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Staphylocidal Action of Thrombin-Induced Platelet Microbicidal Protein Is Influenced by Microenvironment and Target Cell Growth Phase

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Thrombin-induced platelet microbicidal protein (tPMP) is a small, cationic peptide released from rabbit platelets following exposure to thrombin in vitro. This peptide exerts potent in vitro microbicidal activity against a broad spectrum of bloodstream pathogens, including *Staphylococcus aureus*. It is known that the microbicidal actions of other cationic antimicrobial peptides (e.g., neutrophil defensins) are influenced by environmental factors and target cell growth phase. However, whether these parameters affect tPMP microbicidal activity has not been studied. Thus, we assessed the in vitro bactericidal activity of tPMP against two tPMP-susceptible strains, *Bacillus subtilis* ATCC 6633 and *S. aureus* 502A, in various target cell growth phases or under various microenvironmental conditions. The conditions studied included differing bacterial growth phase (logarithmic versus stationary), temperature (range, 4 to 42°C), pH (range, 4.5 to 8.5), cationicity (range, 0.1 mM to 2 M), anionicity (range, 0.08 to 5 μM), and neutral carbohydrates ranging in molecular weight (MW) from 180 to 37,700 (range, 50 to 500 mM) as well as rabbit platelet-free plasma and serum. tPMP staphylocidal activity was greater against logarithmic- than stationary-phase cells. tPMP bactericidal activity against both *B. subtilis* and *S. aureus* was directly correlated with temperature and pH, with microbicidal activity exhibited near the physiological range (37 to 42°C and pH 7.2 to 8.5, respectively). The presence of cations (Na^+, K^+, Ca^{2+}, and Mg^{2+}) decreased tPMP bactericidal activity in a time- and concentration-dependent manner, with complete inhibition at monovalent or divalent cation concentrations of ≥250 or ≥10 mM, respectively. Staphylocidal activity of tPMP was also inhibited by the polyanions polyanetholsulfonic acid and polyaspartic acid, at 0.1 and 0.4 μM, respectively. Coincident exposure with low-MW carbohydrates (glucose, sucrose, and melezitose) did not affect tPMP staphylocidal activity. However, higher-MW carbohydrates (raffinose and dextrans) decreased tPMP activity in a manner directly proportional to their concentration and MW. Solute-mediated inhibition of tPMP bactericidal activity was independent of solute osmolality but directly related to the duration of tPMP-solute coexposure. tPMP enhanced the staphylocidal activities of platelet-free plasma and heat-inactivated serum, while the activity of normal serum was not affected. These collective observations suggest that tPMP retains antimicrobial activities under physiological conditions which are likely to be relevant to host defense in vivo.

Endovascular infections are characterized by a focus of microorganisms adherent to or interiorized within vascular endothelium or endocardium. Examples of such infections include vascular catheter and hemodialysis access site sepsis, infections of vascular grafts and prostheses, and infective endocarditis. The interaction of microbial pathogens with host plasma proteins, endothelial cells, and platelets is believed to be integral in the evolution of such infections. For example, in infective endocarditis, the traditional concept has been that platelets facilitate the induction and propagation of this infection by providing an adhesive surface for bloodborne microorganisms to bind to damaged endocardium (12). In contrast, recent evidence from our laboratory suggests that platelets may play an important role in host defense against endovascular infection. For instance, rabbit platelets have been found to secrete a small, cationic and heat-stable microbicidal peptide (molecular mass, ~8.5 kDa) termed thrombin-induced platelet microbicidal protein (tPMP) (24). tPMP has potent microbiostatic and microbicidal activities against common bloodstream pathogens including *Staphylococcus aureus*, *Staphylococcus epidermidis*, viridans group streptococci, and *Candida albicans* (16, 18, 20, 22–24). In addition, our laboratory has recently utilized the experimental infective endocarditis model to demonstrate that in vitro susceptibility of viridans group streptococci and *C. albicans* to tPMP correlates with reduced microbial proliferation within the endocarditis vegetations (16, 25). Hematogenous dissemination of *C. albicans* from vegetations to extracardiac organs was also reduced in tPMP-susceptible versus tPMP-resistant strains (25).

Data from our laboratory have implicated the bacterial membrane as the principal target for tPMP activity (19, 21). Antimicrobial activities of other membrane-targeting, cationic peptides (e.g., neutrophil defensins) have been shown to be affected by microenvironmental conditions (13, 14). However, the effect of microenvironmental factors on tPMP bactericidal activity is not known. Thus, the aim of the current study was to investigate the influence of bacterial growth phase and a variety of microenvironmental conditions on tPMP bactericidal activity in vitro.

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MATERIALS AND METHODS

Bacterial strains and chemicals. *S. aureus* 502A and *Bacillus subtilis* were obtained from the American Type Culture Collection (ATCC 27217 and 6633, respectively). Stationary-phase cells were grown overnight (14 h) at 37°C. *S. aureus* was cultured on brain heart infusion medium (Difco Laboratories, Detroit, Mich.), while *B. subtilis* was cultured on 6.6% sheep blood agar (Clinical Standards Laboratories, Inc., Rancho Dominguez, Calif.). Mid-logarithmic-phase cells were obtained by inoculating fresh brain heart infusion medium with overnight cells (optical density at 600 nm, 0.05), and the cultures were grown to an optical density at 600 nm of 0.6. For bacterial culture maintenance, cells were grown on sheep blood agar and stored at 4°C.

Analytical-grade chemicals were used for all experiments. Sodium polyanetholsulfonic acid (SPS), sodium polyaspartic acid (SPA), and all carbohydrates (glucose, sucrose, melezitose, raffinose, and dextrans with molecular weights [MWs] of 11,300 and 37,700) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Nisin was kindly provided by Aplin & Barrett Ltd. (Dorset, England), while HNP-1 was purchased from Sigma Chemical Co. All solutions were prepared in sterile, double-distilled water (pH 7.2) except for nisin, which was prepared at pH 5.5.

tPMP. tPMP was prepared from rabbit platelets as previously described (24). In brief, platelets collected from fresh rabbit blood were washed and resuspended in Eagle's minimal essential medium (MEM) (without nonessential amino acids or L-glutamine; pH 7.4) (Irvine Scientific, Santa Ana, Calif.) to a concentration of 10^8 platelets per ml, as determined with a Coulter Counter (Coulter Instruments, Hialeah, Fla.). The platelets were then stimulated with bovine thrombin (1 U/ml; Sigma Chemical Co.) in the presence of 2 mM $CaCl_2$ at 37°C for 30 min. The resulting suspension was centrifuged (3,000 × *g*, 15 min) to sediment platelets, and the tPMP-rich supernatant was stored at 4°C in aliquots. Previous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses of such tPMP preparations indicated that a single staphylocidal, cationic peptide with an apparent molecular mass of ~8.5 kDa is released from the platelets following thrombin stimulation (24). The staphylocidal activity of such preparations is neutralized with concomitant removal of the 8.5-kDa band in SDS-PAGE by anionic filters and resins. Bioactivity can be restored by elution of the same band from the anionic materials (24).

Bactericidal activity of the tPMP preparation was determined as described previously, using *B. subtilis* ATCC 6633 as a highly tPMP-sensitive indicator organism (24). tPMP bioactivity (in units per milliliter) was quantified as the reciprocal of the highest tPMP dilution retaining ≥95% killing of 10^3 CFU of *B. subtilis* inoculum per ml after a 30-min exposure at 37°C. Specific tPMP bioactivity was then estimated as units per milligram of protein, and the value was converted to tPMP concentration, expressed as micrograms per milliliter (2.0 μg/ml = 100 U/ml).

tPMP bactericidal assay. All tPMP bactericidal assays were carried out in essentially identical manners, with selected variations in test conditions as indicated below. Mid-logarithmic-phase cells were collected and washed twice with phosphate-buffered saline (PBS) (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 3.0 mM KCl [pH 7.2]). A final bacterial inoculum of 10^5 to 10^6 CFU/ml was added to either MEM containing tPMP (0.2 μg/ml for *B. subtilis* and 2.0 μg/ml for *S. aureus*) or control MEM buffer alone (pH 7.4). All assays were conducted at 37°C for either 30 min (for *B. subtilis*) or 2 h (for *S. aureus*). Since *B. subtilis* was significantly more susceptible to tPMP than *S. aureus*, *B. subtilis* assays were performed with a lower concentration of tPMP and a shorter incubation time (24). Aliquots were removed from assay tubes at the end of the incubation period and diluted in PBS (pH 7.2). Following the appropriate incubation, the surviving bacterial population was enumerated by quantitative culture on blood agar plates, expressed as the surviving $\log_{10}$ CFU of the initial inoculum per milliliter, and plotted against the different test parameters. All experiments were performed in triplicate on separate days. The final data are mean values ± standard deviations (SD).

Five parameters were varied to investigate their influence on tPMP activity.

(i) Bacterial growth phase. To investigate the effect of target cell growth phase on tPMP bactericidal activity, mid-logarithmic-phase or stationary-phase bacterial cells were exposed to various concentrations of tPMP (for *B. subtilis*, tPMP concentration of 0.02, 0.1, or 0.2 μg/ml; for *S. aureus*, tPMP concentration of 1, 2, or 3 μg/ml) or MEM buffer alone.

(ii) Temperature. The influence of temperature on tPMP bactericidal activity was evaluated by exposing cells to tPMP at 4, 24, 37, and 42°C in parallel. As a control, the microbicidal activities of human neutrophil defensin, HNP-1, and the aminoglycoside gentamicin, each known to be temperature dependent, were also determined over the same temperature range (7, 10). Microbicidal assays for HNP-1 (20 μg/ml) and gentamicin (5 μg/ml) against *S. aureus* were conducted for 1 and 2 h, respectively.

(iii) pH. To study the influence of pH on tPMP activity, bacterial cells were exposed in parallel to tPMP or MEM buffer alone at pH 4.5, 6.0, 7.2, or 8.5. The microbicidal activity of nisin, a cationic, pore-forming lantibiotic (lanthionine-containing antibiotic), is known to be pH dependent (4). Thus, the influence of pH on the microbicidal activity of nisin (10 μg/ml) against *B. subtilis* was determined as a control, across the same pH range at 37°C for 30 min.

(iv) Charged and neutral solutes. *S. aureus* cells were exposed to MEM buffer with or without tPMP in the presence of the following test solutes at the indicated concentrations: monovalent cations, K^+ or Na^+ (50, 100, 250, or 500 mM, as Cl^- salts); divalent cations, Ca^{2+} or Mg^{2+} (2.5, 10, 25, 100, or 250 mM, as Cl^- salts); polyanions, SPS (0.1, 0.5, 1, or 5 μM, as the Na^+ salt) or SPA (0.08, 0.4, 0.8, or 4.0 μM, as the Na^+ salt); carbohydrates, glucose, sucrose, melezitose, or raffinose (50, 100, 150, 250, 300, 400, or 500 mM); and dextrans (polyglucose), with a MW of 11,300 (3.5, 7.0, 10, 14, or 20 mM) or 37,700 (1, 2, 3, or 4 mM). Glycerol, as opposed to the above carbohydrates, is a neutral solute which readily diffuses across the bacterial membrane, producing minimal or no charge and osmotic disturbances to the membrane (6). Thus, the effect of glycerol (0.05, 0.1, 0.25, 0.5, 1.0, or 2.0 M) on tPMP microbicidal activity against *S. aureus* was evaluated as a control. For solutes which inhibited tPMP bactericidal activity, the minimum concentration causing 100% inhibition of tPMP bactericidal activity was defined as the I_{100}.

(v) PFP and serum. One putative site of tPMP activity in vivo is hypothesized to be the luminal surface of damaged or infected vascular endothelium. To assess the influence of various blood components on tPMP activity in vitro, *S. aureus* was exposed to tPMP or MEM buffer containing rabbit platelet-free plasma (PFP) (40% [vol/vol]) or homologous rabbit serum (normal or heat inactivated; 40% [vol/vol]). Plasma was prepared by collecting fresh rabbit blood into a 0.75% (wt/vol) final concentration of sodium citrate anticoagulant. The blood was then centrifuged at 1,200 × *g* for 10 min, and the platelet-rich supernatant was gently filtered twice (pore size, 0.2 μm) to collect the PFP filtrate. To prepare serum, fresh rabbit blood was collected in the absence of anticoagulant and allowed to clot at room temperature. The clot was removed with a sterile swab, and the suspension was centrifuged at 1,200 × *g* for 10 min to collect the serum supernatant. For some experiments, serum was heat inactivated (30 min, 56°C). Plasma and serum were used on the day of preparation.

Relationship between time of tPMP-solute coexposure and inhibition of tPMP staphylocidal activity. To determine if tPMP bactericidal activity was influenced by solutes in a manner dependent on the duration of coincident tPMP-solute exposure, *S. aureus* cells were exposed to tPMP (2.0 μg/ml) or MEM buffer alone at 37°C for 2 h, as described above. At 0, 30, 60, 90, and 120 min of *S. aureus* exposure to tPMP, an aliquot of the reaction mixture was removed and quantitatively cultured on blood agar plates to determine the surviving bacterial population at that time. In parallel, a separate aliquot was obtained and added to either NaCl, $CaCl_2$, $MgCl_2$, SPS, SPA, raffinose, or dextran (MW, 11,300) (37°C) at a final concentration greater than or equal to the I_{100} (predetermined in the studies above). Small volumes of the highly concentrated solute stock solutions were used to prevent a significant change in the final tPMP concentration. The suspensions were then further incubated for a total of 2 h, and the surviving population of cells was quantified by culture on blood agar plates, as described above. The surviving $\log_{10}$ CFU per milliliter was plotted against the time of tPMP-solute coexposure. All experiments were performed in triplicate on separate days. The final data are mean values ± SD.

Measurement of solute osmolality. Osmolality of test solute solutions was measured with a freezing point osmometer (μOsmette; Precision Systems, Inc., Sudbury, Mass.) calibrated with standard solutions of 100, 500, and 1,500 mosmol (Precision Systems, Inc.) before use. All final data are means from at least two separate measurements and are expressed as milliosmoles per kilogram of H_2O ± SD.

RESULTS

Influence of target cell growth phase on tPMP bactericidal activity. Similar to previous data from our laboratory, *B. subtilis* was more susceptible than *S. aureus* to tPMP, even when exposed to lower tPMP concentrations and for shorter incubation periods (Fig. 1) (24). The viability of control cells in MEM buffer was not affected (data not shown). Logarithmic-phase *S. aureus* cells were significantly more susceptible (~25-fold) to tPMP killing than stationary-phase cells, over a tPMP range of 1.0 to 3.0 μg/ml (Fig. 1A). For example, at a tPMP concentration of 3 μg/ml, *S. aureus* logarithmic-phase cells exhibited a decrease in cell viability of 2 $\log_{10}$ CFU/ml compared with a decrease of only 0.5 $\log_{10}$ CFU/ml at stationary phase (Fig. 1A). Bactericidal activity of tPMP against logarithmic- and stationary-phase cells of *B. subtilis* was tPMP concentration dependent (Fig. 1B). However, in contrast to the findings for *S. aureus*, logarithmic- and stationary-phase cells of *B. subtilis* were equally susceptible to tPMP at concentrations between 0.02 and 0.2 μg/ml (Fig. 1B). For instance, at 0.2 μg

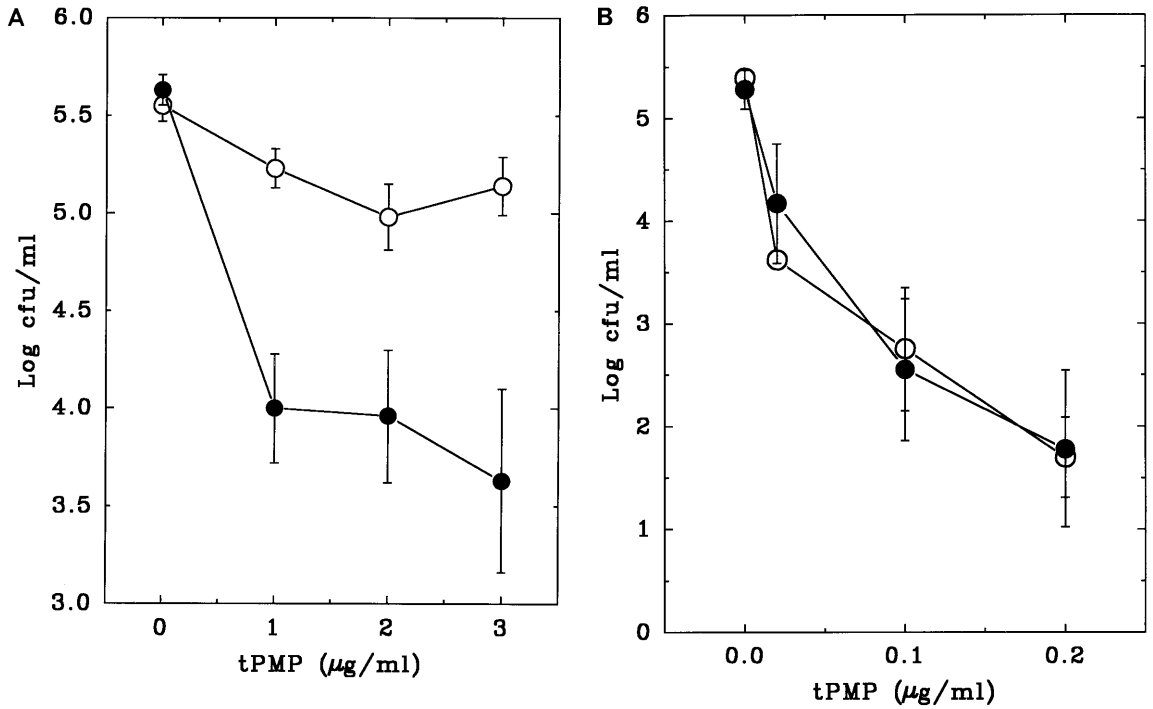

FIG. 1. Dose effect of tPMP bactericidal activity against logarithmic-phase (●) and stationary-phase (○) cells of *S. aureus* (A) and *B. subtilis* (B). Cells of *S. aureus* and *B. subtilis* were exposed to various concentrations of tPMP for 2 h and 30 min, respectively. Mean organism survival ($\log_{10}$ CFU/ml) ± SD was calculated from at least two experiments and plotted against tPMP concentration.

of tPMP per ml, nearly 4 $\log_{10}$ CFU of logarithmic- and stationary-phase *B. subtilis* cells per ml was killed within 30 min.

Influence of temperature on tPMP bactericidal activity. The viability of neither *S. aureus* nor *B. subtilis* in MEM was affected at the various temperatures tested (data not shown). At all temperatures tested, 3 to 5 $\log_{10}$ CFU of the highly tPMP-susceptible *B. subtilis* per ml was killed within 30 min, with a trend toward an enhanced bactericidal effect at 42°C (Fig. 2A). In contrast, tPMP was inactive against the less tPMP-susceptible *S. aureus* at temperatures between 4 and 24°C. At a temperature of 37 or 42°C, tPMP staphylocidal activity increased substantially, with a decrease in staphylococcal viability of 1 or 4 $\log_{10}$ CFU/ml, respectively (Fig. 2A).

As expected, the staphylocidal activities of two control cationic microbicidal compounds, defensin HNP-1 and gentamicin, were also influenced by temperature (Fig. 2B). Similar to the findings for tPMP, the bactericidal activities of both these compounds increased with increasing temperature (Fig. 2B).

Influence of pH on tPMP bactericidal activity. Bactericidal activity of tPMP against both *S. aureus* and *B. subtilis* was inversely related to pH (Fig. 3A). At pHs between 4.5 and 6.0, tPMP activity against *S. aureus* was negligible. However, as the pH was increased to 7.2 or 8.5, staphylocidal activity was reconstituted (Fig. 3A). *B. subtilis* was more susceptible to tPMP than *S. aureus* at all the pHs tested. For example, a decline in *B. subtilis* viability of ~3 $\log_{10}$ CFU/ml was observed at pH 4.5 to 6.0, with further decline in viability observed as the pH was increased from 6.5 to 8.5 (Fig. 3A). At pH 8.5, a decline in cell viability of up to ~5 $\log_{10}$ CFU/ml was observed for *B. subtilis*.

As anticipated, the bactericidal activity of nisin was pH dependent (Fig. 3B). However, in contrast to tPMP, the bactericidal activity of nisin decreased with increasing pH, similar to previous findings (4).

Influence of ions and neutral solutes on tPMP bactericidal activity. Na^+ inhibited tPMP staphylocidal activity in a concentration-dependent manner, with 100% inhibition seen at 250 mM (Fig. 4 and Table 1). For *B. subtilis*, Na^+ also inhibited tPMP killing in a concentration-dependent manner but exhibited a twofold-higher I_{100} (500 mM) (data not shown). Other cations such as K^+, Ca^{2+}, and Mg^{2+} also inhibited tPMP staphylocidal activity in a manner directly proportional to their concentration, with I_{100}s of 250, 25, and 10 mM, respectively (Table 1). For *B. subtilis*, I_{100}s of Ca^{2+} and Mg^{2+} were 4- and 10-fold higher, respectively, than those observed for *S. aureus* (100 mM) (data not shown). tPMP bactericidal activity against *S. aureus* was inhibited by the multivalent anions SPS and SPA at low concentrations (I_{100} = 0.1 and 0.4 μM, respectively; Table 1). Similar to the results for cations, inhibition of tPMP staphylocidal activity by these anions was concentration dependent (data not shown).

The carbohydrates tested had varied effects on tPMP staphylocidal activity. For the lower-MW carbohydrates, such as glucose, sucrose, and melezitose (MW ≤ 504), concentrations up to 500 mM had no effect on tPMP activity against *S. aureus* (Fig. 4; Table 1). However, sugars with higher MWs, such as raffinose (MW, 594.5) and dextrans (MW, 11,300 or 37,700), inhibited tPMP staphylocidal activity in a concentration-dependent manner (Fig. 4; Table 1). The inhibitory capacity of these larger carbohydrates was directly related to their MWs such that as the MW was increased from 595 (raffinose) to 37,700 (dextran), I_{100}s decreased from 600 to 8.3 mM (Table 1). Glycerol (2 M) did not affect tPMP activity against *S. aureus* (Table 1).

Influence of time of tPMP-solute coexposure and inhibition of tPMP staphylocidal activity. The relationship between the time of tPMP-solute coexposure and the degree of inhibition of tPMP activity was studied using the following solutes at the indicated concentrations: Na^+, 250 mM (Cl^- salt); Ca^{2+}, 50 mM (Cl^- salt); Mg^{2+}, 50 mM (Cl^- salt); SPS, 1.5 μM (Na^+ salt); SPA, 1.2 μM (Na^+ salt); raffinose, 600 mM; and dextran

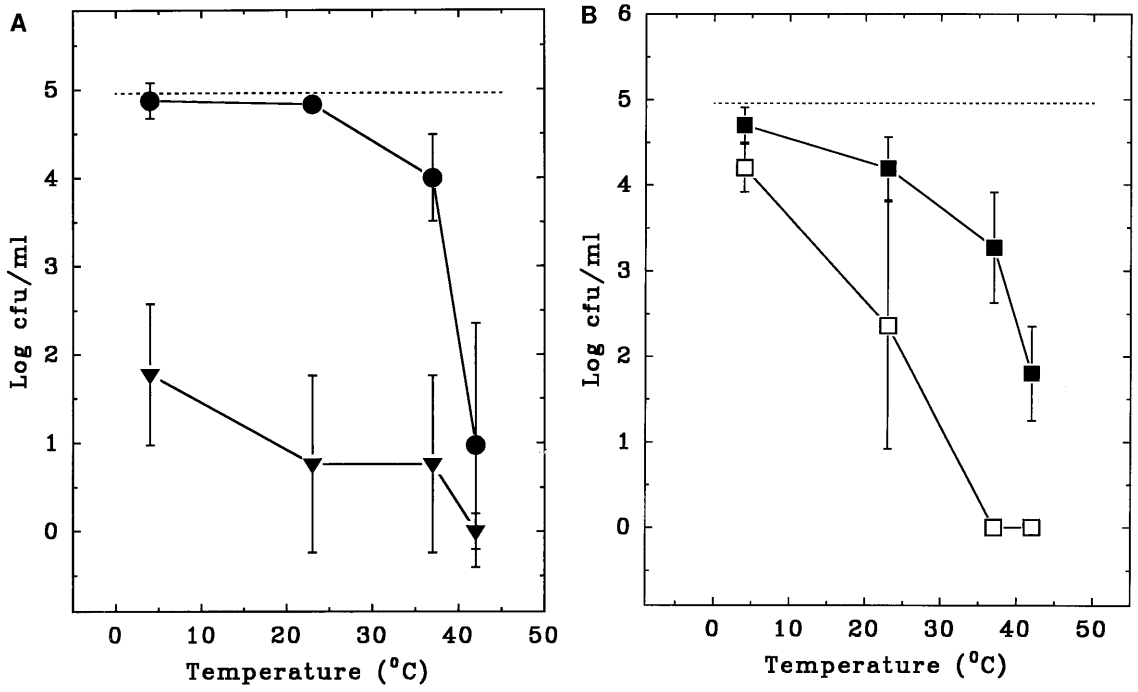

FIG. 2. Influence of temperature on bactericidal activities of tPMP, HNP-1, and gentamicin. (A) Logarithmic-phase cells of *S. aureus* (●) and *B. subtilis* (▼) were exposed to 2.0 (2 h) and 0.2 (30 min) μg of tPMP per ml, respectively, at various temperatures. (B) Logarithmic-phase cells of *S. aureus* were exposed to either 20 μg of HNP-1 per ml for 1 h (■) or 5 μg of gentamicin per ml for 2 h (□) at various temperatures. Mean organism survival ($\log_{10}$ CFU/ml) ± SD was calculated from at least two experiments and plotted against temperature. – – – – –, initial bacterial inoculum.

(MW, 11,300), 20 mM. The inhibition of tPMP staphylocidal activity was determined by comparing bacterial survival obtained after the 2-h incubation with that obtained at the time of solute addition (Fig. 5). Exposure of *S. aureus* to 2.0 μg of tPMP alone per ml for 120 min resulted in a decrease in viable counts of ~1 $\log_{10}$ CFU/ml, while viability of cells in MEM buffer was not affected (Fig. 5; inoculum = 5.7 $\log_{10}$ CFU/ml). The addition of Na^+ (250 mM) to the tPMP-*S. aureus* assay mixtures at the beginning of the incubation (time of coexposure = 120 min) completely protected cells from tPMP-induced killing (Fig. 5). The addition of Na^+ to the assay mixtures after 30, 60, 90, and 120 min of tPMP-*S. aureus* incubation (corresponding to times of coexposure of 120, 90, 60, and 30 min, respectively) protected *S. aureus* cells from further killing in a time-dependent manner (Fig. 5). Maximum inhibition of tPMP staphylocidal activity by Na^+ in these assays was observed at a coexposure time of 120 min, compared with minimum inhibition at a 30-min coexposure time (Fig. 5). No protection by Na^+ was observed when the solute was added at the end of the 120-min incubation (coexposure time = 0 min; Fig. 5). Similar studies with other potential tPMP-inhibiting solutes, including Ca^{2+}, Mg^{2+}, SPS, SPA, raffinose, and dextran (MW, 11,300), yielded similar time-dependent patterns in *S. aureus* protection from tPMP-induced killing (data not shown).

The measured osmolality of the various solutes tested for their inhibitory effect on tPMP bactericidal activity ranged widely, from 2 (for polyanions SPS and SPA) to 600 (for KCl) mosmol/kg of H_2O (Table 1). However, the ability of solutes to inhibit tPMP bactericidal activity was independent of solute osmolality.

Influence of PFP and serum on tPMP bactericidal activity. Normal serum, heat-inactivated serum, or PFP alone decreased *S. aureus* viability by 2.6, 1.5, and 0.8 $\log_{10}$ CFU/ml, respectively, indicating intrinsic staphylocidal activities (Fig. 6). Viability of *S. aureus* cells exposed to tPMP alone was decreased by 1.7 $\log_{10}$ CFU/ml. When *S. aureus* was exposed to tPMP in the presence of either PFP or heat-inactivated serum, ~2.1 $\log_{10}$ CFU of bacteria per ml was killed (Fig. 6). Viability of *S. aureus* cells exposed to tPMP in the presence of normal serum was reduced by 2.5 $\log_{10}$ CFU/ml.

DISCUSSION

Recent studies in our laboratory have implicated the bacterial membrane as the principal target for tPMP activity. First, flow cytometric data suggest that one type of PMP (PMP2) induces permeabilization of the *S. aureus* cell membrane in vitro (21). Second, electron microscopy studies have revealed ultrastructural evidence that tPMP induces rapid and extensive damage of the *S. aureus* cell membrane within minutes of tPMP exposure (19). Despite these membrane effects, cell lysis was not observed over the 120-min exposure studied, indicating that the cell wall was not the likely target of tPMP action (19).

The microbicidal actions of many other membrane-targeting, endogenous antimicrobial peptides, such as nisin and defensins, and also that of the cationic aminoglycoside gentamicin are influenced by various microenvironmental conditions. Such conditions include different pHs, temperatures, and concentrations of extracellular cations (e.g., Na^+, Ca^{2+}, or Mg^{2+}) as well as the target cell growth phase (4, 7, 9, 10, 14). However, the influence of microenvironmental conditions and bacterial growth phase on tPMP bactericidal activity was previously unknown. Therefore, the aim of this study was to investigate the influence of microenvironmental conditions and target cell growth phase on tPMP bactericidal activity in vitro.

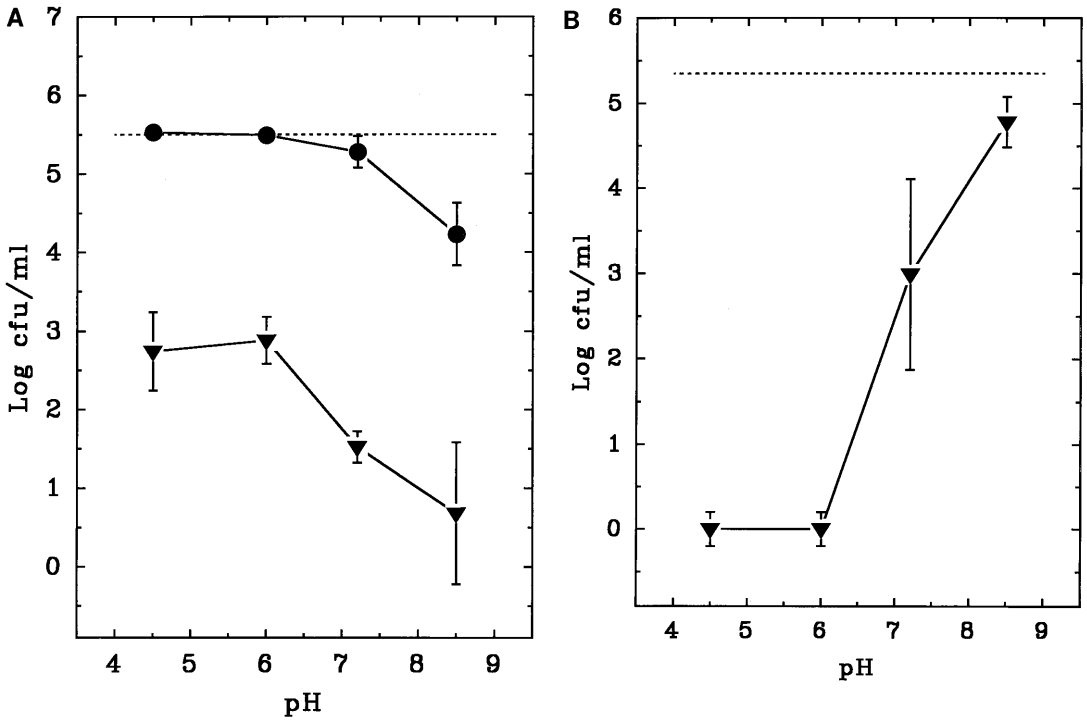

FIG. 3. Influence of pH on bactericidal activities of tPMP and nisin. (A) Logarithmic-phase cells of *S. aureus* (●) and *B. subtilis* (▼) were exposed to 2.0 (2 h) or 0.2 (30 min) μg of tPMP per ml, respectively, at various pHs. (B) Logarithmic-phase cells of *B. subtilis* were exposed to 10 μg of nisin per ml for 30 min at various pHs. Mean organism survival ($\log_{10}$ CFU/ml) ± SD was calculated from at least two experiments and plotted against pH. – – – – –, initial bacterial inoculum.

Several key observations which are potentially relevant to the in vivo microbicidal activity of tPMP were made during this study. Logarithmic-phase *S. aureus* cells were more susceptible to tPMP than stationary-phase cells in vitro. Although not specifically studied in this investigation, stationary-phase bacterial cells typically have a substantially lower transmembrane potential ($\Delta\psi$) than logarithmic-phase cells (5). We have recently shown that a threshold $\Delta\psi$ (less than −100 mV) is associated with staphylococcal susceptibility to tPMP (8). Therefore, it is possible that the decreased tPMP susceptibility of stationary-phase *S. aureus* cells is related to an altered $\Delta\psi$.

Our current findings indicate that tPMP activity is temperature dependent, with the greatest microbicidal activity occurring near the physiological range (37 to 42°C). Of interest, activities of the cationic microbicidal agents HNP-1 and gentamicin were also temperature dependent in a manner similar to that of tPMP, consistent with previous reports (9, 10, 14). tPMP bactericidal activity was also pH dependent, with maximal activity observed at pH 7.2 to 8.5. Of note, the correlation of tPMP activity with both staphylococcal $\Delta\psi$ (noted in a prior study [8]) and pH (shown in the present study) suggests that the net proton motive force (i.e., $\Delta\psi$ and ΔpH) may be a crucial parameter which affects the tPMP susceptibility of a given organism. Such dependence on $\Delta\psi$ and ΔpH for microbicidal activity has been previously documented for other membrane-targeting, endogenous antimicrobial peptides, such as nisin (4, 11). In addition, these observations provide further evidence to support the hypothesis that tPMP exerts antimicrobial activities under physiological conditions in vivo. For instance, the physiological pH and temperature supporting maximal tPMP bactericidal activity are consistent with the putative site of tPMP action in vivo, i.e., the luminal surface of damaged or infected vascular endothelium (2, 3, 24).

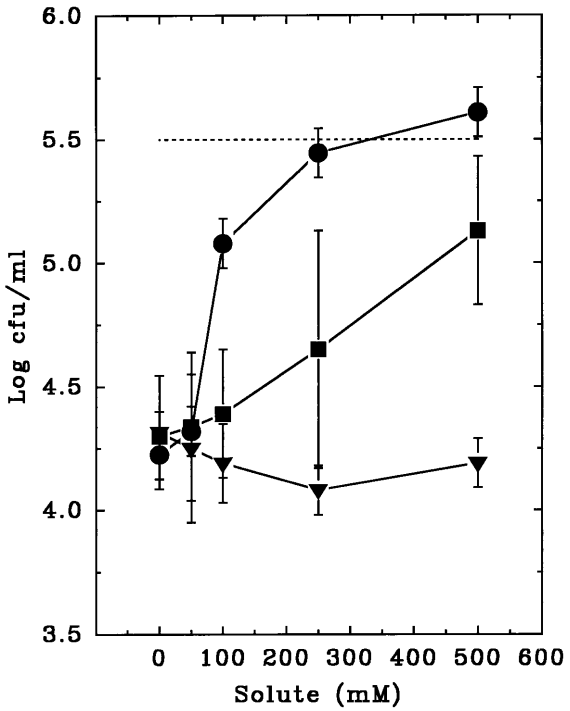

FIG. 4. Effects of NaCl, glucose, and raffinose on tPMP staphylocidal activity. Logarithmic-phase cells of *S. aureus* were exposed to 2.0 μg of tPMP per ml for 2 h in the presence of increasing concentrations of NaCl (●), glucose (▼), or raffinose (■). Mean staphylococcal survival ± SD was calculated from at least two experiments and plotted against solute concentration. Inhibition of tPMP staphylocidal activity was determined by comparing the bacterial survival with the initial inoculum (– – – – –).

TABLE 1. Influence of ionic and neutral solutes on tPMP staphylocidal activity

Solute	I_{100}	Osmolarity[a] (mosmol/kg of H_2O)
tPMP inhibiting		
NaCl	250 mM	500 ± 2
KCl	250 mM	600 ± 3
$CaCl_2$	25 mM	58 ± 2
$MgCl_2$	10 mM	46 ± 7
SPS	0.1 μM	2 ± 4
SPA	0.4 μM	2 ± 4
Raffinose (FW[b] 594.5)	600 mM	350 ± 5
Dextran		
MW, 11,300	20 mM	114 ± 6
MW, 37,700	8.3 mM	93 ± 2
Noninhibiting		
Glucose (FW 180.2)	>500 mM	>564 ± 6
Glucose (FW 342.3)	>500 mM	>611 ± 10
Melezitose (FW 504.4)	>500 mM	
Glycerol	>2 M	

[a] Means ± SD of at least two experiments.
[b] FW, formula weight.

In contrast to *S. aureus*, logarithmic- and stationary-phase cells of *B. subtilis* were equally susceptible to tPMP. Furthermore, at temperatures between 4 and 24°C and at pHs between 4.5 and 6.0, tPMP was active against *B. subtilis*, while it was inactive against *S. aureus* in MEM buffer. As discussed above, *B. subtilis* cells are exquisitely susceptible to tPMP compared with *S. aureus* cells. Thus, it is possible that the intrinsic susceptibility of *B. subtilis* cells accounted for the differences observed between the tPMP susceptibilities of *B. subtilis* and *S. aureus*. The precise mechanisms rendering *B. subtilis* (but not *S. aureus*) cells more susceptible to tPMP at either the logarithmic or the stationary phase of growth and at low temperatures and pHs are being investigated.

Hypothetically, the binding of cationic tPMP to bacterial surfaces may be essential for tPMP microbicidal activity. Membrane-targeting cationic peptides such as defensins bind specifically to anionic membranes, most likely via electrostatic interactions, resulting in eventual membrane permeabilization (17). The microbicidal activities of defensins are inhibited by high concentrations of cations Na^+, Ca^{2+}, Mg^{2+}, and Fe^{2+}, an effect believed to be due to alteration in the binding of defensin to the target bacterial membrane (9, 13, 14). Therefore, we investigated whether tPMP microbicidal activity would be influenced by charged or neutral solutes, varying in osmolality. Our results show that tPMP activity against *S. aureus* was inhibited by the cationic solutes Na^+, K^+, Ca^{2+}, and Mg^{2+} at concentrations 2- to 50-fold above the levels in human plasma (15). Even higher concentrations of Na^+, Ca^{2+}, and Mg^{2+} were required to inhibit tPMP bactericidal activity against *B. subtilis*, corresponding with its greater innate susceptibility to tPMP. The anionic solutes SPS and SPA also inhibited tPMP bactericidal activity. The ability of Na^+, K^+, Ca^{2+}, Mg^{2+}, SPS, and SPA to inhibit tPMP bactericidal activity was directly correlated with the absolute magnitude of solute charge, irrespective of whether the solutes are cationic or anionic. As the various solutes were added to the tPMP bactericidal assays, osmotic pressure against the bacterial surface was concomitantly applied. To investigate whether the inhibition of tPMP staphylocidal activity was related to the osmotic pressure of the solutes tested, the osmolality of the inhibiting solutions at the I_{100} was measured. The results show that the ability of the solutes to inhibit tPMP bactericidal activity was independent of osmolality. Collectively, these data are consistent with the hypothesis that tPMP interacts with the bacterial membrane in a manner reliant upon electrostatic affinity. If this hypothesis is correct, it would suggest that cationic solutes interfere with tPMP bactericidal activity by altering tPMP binding to the bacterial membrane, as demonstrated for HNP-1 (14). Alternatively, it is possible that anionic solutes influence tPMP activity via charge neutralization. We are investigating these possibilities.

The timed-coexposure assays indicated that the inhibition of tPMP staphylocidal activity by ionic solutes was dependent upon the duration of combined tPMP-solute exposure. The time-dependent inhibition of tPMP lethality suggests that tPMP interaction with the target membrane ultimately reaches an irreversible phase, beyond which tPMP-induced target cell death is inevitable.

Low-MW carbohydrates (i.e., glucose, sucrose, and melezitose) did not alter tPMP staphylocidal activity, while those with higher MWs (i.e., raffinose and dextrans) inhibited tPMP activity in a manner which was concentration dependent but osmolality independent. tPMP-carbohydrate coexposure assays indicated that the mode of inhibition by carbohydrate was also dependent upon the duration of tPMP-carbohydrate coexposures. It is not known how high-MW carbohydrates inhibit tPMP activity. One possibility is that viscosity or stearic hin-

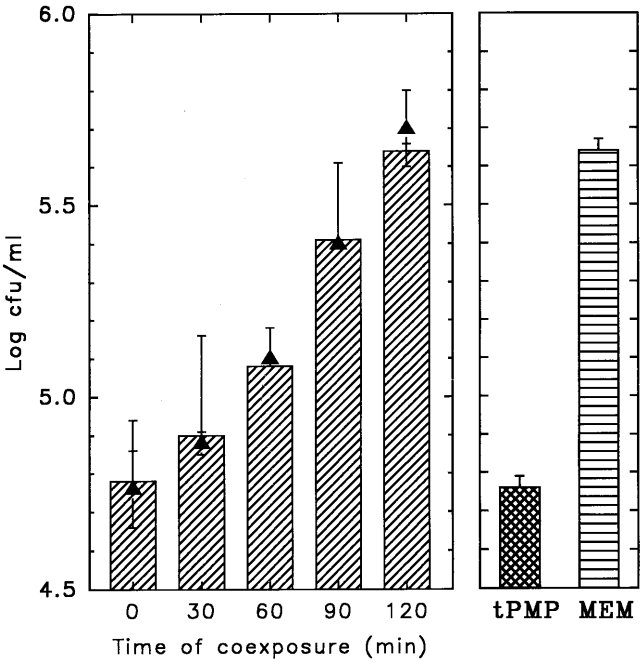

FIG. 5. Influence of tPMP-NaCl coexposure time on inhibition of tPMP staphylocidal activity. Logarithmic-phase cells of *S. aureus* were exposed to 2.0 μg of tPMP per ml for a total of 2 h. At the indicated times during the incubation, staphylococcal survival was enumerated (▲). A final concentration of 250 mM NaCl was also added to an aliquot of the cells, which was then returned to incubation. Staphylococcal survival (log_{10} CFU/ml) at the end of 2 h was determined for all samples (▨). Mean survival ± SD was calculated from at least two experiments and plotted against the time of NaCl exposure. Controls were exposed to tPMP (▩) or MEM buffer (▤) alone.

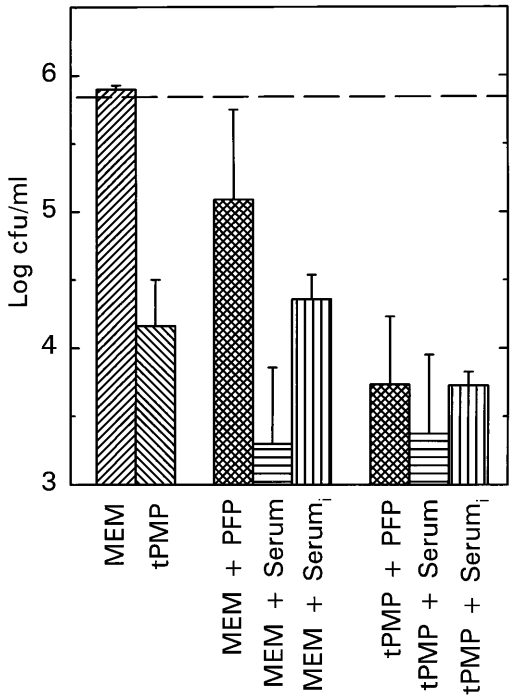

FIG. 6. Effects of PFP and serum on tPMP staphylocidal activity. Logarithmic-phase cells of *S. aureus* were exposed to either 2.0 μg of tPMP per ml or MEM buffer for 2 h in the presence of 40% (vol/vol) PFP, 40% (vol/vol) normal serum, or 40% (vol/vol) heat-inactivated serum (Serum$_i$; 56°C, 30 min). Mean staphylococcal survival ± SD was calculated from at least two experiments and compared with the initial bacterial inoculum (– – – – –).

drance mechanisms may be involved, thus reducing the net degree of interaction between tPMP and the bacterial surface.

PFP, normal serum, or heat-inactivated serum alone exhibited innate staphylocidal activity, which is consistent with previous observations (1). Staphylococcal killing resulting from a combination of tPMP with either PFP or heat-inactivated serum was greater than that caused by any of the agents alone, suggesting an enhanced interaction. Moreover, staphylocidal activity of tPMP in the presence of normal serum was greater than that of tPMP alone, although not different from that of normal serum alone. These data indicate that tPMP and normal serum do not exhibit mutual antagonism, nor does tPMP enhance serum-mediated staphylococcal killing. These observations, in combination with the physiologic temperature and pH which permit tPMP activity, further support the postulate that tPMP likely exerts microbicidal activity in the in vivo milieu of the vascular compartment. Collectively, our findings further substantiate the hypothesis that tPMP is an integral component of host defense against endovascular infection.

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