pH Dependence of Microbe Sterilization by Cationic Antimicrobial Peptides

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Loyola University New Orleans, Department of Chemistry, New Orleans, Louisiana, USA; Tulane University Medical Center, Department of Biochemistry, New Orleans, Louisiana, USA

We recently described a family of cationic antimicrobial peptides (CAMPs) selected from a combinatorial library that exhibited potent, broad-spectrum activity at neutral pH and low ionic strength. To further delimit the utility and activity profiles of these peptides, we investigated the effects of solution conditions, such as pH and ionic strength, on the efficacy of the peptide antimicrobials against a panel of microorganisms. Peptide minimum sterilizing concentrations (MSCs) varied linearly with pH for each subtype within our family of CAMPs for all organisms tested. The peptides were much less effective against Gram-negative bacteria at high pH, consistent with a decrease in net positive charge on the peptides. A similar trend was observed for the fungus Candida albicans. Surprisingly, the opposite pH trend was observed with the Gram-positive Staphylococcus aureus. In addition, an additive ionic strength effect was observed with increasing buffer strengths at identical pH values. The extreme difference in the observed pH behavior between Gram-negative and Gram-positive organisms is attributed to the presence of native charged molecules in the much thicker peptidoglycan layer of the Gram-positive organism. The novel species-specific effects of pH observed here have important implications for applications using CAMPs and for the design of novel CAMPs.

The proliferation of antibiotic resistance has been described as a crisis for more than 20 years due to the increasing number of hospitalizations and deaths attributed to resistant organisms each year (1–3). Discovering and developing new classes of antibiotics is essential to combat this threat. Cationic antimicrobial peptides (CAMPs) are promising drug candidates, having the advantages of broad-spectrum activity, high potency, and a very low incidence of developed resistance (4–7).

Discovered in 1981, CAMPs are a central component of the innate immune system in organisms ranging from invertebrates to plants to humans (8, 9). Although a wide variety of structural motifs are used by various CAMPs (10–12), most share several common characteristics, including small size and an amphipathic sequence containing both hydrophobic and cationic amino acids (7, 8). Most CAMPs also appear to function via a shared mechanism involving the permeabilization of the cytoplasmic membranes of targeted organisms (4–8), although they may have additional intracellular targets (5, 13). In fact, lack of resistance to CAMPs is ascribed to the lack of a distinct macromolecular target and to the large energetic cost in reorganizing the membrane surface of an organism (6, 7, 13).

The peptides used in the current study were selected from a designed combinatorial library (14) using lysis of a simple model membrane as a screen but, remarkably, were found to have broad-spectrum antimicrobial activity and low toxicity (15). The primary structures of these peptides, which have a β-sheet secondary structure in membranes, are shown in Fig. 1. The peptides are named using the nomenclature adopted previously (15) to describe members of this family, which contain a common 9-residue core motif with or without the presence of cationic terminal tripeptide cassettes (see Fig. 1). The asterisks designate the presence of an RRG- or -GRR terminal cassette, and the one-letter codes represent the amino acids found at each of the four varied positions (O) within the 9-residue core sequence, WOLOLOLOY. The peptides used in our studies thus varied between 9 to 15 amino acids in length, where *VDVY* is a 15-amino-acid peptide (RRG WVLDLVLYYGRR) containing both an N-terminal Arg-Arg-Gly and a C-terminal Gly-Arg-Arg and containing V, D, V, and Y, respectively, at the four varied (O) positions. *ARVA is a 12-amino-acid peptide with a single terminal Arg-Arg-Gly cassette, and ARVY is a 9-amino-acid peptide containing A, R, Y, and V at the open positions. Positive charges due to Arg side chains and the N terminus and the sole negative charge (Asp in *VDVY*) are indicated in Fig. 1, as is the amidated C terminus in each peptide.

The lethal step for most CAMPs follows a binding event whereby the positively charged peptides interact with the anionic plasma membranes of targeted microorganisms (4, 6). In addition, there exists the likelihood of interactions with other charged molecules found on the outer layers of the microbial cell wall or lipopolysaccharide layers (10, 16, 17). Once CAMPs bind to the plasma membrane, their imperfectly amphipathic nature allows them to interact via interfacial activity with the nonpolar tails of the membrane lipids (6, 8). Cell death is due to disruption of the membrane rather than by true pore formation (4, 6), although other mechanisms have also been proposed (5, 13).

Due to the well-known problems associated with using peptides as drugs (e.g., solubility, cost, bioavailability), the most obvious applications for CAMPs will be in topical or surface sterilization applications and to help prevent hospital-acquired infections (7, 11). An interesting possibility is the use of tethered CAMPs in surgical applications attached, perhaps in a slow-release form (18), to the surface of artificial joints and implants (19–21). Despite having been studied for over 30 years, the clinical
use of CAMPs is still limited. Clinical trials have been conducted or are in progress for a number of applications, such as skin infections, thrush, diabetic ulcers, and secondary infections related to cystic fibrosis (7, 22). Initial results have been mixed, but promising new candidates continue to be identified (11, 23).

Because CAMPs will often be used in applications where the environment can be variable, it is important to thoroughly understand the effects of environmental factors, such as pH and ionic strength, on the efficacy of CAMPs in directly killing bacteria (11, 23). A number of studies have looked at the effects of pH or salt concentration on the ability of CAMPs to kill microorganisms (24, 25). In most cases, only a narrow range of different pHs or salt concentrations were sampled, although a few studies have been more comprehensive (26, 27). It is well known that higher salt concentrations often interfere with CAMP activity (25, 28, 29), but some CAMPs from marine organisms have been found to tolerate salt concentrations up to 450 mM (30). In the case of cystic fibrosis, lung infection by *Pseudomonas aeruginosa* has been linked to inactivity of CAMPs at the higher salt concentrations found in the lungs of cystic fibrosis patients (29). Other studies have observed that CAMPs work as well or better at lower pH than at neutral pH (26, 31), particularly His-rich peptides such as the clavanins, which contain a number of His residues instead of the more typical Arg or Lys residues (32). In this work, we investigated the effect of a wide range of pH and ionic strength values, both separately and in combination, to gain a more comprehensive understanding of their effect on CAMP activity.

**MATERIALS AND METHODS**

**Bacterial cultures.** Strains of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), and *Candida albicans* (ATCC 90028) were obtained from the American Type Culture Collection (Rockville, MD). The bacterial and yeast cultures were maintained in Trypticase soy broth (TSB) and YPD broth (Difco Laboratories, Detroit, MI), respectively. Overnight cultures of bacteria were grown in 50-ml tubes shaken at 220 rpm at 37°C. Overnight cultures of yeast were grown at 30°C.

**Peptide synthesis.** Bulk peptide synthesis was carried out using Tentagel S peptide amide resin (0.2 mmol/g) on Applied Biosystems Pioneer synthesizer by the standard 9-fluorenylmethoxy carbonyl (Fmoc) solid-phase peptide synthesis method. Peptide cleavage and removal of side-chain protection groups was done by reagent R (90% trifluoroacetic acid [TFA], 5% thioanisole, 3% ethanedithiol, 2% anisole). The crude peptide was dissolved in glacial acetic acid and purified by reverse phase high-performance liquid chromatography (HPLC), using a Waters 600 HPLC pump associated with Waters 486 in-line UV/Vis absorbance detector systems (Millford, MA) and a Dynamax C18 fused silica column (1-cm
TABLE 1 MSCs of CAMPs as a function of pH

<table>
<thead>
<tr>
<th>Organism</th>
<th>pH 4</th>
<th>pH 5</th>
<th>pH 6</th>
<th>pH 7</th>
<th>pH 8</th>
<th>pH 9</th>
<th>pH 5</th>
<th>pH 7</th>
<th>pH 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram negative</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>1.2 ± 0.1</td>
<td>1.5 ± 0.2</td>
<td>5.2 ± 1.1</td>
<td>7.0 ± 0.7</td>
<td>1.3 ± 0.1</td>
<td>1.6 ± 0.2</td>
<td>3.1 ± 0.4</td>
<td>4.8 ± 0.5</td>
<td>2.7 ± 0.2</td>
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<tr>
<td><em>E. coli</em></td>
<td>1.4 ± 0.4</td>
<td>2.2 ± 0.5</td>
<td>2.6 ± 0.3</td>
<td>4.7 ± 0.7</td>
<td>0.5 ± 0.1</td>
<td>1.1 ± 0.3</td>
<td>1.3 ± 0.2</td>
<td>2.9 ± 0.3</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>Gram positive</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>&gt;8</td>
<td>5.3 ± 0.2</td>
<td>3.5 ± 0.6</td>
<td>1.9 ± 0.5</td>
<td>6.8 ± 0.7</td>
<td>3.7 ± 1.0</td>
<td>2.5 ± 0.4</td>
<td>1.1 ± 0.1</td>
<td>&gt;10</td>
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<tr>
<td>Yeast</td>
<td></td>
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<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>3.3 ± 0.3</td>
<td>—</td>
<td>1.1 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>—</td>
<td>7.1 ± 0.3</td>
</tr>
</tbody>
</table>

Ionic strength (M) in 0.1 M buffer

*MSCs of CAMPs as a function of pH

TABLE 2 MSCs of CAMPs as a function of pH

<table>
<thead>
<tr>
<th>Organism</th>
<th>*ARVA</th>
<th>ARYV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>2.6 ± 0.7</td>
<td>4.7 ± 1.7</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>1.5 ± 0.2</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>Gram positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>&gt;10</td>
<td>6.9 ± 1.9</td>
</tr>
<tr>
<td>Ionic strength (M)</td>
<td>0.179</td>
<td>0.181</td>
</tr>
</tbody>
</table>

*NA, not available.

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inner diameter and 30-cm length; Dynamax Inc., Houston, TX). Peptide samples were eluted from the column using a gradient from 0 to 55% acetonitrile (0.1% TFA) and water (0.1% TFA) over 30 min. Predominant peak fractions were collected, and the identity of purified peptides was confirmed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry. The peptide *VDVY* has the sequence RRGWVLDLVLYY WALRLYLVYNH2.

Buffers and ionic strength calculations. In order to make more meaningful comparisons between buffers at different pHs, all buffer solutions were prepared in a liquid test medium (LTM) background containing 1% growth broth in phosphate-buffered saline (PBS). Most pH solutions used in these studies were prepared using an additional 0.1 M concentration of buffer with LTM as the solvent. For purposes of comparison, additional buffers were prepared at a 0.2 M concentration, also in an LTM background. Phosphate buffers were prepared at pHs from 4 to 8, while pH 9 buffer was CHES (N-cyclohexyl-2-aminoethane sulfonic acid). Additional pH 9 buffers were tested using phosphate, CAPS (3-cyclohexylamino-1-propane sulfonic acid), and tricine to rule out specific CHES effects. Buffer controls at pH 10 killed most organisms, and *C. albicans* did not tolerate pH 9 well, limiting the pH range of our analysis. Test mixes in the exact ratios used in the assays were routinely performed to verify the final pH values during the incubation periods. LTM buffer gave a pH value near 5.0 in the assays, except for studies with *S. aureus*, where the value was adjusted such that the pH after mixing was 5.8 during the incubation period. Ionic strengths were calculated in terms of molarity using the equation

where $c_i$ and $z_i$ are the concentration and charge of the various ions in each buffer solution.

Ionic strengths varied from 0.11 M to 0.32 M (see Tables 1 and 2). Effective ionic strengths calculated from extended Debye-Hückel varied from 0.09 M to 0.18 M (33). Net charges on peptides at various pHs were calculated with the Henderson-Hasselbalch equation: pH = $pK_a + \log \left[ \frac{[HA]}{[A^-]} \right]$, using common values for the $pK_a$ s of amino acids in peptides and where HA is the acid form of the buffer and A- is its conjugate base.

**Antimicrobial activity assay.** *E. coli, P. aeruginosa, S. aureus,* and *C. albicans* were grown to mid-logarithmic phase and diluted to 2 × 10^8 CFU/ml with minimal liquid test medium (LTM). We have shown that increasing the experimental cell counts up to 10^11/ml uniformly increases minimum sterilizing concentration (MSC) values by severalfold but does not affect the functional dependence of MSC measurements (15). Assays were performed in sterile Costar 96-well flat-bottom plates (Corning, Lowell, MA). Final assay volume was 170 μl as follows: 60 μl of cell suspension (2 × 10^8 cells/ml in minimal LTM) and 60 μl of 0.1 M pH buffer (0.2 M in some experiments) were added to 50 μl of 0.025% acetic acid containing the peptide. Twelve columns were prepared for each peptide in 0.75-fold serial dilution starting at 10 to 15 μM. Rows were set up in pairs, with and without peptide, to control for the effect of various solution conditions, such as pH and ionic strength, on bacterial growth. Microbial cell suspensions were first incubated at 37°C for 30 min in the presence of only peptide and buffer to test directly the effect of various solution conditions, such as pH and ionic strength, on bacterial growth.
Concentration, to submicromolar concentrations. Opaque wells are overgrown with sequentially across the 12 columns of the plate from about 7 μM peptide down to submicromolar concentrations. Opaque wells are overgrown with *Pseudomonas aeruginosa*, while clear wells indicate complete sterilization at that concentration of *VDVY*. LTM is liquid test medium at pH 5, and pH 4, 7, and 9 buffers were 0.1 M, prepared in an LTM background as described in Materials and Methods.

DISCUSSION

Variation of the initial incubation period with no subsequent growth overnight. Row B contains clear wells, indicating complete sterilization during the incubation period. *VDVY* in LTM is able to completely sterilize organisms, there is a clearly defined trend of increasing MSC with increasing pH, indicating that the peptides work less well at higher pH. In both Fig. 3A and B, *ARVA activity (diamond symbols) is the least sensitive to pH, while ARVY (squares) is the most sensitive to pH. Indeed, in many data sets, ARVY was completely ineffective at pH 9. For all three peptides, there is roughly a 4-fold increase in MSC between the lowest and highest pH values tested. In general, *VDVY* (circles) is much more similar to *ARVA in its effect to pH. Indeed, in many data sets, *ARVA was completely ineffective at pH 9. For all three peptides, there is roughly a 4-fold increase in MSC between the lowest and highest pH values tested. In general, *VDVY* (circles) is much more similar to *ARVA in its activity-versus-pH profiles were collected at higher buffer concentrations are listed in Table 2. Data collected at higher buffer concentrations are listed in Table 2.

**RESULTS**

**Sixty-nine-well pH assay.** Activity-versus-pH profiles were collected for three CAMPs against a panel of microorganisms as described in Materials and Methods. In Fig. 2, the results for a typical pH assay (in this case for *VDVY* against *Pseudomonas aeruginosa*) are displayed. The eight rows (A to H) are matched pairwise such that the buffer conditions are identical in the control and treatment rows. The top row of each pair serves as the buffer control, and the bottom row of each pair contains the antimicrobial peptide to be tested diluted serially across each of the 12 columns of the plate. Following the 30-min incubation period, growth medium is added and the plates are grown overnight at 37°C. Cell survival was evaluated by visual inspection as well as measuring optical density at 600 nm (OD600). Wells were either opaque (OD600 > 0.5), indicating stationary-phase growth, or they were transparent (OD600 < 0.02), indicating no growth (see Fig. 2). Very few wells had intermediate growth, but those that did were assigned a value of 0.5 columns killed. Aliquots from wells with no apparent growth were spread on nutrient agar plates to verify sterility. In all cases, there were few if any CFU in those wells compared to 10^8 CFU/μl in the opaque wells. For most experiments, there were a number of sterile wells, starting from the highest concentration of peptide. The lowest concentration of peptide that prevented cell growth is the minimum sterilizing concentration (MSC). The MSC values were calculated separately for each data point by converting number of columns killed into a concentration value using the relation

\[ MSC = \frac{C \times D^{n-1}}{n} \]

where C is the initial peptide concentration, D is the dilution factor for that experiment, and n is the number of columns killed. Reported MSC values are the average of 8 to 24 data points. Unless indicated otherwise, all error bars are ± 1 standard error (SE).

**Effect of pH on activity versus Gram-negative organisms.** In Fig. 3, the results of pH studies performed with the Gram-negative organisms *Pseudomonas aeruginosa* (Fig. 3A) and *E. coli* (Fig. 3B) are depicted. The MSC values (±1 standard error) for each peptide (see also Table 1) are plotted as a function of pH. For both organisms, there is a clearly defined trend of increasing MSC with increasing pH, indicating that the peptides work less well at higher pH. In both Fig. 3A and B, *ARVA activity (diamond symbols) is the least sensitive to pH, while ARVY (squares) is the most sensitive to pH. Indeed, in many data sets, ARVY was completely ineffective at pH 9. For all three peptides, there is roughly a 4-fold increase in MSC between the lowest and highest pH values tested. In general, *VDVY* (circles) is much more similar to *ARVA in its activity-versus-pH profiles were collected for three CAMPs against a panel of microorganisms as described in Materials and Methods. In Fig. 2, the results for a typical pH assay (in this case for *VDVY* against *Pseudomonas aeruginosa*) are displayed. The eight rows (A to H) are matched pairwise such that the buffer conditions are identical in the control and treatment rows. The top row of each pair serves as the buffer control, and the bottom row of each pair contains the antimicrobial peptide to be tested diluted serially across each of the 12 columns of the plate. Following the 30-min incubation period, growth medium is added and the plates are grown overnight at 37°C. Cell survival was evaluated by visual inspection as well as measuring optical density at 600 nm (OD600). Wells were either opaque (OD600 > 0.5), indicating stationary-phase growth, or they were transparent (OD600 < 0.02), indicating no growth (see Fig. 2). Very few wells had intermediate growth, but those that did were assigned a value of 0.5 columns killed. Aliquots from wells with no apparent growth were spread on nutrient agar plates to verify sterility. In all cases, there were few if any CFU in those wells compared to 10^8 CFU/μl in the opaque wells. For most experiments, there were a number of sterile wells, starting from the highest concentration of peptide. The lowest concentration of peptide that prevented cell growth is the minimum sterilizing concentration (MSC). The MSC values were calculated separately for each data point by converting number of columns killed into a concentration value using the relation

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behavior than to ARYV. For example, *VDVY* and *ARVA* have MSC values near 1.2 μM at pH 4 with *P. aeruginosa* and increase to ~7 μM and ~5 μM, respectively, while ARYV increases from ~2.5 μM to ~8 μM at pH 7 and is unable to completely sterilize the solution at pH 9. In addition, both *VDVY* and *ARVA* seem to be more effective and less pH sensitive against *E. coli* (Fig. 3B) than *Pseudomonas* (Fig. 3A). The *ARVA* peptide at pH 4 versus *E. coli* is the only data to consistently show a submicromolar (0.5 μM) MSC value.

**Effect of pH on activity versus Gram-positive S. aureus.** In Fig. 4, the effect of pH on the activity of peptides against the Gram-positive organism *Staphylococcus aureus* is shown. The MSC value of each peptide is plotted versus pH using the same scheme as described for Fig. 3. Once again, *VDVY* (circles) and *ARVA* (diamonds) exhibit behavior similar to each other and have lower MSC values than ARYV (squares). Remarkably, the exact opposite trend with pH is observed compared to that observed for the Gram-negative organisms tested. The peptides, in contrast to the data in Fig. 3, are most effective (low MSC values) at high pH. At pH 9, both *VDVY* and *ARVA* have an MSC value of ~1 to 2 μM, while ARYV has a value of ~6 μM. The lowest MSC values (at high pH) are very similar to those observed at low pH for the Gram-negative organisms, particularly for *VDVY* and *ARVA* (Fig. 3). The MSC values against *S. aureus* become progressively larger as the pH decreases, moving from 1.1 μM at pH 9 to 6.8 μM at pH 4 (for *ARVA*) and from about 2 μM (for *VDVY*) and 6 μM (for ARYV) at pH 9 to completely ineffective in the sterilization assay at pH 4 (see also Table 1).

**Effect of pH on activity versus Candida albicans.** The sole eukaryote tested was the yeast *C. albicans*. In Fig. 5, the MSC values of our peptides are given as a function of pH. All three peptides worked best in LTM, with *VDVY* (black bars) and *ARVA* (light gray) exhibiting potent MSC values near 1 μM and ARYV (dark gray) a bit higher at ~3 μM. At pH 4, *VDVY* and *ARVA* MSC values were nearly unchanged, but ARYV was increased to nearly 7 μM. At pH 7, which was the highest pH tested, *VDVY* and *ARVA* had increased to ~4 μM and ~2 μM, respectively, while ARYV was unable to sterilize the solution in the initial incubation period before the overnight growth. As observed with the other organisms, *ARVA* remained the least and ARYV the most sensitive to pH. *C. albicans* does not tolerate high pH conditions, so data were not able to be collected at pH 9 (Table 1). In a related set of experiments using a much shorter incubation time of 10 min, we observed that the peptides worked less well at pH 9 than at pH 7. This suggests that the killing behavior of our CAMPs against *C. albicans* follows the pH trend observed for the Gram-negative organisms rather than for *S. aureus*.

**Cumulative effects of pH and ionic strength.** In addition to experiments with 0.1 M buffer in an LTM background (Table 1), experiments were also conducted using 0.2 M buffer in LTM (Table 2). The ionic strengths for each buffer are listed at the bottom of each column in both Tables 1 and 2. Note that the ionic strengths are 15 to 60% higher for the 0.2 M buffers than the 0.1 M buffers at the same pH. In Fig. 6, MSC values for *ARVA* (circles) and ARYV (triangles) against *P. aeruginosa* are plotted as a function of pH at both low (0.1 M; filled symbols) and high (0.2 M; open symbols) buffer concentration. The data at higher buffer concentrations were collected at all six pH values from pH 4 to 9, while the data at low buffer concentrations were collected at only four pH values. For both peptides, the observed trend line is offset to higher MSC values for the 0.2 M buffers (with a similar slope), indicating that the antimicrobial peptides work less well at higher buffer strength despite having an identical pH, presumably due to the higher ionic strengths. In many cases, the MSC values in 0.2 M buffer are nearly halfway between the lower and upper values of each column in both Tables 1 and 2. Note that the ionic strengths for each buffer are listed at the bottom of each column in both Tables 1 and 2.
buffer are nearly double the MSC values in 0.1 M buffer. Above pH 7, ARYV was unable to sterilize the solutions during the incubation period, thus preventing measurement of MSC values (triangle symbols in Fig. 6; compare also Tables 1 and 2).

DISCUSSION

Cationic AMPs (CAMPs) have been studied extensively as part of the ongoing search for new agents with which to combat antibiotic-resistant microorganisms (11). The advantages of CAMPs, such as broad specificity and lack of resistance, are partially offset by disadvantages, such as cost, susceptibility to proteolysis, and possible immunogenicity (6, 7). These disadvantages are common ones shared by most peptide drugs and make it more difficult to use standard delivery methods such as oral administration. For this reason, the initial uses of CAMPs are most likely to be in topical or surface applications (7, 9). Indeed, most clinical trials to date involve the treatment of skin infection or the use of CAMPs in preventing surface colonization and biofilms (11, 23). Topical and surface applications may allow a wider variation in the local environment than would be possible with systemic administration. Thus, a comprehensive understanding of the effect of environmental factors, such as pH, ionic strength, specific ion effects, and surface properties on CAMP activity, is necessary to facilitate the development and application of CAMPs as successful drug therapies (11, 23).

The presence of increased concentrations of ionic species is known to adversely affect the activity of CAMPs. The addition of low mM concentrations of divalent Ca$^{2+}$ and Mg$^{2+}$ ions, for example, has been shown to decrease the activity of mammalian CAMPs, particularly against Gram-negative organisms (7, 25, 34). In order to cross the lipopolysaccharide (LPS) outer membrane of Gram-negative bacteria, the peptides appear to displace divalent ions upon binding LPS and initiate a process described as self-promoted uptake (10, 12, 16, 35, 36). The adverse effects on CAMP activity due to divalent ions occur at a concentration several times lower than predicted by the ionic strength equation and thus fall in the category of specific ion effects.

Any pH study.

Our results at various ionic strengths at constant pH (Fig. 6, Tables 1 and 2) show that the MSC values of all three peptides are adversely affected by increased ionic strength. In these and independent salt studies, we found that ARYV was always the most sensitive to increased ionic strength, while *ARVA was always the least sensitive. In fact, *ARVA displays a substantial degree of salt tolerance, with MSC values in the low micromolar range at ionic strengths of 200 to 300 mM. We attribute the lower salt tolerance of the other two peptides to the small net positive charge of ARYV and to the presence of the negatively charged aspartate in *VDVY* not present in *ARVA.

The Gram-negative organisms *P. aeruginosa* and *E. coli* were readily killed (Table 1 and Fig. 3) by all three CAMPs at low pH and exhibited a linear increase in MSC values as the pH increased. To our knowledge, this is the first report of linear trends in antimicrobial activity as a function of pH for multiple organisms over a broad range of pH. All peptides killed less effectively at higher pHs than at lower pHs. ARYV was inactive at pH 9, and its effectiveness was even compromised at pH 7 in the ionic strength conditions used here. The data for *C. albicans* exhibit similar pH behavior over the pH values amenable to analysis (Fig. 5). These results can be interpreted readily by comparing the net peptide charges of each of our CAMPs across a range of pH values (Table 3). In each case, the peptide net charge is more positive at pH values below neutral pH and become less positively charged as the pH is raised to values of pH 7 or higher. The peptides used in this

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**TABLE 3 Net charge of CAMPs as a function of pH**

<table>
<thead>
<tr>
<th>CAMP</th>
<th>pH 4</th>
<th>pH 5</th>
<th>pH 7</th>
<th>pH 9</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>VDVY</em></td>
<td>+4.5</td>
<td>+4.1</td>
<td>+3.9</td>
<td>+3.1</td>
</tr>
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<td>*ARVA</td>
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<td>+4</td>
<td>+3.9</td>
<td>+3.1</td>
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<tr>
<td>ARYV</td>
<td>+2</td>
<td>+2</td>
<td>+1.9</td>
<td>+1.1</td>
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CAMP molecules are known to interact initially with the anionic phospholipids and phosphomannose found in Gram-positive organisms and plants, which are composed mostly of zwitterionic lipids. Both cell wall outer layers contain charged molecules that could potentially influence CAMP binding (43, 44). In fact, the charged molecules of the Gram-negative LPS and PG layers typically found in E. coli and P. aeruginosa do not have groups with pK\textsubscript{a}s that play a substantial role over the pH range used in our studies. The pK\textsubscript{a} value for most membrane lipid phosphates, for example, is typically in the range of 1 to 2 and thus would not titrate in the pH range of our assays. Thus, we attribute the observed pH effects on MSC values against C. albicans, E. coli, and P. aeruginosa predominately to changes in peptide net charge and observe no complicating factors due to changes in the net charge of cell wall molecules.

The data obtained for the Gram-positive S. aureus are among the most intriguing in our current studies. In Fig. 4 (see also Tables 1 and 2), the pH trend observed for MSC values of all three CAMPs is opposite of that for the Gram-negative organisms and C. albicans. This systematic trend has not been reported for CAMPs in the literature, although in one study (24), hBD2 was found to kill S. aureus more effectively than crotamine at pH 7.5 as opposed to pH 5.5 in a radial diffusion assay. In our studies, despite the decrease in net charge of the peptides with increasing pH (Table 3), which severely affected the performance of the CAMPs against the other three organisms, higher pH allows the CAMPs to kill Staphylococcus much better than at lower pH, and, remarkably, at MSC values comparable to those for the Gram-negative organisms at low pH. In addition, at low pH, where the peptides have their maximum positive charge and kill the other three organisms most effectively, they have their lowest activity (highest MSC values) versus S. aureus and in many cases are completely inactive (see Table 1 and 2) at the highest concentrations used in these studies.

Our results for S. aureus suggest that the CAMPs are able to bind to the inner plasma membrane at higher pH but are less able to do so at low pH. Gram-positive organisms such as S. aureus do not have an anionic LPS layer like the Gram-negative organisms but do have a much thicker (>300-Å) peptidoglycan (PG) layer than is typical for Gram-negative organisms (<100 Å) (45). In addition, many Gram-positive organisms, including S. aureus, contain large amounts of anionic teichoic acids (TA) in their PG layer (46, 47). The TAs contain ribitol phosphates, which give the

![Figure 7](http://aac.asm.org/)

**Figure 7** MSC values as a function of the net charge on the antimicrobial peptide are plotted for data collected using P. aeruginosa and E. coli. Data are shown for *VDVY* (black circles) and *ARVA* (light-gray circles) against P. aeruginosa and for *VDVY* (light-gray triangles) and *ARVA* (dark-gray triangles) against E. coli with trend lines and standard errors.

Study were all amidated at the C terminus and contain one or more arginines as their primary source of positive charge. Arginine has a pK\textsubscript{a} near 12 and thus would remain positively charged at all pH values used in our studies. This leaves the N terminus of each peptide (pK\textsubscript{a} 8) and the sole aspartic acid residue (pK\textsubscript{a} 4) found in peptide *VDVY* as the functional groups most affected by changes in pH. Both *VDVY* and *ARVA* lose about 25% of their positive net charge at pH 9 compared to that at pH 4, and both have noticeably higher (3 to 5×) MSC values (Table 1) against the Gram-negative organisms at pH 9 than at pH 4. ARYV, on the other hand, loses nearly half of its positive charge at pH 9 and is ineffective (MSC > 10 μM) in our assays at pH values above pH 7 (see Tables 1 and 2). In addition, we found that the MSC values for *VDVY* and *ARVA* against E. coli and P. aeruginosa (Fig. 7) exhibit a linear relationship with net charge. The interpretation of the pH behavior of our CAMPs versus Gram-negative organisms and C. albicans is straightforward: the increased net charge on the peptide enhances the electrostatic attraction between the CAMP and the anionic microbial membranes. This is consistent with other observations that increasing the number or clustering of positive charges by changing the amino acid sequence can affect the activity of CAMPs (9, 37, 41).

Candida albicans possesses both an outer cell wall and an inner plasma membrane. The cell wall contains, in addition to polysaccharides, a high content of mannoprotein containing phosphomannose residues which give the cell wall the anionic character necessary to allow C. albicans to be a target for CAMPs (42). The plasma membrane of C. albicans is also anionic, unlike the plasma membranes in cells from multicellular eukaryotes (e.g., animals and plants), which are composed mostly of zwitterionic lipids. The phospholipids and phosphomannose found in C. albicans cannot be protonated over the range of pH used in our studies. The binding event in this case is moderated mainly by the net charge on the peptide and the ionic strength of the solution. The situation is more complicated for Gram-negative organisms due to the presence of both an outer lipopolysaccharide (LPS) layer and a thin peptidoglycan (PG) layer external to the plasma membrane (43). Both cell wall outer layers contain charged molecules that could potentially influence CAMP binding (43, 44). In fact, CAMP molecules are known to interact initially with the anionic LPS layer and in some cases to initiate a process known as self-promoted uptake that often involves distortion of the LPS layer as the CAMPs translocate to the interior of the bacterial cell wall to bind to the anionic prokaryotic plasma membrane (4, 10, 16, 17). The charged molecules of the Gram-negative LPS and PG layers typically found in E. coli and P. aeruginosa are among the most intriguing in our current studies. The pK\textsubscript{a} values for most membrane lipid phosphates, for example, is typically in the range of 1 to 2 and thus would not titrate in the pH range of our assays. Thus, we attribute the observed pH effects on MSC values against C. albicans, E. coli, and P. aeruginosa predominately to changes in peptide net charge and observe no complicating factors due to changes in the net charge of cell wall molecules.

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PG layer of Gram-positive organisms a net negative charge. In a number of Gram-positive species, particularly low-GC species such as S. aureus, the ribitol alcohol groups are esterified to D-Ala residues containing free amino groups (46, 48). We postulate that the positive charges from the amino groups of D-Ala cause the net negative charge on the PG layer of S. aureus to be reduced so that the PG layer is only slightly negative at neutral pH in our studies. In contrast, the thin PG layer of E. coli, for example, is much more negatively charged due to the presence of diaminopimelic acid (DAP) and glutamic acid (45).

Studies have shown that S. aureus with reduced levels of D-Ala in their teichoic acids exhibit an increased sensitivity to CAMPs as well as a reduced rate of sepsis (45, 46, 49). In fact, it has been proposed that one of the main reasons for esterification with D-Ala may be to make CAMPs less effective. Thus, our proposed explanation for the pH behavior observed with S. aureus is as follows. At low pH, the PG layer of S. aureus becomes neutral or even slightly positively charged, causing the CAMPs, despite being more positively charged themselves, to be repelled or have greatly lowered attraction to the PG layer. At high pH, on the other hand, the PG layer actually becomes much more negatively charged as the amino groups of the D-Ala (pKₐ 8.4) (50) become deprotonated, allowing the CAMPs to bind and transit the porous PG layer to kill with a low MSC value, despite the lowered positive charge on the peptides themselves. ARYY with its small net charge at pH 9 (Table 3) is an exception, having a relatively high MSC value near 6 μM. Another factor that could contribute to increased anionic character of the PG layer at high pH is ester hydrolysis, as some D-alanyl esters in TAs are labile (48), with a half-life (t½) of 3.9 h (37°C, pH 8). Variation of the initial incubation period between 30 min and 1 h, however, did not reveal significant differences in MSC values. Intriguingly, the ovine-derived cathelicidins (40) were found to kill the O157:H7 human pathogen strain of E. coli better at pH 7 to 9 than at pH 5 or 6, corresponding to the pattern we observe with S. aureus rather than with E. coli. The likely explanation of this result is that O157:H7 is known to have an LPS layer that has been modified by the addition of free-amino-containing groups, such as glucosamine and ethanolamine (51–53), which would exhibit pH behavior similar to the D-Ala residues in S. aureus. Additional examples of pathogenic organisms altering the net charge of their cell walls to decrease the effectiveness of CAMPs may be observed. Varying the pH from neutrality may increase the effectiveness of CAMPs in such cases.

Our results on the effect of environmental factors such as pH and ionic strength on the effectiveness of CAMPs highlight the importance of the initial binding step on the activity of the peptides under a variety of conditions. This suggests that increasing the percent peptide bound at the outer cell wall is a potential strategy to improve the efficacy of CAMPs rather than focusing only on the plasma membrane. Our results for S. aureus suggest the possibility of manipulating the pH in a species- or strain-dependent fashion as a way of improving CAMP performance in a clinical setting. The observations we report here with regard to the effect of high pH on the activity of our CAMPs, especially the novel pH dependence against Gram-positive versus Gram-negative organisms, suggests a role for charged molecules other than the usual suspects in CAMP binding. Electrostatic interactions between antimicrobial peptides and a variety of charged molecules on microbial membranes and cell walls may play a critical role in antimicrobial activity. These interactions must be taken into account when designing the next generation of cationic antimicrobial peptides.

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