INFECTION AND IMMUNITY, July 1997, p. 2898–2903 Vol. 65, No. 7
0019-9567/97/$04.00+0

Effects of pH and Salinity on the Antimicrobial Properties of Clavanins

IN HEE LEE, YOON CHO, AND ROBERT I. LEHRER*

Department of Medicine, UCLA School of Medicine, Los Angeles, California 90095-1690

Received 30 December 1996/Returned for modification 25 February 1997/Accepted 23 April 1997

Clavanins are histidine-rich, amidated α-helical antimicrobial peptides that were originally isolated from the leukocytes (hemocytes) of a tunicate, *Styela clava*. The activities of clavanin A amide and clavanin A acid against *Escherichia coli*, *Listeria monocytogenes*, and *Candida albicans* were substantially greater at pH 5.5 than at pH 7.4. In contrast, clavanin AK, a synthetic variant of clavanin A acid containing 4 histidine→lysine substitutions exerted substantial activity at both pH 7.4 and pH 5.5. Each of these three clavanins permeabilized the outer and inner membranes of *E. coli* very effectively at pH 5.5, but only clavanin AK did so at pH 7.4. Unlike magainin 1 and cecropin P1, α-helical antimicrobial peptides from frog skin and porcine intestine, respectively, clavanins were broadly effective against gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*, as well as gram-negative organisms. Because clavanins exert substantial antimicrobial activity in 0.1 to 0.3 M NaCl, they provide templates for designing broad-spectrum peptide antibiotics intended to function in extracellular environments containing normal or elevated NaCl concentrations. The pH-dependent properties of histidine-rich antimicrobial peptides may allow the design of agents that would function selectively in acidic compartments, such as the gastric lumen, or within phagolysosomes.

During the past decade, endogenous antimicrobial peptides have become recognized as important, ubiquitous, and ancient contributors to the innate mechanisms which permit animals (4) and plants (5) to resist infection. Most of these host defense peptides are amphipathic and possess either an α-helical or a cystine-stabilized β-sheet structure. Among the α-helical exemplars are magainins (40, 41), cecropins (10, 15), and many cathelin-associated peptides found in mammalian leukocytes (2, 39). The β-sheet peptides include α, β, insect, and plant defensins (5, 7), protegrins (13), and tachyplesins (11).

Clavanins constitute a family of amphipathic, histidine-rich, amidated α-helical peptides found in the hemocytes of a protochordate, the solitary tunicate *Styela clava* (14). Like the magainins of *Xenopus laevis*, to which they show limited primary structural similarity, clavanins contain 23 amino acid residues and manifest potent antimicrobial activity. Because tunicates are marine animals, whereas amphibians such as *Xenopus* live in fresh water, we wondered if clavanins might be more tolerant of salinity than magainin-1. The histidine-rich structures of clavanins also led us to examine the influence of pH on their antimicrobial properties. Our findings provide insights into the relationships between the structure and function of α-helical antimicrobial peptides.

MATERIALS AND METHODS

Synthetic peptides. Clavanin A, clavanin A acid and clavanin AK acid were synthesized by SynPep (Dublin, Calif.) and purified by reverse-phase high-pressure liquid chromatography to virtual homogeneity in our laboratory (data not shown). Clavanin A and clavanin A acid differed only in that the carboxy-terminal phenylalanine was amidated in clavanin A but not in clavanin A acid. Clavanin A acid and clavanin AK were identical except that each histidine residue in the former was changed to a lysine in the latter (see Table 1). Magainin 1 and cecropin P1 were purchased from Bachem (King of Prussia, Pa.) and Sigma (St. Louis, Mo.), respectively. Peptide concentrations were measured by the bicinchoninic acid method (Pierce, Rockford, Ill.).

* Corresponding author. Mailing address: Department of Medicine, Center for the Health Sciences, Box 951690, Los Angeles, CA 90095-1690. Phone: (310) 825-5340. Fax: (310) 206-8766.

Antimicrobial assays. The antimicrobial activity of each peptide was tested by minor modifications of a previously described radial diffusion assay (19). Briefly, washed mid-logarithmic-phase bacteria were trapped in thin agarose gels bearing 3-mm-diameter wells in an evenly spaced five-by-five array. The basal underlay agars contained 9 mM sodium phosphate, 1 mM sodium citrate buffer, 1% (wt/vol) Type I (low electroendosmosis) agarose (A 6013; Sigma), and 0.30 mg of Trypticase soy broth (BBL, Cockeysville, Md.) per ml and were adjusted to pH 5.5, 6.5, or 7.4 prior to sterilization by autoclaving. In some experiments, the underlay gels were supplemented with up to 300 mM NaCl. Stock peptide solutions and serial twofold dilutions were prepared in acidified water (0.01% acetic acid).

Five-microliter samples were introduced as a series of 6 serial twofold dilutions. These ranged in concentration from 3.12 to 200 μg of peptide/ml and in amount from 15.6 ng to 1 μg of peptide/well. After allowing 3 h for diffusion into the underlay, a 10-ml nutrient-rich overlay gel that contained 60 g of Trypticase soy broth plus 1% agarose per liter was poured. After the plates were incubated overnight to allow surviving organisms to form microcolonies, the diameters of the clear zones were measured to the nearest 0.1 mm and graphed (ordinate) against the $\log_{10}$ of the peptide concentration (abscissa). Also, the x intercepts were calculated by a least-mean-squares formula (using $\log_{10}$ transformed peptide concentrations) and these values were used to estimate the MICs of the various peptides.

Permeability of *E. coli* membranes. To examine the ability of antimicrobial peptides to permeabilize the inner and outer membranes of gram-negative bacteria, we simplified a previously described procedure that uses *Escherichia coli* ML-35p (17, 18). The parental strain of the assay organism, *E. coli* ML-35, expressed cytoplasmic β-galactosidase activity constitutively but lacked lactose permease and could not transport β-galactoside substrates through its inner membrane. After transformation by pBR322, the assay strain also expressed periplasmic β-lactamase activity. The ML35p construct allowed outer membrane permeability to be assessed by monitoring the hydrolysis of a bulky chromogenic cephalosphorin called PADAC [7-(thienyl-2-acetamido)-3-(2-(4-*N*,*N*-dimethylaminophenylazo)-pyridiniummethyl)-3-cephem-4-carboxylic acid]. Inner membrane permeability was monitored by measuring the hydrolysis of *o*-nitrophenyl-β-D-galactoside (ONPG).

In our original use of the assay, PADAC and ONPG (both obtained from Calbiochem, LaJolla, Calif.) were added together to a single cuvette and their respective hydrolysis reactions were followed by multiple-wavelength spectrophotometry and deconvolution by a complex set of equations. In the simpler version used here, the assays were performed in 96-well microtiter plates that were monitored every minute at 420 nm with a SpectraMax 250 Microplate Spectrophotometer (Molecular Devices, Sunnyvale, Calif.) using SOFTmax PRO software supplied by the manufacturer. The wells contained 1.5×10^6 washed, stationary-phase *E. coli* ML-35 cells and either 2.5 mM ONPG or 50 μM PADAC but not both. The assays were performed in 10 mM sodium phosphate buffer adjusted to pH 5.5, 6.5, or 7.4. Reactions were started by adding the peptides of interest or an equivalent volume of acidified water to controls. The final incubation volume was 150 μl/well. Assays were run at 37°C with regular shaking cycles (10 s every minute). PADAC (approximately 0.15 mM) and ONPG (25 mM) stock solutions were prepared in 10 mM sodium phosphate

TABLE 1. Primary structures[a]

Peptide	Amino acid sequence	Reference
Clavanin A	VFQFL GKIIH HVGNF VHGFS HVF*	14
Clavanin A acid	VFQFL GKIIH HVGNF VHGFS HVF	
Clavanin AK	VFQFL GKIIK KVGNF VKGFS KVF	
Magainin 1	GI **GK**FL**H** SA**GK**F GKA**F**V GEIMK S	40
Cecropin P1	SWLSK TAKKL ENSAK KRISE GIAIA IQGGP R	15

[a] The sequence of magainin 1 is offset to align it with the clavanins. Magainin residues identical to those in clavanin A are shown in boldface type. The asterisk signifies C-terminal amidation.

buffer, pH 6.5. Stock PADAC concentrations were determined by using a molar extinction coefficient of 43,802 at 570 nm.

E. coli ML-35p was maintained on Trypticase soy agar plates containing 100 μg of ampicillin per ml. Organisms used for antimicrobial testing or membrane permeability assays were picked from a single colony, incubated in 50 ml of sterile Trypticase soy broth for 16 h at 37°C, washed three times with 10 mM sodium phosphate buffer (pH 6.5), adjusted to an optical density at 620 nm of 0.35 (~10^8 CFU/ml), and kept on ice until used. Because β-galactosidase activity was found to vary markedly between pH 5.5 and pH 7.4, we incubated sonicated *E. coli* ML-35p cells (~5×10^6 CFU equivalent) with ONPG at pH 5.5, 6.5, and 7.4 to obtain relative rate factors (4.70 at pH 6.5 and 10.6 at pH 5.5) that were used to normalize ONPG rates for the pH effects. As β-lactamase activity was essentially constant over this pH interval, no correction factors were required.

RESULTS

Peptide composition and purity. The primary amino acid sequences of the synthetic clavanins used in this study are shown in Table 1, which also provides the sequences of magainin 1 and cecropin P1.

Effects of pH. Although the antibacterial activity of the control peptides, magainin 1 and cecropin 1, was unaffected by pHs between 5.5 and 7.4, the pH strongly affected the activity of the amidated and acid forms of clavanin A against *E. coli* ML-35p and *Listeria monocytogenes* EGD (Fig. 1). Note that although their *x* intercepts were similar (around 1 μg/ml), the zone sizes increased progressively as the pH decreased, probably reflecting the effect of pH on the solubility and self-association of these peptides (36a). Clavanin AK produced larger zones at pH 7.4, although its minimal effective concentration (*x* intercept) was close to that of clavanin A amide and acid. We attribute these larger zones to the greater solubility of clavanin AK at a neutral pH.

When tested under low-ionic-strength conditions, clavanin A amide, clavanin A acid, magainin 1, and cecropin P1 showed little activity against *Candida albicans* at pH 7.4 and quite respectable activity at pH 5.5 (Fig. 2). While clavanin AK showed comparably strong activity against *C. albicans* at pH 5.5, it was also reasonably effective at pH 7.4. The differences between these experiments and the results shown in Fig. 1 indicate that organism-specific, pH-dependent factors can affect susceptibility to antimicrobial peptides.

Membrane permeability. The influence of pH on the ability of clavanin A, clavanin A acid, and clavanin AK to permeabilize the outer and inner membranes of *E. coli* ML-35p is shown in Fig. 3. All three clavanins caused extensive and rapid permeabilization of both bacterial membranes at pH 5.5, but only clavanin AK was effective at pH 7.4. At pH 6.5, both clavanin A acid and clavanin A amide were more effective in permeabilizing the outer membrane than the inner membrane, suggesting that these structures are likely to be damaged sequentially rather than concomitantly as previously shown in studies

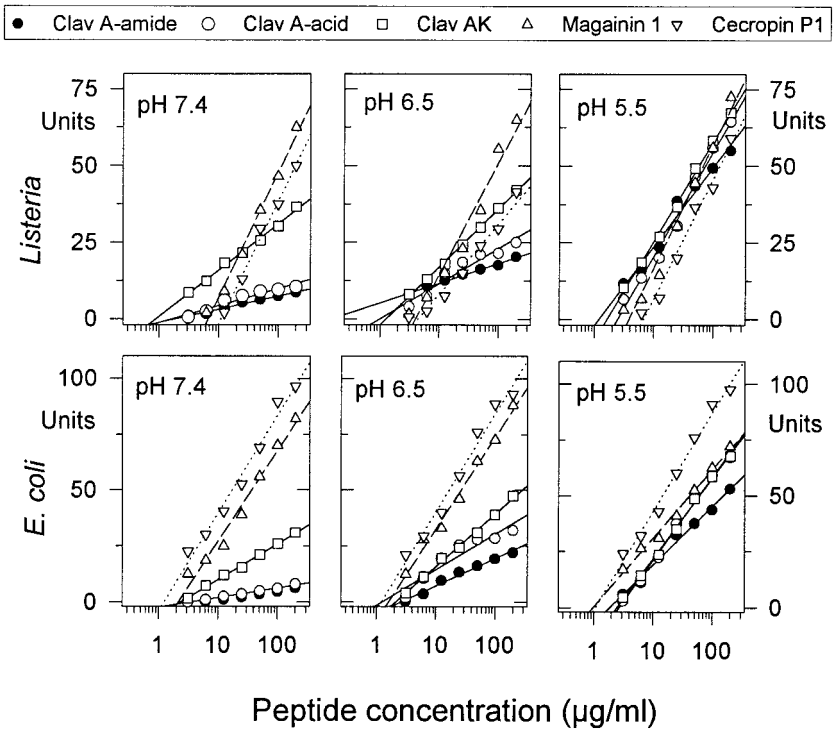

FIG. 1. Effects of pH on antibacterial activity. Synthetic clavanin (Clav) A amide, clavanin A acid, and clavanin AK acid were tested against *E. coli* and *L. monocytogenes* in radial diffusion assays. The underlay gels were prepared at pH 7.4, 6.5, or 5.5, and all contained 10 mM buffer (9:1 phosphate/citrate ratio), 1% agarose, and 0.3 mg of Trypticase soy broth powder/ml. The diameters of the clear zones are shown in units (10 units = 1 mm). The regression lines for the clavanins (solid) and control peptides (broken) were calculated by the least-mean-squares method. The *x* intercept indicates the minimal effective concentration.

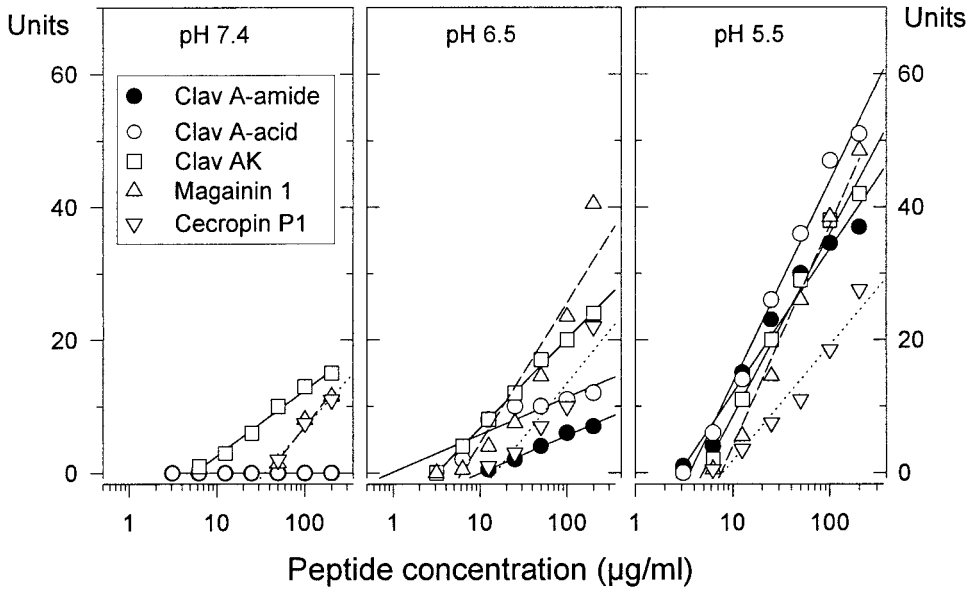

FIG. 2. Effects of pH on antifungal activity. Synthetic clavanin (Clav) A amide, clavanin A acid, and clavanin AK acid were tested against *C. albicans* in radial diffusion assays. The underlay gels were prepared at pH 7.4, 6.5, or 5.5, and all contained 10 mM buffer (9:1 phosphate/citrate ratio), 1% agarose, and 0.3 mg of Trypticase soy broth powder/ml. These gels did not contain any supplemental NaCl. The diameters of the clear zones are shown in units (10 units = 1 mm). The regression lines for the clavanins (solid) and control peptides (broken) were calculated by the least-mean-squares method. The *x* intercept indicates the minimal effective concentration.

with defensins (18). Also at pH 6.5, clavanin A amide acted more rapidly than clavanin A acid in permeabilizing the outer membrane. This kinetic difference suggests that C-terminal amidation, which also occurs in many other antimicrobial peptides, may serve to enhance antimicrobial activity under mildly acidic conditions. Overall, these findings were consonant with the effects of pH on the antimicrobial activity of clavanins against *E. coli* ML-35p shown in Fig. 1.

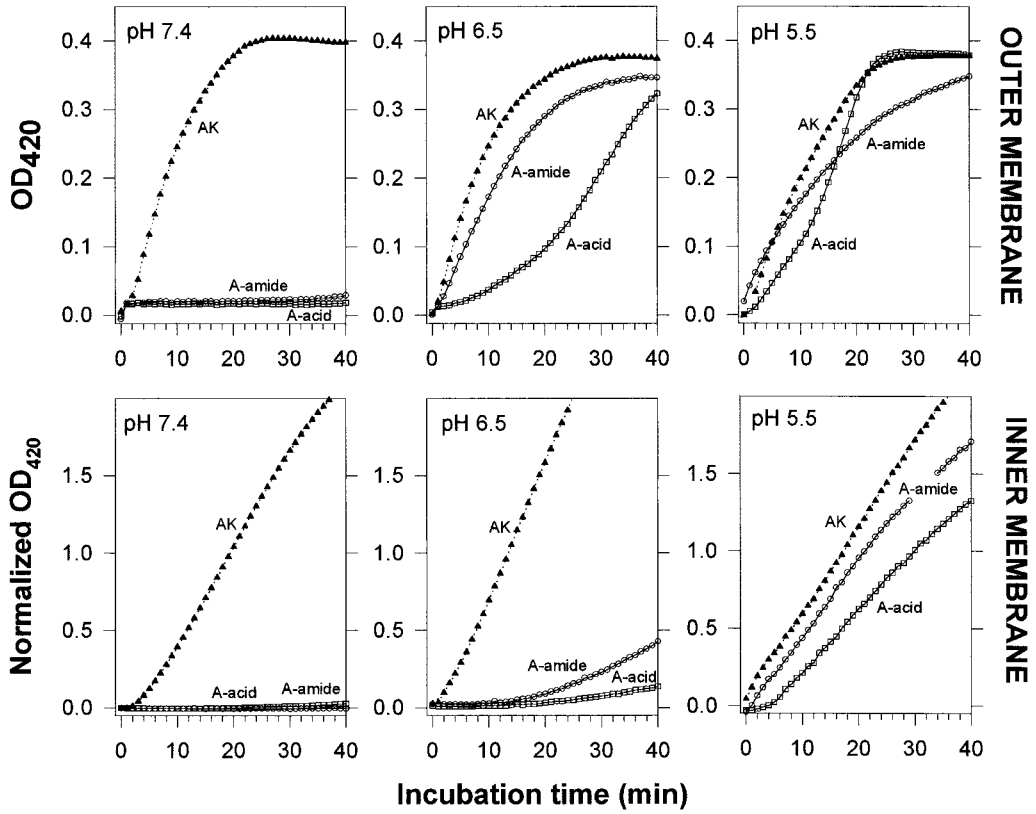

FIG. 3. Effects of pH on membrane permeability. The outer and inner membrane permeabilities of *E. coli* ML-35p were monitored as described in the text. Peptides were tested at final concentrations of 20 μg/ml. ONPG hydrolysis data at pH 6.5 and 5.5 have been normalized to compensate for the effects of reduced pH on the activity of β-galactosidase. OD_{420}, optical density at 420 nm.

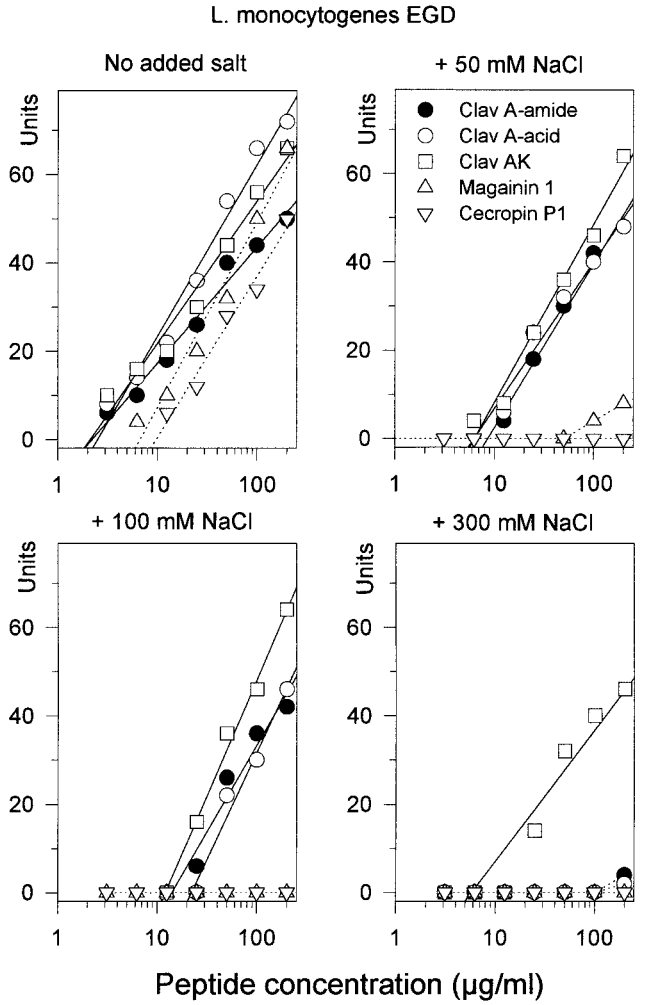

FIG. 4. Effects of salinity on activity against *L. monocytogenes*. Different concentrations of NaCl were added to underlay agars, prepared at pH 5.5, whose compositions were otherwise as described in the legend to Fig. 2. The regression lines for the clavanins (Clav) (solid) and control peptides (broken) were calculated by the least-mean-squares method. The *x* intercept indicates the minimal effective concentration.

Effects of salinity. The antimicrobial activity of magainin 1 and cecropin P1 against *L. monocytogenes* diminished greatly as the NaCl concentration in the underlay gels increased and was ablated when ≥100 mM NaCl was present (Fig. 4). In contrast, all three clavanins retained substantial activity against *L. monocytogenes* in 100 mM NaCl and clavanin AK was also active in 300 mM NaCl. Although adding up to 300 mM NaCl did not impair the antimicrobial activity of cecropin P1 against *E. coli* ML-35p, magainin 1 progressively lost efficacy as the salinity of the underlay gels increased (Fig. 5). Clavanin A and clavanin AK were less susceptible to inhibition by NaCl than was magainin 1, but both were more affected by salinity than was cecropin P1.

Antimicrobial spectrum. We examined the activity of the peptides against a panel of bacteria in assays that were performed at pH 6.5 without supplemental NaCl in the underlay gels (Table 2). We selected low-salt conditions for these assays to afford better support for antibacterial activity by cecropin P1 and magainin 1. As expected from prior studies, magainin 1 and cecropin P1 showed excellent activity against gram-negative bacteria and variable effectiveness against gram-positive bacteria. Neither was very active against the three strains of *Staphylococcus aureus* in our panel, two of which were methicillin resistant (MRSA). In contrast, the clavanins showed more uniform activity and were effective against both gram-positive and gram-negative bacteria.

DISCUSSION

Most antimicrobial peptides are amphipathic, polycationic molecules with highly basic natures that are imparted by multiple arginine and lysine residues without counterbalancing acidic amino acids (23). Often, as in cecropins, clavanins, and protegrins, the net cationicity of antimicrobial peptides is augmented by C-terminal amidation. The positive charge of antimicrobial peptides undoubtedly facilitates their interactions with anionic microbial surface components, such as lipopolysaccharide or various anionic membrane phospholipids. Histidine residues have a pK_a of approximately 6.5. Consequently, histidine-rich peptides like clavanin A have high net positive charges at pH 5.5 yet are relatively uncharged at pH 7.4.

Cecropins were discovered in diapausing pupae of the silk moth, *Hyalophora cecropia* (10), and were later shown to be widely distributed among other insects (for example, see ref-

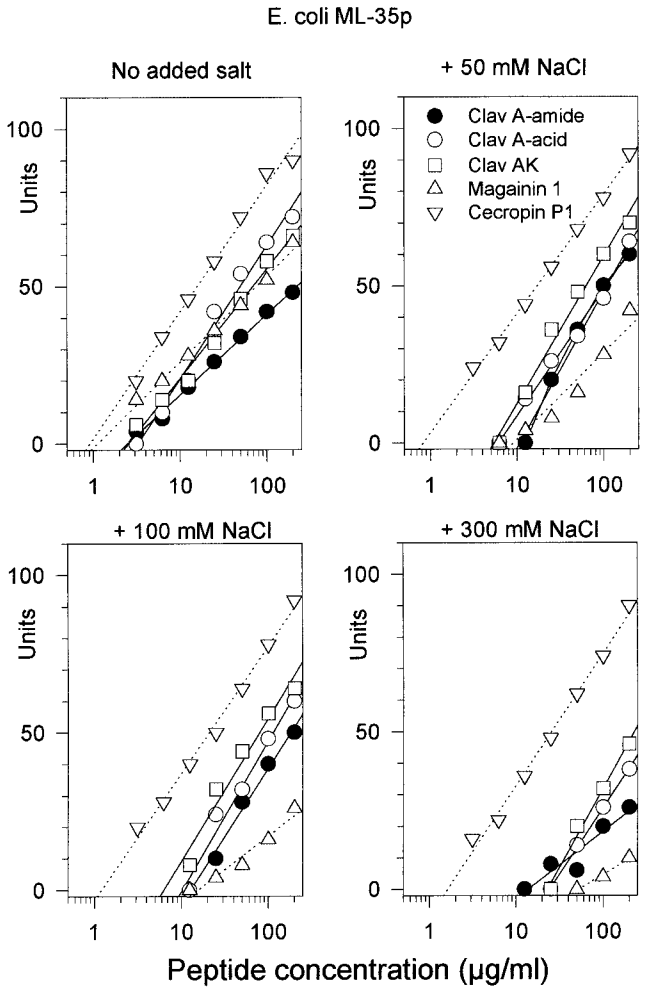

FIG. 5. Effects of salinity on activity against *E. coli* ML-35p. Different concentrations of NaCl were added to underlay agars, prepared at pH 5.5, whose compositions were otherwise as described in the legend to Fig. 2. The regression lines for the clavanins (Clav) (solid) and control peptides (broken) were calculated by the least-mean-squares method. The *x* intercept indicates the minimal effective concentration.

TABLE 2. MICs of various peptides for selected bacteria[a]

Organism	MIC (μg/ml)[b]					Source(s)[c]
	Clavanin A	Clavanin A acid	Clavanin AK	Magainin 1	Cecropin P1	
Gram positive						
S. aureus 930918-3	1.4 ± 0.6	1.7	3.9 ± 1.5	22.0 ± 4.6	22.2 ± 5.5	A
S. aureus 30371 (MRSA)	3.8 ± 1.5	2.7	5.8 ± 0.2	>200	>200	B
S. aureus 28841 (MRSA)	1.4 ± 0.2	1.9	2.5 ± 0.0	94.8 ± 54	>200	B
Enterococcus faecalis CDC 21	1.1 ± 0.3	0.6	2.6 ± 1.9	7.3 ± 1.1	9.4 ± 4.0	B
Enterococcus faecium 94-132	0.2 ± 0.0	0.2	0.3 ± 0.0	2.8 ± 1.4	3.4 ± 2.0	B
E. faecium	0.4 ± 0.2	0.3	0.5 ± 0.1	4.0 ± 1.7	4.0 ± 1.2	B
L. monocytogenes EGD	0.2 ± 0.1	0.4	0.4 ± 0.0	4.6 ± 1.4	4.1 ± 1.0	C
Gram negative						
E. coli ML-35p	2.3 ± 1.4	1.5	1.3 ± 0.4	0.7 ± 0.3	0.5 ± 0.1	D
E. coli mcr 106	1.7	NT	0.7	0.9	0.5	E, F
E. coli BAS 849	0.4 ± 0.1	1.5	0.7 ± 0.1	0.7 ± 0.4	0.3 ± 0.1	E, F
Salmonella typhimurium 14028S	2.1 ± 0.2	1.6	1.5 ± 0.4	1.2 ± 0.7	0.5 ± 0.1	G
S. typhimurium 7953S	0.6 ± 0.2	0.4	1.3 ± 0.4	0.6 ± 0.3	0.4 ± 0.0	G
Klebsiella pneumoniae 2270	4.2 ± 1.6	1.2	2.8 ± 1.3	1.8 ± 0.6	0.5 ± 0.1	A
P. aeruginosa SBI-N	0.4 ± 0.2	1.0	0.9 ± 0.3	0.4 ± 0.1	0.4 ± 0.0	A
P. aeruginosa MR2123	0.4 ± 0.2	0.8	0.7 ± 0.2	0.4 ± 0.0	0.5 ± 0.1	B
P. aeruginosa MR3007	0.8 ± 0.4	1.1	0.9 ± 0.4	0.5 ± 0.2	0.5 ± 0.1	B

[a] The underlay agar plates contained 10 mM sodium phosphate-sodium citrate (9:1) buffer (pH 6.5) and 0.3 mg of Trypticase soy broth powder/ml.
[b] Means ± standard errors of the means. Except for clavanin A acid, which was studied once, the data represent the results from three experiments. NT, not tested.
[c] A, Ian Holder, Shriners Burn Hospital, Cincinnati, Ohio; B, Elizabeth A. Wagar, UCLA Department of Clinical Pathology; C, Ralph van Furth, University of Leiden; D, Sidney Rittenberg, UCLA; E, Spencer Benson, University of Maryland, College Park, Md.; F, Deborah Steinberg, IntraBiotics, Sunnyvale, Calif.; G, Fred Heffron, University of Oregon.

erence 29). Cecropin A, which contains 37 residues and is most effective against gram-negative bacteria, contains two α-helical domains (residues 1 to 21 and 26 to 37) separated by a short proline-containing hinge region (residues 22 to 24). The highly cationic N-terminal domain of cecropin A contains six lysines, one arginine, and two acidic amino acids. Its C-terminal region contains a single lysine and no acidic residues. Cecropin P1, which contains 31 residues and is nonamidated (Table 1), was isolated from the small intestines of pigs and is thought to be a mammalian analog of the insect cecropins (15). Like insect cecropins, cecropin P1 contains a lysine-rich, cationic N-terminal region and has a characteristic tryptophan residue in position 2. Although as active as insect cecropins against gram-negative bacteria, cecropin P1 is less active against gram-positive bacteria (15).

Magainin 1, a positively charged peptide that assumes an α-helical structure in the presence of anionic liposomes and structure-promoting solvents (12, 24), manifests broad-spectrum antimicrobial activity in vitro (40, 41). It contains 23 amino acid residues and is nonamidated, and its sequence is identical to PGS, one of the array of small peptides in the cutaneous granular glands of *X. laevis* (9, 34). Magainins form anion channels in patch-clamped lipid bilayers (6) and bind to phospholipid vesicles, where they self-associate, insert, and form pentameric pores that allow leakage of internal contents (24–26).

Although clavanin A appears in many respects to be a typical α-helical antimicrobial peptide (14), it is unusual in that its cationicity derives primarily from histidines rather than from arginine or lysine residues. Only a few other histidine-rich antimicrobial peptides have been described, including histatins (21) and a peptide which corresponds to residues 20 to 44 of CAP37/azurocidin (NQGRHFCGGALIHARFVMTAASC FQ) and may form the antimicrobial domain of this lipopolysaccharide-binding, antimicrobial protein of human neutrophils (28). Like the clavanins described in this report, these other histidine-rich antimicrobial peptides also show greater antimicrobial activity at pH 5.0 to 5.5 than at a neutral pH (20, 28).

Histatins are a family of peptides secreted by the human parotid gland. They have also been called histidine-rich polypeptides (3, 27) and usually appear as six distinct main bands on polyacrylamide gels (1). Several of these bands are proteolytic products (37) of shared peptide precursors (32) encoded by genes located on chromosome 4q13 (35). The major family members—histatins 1, 3, and 5—contain 38, 32, and 24 amino acid residues, respectively (38), and exhibit antibacterial and anticandidal properties (37, 38).

The candidacidal activity of histatin 3 has been attributed to a region containing KFHEKHHSHRG, comprising residues 13 to 24 of the holopeptide (37). A candidacidal C-terminal fragment of histatin 5, GYKRKFHEKHHSHRGY (residues 9 to 24), adopts a largely α-helical conformation in lipid-like environments which simulate cell membranes (30). Histatins are also being used as templates for designing α-helical peptides that are active against *C. albicans* (31, 42).

We initiated the studies reported herein to examine the effects of pH and salinity on the antimicrobial properties of clavanin A, an α-helical antimicrobial peptide found in the hemocytes of *S. clava*, a marine protochordate (tunicate). In unpublished experiments that will be described elsewhere, we have localized hemocyte clavanins by immunoelectron microscopy to the cytoplasmic granules of certain hemocytes, including avidly phagocytic morula cells. It is noteworthy that morula cells from the tunicate *Ascidia ceratodes* were recently reported to have cytoplasmic vacuoles with pHs of 5.0 (16). Such acidic conditions are likely also to exist in the phagocytic vacuoles of tunicate hemocytes and should support optimal antimicrobial activity by these histidine-rich granule-associated peptides after their delivery via postphagocytic degranulation.

Taken in conjunction with studies of the other histidine-rich antimicrobial peptides mentioned above, the data described in this report indicate that strategically placed histidine residues endow antimicrobial peptides with pH-dependent antimicrobial activity which can render them active at low pH and relatively inactive at neutral pH. Although the design of clavanin A probably relates to its ability to function optimally within highly acidic vacuoles, histidine-rich polypeptides may be especially useful templates for developing antimicrobial peptides

intended to function within acidic environments such as the gastric lumen, vagina (for example, see reference 22), or cariogenic dental foci (36) of mammals. The relative lack of activity of histidine-rich peptides at a neutral pH could prove to be especially advantageous by limiting their activity to such sites.

Since the broad antimicrobial spectrum of clavanins includes both *S. aureus* and *Pseudomonas aeruginosa*, and since they retain antimicrobial activity in high NaCl environments, clavanins might also provide useful starting points for designing peptides for topical delivery to the lungs of patients with cystic fibrosis. In this condition, the increased salinity of bronchopulmonary fluids appears to promote colonization by organisms such as *Pseudomonas* and *S. aureus*, presumably by reducing the efficacy of endogenous antimicrobial molecules of pulmonary mucosal surfaces (33).

ACKNOWLEDGMENTS

This work was supported in part by grant AI 22839 from the National Institutes of Health.

We thank Lorenzo Menzel for expert technical assistance.

REFERENCES

1. **Azen, A. E.** 1973. Properties of salivary basic proteins showing polymorphism. Biochem. Genet. **9:**69–86.
2. **Bagella, L., M. Scocchi, and M. Zanetti.** 1995. cDNA sequences of three sheep myeloid cathelicidins. FEBS Lett. **376:**225–228.
3. **Baum, B. J., J. L. Bird, and R. W. Longton.** 1977. Polyacrylamide gel electrophoresis of human salivary histidine-rich polypeptides. J. Dent. Res. **56:** 1115–1118.
4. **Boman, H. G.** 1995. Peptide antibiotics and their role in innate immunity. Annu. Rev. Immunol. **13:**61–92.
5. **Broekaert, W. F., F. R. Terras, B. P. Cammue, and R. W. Osborn.** 1995. Plant defensins: novel antimicrobial peptides as components of the host defense system. Plant Physiol. (Rockville) **108:**1353–1358.
6. **Duclohier, H., G. Molle, and G. Spach.** 1989. Antimicrobial peptide magainin I from *Xenopus* skin forms anion-permeable channels in planar lipid bilayers. Biophys. J. **56:**1017–1021.
7. **Ganz, T., and R. I. Lehrer.** 1994. Defensins. Curr. Opin. Immunol. **6:**584–589.
8. **Gazit, E., A. Boman, H. G. Boman, and Y. Shai.** 1995. Interaction of the mammalian antibacterial peptide cecropin P1 with phospholipid vesicles. Biochemistry **34:**11479–11488.
9. **Giovannini, M. G., L. Poulter, B. W. Gibson, and D. H. Williams.** 1987. Biosynthesis and degradation of peptides derived from *Xenopus laevis* prohormones. Biochem. J. **243:**113–120.
10. **Hultmark, D., A. Engstrom, H. Bennich, R. Kapur, and H. G. Boman.** 1982. Insect immunity: isolation and structure of cecropin D and four minor antibacterial components from Cecropia pupae. Eur. J. Biochem. **127:**207–217.
11. **Iwanaga, S., T. Muta, T. Shigenaga, Y. Miura, N. Seki, T. Saito, and S. Kawabata.** 1994. Role of hemocyte-derived granular components in invertebrate defense. Ann. N. Y. Acad. Sci. **712:**102–116.
12. **Jackson, M., H. H. Mantsch, and J. H. Spencer.** 1992. Conformation of magainin-2 and related peptides in aqueous solution and membrane environments probed by Fourier transform infrared spectroscopy. Biochemistry **31:**7289–7293.
13. **Kokryakov, V. N., S. S. Harwig, E. A. Panyutich, A. A. Shevchenko, G. M. Aleshina, O. V. Shamova, H. A. Korneva, and R. I. Lehrer.** 1993. Protegrins: leukocyte antimicrobial peptides that combine features of corticostatic defensins and tachyplesins. FEBS Lett. **327:**231–236.
14. **Lee, I. H., C. Zhao, Y. Cho, S. S. L. Harwig, E. L. Cooper, and R. I. Lehrer.** Clavanins, α-helical antimicrobial peptides from tunicate hemocytes. FEBS Lett., in press.
15. **Lee, J. Y., A. Boman, C. X. Sun, M. Andersson, H. Jornvall, V. Mutt, and H. G. Boman.** 1989. Antibacterial peptides from pig intestine: isolation of a mammalian cecropin. Proc. Natl. Acad. Sci. USA **86:**9159–9162.
16. **Lee, S., K. Nakanishi, and K. Kustin.** 1990. The intracellular pH of tunicate blood cells: *Ascidia ceratodes* whole blood, morula cells, vacuoles and cytoplasm. Biochim. Biophys. Acta **1033:**311–317.
17. **Lehrer, R. I., A. Barton, and T. Ganz.** 1988. Concurrent assessment of inner and outer membrane permeabilization and bacteriolysis in E. coli by multiple-wavelength spectrophotometry. J. Immunol. Methods **108:**153–158.
18. **Lehrer, R. I., A. Barton, K. A. Daher, S. S. Harwig, T. Ganz, and M. E. Selsted.** 1989. Interaction of human defensins with *Escherichia coli*. Mechanism of bactericidal activity. J. Clin. Invest. **84:**553–561.
19. **Lehrer, R. I., M. Rosenman, S. S. Harwig, R. Jackson, and P. Eisenhauer.** 1991. Ultrasensitive assays for endogenous antimicrobial polypeptides. J. Immunol. Methods **137:**167–173.
20. **MacKay, B. J., L. Denapitaya, V. J. Iacono, S. B. Krost, and J. J. Pollock.** 1984. Growth-inhibitory and bactericidal effects of human parotid salivary histidine-rich polypeptides on *Streptococcus mutans*. Infect. Immun. **44:**695–701.
21. **MacKay, B. J., J. J. Pollock, V. J. Iacono, and B. J. Baum.** 1984. Isolation of milligram quantities of a group of histidine-rich polypeptides from human parotid saliva. Infect. Immun. **44:**688–694.
22. **Mahmoud, E. A., L. O. Svensson, S. E. Olsson, and P. A. Mardh.** 1995. Antichlamydial activity of vaginal secretion. Am. J. Obstet. Gynecol. **172:** 1268–1272.
23. **Maloy, W. L., and U. P. Kari.** 1995. Structure-activity studies on magainins and other host defense peptides. Biopolymers **37:**105–22.
24. **Matsuzaki, K., M. Harada, T. Handa, S. Funakoshi, N. Fujii, H. Yajima, and K. Miyajima.** 1989. Magainin 1-induced leakage of entrapped calcein out of negatively-charged lipid vesicles. Biochim. Biophys. Acta **981:**130–134.
25. **Matsuzaki, K., O. Murase, H. Tokuda, S. Funakoshi, N. Fujii, and K. Miyajima.** 1994. Orientational and aggregational states of magainin 2 in phospholipid bilayers. Biochemistry **33:**3342–3349.
26. **Matsuzaki, K., O. Murase, and K. Miyajima.** 1995. Kinetics of pore formation by an antimicrobial peptide, magainin 2, in phospholipid bilayers. Biochemistry **34:**12553–12559.
27. **Oppenheim, F. G., Y.-C. Yang, R. D. Diamond, D. Hyslop, G. D. Offner, and R. F. Troxler.** 1988. Histatins, a novel family of histidine-rich proteins in human parotid secretions. J. Biol. Chem. **261:**7472–7477.
28. **Pereira, H. A., I. Erdem, J. Pohl, and J. K. Spitznagel.** 1993. Synthetic bactericidal peptide based on CAP37: a 37-kDa human neutrophil granule-associated cationic antimicrobial protein chemotactic for monocytes. Proc. Natl. Acad. Sci. USA **90:**4733–4737.
29. **Petersen, U. M., G. Bjorklund, Y. T. Ip, and Y. Engstrom.** 1995. The dorsal-related immunity factor, Dif, is a sequence-specific trans-activator of Drosophila Cecropin gene expression. EMBO J. **14:**3146–3158.
30. **Raj, P. A., S. D. Soni, and M. J. Levine.** 1994. Membrane-induced conformation of an active candidacidal fragment of salivary histatins. J. Biol. Chem. **269:**9610–9619.
31. **Ramalingam, K., T. L. Gururaja, N. Ramasubbu, and M. J. Levine.** 1996. Stabilization of helix by side-chain interactions in histatin-derived peptides: role in candidacidal activity. Biochem. Biophys. Res. Commun. **225:**47–53.
32. **Sabatini, L. M., and E. A. Azen.** 1989. Histatins, a family of salivary histidine-rich proteins, are encoded by at least two loci (His1 and His2). Biochem. Biophys. Res. Commun. **160:**495–502.
33. **Smith, J. J., S. M. Travis, E. P. Greenberg, and M. J. Welsh.** 1996. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. Cell **85:**229–236.
34. **Terry, A. S., L. Poulter, D. H. Williams, J. C. Nutkins, M. G. Giovannini, C. H. Moore, and B. W. Gibson.** 1988. The cDNA sequence coding for prepro-PGS prepro-magainins and aspects of the processing of this prepropolypeptide. J. Biol. Chem. **263:**5745–5751.
35. **VanderSpek, J. C., H. E. Wyandt, J. C. Skare, A. Milunsky, F. G. Oppenheim, and R. F. Troxler.** 1989. Localization of the genes for histatins to human chromosome 4q13 and tissue distribution of the mRNAs. Am. J. Hum. Genet. **45:**381–387.
36. **van Houte, J., J. Lopman, and R. Kent.** 1996. The final pH of bacteria comprising the predominant flora on sound and carious human root and enamel surfaces. J. Dent. Res. **75:**1008–1014.

36a. **Waring, A., and R. I. Lehrer.** Unpublished data.

37. **Xu, L., K. Lal, R. P. Santarpia III, and J. J. Pollock.** 1993. Salivary proteolysis of histidine-rich polypeptides and the antifungal activity of peptide degradation products. Arch. Oral Biol. **38:**277–283.
38. **Xu, T., S. M. Levitz, R. D. Diamond, and F. G. Oppenheim.** 1991. Anticandidal activity of major human salivary histatins. Infect. Immun. **59:**2549–2554.
39. **Zanetti, M., R. Gennaro, and D. Romeo.** 1995. Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. FEBS Lett. **374:**1–5.
40. **Zasloff, M.** 1988. Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc. Natl. Acad. Sci. USA **84:**5449–5453.
41. **Zasloff, M., B. Martin, and H. C. Chen.** 1988. Antimicrobial activity of synthetic magainin peptides and several analogues. Proc. Natl. Acad. Sci. USA **85:**910–913.
42. **Zuo, Y., T. Xu, R. F. Troxler, J. Li, J. Driscoll, and F. G. Oppenheim.** 1995. Recombinant histatins: functional domain duplication enhances candidacidal activity. Gene **161:**87–91.

Editor: P. E. Orndorff