Gramicidin-Induced Enhancement of Transbilayer Reorientation of Lipids in the Erythrocyte Membrane†

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ABSTRACT: Incorporation of the channel-forming antibiotic gramicidin into the membrane of human erythrocytes highly (up to 30-fold) enhances rates of reorientation (flip) of lysophosphatidylcholine and palmitoylcarnitine to the inner membrane layer after their primary incorporation into the outer layer. Despite the high increase of flip rates by gramicidin, the asymmetric orientation of the inner membrane layer phospholipids phosphatidylethanolamine and phosphatidylserine is stable as demonstrated by the lack of accessibility of these lipids toward cleavage by exogenous phospholipase A_2. On the other hand, gramicidin enhances the rate of cleavage of outer membrane layer phosphatidylcholine by phospholipase A_2, which indicates changes in the packing of phosphatidylcholine following gramicidin binding. The increase of flip becomes detectable when about 10^5 copies of gramicidin per cell have been bound (gramicidin to membrane phospholipid ratio of 1:2000). This is a 1000-fold higher concentration than that required for an increase of K^+ permeability mediated by the gramicidin channel. Acceleration of flip is thus not simply correlated with channel formation. The enhancement of flip is markedly dependent on structural details of gramicidin. Formylation of its four tryptophan residues abolishes the effect. Even at high concentrations of formylated gramicidin at which the extents of binding of native and of formylated gramidicin to the membrane are comparable, no flip acceleration is produced. Enhancement of flip by gramicidin occurs after a temperature-dependent lag phase. At 37 °C, flip rates begin to increase within a few minutes and at 25 °C, only after 3 h. This lag phase is most likely not due to limitations by the rate of binding of gramicidin to the membrane. At gramicidin concentrations at which flip is enhanced, gramicidin, but not Trp-N-formylated gramicidin, produces aqueous leaks in the erythrocyte membrane permeable to large ions (choline, oxalate) and nonelectrolytes (erythritol, sucrose). In the following paper, flip acceleration and production of aqueous leaks by gramicidin are shown to be accompanied by the formation of hexagonal H_{II} lipid structures, and the relationship between the phenomena is discussed [Tournois, H., Leunissen-Bijvelt, J., Haest, C. W. M., De Gier, J., & De Kruijff, B. (1987) *Biochemistry* (following paper in this issue)].

The low rate of transbilayer reorientation of phospholipids in the human erythrocyte is highly enhanced by the incorporation into the membrane of the channel-forming polyene antibiotic amphotericin B (Schneider et al., 1986a), whereas pimaricin, an antibiotic with a shorter hydrophobic polyene chain, neither produces channels nor enhances flip (Haest et al., 1986). Incorporation into the membrane of channel-forming bacterial proteins like α-toxin from *Staphylococcus aureus* and cytotoxin from *Pseudomonas aeruginosa* produces flip enhancement, too (Schneider et al., 1986a). Enhancement of flip rates by the channel formers has been suggested (Schneider et al., 1986a) to result from a thinning of the lipid bilayer at the channel–lipid interface and a consecutive reduction of the hydrophobic barrier for the polar phospholipid head groups. Another mechanism proposed to induce an increase of the transbilayer reorientation of phospholipids is a reversible formation of nonbilayer structures in the lipid bilayer (Cullis & De Kruijff, 1978). This proposition was supported by model membrane experiments, which revealed an increase of flip rates of phospholipids under conditions that induced nonbilayer structures (Gerritsen et al., 1980; Noordam et al., 1981; Van Duijn et al., 1986). Recently, it was shown (Van Echtheld et al., 1981) that the hydrophobic pentadecapeptide gramicidin, whose channel structure is assumed to be a helical dimer (*N*-formyl end to *N*-formyl end) (Urry et al., 1971), destabilizes the bilayer structure of dispersed diacyllipids and induces formation of hexagonal phases [for a review, see Killian and De Kruijff (1986)]. This bilayer destabilizing effect is dependent on the length of the fatty acyl chain of the phospholipids and is produced for phospholipids of varying polar head groups. Studies on model membranes of dioleoylphosphatidylcholine have demonstrated that formylation of the four tryptophan residues of gramicidin, which are located at the C-terminal end of the molecule, completely blocks formation of hexagonal structures (Killian et al., 1985). This indicates the importance of tryptophans for the bilayer destabilizing effect. In the present paper, we demonstrate that gramicidin, but not its formylated derivative, enhances flip rates of phospholipids and produces in addition to cation-selective channels unspecific membrane leaks in the human erythrocyte membrane. In the following paper (Tournois et al., 1987), it is demonstrated that this effect is paralleled by an induction of hexagonal structures in dispersions of erythrocyte lipids as well as in erythrocyte membranes.

Materials and Methods

Materials. Fresh human blood anticoagulated with citrate was obtained from the local blood bank and used within 4 days. Erythrocytes were isolated by centrifugation, the buffy coat was removed, and cells were washed 3 times with isotonic saline. Gramicidin and *Naja naja* phospholipase A_2 were

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obtained from Sigma (Deisenhofen), bee venom phospholipase A_2 and fatty acid free bovine serum albumin were from Boehringer (Mannheim), and Dextran 4 was from Serva (Heidelberg). [*methyl*-^{14}C]Choline chloride, [U-^{14}C]erythritol, [U-^{14}C]oxalic acid, 1-[1-^{14}C]palmitoyllysophosphatidylcholine (specific activity 1.67 GBq/mmol), and L-1-[1-^{14}C]oleoyllysophosphatidylcholine (specific activity 2.07 GBq/mmol) were purchased from Amersham (Braunschweig), and L-[1-^{14}C]palmitoylcarnitine (specific activity 2.04 GBq/mmol) was from NEN (Dreieich). ^{14}C-Labeled gramicidin (specific activity 2.11 GBq/mmol) was prepared as described in the following paper (Tournois et al., 1987). Trp-N-formylated gramicidin and ^{14}C-labeled formylated gramicidin (specific activity 1.01 MBq/mmol) were prepared by formylation of the four tryptophan residues of the corresponding precursor gramicidins according to Killian et al. (1985). Gramicidins A, B, and C were purified from commercial gramicidin by high-performance liquid chromatography (HPLC)[1] (Killian et al., 1987).

Incubations. One volume of washed erythrocytes was suspended in 9 volumes of a medium containing (concentration in millimoles per liter) KCl (90), NaCl (44), and Na_2HPO_4/NaH_2PO_4 (12.5) (pH 7.4). To protect gramicidin-treated cells against colloid-osmotic lysis, incubations were carried out in media supplemented with 25 mmol·L^{-1} Dextran 4 (M_r 4000–6000) (medium A).

Quantification of Cell-Bound Gramicidin. Erythrocyte suspensions in medium A (hematocrit 10%) were incubated with ^{14}C-labeled gramicidin or its Trp-N-formylated derivative and increasing amounts of the corresponding nonlabeled analogue dissolved in dimethyl sulfoxide (final concentration 1% v/v). After 4 min of incubation at 37 °C, samples were collected to quantify radioactivity of the suspension. The difference between added radioactivity and radioactivity measured in the cell suspension was taken as the radioactivity left at the vessel wall. Subsequently, erythrocytes were pelleted and samples taken to quantify radioactivities in the supernatant. The difference between the radioactivity in the suspension and that in the supernatant represents the cell-bound radioactivity.

Measurements of Flip Rates. Small portions of [^{14}C]-lysophosphatidylcholine or [^{14}C]palmitoylcarnitine dissolved in methanol were dried, and 100-μL samples of a 1:1 suspension of erythrocytes in medium A were added to load (2 min, 22 °C) the outer membrane layer with the labeled lipids (about 20 nmol/mL of packed cells, 0.5 mol % of total membrane phospholipid). The erythrocyte suspension (100 μL) was transferred to a centrifuge tube and mixed with medium A (800 μL, 4 °C). After centrifugation, cells were resuspended in medium A (10% hematocrit, 4 °C), and gramicidin was added from stock solutions in dimethyl sulfoxide (this solvent at a concentration of 1% v/v does not affect the flip rates). Since the flip rate is very slow at 4 °C, the flip process could be considered to be started by warming (3 min) of the cell suspension to 37 °C.

Reorientation of labeled lysophosphatidylcholine or palmitoylcarnitine to the inner membrane layer after their primary incorporation into the outer layer was quantified by measuring the time-dependent decrease of the fraction of these lipids extractable by albumin according to Bergmann et al. (1984a) with minor modifications. Shortly, 50-μL samples of the suspension were taken at different time intervals and diluted with 400 μL of ice-cold medium. Two 200-μL samples of this suspension were centrifuged (20 s, 14000*g*), and the supernatant was removed. One sample of cells serving as the control was mixed with 200 μL of H_2O and pipetted into scintillation fluid to determine the total counts taken up. The other sample was treated for 2 min with 200 μL of a solution of 1.5% albumin in medium A (4 °C) to extract labeled lipids from the outer membrane layer. After centrifugation and removal of the supernatant, the extraction procedure was repeated to increase the efficiency of extraction. Subsequently, cells were washed with 800 μL of medium to remove residual albumin and then transferred to scintillation fluid like the control. The ratio of the counts in the albumin-extracted cells normalized to the total counts in the control sample served as a measure of labeled lipid reoriented to the inner membrane layer. From the increase of this ratio with time, flip rate constants were calculated by assuming a two-compartment system (Bergmann et al., 1984a). In order to minimize effects of acylation of labeled lysophosphatidylcholine to its diacyl analogue on the kinetics of equilibration of lysophosphatidylcholine between the inner and the outer membrane layer, calculations of rate constants for flip of lysophosphatidylcholine were done on nonextractable fractions of radioactivity in which acylation was <20% of this fraction. Acylation was determined after extraction of the lipids from the cells (Rose & Oklander, 1965) followed by thin-layer chromatographic separation of phospholipids (Bergmann et al., 1984b) and scanning of the plate for radioactivity using a TLC linear analyzer of Berthold (Wildbad, FRG).

Temperature Dependence and Reversibility of Gramicidin-Induced Flip Enhancement. In experiments on the temperature dependence and reversibility of gramicidin-induced flip, the experimental protocol for flip measurements described above was slightly modified. Gramicidin was added to the erythrocyte suspension and the suspension warmed up to the desired temperature prior (3 min) to the loading of the cells (<3 min to reach saturation) with [^{14}C]palmitoylcarnitine or [^{14}C]palmitoyllysophosphatidylcholine (hematocrit 10%). Furthermore, after being loaded with the labeled lipid, the suspension was not transferred to a new tube, but flip was directly measured as described above.

Accessibility of Membrane Phospholipids toward Cleavage by Phospholipase A_2. Erythrocytes were suspended in 10 volumes of medium (pH 7.4) containing (concentration in millimoles per liter) KCl (90), NaCl (45), Hepes (10), $MgCl_2$ (0.25) (medium B), and either sucrose or Dextran 4 (40). After addition of gramicidin, the suspension was incubated for 10 min at 37 °C. Subsequently, half of the suspension was treated with phospholipase A_2 from bee venom (10 IU/mL of cells) in the presence of 0.25 mmol·L^{-1} $CaCl_2$ and the other half with phospholipase A_2 from *Naja naja* (20 IU/mL of cells) in the presence of 5 mmol·L^{-1} $CaCl_2$. After increasing time periods, phospholipase activities were blocked by supplementation of samples of the suspensions with 1 and 10 mmol·L^{-1} EDTA, respectively, followed by direct mixing with 5.5 volumes of 2-propanol (15 min) and 3.5 volumes of chloroform (15 min) to extract lipids (Rose & Oklander, 1965). Phospholipids were separated by two-dimensional thin-layer chromatography (silica gel 60, Merck No. 5715, Darmstadt) using chloroform/methanol/ammonia (25%)/H_2O (90:54:6:5) and chloroform/methanol/acetic acid/H_2O (60:30:12:2). Diacyl- and lysophospholipid-containing spots

[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; LPC, lysophosphatidylcholine; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; H_{II}, hexagonal phase of type II; TLC, thin-layer chromatography.

were scraped from the plates, and phospholipid content was quantified by phosphate analysis.

Estimations of Permeability. (*A*) K^+ *Efflux.* In order to assess the K^+ permeability mediated by the gramicidin channel, net efflux of intracellular K^+ from the erythrocytes was determined by following the time-dependent appearance of K^+ ions in the suspension medium. To this end, erythrocytes were washed 3 times with an isotonic solution of choline chloride and resuspended in 9 volumes of isotonic choline chloride buffered with 0.1 mmol/L imidazole (37 °C, pH 7.4). Subsequently, gramicidin was added and K^+ leakage from the cells measured continuously using a K^+-sensitive combined glass electrode (Ingold PK 401-NS-K7).

(*B*) *Net Uptake of Solutes and Water* (*Colloid-Osmotic Lysis*). Erythrocytes made leaky to otherwise impermeable solutes by the introduction of gramicidin into the membrane take up the solutes and water, swell, and undergo colloid-osmotic lysis. In one type of experiment, the inverse of the half-time of lysis of cells incubated with gramicidin in saline media [NaCl 150/phosphate buffer 1 (mmol·L^{-1})] served as a measure of the induced permeability.

In another type of experiment, the colloid-osmotic nature of the lysis and the selectivity of the unspecific leak induced by high concentrations of gramicidin were checked by means of the suppression of lysis by solutes that do not pass the leak pathway and thereby can counterbalance the osmotic pressure of intracellular impermeable molecules (hemoglobin, organic phosphates). To this end, samples of erythrocytes were suspended in 10 volumes of media containing (concentrations in millimoles per liter) NaCl (150), Hepes (5), and either mannitol, sucrose, or Dextran 4 (40) to protect the cells against colloid-osmotic lysis. Erythrocytes suspended in this medium without protectant served as a control. After addition of gramicidin, the time-dependent increase of hemolysis was followed by quantification of hemoglobin released into the medium after centrifugation of the cells. The retardation of lysis by the tentative protectant—relative to lysis in the absence of protectant—served as a semiquantitative measure of permeability for the protectants.

(*C*) *Tracer Efflux.* Tracer fluxes of [^{14}C]choline, [^{14}C]-erythritol, and [^{14}C]oxalate were determined as described by Deuticke et al. (1983). Briefly, cells were loaded with labeled and unlabeled (1 mmol/L) test permeant in medium A, centrifuged, and washed once with tracer-free medium at 0 °C. Efflux was started by injecting the cells into tracer-free medium (37 °C) containing 40 mmol·L^{-1} Dextran 4 (M_r 4000–6000) for osmotic protection. For measurements of erythritol and oxalate fluxes, cytochalasin B (10 μmol·L^{-1}) or 4,4′-dinitrostilbene-2,2′-disulfonate (1 mmol·L^{-1}) was added to inhibit the carrier-mediated components of the fluxes. Gramicidin or Trp-N-formylated gramicidin dissolved in dimethyl sulfoxide was added 30 s later under vigorous stirring. Samples were taken at appropriate intervals and centrifuged (30 s, 12000*g*), and the supernatants were counted for radioactivity. Flux rate coefficients and permeabilities were calculated as described (Deuticke et al., 1983).

Results

Flip Enhancement by Gramicidin. Lysophosphatidylcholine (or palmitoylcarnitine) when added to an erythrocyte suspension is incorporated into the outer membrane layer of the erythrocyte from where it can be extracted by albumin (Bergmann et al., 1984a). In the presence of only trace amounts of these ^{14}C-labeled lipids, no perturbation of the membrane lipid domain will occur. Under our experimental conditions (hematocrit 10%), essentially no labeled lipid is left in the medium (<1%). After reorientation of the lipid probe to the inner membrane layer at 37 °C, its extractability by albumin (2 × 2 min, 4 °C) becomes lost. By measurement of the time-dependent decrease of the extractability of the lipid probe by albumin, the rate of reorientation of the lipid from the outer to the inner membrane layer (flip) can be quantified in native (Bergmann et al., 1984b) as well as in modified (Haest et al., 1983b; Dressler et al., 1983, 1984; Bergmann et al., 1984a; Schneider et al., 1986a) erythrocytes.

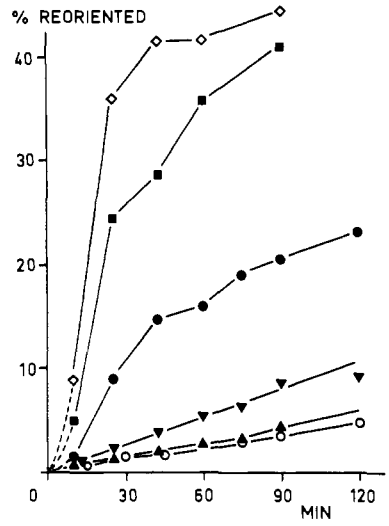

FIGURE 1: Gramicidin-induced enhancement of the transmembrane reorientation of palmitoyllysophosphatidylcholine (37 °C) in human erythrocytes. Time-dependent reorientation of [^{14}C]palmitoyllysophosphatidylcholine from the outer to the inner membrane layer in the presence of varying concentrations of gramicidin as measured by the increase of the fraction of lysophosphatidylcholine nonextractable by albumin: 0 (○), 0.25 (▲), 0.5 (▼), 1 (●), 2.5 (■), and 5 (◇) μmol of gramicidin/L of suspension.

The flip of palmitoyllysophosphatidylcholine is a slow process (Figure 1) with a half-time of 11 h at 37 °C (Bergmann et al., 1984b). The flip process becomes dramatically enhanced by addition of increasing concentrations of gramicidin (Figure 1). The first increase of flip rate is observed at concentrations of gramicidin >0.25 μmol·L^{-1} (37 °C, hematocrit 10%). In addition to flip acceleration, gramicidin seems to affect the stationary distribution of lysophosphatidylcholine between the inner and the outer membrane layer. In the presence of 5 μmol·L^{-1} gramicidin, after 1 h of flip the fraction of lysophosphatidylcholine in the inner layer, which can be calculated from the albumin-inextractable fraction after correction for acylation, increases from 0.3 in native cells (Bergmann et al., 1984b) to at least 0.4. This indicates an effect of gramicidin on the distribution of lysophosphatidylcholine between inner and outer membrane layers. From the time dependence of the reorientation of lysophosphatidylcholine to the inner membrane layer, flip rate constants can be calculated (Bergmann et al., 1984a). As Figure 2 shows, gramicidin produces a concentration-dependent enhancement of flip rates of palmitoyl- and oleoyllysophosphatidylcholine as well as of palmitoylcarnitine.

To correlate flip enhancement with numbers of gramicidin molecules bound per cell, binding studies using labeled gramicidin were carried out. Data compiled in Table I show that the fraction of bound gramicidin depends on the concentration of gramicidin. With increasing concentration of gramicidin, the fraction of membrane-bound gramicidin increases (Table I), while the fractions of gramicidin in the medium and at the vessel wall decrease. This is in agreement with an observation of Kemp et al. (1972). A decrease of the fraction of labeled

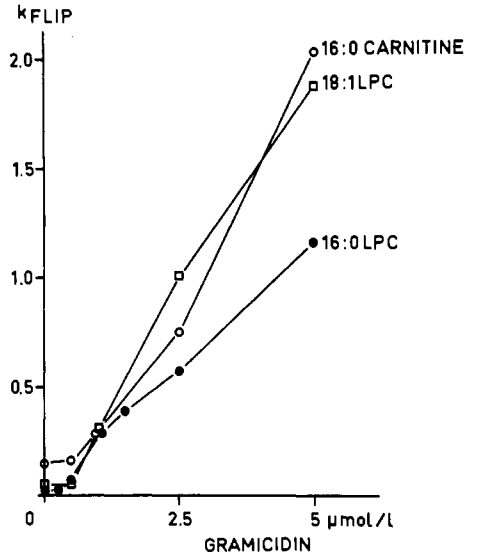

FIGURE 2: Concentration-dependent increase of flip rate constants (37 °C) for palmitoyllysophosphatidylcholine (16:0 LPC) (●), oleoyllysophosphatidylcholine (18:1 LPC) (□), and palmitoylcarnitine (○). Rate constants for reorientation [K_{flip} (h^{-1})] were calculated according to Bergmann et al. (1984a) by using a value of 0.3 for the fraction of lysophosphatidylcholine in the inner membrane layer at infinite time for gramicidin concentrations up to 1 μmol·L^{-1}. For higher gramicidin concentrations, a value of 0.5 was used. A value of 0.5 was also used for palmitoylcarnitine. The data represent mean values from up to six experiments.

Table I: Extent of Binding of Gramicidin and Its Trp-Formylated Derivative to Erythrocytes at Increasing Concentrations[a]

	% of radioact. for gramicidin (μmol·L^{-1} of suspension)					
compartment	0.2	0.5	1	2.5	5	34
membrane	37	41	46	51	58	87
medium	31	29	32	32	29	4
vessel wall	32	30	22	17	13	9

	% of radioact. for Trp-N-formylated gramicidin (μmol·L^{-1} of suspension)	
compartment	32	64
membrane	73	78
medium	10	6
vessel wall	17	16

[a] The results represent the mean value of up to four experiments.

gramicidin in the supernatant at higher concentrations is not the result of sedimentation of gramicidin due to its low water solubility, since in the absence of cells no radioactivity could be pelleted by centrifugation (data not shown).

In further studies, we investigated the temperature dependence of the induced flip. At 37 and 33 °C, gramicidin enhances the flip rate of palmitoylcarnitine considerably and almost instantaneously (Figure 3). Conversely, at 29 °C, flip enhancement is not observed during the first 45 min of gramicidin treatment, but enhancement of reorientation of palmitoylcarnitine gradually occurs after this lag phase. At 25 °C, the lag phase is much longer. The flip remains at the control level during the first 3 h of the gramicidin treatment but then starts to increase (Figure 3 insert). Pretreatment of cells with gramicidin at 37 °C for 15 min and subsequent flip measurement at 25 °C abolished the lag phase (data not shown). For the flip of palmitoyllysophosphatidylcholine, the same temperature dependence of the onset of the gramicidin-induced flip was found as for the flip of palmitoylcarnitine

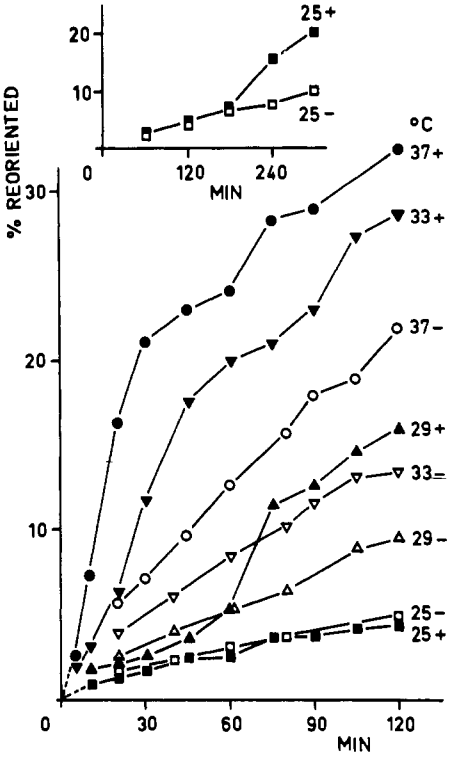

FIGURE 3: Influence of temperature on the flip rate enhancement by gramicidin. Erythrocytes loaded with ^{14}C-labeled palmitoylcarnitine were suspended in medium A of 25 (□, ■), 29 (△, ▲), 33 (▽, ▼), and 37 (○, ●) °C in the presence (+, closed symbols) or absence (–, open symbols) of gramicidin (2.5 μmol·L of suspension). Insert: Lag phase for gramicidin-induced flip enhancement at 25 °C.

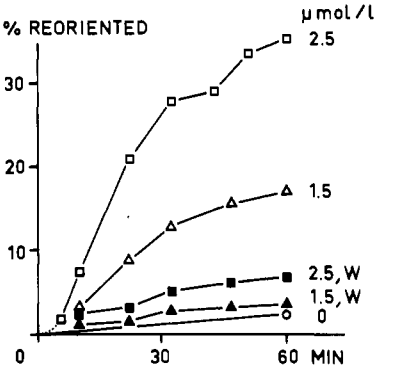

FIGURE 4: Reversibility of the gramicidin-induced enhancement of flip rates. Erythrocyte suspensions were incubated (3 min, 37 °C) with 1.5 (△, ▲) or 2.5 (□, ■) μmol of gramicidin/L. Subsequently, one portion of the cell suspension was directly loaded with ^{14}C-labeled palmitoyllysolecithin (open symbols) and the other one after two washings (W) with medium A at 4 °C and resuspension in this medium (closed symbols). Flip was then measured at 37 °C. For comparison, the flip for nontreated cells (○) is given.

(data not shown). Since the fraction of membrane-bound antibiotic is quite similar at 0 and at 37 °C (data not shown), the temperature-dependent lag phase of flip enhancement is not due to a temperature dependence of gramicidin binding to the cells.

Further experiments aimed to check the reversibility of the flip enhancement induced by gramicidin. In analogy to the reversibility of the flip enhancement by the channel-forming polyene antibiotic amphotericin B (Schneider et al., 1986a), the increase of flip rates by gramicidin could be reversed to a high extent by washing of the cells within 3 min after addition of gramicidin at 37 °C (Figure 4). Binding experiments demonstrate that gramicidin, although incompletely, can be removed from the cells by washing. However, pretreatment

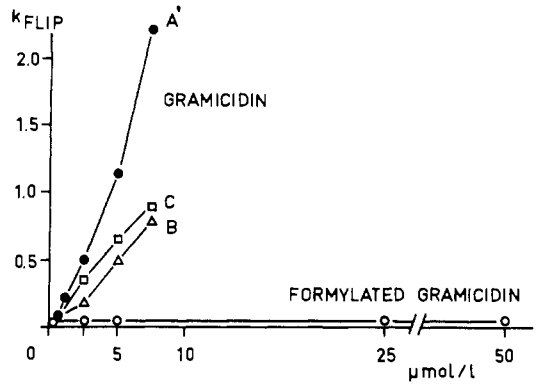

FIGURE 5: Flip enhancement by gramicidins B and C and lack of effect of Trp-formylated gramicidin. Erythrocytes were loaded with [^{14}C]palmitoyllysophosphatidylcholine and resuspended in medium A. Varying concentrations (μmol·L^{-1} of suspension) of commercial gramicidin (A'), of gramicidin B, of gramicidin C, or of Trp-N-formylated gramicidin were then added. Subsequently, flip rates (h^{-1}) were measured in the presence of gramicidin and rate constants calculated (Bergmann et al., 1984a).

for 15 min at 37 °C, which exceeds the lag phase, results in a complete loss of reversibility of the gramicidin effect, whereas the amphotericin effect on flip can still be reversed to a high extent after a 1-h pretreatment (Schneider et al., 1986a). Loss of reversibility of the gramicidin-induced flip enhancement indicates that the state of association of gramicidin with the membrane is altered in the course of the lag phase.

To characterize the specificity of the flip enhancement by gramicidin, the effects on flip rates of the three purified components A, B, and C contained in commercial gramicidin (Glickson et al., 1972) as well as Trp-N-formylated gramicidin were investigated. Purified gramicidin A, which is the major component of the commercial mixture, had essentially the same flip-enhancing effect as commercial gramicidin (data not shown). Gramicidins B and C, which produce channels (Bamberg et al., 1976) but differ from gramicidin A in that tryptophan in position 11 is replaced by phenylalanine and tyrosine, respectively (Glickson et al., 1972), also enhanced flip rates albeit to a lower extent than commercial gramicidin (A'), gramicidin B being the least effective (Figure 5). The Trp-N-formylated analogue was prepared from the natural gramicidin mixture by formylation of its four tryptophan residues. Trp-N-formylated gramicidin differs from gramicidin in its efficiency to induce K^+ leakage. At least 10-fold higher concentrations of the Trp-formylated analogue than of gramicidin are required to produce the same increase of K^+ leak permeability (Figure 6). Formation of a K^+ leak by the analogue is not due to small amounts of nonformylated gramicidin remaining after derivatization. HPLC demonstrated an essentially complete (>99%) formylation (data not shown). Interestingly, the formylated analogue does not enhance flip rates even at 10 times the concentration of gramicidin that produces a 30-fold increase of flip rate (Figure 5).

Since the lack of flip acceleration and the low efficiency of induction of K^+ leakage by the Trp-N-formylated derivative might be due to a low affinity for the membrane, binding studies with the analogue were also carried out. Binding of the derivative could only be studied at higher concentrations due to its low specific radioactivity. At these higher concentrations (at which gramicidin, but not the Trp-N-formylated analogue, produces flip acceleration), the fraction of the membrane-bound formylated analogue was comparable to that of gramicidin (Table I). Thus, lack of flip enhancement in the presence of Trp-N-formylated gramicidin is not the result of differences in the primary binding to the membrane but is

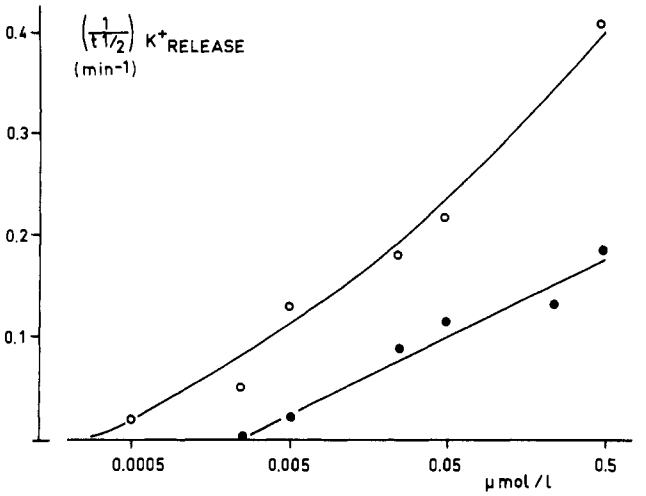

FIGURE 6: Loss of K^+ from erythrocytes into isotonic choline chloride solution induced by various concentrations of gramicidin (○) and of its Trp-N-formylated derivative (●). The appearance of K^+ in the external medium was followed by a K+-sensitive electrode. From the curves, half-times of K^+ release ($t_{1/2}$) were calculated. The inverse of $t_{1/2}$ was taken as a measure of permeability.

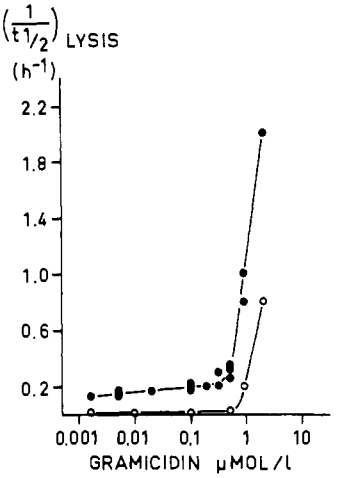

FIGURE 7: Rates of lysis of erythrocytes as a function of gramicidin concentration. Erythrocytes were incubated in phosphate-buffered (1 mmol·L^{-1}) isotonic saline (hematocrit 5%) containing gramicidin in the absence (●) or presence (○) of sucrose (40 mmol·L^{-1}). Rates of hemolysis are defined as the reciprocal of the time required for 50% lysis. Note the abrupt transition from a slow, sucrose-suppressible to a fast, highly concentration-dependent but non-sucrose-suppressible lysis at about 0.5 μmol of gramicidin/L^{-1} suspension.

due to differences in the interaction of gramicidin and of its analogue with membrane constituents.

Formation of Unspecific Membrane Leaks by Gramicidin. Leakage of K^+ from gramicidin-treated cells is observed at antibiotic concentrations much below those required for flip enhancement (compare Figures 6 and 2). A simple relationship between the formation of cation-permeable channels and of "flip sites" is therefore unlikely. Since gramicidin channels (internal diameter 0.4–0.5 nm) are selective for small monovalent cations and H_2O (Hladky & Haydon, 1984), erythrocytes lyse slowly in saline media in the presence of low concentrations of gramicidin (Figure 7) due to swelling following the coupled uptake of alkali cations via the channel and of chloride via the conductance pathway of band 3 (Cass & Dalmark, 1979). This colloid-osmotic lysis can be fully suppressed by sucrose (44 mmol·L^{-1}) to which the channel is impermeable. At higher concentrations of gramicidin (>0.5 μmol·L^{-1} suspension, hematocrit 10%), rates of lysis increase much more with gramicidin concentration than in the lower

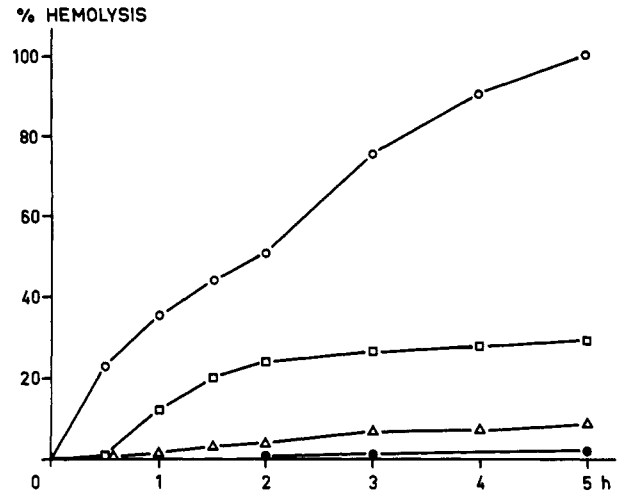

FIGURE 8: Protection of cells against gramicidin-induced lysis by nonelectrolytes of varying size. Erythrocytes were suspended in Hepes-buffered isotonic saline (hematocrit 10%) containing gramicidin (3.5 μmol·L^{-1} of medium), and the time course of hemolysis was measured in the absence (○) or presence of different solutes of varying molecular weights: (□) mannitol; (△) sucrose; and (●) Dextran 4.

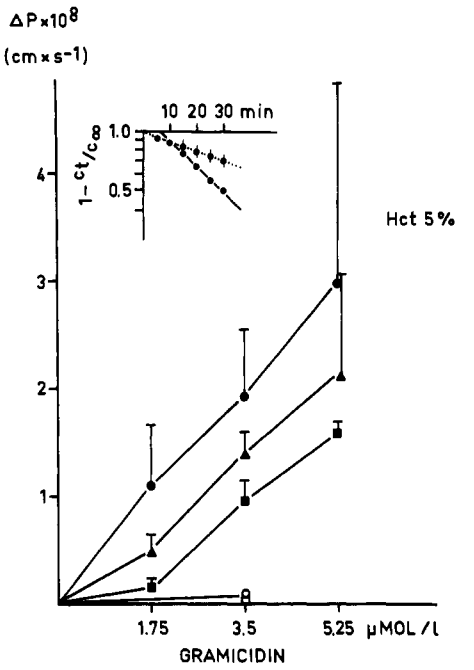

FIGURE 9: Leak permeabilities for various solutes induced in erythrocytes by high concentrations of gramicidin. Cells were loaded with labeled test solutes, and efflux was measured (hematocrit 5%) as described under Materials and Methods, in the presence of gramicidin (closed symbols) or of Trp-N-formylated gramicidin (open symbols): (○, ●) choline; (□, ■) oxalate (in the presence of DNDS, 1 mmol·L^{-1}); (△, ▲) erythritol (in the presence of cytochalasin B, 10 μmol·L^{-1}). Mean values (±SEM) from four to eight experiments are given. Insert: Time course of efflux of choline in the absence (◖) or presence (●) of gramicidin (added at 30 s), illustrating the time lag in the onset of leak formation.

concentration range, and sucrose loses its protective influence (Figure 7). This suggests the formation of an additional pathway of leak permeability. This additional leak pathway discriminates permeants according to size as indicated by differences in the protection by large nonelectrolytes of varying molecular weight (Figure 8). Dextran 4 suppresses hemolysis much better than sucrose and sucrose better than mannitol. Interestingly, protection by the nonelectrolytes was not complete. A fraction of the cell population lysed even in the presence of protectant. This fraction decreased with the size of the protectant at a given concentration of gramicidin (Figure 8) and increased with the concentration of antibiotic for a given protectant (data not shown).

Tracer flux measurements (Figure 9) more directly demonstrated that an unspecific leak permeability is induced in the erythrocyte membrane by gramicidin. Three types of solutes which do not permeate the gramicidin channel (Hladky & Haydon, 1984)—a large cation (choline), a divalent anion (oxalate), and a large hydrophilic nonelectrolyte (erythritol)—passed the erythrocyte membrane at a considerable rate at high concentrations of gramicidin. The normal routes of transfer of these solutes either are very slow (choline; Ashari, 1966) or had been blocked by inhibitors (see Materials and Methods). The effect increases with the gramicidin concentration. In contrast, Trp-N-formylated gramicidin does not produce such a leak permeability (Figure 9).

Formation of unspecific leaks in the presence of gramicidin is not instantaneous but takes about 7–10 min at 37 °C after addition of the gramicidin, as indicated by the graphs for the pseudo-first-order efflux kinetics (Figure 9 insert). Such a lag phase can also be derived from Figure 1 for flip enhancement by gramicidin. The extent of induced leakiness scatters considerably. We think this is due to the variable incorporation of the antibiotic which is not well dispersible in water, thereby forming aggregates that are unable to enter the membrane rapidly after injection of gramicidin solution into the suspension. These results demonstrate that the formation of unspecific leaks in erythrocytes starts at gramicidin concentrations at which flip rates becomes enhanced, i.e., in a range of concentrations far above those required to produce cation-selective channels.

Effect of Gramicidin on the Transbilayer Asymmetry of Phospholipids. Further experiments aimed to check possible consequences of flip enhancement for the transbilayer distribution of phospholipids in the erythrocyte membrane as assessed by the phospholipase technique. Figure 10 shows that the accessibility of the outer membrane layer phosphatidylcholine toward cleavage by phospholipase A_2 from bee venom or from *Naja naja* is enhanced by addition of gramicidin. This finding indicates changes of the organization of outer membrane layer phospholipids. Trp-N-formylated gramicidin did not enhance the rate of phosphatidylcholine cleavage (data not shown), which is in line with its lack of effect on flip rates. In parallel to the flip enhancement by gramicidin, which starts after a temperature-dependent delay, the phosphatidylcholine cleavage by phospholipase A_2 (bee venom, 10 min) gradually increased from 15% to a maximum of 60–65% after a 1-h pretreatment of cells with gramicidin (5 μmol·L^{-1}) at 25 °C (data not shown). Thus, enhancement of phosphatidylcholine cleavage by gramicidin is correlated to the formation of the structural defects acting as "flip sites" in the membrane lipid domain. No evidence for an acceleration of phosphatidylcholine flip from the inner [according to Verkleij et al. (1973), 25% of the phosphatidylcholine is contained in this layer] to the outer membrane layer could be derived from the extent of phosphatidylcholine cleavage, which did not exceed 70% before hemolysis (>3%) occurred.

Despite high rates of phosphatidylcholine cleavage by the lipase and a 30-fold increase of flip rate of lysophosphatidylcholine, no significant increase of cleavability of inner layer phosphatidylethanolamine (Figure 10) and phosphatidylserine (data not shown) was observed in gramicidin (5 μmol·L^{-1} of medium) treated cells. In most studies, sucrose was used to protect the cells against hemolysis, since the better protectant Dextran 4 appeared to have a stimulatory effect on the phospholipase itself. In order to check cleavability of

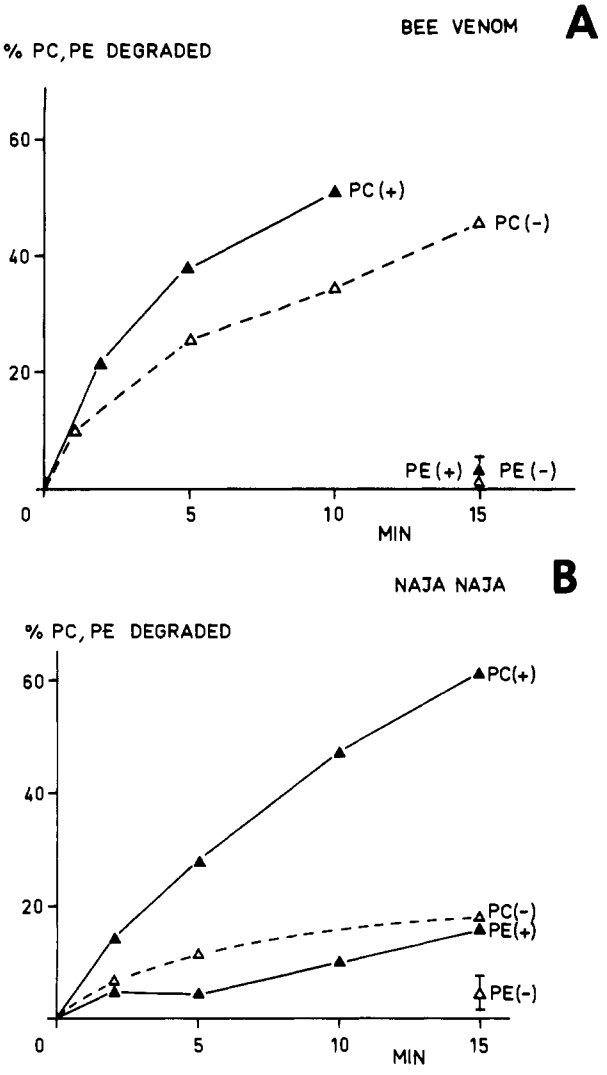

FIGURE 10: Gramicidin-induced enhancement of cleavage of membrane phospholipds by phospholipase A_2. Erythrocytes in medium B supplemented with sucrose (40 mmol·L^{-1}) were incubated either without (△) or with (▲) gramicidin (5 μmol·L^{-1} of medium) for 10 min at 37 °C. After the addition of Ca^{2+}, the cells were treated with phospholipase A_2 from bee venom (A) or *Naja naja* (B) in the presence of the antibiotic for increasing time periods. PC, phosphatidylcholine; PE, phosphatidylethanolamine.

phospholipids under more extreme conditions of modification and phospholipase exposure (45 min) in the absence of lysis, some studies were done with Dextran 4 as a protectant. In this case, acceleration of phosphatidylcholine cleavage in the presence of gramicidin, although much less pronounced than in the presence of sucrose, was observed, too. The maximal amount of phosphatidylethanolamine cleavable in the presence of gramicidin before hemolysis exceeds 3% of the cell population varied between ≤5% and 21% (n = 12). No cleavage of phosphatidylserine could be detected. Extension of the time of pretreatment of cells with gramicidin from 10 to 60 min, which would increase the time to establish a possible new equilibrium distribution, did not enhance the accessibility of inner membrane layer phospholipids either (Haest & Classen, 1986).

Discussion

Analogues of endogenous diacylphospholipids such as lysophospholipids, acylcarnitines, and platelet activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) can be used to investigate the process of transbilayer reorientation of phospholipids in the erythrocyte membrane (Bergmann et al., 1984a,b; Haest et al., 1986; Schneider et al., 1986a,b). The half-times for the flip from the outer to the inner membrane layer of various lysophospholipids are of the same order of magnitude as those for diacylphosphatidylcholines of varying fatty acid composition (Haest et al., 1986; Middelkoop et al., 1986). Moreover, the steady-state distribution of lysophospholipids of varying polar head-group structure between inner and outer membrane layers mimics the asymmetric distribution of their endogenous diacyl analogues (Haest et al., 1983a, 1986).

The slow flip rate of palmitoyllysophosphatidylcholine at 37 °C ($t_{1/2}$ = 11 h) can be highly enhanced by chemical modification of membrane proteins (Bergmann et al., 1984a; Haest et al., 1983b; Mohandas et al., 1982), by reversible electric breakdown of the membrane barrier (Dressler et al., 1983), or by insertion of compounds such as local anesthetics, channel-forming polyene antibiotics such as amphotericin, or bacterial cytolytic proteins (Schneider et al., 1986a; Haest et al., 1986). We have now demonstrated that the hydrophobic pentadecapeptide gramicidin, which produces cation-selective channels [internal diameter 0.4–0.5 nm (Hladky & Haydon, 1984)], highly accelerates flip rates, too. The possibility that a loss of extractability of lysophosphatidylcholine or palmitoylcarnitine by albumin is due to specific binding of the lipid probes to gramicidin, which has been found for gramicidin/lysophosphatidylcholine mixtures (Killian et al., 1983) and might simulate high flip rates, seems unlikely for a number of reasons. First, addition of albumin at 37 °C after 30 min of palmitoylcarnitine flip in the presence of gramicidin instantaneously removes a fraction of palmitoylcarnitine from the cells, probably located in the outer membrane layer, followed by a time-dependent removal of the remaining fraction, probably originally located in the inner layer and becoming accessible to albumin extraction after reorientation to the outer membrane surface. Second, essentially all of the lysophosphatidylcholine that becomes nonextractable by albumin from the cells after a 45-min flip period (37 °C) in the presence of gramicidin (2.5 μmol·L^{-1}) could be removed from the membrane by albumin following hemolysis of the cells (data not shown). Third, enhancement by gramicidin of reorientation of [^{14}C]palmitoyllysophosphatidylcholine to the inner membrane layer is demonstrated by acceleration of the incorporation of radioactivity derived from lysophosphatidylcholine into diacylphosphatidylcholine, a process taking place at the inner membrane surface (Renooij et al., 1974). After 90 min of flip at 37 °C in the presence of gramicidin (2.5 μmol·L^{-1}), 11% of the radioactivity is found in the diacylphosphatidylcholine fraction (data not shown), whereas in control cells in the absence of gramicidin a maximum of 3%, which is the fraction reoriented to the inner membrane surface (Bergmann et al., 1984b), can be acylated. It should be considered that at high flip rates formation of diacylphosphatidylcholine is probably limited by the acylation process.

In all membrane modifications that we studied previously, a marked increase (>30-fold) of flip rates was accompanied by a partial loss of the asymmetric orientation of the aminophospholipids phosphatidylethanolamine and phosphatidylserine to the inner membrane surface (Haest et al., 1978; Dressler et al., 1983; Bergmann et al., 1984a; Schneider et al., 1986a). In contrast, gramicidin has only minor effects on aminophospholipid orientation [Figure 8 and Haest and Classen (1986)]. This stability of orientation in spite of high flip rates is new support for our concept of the existence of stabilizing forces at the inner membrane surface that suppress

a spontaneous reorientation of aminophospholipids into the outer membrane layer (Haest et al., 1978; Haest, 1982; Dressler et al., 1984). In our concept, the ATP-dependent translocation of aminophospholipids from the outer to the inner membrane layer reported by Devaux and collaborators (Zachowski et al., 1986) serves to shorten the residence time of the aminophospholipids in the outer layer when they escape from the inner membrane layer by chance.

The first enhancement of flip by gramicidin is observed at a concentration of about 0.5 $\mu mol \cdot L^{-1}$ (1 $\mu g/mL$) of suspension. Considering an incorporation of about 40% of the gramicidin added to the cell suspension (Table I), this represents 2 nmol of gramicidin per milliliter of packed cells, equivalent to 1.2×10^5 copies per cell, or a gramicidin to phospholipid ratio of about 1:2000 on the basis of 4.25 μmol of phospholipid per milliliter of packed cells (Broekhuyse, 1974).

Flip site formation is unlikely to be due to perturbation of the lipid bilayer at the interface between single channels and their surrounding lipids for two reasons. First, K^+ conducting channels are already detectable at a concentration of gramicidin 1000-fold lower (Figure 6) than that required for an increase of flip rates (Figure 2). Second, Trp-N-formylated gramicidin does not enhance flip rate, even at concentrations at which incorporation in the hydrophobic barrier is demonstrated by induction of a fast K^+ release. The question thus arises how gramicidin increases flip rates. The enhancement of flip rates by gramicidin is accompanied by formation of rather large unspecific aqueous leaks (Figures 8 and 9) on top of the cation-selective channel. The leaks induce permeabilities of the order of $(0.5–3) \times 10^{-8}$ $cm \cdot s^{-1}$ and are permeable to anions, cations, and large nonelectrolytes up to the size of at least sucrose ($r_{SE} \sim 0.5$ nm) while Dextran 4 (mean radius about 1.7 nm) hardly can pass this pathway. Similar leaks have been shown to accompany the flip enhancement by the SH-oxidizing agent diamide (Deuticke et al., 1983), by the radical-forming agent *tert*-butyl hydroperoxide (Deuticke et al., 1986), by electric breakdown (Schwister & Deuticke, 1985), and by local anesthetics (B. Deuticke, unpublished results). In these cases, lateral lipid phase separations including formation of nonbilayer phases or an induced mismatched between intrinsic proteins and lipids have been discussed (Deuticke et al., 1983; Haest et al., 1983b; Schwister & Deuticke, 1985).

In the case of gramicidin's potency to induce the formation of flip sites and unspecific leaks, the capacity of the antibiotic to perturb the lipid bilayer should be considered. Gramicidin has been shown to induce hexagonal H_{II} phases in lipid bilayers (Killian & De Kruijff, 1986). In artificial lipid membranes, the presence of nonbilayer, e.g., H_{II} phases has long been known to go along with high flip rates of phospholipids and the occurrence of a permeation pathway for polar solutes (Gerritsen et al., 1980; Noordam et al., 1981). As shown in the following paper (Tournois et al., 1987), gramicidin indeed produces hexagonal phases in lipid bilayers prepared from total erythrocyte lipids as well as in isolated erythrocyte membranes. However, H_{II} phase induction by gramicidin occurs at a much higher ratio of gramicidin to phospholipid than that required to produce flip sites. Formation of the H_{II} phase can thus not be responsible for the observed flip enhancement. Therefore, other membrane structural changes induced by gramicidin occurring already at a lower gramicidin to phospholipid ratio and possibly ending up in H_{II} phase formation at higher ratios should be considered. All three—the high capacity of gramicidin to form cation-selective channels, its effect on the formation of hexagonal phases, and its accelerating effect on flip rate—are specific for the native antibiotic. Formylation of the four tryptophan residues of gramicidin abolishes the formation of hexagonal H_{II} lipid structures (Killian et al., 1985), eliminates enhancement of flip rate (Figure 5), and reduces cation-selective channel formation (Figure 6). This lack of effect of Trp-N-formylated gramicidin is not due to a possible low extent of membrane binding (Table I). On the other hand, both gramicidin B and gramicidin C, which contain the aromatic amino acids phenylalanine and tyrosine instead of tryptophan in position 11 of the peptide chain (Glickson et al., 1972) and induce smaller or similar conductivities than gramicidin (Bamberg et al., 1976), produce hexagonal lipid structures, albeit to a lesser extent (Killian et al., 1987), as well as flip sites (Figure 5). Induction of hexagonal lipid structures by gramicidin has been claimed to depend on the aggregation of gramicidin in the membrane (Killian & De Kruijff, 1985a,b). A time- and temperature-dependent formation of gramicidin clusters could explain the temperature-dependent lag phase before flip acceleration and formation of unspecific leaks occurs. Moreover, it could also explain the disappearance of the lag phase after a short pretreatment (15 min) of the cells at 37 °C in flip kinetics at lower temperatures. In this concept, the observation that reversibility of the gramicidin-induced flip acceleration by washing of the cells is lost during the lag phase would mean that gramicidin clusters cannot be removed from the cells by washing. Formation of gramicidin clusters, which are essential intermediates in the formation of hexagonal H_{II} phases, could produce structural defects in the bilayer functioning as flip sites and as a nonspecific permeability pathway for ions and nonelectrolytes, as will be discussed in the following paper in this issue (Tournois et al., 1987).

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