Lipid−Peptide Communication in Fluid Bilayers

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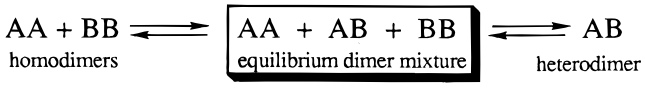
Abstract: The influence of channel and nonchannel forms of gramicidin A (*c*-gA and *nc*-gA, respectively) on the mixing behavior of phospholipids derived from 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) has been examined in the physiologically relevant fluid phase by means of nearest-neighbor recognition methods (Davidson, S. K. M.; Regen, S. L. *Chem. Rev.* **1997**, *97*, 1269). Thus, when disulfide-based dimers were allowed to undergo monomer exchange at 60 °C in the presence of 5 mol % of *nc*-gA, the molar ratio of heterodimer to each homodimer was 1.55 ± 0.07; replacement of 50% of the exchangeable monomers with 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (a chemically inert diluent having an intermediate chain length and hydrophobicity) eliminated the ability of *nc*-gA to induce NNR. In sharp contrast, the presence of *c*-gA, at concentrations as high as 9 and 17 mol %, left the phospholipids in a randomly arranged state. The ability of gramicidin A to "communicate" its conformation to the surrounding phospholipids implies that lateral transmission of conformational information should also be possible between lipids and proteins in biological membranes.

Introduction

Biological membranes are of fundamental importance to living cells by serving as selective barriers for transport, as fluid matrixes for biosynthetic transformations, and as boundaries for the transfer of energy and information.[1] While the general bilayer structure of biological membranes is now well-established, their two-dimensional organization remains poorly defined.[2,3] One intriguing question, in this regard, is whether membrane proteins "communicate" with the lipid framework. Specifically, can conformational changes of membrane proteins modulate the lateral distribution of the surrounding phospholipids? In this paper we show, by use of a model system, that such communication is possible; i.e., *we present compelling evidence for lipid−peptide communcation in fluid bilayers*.

The technique that we have used to probe phospholipid mixing ("nearest-neighbor recognition", NNR) involves the generation and analysis of equilibrium mixtures of phospholipid dimers.[4] In brief, two phospholipids (**A** and **B**) are first converted into exchangeable, disulfide-linked homodimers (**AA** and **BB**) and the corresponding heterodimer (**AB**). Subsequent vesicle formation, using an equimolar mixture of the two homodimers, followed by monomer exchange via thiolate−disulfide interchange leads to an equilibrium mixture. A similar reaction that is carried out with vesicles made from pure heterodimer ensures that an equilibrium point has been reached. When the resulting dimer composition is statistical (i.e., when the molar ratio of heterodimer to each homodimer is 2.0), and when there is no driving force for transmembrane asymmetry (i.e., an uneven distribution of phospholipids between the inner and outer monolayer of the bilayer), this finding establishes that the monomeric components are randomly distributed throughout the membrane. When homodimers are favored (i.e., when NNR is present), lateral heterogeneity may or may not exist. If the intramolecular and intermolecular forces within the membrane are similar in magnitude, then NNR also indicates the presence of lateral heterogeneity.



Experimental Section

General Methods. All of the general methods that have been used in this study were similar to those previously described.[5] Unless stated otherwise, all reagents were obtained from commerical sources and used without further purification. All synthetic transformations were carried out under an argon atmosphere. Just prior to carrying out a NNR experiment, an appropriate vesicle dispersion was degassed with an aspirator for 20 min and the residual traces of organic solvent were removed by dialysis (Spectra/Por Membrane, MWCO 6000−8000) under an argon atmosphere with two 200 mL portions of degassed 10 mM Tris−HCl buffer (pH 7.4, 150 mM NaCl, 2.0 mM NaN_3) over the course of 15 h.

Digestion of Large Unilamellar Vesicles by Phospholipase A_2. Vesicles were formed by reverse phase evaporation methods by using procedures similar to those described in the text, where 5% of the nonchannel form of gramicidin A was included. The dispersion was then subjected to digestion by phospholipase A_2 (*Naja naja*) by using experimental procedures that were very similar to those previously described.[6]

Reaction of Large Unilamellar Vesicles With DTNB. Using procedures similar to those described in the text, large unilamellar vesicles were prepared from **4** (0.6 μmol), **5** (0.6 μmol), and nonchannel gA (0.06 μmol) plus 2 mL of 10 mM Tris−HCl buffer (pH 5.0, 150 mM NaCl, 2.0 mM NaN_3, and 5.0 mM EDTA). To a disposable

(1) Gennis, R. B. *Biomembranes: Molecular Structure and Function*; Springer-Verlag: New York, 1989.
(2) Tocanne, J.-F.; Cezanne, L.; Lopez, A.; Piknova, B.; Schram, V.; Tournier, J.-F.; Welby, M. *Chem. Phys. Lipids* **1994**, *73*, 139.
(3) Welti, R.; Glaser, M. *Chem. Phys. Lipids* **1994**, *73*, 121.
(4) (a) Krisovitch, S. M.; Regen, S. L. *J. Am. Chem. Soc.* **1992**, *114*, 9828. (b) Davidson, S. K. M.; Regen, S. L. *Chem. Rev.* **1997**, *97*, 1269.
(5) Uragami, M.; Dewa, T.; Inagaki, M.; Hendel, R. A.; Regen, S. L. *J. Am. Chem. Soc.* **1997**, *119*, 3797.
(6) Inagaki, M.; Shibakami, M.; Regen, S. L. *J. Am. Chem. Soc.* **1997**, *119*, 7161.

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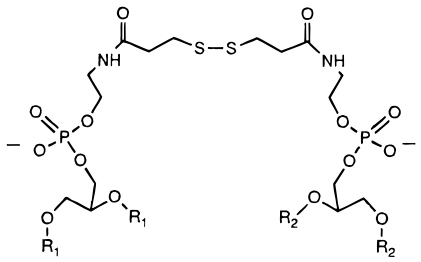
polystyrene cuvette was then added 1.1 mL of a 1 mM solution of DTNB (10 mM Tris−HCl buffer, pH 7.4, 150 mM NaCl, 2.0 mM NaN_3, and 5.0 mM EDTA). After the solution was incubated for 2 min at 30 °C, an aliquot of the vesicle dispersion (100 μL) was added and the absorbance at 412 nm recorded as a function of time. Similar experiments were carried out in which the buffer solution initially contained 20% ethanol (v/v) (prior to vesicle addition) in order to obtain a value for the total thiol content. Concentrations of released dye were calculated by use of appropriate calibration curves.

Results and Discussion

Choice of Exchangeable Phospholipids and Peptide. The specific dimers that were used in the present investigation (**1**, **2**, and **3**) have previously been described.[4] In the fluid bilayer state (60 °C), thiolate−disulfide interchange creates a random arrangement of the individual monomer units.[4] Gramicidin A (gA) was selected as a protein model based on its strong hydrophobicity, its structural simplicity, and its ability to adopt two distinct conformations: i.e., a *channel* form (two helical monomers that extend across a lipid bilayer, being joined at the N-termini in the center of the membrane) and a *nonchannel* form (a double-helical head-to-tail dimer that is unable to span the bilayer).[1,7−9] Since the nonchannel form (*nc*-gA) can be converted into the channel form (*c*-gA) *in situ*, gA serves as a useful model for exploring the possibility of lipid−protein communication.



1, R_1=R_2= $CO(CH_2)_{12}CH_3$
2, R_1=R_2= $CO(CH_2)_{16}CH_3$
3, R_1= $CO(CH_2)_{12}CH_3$; R_2= $CO(CH_2)_{16}CH_3$

Assembly of Vesicles Containing Channel and Nonchannel Forms of Gramicidin A. In a typical vesicle preparation (reverse phase evaporation method), a chloroform solution composed of **1** (0.3 μmol), **2** (0.3 μmol), and gA (0.12 μmol, 9 mol %) was added to a test tube and the solvent removed under a stream of argon.[10] Addition of diisopropyl ether (270 μL), chloroform (100 μL), and Tris−HCl buffer (35 μL of a 3.3 mM Tris−HCl buffer that was made from 10 mM Tris−HCl, 150 mM NaCl, 2 mM NaN_3, pH 7.4), followed by vortex mixing, mild sonication (bath-type, 3 min), and concentration under reduced pressure (63 °C), afforded a white gel. Subsequent collapse of the gel by vortex mixing, dilution with 2.0 mL of 10 mM Tris−HCl buffer (pH 7.4), and dialysis (2 × 200 mL of this same Tris−HCl buffer, 15 h) under an argon atmosphere afforded a stock dispersion. Gel filtration of an aliquot (Sephadex G-25 M) and analysis of the void volume for phospholipid and gA content (phosphorus, UV) indicated that peptide incorporation into the vesicular membranes was quantitative.[8] Examination by CD revealed the presence of *nc*-gA, having a large negative peak at 229 nm, a weak positive peak at 218 nm, and a positive ellipticity below 208 nm (Figure 1A).[11,12] Vesicles containing *c*-gA were prepared by use of similar procedures except that (i) trifluoroethanol was used to introduce the peptide to the chloroform solution of phospholipids and (ii) the dialysis was carried out at 60 °C. Evidence for the presence of the channel conformation was obtained by CD, where positive peaks at 218 and 235 nm, a weak negative inflection at 229 nm, and negative ellipticity below 208 nm were apparent (Figure 1B).[11]

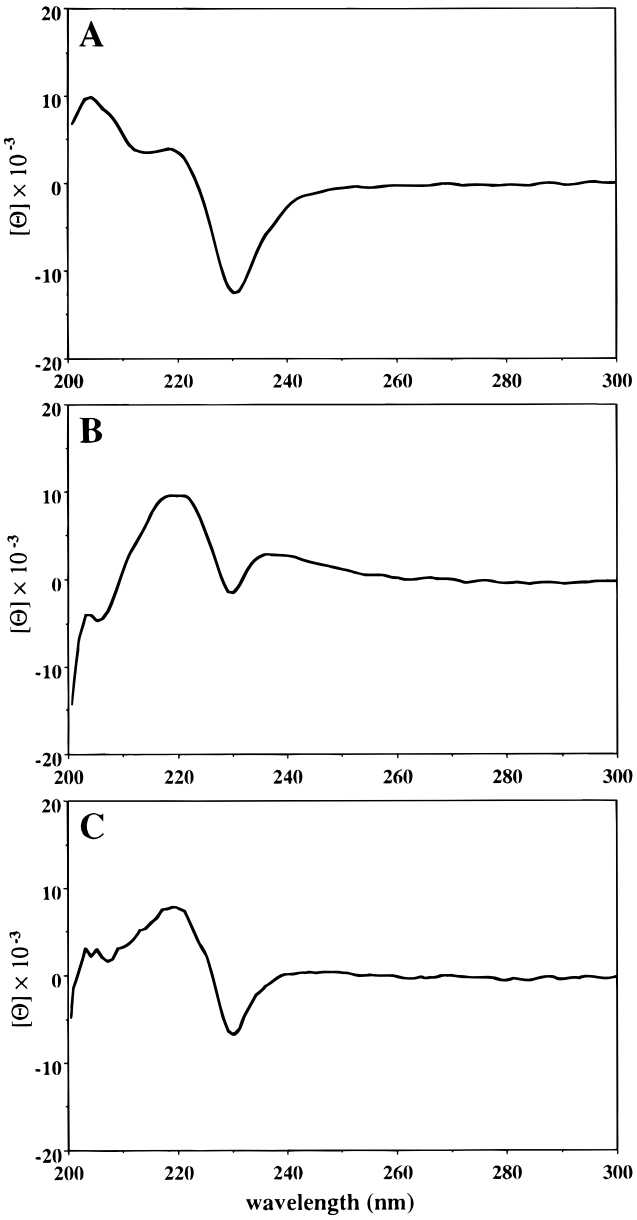


Figure 1. CD spectra of vesicles containing an equimolar mixture of **1** and **2** plus 9 mol % of gramicidin in (A) the nonchannel form, (B) the channel form, and (C) the nonchannel form that has been incubated for 15 h at 60 °C.

(7) Gramicidin A (*Bacillus brevis*) is a hydrophobic pentadecapeptide having the following primary sequence: formyl-L-Val_1-Gly_2-L-Ala_3-D-Leu_4-L-Ala_5-D-Val_6-L-Val_7-D-Val_8-L-Trp_9-D-Leu_{10}-L-Trp_{11}-D-Leu_{12}-L-Trp_{13}-D-Leu_{14}-L-Trp_{15}-ethanolamine. The peptide was purchased from Fluka (≥90%) and used directly.

(8) Cox, K. J.; Ho, C.; Lombardi, J. V.; Stubbs, C. D. *Biochemistry* **1992**, *31*, 1112.

(9) Muller, J. M.; van Ginkel, G.; van Fassen, E. E. *Biochemistry* **1996**, *35*, 488.

(10) *Liposomes: A Practical Approach*; New, R. R. C., Ed.; Oxford University Press: New York, 1990.

(11) Killian, J. A.; Prasad, K. U.; Hains, D.; Urry, D. W. *Biochemistry* **1988**, *27*, 4848.

(12) CD spectra were obtained on an AVIV 62ADS CD spectrometer that was equipped with computer-controlled data acquisition and analysis. The path length was 1 mm, and vesicles without gA were taken as the baseline.

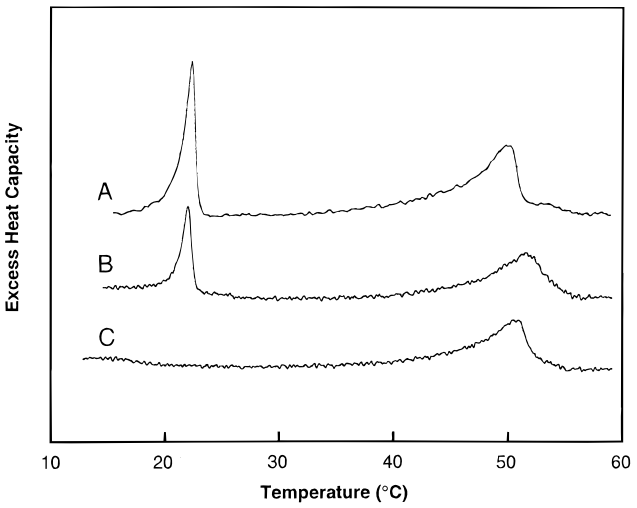


Figure 2. High-sensitivity excess heat capacity profile for large unilamellar vesicles made from **1**/**2** (1/1, mol/mol) (A) in the absence of peptide, (B) in the presence of *nc*-gA (1 mol %), and (C) in the presence of *nc*-gA (5 mol %).

Table 1. Nearest-Neighbor Recognition in Fluid Phospholipid Membranes Containing Gramicidin A[a]

gramicidin A (mol %)[b]	conformation	heterodimer[c]/ homodimer
none[d]		1.98 ± 0.05
9	channel	1.98 ± 0.04
17	channel	2.04 ± 0.03
1	nonchannel	1.58 ± 0.05
5	nonchannel	1.55 ± 0.07
5[e]	nonchannel	2.00 ± 0.07
9	nonchannel	1.34 ± 0.02
13	nonchannel	1.28 ± 0.03

[a] All NNR experiments were carried out at 60 °C; chemical equilibrium was generally reached in ca. 3 h. [b] Mole percentage of gramicidin A, where each dimer is counted as 2 moles of phospholipid. [c] Molar ratio of heterodimer to each homodimer ± two standard deviations. [d] See ref 4. [e] DPPC (50 mol %) was included as a diluent.

To ensure that NNR experiments were carried out in the physiologically relevant fluid phase, the influence of gA on the melting behavior of bilayers composed of **1** plus **2** (1/1, mol/mol) was first examined by high-sensitivity differential scanning calorimetry (*hs*-DSC, Figure 2). Incorporation of 1 mol % of *nc*-gA resulted in a decrease in intensity of the lower melting endotherm. In addition, the higher melting endotherm was broadened and shifted from 50.1 °C to 53.0 °C. As a point of reference, the gel to liquid-crystalline phase transition temperatures (T_m) for pure **1** and **2** are 22.7 and 55.4 °C, respectively.[4] Inclusion of 5 mol % of *nc*-gA led to substantial broadening of the lower melting endotherm as well as a shift to lower temperatures, and a return of the higher melting endotherm to its original temperature. These results imply that *nc*-gA favors association with **1** in the gel−fluid coexistence region. Identical results were obtained with *c*-gA (see Supporting Information).

Nearest-Neighbor Recogntion. By using procedures similar to those previously described, NNR experiments were performed with bilayers containing varying concentrations of *c*-gA and *nc*-gA; our principal results are summarized in Table 1.[13] In the presence of *c*-gA, a statistical mixture of dimers was obtained, indicating a random lateral distribution of the phospholipids.

(13) Vigmond, S. J.; Dewa, T.; Regen, S. L. *J. Am. Chem. Soc.* **1995**, *117*, 7838.

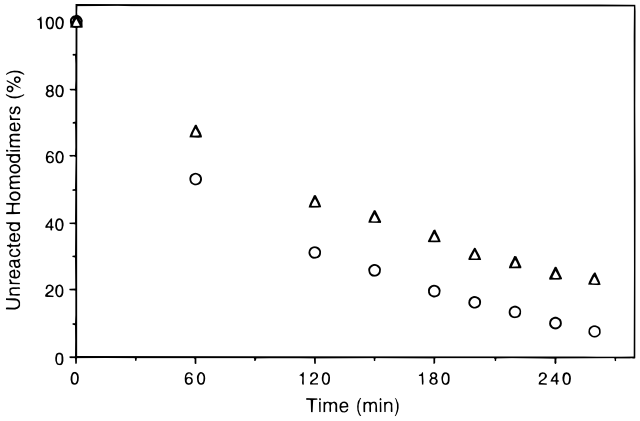


Figure 3. Plot of unreacted (○) **1** and (△) **2** as a function of time for vesicles containing *nc*-gA (5 mol %) that have been exposed to phospholipase A_2.

In sharp contrast, NNR was clearly detected when *nc*-gA was present, i.e., the molar ratio of heterodimer to each homodimer was significantly less than 2.0. When 50% of the exchangeable monomers was replaced by 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), a chemically inert diluent having an intermediate chain length and hydrophobicity, the ability of *nc*-gA to induce NNR was eliminated.[13,14]

Transmembrane Symmetry. Although one would not expect to observe transmembrane asymmetry in large unilamellar vesicles of the type used herein (average diameter of 10 000 Å, dynamic light scattering), we attempted to analyze the outer monolayer leaflet of vesicles that were formed from an equimolar mixture of **1** and **2**, plus *nc*-gA (5 mol %) via digestion with phospholipase A_2.[15−17] Analysis of the dimer content as a function of time (TLC, phosphorus analysis) revealed over-digestion (Figure 3). Since a well-defined break in the kinetics at 50% digestion was not apparent (indicative of exclusive hydrolysis of the outer monolayer), these experiments do not provide insight into the transmembrane distribution of the lipids. It is noteworthy that in the absence of the peptide, bilayers that are made from an equimolar mixture of **1** and **2** show the expected 50% hydrolysis of each homodimer.[18] The precise reason for over-digestion in the presence of this peptide is not presently clear.

In a related series of experiments, "monomer-analogous" vesicles were prepared from an equimolar mixture of **4** and **5**.[19] Subsequent reaction with an excess of 5,5′-dithiobis(2-nitrobenzoic acid), DTNB, and thiol analysis as a function of time (UV−vis) showed that exactly 50% of the thiol-containing monomer (**5**) was present in the outer monolayer, i.e., only half of the total thiols that were present underwent reaction (Figure 4A).[20]

(14) The equilibrium dimer ratio was derived from experiments with heterodimer-based vesicles.

(15) Kumar, A.; Gupta, C. M. *Biochemistry* **1985**, *24*, 5157.

(16) Runquist, E. A.; Helmkamp,G. M., Jr. *Biochim. Biophys. Acta* **1988**, *940*, 10.

(17) Vesicles were also examined by optical microscopy on an Olympus IX-70 microscope in bright field or Normarski DIC configurations, equipped with a 100× UplanApo (NA = 1.35) oil imersion objective. In the absence of peptide, spherical particles were readily apparent having a mean diameter of 1.26 ± 0.36 μm. In the presence of 2% *c*-gA or 2% *nc*-gA, spherical particles were observed having mean diameters of 0.93 ± 0.24 and 0.98 ± 0.39 μm, respectively. Mean diameters and standard deviations were determined by measuring at least 90 vesicles.

(18) Shibakami, M.; Inagaki, M.; Regen, S. L. *J. Am. Chem. Soc.* **1997**, *119*, 12354.

(19) Compounds **4** and **5** were prepared by using procedures similar to those previously described.[4] Phospholipid **4** had the expected ^{1}H NMR spectrum; HRMS for $(C_{37}H_{71}NO_9PS)^+$ calcd 736.4587, found 736.4585.

(20) Ganong, B. R.; Bell, R. M. *Biochemistry* **1984**, *23*, 4977.

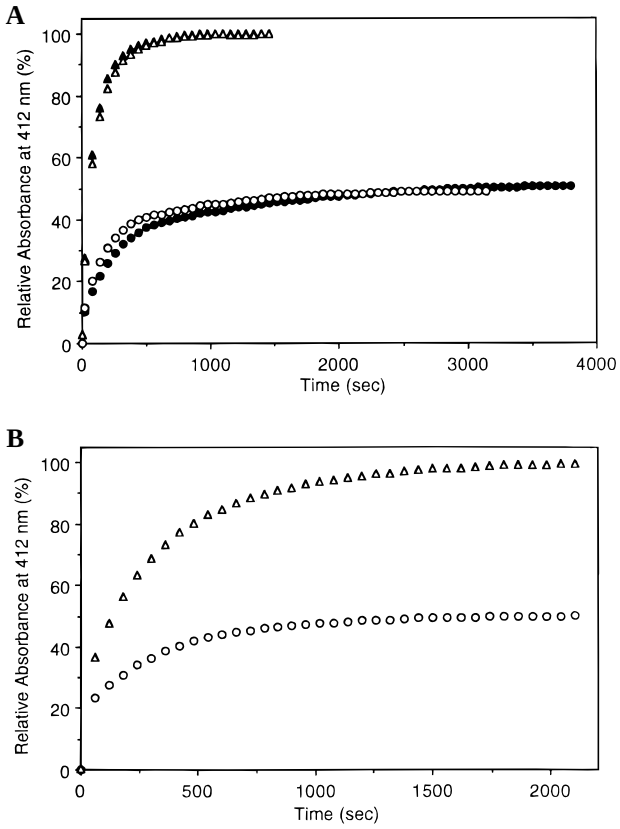
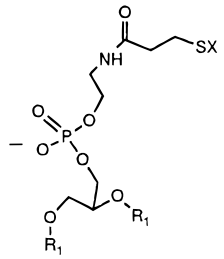


Figure 4. Plot of absorbance at 412 nm as a function of time for the reaction of vesicles made from an equimolar mixture of **4** and **5** (A) without peptide or (B) plus *nc*-gA (5 mol %), with an excess of DTNB in the (○) absence and (△) presence of 30% ethanol.

Similar experiments that were carried out with vesicles composed of **4**/**5**/*nc*-gA (10/10/1, mol/mol/mol) gave similar results (Figure 4B). Thus, the presence of the peptide leaves **5** evenly distributed across such bilayers.



4, R_1= $CO(CH_2)_{12}CH_3$; X=CH_3
5, R_1= $CO(CH_2)_{16}CH_3$; X=H

Lipid−Peptide Communication. The observation that *nc*-gA induces significant NNR, coupled with the fact that such recognition is eliminated in the presence of a diluent (DPPC), provides compelling evidence that the peptide generates a nonrandom lateral distribution of the phospholipids.[4,13] The added fact that *c*-gA leaves the phospholipids randomly distributed further indicates that *lipid mixing is controlled by the conformation of the peptide*. While the reaction times that were needed in order to reach chemical equilibrium were sufficiently short, such that negligible *nc*-gA to *c*-gA conversion had taken place (as indicated by the fact that dimer ratios reached a plateau with time), prolonged incubation at 60 °C (15 h) produced significant concentrations of the channel conformation (Figure 1C).

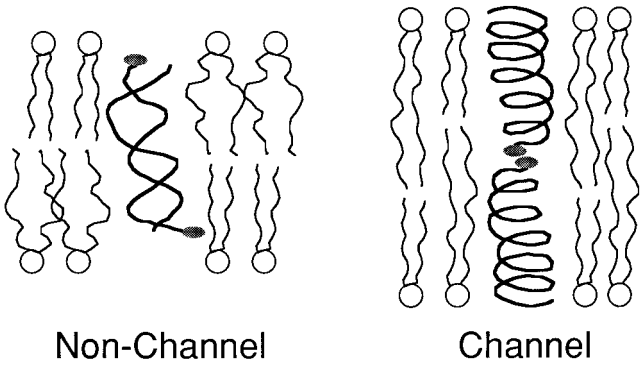


Figure 5. Styllized illustration of vesicle membranes derived from **1** plus **2** containing *nc*-gA or *c*-gA; the former depicts **2** in a hypothetical "crumpled" state.

Although the detailed mechanism by which *nc*-gA promotes lateral heterogeneity remains to be established, we presently favor a model that is based on membrane thinning. Specifically, we envision that by reducing the average thickness of the bilayer, *nc*-gA forces the relatively long phospholipids (i.e., **2**) into a "crumpled" state in order to maximize hydrophobic contact. Thus, we hypothesize that crumpled phospholipids favor other crumpled phospholipids as nearest-neighbors, i.e., **2** is "kicked out" of regions of the membrane that contain the more cylindrical-shaped monomer units of **1** (Figure 5).[21,22] The fact that a significant level of NNR is observed with peptide concentrations as low as 1% is remarkable, but not entirely surprising in view of the effectiveness of this peptide in altering the melting behavior of these lipids (vide ante).

Biological Implications. The ability of gA to communicate its conformation to the surrounding phospholipids implies that lateral transmission of conformational information should also be possible between lipids and proteins in biological membranes. It is on this basis that we hypothesize that such lipid−protein communication plays an important role in some of the most fundamental of cellular process, especially those that involve a substantial reorganization of membrane structure, e.g., fusion and endocytosis. Studies that are currently in progress are aimed at probing the effects of small cationic peptides (i.e., models for peripheral proteins) on the lateral distribution of phospholipids in the physiologically relevant fluid phase. The results of these studies will be reported in due course.

Acknowledgment. We are grateful to the American Cancer Society (Grant BE-205) for support of this research. We are also grateful to Dr. Luke E. Dewalt (Lehigh University) for assistance with the optical microscopy.

Supporting Information Available: High-sensitivity excess heat capacity profile for large unilamellar vesicles made from **1**/**2** (1/1, mol/mol) in the absence of peptide and in the presence of 1 and 5 mol % of *c*-gA (1 page, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(21) Fluorescence polarization measurements (60 °C, 10 min, 15 μM phospholipid) also indicate that *nc*-gA produces a more ordered and compact bilayer. Thus, inclusion of 1,6-diphenyl-1,3,5-hexatriene (DPH, 0.25 mol %) in vesicles containing *c*-gA (9 mol %) revealed a level of anisotropy (0.070 ± 0.010) that was identical to that found in the absence of peptide (0.079 ± 0.006). In sharp contrast, the anisotropy associated with *nc*-gA (9 mol %) was significantly greater (0.137 ± 0.009).[8−10]

(22) An interesting alternate model (proposed by a Reviewer) is that the longer phospholipid may preferentially orient around *nc*-gA with the chain ends filling in any voids around the end of the peptide.