unspecified' target molecules of gram negative bacteria, we investigated the possibility that a stereospecific recognition may exist between the two, as for instance between peptide hormones and their receptors. It has been shown that synthetic all-D enantiomers of such hormones are biologically inactive (29).

The all-D apidaecin enantiomer was therefore synthesized for further study. Previous analyses of synthetic all-D antibacterial (lytic) peptides cecropin, magainin and melittin, indicated that they exhibit undiminished activities as compared to the natural all-L counterparts, as they were able to form channels in lipid bilayers and kill bacteria (10,11). In sharp contrast, all-D apidaecin was found to be totally inactive to inhibit growth of *E. coli* ATCC 11775 in culture, even at extremely high doses (800 μg/ml; see table 1). When assayed in 10% strength LB medium, this concentration exceeds the MIC of all-L apidaecin by four orders of magnitude (data not shown). In addition, when tested to inhibit growth on agar plates of the twelve most apidaecin-sensitive bacterial strains [1], again, total lack of activity was observed (table 2).

As the highest purity D-amino acids were used in the synthesis of all-D apidaecin and because the peptide mass and sequence were absolutely, positively correct we have very strong evidence that this biologically inactive peptide is the true all-D isoform of apidaecin and that, therefore, the antagonistic effects of apidaecin on the growth of gram negative bacteria involve stereoselective recognition of a chiral cellular target. While some proline-bond racemization will undoubtedly

[1] P. Casteels, J. Romagnolo, H. Erdjument-Bromage and P. Tempst, manuscript in preparation.

Table 2. **Antibacterial activities of all-L and all-D apidaecin.** Listed are the results of agar plate inhibition zone assays. Bacterial strain identifications (ATCC #) are given; specific growth conditions can be found in "Materials and Methods". Peptides were applied in 3mm wells, 25 nanomoles per well. The values express the diameter of the inhibition zone. (-) denotes that no inhibition was observed.

Bacteria	Inhibition zone all-L	all-D	Bacteria	Inhibition zone all-L	all-D
	mm			*mm*	
Enterobacteriaceae			'Other' bacteria		
Escherichia coli 11775	15	-	*Agrobacterium tumefaciens* 15955	18	-
Erwinia amylovora 15580	17	-	*Francisella tularensis* 6223	14	-
Klebsiella pneumoniae 13883	11	-	*Pseudomonas syringae* NCPPB 1106	16	-
Salmonella typhimurium 14028	14	-	*Rhizobium meliloti* 10310	17	-
Shigella dysenteriae 13313	15	-	*Haemophilus influenzae* 19418	10	-
Yersinia enterocolitica 9610	12	-	*Acinetobacter calcoaceticus* 49137	6	-

occur in free apidaecin, the entire peptide could get locked into a more rigid structure once bound to its target molecule.

Conclusions--Structurally, apidaecin-type peptides (currently seventeen different isoforms, all isolated from hymenopteran insects[1]) form a very distinct group. Although the isolation of several other Arg-Pro-rich antibacterial peptides has been described (2,4,15,30), none show appreciable sequence homology to apidaecins. Moreover, PR-39 is amidated (15), drosocin is glycosylated (2) and Bac 5 and 7 were shown to be lytic peptides (28), all in contrast to apidaecin structure and function. All data in the present study are consistent with the view that apidaecin functions through a none-poreforming mechanism that requires stereospecific recognition of target molecules. As such, this peptide is also very unique, functionally, among antibacterials produced by multicellular organisms. While the inactivity of all-D apidaecin may cast some doubts on its development into a useful therapeutic, identification of its putative OM (inward) translocation machinery and the ultimate targets, and specific sites of interaction with each, may provide the scientific basis for rational design of novel antimicrobial drug compounds, peptides or other.

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