Potassium Flux through Gramicidin Ion Channels Is Augmented in Vesicles Comprised of Plasmenylcholine: Correlations between Gramicidin Conformation and Function in Chemically Distinct Host Bilayer Matrices[†]

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ABSTRACT: The functional role of distinct phospholipid subclasses and molecular species in modulating gramicidin-mediated K^+ flux was characterized through quantification of changes in the fluorescence intensity of ion specific fluorescent probes encapsulated inside vesicles comprised of individual molecular species of plasmenylcholine and phosphatidylcholine. The rate constant of gramicidin-mediated K^+ ion flux across bilayers comprised of 1-*O*-(*Z*)-hexadec-1′-enyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphocholine (plasmenylcholine) was 18.9 ± 1.7 s^{-1}, while that present across bilayers comprised of 1-hexadecanoyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphocholine (phosphatidylcholine) was 12.3 ± 1.5 s^{-1}. The observed changes were not due to alterations in the nature of the *sn*-2 aliphatic chain or the net surface charge present at the membrane interface and were unaltered by the addition of several amphiphilic agents (including charged amphiphiles), suggesting that the observed alterations specifically reflect changes in channel function which result from the covalent alteration of host phospholipid in the proximal portion of the *sn*-1 aliphatic chain (i.e., phospholipid subclass-specific alterations). Addition of cholesterol to bilayer matrices comprised of plasmenylcholine resulted in dose-dependent attentuation of the rate of gramicidin-mediated K^+ flux, but did not alter the rate of gramicidin-mediated K^+ flux in membranes comprised of phosphatidylcholine. Gramicidin ion channels experience distinct environments in membranes comprised of phosphatidylcholine and plasmenylcholine host lipids demonstrated by both the different fluorescence anisotropies of endogenous tryptophan residues and the different C=O stretching frequencies of intramonomer carbonyls in gramicidin incorporated into these two choline glycerophospholipid subclasses. Collectively, these results demonstrate the importance of the aggregate properties of host−guest complexes comprised of ion channels and phospholipids in biologic membranes as a primary determinant of ion channel function and identify a likely mechanism contributing to the predominance of plasmalogen molecular species in electrically active membranes.

Electrically active membranes in many cell types (e.g., the sarcolemmal membrane in myocytes) are highly enriched in plasmalogen molecular species (Gross, 1984, 1985). Plasmalogen molecular species contain a vinyl ether bond in the proximal portion of their *sn*-1 aliphatic constituent and thus differ from their diacyl phospholipid counterparts by the presence of two sp^2 carbons at the hydrophobic−hydrophilic interface. The resultant alterations in critical geometric relationships, dynamics, and physical properties engendered by the vinyl ether bond at the membrane interface are now well established (Pak et al., 1987; Han & Gross, 1990; Chen et al., 1993). The accommodation of transmembrane ion channels in a phospholipid bilayer matrix formally represents one specialized subset of host−guest chemistry. A fundamental principle of host−guest chemistry asserts that the functional characteristics of the host−guest complex reflect properties that are unique to the aggregate and are distinct from its constituent parts. Since transmembrane ion channels are resident guests in bilayer matrices comprised of specialized phospholipids, one potential reason underlying the predominance of plasmalogens in electrically active membranes is their ability to facilitate the function of ion channels through specific host−guest interactions.

Gramicidin is a linear pentadecapeptide isolated from *Bacillus brevis* which forms transmembrane channels that have served as prototypical models for studying the molecular mechanisms underlying transmembrane ion permeation, lipid−protein interactions, and the conformational dynamics of transmembrane ion channels (Pullman et al., 1988; Cornell et al., 1988; Killian, 1992). Despite considerable efforts to elucidate the role that the lipid matrix plays in modulating the structure and function of the gramicidin ion channel, the functional sequelae of establishing a host−guest relationship between the major phospholipid in electrically active membranes (i.e., plasmalogens) and any transmembrane ion channel have not yet been explored. We now report that plasmalogen molecular species facilitate gramicidin-mediated potassium flux, that these effects reflect a distinct structural and dynamic motif of gramicidin in membranes comprised of plasmalogen molecule species, that the observed functional changes in channel kinetics are independent of alterations in the bulk physical properties of the membrane bilayer, and that cholesterol substantially modulates the efficiency of

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gramicidin-mediated potassium flux in membranes comprised of plasmenylcholine but not in membranes comprised of phosphatidylcholine.

MATERIALS AND METHODS

Materials. 1-Hexadecanoyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphocholine (PO PhosCho),[1] 1-hexadecanoyl-2-eicosa-5′,8′,11′,14′-tetraenoyl-*sn*-glycero-3-phosphocholine (PA PhosCho), and 1-hexadecanoyl-*sn*-glycero-3-phosphocholine (LPhosCho) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Homogeneous 1-*O*-(*Z*)-hexadec-1′-enyl-*sn*-glycero-3-phosphocholine (LPlasCho), 1-*O*-(*Z*)-hexadec-1′-enyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphocholine (PO PlasCho), and 1-*O*-(*Z*)-hexadec-1′-enyl-2-eicosa-5′,8′,11′,14′-tetraenoyl-*sn*-glycero-3-phosphocholine (PA PlasCho) were prepared as described previously (Han et al., 1992). The purity of these compounds was substantiated by thin-layer chromatography, straight-phase HPLC, and electrospray ionization mass spectroscopy. [^{3}H]Dipalmitoylphosphatidylcholine and [^{14}C]inulin were supplied by NEN (Boston, MA). Arachidonic acid was purchased from Nu Chek Prep, Inc. (Elysian, MN). Tetramethylammonium chloride and 2,2,2-trifluoroethanol (99.5%) (TFE) were obtained from Aldrich Chemical Co. (Milwaukee, WI). The fluorescent probe potassium-binding benzofuran isophthalate (PBFI) was purchased from Molecular Probes, Inc. (Eugene, OR). Most other chemicals including a mixture of naturally occurring gramicidins (approximately 80% gramicidin A, 5% gramicidin B, and 15% gramicidin C) were obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of Samples for Stopped-Flow Fluorescence Spectroscopy. Stock solutions of choline glycerophospholipids (10.5 μmol) in $CHCl_3$/MeOH (2:1) were added to tubes containing in addition either arachidonic acid, lysophospholipids, lidocaine, or cholesterol as indicated to form the stated mixtures. Solutions containing the indicated lipids were dried under a nitrogen stream to thin films prior to the addition of 0.0025 mol % gramicidin in TFE. The total volume of TFE in each preparation was adjusted to 0.5 mL, and the mixtures were subsequently incubated for 1 h at 37 °C to assure that gramicidin was present in the channel conformation ($\beta^{6.3}$-helix) (LoGrasso et al., 1988; Killian et al., 1988; Bañó et al., 1991). Next, the TFE was evaporated under a nitrogen stream and subsequently evacuated (10 mTorr) overnight to ensure its complete removal. Lipid mixtures were hydrated in 0.8 mL of buffer [300 mM $(CH_3)_4$NCl, 10 mM MOPS, 5 mM Tris·HCl, 0.01 mM EDTA, and 100 mM LiCl, pH 7.0 at 25 °C] containing 300 μM PBFI for 0.5 h prior to vigorous vortexing to form multilamellar vesicles (MLVs). Freeze-thawed MLVs were obtained by alternately freezing the MLVs in liquid nitrogen for 3 min and thawing in a 37 °C water bath (repeated five times). The resulting multilamellar vesicle dispersion was then passed through an extrusion device (Lipex Biomembranes, Inc., Vancouver, Canada) to form large unilamellar vesicles (LUVETs). Extrusion of MLVs was performed through 25-mm polycarbonate filters (0.1-μm pore size) (Nucleopore Corp., Pleasanton, CA). Iterative passage of vesicles (10 times) resulted in the formation of LUVETs with a diameter of 0.1 μm and a trapped volume of $\approx$1.7 μL/μmol of lipid (Hope et al., 1985; Mayer et al., 1986). The unencapsulated PBFI probe (i.e., free probe in solution) was removed by gel filtration chromatography employing Sephadex G-50 (medium) spin columns. Briefly, Sephadex G-50 was preswollen in buffer [300 mM $(CH_3)_4$NCl, 10 mM MOPS, 5 mM Tris·HCl, 0.01 mM EDTA, and 100 mM LiCl, pH 7.0 at 25 °C] and packed dry in a 3 mL disposable syringe (Pick, 1981). An aliquot of passaged vesicles (200 μL) was loaded onto the column and eluted by centrifugation of the spin column for 4 min at $200g_{max}$. Phospholipid mass was quantified by acid methanolysis and capillary gas chromatography (Gross, 1984; Fink & Gross, 1984). Gramicidin content (in vesicles putatively containing 2 mol % gramicidin) was assessed by measuring the absorbance at 280 nm (25 °C) after dilution of the sample in methanol [a molar extinction coefficient of 20 787 M^{-1} cm^{-1} was determined in our study, which approximates the value of 20 700 M^{-1} cm^{-1} previously reported (Killian et al., 1988)]. The final lipid/gramicidin molar ratio was nearly equal to that of the initial mixtures and was individually determined for each of the subclasses and molecular species utilized in this study.

Determination of Surface Area to Volume Ratio Present in Vesicles Comprised of Different Phospholipid Subclasses. The surface area to volume ratio of different choline glycerophospholipid subclasses was determined as previously described (Chen et al., 1993). Briefly, unilamellar vesicles containing [^{3}H]dipalmitoylphosphatidylcholine were prepared in the presence of [^{14}C]inulin by sonication. Sample aliquots (200 μL) were next loaded onto Sephadex G-50 columns packed in 3-mL disposable syringes, and vesicles were eluted by centrifugation at $200g_{max}$ for 4 min. This procedure was repeated once more to remove traces of unincorporated inulin. Next, [^{3}H] and [^{14}C] were quantified in aliquots of column eluents by scintillation spectrometry, and the ratios of radioactivity in entrapped inulin to the radioactivity in phospholipids were utilized for comparisons of relative vesicle sizes.

Stopped-Flow Experiments. Stopped-flow measurements were performed utilizing an SLM 4800C spectrofluorometer equipped with a stopped-flow apparatus (SLM Instruments, Urbana, IL) possessing an intrinsic dead time of $\approx$4 ms. The temperature was maintained through a circulating water bath and monitored with an indwelling thermistor (Model YSI 421, VWR Scientific). Vesicles containing encapsulated PBFI probe were illuminated by utilizing an ozone-free 450-W xenon arc lamp powered by a stabilized DC power supply. Fluorescence emission was assessed through a 470-nm cutoff filter (Schott Glass, Duryea, PA) with excitation and emission wavelengths of 350 nm (8-nm slit width) and 508 nm (8-nm slit width), respectively. The resultant signal was recorded at an acquisition rate of 1 point/ms utilizing an interfaced IBM PC/AT computer. Stopped-flow experiments were performed by mixing 0.082 mL of LUVETs ($\sim$6 mg of phospholipid/mL) containing 300 μM encapsulated

[1] Abbreviations: AA, arachidonic acid; Chol, cholesterol; EDTA, ethylenediaminetetraacetic acid; LPhosCho, 1-hexadecanoyl-*sn*-glycero-3-phosphocholine; LPlasCho, 1-*O*-(*Z*)-hexadec-1′-enyl-*sn*-glycero-3-phosphocholine; LUVETs, large unilamellar vesicles by extrusion techniques; MLVs, multilamellar vesicles; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; PA PhosCho, 1-hexadecanoyl-2-eicosa-5′,8′,11′,14′-tetraenoyl-*sn*-glycero-3-phosphocholine; PA PlasCho, 1-*O*-(*Z*)-hexadec-1′-enyl-2-eicosa-5′,8′,11′,14′-tetraenoyl-*sn*-glycero-3-phosphocholine; PBFI, potassium-binding benzofuran isophthalate; PO PhosCho, 1-hexadecanoyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphocholine; PO PlasCho, 1-*O*-(*Z*)-hexadec-1′-enyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphocholine; TFE, trifluoroethanol.

PBFI with an equal volume of buffer containing 100 mM KCl (substituted for LiCl) at 37 °C to give the specified inward potassium and outward lithium ionic gradients. K^+ influx through the channels was measured by quantifying the temporal alterations of PBFI fluorescence intensity with PBFI·K^+ enhanced fluorescence (Minta & Tsien, 1989).

Analyses of Ion Permeability across Biological Membranes. Since K^+ influx through gramicidin channels is accompanied by the opposite flow of Li^+ ion to offset charge separation, for permeation by solubility diffusion, the only driving force for K^+ movement is the concentration gradient over the thickness of the bilayer. Thus, the expression for permeability is a simple first-order exponential relationship, as the captured PBFI probe is only sensitive to K^+, and initially K^+ is only present outside the vesicle (Jain, 1988):

$$C = C_0(1 - e^{-\kappa t}) \tag{1}$$

where K^+ concentrations inside and outside the vesicle are C and C_0, respectively, and κ is the K^+ rate constant regarding K^+/Li^+ exchange and is related to P as

$$P = \kappa\frac{V}{A} = \kappa\frac{r}{3} \tag{2}$$

where V and A are the volume and surface area of a vesicle, respectively, and $r = 50$ nm for LUVETs passaged through 100-nm filters.

PBFI fluorescence intensities, I, were found to depend hyperbolically on $[K^+]$ as discussed by Ježek et al. (1990):

$$I = I_0 + \frac{\alpha P_T}{1 + \frac{K_{eq}}{C}} \tag{3}$$

or

$$I - I_0 = I_{max} - I_0 - \frac{K_{eq}(I - I_0)}{C} \tag{4}$$

where I_0 is the baseline intensity, I_{max} is the fluorescence intensity at $K_{eq}/C \rightarrow 0$, P_T is the total probe concentration, K_{eq} is the dissociation constant of the K^+−probe complex, and α is a proportionality coefficient. Insertion of eq 1 into eq 3 leads to

$$I = I_0 + \frac{1}{k_1 + \frac{1}{k_2 - k_3 e^{-\kappa t}}} \tag{5}$$

where k_1, k_2, and k_3 are proportionality coefficients.

Temporal alterations in PBFI fluorescence were analyzed utilizing eq 5 through curve fitting (Sigma Plot program, version 5.0) to derive K^+ rate constants regarding K^+/Li^+ exchange (κ) and corresponding permeability coefficients (P).

Fluorescence Steady-State Anisotropy. Choline glycerophospholipids (PO PhosCho or PO PlasCho) in $CHCl_3$/MeOH (2/1, v/v) were dried under a nitrogen stream to thin films prior to the addition of 1 mol % gramicidin in TFE. The total volume of TFE in each preparation was adjusted to 0.5 mL, and the mixtures were incubated for 1 h at 37 °C to assure that gramicidin was present in the channel conformation ($\beta^{6.3}$-helix) (LoGrasso et al., 1988; Killian et al., 1988; Bañó et al., 1991). Next, the TFE was evaporated under a nitrogen stream prior to evacuation at high vacuum (10 mTorr) overnight to ensure complete removal of solvent. Lipid mixtures were suspended in 2 mL of buffer [300 mM $(CH_3)_4NCl$, 10 mM MOPS, 5 mM Tris·HCl, and 0.01 mM EDTA, pH 7.0 at 25 °C; previously degassed with nitrogen] to yield a solution which contained 1.3 mM lipid. Small unilamellar vesicles were obtained by high-energy sonication under a nitrogen atmosphere [3 min at a power level of 1.5 employing a 50% duty cycle from a Vibra Cell Model VC600 sonicator (Sonics Materials, Inc., Danbury, CT) followed by cooling on ice]. Next, an additional 3-min sonication was performed utilizing parameters identical to those described above. Fluorescence steady-state anisotropy of the tryptophans present in gramicidin was measured utilizing an SLM 4800C spectrofluorometer in the L-format as described by Lakowicz (1983). Gramicidin was excited at 303 nm (8-nm slit width), and emission intensity was monitored at 345 nm (8-nm slit width, through a 335-nm filter). Vesicles lacking gramicidin were prepared in an identical fashion to those containing gramicidin to correct for background scattering.

FT-IR Spectroscopy. Choline glycerophospholipids (5 mg) in $CHCl_3$/MeOH (2/1, v/v) were first dried under a nitrogen stream; then 3 mol % gramicidin in TFE was added. The mixture in TFE was incubated for 1 h at 37 °C prior to evaporation under a N_2 stream and subsequent evacuation at 10 mTorr overnight to ensure the complete removal of TFE. Lipid mixtures were initially hydrated in 50 μL of Milli-Q distilled H_2O and subsequently bath sonicated (Branson Cleaning Equipment Company, Shelton, CT) for 1 h at room temperature. The resulting suspension was evenly deposited onto a AgCl window for 1 h, and a second AgCl window was placed above the first. Samples lacking gramicidin were prepared in an identical manner. Infrared spectra were acquired at room temperature on a Galaxy 6020 spectrometer (Mattson Instruments, Inc., Madison, WI) equipped with a mercury cadmium telluride detector. The instrument resolution was 1 cm^{-1}, and 500 scans were collected with triangular apodization.

RESULTS

Utilization of Alterations in the Fluorescence Emission of Ion-Specific Probes To Quantify Gramicidin-Mediated K^+ Flux across Vesicular Membranes. Measurements of the kinetics of ion flux through gramicidin ion channels were based upon increases in fluorescence intensity after specific recognition of monovalent cations by complexation with fluorophore. Essential to the success of this process was the efficacious removal of untrapped PBFI probe by gel filtration chromatography as well as the diffusion-limited response of probe to the presence of K^+. As anticipated, the intensity of PBFI fluorescence was hyperbolic with respect to potassium concentration (i.e., a linear plot was obtained between $I - I_0$ and $(I - I_0)/C$ according to eq 4 in Materials and Methods (Figure 1)). Since the magnitude of the fluorescence signal was dependent not only on the number of vesicles which have functional channels but also on the amount of probe captured within the vesicles, the trapped volumes of vesicles comprised of PO PhosCho or PO PlasCho (with or without an additional 30 mol % of cholesterol) were measured by examining the ratio of the amount of [^{14}C]inulin captured inside the respective ^{3}H-labeled phospholipid comprised of the vesicle surface. The

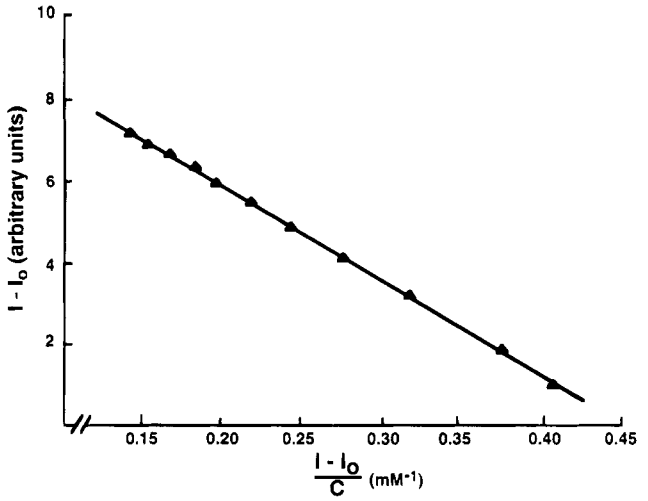

FIGURE 1: Relationship of alterations in the PBFI fluorescence intensity to alterations in K^+ concentration. PBFI (1 μM) was added to a cuvette containing 2 mL of buffer [300 mM $(CH_3)_4NCl$, 10 mM MOPS, 5 mM Tris·HCl, and 0.01 mM EDTA, pH 7.0 at 25 °C), and various K^+ concentrations (spanning those employed in this study) were generated by addition of aliquots of KCl stock solution. The fluorescence intensity of PBFI was measured at 37 °C with excitation at 350 nm and emission at 508 nm as described in Materials and Methods. Data were plotted according to eq 4.

results demonstrated that the trapping efficiency is almost identical (within 5%) for each subclass of phospholipids or their binary dispersions with cholesterol.

Alterations in Gramicidin-Mediated K^+ Flux in Vesicles Comprised of Different Phospholipid Subclasses. The rate of gramicidin-mediated K^+ flux was assessed using a stopped-flow apparatus by quantitation of the temporal course of alterations in fluorescence intensity of the PBFI·K^+ complex after the acute introduction of a K^+ and Li^+ concentration gradient across the unilamellar vesicles. Since the disturbed system reached equilibrium very quickly, only the first 100 ms of data was used in the derivation of the K^+ rate constants regarding K^+/Li^+ exchange (κ) and permeability coefficients (P). To compare the effects of phospholipid subclass composition on the rate of gramicidin-mediated potassium flux, vesicles comprised of individual phospholipid molecular species and subclasses were constructed which contained gramicidin ion channels as substitutional impurities (i.e., $\leq$ 1 functional channel/vesicle based on a statistical distribution of the numbers of vesicles and gramicidin dimers present). After the introduction of a K^+ gradient across the unilamellar vesicle, alterations in PBFI fluorescence in vesicles comprised of PO PlasCho were substantially faster than those manifest in vesicles comprising PO PhosCho, demonstrating a phospholipid subclass dependent modulation of ion channel function (Figure 2A). Similarly, after the introduction of a K^+ gradient, the rate of change in PBFI fluorescence was greater in vesicles comprised of PA PlasCho than that manifest in vesicles comprised of PA PhosCho (Figure 2B). Calculation of the rate constants (κ) and the permeability coefficients (P) from these experiments demonstrated statistically significant differences between the flux of potassium through gramicidin ion channels in vesicles comprised of plasmalogen molecular species and their diacylphospholipid counterparts (Figure 3). Furthermore, although alterations in the rate of gramicidin-mediated K^+ transport were apparent in vesicles comprised of each phospholipid subclass, no significant differences were present between vesicles comprised of distinct individual molecular species of each subclass (i.e., no rate differences were present in vesicles comprised of plasmalogens or diacylphospholipids containing oleic acid vs arachidonic acid at the *sn*-2 position). Thus, these results demonstrate that the observed alterations in the rate of K^+ flux largely reflected differences in channel function resulting from the covalent alteration present in the proximal portion of the *sn*-1 aliphatic chain at (or near) the membrane interface (i.e., phospholipid subclass) and were independent of the degree of unsaturation of the *sn*-2 fatty acyl chain and, therefore, independent of alterations in membrane dynamics and conformation in the membrane interior (Figure 3).

The absolute magnitude of the change in PBFI fluorescence reflects the number of vesicles containing functional

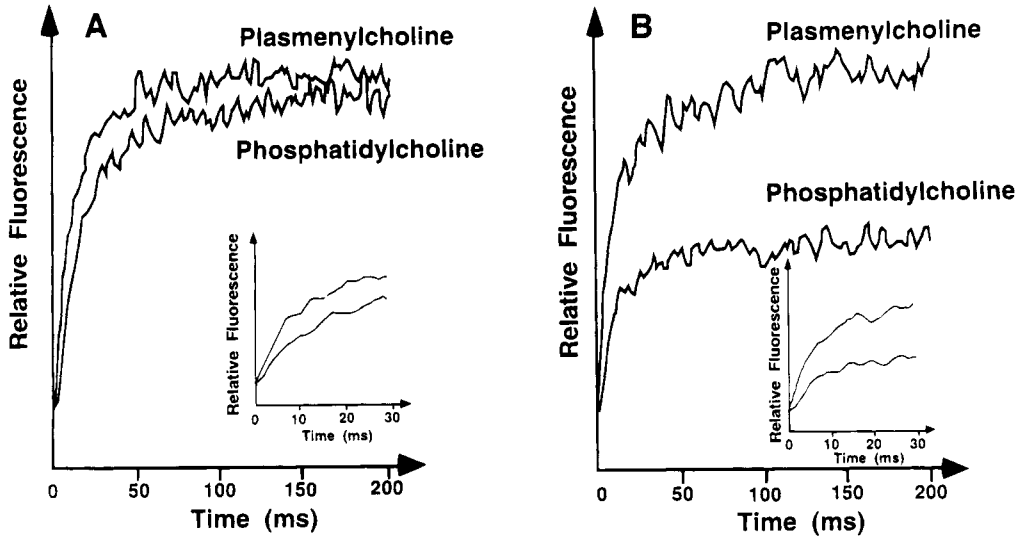

FIGURE 2: Flux of K^+ through gramicidin ion channels reconstituted in membranes comprised of plasmenylcholine or phosphatidylcholine. LUVETs (~6 mg phospholipid/mL) comprised of either 1-palmitoyl-2-oleoylplasmenylcholine or phosphatidylcholine molecular species (Panel A) or 1-palmitoyl-2-arachidonoyl plasmenylcholine or phosphatidylcholine molecular species (Panel B) were rapidly mixed in a stopped-flow apparatus with isoosmotic buffer in which 100 mM KCl was substituted for LiCl to generate a specified inward K^+ and an outward Li^+ gradient. Alterations in the time course of PBFI fluorescence intensity (reflecting K^+ influx) were recorded at 508 nm with the excitation wavelength set at 350 nm as described in Materials and Methods. Insets are the fluorescence tracings of the first 30 ms.

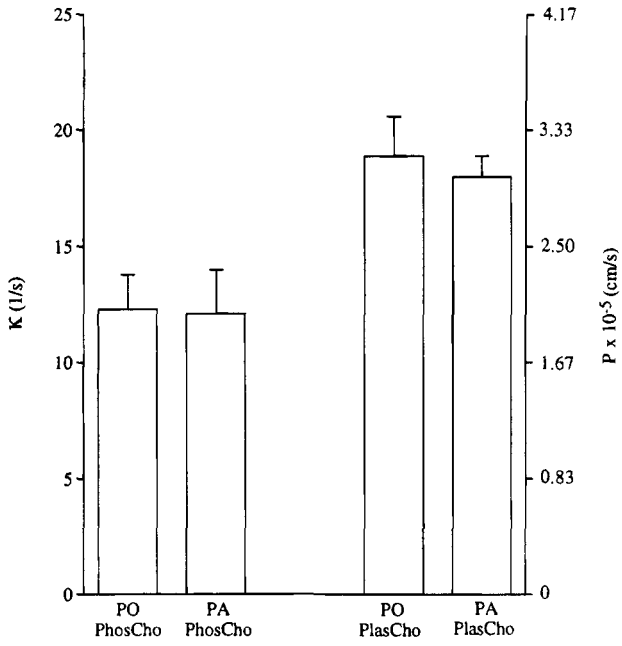

FIGURE 3: Comparisons of the rate constants and permeability coefficients of gramicidin-mediated K^+ flux across phosphatidylcholine or plasmenylcholine membranes. LUVETs (~6 mg of phospholipid/mL) were mixed with an isoosmotic buffer in which 100 mM KCl was substituted for LiCl to generate an inward K^+ and an outward Li^+ gradient. K^+ influx through gramicidin channels was quantified by curve-fitting the time course of alterations in PBFI fluorescence (according to eq 5) to obtain rate constants (κ) and permeability coefficients (P) for gramicidin-mediated K^+ flux in PO PhosCho, PA PhosCho, PO PlasCho, or PA PlasCho membranes as indicated. Data represents the mean ±SD of three independent preparations.

ion channels and the amount of encapsulated probe in each vesicle. The absolute magnitude of fluorescence changes in vesicles comprised of PO PhosCho, PO PlasCho, and PA PlasCho were similar while the magnitude of fluorescence change in vesicles comprised of PA PhosCho was approximately one-half of that manifest for the other phospholipid molecular species examined. Increasing the gramicidin concentration in vesicles comprised of PA PhosCho resulted in an increase in the absolute magnitude of the fluorescence change to the levels present utilizing the other phospholipid molecular species demonstrating that a similar number of vesicles containing a similar amount of the captured PBFI probe were present. Since the number of vesicles and the concentration of encapsulated fluorophore are similar in each case, these results demonstrate that the probability of having a functional channel in PA PhosCho vesicles is approximately one-half of that for the other phospholipid molecular species examined.

Effect of Cholesterol on Gramicidin-Mediated K^+ Flux in Vesicles Comprised of Plasmenylcholine or Phosphatidylcholine. The effect of cholesterol on the rate (i.e., channel function) and the amount (i.e., probability of channel formation) of gramicidin-mediated K^+ influx was assessed by quantifying the rate of PBFI fluorescence change after the introduction of a K^+ gradient utilizing a stopped-flow fluorescence spectrometer. Cholesterol had substantive effects on reducing the magnitude of PBFI fluorescence change in vesicles comprised of either plasmenylcholine or phosphatidylcholine (Figure 4). Thus, the probability of forming a functional channel in vesicles containing cholesterol was substantially less than that manifest in vesicles lacking cholesterol. The observed differences in fluorescence intensity were not due to alterations in the internal volume of the vesicles or the amount of PBFI fluorescent probe captured within the vesicle, since elevation of the gramicidin concentration to a very high level (e.g., 1 mol %), addition of potassium, and subsequent sonication resulted in identical alterations in the fluorescence intensity in vesicles comprised of phosphatidylcholine alone or of binary dispersions of phosphatidylcholine and cholesterol (30 mol %) (data not shown).

Calculation of the rate of K^+ influx after the introduction of a potassium gradient demonstrated that cholesterol substantially attenuated the rate of K^+ influx in vesicles comprised of plasmenylcholine, but did not attenuate the rate of K^+ influx in vesicles comprised of phosphatidylcholine,

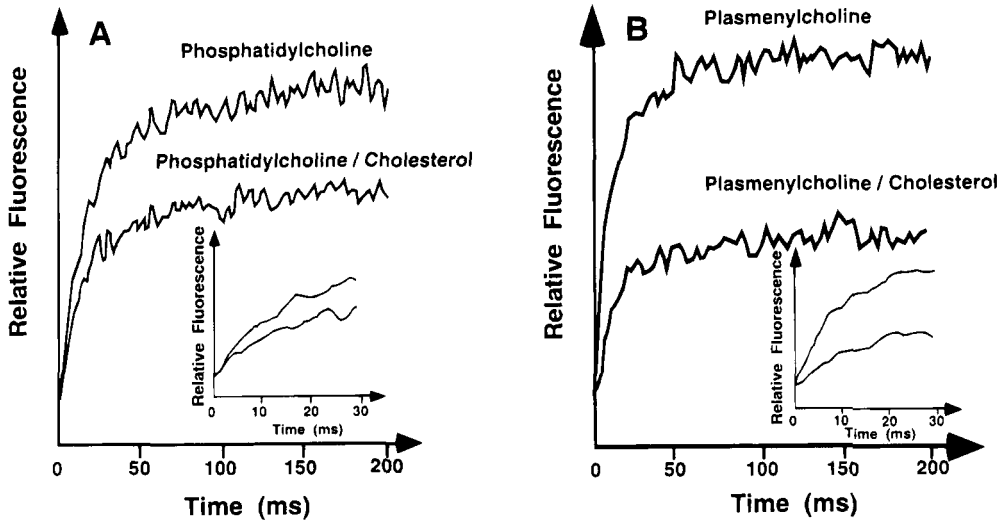

FIGURE 4: Effects of cholesterol on the kinetics of K^+ flux through gramicidin ion channels in membranes comprised of phosphatidylcholine or plasmenylcholine. LUVETs (~6 mg phospholipid/mL) comprised of PO PhosCho (Panel A) or PO PlasCho (Panel B) in the absence or presence of 30 mol % cholesterol were rapidly mixed at 37 °C in a stopped-flow apparatus with an isoosmotic buffer in which 100 mM KCl was substituted for LiCl to generate specified inward K^+ and outward Li^+ gradients. Alterations in the time course of PBFI fluorescence intensity (reflecting K^+ influx) were recorded at 508 nm with the excitation wavelength set at 350 nm as described in Materials and Methods. Insets are the fluorescence tracings of the first 30 ms.

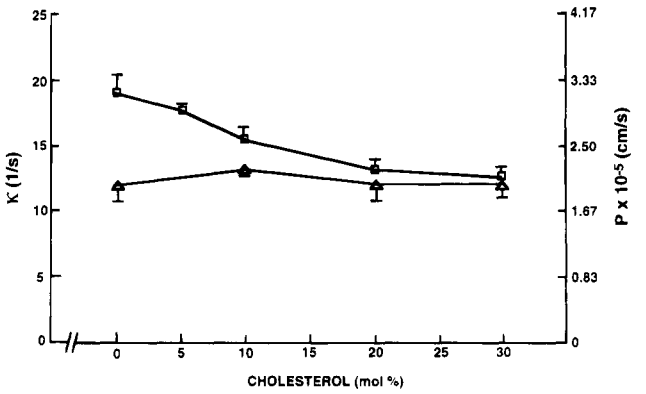

FIGURE 5: Effects of cholesterol on rate constants and permeability coefficients of gramicidin-mediated K^+ flux in vesicles comprised of plasmenylcholine or phosphatidylcholine. LUVETs (~6 mg of phospholipid/mL) were rapidly mixed in a stopped-flow apparatus with an isoosmotic buffer in which 100 mM KCl was substituted for LiCl to give specified inward K^+ and outward Li^+ gradients. K^+ influx through gramicidin channels was quantified by curve-fitting the time course of alterations in PBFI fluorescence according to eq 5 to obtain K^+ rate constants (κ) and permeability coefficients (P) in either phosphatidylcholine (△) or plasmenylcholine (□) membranes in the presence of 0, 5, 10, 20, or 30 mol % cholesterol. Data represents the mean ±SD of three independent preparations.

even in the presence of concentrations of cholesterol similar to those found in the sarcolemmal membrane (Pak et al., 1992) (Figure 5). Collectively, these results demonstrate that cholesterol possesses separate and distinct effects on gramicidin-mediated K^+ flux in vesicles comprised of plasmenylcholine in comparison to vesicles comprised of phosphatidylcholine.

Effects of Amphiphiles on Gramicidin-Mediated K^+ Influx in Vesicles Comprised of Plasmenylcholine or Phosphatidylcholine. Addition of 5 mol % arachidonic acid or 10 mol % lysophosphatidylcholine, lysoplasmenylcholine, or lidocaine had no measurable effect on the rate of K^+ influx into vesicles comprised of phosphatidylcholine or plasmenylcholine (Figure 6). Furthermore, the number of functional channels (as reflected by the magnitude of the fluorescence change) present were similar in vesicles comprised of phosphatidylcholine or plasmenylcholine in the absence or presence of arachidonic acid, lysophosphatidylcholine, lysoplasmenylcholine, or lidocaine. Since addition of arachidonic acid or either subclass of lysophospholipids has profound effects on the fluidity of membranes comprised of plasmenylcholine or phosphatidylcholine (Han & Gross, 1991; Chen et al., 1993), and since no alterations in the rate of gramicidin-mediated K^+ permeability were observed, these results demonstrate that the observed alterations in gramicidin-mediated K^+ flux were not due to changes in the bulk physical properties of the membrane lipids (i.e., packing, dynamics, surface charge, etc.), but rather were the result of specific interactions between the phospholipid host and the guest (i.e., ion channel).

Comparison of Alterations in Fluorescence Steady-State Anisotropy of Gramicidin Tryptophan Fluorophore in Vesicles Comprised of Phosphatidylcholine or Plasmenylcholine. Fluorescence anisotropy measurements were made by exciting the red edge of the tryptophan absorbance in gramicidin (303 nm) to avoid tryptophan homotransfer and self-quenching (Weber, 1960). Comparisons of the fluorescence anisotropy of gramicidin tryptophans incorporated into the membranes as a substitutional impurity (1/100) demonstrated that the tryptophan residues experience different environments in membranes comprised of PO PhosCho in comparison to PO PlasCho at all temperatures studied (Figure 7). The motion of the tryptophans in gramicidin is more restrained in phosphatidylcholine membranes as compared to plasmenylcholine membranes, assuming that only small changes in the average fluorescence lifetime of tryptophans are present, which seems likely on the basis of analogous studies by Scarlata (1988) who demonstrated only modest changes in the lifetimes of gramicidin tryptophans during large alterations in the bulk physical properties of the membrane host (e.g., passage through the phase transition, alteration of molecular species of phospholipid, etc.).

Comparison of Motional Modes of Gramicidin in Vesicles Comprised of Phosphatidylcholine or Plasmenylcholine as

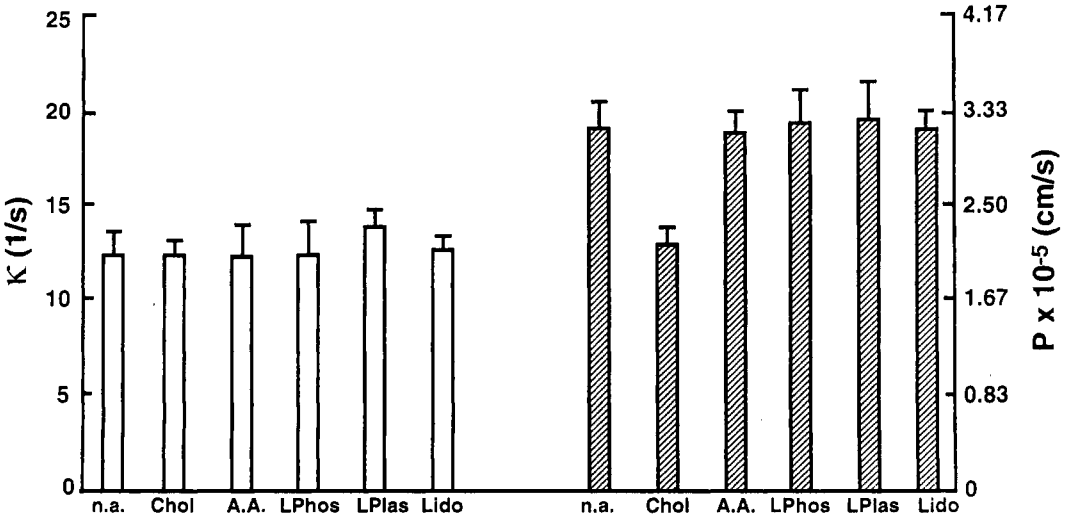

FIGURE 6: Effects of amphiphilic agents on gramicidin-mediated K^+ flux in vesicles comprised of phosphatidylcholine or plasmenylcholine. LUVETs (~6 mg of phospholipid/mL) were rapidly mixed in a stopped-flow apparatus with isoosmotic buffer in which 100 mM KCl was substituted for LiCl to give specified inward K^+ and outward Li^+ gradients. Gramicidin-mediated K^+ influx was quantified by curve-fitting the alterations in PBFI fluorescence according to eq 5 to yield K^+ rate constants (κ) and permeability coefficients (P) in phosphatidylcholine (open bars) or plasmenylcholine (hatched bars) membranes in the absence (n.a.) or presence of 30 mol % cholesterol (Chol), 5 mol% arachidonic acid (A.A.), 10 mol % lysophosphatidylcholine (LPhos), 10 mol % lysoplasmenylcholine (LPlas), or 10 mol % lidocaine (Lido). Data represents the mean ±SD of three independent preparations.

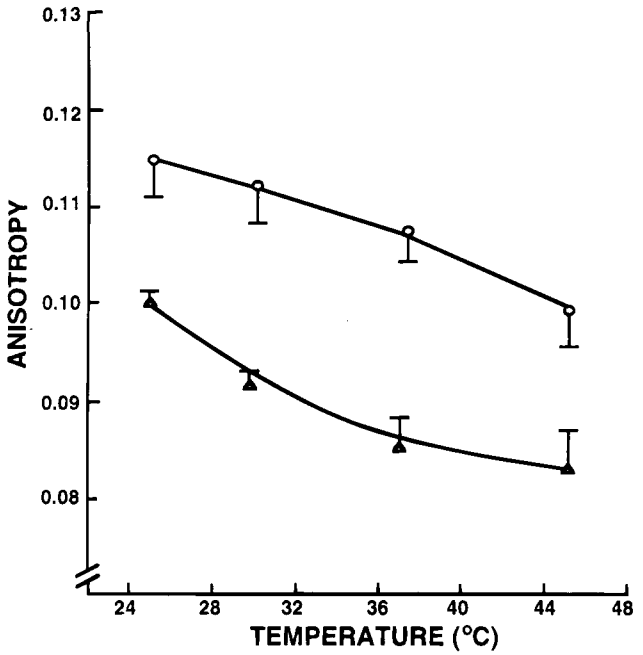

FIGURE 7: Comparisons of the steady-state anisotropy of endogenous tryptophan residues in gramicidin ion channels present in phosphatidylcholine or plasmenylcholine membranes. Vesicles comprised of PO PhosCho (○) or PO PlasCho (△) containing 1 mol % gramicidin were prepared by sonication to yield a 1.3 mM lipid solution. The steady-state fluorescence anisotropy of the endogenous tryptophans present in gramicidin was measured at 345 nm with the excitation wavelength set at 303 nm. Vesicles lacking gramicidin were prepared in exactly the same way to correct for background scattering. Temperatures during the experiments were varied between 25 and 45 °C. Data represents the mean ±SD of three independent preparations.

Assessed by FT-IR. The C=O stretching regions in infrared spectra of gramicidin can be used to obtain information on the conformation of gramicidin in membrane bilayers (Sychev & Ivanov, 1982; Urry et al., 1983). Initial experiments compared the C=O stretching frequencies in membranes comprised of phosphatidylcholine or plasmenylcholine in the absence of gramicidin. Substantial differences in the carboxyl stretching frequency for PO PhosCho (1738 cm^{-1}) compared to PO PlasCho (1732 cm^{-1}) were present (Figure 8). Incorporation of 3 mol % gramicidin into either PO PhosCho or PO PlasCho membranes resulted in no demonstrable shift of C=O resonances. However, the amide I band corresponding to the intramonomer carbonyls in gramicidin channels (Buchet et al., 1985) is slightly blue shifted in PO PhosCho compared to that in PO PlasCho membranes (1637 vs 1635 cm^{-1} for PO PhosCho and PO PlasCho, respectively) (Figure 8). Information from the band corresponding to the carbonyls at the head-to-head junction or entrance of gramicidin channels (~1670 cm^{-1}) (Buchet et al., 1985) was not accessible since it is overlapped with the broad water deformation band and the band from double bond stretching in phospholipids.

DISCUSSION

The results of the present study demonstrate that gramicidin-mediated K^+ flux is more rapid in vesicles comprised of plasmenylcholine than in vesicles comprised of phosphatidylcholine. These alterations in the function of the gramicidin ion channel are due, at least in part, to its altered conformation in membranes comprised of phosphatidylcholine compared to plasmenylcholine as assessed by changes

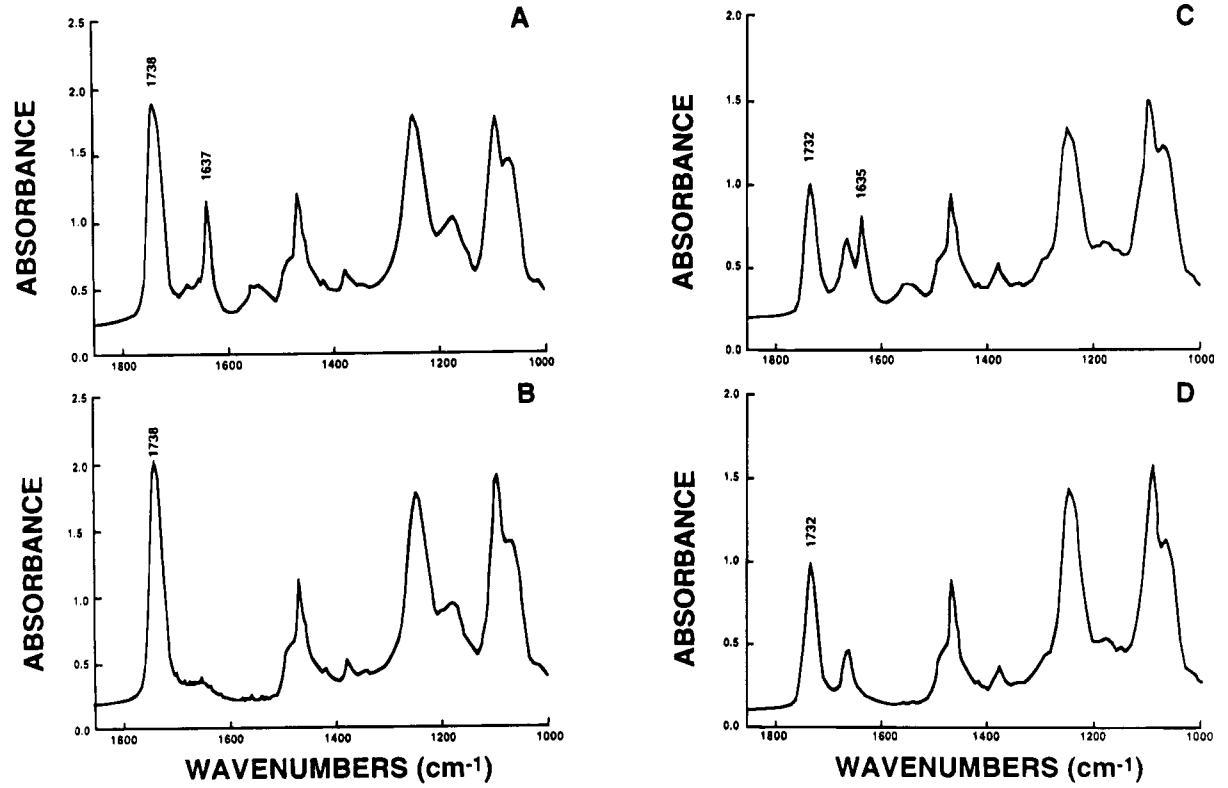

FIGURE 8: Comparisons of FT-IR C=O stretching regions in phosphatidylcholine or plasmenylcholine membranes in the presence or absence of gramicidin ion channels. Lipid suspensions (5 mg) of PO PhosCho/3 mol % gramicidin (Panel A), PO PhosCho (Panel B), PO PlasCho/3 mol % gramicidin (Panel C), or PO PlasCho (Panel D) were subjected to bath sonication and were evenly deposited onto AgCl windows to form a sticky film of hydrated samples. Data (500 scans) were acquired on a Galaxy 6020 spectrometer using triangular apodization and 1-cm^{-1} instrument resolution. The C=O stretching bands in phospholipids are present at ≈1735 cm^{-1}; the amide I band corresponding to the intramonomer carbonyls in gramicidin channels is present at ≈1635 cm^{-1}.

in the fluorescence anisotropy of endogenous gramicidin tryptophan residues and the differential intramonomer carbonyl stretching frequencies. The K^+ permeability coefficients derived in this study ($\sim 2 \times 10^{-5}$ cm/s) are about 1 order of magnitude smaller than the diffusional permeability coefficient of H_2O in vesicles containing similar amounts of gramicidin (Ye & Verkman, 1989). Although the kinetics of the movement of H_2O molecules across the ion channel is an important determinant of channel transmembrane ion permeability, the presence of Li^+ can attenuate K^+ movement across the gramicidin channel due to interactions of Li^+ with the channel wall (Dani & Levitt, 1981). Thus, the kinetic comparisons between ion channel function in membranes comprised of different lipid compositions reflects, at least in part, the conjoint interactions of both K^+ and Li^+ ion with the gramicidin ion channel in each lipid milieu.

Ions in the channel are located in energy minima, with positions determined by the superposition of long-range electrostatic forces and local interactions between the ions and the channel wall (Andersen, 1984). Previous work has demonstrated the existence of monovalent cation binding sites near the mouth of the channel (Hladky & Haydon, 1984; Jordan, 1988; Olah et al., 1991) and the motionally restricted tryptophan environments at the peptide−lipid interface of gramicidin channels (Mukherjee & Chattopadhyay, 1994). We have previously demonstrated substantial differences in the conformation and dynamics of plasmenylcholine and phosphatidylcholine membranes which are particularly pronounced at the membrane interface (Pak et al., 1987; Han & Gross, 1990; Chen et al., 1993). Since a rate-determining step in transmembrane ion flux in the gramicidin−lipid system is the entry of ion into the mouth of the channel (Läuger, 1976; Levitt, 1985; Dani, 1986; Chiu & Jakobsson, 1989), and since the conformation and dynamics of plasmenylcholine and phosphatidylcholine are different at the membrane interface, the finding that the function of gramicidin ion channels is substantially different in vesicles comprised of these two choline glycerophospholipid subclasses is not unexpected. Furthermore, since tryptophan residues in gramicidin occupy regions near the membrane interface, in close proximity to ion binding sites in the channel (Urry et al., 1982a,b; Scarlata, 1991), alterations in tryptophan fluorescence provide information on phospholipid subclass induced alterations in channel conformation and mobility. Results from fluorescence anisotropy measurements demonstrate that the endogenous tryptophan residues in gramicidin present in membranes comprised of phosphatidylcholine are less mobile than those present in membranes comprised of plasmenylcholine. The increase in tryptophan rotational motion in plasmenylcholine could be caused by a destabilization of hydrogen bonds between the indole hydrogens and the hydrophilic bilayer surface (Scarlata, 1991; Hu et al., 1993) due to the more upright head group geometry in plasmenylcholine (Han & Gross, 1990) or the lack of fatty acyl carbonyl oxygen at the *sn*-2 position. However, the membrane interface in plasmenylcholine is substantially more immobilized than that in phosphatidylcholine as assessed by multiple independent techniques including NMR, ESR, and fluorescence spectroscopy (Pak et al., 1987; Chen et al., 1993). Thus, it seems clear that a rigid matrix scaffolding in the host does not, of necessity, dictate that a corresponding rigidity is present in the guest. Rather, it appears that the boundary conditions and the "dynamic impedance matching" at the host−guest interface represent the critical determinant of guest (i.e., channel in the present case) dynamics and function. Furthermore, Fourier transform infrared spectroscopy of the C=O stretching frequency of intramonomer carbonyls in gramicidin channels is 2 cm^{-1} lower in plasmenylcholine than in phosphatidylcholine membranes. The results imply the presence of a stronger interaction between channel and phospholipids in the interior of membranes comprised of phosphatidylcholine than those comprised of plasmenylcholine, resulting in the subclass-selective partial ablation of intramolecular hydrogen bonds in the channel. We speculate that enhanced mobility of the gramicidin ion channel (especially at the membrane interface) may be necessary to facilitate optimal ion flux through membrane bilayers and that the structure of plasmalogens is involved, at least in part, in the optimization of the mode of host−guest interaction to facilitate the function of this and potentially other transmembrane ion channels. Thus, it seems possible that one reason underlying the predominance of plasmalogens in electrically active membranes is their ability to facilitate ion channel function.

Cholesterol possessed disparate effects on gramicidin-mediated K^+ flux in vesicles comprised of plasmenylcholine compared to vesicles comprised of phosphatidylcholine. Although cholesterol substantially decreased the rate of gramicidin-facilitated K^+ flux in plasmenylcholine vesicles, no effects of cholesterol on the function of formed channels were identified in vesicles comprised of phosphatidylcholine. These results are entirely compatible with prior work demonstrating that gramicidin ion channels do not bind to cholesterol (Mickus et al., 1992) and that the effects of cholesterol must therefore necessarily be mediated by its effects on phospholipid dynamics and conformation.

Prior work has demonstrated large differences in the membrane dipole potential present in membranes comprised of plasmenylcholine compared to phosphatidylcholine (Shah & Schulman, 1965; Smaby et al., 1983). Alterations in the membrane dipole potential can alter the free energy profile of interaction between charged moieties (or discrete dipoles) in a protein and the membrane. Accordingly, such alterations in free energy profiles may contribute to changes in the conformation of gramicidin in membranes comprised of each subclass as well as alter the free energy profiles of solvated cation and its dynamics as it traverses the ion channel. Both of these effects could potentially alter ion channel function. However, it is important to note that addition of surface charge (as in the case of experiments where arachidonate was added) does not, by itself, substantially alter channel kinetics, suggesting that the channel can effectively shield the effects of dipole potentials through internal solvation.

Collectively, the present results demonstrate the importance of subclass-specific membrane alterations in the function of transmembrane proteins and underscore the differential effects of cholesterol on channel function in membranes comprised of each choline glycerophospholipid subclass. The demonstration that membranes comprised of plasmalogen molecular species augment ion channel function may underlie the evolutionary persistence of the vinyl ether linkage in nature, explain the enrichment of plasmalogens in electrically active membranes, and provide insight into the importance of phospholipid subclass-mediated alterations in transmembrane protein conformation and function.

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