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Daptomycin Disrupts Membrane Potential in Growing *Staphylococcus aureus*

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Daptomycin (LY146032) caused a calcium-dependent dissipation of the membrane potential ($\Delta\Psi$) in *Staphylococcus aureus* without noticeably affecting the chemical gradient (ΔpH) across the membrane. The effect of daptomycin on membrane energization may account for many of the inhibitory effects on macromolecular biosyntheses and membrane function reported for this antibiotic. Our evidence indicates that the bactericidal activity of daptomycin is dependent on an available $\Delta\Psi$.

Previous studies have demonstrated effects of daptomycin (LY146032), a calcium-requiring cyclic lipopeptide antibiotic, on both peptidoglycan synthesis and membrane integrity in gram-positive bacteria (4, 7, 11, 12). No specific step in cell wall formation sensitive to inhibition by daptomycin has been identified, but membrane disruption as evidenced by leakage of intracellular potassium (6, 7) activity against an L form of *Staphylococcus aureus* (4), and the calcium-dependent interaction between daptomycin analogs and phospholipid vesicles (14, 15) suggests a membrane-associated target for this antibiotic.

In the present study, we examined the effects of daptomycin on membrane energetics (i.e., the electrochemical proton gradient) in *S. aureus* under conditions where daptomycin is bactericidal. The results of this study and a preliminary report (3) indicate that daptomycin can dissipate membrane potential ($\Delta\Psi$) without affecting the chemical gradient (ΔpH). These findings provide, for the first time, a coherent explanation for the multiplicity of antibacterial effects attributed to this antibiotic, including inhibition of peptidoglycan biosynthesis (6).

MATERIALS AND METHODS

Bacteria. *S. aureus* FDA strain 209P (ATCC 6538P) was used throughout this study. Cultures were stored at −135°C.

Flow cell measurements. $\Delta\Psi$ and ΔpH were estimated by using the flow cell technique described by Ramos et al. (19). Briefly, the apparatus (Bel-Art Products, Pequannock, N.J.) consisted of a lucite block containing an upper and lower chamber separated by a dialysis membrane having a molecular weight cutoff of 6,000 to 8,000. The upper chamber was open to the atmosphere, and the bottom chamber contained influx and efflux portals for the broth medium used. Measurements were made on cells suspended in Mueller-Hinton II (MHII) broth (adjusted to contain 40 to 50 mg of calcium per liter; Baltimore Biological Laboratories). In one experiment, MH broth (without calcium supplement) was used. Experiments were initiated by adding either [^{14}C]tetraphenylphosphonium bromide (TPP^+; 19.2 mCi/mmol), a lipophilic cation that equilibrates across the cytoplasmic membrane with $\Delta\Psi$, or [^{14}C]acetylsalicylic acid (ASA; 57.4 mCi/mmol), a weak acid that equilibrates with the transmembrane pH gradient (interior alkaline) and allows measurement of ΔpH (both from New England Nuclear) to the upper chamber to a final concentration of 0.5 μCi/ml. After equilibration of the isotope between the chambers at room temperature, cells were added to give a final concentration of 10^{10} CFU/ml. Antibiotics and other test compounds were added to the upper chamber after an additional 15 min of incubation. The total volume of the reaction mix in the upper chamber was 0.5 ml; the volume of the lower chamber was 1.0 ml.

Two-milliliter fractions were collected during the course of the experiment from the lower chamber at a flow rate of 2 ml/min. Radioactivity was determined from 1-ml aliquots mixed with 9 ml of Aquassure (DuPont) counted in a Beckman model LS3811 liquid scintillation counter.

In some experiments, steady-state distribution data from the [^{14}C]TPP^+ flow cell experiments were used to calculate $\Delta\Psi$ by using the Nernst equation: $\Delta\Psi = 58.8 \log([TPP^+_{in}/TPP^+_{out}])$ (19). Concentration gradients were calculated on the basis of a *S. aureus* cell volume of 4.2 μl of intracellular fluid per mg of cell protein (16).

Daptomycin stability. Stability of daptomycin to various pH conditions was tested by dissolving the antibiotic in MHII broth adjusted to different pH values with either 5.0 N NaOH or 5.0 N HCl. Microbiological activity was determined by a disk diffusion assay on swabbed agar plates incubated overnight at 35°C with *S. aureus* 209P (10^8 CFU/ml of agar) as the indicator organism.

Chemicals. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 2,4-dinitrophenol (DNP), nigericin, *N,N'*-dicyclohexylcarbodiimide (DCCD), nisin, and valinomycin were purchased from Sigma Chemical Corporation, St. Louis, Mo. Cefamandole, daptomycin, erythromycin, tobramycin, and vancomycin were obtained from Eli Lilly & Co., Indianapolis, Ind. The MICs of some of these compounds in both MH and MHII, respectively, for *S. aureus* 209P were as follows (in micrograms per milliliter): CCCP, 8.0 and 2.0; DNP, 32 and 32; nisin, 2.0 and 4.0; valinomycin (+10 mM K^+), 32 and 2.0; cefamandole, 2.0 and 1.0; daptomycin, 8.0 and 0.25; erythromycin, 0.5 and 0.5; tobramycin, 2.0 and 2.0; and vancomycin, 1.0 and 1.0.

RESULTS

Daptomycin's ability to perturb $\Delta\Psi$ and ΔpH was measured in the flow cell. Under the conditions of these experiments, the ratio of daptomycin (100 μg/ml) to cells (10^{10}

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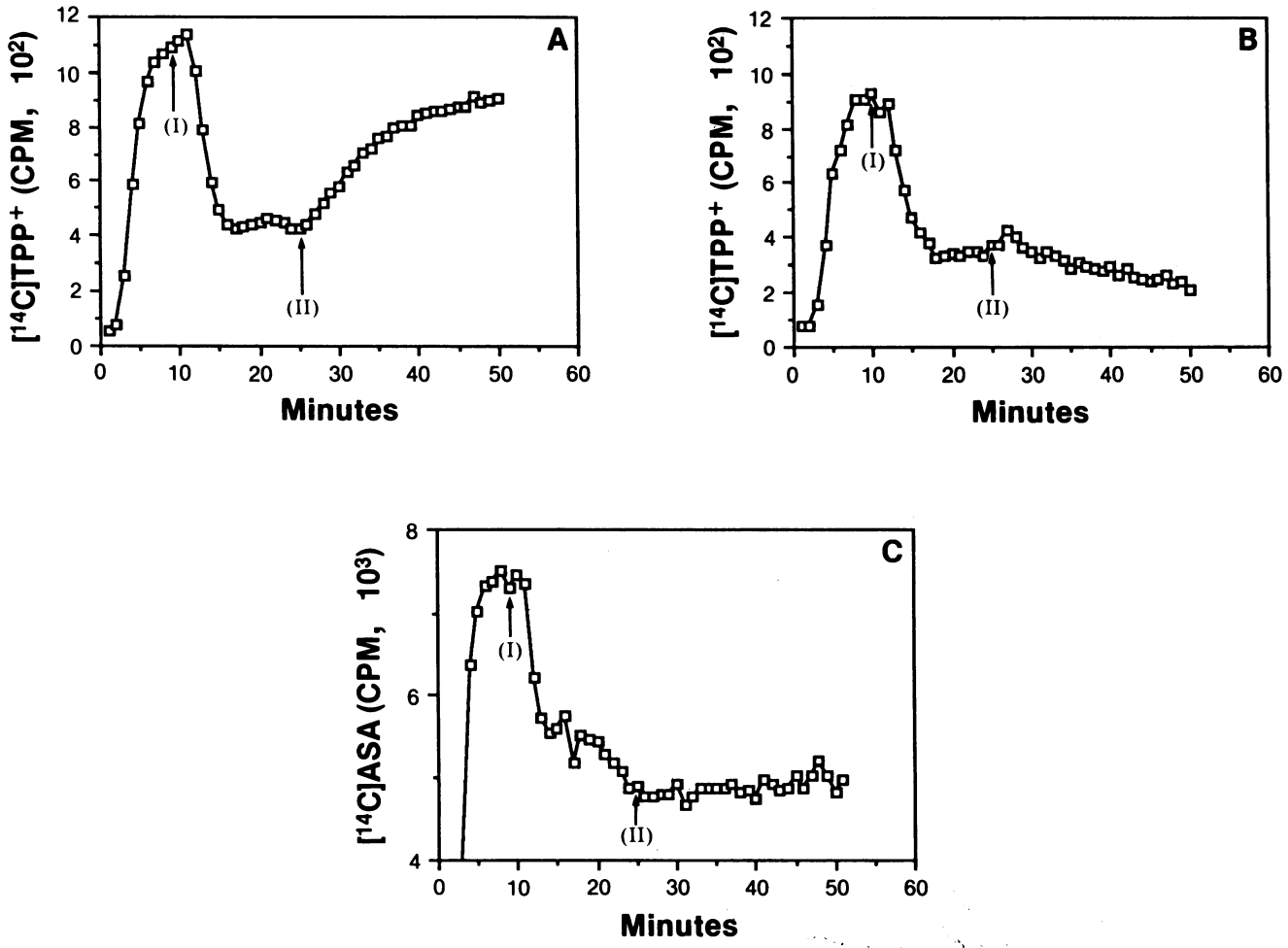

FIG. 1. Effect of daptomycin on ΔΨ and ΔpH. Cells were grown in MHII broth (A and C) or in MH broth (B) to late log phase, harvested, and resuspended in the same medium. A 50-μl aliquot was added (arrow I) to the upper chamber of the flow cell following equilibration at room temperature with either [^{14}C]TPP$^+$ at pH 7.5 (A and B) or [^{14}C]ASA at pH 5.0 (C) (see Materials and Methods). Daptomycin was added (arrow II) to give a final concentration of 100 μg/ml. Under these conditions, the concentration ratio of daptomycin to cells was several orders of magnitude less than that encountered in standard broth susceptibility assays (18). Samples were collected, and radioactivity was measured as described in Materials and Methods.

CFU/ml) was approximately 200-fold less than that found at the MIC in a typical broth dilution test. The addition of daptomycin (100 μg/ml) to cells after equilibration of [^{14}C]TPP$^+$ resulted in a rapid release of the lipophilic cation (Fig. 1A). Release of [^{14}C]TPP$^+$ in the calcium-deficient medium was very low and transient (Fig. 1B). In contrast to the effects on [^{14}C]TPP$^+$, daptomycin, in the presence of calcium, had no effect on [^{14}C]ASA accumulated at pH 5.0 (Fig. 1B). These results indicate that daptomycin induced a specific, calcium-dependent dissipation of ΔΨ in *S. aureus*. No effect on accumulated [^{14}C]TPP$^+$ was observed when cefamandole, vancomycin, erythromycin, or tobramycin was tested, each at 100 μg/ml, in the same manner (Fig. 2).

The observed effect of daptomycin upon ΔΨ was compared with the action of a variety of membrane-active compounds. Figure 3 illustrates dissipation of ΔΨ as a function of time by daptomycin and several other compounds known to affect energized bacterial membranes. Fifteen minutes after addition of daptomycin (100 μg/ml), membrane potential had decreased by 50 mV (Fig. 3A). A comparable reduction in ΔΨ was observed with DNP (25 μg/ml) and CCCP (25 μg/ml). Valinomycin (10 μg/ml in the presence of K^+) and nisin (100 μg/ml) dissipated ΔΨ more rapidly and to a greater extent than did daptomycin. Nigericin (100 μg/ml), which is known to collapse ΔpH (1), had no effect on ΔΨ in this experiment. DCCD hyperpolarizes the cytoplasmic membrane of *S. aureus* and increases ΔΨ (21). As shown in Fig. 3B, the addition of DCCD (5 μg/ml) increased ΔΨ, which was subsequently dissipated by the addition of daptomycin.

The relationship between membrane energization and the bactericidal activity of daptomycin is demonstrated in Fig. 4. Membrane potential was varied by adjusting the external pH (Fig. 4A) of the medium. At pH 7.5, where ΔΨ was −165 mV, 1 μg of daptomycin per ml reduced the number of viable cells by 1 log in approximately 5 min (Fig. 4B). At lower external pH values at which membrane potential was reduced, the bactericidal activity of daptomycin was likewise reduced (Fig. 4C through E). At pH 6.0, the time required for a 1 log reduction increased nearly 10-fold. At pH 5.0, where ΔΨ was about −100 mV, the reduction in cell count was limited to less than 1 log after 2 h of incubation. Lowering

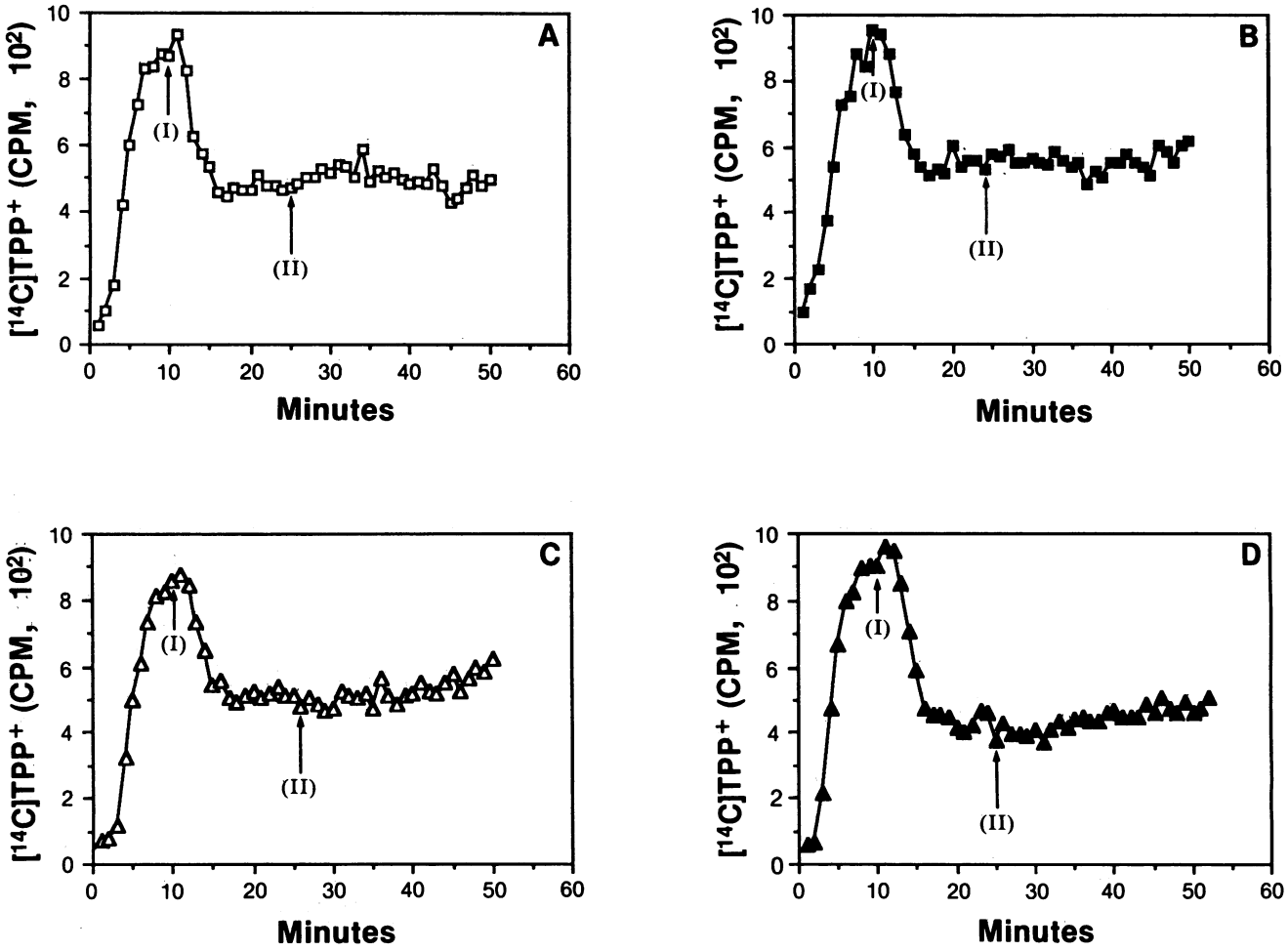

FIG. 2. Effect of different antibiotics on $[^{14}C]TPP^+$ distribution. Cefamandole (A), erythromycin (B), tobramycin (C), and vancomycin (D) were tested at 100 μg/ml exactly as described in the legend to Fig. 1.

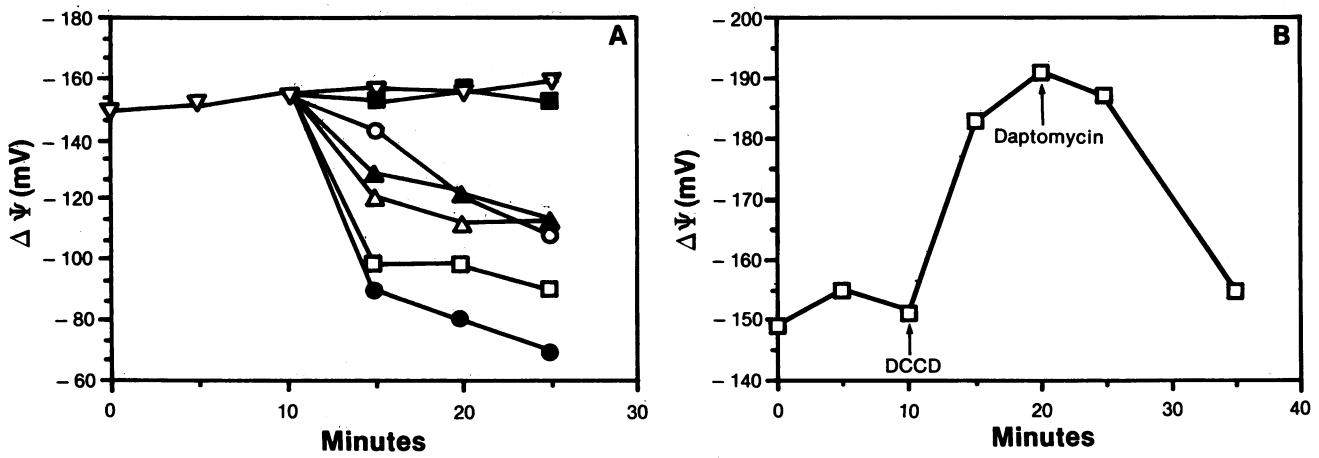

FIG. 3. Effect of daptomycin and membrane-active compounds on ΔΨ. Experimental conditions were as described in the legend to Fig. 1. ΔΨ was calculated as explained in Materials and Methods. (A) Comparison of nigericin (■; 100 μg/ml), daptomycin (○; 100 μg/ml), CCCP (▲; 25 μg/ml), DNP (△; 25 μg/ml), valinomycin (□; 10 mg/ml in the presence of 10 mM K^+), nisin (●; 100 mg/ml), and control (▽; no added compound). Test compounds were added at the 10-min mark. (B) Same as in panel A except that DCCD (5 μg/ml) was added at 10 min and daptomycin (100 μg/ml) was added 10 min after the addition of DCCD.

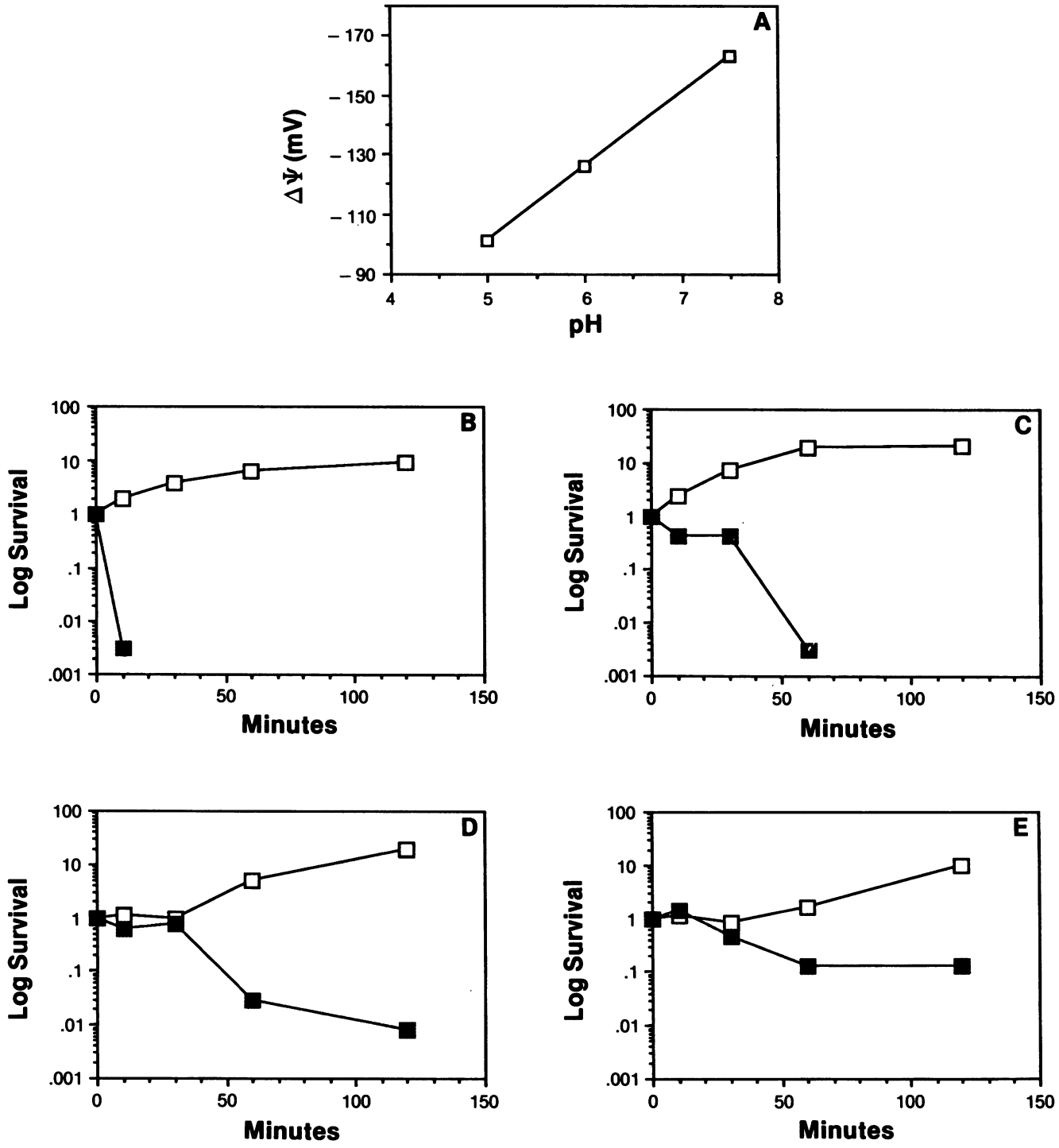

FIG. 4. Effect of membrane potential on the lethal effects of daptomycin. Log-phase *S. aureus* grown in MHII broth was diluted in fresh medium (adjusted to different pH values) to give 10^6 CFU/ml. $\Delta\Psi$ was measured at time zero as described in Materials and Methods. Cell suspensions were incubated at 35°C in a shaking water bath, and 100-μl aliquots were removed as indicated and diluted 10-fold in medium of the same pH. Aliquots (50 μl) were plated on MHII agar (pH 7.2) for colony counting. (A) Relationship between pH and $\Delta\Psi$; (B through E) bactericidal activity of daptomycin at pH 7.5 (B), pH 7.0 (C), pH 6.0 (D), and pH 5.0 (E). Symbols: □, no daptomycin; ■, 1.0 μg of daptomycin per ml.

the pH had much less effect on the growth rate of control cells; throughout the pH range used in these experiments, the doubling time was 30 min. The activity of daptomycin, measured by using a microbiological assay (see Materials and Methods), was unaffected by exposure to the pH range employed in this experiment.

DISCUSSION

The flow cell experiments using TPP^+ and ASA as probes of the electrochemical gradient in *S. aureus* showed that daptomycin induced a calcium-dependent release of TPP^+ with no observable effect on accumulated ASA. The results indicate that daptomycin, in the presence of calcium, collapsed $\Delta\Psi$ with no measurable effect on ΔpH. A similar effect of daptomycin on $\Delta\Psi$ in *Bacillus megaterium* has been observed (5). The effect of daptomycin on $\Delta\Psi$ was similar to that demonstrated by a variety of compounds including the uncouplers DNP and CCCP (17); a potassium ionophore, valinomycin (1); and nisin, a peptide antibiotic which causes nonselective ion efflux (20). The effect of daptomycin, i.e., loss of accumulated TPP^+, was not seen when nigericin, an ionophore which collapses ΔpH (1), was tested under identical conditions. Antibiotics that inhibit protein synthesis (erythromycin and tobramycin) or peptidoglycan formation (cefamandole and vancomycin) had no effect on accumulated TPP^+.

Maintenance of an appropriately energized cytoplasmic membrane is fundamental to the survival and growth of bacterial cells. Without the electrochemical gradient-induced proton motive force, cells cannot synthesize ATP or concentrate nutrients needed for growth. Because of the singular importance of an energized membrane, the ability of daptomycin to collapse $\Delta\Psi$ may explain the seemingly disparate effects reported for this antibiotic. For example, a recent study showed that MICs of daptomycin inhibited protein, RNA, DNA, peptidoglycan, and lipid biosynthesis in *S. aureus* with equal facility (9). The same report proposed that the target of daptomycin in *Enterococcus faecalis*, *Enterococcus faecium*, and *Bacillus subtilis* was lipoteichoic acid biosynthesis. Earlier reports (6, 11) showed that daptomycin inhibited peptidoglycan biosynthesis in *S. aureus* and *E. faecalis* without affecting the formation of other macromolecules. A recent study (5) presents evidence that the previously observed effects of daptomycin on formation of peptidoglycan precursors in *S. aureus* and *B. megaterium* (6, 7) may have been due to inhibition of active transport of certain amino acids. Induction of potassium efflux in *S. aureus* and *B. megaterium* (6), and interaction with planar bilayer membranes (13) and phospholipid vesicles (14, 15) by daptomycin are indicative of a membrane-localized target for this antibiotic. All of these observations are consistent with the hypothesis that the initial antibacterial event effected by daptomycin is a disruption of membrane energization.

The antimicrobial effects of daptomycin are dependent on the presence of calcium (8, 11, 12); other monovalent and divalent cations do not substitute (11). The effects of daptomycin on peptidoglycan and other macromolecular biosyntheses, as well as efflux of intracellular potassium, have been demonstrated in the presence of calcium. Results reported here demonstrate that the daptomycin-induced collapse of $\Delta\Psi$ does not occur in the absence of this cation. Lakey and Ptak (15) showed that daptomycin was bound only weakly to phospholipid vesicles in calcium-free solutions. These investigators also presented evidence that addition of calcium to the vesicles initiated a dramatic penetration of daptomycin deeper into the lipid bilayer. Canepari et al. (9) have reported evidence that daptomycin does not penetrate to the bacterial cytoplasm. It is reasonable to predict that calcium binding to a specific site on the peptide moiety of daptomycin (15) facilitates association of the antibiotic with the cytoplasmic membrane, where it disrupts membrane energetics and, ultimately, causes cell death.

The lethal effect of daptomycin may require a membrane potential. When $\Delta\Psi$ was lowered to about -100 mV, the lethal effects of daptomycin were severely impaired (Fig. 4E). Less severe decreases in $\Delta\Psi$ resulted in concomitant reductions in rates of killing compared with that in cells where $\Delta\Psi$ was -165 mV (Fig. 4B to D). This is consistent with the observation that *S. aureus* suspended in Tris buffer (pH 7.0) was not killed by daptomycin (2) and is in accord with the finding of Ruhr and Sahl (20) indicating that starved cells had a lower $\Delta\Psi$ and were therefore less susceptible to the lethal effect of nisin. The apparent inability of daptomycin to inhibit protein, RNA, or DNA biosynthesis in nongrowing cells suspended in phosphate-buffered saline (6) also could be explained as because of a lack of sufficient membrane potential under the conditions employed. The membrane effects of nisin (20) and the uptake and cidal activity of aminoglycoside antibiotics (10, 21) require a $\Delta\Psi$. Daptomycin may similarly require a $\Delta\Psi$ in order to reach and interact with a specific site in the cytoplasmic membrane.

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