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Inactivation of viruses infecting ectothermic animals by amphibian and piscine antimicrobial peptides

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Abstract

The ability of five purified amphibian antimicrobial peptides (dermaseptin-1, temporin A, magainin I, and II, PGLa), crude peptide fractions isolated from the skin of *Rana pipiens* and *R. catesbeiana*, and four antimicrobial peptides (AMPs) from hybrid striped bass (piscidin-1N, -1H, -2, and -3) were examined for their ability to reduce the infectivity of channel catfish virus (CCV) and frog virus 3 (FV3). All compounds, with the exception of magainin I, markedly reduced the infectivity of CCV. In contrast to CCV, FV3 was 2- to 4-fold less sensitive to these agents. Similar to an earlier study employing two other amphibian peptides, the agents used here acted rapidly and over a wide, physiologically relevant, temperature range to reduce virus infectivity. These results extend our previous findings and strongly suggest that various amphibian and piscine AMPs may play important roles in protecting fish and amphibians from pathogenic viruses.

Keywords: Antimicrobial peptides; Channel catfish virus; Herpesvirus; Frog virus 3; Iridovirus; Ectothermic organisms; Amphibian decline; Temporin; Dermaseptin; Magainin; Piscidins; Ranatuerin

Introduction

Antimicrobial peptides (AMPs) are low-molecular-weight molecules (approximately 1–5 kDa) possessing potent inhibitory activity against a wide range of bacteria, fungi, and viruses. To date, more than 750 AMPs have been isolated from a phylogenetically diverse collection of organisms including mammals, fish, amphibians, and insects (Boman, 1995; Brogden et al., 2003; Hancock and Chapple, 1999; Lehrer et al., 1993; Nicolas and Mor, 1995; Tossi; Zasloff, 2002). The majority of AMPs are cationic, amphipathic molecules, which can be loosely grouped into categories that reflect their primary and secondary structure (Zasloff, 2002): alpha helical peptides (e.g., cecropins); proline- (e.g., bactenecins), glycine- (e.g., attacins), or histidine-rich (e.g., histatins) peptides, and peptides with one (e.g., ranatuerins) or more (e.g., protegrins and defensins) disulphide bonds. In contrast, there are smaller groups of AMPs composed of anionic peptides, aromatic dipeptides, processed forms of oxygen-binding proteins, and peptides derived from neuropeptide precursors (Brogden et al., 2003; Salzet, 2002; Stefano et al., 1998). For example, enkelytin, a 31-amino-acid peptide containing seven glutamic acid residues, is generated by cleavage of proenkephalin-A (Goumon et al., 1998), and three ovine heptapeptides were identified that contain six to seven aspartic acid residues (Brogden et al., 1996). Among the larger class of cationic AMPs, the principal structural feature common to all is their ability to adopt an amphipathic configuration in which clusters of hydrophobic and cationic amino acids are spatially organized such that the molecule possesses discrete hydrophobic and hydrophilic faces (Oren and Shai, 1998). It is thought that AMPs exert their antimicrobial effects by interacting with negatively charged phospholipid headgroups present on the outermost leaflet of bacterial mem-

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branes via their cationic (hydrophilic) surfaces. This interaction leads to lipid displacement, changes in membrane structure, and, in some cases, entry of the peptide into the interior of the bacterial cell (Oren and Shai, 1998; Zasloff, 2002; Zhao et al., 2002). Although the precise mechanism is unclear, killing is thought to involve one or more of the following: fatal membrane depolarization, membrane pore formation leading to loss of intracellular contents, induction of hydrolases, disturbance of membrane function, and specific damage to critical intracellular targets. In contrast to the deleterious effect of AMPs on bacterial membranes, eukaryotic cells are relatively resistant to destabilization due to the presence of cholesterol within the plasma membrane and by the absence of negatively charged phospholipids on the outermost leaflet of the plasma membrane (Lichtenstein, 1991; Oren and Shai, 1998).

Although it has been known for some time that AMPs show broad antibacterial and antifungal activity in vitro, recent in vivo studies support a role for AMPs in protection from overt disease. For example, mice expressing human defensin 5 survived infection with *Salmonella typhimurium*, whereas normal mice lacking this AMP succumbed to infection within 2 days (Salzman et al., 2003). In another study, mice with a disruption within the gene encoding the AMP cathelicidin developed persistent infections following exposure to β-hemolytic Group A *Streptococcus*, whereas wild-type mice were resistant to infection (Nizet et al., 2001). In addition to playing a direct role in pathogen destruction, AMPs are also thought to enhance immune defenses by modulating innate and acquired immune responses (Salzet, 2002; Stefano et al., 1998; Yang et al., 2002). Thus, the dual role of AMPs as effectors of innate immunity and inducers of both innate and adaptive responses may place AMPs at the center of immune defense strategies.

Although most studies to date have focused on protection against bacterial and fungal pathogens, AMPs have also been shown to be effective against viral pathogens (Daher et al., 1986; Lehrer et al., 1985; Yasin et al., 2000; Zhang et al., 2002). The defensin human neutrophil peptide -1 (HNP-1) inactivated enveloped viruses such as herpes simplex virus types 1 and 2 (HSV-1, -2), vesicular stomatits virus (VSV), and influenza A virus, but had little to no effect against another herpesvirus, cytomegalovirus (CMV), as well as two non-enveloped viruses, ECHO virus 11 and reovirus type 3 (Daher et al., 1986). In a similar manner, two additional defensins, rabbit lung macrophage peptides MCP-1 and MCP-2, demonstrated in vitro neutralizing activity against HSV-2, VSV, and influenza A, but showed little activity against CMV, reovirus type 3, or ECHO virus 11 (Lehrer et al., 1985). Interestingly, several similar peptides were markedly less effective against HSV-1 indicating that small differences in the primary structure of the AMP may produce major differences in antiviral activity (Lehrer et al., 1985). Recently human α-defensins -1, -2, and -3 were suggested to possess anti-HIV-1 activity and to account for some, but not all, of the non-lytic, anti-HIV activity of CD8 T cells (Chang et al., 2003; Zhang et al., 2002). These results indicate that although the degree of viral inactivation varies markedly with the type of AMP and the species of virus, AMPs likely play an important role in antiviral immunity.

Although AMPs were first isolated from amphibian skin over 30 years ago (Csordas and Michl, 1970), it was not until recently that the effects of amphibian AMPs have been measured against amphibian pathogens. Recent studies showed that a panel of amphibian AMPs (magainin -I and -II, CPF, PGLa, dermaseptin, and ranalexin) inhibited the growth of the fungi *Batrachochytrium dendrobatidis* and *Basidiobolus ranarum*, although having no effect on the bacterium *Aeromonas hydrophilia* (Rollins-Smith et al., 2002b). Other peptides from North American ranid frogs and the European frog *Rana temporaria* were also shown to inhibit the growth of *B. dendrobatidis* (Rollins-Smith et al., 2002a, 2002c, 2003). These results are ecologically significant because *B. dendrobatidis* is the fungal agent recently linked to the global decline of amphibians (Daszak et al., 1999). In addition to amphibians, AMPs have also been detected in several species of fish (Cole et al., 1997; Fernandes et al., 2004; Noga and Silphaduang, 2003; Oren and Shai, 1996; Silphaduang and Noga, 2001; Smith et al., 2000), for example, winter flounder *Pleuronectes americanus* (pleurocidin), rainbow trout *Oncorhynchus mykiss* (an unnamed 3-kDa cationic peptide and a 60-residue cleavage product of histone H1 termed oncorhyncin II), Moses sole *Pardachirus marmoratus* (pardaxin), and hybrid striped bass *Marone saxatilis* × *M. chrysops* (piscidin). As with amphibian AMPs, fish AMPs inactivated many mammalian and fish bacterial pathogens including *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphlycoccus aureus*, *A. hydrophilia*, *A. salmonicida*, and *Streptococcus iniae* (Cole et al., 1997; Oren and Shai, 1996; Silphaduang and Noga, 2001; Smith et al., 2000).

Given the potential importance of AMPs to antiviral immunity, we examined the ability of a panel of amphibian and piscine AMPs to inactivate two viral pathogens of ectothermic animals, channel catfish virus (CCV, family *Herpesviridae*, genus unassigned, Hanson, 2002) and frog virus 3 (FV3, family *Iridoviridae*, genus *Ranavirus*, Chinchar, 2002). Our results indicate that amphibian and piscine AMPs inactivate both pathogens, albeit with different efficiencies. Moreover, inactivation is rapid and occurs, in contrast to mammalian AMPs, over a wide temperature range. These results suggest that AMP-mediated viral inactivation may play an important part in protection from viral disease.

Results

Antiviral activity of single amphibian peptides

In initial experiments, we examined the ability of seven AMPs, originally isolated from amphibians, to inactivate CCV, an enveloped virus that is a major pathogen of

fingerling channel catfish (Hanson, 2002). Figs. 1 and 2 show the effects of varying concentrations of four amphibian AMPs (dermaseptin, temporin A, PGLa, and magainin II) on CCV infectivity. These results, summarized in Table 2, indicate that dermaseptin ($[i]_{50}$ = 3 μM) and temporin A ($[i]_{50}$ = 15 μM) were more effective than PGLa ($[i]_{50}$ = 100 μM) and magainin II ($[i]_{50}$ = 48 μM) in reducing the infectivity of CCV. Surprisingly, magainin I appeared to have only limited ability to inactivate CCV (data not shown). Based on these results, we examined the ability of dermaseptin and temporin A, the two most active compounds, to reduce the infectivity of FV3, a non-enveloped virus that is the type species of the genus *Ranavirus* and an emerging pathogen of frogs (Chinchar, 2002; Daszak et al., 1999). As shown in Fig. 1, both dermaseptin and temporin A reduced the infectivity of FV3, although AMP concentrations 3- to 4-fold higher than those required for CCV inactivation were needed. Because AMPs are thought to act by membrane perturbation, the higher AMP concentrations required for FV3 inactivation may reflect the fact that CCV is an enveloped virus, whereas FV3 is non-enveloped

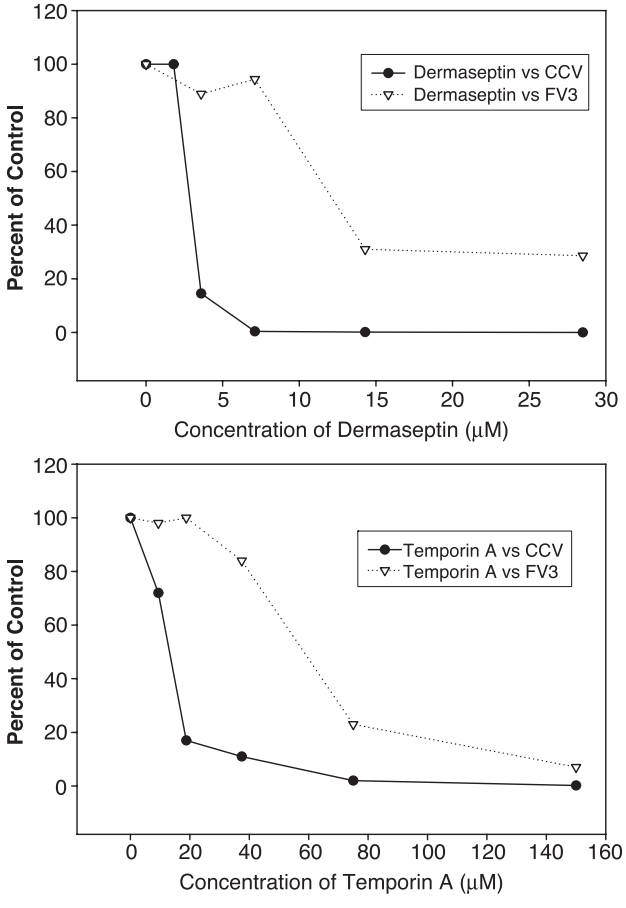

Fig. 1. Effect of dermaseptin-1 and temporin A on the infectivity of CCV and FV3. CCV and FV3 were incubated in the presence of the indicated concentrations of peptides for 60 min at 26 °C and the residual infectivity was determined by plaque assay. Upper panel: dermaseptin-1 vs. CCV and FV3; lower panel: temporin A vs. CCV and FV3. A representative experiment is shown.

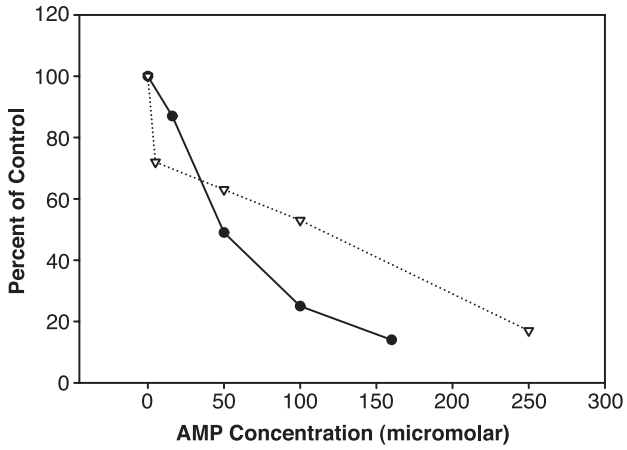

Fig. 2. Effect of magainin II and PGLa on CCV infectivity. CCV was incubated in the presence of the indicated concentrations of either magainin II (filled circles) or PGLa (open triangles) for 60 min at 26 °C and the residual infectivity determined by plaque assay. The data shown here represent the means from two to five replicative experiments.

(Chinchar et al., 2001). Finally, in addition to studies using purified AMPs, crude peptide preparations from *Rana pipiens* and *R. catesbeiana* were also shown to inactivate CCV (Fig. 3).

Synergistic effect of AMPs

Next, we determined whether synergy occurred when virions were incubated in the presence of AMPs from different structural classes. Increased inactivation was detected when CCV was treated with a combination of ranatuerin-2P and magainin II (Fig. 4), dermaseptin and ranatuerin-2P (data not shown), or dermaseptin and esculentin-2P (data not shown). However, because about the same level of inactivation was achieved by simply doubling the concentration of each individual AMP, apparently, the

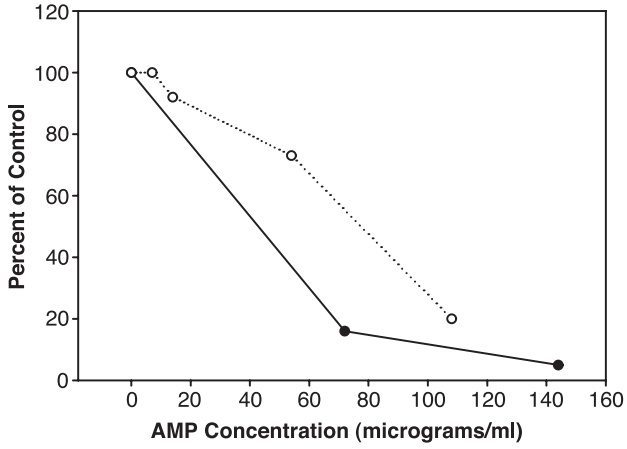

Fig. 3. Effect of a crude preparation of amphibian skin peptides on CCV infectivity. Partially purified peptides prepared from the skin of *R. catesbeiana* (open circles) and *R. pipiens* (filled circles) were monitored for their ability to inactivate CCV as described above. A representative experiment is shown.

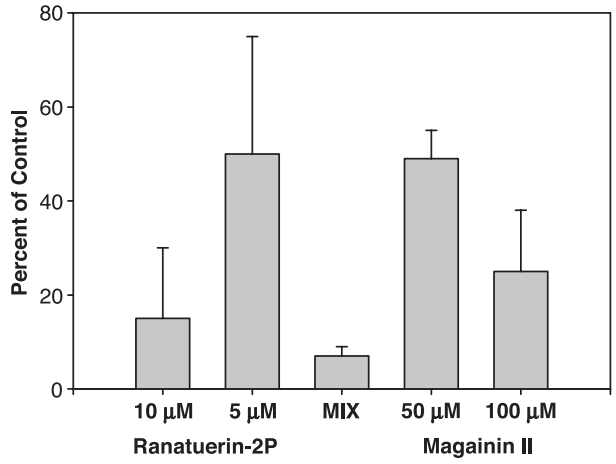

Fig. 4. Interaction between various antimicrobial peptides. Effect of ranatuerin-2P and magainin II, singly and in combination, on the infectivity of CCV. The concentration of each AMP used is indicated below the bar; "MIX" indicates 5 µM R2P and 50 µM magainin II. Shown is the mean and standard deviation of two experiments.

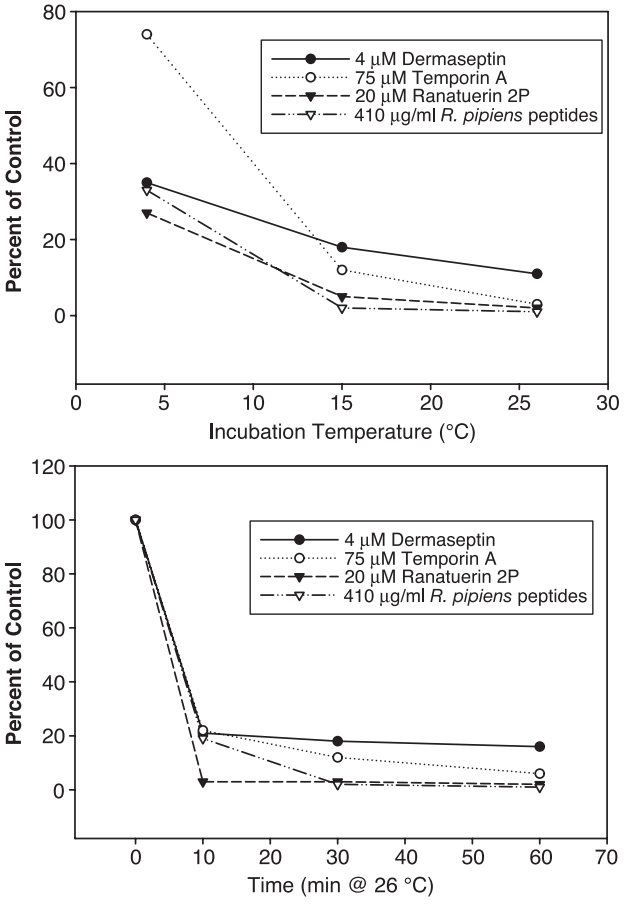

Fig. 5. Effect of temperature and time on the inactivation kinetics of various amphibian antimicrobial peptides. Upper panel: effect of temperature on the inactivation of CCV; lower panel: effect of increasing incubation time on inactivation of CCV. Concentrations of peptides were chosen which reduced CCV infectivity by >90% following a 60-min incubation at 26 °C. The means of two to four replicative experiments are shown. Sample standard deviations were generally <10%.

increased inactivation of CCV represents an additive, rather than synergistic, effect. These results are in contrast to previous findings in which combinations of magainin II and PGLa showed clear evidence of synergy when tested against *B. dendrobatidis* (Rollins-Smith et al., 2002b).

Effect of temperature on activity of AMPs

Previously, we showed that two AMPs from amphibians, ranatuerin-2P (R2P) and esculentin-2P (E2P) rapidly inhibited CCV at both "normal" environmental temperatures (26 °C) and at two lower (4 and 14 °C), but still physiologically relevant, temperatures (Chinchar et al., 2001). To determine if representative members of this new panel of AMPs behaved in a similar fashion, we monitored the ability of temporin A, dermaseptin, a crude peptide fraction from *R. pipiens*, and R2P to inactivate CCV over a range of temperatures and times. In the first series of experiments, inactivation was monitored over a range of physiologically relevant temperatures. As seen in Fig. 5 (upper panel), all four AMPs were about as active at 14 °C as they were at 26 °C. Moreover, except for temporin A, they retained considerable activity at 4 °C. In the next study, inactivation was monitored following incubation for increasing periods of time at 26 °C. Consistent with earlier results, most infectivity was lost within 10 min (Fig. 5, bottom panel) indicating that the AMPs acted rapidly to inactivate infectivity.

Antiviral activity of a novel class of fish AMPs

Lastly, we examined the impact of four piscine AMPs on CCV infectivity. The four AMPs studied were originally isolated from hybrid striped bass (*M. saxatilis* × *M. chrysops*) and shown to be effective against a variety of bacterial pathogens (Silphaduang and Noga, 2001). The four piscidins are structurally very similar to each other (Table 1). Two of them, piscidin-1N and piscidin-1H, differ only in the presence or absence of an amidated C-terminus, a feature that is found in naturally occurring

Table 1
Primary structure of antimicrobial peptides[a]

Temporin A	FLPLIG**R**VLSGIL.NH_2
Dermaseptin-1	ALW**K**TML**KK**LGTMAL**H**AG**K**AALGAAADTISQGTQ
Magainin I	GIG**K**FL**H**SAG**K**FG**K**AFVGEIM**K**S
Magainin II	GIG**K**FL**H**SA**KK**FG**K**AFVGEIMNS
PGLa	GMAS**K**AGAIAG**K**IA**K**VAL**K**AL.NH_2
Piscidin-1	FF**HH**IF**R**GIV**H**VG**K**TI**HR**LVTG[b]
Piscidin-2	FF**HH**IF**R**GIV**H**VG**K**TI**HK**LVTG
Piscidin-3	FI**HH**IF**R**GIV**H**AG**R**SIG**R**FLTG
Ranatuerin-2P	GLMDTV**K**NVA**K**NLAG**H**MLD**K**LK<u>C</u>**K**ITG<u>C</u>
Esculentin-2P	GFLSIF**R**GVA**K**FAS**K**GLG**K**DLA**R**LGVNLVA<u>C</u>**K**IS**K**Q<u>C</u>

[a] Basic amino acids are shown in bold typeface, and cysteine residues involved in disulfide bond formation are underlined.
[b] Because natural piscidins sometimes have an amidated carboxy terminus, piscidin-1 has been synthesized in both the amide (piscidin-1N) and acid (piscidin-1H) forms.

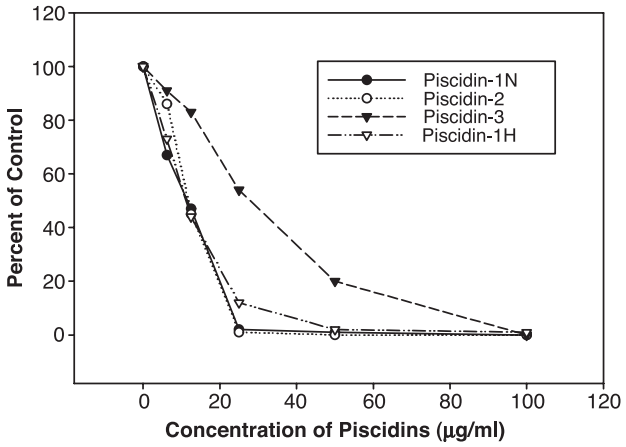

Fig. 6. Effect of piscidins on the infectivity of CCV. CCV was incubated in the presence of the indicated concentrations of piscidin-1H, -1N, -2, or -3 at 26 °C for 60 min and assayed for residual infectivity by plaque assay. The data depict the means of two to three experiments.

piscidins, and piscidins-1 and -2 differ only in an R→K substitution at amino acid position 18 (Silphaduang and Noga, 2001). Fig. 6 indicates that all four piscidins possessed marked antiviral activity and Table 2 shows that they are as active on a molar basis as dermaseptin and temporin A. In a follow up to these studies, we also examined the influence of incubation time and temperature on loss of infectivity. As with the amphibian-derived peptides, the four piscidins tested acted rapidly and over a wide temperature range (Fig. 7). Experiments monitoring the ability of piscidins to inactivate FV3 showed that, as with the above studies, FV3 inactivation took place, but that a higher concentration of piscidin was required to achieve inactivation of FV3 compared to CCV (Table 2).

Effect of AMPs on host cells

Although exhibiting marked antiviral activity, the two most active AMPs tested did not appear to be cytolytic for uninfected cells. As shown by trypan blue staining and visual inspection of cell cultures, dermaseptin-1 (0.1–11.4 μM) and piscidin-1N (0.25–25 μg/ml) did not kill CCO cells following exposure to either compound for as long as 20 h (data not shown). Interestingly, despite the apparent lack of cytolytic activity, we detected about a 20% decrease (average of four experiments) in the ability of CCO cells to metabolize MTT following a 3-h exposure to 25 μg/ml piscidin-1N or -2. In contrast, CCO cells exposed to 5.7 μM dermaseptin-1 showed essentially no decrease in their ability to metabolize MTT. Collectively, these results suggest that AMPs, at concentrations that reduce viral infectivity by >90%, are not cytolytic for host cells.

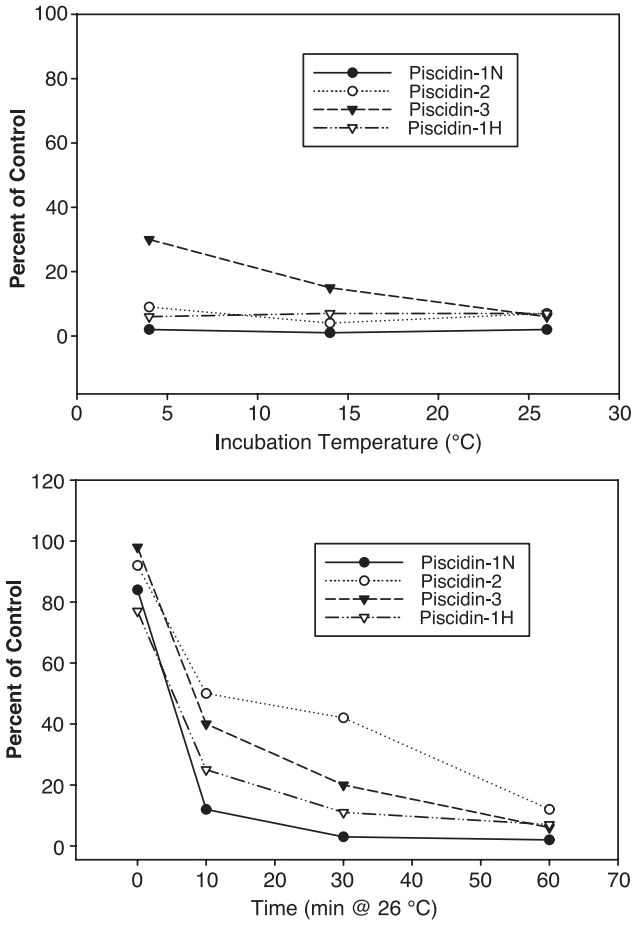

Fig. 7. Effect of temperature and time on the inactivation of CCV by Piscidin-1, -2, and -3. CCV was incubated in the presence of 25 μg/ml Piscidin-1N, -1H, and -2, or 50 μg/ml Piscidin-3, for 60 min at 4°, 14°, or 26 °C (Upper Panel) or for the indicated period of time at 26 °C (Lower Panel). Residual infectivity was determined by plaque assay, and the means of two to three experiments are shown.

Table 2
Antiviral activity of amphibian and piscine AMPs

Peptide	$[i]_{50}$[a]	
	CCV	FV3
Dermaseptin-1	3 μM	12 μM
Temporin A	15 μM	58 μM
Magainin II	48 μM	ND
PGLa	100 μM	ND[b]
R. catesbeiana peptides	78 μg/ml	ND
R. pipiens peptides	42 μg/ml	ND
Piscidin-1N	4 μM	13 μM
Piscidin-1H	4 μM	13 μM
Piscidin-2	4 μM	13 μM
Piscidin-3	11 μM	16 μM

[a] $[i]_{50}$ denotes the concentration of AMP that reduced viral infectivity by 50%. The values shown represent the average of two or more experiments.
[b] ND denotes "not determined."

Discussion

The above results confirm and extend our earlier work and demonstrate that an expanded panel of amphibian and piscine AMPs possessed marked antiviral activity. The

presence of AMPs in both invertebrates and vertebrates suggests that they are an evolutionary ancient weapon in the continuing fight against microbial invaders (Stefano et al., 1998; Zasloff, 2002), and likely play a role in protecting lower vertebrates from infection. As seen previously, CCV was more sensitive to inactivation than FV3, a result which may reflect the enveloped character of the former (Chinchar et al., 2001). However, although most FV3 particles lack a conventional viral envelope, FV3 possesses a lipid membrane located between the outer capsid coat and the inner DNA core (Chinchar, 2002). Whether the ability of the tested AMPs to reduce the infectivity of FV3 is due to their capacity to interact with this essential lipid membrane, or whether it reflects interaction between AMPs and the protein shell itself is not known. We favor the former mechanism because non-enveloped mammalian viruses such as reovirus 3 and ECHO virus 11 are resistant to AMP-mediated inactivation (Daher et al., 1986; Lehrer et al., 1985). However, sensitivity of a given virus to an AMP is not simply determined by the presence or absence of an envelope because enveloped viruses demonstrated a 1000-fold difference in sensitivity to HNP-1 (Daher et al., 1986).

The mechanism by which AMPs reduce virus infectivity remains to be determined. In contrast to the work of Daher et al. (1986), our previous electron microscopic study showed that R2P and E2P appeared to destabilize CCV, whereas treated FV3 remained ostensibly intact (Chinchar et al., 2001). These results were surprising because, although one might have expected that AMPs would destabilize the CCV envelope, one would have predicted that the resulting viral nucleocapsids would have remained intact. This did not appear to be the case as both the envelope and underlying nucleocapsid were destabilized. Furthermore, because the lipid component of the viral envelope is derived from the host cell's plasma membrane, and because the latter is relatively resistant to AMP attack, it is unclear why the viral envelope should be degraded. Perhaps the presence of viral proteins or externally orientated phosphadtidylserine within the viral envelope alters its properties and enhances its susceptibility to AMP attack.

As seen earlier, amphibian and piscine AMPs were active over a greater temperature range than mammalian AMPs. For example, in assays involving HSV-1 and the mammalian AMP HNP-1, there were successive 10-fold drops in activity as the incubation temperature was progressively lowered from 42 to 37 °C, 37 to 26 °C, and 26 to 14 °C (Daher et al., 1986). Similar losses in antiviral activity were also seen with MCP-1 and MCP-2 (Lehrer et al., 1985). In contrast to mammalian AMPs, ectothermic AMPs showed about as much as activity at 14 °C as they did at 26 °C, and retained considerable activity at temperatures as low as 4 °C. The wider temperature range over which amphibian and piscine AMPs appear to function may reflect the need for amphibian and piscine AMPs to provide protection over a wider range of environmentally relevant temperatures, whereas mammalian AMPs are active only within a narrow physiological temperature range.

Despite increasing evidence for their ability to inactivate viruses in vitro, a definitive role for AMPs in protecting amphibians and fish from virus infection has not yet been established. Moreover, determining the precise role that various AMPs play in protection from infectious disease will be challenging. For example, treatments (e.g., mild electrical stimulation or exposure to adrenergic agents) designed to release AMPs and generate AMP-deficient animals may also suppress other aspects of innate and acquired immunity and make it difficult to determine the relative contribution of AMPs. However, if, as suggested for mammals, amphibian and piscine AMPs also play a role in regulating innate and adaptive immunity (Yang et al., 2002), then one can envision a scenario in which AMPs act as a first line of defense and block virus infection by direct inactivation, or, if that is not successful, control and eliminate infection by upregulating additional innate and adaptive responses.

Methods

Cells and viruses

The propagation of FV3 in fathead minnow (FHM) cells (ATCC No. CCL 42) and of CCV in channel catfish ovary (CCO) cells (Bowser and Plumb, 1980) has been described previously (Chinchar et al., 2001). Virus samples were prepared in either Dulbecco's modified Eagle medium (DMEM) containing 4% fetal calf serum (FCS) or in RSB (10 mM Tris–HCl, pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$).

Antimicrobial peptides

Temporin A was synthesized as previously described (Wade et al., 2001). With the exception of PGLa, which was a gift from Magainin Pharmaceuticals (Plymouth Meeting, PA), the other amphibian peptides used in this study were either synthesized by Sigma/Genosys (ranatuerin-2P) or purchased from Sigma (St. Louis, MO) (dermaseptin-1, magainin I, magainin II). Amphibian AMPs were dissolved in sterile water at concentrations ranging from 114 to 1000 μM, and stored at −80 °C. The piscidins employed in this study were synthesized using Fmoc chemistry on a Rainin Symphony synthesizer. Piscidin stock solutions were prepared at 4 mg/ml in 0.01% acetic acid and stored at −80 °C. Working solutions of all peptides were prepared by dilution in sterile water and the indicated concentrations tested for their ability to reduce virus infectivity as described below. The primary amino acid sequences of the AMPs used in this study are shown in Table 1.

Amphibian skin secretions

Crude peptide fractions were prepared from *R. catesbeiana* and *R. pipiens* as follows. Individual frogs were injected bilaterally with 2 nmol/g body weight of [+/−]-norepinephrine hydrochloride in a volume of 0.1 ml per 10 g frog weight via the dorsal lymph sac. Immediately following injection, the frog was placed in 100 ml of collection buffer (25 mM NaCl, 25 mM sodium acetate, pH 7.0) (Giovannini et al., 1987), and skin secretions were allowed to accumulate in the buffer for 15 min. Following release of skin secretions, the frog was removed, and the buffer was acidified by addition of 1.0 ml of trifluoroacetic acid (TFA). The skin secretions were partially purified by passage over a C-18 Sep-Pak cartridge (Waters Corporation, Milford, MA) as previously described (Goraya et al., 1998, 2000). Protein concentration was determined using the Micro BCA* Assay (Pierce, Rockford, IL) following manufacturer's instructions except that Bradykinin (RPPGFSPFR) (Sigma) was used to establish a standard curve (Smith et al., 1985).

Assay of residual virus infectivity

The efficacy of individual AMPs in reducing virus infectivity was determined by plaque assay. Briefly, 10 μl of virus (containing between 10^6 and 10^8 PFU/ml) were mixed with an equal volume of AMP (or sterile water) and incubated at 26 °C for 1 h. At that time, samples were diluted with 1 ml of DMEM containing 4% FCS and stored on ice. Samples were subsequently serially diluted 10-fold and the residual virus titer determined by plaque assay on either CCO cells (for CCV) or FHM cells (for FV3). In some cases, residual levels of virus infectivity were determined by $TCID_{50}$ assay. Individual inactivation experiments were performed two or more times. Virus titers are expressed as "percentage of control" [i.e., (virus + peptide/virus + water) × 100]. Addition of the AMP to the virus inoculum did not change the color of the growth medium in which the virus was prepared, suggesting that decreases in infectivity were not due to changes in pH. Moreover, plaque assay confirmed that the dilute acid present within the piscidin samples did not reduce viral infectivity (data not shown).

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