Cholesterol-Modulated Lipid−Peptide Communication in Fluid Bilayers

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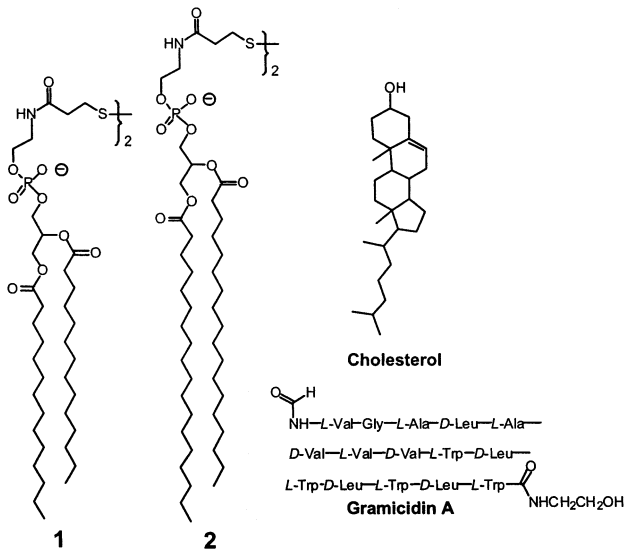
Received October 18, 2002

Cholesterol has been found to influence the relative effectiveness of channel (c-gA) and nonchannel (nc-gA) forms of gramicidin A in inducing the clustering of like phospholipid monomers of exchangeable dimers derived from 1,2-dimyristoyl-*sn*-glycerol-3-phosphoethanolamine and from 1,2-distearoyl-*sn*-glycerol-3-phosphoethanolamine (i.e., **1** and **2**, respectively) in fluid bilayers. Thus, when cholesterol concentrations were increased in vesicles made from equimolar mixtures of **1** and **2** containing 9 mol % nc-gA, a decrease in the preference for like phospholipids to become nearest neighbors was observed. In sharp contrast, for analogous membranes that contained c-gA, the introduction of cholesterol resulted in a significant increase in nearest-neighbor recognition, similar to what was found in the absence of the peptide. A model that accounts for this behavior and the biological implications of these findings are briefly discussed.

Introduction

In order for an enveloped virus to infect a mammalian cell, it must first fuse with its plamsa membrane. In the case of HIV-1, such fusion is controlled by a glycoprotein complex (gp120−gp41), which is included in the viral envelope. Specifically, when gp120 binds to a cellular receptor, this triggers a change in the conformation of the membrane-spanning gp41 segment, and an activation of its fusogenic properties.[1−6] Recently, it has been shown that a reduction in cholesterol levels in HIV-1 particles reduces their ability to infect cells.[7] The molecular basis for this effect, however, is not well understood. Since membrane fusion involves a major reorganization of the lipid bilayer, and since cholesterol is a major component of the viral membrane, it seems plausible that cholesterol concentrations may influence the ability of the gp41 unit to be reshaped by the surrounding lipids. In a broader context, cholesterol concentrations may also influence other signaling processes that rely on the communication between protein conformation and lipid arrangement.

In this paper, we provide the first experimental evidence that cholesterol can modulate the interaction between a membrane-bound peptide and the surrounding phospholipids. For this purpose, we have measured the mixing behavior of exchangeable monomers of 1,2-dimyristoyl-*sn*-glycerol-3-phosphoethanolamine and 1,2-distearoyl-*sn*-glycerol-3-phosphoethanolamine (**1** and **2)** in vesicles containing the channel (c-gA) and nonchannel (nc-gA) forms of gramicidin A, plus varying concentrations of cholesterol. In previous studies, we have shown that nc-gA (but not c-gA) induces nonideal mixing of these monomers, where like monomers become favored nearest neighbors.[8] A detailed examination of this system has further indicated that the driving force for nonideal mixing involves the formation of a complex between nc-gA and the longer, exchangeable monomer units of **2**.[9] Because this system exhibits lipid−peptide communication, and because such communication can be quantified, it is particularly well-suited for demonstrating the effects of added cholesterol.



Experimental Section

Nearest-Neighbor Recognition Analysis. In a typical liposome preparation, a test tube was charged with a chloroform solution that contained 0.24 μmol of **1a**, 0.24 mmol of **2a**, and 0.095 mmol of cholesterol. The chloroform was then evaporated by passing a stream of argon over the solution, thereby leaving a thin film of the lipid mixture. When nc-gA was included in the membrane, a chloroform solution containing 0.095 μmol of the peptide was added prior to solvent evaporation. In contrast, when c-gA was to be included, a trifluoroethanol solution containing 0.095 μmol of the peptide was added prior to solvent evaporation. The lipid mixture (containing 9 mol % cholesterol and 9 mol % peptide in the example given) was then dissolved in 80 μL of chloroform, diluted with 216 μL of diisopropyl ether, and mixed by vortexing. Subsequent addition of 27 μL of a 3.3 mM Tris-HCl buffer (pH 7.4, 50 mM NaCl, 0.667 mM NaN_3) produced an emulsion. After the emulsion was sonicated for 3 min, using a mild (bath type) sonicator, the organic phase was removed by

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10.1021/la026717c CCC: $22.00
Published on Web 11/12/2002

Table 1. Mixing Behavior of Monomers of 1 and 2

cholesterol (mol %)[a]	peptide	dimer ratio[b]
0		1.98 ± 0.06
9		1.98 ± 0.06
29		1.55 ± 0.04
40		1.47 ± 0.08
0[c]	c-gA	1.98 ± 0.04
9	c-gA	1.90 ± 0.03
29	c-gA	1.69 ± 0.05
40	c-gA	1.57 ± 0.06
0	nc-gA	1.34 ± 0.02
9	nc-gA	1.80 ± 0.02
29	nc-gA	1.81 ± 0.02
40	nc-gA	1.83 ± 0.06

[a] The mol % of cholesterol is based on the total phospholipid that is present, where each mole of phospholipid dimer "counts" as 2 mol of phospholipid. When included in the bilayers, 9 mol % of c-gA or nc-gA is also based on the total phospholipid content. [b] Equilibrium molar ratio of **3**/**2** obtained at 60 °C. [c] Taken from ref 8.

gentle evaporation at 60 °C under reduced pressure, resulting in a white gel at the bottom of the test tube. The gel was then collapsed by vigorous vortex mixing for 40 min, and 1.6 mL of additional buffer (pH 7.4, 10 mM Tris-HCl, 150 mM NaCl, and 2.0 mM NaN_3) was added dropwise with vortex mixing. The dispersion was then degassed with an aspirator for 1 min, and the residual traces of organic solvent were removed by dialysis (Spectra/Por Membrane, MWCO 6000−8000) under an argon atmosphere, using two 100-mL portions of degassed 10 mM Tris-HCl buffer (pH 7.4, 150 mM NaCl, 2.0 mM NaN_3) over the course of 18 h.

Thiolate−disulfide interchange reactions were initiated, after the dispersions had equilibrated at 60 °C, by injecting 34 μL of an aqueous solution containing 1 M NaOH and 4.8 mM dithiothreitol (1.6 equiv) and brief vortex mixing. Under these conditions, the pH of the dispersion was 8.5. All dispersions were maintained under an argon atmosphere throughout the course of the interchange reactions. Aliquots (0.240 mL) were withdrawn as a function of time and quenched with 50 μL of 0.03 M HCl (final pH 5.0). After removal of water under reduced pressure, the residue was triturated with 2 mL of chloroform and centrifuged. The chloroform was then removed under reduced pressure to yield a clear film, which was, subsequently, dissolved in 10 μL of chloroform and 90 μL of the mobile phase that was used for high-performance liquid chromatography (HPLC) analysis. Under the conditions used, the extent of reduction of the lipid dimers by dithiothreitol was less than ca. 10%.

Analysis of Dimer Distributions by HPLC. Mixtures of lipid dimers were analyzed by HPLC using a Beckman Ultrasphere C18 reverse phase column (4.6 × 250 mm, 5 μm particle size). The flow rate was 0.9 mL/min, and the column temperature was maintained at 31.0 °C. Peaks were monitored at 205 nm by use of a Waters 996 photodiode array detector. Data were collected and processed using a Millennium workstation (Waters Corp.). The mobile phases that were used in these analyses were composed of 10 mM tetrabutylammonium acetate (TBA) in denatured ethanol/water/hexane in volume ratios of (83/11/6, v/v/v).

Results

Phospholipid Mixing As Measured by the Nearest-Neighbor Recognition Method. The technique that we have used in this study to probe lipid mixing is the nearest-neighbor recogntion (NNR) method. In essence, NNR measurements can detect and quantify the thermodynamic tendency of the exchangeable monomers of **1** and **2** to become nearest neighbors.[10] In brief, **1** and **2** are allowed to undergo monomer interchange via thiolate−disulfide displacement reactions. Equilibrium dimer distributions are then analyzed as formal, noncovalent bonds between pairs of adjacent lipids. Thus, a membrane that

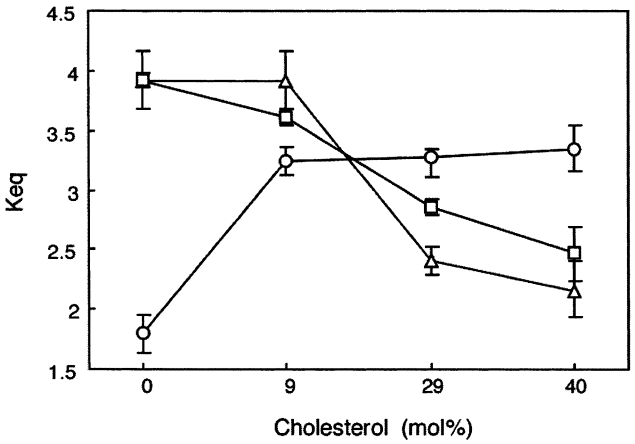

Figure 1. Plot of K versus mol % of sterol present in bilayers containing equilibrium concentrations of **1a**, **2a**, and **3** in the absence of peptide (△) and in the presence of 9 mol % c-gA (□) or 9 mol % nc-gA, (○).

is derived from of **1** and **2** is analyzed as an equilibrium mixture of **1**, **2**, and the corresponding heterodimer (**3**, not shown) according to eqs 1 and 2. Here, K is the equilibrium constant, and [**1**], [**2**], and [**3**] are the equilibrium concentrations of the phospholipid dimers. If the exchangeable monomers are ideally mixed, then the observed dimer distribution would be statistical. Thus, if the membrane were prepared from an equimolar mixture of **1** and **2**, the equilibrium mole ratio of **1**/**3**/**2** would be 1/2/1), and the equilibrium constant would be equal to 4. If a thermodynamic preference for homoassociations existed, then the value of K would be less than 4; favored heteroassociations would be indicated by a value of K that is greater than 4. As discussed elsewhere, although the NNR method involves the use of exchangeable dimers, it provides thermodynamic information that relates to nearest-neighbor interactions between *individual* lipid monomers.[10]

$$\mathbf{1} + \mathbf{2} \overset{K}{\rightleftharpoons} 2\,\mathbf{3} \tag{1}$$

$$K = [\mathbf{3}]^2/([\mathbf{1}][\mathbf{2}]) \tag{2}$$

Nearest-Neighbor Preferences. Using experimental procedures similar to those previously described, NNR measurements were carried out using large unilamellar vesicles (reverse phase evaporation method) composed of an equimolar mixture of **1** and **2**, 9 mol % of nc-gA (or c-gA), and varying concentrations of cholesterol.[8,11] To ensure that equilibrium was reached in each case, similar experiments were carried out using vesicles derived from the heterodimer, **3**, plus the same percentages of peptide and sterol. Equilibrium distributions were then determined by averaging dimer ratios from at least three different reaction times for the homodimer- and heterodimer-based vesicles.

In Table 1 are shown the nearest-neighbor preferences (expressed as molar ratios of heterodimer/homodimer, **3**/**2**) for the different membranes investigated. Figure 1 shows plots of the corresponding equilibrium constants (K) as a function of the mol % of cholesterol. In the absence of peptide, a preference for homophospholipid association is clearly evident, but only when the sterol concentration exceeds 9 mol %. For bilayers containing c-gA, a preference for homophospholipid association also increases as the

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sterol concentration is increased; this increase is very similar to that found in the absence of peptide. In sharp contrast, for bilayers containing nc-gA, inclusion of 9 mol % cholesterol leads to a significant *reduction* in the preference for homophospholipid association, which remains essentially constant as the sterol content is raised to 29 and to 40 mol %.

Discussion

The ability of cholesterol to promote the apparent homophospholipid association in bilayers made from **1** and **2** is due to a strong preference of cholesterol to have two or more of the longer phospholipids (i.e., monomers of **2**) as nearest neighbors (see ref 11). Such a preference, in a sense, may be viewed as the formation of a "condensed complex".[12] The present results show, however, that this effect becomes signficant only at cholesterol concentrations exceeding 9 mol %. The fact that cholesterol maintains its ability to promote this nearest-neighbor recognition in bilayers that contain 9 mol % c-gA also shows that this peptide has no selectivity whatsover toward either of the two phospholipids or toward cholesterol. In fact, c-gA appears to behave in this system as an inert diluent.

In contrast, even though both cholesterol and nc-gA, when acting alone, do induce apparent homophospholipid association in bilayers of **1** and **2**, the combination of the two is significantly less effective than the presence of either one alone. Such behavior can be readily accounted for, if one considers that the mode of action of cholesterol and nc-gA in segregating **1** and **2** is exactly the same; that is, both form complexes, selectively, with **2**. Thus, by virtue of this same property, nc-gA should also be able to form complexes with cholesterol. In other words, the peptide is in strong competition for **2** with cholesterol. Thus, by formation of nc-gA/cholesterol complexes, the amount of both of these agents that is available for binding with **2** is reduced. Our data show that the affinity of cholesterol and nc-gA toward each other is stronger than their affinity toward **2**; hence their mixture results in a higher percentage of uncomplexed **2** and a lower degree of apparent nearest-neighbor recognition.

The ability of cholesterol to influence the relative effectiveness of channel and nonchannel forms of gramicidin A in controlling the mixing properties of monomers of **1** and **2** reported herein is significant. Whether or not cholesterol can affect the communication between membrane proteins and the surround lipids in biological membranes—e.g., gp120−gp41 and the lipids in the HIV-1 viral envelope—remains to be established, but our results in these model systems suggest that it could very well account for the effects of cholesterol on HIV-1 infectivity.

Acknowledgment. We are grateful to the National Institutes of Health (PHS GM56149) for support of this research.

LA026717C

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