BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **244,** 253–257 (1998)
ARTICLE NO. RC988159

Mechanism of Action of the Antimicrobial Peptide Buforin II: Buforin II Kills Microorganisms by Penetrating the Cell Membrane and Inhibiting Cellular Functions

Chan Bae Park, Hun Sik Kim, and Sun Chang Kim[1]

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1 Yusong-gu Kusong-dong, Taejeon 305-701, Korea

Received January 6, 1998

The mechanism of action of buforin II, which is a 21-amino acid peptide with a potent antimicrobial activity against a broad range of microorganisms, was studied using fluorescein isothiocyanate (FITC)-labeled buforin II and a gel-retardation experiment. Its mechanism of action was compared with that of the well-characterized magainin 2, which has a pore-forming activity on the cell membrane. Buforin II killed *Escherichia coli* without lysing the cell membrane even at 5 times minimal inhibitory concentration (MIC) at which buforin II reduced the viable cell numbers by 6 orders of magnitude. However, magainin 2 lysed the cell to death under the same condition. FITC-labeled buforin II was found to penetrate the cell membrane and accumulate inside *E. coli* even below its MIC, whereas FITC-labeled magainin 2 remained outside or on the cell wall even at its MIC. The gel-retardation experiment showed that buforin II bound to DNA and RNA of the cells over 20 times strongly than magainin 2. All these results indicate that buforin II inhibits the cellular functions by binding to DNA and RNA of cells after penetrating the cell membranes, resulting in the rapid cell death, which is quite different from that of magainin 2 even though they are structurally similar: a linear amphipathic α-helical peptide.

Antimicrobial peptides have received increasing attention in recent years as their contribution to host defense mechanisms in the animal kingdom is gradually becoming appreciated (1). More than 2,000 antimicrobial peptides have been isolated from diverse biological sources, including amphibians (2, 3), insects (4), mammals (5), fish (6), plants (7) and prokaryotes (8). These peptides possess antimicrobial activities against bacteria, fungi and enveloped viruses with little or no cytolytic activity (9, 10). In most cases, these peptides have the common features of being highly basic, due to the presence of multiple arginine and lysine residues, and of forming amphipathic structures (11). The amphipathic nature of these peptides presumably underlies their biological activities which enables them to associate with lipid membranes and disrupt normal membrane functions. The mechanism of action has been investigated for many of antimicrobial peptides, including cecropin A (12, 13), several defensins (14) and magainins (15, 16). The killing mechanism found for most peptides investigated consists of attacks on the outer and inner membranes, ultimately resulting in lysis of the bacteria. Channel formation in artificial membranes was demonstrated for cecropins (17), defensins (18) and magainins (19, 20). Proline-arginine-rich peptides, however, act differently, because the high concentration of proline is incompatible with amphipathic structure formation. Indeed, PR-39 does not have a pore-forming activity but does inhibit the macromolecular synthesis (21). Therefore, there is still considerable debate on the precise mechanism.

Recently a novel 39-amino acid residue peptide (termed buforin I) was isolated from the stomach tissue of the Asian toad, *Bufo bufo garagriozans*, and a more potent buforin II consisting of 21 amino acids was derived from buforin I (3). Buforin II showed a strong antimicrobial activity against a broad spectrum of microorganism, including Gram-positive and Gram-negative bacteria, as well as fungi without any significant hemolytic activity (3). The structure study of buforin II using nuclear magnetic resonance showed that the peptide adopted an amphipathic helix in a hydrophobic environment (22). The structure of buforin II predicted

[1] Corresponding author. Mailing address: Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1 Yusong-gu Kusong-dong, Taejeon 305-701, Korea. Fax: 82-42-869-2610. E-mail: sckim@sorak.kaist.ac.kr.

0006-291X/98 $25.00

a membrane-active function. In this study, we investigated the mechanism of action of buforin II and compared it with the membrane-active antimicrobial peptide, magainin 2.

MATERIALS AND METHODS

Materials. Fluorescein isothiocyanate (FITC) was purchased from Calbiochem-Novabiochem Corp. (San Diego, CA) and magainin 2 from Sigma (St. Louis, MA). High-performance-liquid-chromatography (HPLC) grade acetonitrile was obtained from Merck (Rahway, NJ). All other reagents were of analytical grade.

Peptide synthesis. Buforin II was synthesized by the solid-phase synthesis method on a Milligen 9050 Pepsynthesizer according to fluoren-9-methyloxycarbony (Fmoc)-polypeptide active ester chemistry. The synthesized peptide was purified by C18 reverse-phase HPLC and the purity was confirmed by amino acid analysis and matrix associated laser desorption ionization (MALDI) mass spectroscopy.

Antimicrobial assays. The antimicrobial activity of the peptides against *Escherichia coli* was determined as described by Park *et al.* (3). 20 ml culture of *E. coli* cells in the mid-logarithmic phase was washed with cold 10 mM sodium phosphate buffer, pH 7.4 (NAPB) and resuspended in 10 ml of cold NAPB. A volume containing 1×10^6 bacterial CFU was added to 6 ml of underlayer agar [10 mM sodium phosphate, 1% (v/v) trypticase soy broth (TSB), 1% agarose, pH 6.5] and the mixture was poured into a Petri dish. Samples were added directly to the 3-mm wells made on the solidified underlayer agar. After incubation at 37 °C for 3 h, the underlayer agar was covered with nutrient-rich top agar overlay and incubated overnight at 37 °C. The antimicrobial activity was determined by observing the zone of suppression of bacterial growth around the 3-mm wells. The lowest concentration of antimicrobial peptide, which showed visible suppression of growth, was defined as the minimal inhibitory concentration (MIC). The bactericidal and lytic activities of the antimicrobial peptides were assessed against *E. coli* as described by Boman *et al.* (21). 150 μl of 10^7 CFU/ml of *E. coli* cells in the mid-logarithmic phase was mixed with the same volume of 10 mM NAPB containing 40 μg/ml buforin II or 500 μg/ml magainin 2, and incubated at 37 °C. Aliquots (5 μl) were withdrawn at time intervals, adequately diluted, plated on a trypticase soy agar (TSA) plate and then the colonies were counted after incubation overnight at 37 °C. The lysis of cell was assessed by measuring the optical density of the remaining culture at 630 nm using a spectrophotometer (BioRad, CA).

FITC-labeling of peptides. Two milligram of each peptide was dissolved in 1 ml of 0.02 M Na_2CO_3-$NaHCO_3$ buffer, pH 9.1, containing 0.02 M NaCl. To the peptide solution, 500 μl of 1% (w/v) FITC dissolved in acetone was added, and the reaction mixture was left at 25 °C for 2 h. The reaction was terminated by rapid passage of the reaction mixture through a 2×30 cm column of Sephadex G-10, which was equilibrated with isotonic phosphate-buffered saline, pH 7.4. After gel permeation chromatography, the active eluates were further purified by C18 reverse-phase HPLC. The purified FITC-labeled peptides were dried under vacuum and then resuspended in water. Their concentrations were determined by amino acid analysis and their purity was assessed by MALDI mass spectroscopy.

Confocal laser scanning microscopy. *E. coli* cells were grown overnight on a TSA plate at 37 °C, then collected from the plate with a sterile plate loop and suspended in 20 ml of TSB. After incubation at 37 °C for 6 h, 20 ml of *E. coli* cells in the mid-logarithmic phase was harvested by centrifugation, washed with 10 mM NAPB and resuspended in 10 ml of the same buffer. To immobilize *E. coli* cells on a glass slide, a poly-L-lysine coated glass slide was immersed in 10 mM NAPB, containing 10^7 CFU/ml of *E. coli*. After 30 min, the slide was rinsed with 10 mM NAPB. Adequately diluted FITC-labeled antimicrobial peptides were added to the slide glass and laser scanning confocal microscopy was carried out to examine the interaction between FITC-labeled antimicrobial peptides and cell walls with a Zeiss Axiovert 135M confocal microscope (Zeiss, Germany) equipped with a laser. Fluorescent images were obtained with a 488 nm bandpass filter for excitation of FITC. Software merging of images was carried out using the COMOS software (Zeiss, Germany). Images were recorded using a Screenstar film recorder (Zeiss, Germany).

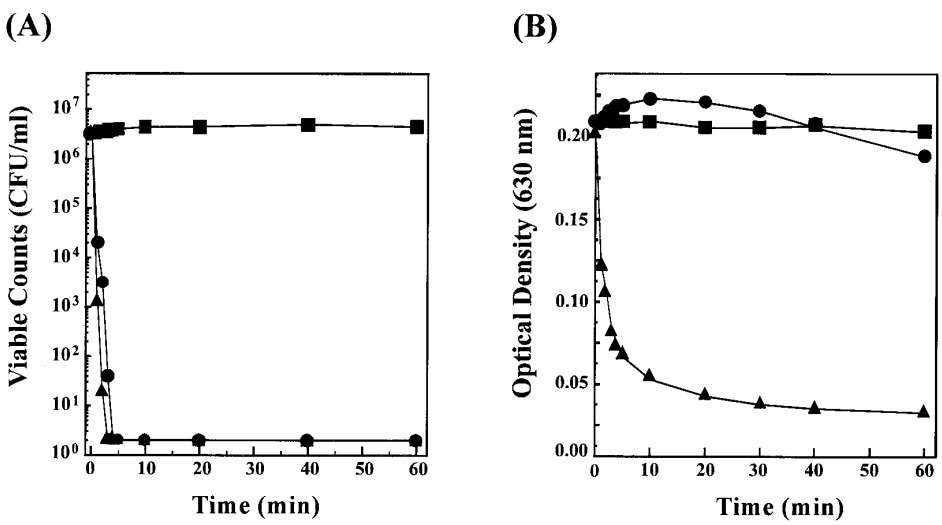

FIG. 1. Comparison of the bactericidal and lytic activity of buforin II with that of magainin 2. 150 μl of 10^7 CFU/ml of *E. coli* cells was incubated at 37 °C with the same volume of each antimicrobial peptide dissolved in 10 mM NAPB: ■, water; ●, 40 μg/ml of buforin II; ▲, 500 μg/ml of magainin 2. (A) Aliquots were removed at time intervals, diluted and plated on a TSA plate for the viable cell counts. (B) The lysis of cell was assessed by measuring the optical density of the remaining mixture at 630 nm.

DNA/RNA binding assay. Gel-retardation experiments were performed by mixing 100 ng of the plasmid DNA (pBluscriptII SK^+) or 2 μg of the yeast total RNA with increasing the amount of peptides in 20 μl of binding buffer [5% glycerol, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1mM DTT, 20 mM KCl and 50 μg/ml BSA]. The reaction mixtures were incubated at room temperature for 1 h. Subsequently, 4 μl of native loading buffer was added [10% Ficoll 400, 10 mM Tris-HCl (pH 7.5), 50 mM EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol] and an aliquot of 12 μl was applied to a 1% agarose gel electrophoresis in 0.5×Tris borate-EDTA buffer. The plasmid DNA used in this experiment was purified by ultracentrifugation in CsCl gradients to select the closed circular DNA. Yeast total RNA was prepared by the guanidium thiocyanate method (23) and resuspended in diethyl pyrocarbonate (DEPC)-treated water.

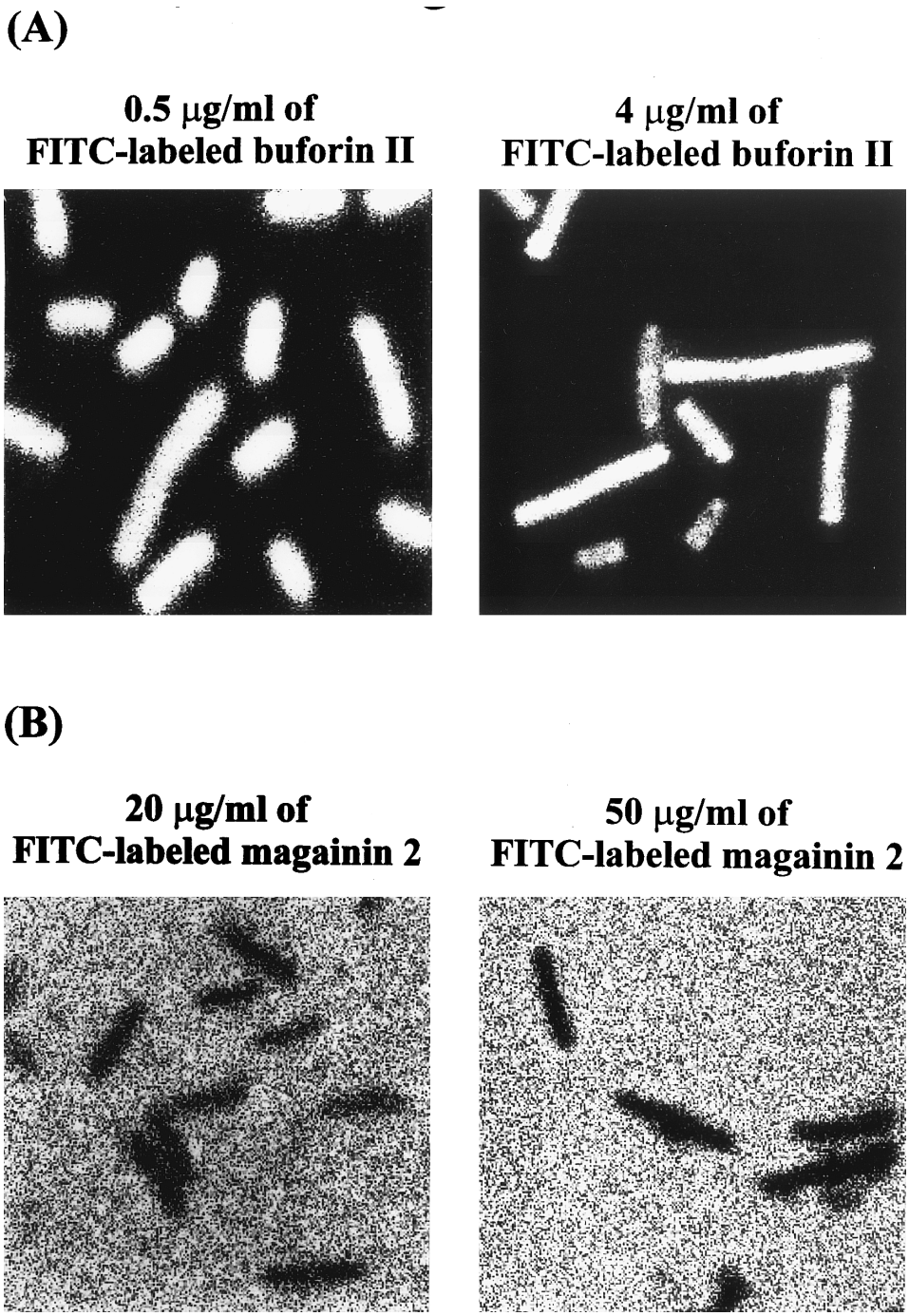

FIG. 2. Confocal microscopic images of *E. coli* reacted with FITC-labeled peptides. *E. coli* cells in the mid-logarithmic phase were washed and diluted to 10^6 CFU/ml in 10 mM NAPB. Cells were immobilized on a glass slide. The immobilized cell was then reacted with various amounts of FITC-labeled buforin II (A) and FITC-labeled magainin 2 (B).

RESULTS

Bactericidal activity and cell lysis. The mechanism of action of buforin II was studied by examining its bactericidal and cell lytic activity against *E. coli.* To compare its mechanism of action with that of a membrane-active antimicrobial peptide, the same experiment was performed with magainin 2. The bactericidal and cell lytic activities of buforin II and magainin 2 against *E. coli* are shown in Fig. 1. The optical density of the cells at 630 nm decreased rapidly upon addition of 250 μg/ml (5×MIC) of magainin 2 and the number of viable cells dropped by 6 orders of magnitude. However, when the cells were treated with 20 μg/ml (5×MIC) of buforin II, there was no change in optical density at 630 nm even though the number of viable cells decreased by 6 orders of magnitude. These results indicate that both antimicrobial peptides have the bactericidal activity but with a different killing mechanism of action. It seems that buforin II kills the bacteria without lysing the cells, but magainin 2 lyses the cell to death.

Confocal laser scanning microscopy. To examine the target sites of buforin II and magainin 2 in *E. coli,* they were labeled with FITC and visualized under the confocal microscopy. The FITC-labeled peptides, purified using gel-permeation column and C18 reverse-phase HPLC, were about 95% homogeneous by MALDI mass spectroscopy (data not shown). Labeling buforin II and magainin 2 with FITC did not give any affect on the antimicrobial activity of buforin II and magainin 2. FITC-labeled buforin II penetrated the cell membrane and accumulated in the cytoplasm of the cell immediately after addition to the cells below or at its MIC (Fig. 2A), whereas FITC-labeled magainin 2 remained on the cell wall under the same condition (Fig. 2B). These results indicate that the major working place of buforin II is the cytoplasm of the cell, not the membrane.

DNA/RNA binding. In an attempt to clarify the molecular mechanism of action, we examined the binding properties exerted by buforin II and magainin 2 on DNA or RNA. The DNA or RNA binding abilities of buforin II and magainin 2 were examined by analyzing the electrophoretic mobility of DNA or RNA bands at the various weight ratios of peptides to DNA or RNA on an agarose gel (1%, w/v). Buforin II inhibited the migration of DNA and RNA above the weight ratio of 4.0 (Fig. 3A) and 0.2 (Fig. 3B), respectively, and magainin 2 suppressed the migration of DNA and RNA above the weight ratio of 100 and 5.0, respectively. This result indicates that buforin II binds to DNA and RNA at least over 20 times tightly than magainin 2.

DISCUSSION

The mechanism of action of the antimicrobial peptide buforin II was studied against *E. coli* in this study.

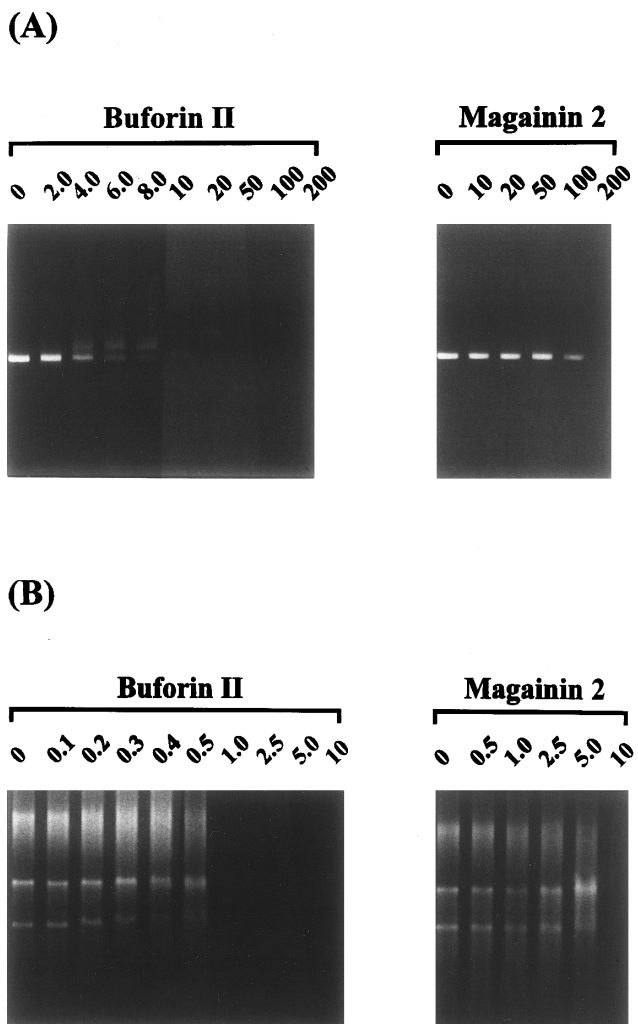

FIG. 3. Gel retardation assays. Binding was assayed by the inhibitory effect of peptides on the migration of DNA or RNA. Various amounts of peptides were incubated with 100 ng of plasmid DNA (A) or 2 μg of yeast RNA (B) at room temperature for 1 h and the reaction mixtures were applied to a 1% agarose gel electrophoresis. The weight ratio (peptide:DNA) is indicated above each lane.

Structurally buforin II belongs to one major group of antimicrobial peptides: a linear amphipathic α-helical peptide without cysteine. The members of this group include cecropins (13), magainins (24) and dermaseptin (25). Recent studies on several antimicrobial peptides of this family suggested that the peptides killed microorganisms either by forming pores and increasing the permeability (26) or by disintegration of the cell membrane (27). In our experiment, we found that magainin 2 killed bacteria by a rapid lysis of cells and the FITC-labeled magainin 2 did not penetrate the cell membrane, which was in good agreement with the results of the earlier studies (20). However, despite the structural similarity to magainin 2, buforin II showed a rapid killing of bacteria without lysing the cells and the

FITC-labeled buforin II penetrated the cell membrane even below its MIC. Thus it seems that the target site of buforin II is the cytoplasm of the cell. What does then buforin II do inside the cell? Recent studies suggested that several antimicrobial peptides such as PR-39 and tachyplesin I bound tightly to DNA or RNA and inhibited the macromolecular synthesis of the cell (28, 21). PR-39, the proline-arginine-rich peptide, is known to stop the synthesis of protein or DNA after penetrating the cytoplasm of the cell (21). Buforin II may kill microorganisms in a way similar to that of PR-39. However, there were some differences. PR-39 requires a lag period to penetrate the outer membrane of bacteria (21). However, buforin II showed a rapid killing of the bacteria without a lag period. Furthermore, the voltage-dependent channel formation in artificial membranes was also observed for buforin II (unpublished data). In the gel-retardation experiment, it was confirmed that buforin II bound to DNA and RNA with over 20 times higher affinity than magainin 2. Although a detailed experiment on the interaction between buforin II and nucleic acids *in vivo* is needed, the present results lead us to believe that buforin II inhibits the cellular functions by binding to DNA and RNA of cells after penetrating the cell membranes, resulting in the rapid cell death.

ACKNOWLEDGMENTS

This work was partially supported by the grants from the Korea Science and Engineering Foundation (KOSEF) through Research Center for New Bio-Materials in Agriculture at Seoul National University, Health Technology Planning and Evaluation Board (HTDEB) and Ministry of Agriculture, Forestry and Fisheries-Special Grants Research Program (MAFF-SGRP).

REFERENCES

1. Merrifield, R. B., Juvvadi, P., Anreu, D., Ubach, J., Boman, A., and Boman, H. G. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3449–3453.
2. Steinborner, S. T., Waugh, R. J., Bowie, J. H., Wallace, J. C., Tyler, M. J., and Ramsay, S. L. (1997) *J. Pept. Sci.* **3,** 181–185.
3. Park, C. B., Kim, M. S., and Kim., S. C. (1996) *Biochem. Biophys. Res. Commun.* **218,** 408–413.
4. Rees, J. A., Moniatte, M., and Bulet, P. (1997) *Insect Biochem. Mol. Biol.* **27,** 1413–422.
5. Skerlavaj, B., Gennaro, R., Bagella, L., Merluzzi, L., Risso, A., and Zannetti, M. (1996) *J. Biol. Chem.* **271,** 28375–28381.
6. Park, C. B., Lee, J. H., Park, I. Y., Kim, M. S., and Kim., S. C. (1997) *FEBS Lett.* **218,** 408–413.
7. Marcus, J. P., Goulter, K. C., Green, J. L., Harrison, S. J., and Manners, J. M. (1997) *Eur. J. Biochem.* **244,** 743–749.
8. Green, G., Dicks, L. M., Bruggeman, G., Vandamme, E. J., and Chikindas, M. L. (1997) *J. Appl. Microbiol.* **83,** 127–132.
9. Nicolas, P., and Mor, A. (1995) *Annu. Rev. Microbiol.* **49,** 277–304.
10. Boman, H. G. (1995) *Annu. Rev. Immunol.* **13,** 61–92.
11. Maloy, W. L., and Kari, U. P. (1995) *Biopolymers* **35,** 105–122.
12. Silvestro, L., Gupta, K., Weiser, J. N., and Awelsen, P. H. (1997) *Biochemistry* **36,** 11452–11460.
13. Steiner, H., Hltmark, D., Engstrom A., Bennich, H., and Boman, H. G. (1991) *Nature* **292,** 246–248.
14. Wimley, W. C., Selstedt, M. E., and White, S. H. (1997) *Protein Sci.* **3,** 1362–1373.
15. Westerhoff, H. V., Juretic, D., Hendler, R. W., and Zasloff, M. (1989) *Biochim. Biophys. Acta* **975,** 361–369.
16. Matsuzaki, K., Sugishita, K., Harada, M., Fujii, N., and Miyajima, K. (1997) *Biochim. Biophys. Acta* **1327,** 119–130.
17. Bechinger, B. (1997) *J. Membr. Biol.* **156,** 197–211.
18. Maget-Dana, R., and Ptak, M. (1997) *Biophys. J.* **73,** 2527–2533.
19. Cruciani, R. A., Barker, J. L., Zasloff, M., Chen, H. C., and Colamonici, O. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3792–3796.
20. Matsuzaki, K., Murase, O., Fujii, N., and Miyajima, K. (1995) *Biochemistry* **30,** 6521–6526.
21. Boman, H. G., Agerberth, B., and Boman, A. (1993) *Infect. Immun.* **61,** 2978–2984.
22. Yi, G., Park, C. B., Kim, S. C., and Cheong, C. (1996) *FEBS Lett.* **398,** 87–90.
23. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
24. Zasloff, M. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 5449–5453.
25. Mor, A., Nguyen, V. H., Delfour, A., Migliore-Samour, D., and Nicolas, P. (1991) *Biochemistry* **30,** 8824–8830.
26. Cruciani, R. A., Barker, J. L., Durell, S. R., Raghunathan, G., Guy, H. R., Zasloff, M., and Stanley, E. F. (1992) *Eur. J. Pharmacol.* **226,** 287–296.
27. Oren, Z., and Shai, Y. (1996) *Eur. J. Biochem.* **237,** 303–310.
28. Yonezawa, A., Kuwahara, J., Fujii, N., and Sugiura, Y. (1992) *Biochemistry* **31,** 2998–3004.