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The Cytoplasmic Membrane Is a Primary Target for the Staphylocidal Action of Thrombin-Induced Platelet Microbicidal Protein

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Thrombin-induced platelet microbicidal protein (tPMP-1) is a small, cationic peptide released from rabbit platelets exposed to thrombin in vitro. tPMP-1 is microbicidal against a broad spectrum of bloodstream pathogens, including *Staphylococcus aureus*. Preliminary evidence suggests that tPMP-1 targets and disrupts the staphylococcal cytoplasmic membrane. However, it is not clear if the cytoplasmic membrane is a direct or indirect target of tPMP-1. Therefore, we assessed the in vitro activity of tPMP-1 versus protoplasts prepared from logarithmic-phase (LOG) or stationary-phase (STAT) cells of the genetically related *S. aureus* strains 19S and 19R (tPMP-1 susceptible and resistant, respectively). Protoplasts exposed to tPMP-1 (2 μg/ml) for 2 h at 37°C were monitored for lysis (decrease in optical density at 420 nm) and ultrastructural alterations (by transmission electron microscopy [TEM]). Exposure to tPMP-1 resulted in substantial lysis of LOG but not STAT protoplasts of 19S, coinciding with protoplast membrane disruption observed by TEM. Thus, it appears that tPMP-1-induced membrane damage is influenced by the bacterial growth phase but is independent of the staphylococcal cell wall. In contrast to 19S, neither LOG nor STAT protoplasts of 19R were lysed by tPMP-1. tPMP-1-induced membrane damage was further characterized with anionic planar lipid bilayers subjected to various *trans*-negative voltages. tPMP-1 increased conductance across bilayers at −90 mV but not at −30 mV. Once initiated, a reduction in voltage from −90 to −30 mV diminished conductance magnitude but did not eliminate tPMP-1-mediated membrane permeabilization. Therefore, tPMP-1 appears to directly target the staphylococcal cytoplasmic membrane as a primary event in its mechanism of action. Specifically, tPMP-1 likely leads to staphylococcal death, at least in part by permeabilizing the bacterial membrane in a voltage-dependent manner.

Mammalian platelets are believed to be integral components of the host defense against hematogenous pathogens, in part through the release of endogenous antimicrobial peptides at sites of endovascular damage (29, 34, 36, 39, 40). In rabbits, these antimicrobial peptides have been termed platelet microbicidal proteins (PMPs) (36). One such protein, thrombin-induced PMP-1 (tPMP-1), is released from rabbit platelets following thrombin stimulation in vitro (40). tPMP-1 exhibits potent antimicrobial activity against common bloodstream pathogens, including *Staphylococcus aureus*, *Staphylococcus epidermidis*, viridans streptococci, and *Candida albicans* (29, 34, 36, 37).

A number of other endogenous microbicidal peptides, such as neutrophil defensins, are believed to kill bacteria by permeabilizing the cytoplasmic membrane in a voltage-dependent manner (10, 15, 19). tPMP-1 is similar to such peptides in that it is also small (8.5 kDa) and cationic and exhibits maximum activity at neutral pH and low ionicity (17, 26, 36). In addition, the transmembrane potential ($\Delta\Psi$) across the staphylococcal membrane appears to influence tPMP-1 microbicidal activity, with a $\Delta\Psi$ more negative than −100 mV associated with increased activity (16).

Recent transmission electron microscopy (TEM) and flow cytometry studies indicate that tPMP-1 causes staphylococcal membrane damage and permeabilization, respectively (35, 41). These data implicate the staphylococcal membrane as a likely primary target of tPMP-1 activity. Logarithmic-phase (LOG) cells of tPMP-1-susceptible staphylococcal strains were previously found to be more susceptible to tPMP-1 than stationary-phase (STAT) cells, indicating that the cellular growth phase influences the susceptibility of these strains to tPMP-1 (17). However, LOG and STAT cells of tPMP-1-resistant strains remained equally resistant to tPMP-1 (data not published). Thus, we hypothesize that (i) the staphylococcal cytoplasmic membrane is a direct target of tPMP-1 and (ii) LOG and STAT protoplasts of tPMP-1-susceptible versus -resistant *S. aureus* strains would differ substantially in their response to tPMP-1 exposure. To address these hypotheses, we studied the effect of tPMP-1 on LOG and STAT protoplasts and, in vitro, on planar lipid bilayers.

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MATERIALS AND METHODS

Staphylococcal protoplasts. Stable staphylococcal protoplasts were prepared from LOG or STAT whole cells of *S. aureus* 19S or 19R (tPMP-1 susceptible and

resistant, respectively) (38). Strains 19S and 19R are genetically related and have been well characterized previously (38). In brief, 19R is a stable tPMP-1-resistant variant of 19S (a clinical isolate), derived by serial in vitro passage in tPMP-1 (38). tPMP-1 susceptibility testing of 19S and 19R previously revealed that the percent survival of a 10^3-CFU/ml inoculum following 2 h of exposure to tPMP-1 (2 μg/ml) at 37°C was ≤10 and ≥90%, respectively (38). These strains are indistinguishable by biotyping, antibiotic susceptibility profiles, genomic DNA pulse-field gel electrophoresis patterns, and surface protein immunoblotting (38). Unlike strain JB-1, which is a small-colony variant used in our previous experiment, 19R exhibits normal colony morphology, is gentamicin susceptible (MIC = 1 μg/ml versus 6 μg/ml for JB-1), and exhibits only a modest lowering of transmembrane $\Delta\Psi$ from the parental level (−30 mV less than parental versus −50 mV for JB-1) (3, 16).

LOG or STAT cells were grown in brain heart infusion medium (Difco Laboratories, Detroit, Mich.) at 37°C for 4 or 16 h, respectively, washed, and then resuspended to an optical density at 600 nm (OD_{600}) of ~1.0 in phosphate-buffered saline (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl, 3 mM KCl [pH 7.2]). A 25-ml volume of this suspension was pelleted by centrifugation (5,000 × *g* for 15 min) and resuspended in 600 μl of digestion buffer (20% [wt/vol] sucrose, 0.05 M Tris-HCl, 0.145 M NaCl [pH 7.6]). The staphylococcal cell wall was then digested with lysostaphin (34 μg/ml; Applied Microbiology) in the presence of DNase I (16 μg/ml; Boehringer Mannheim) for 1 h at 37°C. The sucrose-stabilized protoplasts were collected by centrifugation (10,000 rpm for 15 min), and resuspended in fresh digestion buffer. The adequacy of cell wall digestion was confirmed by (i) Gram staining to ensure that the preparation consisted of negatively stained round protoplasts rather than gram-positive cocci and (ii) hypoosmotic shock in ice-cold 0.05 M Tris-HCl to induce protoplast lysis, as detected by a rapid decrease in the OD_{420} (Milton Roy Spectronic 401 spectrophotometer) (24). All protoplast preparations were kept at room temperature and used within 24 h of preparation.

Thrombin-induced platelet microbicidal protein. Preparations containing tPMP-1 were prepared by stimulating fresh rabbit platelets with bovine thrombin in glutamine-free Eagle's minimal essential medium (MEM [pH 7.4]; Irvine Scientific) as described elsewhere (36). Previous sodium dodecyl sulfate-polyacrylamide gel electrophoresis of such tPMP-1 preparations indicated that a predominant staphylocidal cationic peptide with an apparent molecular mass of ~8.5 kDa is released from the platelets following thrombin stimulation (36). The staphylocidal activity of such preparations is neutralized with concomitant removal of the 8.5-kDa band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis by anionic filters and resins. Bioactivity can be restored by elution of the same band from the anionic materials (36). Furthermore, reversed-phase high pressure-liquid chromatography (RP-HPLC) of the tPMP-1 preparations has demonstrated that tPMP-1 is the predominant antimicrobial component in such preparations (40).

The bactericidal activity of the tPMP-1 preparations was determined as described previously with *Bacillus subtilis* ATCC 6633, a highly tPMP-1-susceptible indicator organism (36). In brief, *B. subtilis* was grown on 6.6% (vol/vol) sheep blood agar (Clinical Laboratories, Detroit, Mich.) for 14 h at 37°C. The cells were collected and washed twice in phosphate-buffered saline (pH 7.2). A final inoculum of 10^3 CFU of *B. subtilis* per ml was added to microtiter plate containing serially diluted tPMP-1 in MEM buffer (range, 1:1 to 1:1,024). The percentage of the initial bacterial inoculum surviving 30 min of tPMP-1 exposure at 37°C was determined by quantitative culture. tPMP-1 bioactivity (Units per milliliter) was then quantified as the reciprocal of the highest tPMP-1 dilution retaining ≥95% killing of the *B. subtilis* inoculum. Specific tPMP-1 bioactivity was then estimated as units per milligram of protein, and the value was converted to tPMP-1 concentration expressed as micrograms per milliliter (2.0 μg/ml = 100 U/ml).

Purified tPMP-1 was used in planar lipid bilayer studies. The tPMP-1 preparation above was homogeneously purified by gel filtration and RP-HPLC, as recently described (40). The purity of tPMP-1 was then confirmed by analytical RP-HPLC and acid-urea polyacrylamide gel electrophoresis (40).

Protoplast and cell lysis assays. The ultrastructural effect of tPMP-1 on protoplasts was assessed by exposing LOG or STAT protoplasts of 19S or 19R (final OD_{420}, 0.5 to 0.6) to semipurified tPMP-1 (2.0 μg/ml) for 3 h at 37°C. All protoplasts were stabilized in medium containing 20% (wt/vol) sucrose during the assays. Protoplast lysis was determined spectrophotometrically by measuring the OD_{420} over time. Data obtained were then normalized against their respective MEM controls, and changes in the absorbance over time were determined. A decrease in OD_{420} was interpreted to represent protoplast lysis. A minimum of three separate experiments were performed for each LOG or STAT protoplast lysis assay.

TEM. TEM was used to visually assess the ultrastructural effects of tPMP-1 on staphylococcal protoplasts in vitro. Staphylococcal protoplasts were exposed to tPMP-1 (2.0 μg/ml) at 37°C as described above. The resulting protoplasts or protoplast lysates were then collected in Eppendorf tubes from 1.0-ml samples by centrifugation (7,000 × *g* for 15 min) and fixed for 12 h at 24°C (in darkness) with 0.5% (wt/vol) osmium tetroxide (Sigma Chemical Co., St. Louis, Mo.) prepared in Michaelis Veronal-acetate buffer (MVAB; 0.24 M sodium acetate, 0.14 M sodium Veronal, 0.58 M NaCl [pH 6.1]) containing 0.05% (wt/vol) tryptone broth (Difco Laboratories). The fixative was replenished at 15 and 30 min. Following fixation, protoplasts were collected (10,000 rpm for 15 min) and stabilized in 0.5 ml of 2% (wt/vol) molecular biology grade agarose prepared in MVAB (43°C). Upon solidifying, the agarose forms were macroscopically sectioned into cubes of 1 mm^3 and stained in 1% (wt/vol) uranyl acetate (Aldrich Chemical Co., Allentown, Pa.) in MVAB for 2 h at 24°C. Stained macrosections were then washed for 30 min with two fresh changes of MVAB buffer and prepared for TEM by the following standard method. The samples were post-fixed for 90 min in 1% (wt/vol) osmium tetroxide prepared in cacodylate buffer and then washed in cacodylate buffer and in distilled water. The agar blocks were serially dehydrated in increasing concentrations of HPLC-grade acetone (range, 40 to 100% [vol/vol]) before being embedded in Eponate resin. The resin blocks were polymerized overnight at 70°C, thinly sectioned, and mounted on 400-mesh copper grids before being poststained with uranyl acetate and lead citrate (1% [wt/vol] in MVAB). The thin sections were examined under a JEOL 100B transmission electron microscope with an accelerating voltage of 80 kV.

Planar bilayer experiments. Membrane permeabilization studies were performed by planar lipid bilayer techniques. Previous studies of many cationic peptides (e.g., defensins) have revealed that they readily permeabilize negatively charged lipid bilayers (10). Since tPMP-1 is cationic under the physiological range of pH, we hypothesized that this peptide may also directly permeabilize anionic membranes. The apparatus for the analysis of bilayer conductance consisted of a Teflon chamber with two separate compartments, which contained 1 M KCl (pH 7.0) and were connected by a conduit (0.2 mm^2). Black lipid bilayer membranes were formed across this space by using 1.5% (wt/vol) of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) (7:3) (Avanti Polar Lipids Inc., Birmingham, Ala.) in *n*-decane. This ratio of lipid constituents was used because it reproducibly yields stable, net anionic bilayers. A voltage potential was generated across the membrane with a pair of calomel electrodes (Metrohm). One of the electrodes was linked to a direct-current voltage source (Omnical 2000; W. P. Instruments), and the complementary electrode was connected to the measuring circuit, consisting of a current amplifier and chart recorder. Voltage orientation was defined by the addition of HPLC-purified tPMP-1 (40 ng/ml) to one side of the membrane (designated the *cis* side). A *trans*-negative potential (indicated by a minus sign) was generated by applying a negative potential to the compartment opposite to the *cis* side (i.e., the *trans* side). All experiments were performed at room temperature (~23°C), and conductance across the membrane recorded in nanosiemens. Gramicidin A (4 μg/ml), a voltage-independent channel-forming ionophore, was used as a positive control for membrane permeabilization at a transmembrane voltage of −60 mV (13).

RESULTS

tPMP-1 induction of staphylococcal protoplast lysis. The ability of tPMP-1 to induce lysis of staphylococcal protoplasts was determined spectrophotometrically (by measuring the OD_{420}) over time. A decrease in protoplast OD_{420} was interpreted as protoplast lysis. Untreated LOG or STAT protoplasts of 19S or 19R in MEM buffer exhibited minor degrees of spontaneous lysis (change in OD_{420} = 0.02 to 0.05) over the 3-h study period (data not shown). No substantial differences were observed among these controls. tPMP-1 induced the greatest extent of lysis in 19S LOG protoplasts, with a mean decrease of ~0.2 absorbance unit (Fig. 1A). Lysis of the protoplasts was observed after 30 min of exposure to tPMP-1, and the decline in absorbance thereafter was rapid and progressive (Fig. 1B). 19S STAT protoplasts were significantly less susceptible to lysis by tPMP-1 than were 19S LOG protoplasts (Fig. 1), exhibiting only a slight decrease in absorbance over 3 h of exposure to tPMP-1. 19R LOG and STAT protoplasts were also significantly less susceptible to lysis than were 19S LOG protoplasts. Both were relatively resistant to lysis compared to 19S LOG protoplasts (Fig. 1A).

tPMP-1 disruption of staphylococcal protoplast membranes. The ultrastructural consequences of exposing staphylococcal protoplasts to tPMP-1 were visualized by TEM. Control protoplasts of 19S LOG (Fig. 2A), 19S STAT, 19R LOG, and 19R STAT (data not shown) in MEM buffer alone remained relatively intact during the 3-h experiment. No significant differences in the ultrastructural appearance between 19S and 19R control protoplasts were observed. At 0 min, LOG and STAT protoplasts of 19S and 19R in tPMP-1 appeared intact and comparable to controls in MEM buffer (data not shown). Damage to 19S LOG protoplast membranes was observed after a 15-min exposure to tPMP-1, with staphylococcal

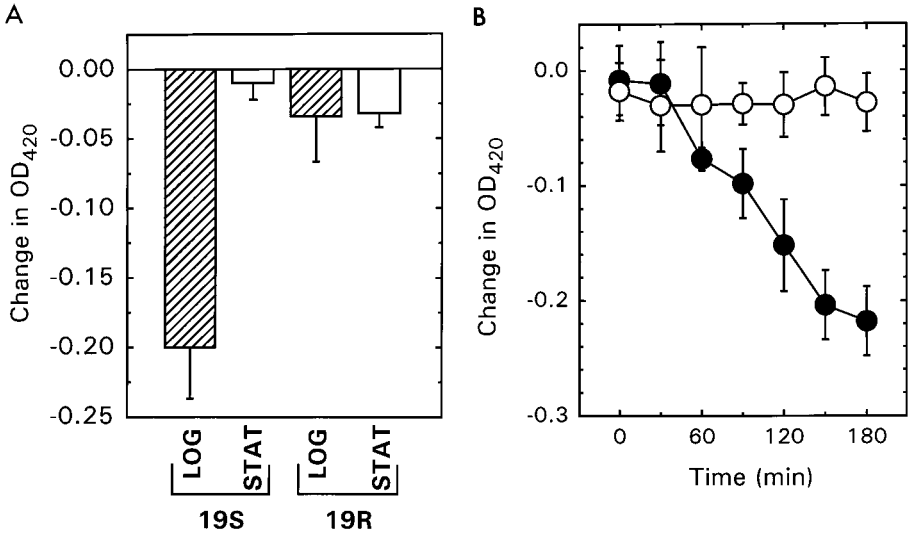

FIG. 1. Effect of tPMP-1 on protoplast absorbance. The OD_{420} of LOG or STAT protoplasts of 19S or 19R during exposure to crude tPMP-1 for 3 h at 37°C was determined as described in the text (initial OD_{420}~0.55). A decrease in absorbance was indicated as a negative value of OD_{420}. The data shown represents the mean (± standard deviation) of at least three separate experiments. (A) Total change in absorbance after 3 h of exposure to tPMP-1; (B) temporal change in the optical density of LOG (●) or STAT (○) protoplast suspensions of 19S.

membranes disrupted at single or multiple sites (Fig. 2B and C). 19S LOG protoplast membranes exhibited the most extensive damage compared with either 19S STAT, 19R LOG, or 19R STAT protoplasts (Fig. 2). Of note, minor degrees of membrane damage were observed in 19S STAT protoplasts (Fig. 2D), whereas 19R LOG and STAT protoplasts remained essentially intact during tPMP-1 exposure (Fig. 2E and F).

tPMP-1 permeabilization of planar lipid bilayers. Membrane permeabilization due to gramicidin A was consistent with that reported previously, confirming the utility of our lipid bilayer model system (data not shown) (13). Membranes in buffer alone remained stable and exhibited minimal conductance for up to 40 min when maintained at a transmembrane potential of either −30 or −90 mV (data not shown). No change in membrane conductance was observed for 40 min at either −30 or −90 mV when 0.01% (vol/vol) acetic acid buffer (pH 5.5) was added in an excess volume to the *cis* side of the membrane (Fig. 3A). Subsequent addition of purified tPMP-1 (final concentration of 40 ng/ml in 0.01% [vol/vol] acetic acid buffer [pH 5.5]) to the same compartment at a transmembrane potential of −30 mV produced no change in conductance over the ensuing 40 min (Fig. 3B). However, as the transmembrane voltage was increased from −30 to −90 mV, conductance due to tPMP-1 was observed to increase within 20 min (Fig. 3C). Fluctuations in conductance ranging from 0.21 to 1.61 nS (mean, ~1 nS), typically lasting for millisecond durations, were noted. Once initiated, decreasing the voltage from −90 to −30 mV diminished but did not abolish membrane conductance (mean, ~0.2 nS) (Fig. 3D). Likewise, further reduction of the voltage to 0 mV for 5 min and then back to −30 mV did not eliminate conductance (data not shown).

DISCUSSION

tPMP-1 is believed to play an important role in host defense against the induction and progression of endovascular infections. Endothelial cells damaged or colonized by microorganisms release tissue factor, which in turn generates thrombin from prothrombin (8, 9). We and others have recently shown that thrombin prompts the release of tPMPs and analogous peptides from rabbit and human platelets in vitro, respectively (30, 31, 40). Furthermore, recent evidence indicates that microorganisms themselves may also stimulate the release of PMPs or tPMPs from platelets during platelet aggregation (1). Thus, we have hypothesized that tPMPs are released from platelets at sites of endovascular damage as a component of host defense to locally kill or limit the proliferation of colonizing pathogens (39).

Many cationic peptides appear to target the bacterial membrane in a voltage-dependent manner. However, their precise mechanisms of action appear to vary considerably. For example, several different membrane permeabilization models have been proposed for the mechanism of action of cationic peptides, including the barrel-stave and the generalized "carpet" models (4, 5, 21). Furthermore, the lantibiotic nisin (a lanthionine-containing antimicrobial peptide) forms pores in target membranes only when the voltage across the membrane is in a *trans*-negative orientation (23). In contrast, a structurally related lantibiotic, subtilin, induces membrane permeabilization with both *trans*-positive and *trans*-negative voltages (25). Interestingly, cationic bacteriocins, such as pediocin JD, appear to destabilize target membranes in a voltage-independent manner (7). Recent work in our group indicates that tPMP-1 may permeabilize target staphylococcal membranes without depolarizing them, unlike other cationic peptides (41). The present studies therefore provide important insights into the mechanism of action of this new family of cationic peptides, which may translate to other antimicrobial peptides.

tPMP-1 is similar to other membrane-targeting cationic antimicrobial peptides in structure and microbicidal activity (10, 15, 19). Therefore, we postulated that tPMP-1, like these other peptides, would also permeabilize the bacterial cytoplasmic membrane in its mechanism of action. Our past investigations, with *S. aureus* whole cells as the model organism, supported this hypothesis. First, a staphylococcal mutant defective in the generation of an intact transmembrane potential ($\Delta\Psi$) exhibited reduced susceptibility to the microbicidal actions of tPMP-1, compared to its parent strain with an intact $\Delta\Psi$ (16). Second, in vitro conditions which reduced bacterial energetics (e.g., low temperature and stationary-phase growth) also sub-

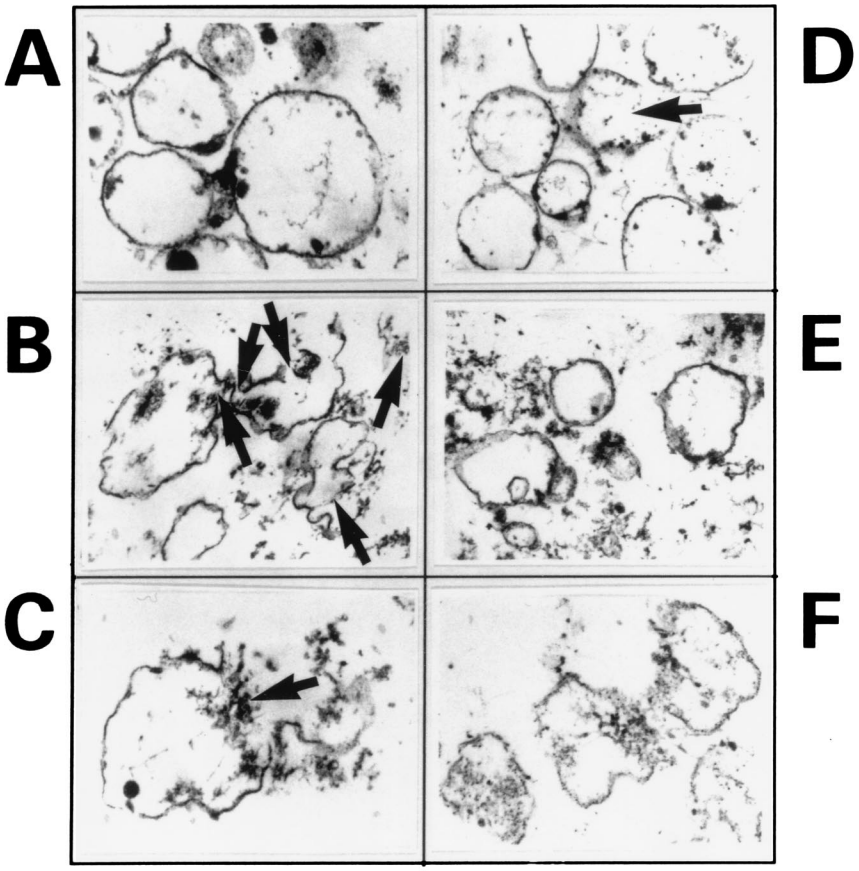

FIG. 2. TEM of tPMP-1-exposed protoplasts. Protoplasts of 19S (LOG or STAT) were exposed to either crude tPMP-1 or MEM buffer. Samples were then analyzed by TEM after 15 min of exposure. (A) Intact 19S LOG protoplast membranes in MEM buffer (magnification, ×136,000); (B) damaged 19S LOG protoplast membranes in tPMP-1 (note the disruption of protoplast membranes at multiple sites); (C) damaged 19S LOG protoplast membrane in tPMP-1 (magnification, ×136,000); (D) partially damaged 19S STAT protoplast membranes in tPMP-1; (E) intact 19R LOG protoplast membranes in tPMP-1; (F) intact 19R STAT protoplast membranes in tPMP-1. Magnification, ×90,750 (except where otherwise indicated).

stantially decreased staphylococcal susceptibility to tPMP-1 (17). Additionally, flow cytometry data indicated that tPMP-1 rapidly permeabilized the staphylococcal membrane to propidium iodide, which has a molecular diameter of 2 nm (41). Finally, TEM of whole staphylococcal cells revealed that tPMP-1 induced membrane disruption within minutes of peptide addition, with the absence of substantial changes in cell wall ultrastructure (35). Of interest, Shimoda et al. have recently demonstrated by TEM that defensins also selectively disrupt the staphylococcal membrane (27). Although suggestive, the above observations did not provide direct evidence to support the notion that tPMP-1 permeabilizes microbial membranes. Thus, the present studies were carried out with cell wall-free staphylococcal protoplasts and planar lipid bilayers to specifically study the effect of tPMP-1 on these membrane targets.

Data obtained from the current investigation strongly support the hypothesis that tPMP-1 directly targets and permeabilizes the staphylococcal cytoplasmic membrane. Staphylococcal protoplasts were lysed by tPMP-1, as detected spectrophotometrically. TEM analysis of the protoplasts further confirmed that tPMP-1 disrupts protoplast membranes at one or more sites. Of note, these data also support the concept that an intact staphylococcal cell wall is not required by tPMP-1 to elicit its membrane effects. Susceptibility of the protoplasts to lysis by tPMP-1 directly reflects the known tPMP-1-susceptibility of whole staphylococcal cells from which the protoplasts were prepared. For example, our previous observations indicate that LOG cells of tPMP-1-susceptible strains are more susceptible to tPMP-1 killing than are STAT cells (17). In this study, protoplasts derived from LOG cells of 19S (tPMP-1 susceptible) were more susceptible to lysis by tPMP-1 than were those derived from STAT cells of the same strain, consistent with whole-cell data. Furthermore, protoplasts derived from 19R (tPMP-1 resistant) were less susceptible to lysis than were protoplasts derived from 19S, reflecting the tPMP-1 susceptibilities of strains 19R and 19S, respectively. Thus, it appears that the staphylococcal susceptibility to tPMP-1 may be influenced, at least in part, by the intrinsic properties of its cytoplasmic membrane. Differences in membrane lipid composition and $\Delta\Psi$ of LOG and STAT cells have been reported before, which may explain the differences in their susceptibilities to tPMP-1 (14, 28, 33). Further, our comparison of tPMP-1-susceptible and tPMP-1-resistant staphylococcal strain pairs indicated that their cytoplasmic membranes are significantly different in bioenergetics, fatty acid and lipid content, and fluidity (2, 3). Thus, it is possible that membrane characteristics contribute to alterations in cationic peptide susceptibility.

Many investigators have used the planar (black) lipid bilayer to study the peptide-membrane interactions of various endogenous antimicrobial peptides, such as cecropins, defensins, and

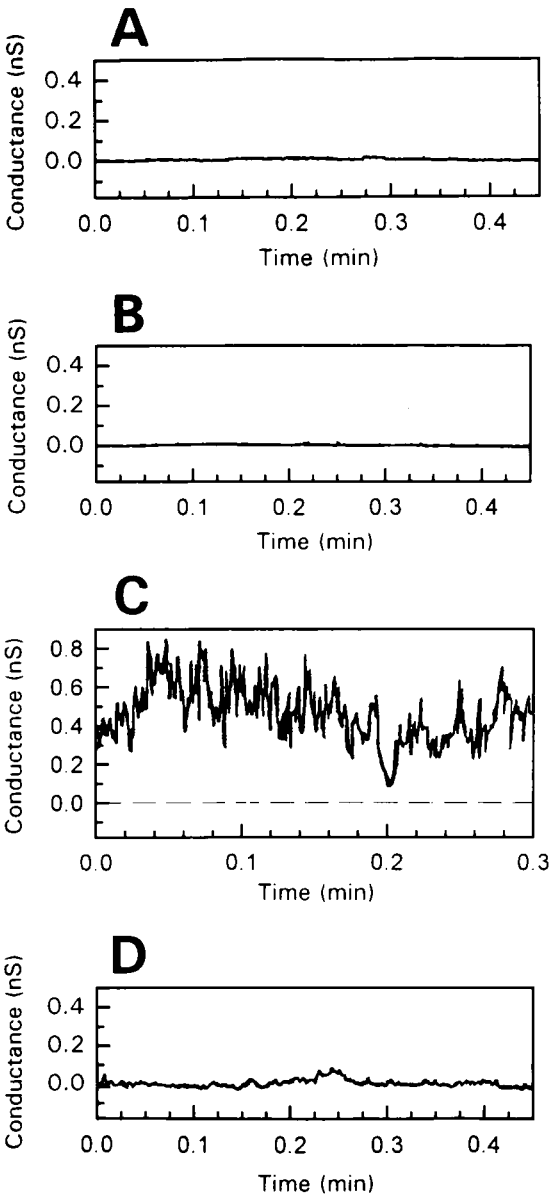

FIG. 3. Permeabilization of planar lipid bilayers by purified tPMP-1. (A) Tracing obtained when an excess volume of 0.01% (vol/vol) acetic acid buffer (pH 5.5) was added to the *cis* side of a planar lipid bilayer membrane held at −90 mV. The voltage was then decreased to −30 mV, and purified tPMP-1 (40 ng/ml) was added to the same compartment. (B to D) Conductance tracings were obtained when the voltage across the membrane was altered in the following sequence: −30 mV (B), −90 mV (C), and −30 mV (D). The experiment was repeated at least once.

lantibiotics (6, 15, 18, 23). These peptides form discrete voltage-gated channels in target membranes, which display distinct electrophysical characteristics. The present planar lipid bilayer studies indicate that tPMP-1 has the ability to permeabilize membranes in a voltage-dependent manner. At a low transmembrane voltage (−30 mV), tPMP-1 did not cause the membrane permeabilization noted at a higher voltage (−90 mV). This finding is consistent with our previous observation that tPMP-1 staphylocidal activity is correlated to $\Delta\Psi$ (16, 41). The present study indicated that once membrane permeabilization was initiated by tPMP-1, altering the transmembrane voltage influenced but did not eliminate tPMP-1 activity. Thus, it appears that initiation of membrane permeabilization by tPMP-1 is voltage dependent but the continuation of its effect is not voltage driven.

The formation of channels by cationic peptides in target membranes has been proposed to occur via the barrel-stave mechanism, whereby membrane-inserted monomers oligomerize in the membrane to form stable pores (21). A different model of membrane permeabilization, namely, the carpet effect, has also been postulated for several cationic peptides including the amphibian peptide magainin (4, 5). According to this model, peptide monomers bind to the surface of target membranes by hydrophobic interactions. As a threshold peptide concentration is reached, the membrane becomes unstable, leading to increases in membrane permeabilization. In the present study, lipid bilayers exposed to tPMP-1 revealed conductances with heterogeneous fluctuations which lacked the discrete, stepwise increases typical of channel-forming peptides (15, 18, 23). Therefore, tPMP-1 may not form classical voltage-gated membrane channels. Instead, it is likely that tPMP-1 induces nonuniform membrane permeabilization such as that proposed in the carpet model.

Previous flow cytometry data indicate that functional membrane permeabilization is initiated within seconds of tPMP-1 addition to whole staphylococcal cells (41). However, concurrent viability data in the same experiments indicate that a significant extent of cell death does not occur until after 30 min of exposure to tPMP-1 (41). The present protoplast TEM and spectrophotometric studies indicate that structural membrane disruption by tPMP-1 occurs 15 to 30 min after exposure, consistent with previous TEM data of whole staphylococcal cells (35). Studies of the mechanism of action of other cationic, membrane-permeabilizing antimicrobial peptides revealed possibilities of secondary targets beyond the cytoplasmic membrane. The wide variety of metabolic changes observed include alterations in cell transport; inhibition of DNA, RNA, and protein synthesis; and decreases in translation efficiency (11, 12, 20, 22, 32). Thus, it is reasonable to hypothesize that during the 15- to 30-min lag period between functional membrane permeabilization and structural membrane disruption by tPMP-1, the peptide could be targeting a secondary site, which ultimately contributes to killing of the bacteria. We are aware that LOG and STAT cells may also vary in other aspects of cell physiology besides membrane lipid composition and $\Delta\Psi$ (e.g., macromolecular or protein synthesis), which may be important in their relative susceptibility to tPMP-1.

Studies are in progress to further characterize the mechanism of membrane permeabilization by tPMP-1 with planar lipid bilayers and liposomes. In these investigations, membranes with different lipid compositions are being used to mimic the lipid content of isogenic tPMP-1-susceptible and -resistant staphylococcal strains (2). We are also investigating the effect of tPMP-1 on other cellular functions (e.g., macromolecular synthesis) in an attempt to identify possible secondary targets. By determining the bactericidal target of endogenous antimicrobial peptides such as tPMP-1, we may improve our understanding of the mechanism of action of these agents. This information may facilitate the development of new antimicrobial agents with improved specificity and potency.

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