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Correlation of Daptomycin Bactericidal Activity and Membrane Depolarization in *Staphylococcus aureus*

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The objective of this study was to further elucidate the role of membrane potential in the mechanism of action of daptomycin, a novel lipopeptide antibiotic. Membrane depolarization was measured by both fluorimetric and flow cytometric assays. Adding daptomycin (5 μg/ml) to *Staphylococcus aureus* gradually dissipated membrane potential. In both assays, cell viability was reduced by >99% and membrane potential was reduced by >90% within 30 min of adding daptomycin. Cell viability decreased in parallel with changes in membrane potential, demonstrating a temporal correlation between bactericidal activity and membrane depolarization. Decreases in viability and potential also showed a dose-dependent correlation. Depolarization is indicative of ion movement across the cytoplasmic membrane. Fluorescent probes were used to demonstrate Ca^{2+}-dependent, daptomycin-triggered potassium release from *S. aureus*. Potassium release was also correlated with bactericidal activity. This study demonstrates a clear correlation between dissipation of membrane potential and the bactericidal activity of daptomycin. A multistep model for daptomycin's mechanism of action is proposed.

Daptomycin is a novel lipopeptide antibiotic in late-stage clinical development for the treatment of serious gram-positive infections. Daptomycin exhibits rapid in vitro bactericidal activity against clinically significant strains of gram-positive pathogens including hemolytic streptococci, methicillin-resistant *Staphylococcus aureus*, and vancomycin-resistant enterococci (4, 10, 12, 14, 19, 22, 23). Daptomycin acts at the cytoplasmic membrane of susceptible bacteria (8), as demonstrated by binding and fractionation studies. Additionally, the activity of daptomycin is dependent on the presence of physiologic levels of free calcium ions (50 mg/liter).

Debate over daptomycin's mechanism of action has continued for more than a decade. One hypothesis suggests that daptomycin bactericidal activity is mediated by inhibition of lipoteichoic acid (LTA) biosynthesis (7, 8). However, a recent investigation has failed to find evidence of a role for LTA in the mechanism of action of daptomycin in *S. aureus* or *Enterococcus faecalis*, suggesting that the in vitro bactericidal activity of daptomycin is independent of LTA biosynthesis (V. Laganas, J. Alder, and J. A. Silverman, submitted for publication). A second proposed mechanism of action for daptomycin is that the antibiotic causes dissipation of bacterial membrane potential, resulting in disruption of multiple aspects of cellular function (1, 2). Bactericidal activity via disruption of membrane potential is the proposed mechanism of action for a variety of antimicrobial peptides, including the pore-forming antibiotic nisin (18, 20). We wished to further investigate the role of bacterial membrane potential in the mechanism of action of daptomycin. In this study, we demonstrate a significant correlation between membrane depolarization and bactericidal activity. Furthermore, we demonstrate that one possible mechanism of membrane depolarization involves K^+ release by bacteria on daptomycin exposure (J. A. Silverman, N. G. Perlmutter, and H. M. Shapiro, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-1800, 2001).

MATERIALS AND METHODS

Strains, media, and antibiotics. *S. aureus* ATCC 29213 was grown in Mueller-Hinton broth (Becton Dickinson, Cockeysville, Md.) supplemented with 50 mg of Ca^{2+}/liter (MHBc). The MIC of daptomycin was determined as previously described (21). Daptomycin was provided by Cubist Pharmaceuticals, Inc. (Lexington, Mass.); nisin and valinomycin were obtained from Sigma Chemical Company (St. Louis, Mo.). Using MHBc and Mueller-Hinton agar, bacterial cell viability was determined by following National Committee for Clinical Laboratory Standards guidelines (16).

Fluorimeter assay for membrane potential. Membrane potential was measured by using a fluorescent assay based on the method of Wu and Hancock (24). *S. aureus* cultures grown to the early exponential phase (optical density at 600 nm [OD_{600}], 0.2 to 0.3) in MHBc were treated with no antibiotic (control), daptomycin (as indicated), nisin (25 μg/ml), or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (10 μM) at 37°C. Samples (2 ml) were transferred to a polystyrene fluorimeter cuvette (VWR International, West Chester, Pa.) containing a stir bar and placed in the heated (37°C) sample chamber of an AMINCO-Bowman series 2 luminescence fluorimeter (Thermo Spectronic, Rochester, N.Y.). The cells were excited at 622 nm, and the fluorescence emission was collected at 670 nm. Background data were collected for 30 s before the addition of $DiSC_3(5)$ (3,3′-dipropylthiadicarbocyanine iodide; Molecular Probes, Eugene, Oreg.) to a final concentration of 100 nM. Data were collected for an additional 5 min after the addition of the fluorescent dye.

In a modified form of the assay described above, bacteria were grown to the early exponential phase in culture flasks (OD_{600}, 0.2 to 0.3) and 2.5-ml aliquots were transferred to 14-ml round-bottom polystyrene tubes. The bacterial cells were subsequently treated with no antibiotic (control), daptomycin (as indicated), or nisin (25 μg/ml) at 37°C. At appropriate time points, $DiSC_3(5)$ (100 nM final concentration) was added directly to the round-bottom tubes and the samples were incubated for an additional 5 min at 37°C. Samples (2 ml) were transferred to cuvettes without a stir bar, cells were excited at 622 nm, and the fluorescence emission was collected at 670 nm for 10 s.

Flow cytometry. The membrane potential was determined by flow cytometry by using a laboratory-built dual-laser instrument, as previously described (17), with $DiOC_2(3)$ (3,3′-diethyloxacarbocyanine iodide; Molecular Probes). Exponential-phase (OD_{600} = 0.3) *S. aureus* cultures grown in MHBc were treated with no antibiotic (control), 5-μg/ml daptomycin, or 25-μg/ml nisin at 37°C. At the

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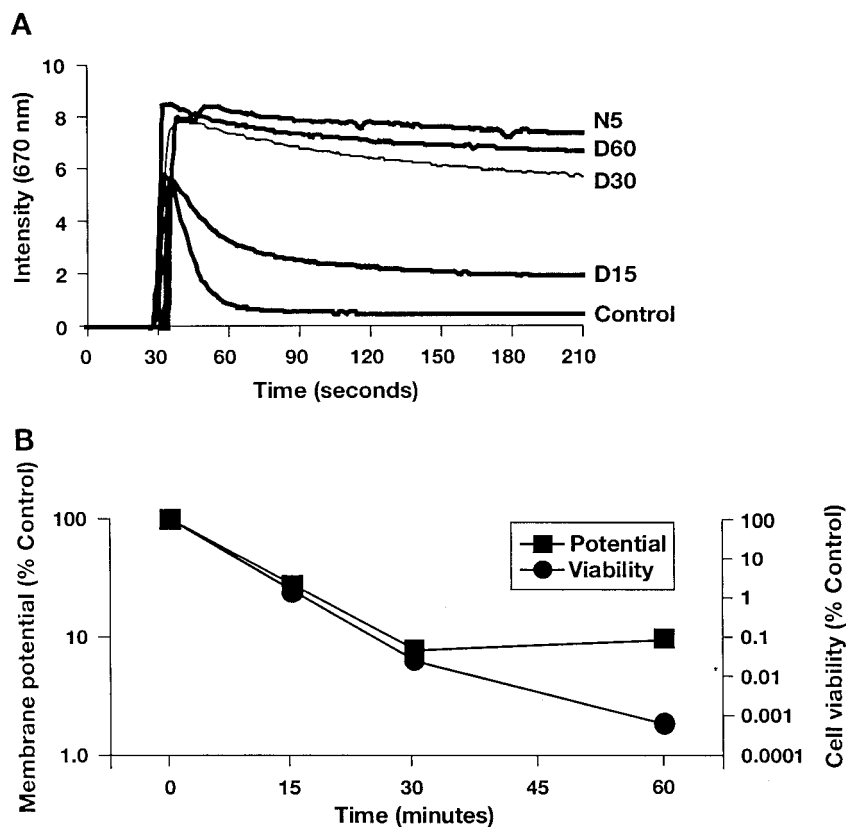
FIG. 1. Daptomycin dissipates membrane potential in *S. aureus*, as measured by fluorimetry. *S. aureus* cell samples were incubated for 0 (control), 15 (D15), 30 (D30), or 60 (D60) min with 5 μg of daptomycin/ml or for 5 min with 25 μg of nisin (N5)/ml. (A) Individual traces. (B) Changes in membrane potential and cell viability, expressed as percentages of the untreated control.

indicated time points, samples were stained for 4 min at room temperature with 30 μM DiOC$_2$(3) and 100 nM To-Pro-3 iodide (Molecular Probes). Flow cytometry was performed on 5,000 *S. aureus* cells per run by using the 488-nm beam from an argon ion laser to excite the DiOC$_2$(3); the green fluorescence was detected through a 530- to 20-nm bandwidth band-pass filter, and the red fluorescence was detected through a 610- to 19-nm bandwidth band-pass filter. The membrane potential was measured as the intensity ratio of the red fluorescence (a membrane potential-dependent signal) and the green fluorescence (a membrane potential-independent signal). The 633-nm beam from a helium-neon laser was used to excite To-Pro-3 iodide; elevated far red fluorescence from this dye (detected through a 695-nm long-pass filter) is indicative of membrane damage.

Potassium ion release assay. Potassium ion release assays were performed by following the method of Herranz et al. (11). *S. aureus* was grown to the exponential phase in MHBc (OD$_{600}$, 0.3) and washed twice with and resuspended in a one-fifth volume of 10 mM HEPES (pH 7.2) and 0.5% glucose. Samples (2 ml) were transferred to a polystyrene fluorimeter cuvette containing a stir bar and placed in the heated (37°C) fluorimeter sample chamber. Bacterial cells were excited at 346 nm, and the fluorescence emission was collected at 505 nm for 30 s before the addition of 1,3-benzenedicarboxylic acid, 4,4′-[1,4,10,13-tetraoxa-7,16-diazacyclooctadecane-7, 16-diylbis(5-methoxy-6,2-benzofurandiyl)]bis (PBFI; Molecular Probes) to a 1 μM final concentration. Data were collected for an additional 2 min to establish a baseline signal before the addition of test compounds. For the control sample, 10 μg of valinomycin/ml was added to the *S. aureus* cells and followed 15 min later by K$^+$ at a 1 mM final concentration to demonstrate continued indicator responsiveness. For some daptomycin-treated samples, bacterial cells were incubated for 5 min after the addition of daptomycin (1 to 10 μg/ml) and before the addition of Ca^{2+} (50 mg/liter, final concentration). Data were normalized relative to the fluorescent signal before the addition of calcium (average of the last 10 values prior to addition).

Statistical methods. In the fluorimeter membrane potential experiments, some data were expressed as a percentage of the control, determined by the following: [(max/min)$_{control}$/(max/min)$_{sample}$] × 100%, where max was defined as the max-

imum fluorescent signal in the trace (usually <10 s after dye addition) and min was defined as the minimal signal (the average signal of the last 10 s of the trace) for the control or antimicrobial agent-treated samples. In flow cytometry experiments, some data were expressed as the percent polarized, which was defined as the total number of cells with a fluorescence intensity ratio of >135 arbitrary units divided by the total number of cells counted ($n = 5,000$).

RESULTS

Membrane depolarization correlates with bactericidal activity. Research has previously demonstrated that daptomycin is capable of reducing the membrane potential of *S. aureus* (1). However, these studies were performed under conditions in which it was impossible to monitor effects on cell viability and at relatively high (100 μg/ml) drug concentrations. We have reexamined daptomycin's effects on membrane potential with an assay designed to allow simultaneous monitoring of potential and viability under conditions similar to those used for MIC or bactericidal testing. The assay uses DiSC$_3$(5), a membrane potential-sensitive fluorescent probe. The fluorescence of DiSC$_3$(5) decreases as the dye partitions to the surface of polarized cells; depolarization prevents partitioning and can release bound dye into the media. Hence, control cells (no drug treatment) produce a low signal and depolarized cells produce a high signal (24). As shown in Fig. 1A, the addition of daptomycin at 5 μg/ml (approximately eight times the MIC) gradually dissipated the membrane potential in *S. aureus*. Full
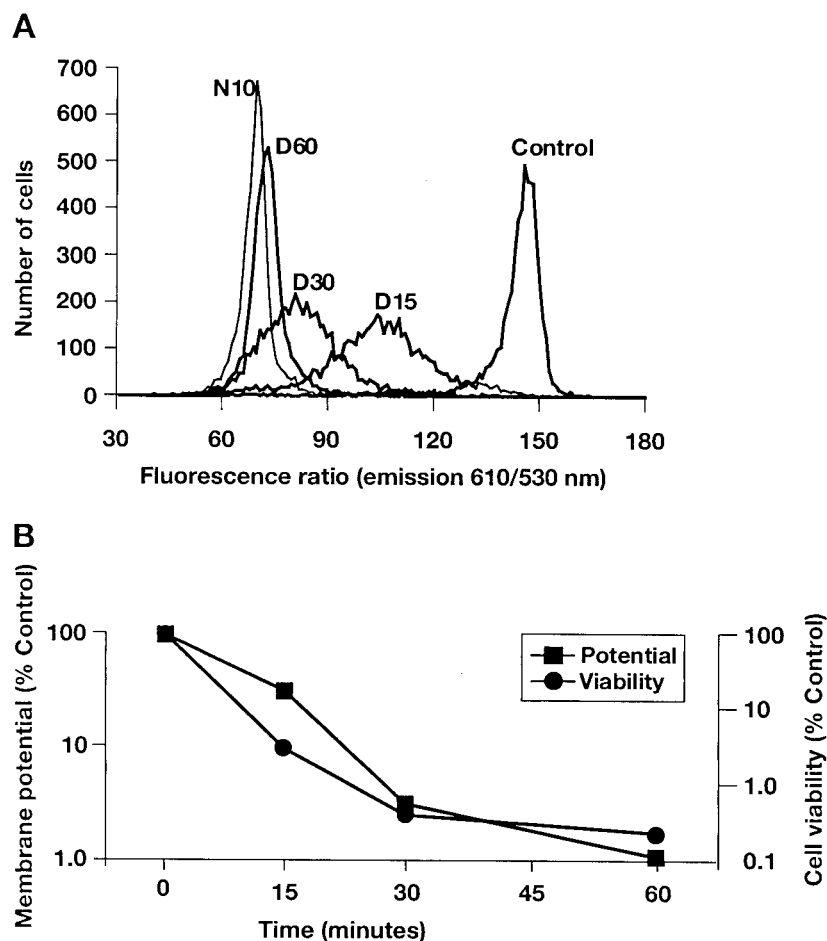
FIG. 2. Daptomycin dissipates membrane potential in *S. aureus*, as measured by flow cytometry. *S. aureus* cell samples were incubated for 0 (control), 15 (D15), 30 (D30), or 60 (D60) min with 5 μg of daptomycin/ml or for 10 min with 25 μg of nisin (N10)/ml. (A) Individual traces. (B) Changes in membrane potential (as measured by the ratio of fluorescence emission intensities at 610 and 530 nm) and cell viability, both expressed as percentages of the untreated control.

depolarization required 30 to 60 min compared with <5 min for the pore-forming antimicrobial nisin (25 μg/ml, four times the MIC) (Fig. 1A) and the proton ionophore CCCP (data not shown). Cell viability decreased in parallel with the changes in membrane potential (Fig. 1B), demonstrating a temporal correlation between these two processes. After 30 min, <0.1% of cells remained viable and membrane potential was reduced to <10% of that of the control. The apparent lack of correlation between membrane potential and cell viability at the 60-min time point probably reflects the limited sensitivity of the depolarization assay.

To confirm the results observed with the fluorimetric assay, membrane potential was also studied via flow cytometry with DiOC$_2$(3), a membrane potential-sensitive dye capable of ratiometric measurements of bacterial membrane potential (17). Similar to the fluorometric analysis, flow cytometric assays were performed concurrently with cell viability measurements. Daptomycin gradually dissipated the membrane potential in this assay, with full membrane depolarization observed between 30 and 60 min (Fig. 2A). This timeframe was consistent with the fluorometric analysis results. Further examination of the flow cytometry data suggested that the entire population of

S. aureus cells was progressively depolarized, resulting in a wide but unimodal distribution. This distribution suggests that membrane potential in individual bacteria is lost gradually rather than instantaneously. As demonstrated with the fluorometric analysis, there was also a close correlation between the kinetics of membrane depolarization and cell viability (Fig. 2B). Cell viability was reduced to <1% and membrane potential was <10% of that of the control. The sensitivity of the flow cytometry assay allowed the correlation to be demonstrated throughout the 60-min time course. Membrane permeability was monitored simultaneously by measuring the uptake of the membrane-impermeant fluorescent indicator To-Pro-3 iodide. To-Pro-3 iodide fluorescence was not increased in cells treated with daptomycin for as long as 60 min, indicating that membrane depolarization was not caused by rupture or lysis of the membrane (data not shown).

Recently, published studies have demonstrated that some antimicrobial peptides generally believed to act via disruption of membrane function may actually have intracellular targets and that membrane depolarization may only occur in response to artificially high drug concentrations (9). Therefore, we used low doses of daptomycin (one to four times the MIC [MIC =
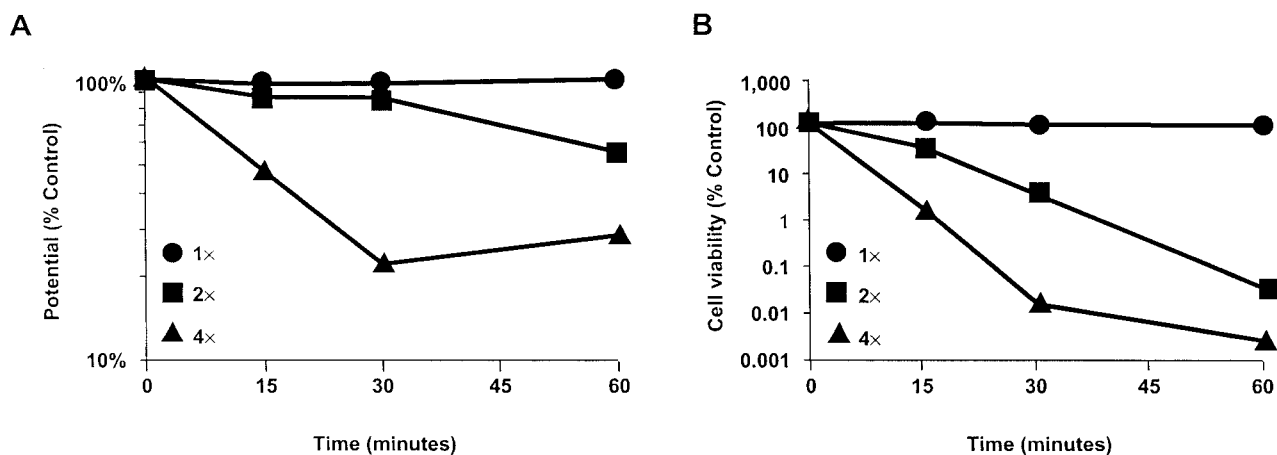
FIG. 3. Dose response for membrane potential and cell viability. Membrane potential (A) and cell viability (B) were measured by using the fluorimetry assay after daptomycin treatment at one, two, and four times the MIC. Results from both assays were graphed as percentages of the untreated control.

0.78 μg/ml]) to measure daptomycin bactericidal activity and membrane depolarization in the fluorimeter assay (results are shown in Fig. 3). At daptomycin doses as low as the MIC, bactericidal activity and membrane depolarization were correlated for daptomycin-treated *S. aureus* cells. The activity of low daptomycin doses was confirmed by using the flow cytometry assay (data not shown).

Daptomycin triggers potassium efflux in *S. aureus*. Dissipation of membrane potential requires ion movement across the cytoplasmic membrane. Using the K^+-sensitive fluorescent probe PBFI, we examined the ability of daptomycin to trigger potassium release. The addition of PBFI to a suspension of *S. aureus* cells yielded a stable fluorescent signal (Fig. 4). Adding the potassium ionophore valinomycin resulted in an immediate signal increase consistent with the release of intracellular K^+ into the surrounding medium. Adding daptomycin to *S. aureus* also triggered a similar signal increase (Fig. 5A), accompanied by a cell viability decrease. This result suggests that one component of the mechanism of action of daptomycin is the release of K^+. The antibacterial activity of daptomycin is dependent on the physiologic levels of calcium ions. Daptomycin-dependent K^+ release was observed only in the presence of Ca^{2+}. As illustrated in Fig. 5B, incubation of *S. aureus* cells for 10 min with 5-μg/ml daptomycin in the absence of Ca^{2+} resulted in no significant increase in signal or decrease in cell viability. (The modest change in fluorescence intensity detected upon addition of daptomycin also occurred in the absence of bacterial cells [data not shown].) Adding calcium to the daptomycin-containing sample resulted in an immediate increase in fluorescence, consistent with a rapid release of K^+. This was accompanied by a rapid loss of cell viability, as measured 15 min after the addition of calcium. Finally, as demonstrated in the membrane potential experiments, there was a clear correlation between bactericidal activity and the extent and initial rate of K^+ release at daptomycin doses between 10 and 1 μg/ml (Fig. 6), although the limited sensitivity of the assay compresses the dose response.

DISCUSSION

Daptomycin is a novel antibiotic with rapid in vitro bactericidal activity against gram-positive organisms. Although the mechanism of action has not been fully elucidated, previous studies have suggested that daptomycin activity results in the dissipation of cytoplasmic membrane potential (1). In these earlier studies, membrane potential was monitored in *S. aureus* by using the equilibration of the membrane-permeant anion tetraphenylphosphonium bromide with bacterium and daptomycin concentrations of 10^{10} CFU/ml and 100 μg/ml, respectively (1). Bacterial viability measurements were not made and no correlation could be established between membrane depolarization and daptomycin bactericidal activity. Therefore, we measured membrane potential under conditions typically used for determining the time-kill kinetics of daptomycin—10^6 to 10^7 CFU of mid-exponential-phase cells/ml in MHBc, with daptomycin concentrations of one to eight times the MIC.

Exposure to daptomycin gradually (within 30 to 60 min) dissipated the membrane potential in *S. aureus* cells. The relatively slow depletion of membrane potential suggests a novel mechanism of action compared with other antimicrobials. For example, the pore-forming peptide nisin and the proton ionophore CCCP disrupted the membrane potential in <5 min. The mechanism of action of daptomycin also differs from that of some antimicrobial peptides (e.g., human neutrophil peptide 1) that are capable of rapid depolarization of the cytoplasmic membrane but do not induce cell death for 1 to 2 h (26, 27). It has been suggested that membrane depolarization is not the primary target of some antimicrobial peptides but that depolarization is required to facilitate antibiotic entry into the bacteria for expression of activity (9). This requirement could account for a lag time between membrane depolarization and bacterial killing. The lack of any kinetic separation between depolarization and killing in daptomycin-treated cells strongly suggests that depolarization is the primary mechanism of action. The correlation between membrane depolarization and bactericidal killing observed even at one to two times the MIC
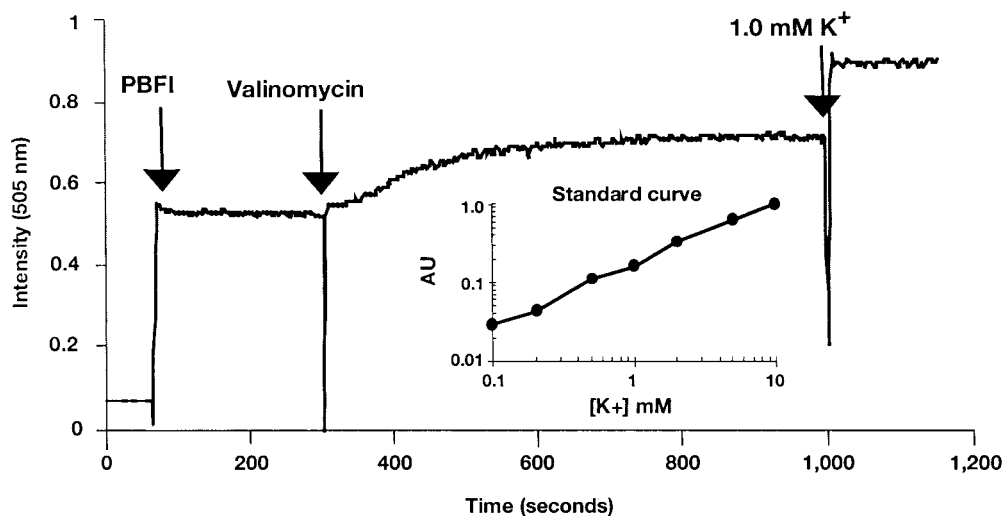
FIG. 4. Potassium release assay. PBFI (1 μM), valinomycin (10 μg/ml), and KCl (1 mM final concentration) were added at the indicated time points to *S. aureus* in HEPES-glucose solution. AU, arbitrary unit.

further supports this idea and suggests that membrane depolarization is not the result of artificially high antibiotic concentrations. The consistent correlation between these two activities strongly suggests that membrane depolarization is an inherent part of the mechanism of action of daptomycin.

Establishing the importance of membrane depolarization in daptomycin's bactericidal activity prompted an attempt to begin to discern the detailed mechanism of cell depolarization. Bacteria maintain membrane potential by establishing multiple ion gradients across the cytoplasmic membrane. Because
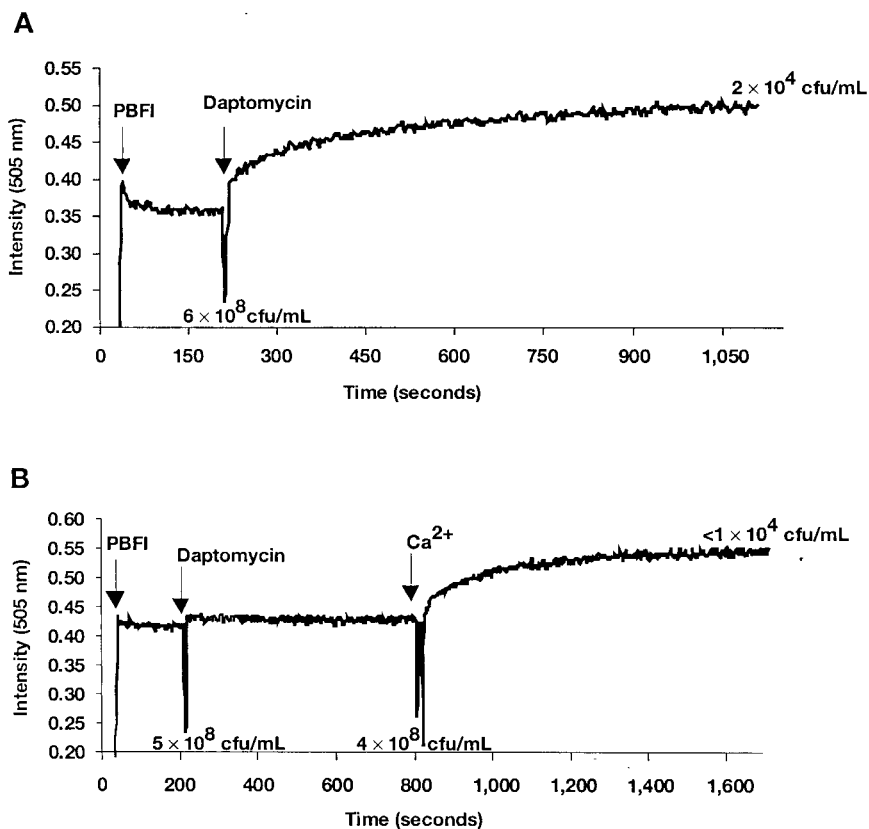

FIG. 5. Daptomycin triggers potassium release from *S. aureus*. (A) PBFI (1 μM) and daptomycin (5 μg/ml) were added to *S. aureus* in HEPES-glucose solution containing 1 mM CaCl₂ at the indicated time points. (B) PBFI (1 μM), daptomycin (5 μg/ml), and Ca²⁺ (50 mg/liter) were added to *S. aureus* at the indicated time points. For both experiments, viable cell counts were determined at the indicated time points.
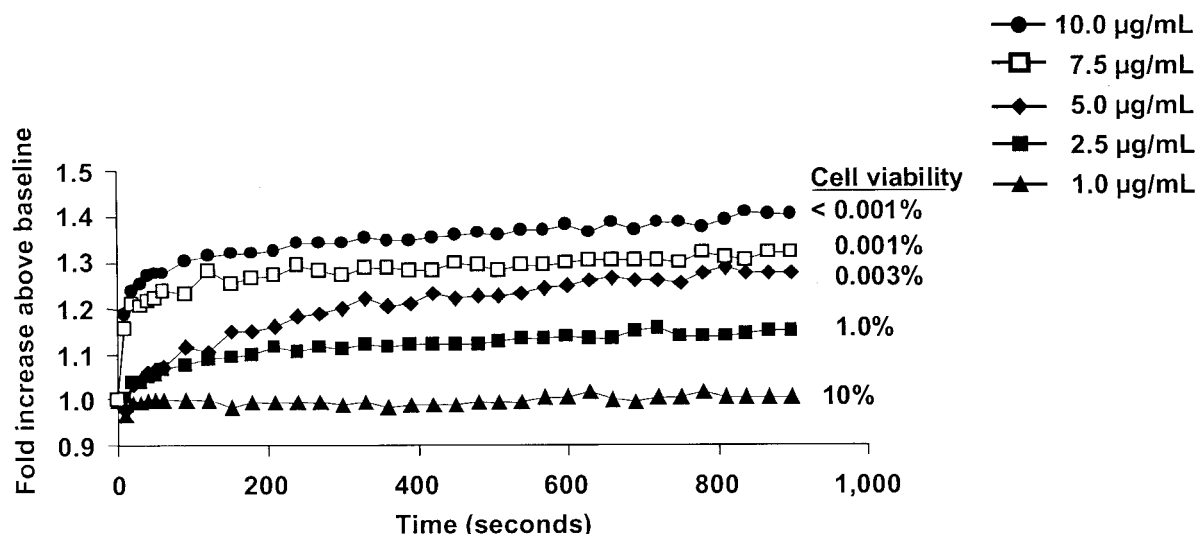
FIG. 6. Bactericidal activity and potassium release. Potassium release was triggered by the addition of 1 mM $CaCl_2$. Data were normalized to the pre-calcium addition baseline values. The cell viability determined 15 min after calcium addition is indicated for each daptomycin dose level.

proper maintenance of the K^+ gradient is important to bacterial viability, we measured K^+ release during exposure to daptomycin in *S. aureus*. Adding daptomycin resulted in a calcium-dependent release of K^+ in *S. aureus*. This calcium-dependent activity supports previous observations indicating that physiologic concentrations of calcium are required for daptomycin activity (3, 13). In addition, potassium release and viability loss were correlated. In these experiments, bactericidal activity was more rapid compared than in membrane depolarization studies. This increased activity resulted from the use of HEPES-glucose assay media in these experiments as opposed to the rich media (MHBc) used in the membrane potential assays. The analysis of the K^+ gradient during daptomycin exposure suggests that K^+ release may be responsible for membrane depolarization or that K^+ may be one of several ions released during membrane depolarization by daptomycin.

We have demonstrated a clear correlation between daptomycin bactericidal activity and membrane potential dissipation and the calcium-dependent release of potassium from daptomycin-exposed cells. We have proposed a multistep model for

the mechanism of action of daptomycin based on the results of these and previously published experiments (Fig. 7). During step 1 of the process, daptomycin inserts into the cytoplasmic membrane of bacteria, as indicated by whole-cell and artificial membrane studies (8, 13). Artificial bilayer studies suggest that insertion can occur in the absence of a bacterium-specific component; however, the possible existence of a gram-positive specific receptor for daptomycin has not been ruled out. Step 2 postulates an as-yet-unproven oligomerization event: daptomycin oligomers could form ion channels, larger nonspecific pores, or irregular aggregate structures (25). The latter have been implicated in the mechanism of action of iturin A, an antifungal membrane-active lipopeptide (15). Alternatively, a cellular component could contribute to this process (5, 6). Formation of any of these structures disrupts the functional integrity of the membrane and triggers a release of intracellular ions (e.g., K^+) leading to rapid cell death (step 3). Additional whole-cell and in vitro studies are being conducted to understand the behavior of other ions (e.g., Na^+ and H^+) and larger molecules (e.g., ATP and proteins) in the presence of
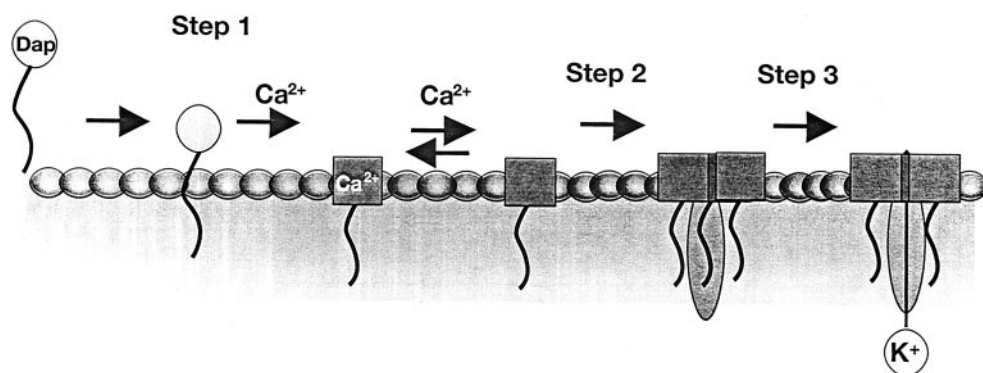

FIG. 7. Proposed model for the bactericidal mechanism of action of daptomycin. Daptomycin (Dap) inserts into the bacterial cytoplasmic membrane in a calcium-dependent fashion and is followed by oligomerization and disruption of the functional integrity of the cytoplasmic membrane (as described in the text), triggering a release of intracellular ions and rapid cell death.

daptomycin to determine the nature of the proposed ion-conducting structure.

These studies support the hypothesis that the in vitro bactericidal activity of daptomycin is a result of cytoplasmic membrane potential dissipation in gram-positive organisms. This mechanism of action is novel compared with classes of antibiotics currently marketed; the relatively gradual rates of depolarization suggest the mechanism of action is also novel compared with other membrane-targeted compounds. Daptomycin may provide another critical line of defense against increasingly antibiotic-resistant gram-positive pathogens.

ACKNOWLEDGMENTS

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