

# The Lantibiotic Nisin Induces Transmembrane Movement of a Fluorescent Phospholipid

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**Nisin is a pore-forming antimicrobial peptide. The capacity of nisin to induce transmembrane movement of a fluorescent phospholipid in lipid vesicles was investigated. Unilamellar phospholipid vesicles that contained a fluorescent phospholipid (1-acyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl}-sn-glycero-3-phosphocholine) in the inner leaflet of the bilayer were used. Nisin-induced movement of the fluorescent phospholipid from the inner leaflet to the outer leaflet of the membrane reached stable levels, which were dependent on the concentration of nisin added. The rate constant  $k$  of this nisin-induced transmembrane movement increased with the nisin concentration but was not dependent on temperature within the range of 5 to 30°C. In contrast, the rate constant of movement of fluorescent phospholipid from vesicle to vesicle strongly depended on temperature. The data indicate that nisin transiently disturbs the phospholipid organization of the target membrane.**

Asymmetric distribution of phospholipids over the inner and outer leaflets of biological membranes is essential for membrane function (28, 29). In eukaryotic membranes, the phospholipid distribution is maintained by proteins termed “flippases” and by interactions with cytoskeletal proteins (12). For instance, the ATP-dependent flippase in erythrocytes translocates amino phospholipids from the outer leaflet to the inner leaflet of the membrane (36). Also, MDR P-glycoprotein translocates phospholipid (43). In bacterial membranes, little is known about the distribution of phospholipids over the inner and outer leaflets. Rapid movement of fluorescent phospholipids across the inner membrane of *Escherichia coli* has been demonstrated and is probably a protein-mediated process (14). Movement of a fluorescent phospholipid from the outer leaflet to the inner leaflet was demonstrated in the membrane of *Bacillus megaterium* (13).

Channel-forming cytotoxins (from *Staphylococcus aureus* [ $\alpha$ -phage-toxin] and *Pseudomonas aeruginosa*) in addition to relatively small “pore-forming” antimicrobial peptides, such as melittin, the synthetic peptide GALA (10), and mastoparan X (24), are known to cause movement of membrane phospholipids from one leaflet to the other (35). In the cases mentioned above, the peptide-induced phospholipid movement might reflect an important mechanistic aspect of pore formation. For example, fusion of the inner and outer leaflets of the target membrane may play a role such as that proposed for magainin-induced “toroidal” pores (22).

Nisin is a cationic peptide antibiotic of 34 amino acids that contains five intramolecular rings. Its genetics (18), synthesis regulation (19), and structure (21, 39–42) are known, and several mutants have been generated (16–18, 20). Because of its antimicrobial activities, of which permeabilization of the membranes of the target cells is the most important (25), nisin is used as a food preservative. Nisin preferentially interacts with anionic phospholipid-containing (3, 8, 9) membranes, tran-

siently enhancing the permeability for small ions and solutes (5, 11, 15, 27, 31–34). We previously proposed a “wedge-like” model for pore formation by nisin (9, 26). The positively charged carboxyl terminus of nisin, together with the bound lipids, enter into the membrane. Both the transmembrane electrical potential ( $\Delta\psi$ ) (9, 32, 33) and the  $\Delta\text{pH}$  (*cis* acid) stimulate nisin-mediated membrane permeabilization (26). In this study, we demonstrate that nisin induces rapid movement of a fluorescent phospholipid from the inner leaflet to the outer leaflet of unilamellar phospholipid vesicles. This provides support for nisin-induced disturbance of the phospholipid organization in the membrane, as proposed previously in the wedge-like model for nisin-induced pore formation.

## MATERIALS AND METHODS

**Materials.** Nisin was obtained as a gift from Aplin & Barrett. Dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), 1- $\alpha$ -phosphatidylethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rh-PE), 1-acyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl}-sn-glycero-3-phosphocholine ( $C_6$ -NBD-PC), and 1-acyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl}-sn-glycero-3-[phospho-rac-(1-glycerol)] ( $C_6$ -NBD-PG) were obtained from Avanti Polar Lipids. The 1-acyl chains of  $C_6$ -NBD-PC and  $C_6$ -NBD-PG represent the fatty acid content of egg lysophosphatidylcholine.

**Preparation of small unilamellar phospholipid vesicles.** Phospholipids dissolved in organic solvent were dried under nitrogen. Subsequently, a small volume of ethanol was added, and the lipids were dried again. Multilamellar vesicles were formed by vortexing in the presence of buffer and glass beads. Small unilamellar vesicles were obtained by sonication (tip diameter, 3 mm) (Soniprep 150; Beun de Ronde, Abcoude NL) of the multilamellar vesicles under the following conditions: 30 min, 10- $\mu\text{m}$  amplitude, pulses of 30 s on and 30 s off, incubation on ice under a stream of nitrogen. Metal particles and large vesicles were removed by centrifugation for 15 min at 80,000  $\times g$  at 4°C. Vesicles composed of DOPC-Rh-PE (98:2 [wt/wt]) were prepared by ethanol injection (2), followed by dialysis to remove ethanol.

**Preparation of large unilamellar phospholipid vesicles with symmetric transmembrane distribution of  $C_6$ -NBD-phospholipids.** Phospholipid vesicles labelled with 2% (wt/wt)  $C_6$ -NBD-phospholipid were prepared by reverse-phase evaporation (38), followed by extrusion performed 11 times through 400-nm-pore-size polycarbonate filters (Avestin) in either piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES), or phosphate buffer (pH 7.0) containing 0.21 M sucrose and 50 mM  $K^+$  or 50 mM  $Na^+$ . Extravesicular sucrose was removed by dialysis against the same buffer without sucrose.

**Preparation of large unilamellar phospholipid vesicles with  $C_6$ -NBD-lipids exclusively in the inner leaflet.** Vesicles prepared as in the preceding paragraph, which contain  $C_6$ -NBD-phospholipid in both inner and outer membrane leaflets, were incubated four times at 20°C with sonicated small DOPG vesicles that do

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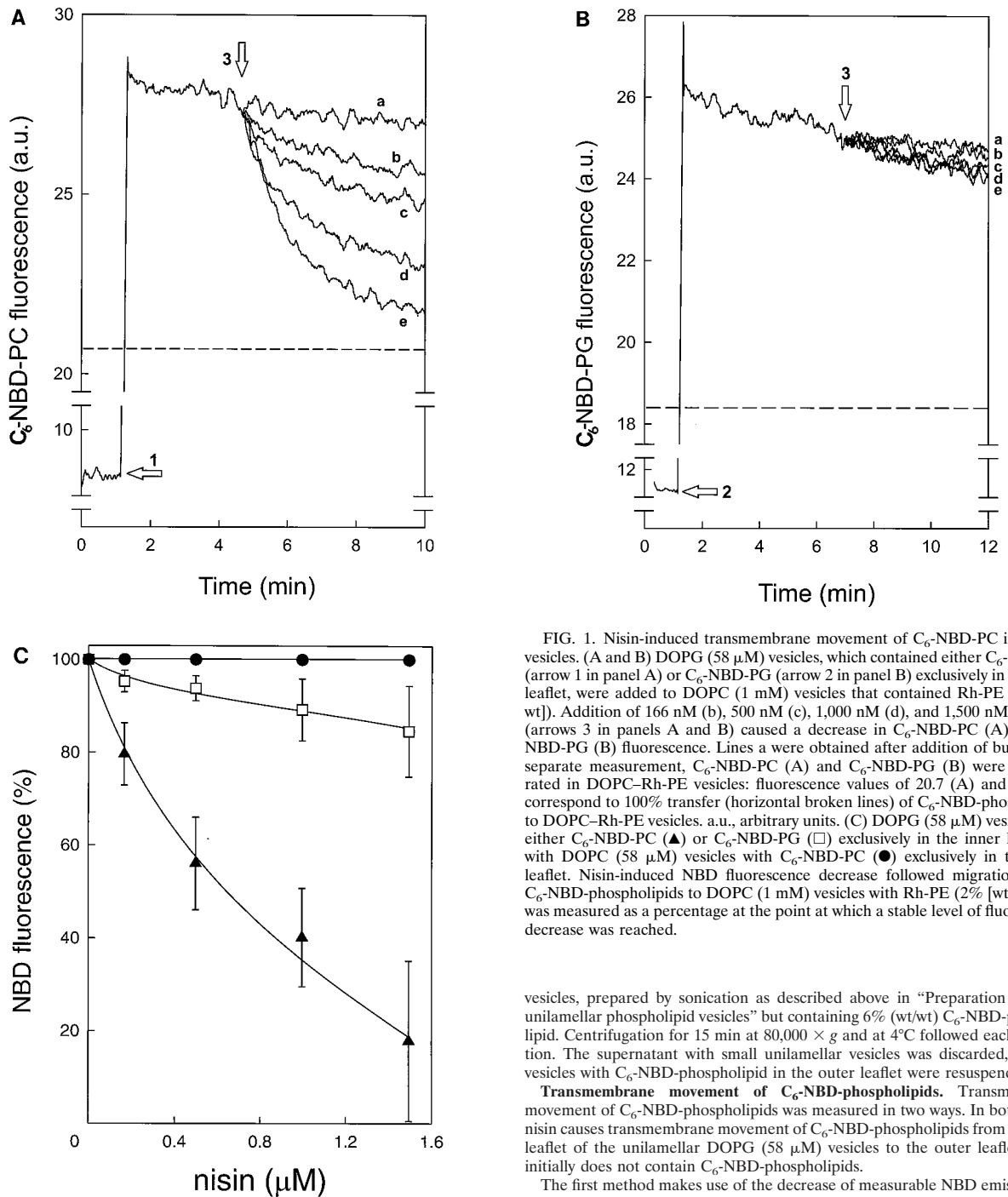


FIG. 1. Nisin-induced transmembrane movement of C<sub>6</sub>-NBD-PC in DOPG vesicles. (A and B) DOPG (58 μM) vesicles, which contained either C<sub>6</sub>-NBD-PC (arrow 1 in panel A) or C<sub>6</sub>-NBD-PG (arrow 2 in panel B) exclusively in the inner leaflet, were added to DOPC (1 mM) vesicles that contained Rh-PE (2% [wt/wt]). Addition of 166 nM (b), 500 nM (c), 1,000 nM (d), and 1,500 nM (e) nisin (arrows 3 in panels A and B) caused a decrease in C<sub>6</sub>-NBD-PC (A) and C<sub>6</sub>-NBD-PG (B) fluorescence. Lines a were obtained after addition of buffer. In a separate measurement, C<sub>6</sub>-NBD-PC (A) and C<sub>6</sub>-NBD-PG (B) were incorporated in DOPC-Rh-PE vesicles: fluorescence values of 20.7 (A) and 18.4 (B) correspond to 100% transfer (horizontal broken lines) of C<sub>6</sub>-NBD-phospholipid to DOPC-Rh-PE vesicles. a.u., arbitrary units. (C) DOPG (58 μM) vesicles with either C<sub>6</sub>-NBD-PC (▲) or C<sub>6</sub>-NBD-PG (□) exclusively in the inner leaflet or with DOPC (58 μM) vesicles with C<sub>6</sub>-NBD-PC (●) exclusively in the inner leaflet. Nisin-induced NBD fluorescence decrease followed migration of the C<sub>6</sub>-NBD-phospholipids to DOPC (1 mM) vesicles with Rh-PE (2% [wt/wt]) and was measured as a percentage at the point at which a stable level of fluorescence decrease was reached.

not contain C<sub>6</sub>-NBD-phospholipid, essentially as described previously (10). Each incubation was followed by centrifugation for 15 min at 80,000 × g and at 4°C, after which the supernatant with the sonicated small DOPG vesicles was discarded and the DOPG-C<sub>6</sub>-NBD-phospholipid vesicles were resuspended. The incubation with sonicated small DOPG vesicles was at a 5- to 10-fold (first incubation) or one- to twofold (subsequent three incubations) excess quantity over the amount of total phospholipid present in the DOPG-C<sub>6</sub>-NBD-phospholipid vesicles.

**Preparation of large unilamellar phospholipid vesicles with C<sub>6</sub>-NBD-phospholipids exclusively in the outer leaflet.** DOPG vesicles without C<sub>6</sub>-NBD-phospholipids were prepared as described above by reverse-phase evaporation (38) followed by extrusion. These acceptor vesicles were incubated twice during 1 h at 20°C with twice the amount (on a phospholipid basis) small unilamellar donor

vesicles, prepared by sonication as described above in "Preparation of small unilamellar phospholipid vesicles" but containing 6% (wt/wt) C<sub>6</sub>-NBD-phospholipid. Centrifugation for 15 min at 80,000 × g and at 4°C followed each incubation. The supernatant with small unilamellar vesicles was discarded, and the vesicles with C<sub>6</sub>-NBD-phospholipid in the outer leaflet were resuspended.

**Transmembrane movement of C<sub>6</sub>-NBD-phospholipids.** Transmembrane movement of C<sub>6</sub>-NBD-phospholipids was measured in two ways. In both assays, nisin causes transmembrane movement of C<sub>6</sub>-NBD-phospholipids from the inner leaflet of the unilamellar DOPG (58 μM) vesicles to the outer leaflet, which initially does not contain C<sub>6</sub>-NBD-phospholipids.

The first method makes use of the decrease of measurable NBD emission due to energy transfer to Rh-PE. Nisin-induced transmembrane movement of C<sub>6</sub>-NBD-phospholipids to the outer leaflet of DOPG (58 μM) vesicles is followed by the diffusion of C<sub>6</sub>-NBD-phospholipid to DOPC-Rh-PE (98:2 [wt/wt]) (1 mM DOPC) vesicles, resulting in a decrease of the NBD emission, which was measured as a percentage at the point at which a stable level of fluorescence decrease was reached. The NBD fluorescence measured when vesicles composed of C<sub>6</sub>-NBD-phospholipid-DOPC-Rh-PE were prepared was taken as the 100% control of fluorescence decrease.

In the second assay, transmembrane movement of C<sub>6</sub>-NBD-phospholipids from the inner leaflet to the outer leaflet of DOPG vesicles is measured in the absence of quenching DOPC-Rh-PE vesicles. The NBD fluorescence increase due to shielding of NBD groups in the outer leaflet from the aqueous phase by membrane-bound nisin is measured (9). Analysis of the kinetics of transmembrane movement was performed by assuming second-order kinetics and by using the table curve program (Jandel). The NBD fluorescence increase was fitted according to the equation that gave the best fit:  $y = a + b(1 - e^{-kx}) + d(1 -$

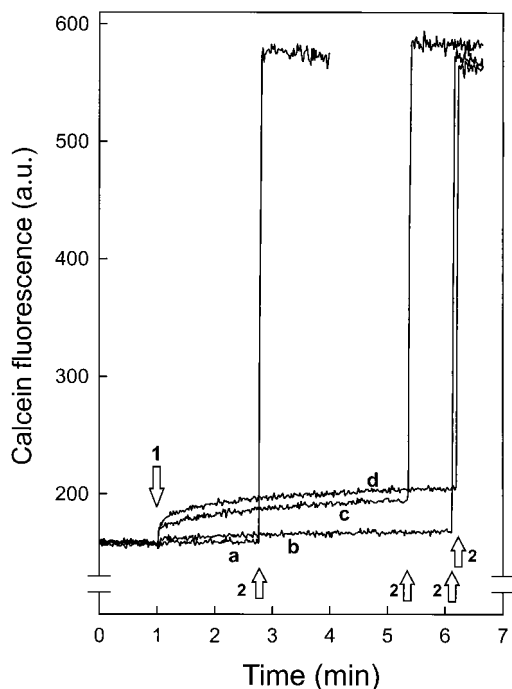


FIG. 2. Nisin-induced release of calcein from phospholipid vesicles. DOPG (58  $\mu\text{M}$ ) vesicles loaded with calcein were incubated with nisin. At arrow 1, nisin at either 166 (a), 500 (b), 1,000 (c), or 1,500 (d) nM was added, followed by addition of 0.2% Triton X-100 (arrows 2). a.u., arbitrary units.

$e^{-kt}$ . The value of the second rate constant  $e$  was very small (generally between  $10^{-2}$  and  $10^{-5}$  times smaller than  $k$ ) compared to the first rate constant  $k$ . In parallel experiments, the NBD fluorescence increase was measured for DOPG vesicles with symmetric transbilayer distribution of  $C_6$ -NBD-phospholipid.

All fluorescence measurements were performed at 30°C, unless otherwise indicated, using a Perkin Elmer LS-50 spectrofluorimeter. For the NBD fluorescence, excitation and emission wavelengths were 475 and 530 nm, respectively. The slit widths were set at 4 nm.

**Transfer of  $C_6$ -NBD-phospholipids between phospholipid vesicles.** Interbilayer movement of lipids was measured as follows: DOPG-Rh-PE vesicles (98:2 [wt/wt]) containing either 2% (wt/wt)  $C_6$ -NBD-PG or  $C_6$ -NBD-PC were incubated with DOPG or DOPC vesicles without fluorescent probe. Rate constants of phospholipid transfer were derived from the NBD fluorescence increase.

**Calcein leakage.** Liposomes were prepared by reverse-phase evaporation (38) in a solution containing 0.21 M sucrose, 8 mM calcein, and 50 mM K-PIPES, pH 7.0. After dialysis against 50 mM K-PIPES (pH 7.0), external calcein was removed by centrifugation for 15 min at  $80,000 \times g$  and 4°C, followed by two washes (each wash done by resuspension and then centrifugation). The integrity of the phospholipid vesicles was assayed by measuring the fluorescence increase after the addition of nisin and after subsequent addition of 0.2% Triton X-100 to calcein-loaded phospholipid vesicles. Excitation and emission wavelengths were 470 and 520 nm, respectively, and the slit widths were 2.5 nm.

**Phosphate determinations.** Phosphate determinations were performed by the method of Rouser et al. (30).

## RESULTS

In the present study, we investigated the capacity of nisin to induce transmembrane movement of the  $C_6$ -NBD-phospholipid. In a first assay, unilamellar DOPG vesicles which contained  $C_6$ -NBD-phospholipids in the inner leaflet were incubated with an excess of DOPC-Rh-PE vesicles. Nisin is known to bind to negatively charged phospholipids, like DOPG, but not to zwitterionic DOPC phospholipids (9). After nisin-mediated movement to the outer leaflet of the membrane, the  $C_6$ -NBD-phospholipids diffused to the Rh-PE-containing DOPC vesicles. Once inserted into these vesicles, the NBD emission was largely used for the excitation of rhodamine, which gave rise to a decrease in the emission of  $C_6$ -NBD-PC (Fig. 1A and C) and  $C_6$ -NBD-PG (Fig. 1B and C). In the absence of nisin or when DOPC vesicles (Fig. 1C) were used instead of DOPG vesicles, no decrease in the NBD fluorescence was observed. This implied that the acidic phospholipid DOPG was needed for the nisin-induced transmembrane movement of the  $C_6$ -NBD-phospholipid. Interestingly, the decrease in NBD fluorescence was much greater for  $C_6$ -NBD-PC than for  $C_6$ -NBD-PG (Fig. 1C). Control experiments showed that nisin did not inhibit the transfer of either  $C_6$ -NBD-PG or  $C_6$ -NBD-PC from DOPG vesicles towards DOPC vesicles. These control experiments were performed by following the increase in NBD fluorescence due to transfer of  $C_6$ -NBD-phospholipid from DOPG (58  $\mu\text{M}$ )- $C_6$ -NBD-phospholipid (2% [wt/wt]) in both leaflets)-Rh-PE (2% [wt/wt] in both leaflets) vesicles, in which the NBD fluorescence was quenched by the Rh-PE, towards DOPC (1 mM) vesicles (data not shown).

To exclude the possibility that the  $C_6$ -NBD-phospholipids in the inner leaflet diffused to the acceptor vesicles through an

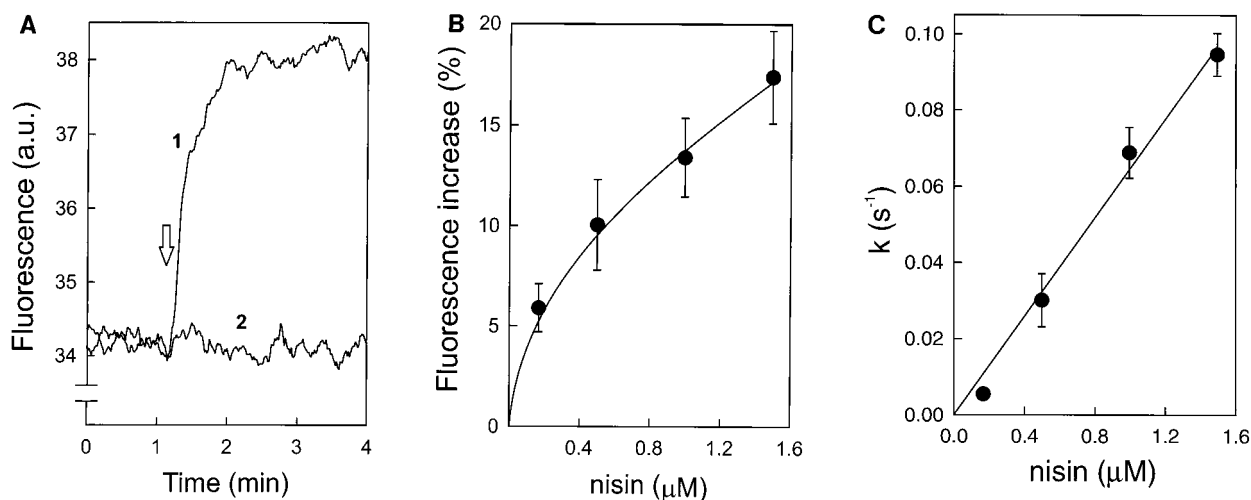


FIG. 3. Nisin induced a transmembrane movement of  $C_6$ -NBD-PC. In panel A, at the arrow, 500 nM nisin (line 1) or buffer (line 2) was added to DOPG (58  $\mu\text{M}$ ) vesicles with  $C_6$ -NBD-PC exclusively in the inner leaflet, and the increase in the NBD fluorescence was recorded. The same experiment was performed with various concentrations of nisin. At each nisin concentration a different plateau level of fluorescence increase (B) and a different rate constant (C) were measured.

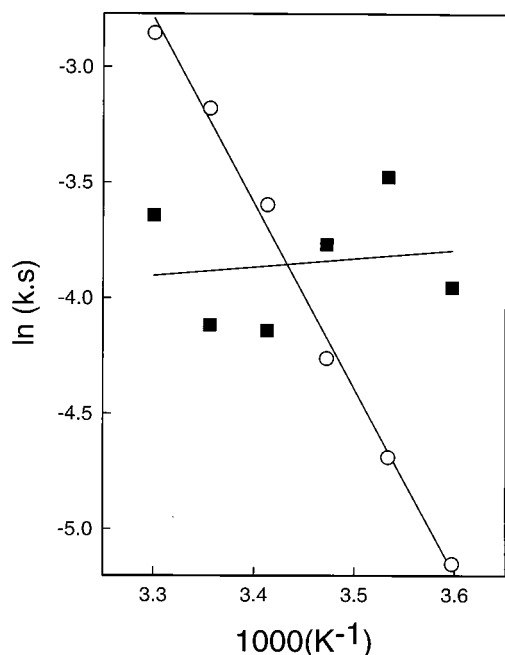


FIG. 4. Temperature dependence of the rate constants of transmembrane movement and inters vesicle movement of  $C_6$ -NBD-PC. Nisin (500 nM) was added to DOPG (58  $\mu$ M) vesicles, with  $C_6$ -NBD-PC (■) exclusively in the inner leaflet. Inters vesicle movement was measured by incubating DOPG (58  $\mu$ M)-Rh-PE- $C_6$ -NBD-PC vesicles with pure DOPG (174  $\mu$ M) vesicles (○). Lines were obtained by regression. Standard deviations of the absolute values of the rate constants were around 17% for transmembrane movement and around 2% for inters vesicle movement.

aqueous pore formed by nisin instead of via a transmembrane movement, the integrity of the vesicles was determined by following the nisin-induced leakage of the fluorophore calcein. Under conditions where extensive transmembrane movement of  $C_6$ -NBD-PC took place, nisin-induced calcein leakage was only moderate and limited to around 10% at the highest nisin concentration (Fig. 2). This demonstrated that the decrease in  $C_6$ -NBD-PC fluorescence, as observed in Fig. 1, was the result of a transmembrane movement of the  $C_6$ -NBD-PC. The small decrease in  $C_6$ -NBD-PG fluorescence (Fig. 1C) was, however, only slightly larger than that observed for calcein leakage. Therefore, no conclusion could be drawn on the nature of the nisin-induced  $C_6$ -NBD-PG movement. As observed previously (10), control experiments with phospholipid vesicles containing the  $C_6$ -NBD-phospholipids exclusively in the outer leaflet showed in the absence of nisin a rapid maximal decrease in fluorescence due to diffusion of the  $C_6$ -NBD-phospholipid to the quenching Rh-PE-DOPC acceptor vesicles.

In order to enable a more direct analysis of the transmembrane movement, experiments were performed with DOPG vesicles containing the  $C_6$ -NBD-phospholipid in the inner leaflet, without the addition of Rh-PE-DOPC acceptor vesicles. In this case, nisin-induced movement of  $C_6$ -NBD-phospholipid to the outer leaflet caused an increase in NBD fluorescence. This is due to shielding of the NBD group from the aqueous environment by the membrane-bound nisin (9). The NBD group in  $C_6$ -NBD-phospholipids loops back to the phospholipid head group (1, 6, 7). Indeed, addition of nisin to DOPG vesicles with  $C_6$ -NBD-PC (Fig. 3A and B) or with  $C_6$ -NBD-PG (data not shown) exclusively in the inner leaflet caused an increase in the NBD fluorescence. Analyses of the kinetics of the fluorescence increase revealed that at all nisin concentrations, the rate con-

stant  $k$  was lower for  $C_6$ -NBD-PC (Fig. 3C) than for  $C_6$ -NBD-PG; in the latter case, the rate constant ranged from  $0.046 \pm 0.007 \text{ s}^{-1}$  at 166 nM nisin to  $0.194 \pm 0.032 \text{ s}^{-1}$  at 1.5  $\mu$ M nisin (data not shown). In contrast, addition of the nisin to vesicles with symmetric distribution of  $C_6$ -NBD-PC or  $C_6$ -NBD-PG resulted in a very rapid increase in fluorescence, which reached a higher plateau within less than a second. This was observed previously for vesicles with symmetric distribution of head group-labelled NBD-phosphatidylethanolamine (9).

Nisin pore formation is known to depend on the proton motive force. We investigated the effect of a valinomycin-induced membrane potential ( $\Delta\Psi$ ) on transmembrane movement of  $C_6$ -NBD-PC. Nisin was added to DOPG vesicles with  $C_6$ -NBD-phospholipid exclusively in the inner leaflet, and the fluorescence increase was recorded. Neither for  $C_6$ -NBD-PC nor for  $C_6$ -NBD-PG was any effect of the  $\Delta\Psi$  on the extent or rate of fluorescence increase observed (data not shown). Under the conditions used, nisin-induced dissipation of the  $\Delta\Psi$  was much faster than the rate of nisin-induced NBD fluorescence increase (data not shown). The rapid dissipation of the  $\Delta\Psi$  therefore prevented the detection of a possible effect of the  $\Delta\Psi$  on nisin-induced transmembrane movement of  $C_6$ -NBD-phospholipid.

The effect of temperature on the transmembrane movement of  $C_6$ -NBD-phospholipid was investigated by using the assay with the  $C_6$ -NBD-phospholipid exclusively in the inner leaflet. The nisin-induced transmembrane movement of  $C_6$ -NBD-PC was not affected by temperature (Fig. 4). As a control, the temperature dependence of the transfer of  $C_6$ -NBD-PC from  $C_6$ -NBD-PC-Rh-PE-DOPG vesicles to DOPG vesicles was measured. In contrast to the rate constant of transmembrane movement, the rate constant of the movement of  $C_6$ -NBD-PC from vesicle to vesicle was strongly affected by temperature (Fig. 4). Also, the nisin-induced  $C_6$ -NBD-PG movement observed when nisin is added to vesicles in which the inner leaflet contains  $C_6$ -NBD-PG was dependent on temperature. At 500 nM nisin,  $k$  increased from  $0.017 \pm 0.003 \text{ s}^{-1}$  at 5°C to  $0.066 \pm 0.012 \text{ s}^{-1}$  at 30°C (data not shown), which further concurs with the suggestion that the  $C_6$ -NBD-PG movement occurs via a mechanism that is different from that of the  $C_6$ -NBD-PC transmembrane movement.

## DISCUSSION

The present work shows that nisin induces movement of a fluorescent phospholipid,  $C_6$ -NBD-PC, from the inner leaflet of unilamellar phospholipid vesicles to the outer leaflet. Nisin is a pore-forming antimicrobial peptide. However, various lines of evidence indicate that  $C_6$ -NBD-PC undergoes transmembrane movement and does not pass through an aqueous pore formed by nisin. First, the extent of calcein leakage was very low compared to the transmembrane movement of  $C_6$ -NBD-PC. Nisin Z forms an anion-selective pore in DOPG vesicles (3). This excludes the theoretical possibility that the negative

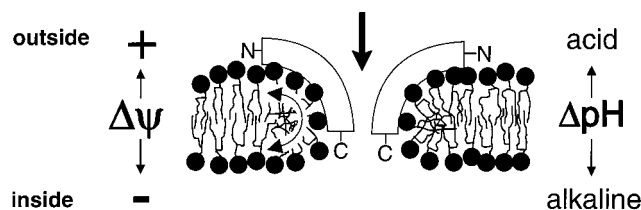


FIG. 5. Model of altered phospholipid organisation after insertion of nisin.

charge of calcein (and/or C<sub>6</sub>-NBD-PG) might limit its passage through a nisin pore due to electrostatic repulsion between DOPG and calcein (and/or C<sub>6</sub>-NBD-PG). The calcein leakage experiments also demonstrated that the vesicles remained intact under the conditions employed. Second, movement of C<sub>6</sub>-NBD-PG was much more restricted than that of C<sub>6</sub>-NBD-PC (Fig. 1C), whereas the rate constant of C<sub>6</sub>-NBD-PG movement was higher than that of C<sub>6</sub>-NBD-PC. Third, the rate constant of transmembrane movement of C<sub>6</sub>-NBD-PC markedly increased with the nisin concentration (Fig. 3C). Similarly, another study (35) showed that the (low) rate constant of transmembrane movement of lysophospholipid in erythrocytes increased with cytotoxin (of *S. aureus* and of *P. aeruginosa*) concentration. Finally, the rate constant of intervesicle movement of C<sub>6</sub>-NBD-PC increased strongly with the temperature, while the rate constant of transmembrane movement of C<sub>6</sub>-NBD-PC was not affected by temperature. Similar results were found in experiments on flippase activity in *B. megaterium* membrane vesicles. In that study, the rate constant of transmembrane movement of phospholipid was largely independent of temperature, whereas the rate constant of intervesicle transport was dependent on temperature (13).

Why was the nisin-induced transbilayer movement of lipids restricted to C<sub>6</sub>-NBD-PC without having an equal effect on the movement of C<sub>6</sub>-NBD-PG? The lack of the C<sub>6</sub>-NBD-PG movement was possibly due to the immobilization of the anionic PG by the cationic nisin as previously suggested by <sup>31</sup>P nuclear magnetic resonance experiments (9). The relative nisin-induced C<sub>6</sub>-NBD-PG movement hardly exceeded the calcein leakage. Therefore, the nature of this limited movement is not clear at this time.

What is the mechanism of nisin-induced transmembrane movement of C<sub>6</sub>-NBD-PC? In all likelihood, transmembrane movement of C<sub>6</sub>-NBD-PC is the consequence of insertion of part(s) of the nisin molecule into the membrane with concomitant disturbance of the membrane (Fig. 5). Insertion of nisin into lipid monolayers (3, 8) has been demonstrated, and the C terminus of nisin is known to insert deeply into the membrane (23). A study in which a His-tagged nisin was used indicated that the C-terminal part of nisin had the ability to translocate across the membrane (44). Physicochemical measurements on the interaction of nisin with *Listeria* lipid vesicles also indicated an insertion of the C-terminal part of nisin into the membrane (45). In equilibrium, however, nisin seemed to be oriented parallel with the membrane surface, assuming a surface-bound state (4). The wedge-like model (Fig. 5) invokes a proton motive force-driven insertion of the C-terminal part of nisin (9, 26). It could well be that the insertion of the C-terminal region of nisin induced temporary fusion joints that permit transmembrane movements.

In the experiments in which C<sub>6</sub>-NBD-PC moves to the outer leaflet and subsequently to Rh-PE-containing vesicles, nisin concentration-dependent stable levels of movement were reached after 5 min (data not shown). Supposing a surface area of 0.716 nm<sup>2</sup>/DOPG molecule (37) and using a phospholipid/nisin ratio that ranges from 39 to 438, the number of molecules per vesicle was between 1,602 and 18,000. Therefore, the observed saturation of the fluorescence decrease was probably not due to a low nisin/phospholipid ratio. Apparently, nisin only transiently induced transmembrane movement of C<sub>6</sub>-NBD-phospholipid. Within the range used, the rate constant of transmembrane movement increased with the nisin concentration (Fig. 3C). This confirms that nisin indeed bound to all vesicles and that binding of additional nisin cooperatively enhanced transmembrane movement of C<sub>6</sub>-NBD-PC. This phenomenon might be the result of aggregation of nisin molecules.

In conclusion, the present study shows for the first time that nisin transiently induces rapid transmembrane movement of C<sub>6</sub>-NBD-PC. This nisin-induced transmembrane movement indicates that a transient disturbance of the phospholipid organization of the membrane takes place locally during nisin-induced membrane permeabilization.

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