Optimization of Receptor-G Protein Coupling by Bilayer Lipid Composition II

FORMATION OF METARHODOPSIN II-TRANSCLUDIN COMPLEX*

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The visual transduction system was used as a model to investigate the effects of membrane lipid composition on receptor-G protein coupling. Rhodopsin was reconstituted into large, unilamellar phospholipid vesicles with varying acyl chain unsaturation, with and without cholesterol. The association constant (K_a) for metarhodopsin II (MII) and transducin (G_t) binding was determined by monitoring MII-G_t complex formation spectrophotometrically. At 20 °C, in pH 7.5 isotonic buffer, the strongest MII-G_t binding was observed in 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (18:0,22:6 PC), whereas the weakest binding was in 1-oleoyl-2-oleoyl-sn-glycero-3-phosphocholine (18:0,18:1 PC) with 30 mol% cholesterol. Increasing acyl chain unsaturation from 18:0,18:1PC to 18:0,22:6PC resulted in a 3-fold increase in K_a. The inclusion of 30 mol% cholesterol in the membrane reduced K_a in both 18:0,22:6PC and 18:0,18:1 PC. These findings demonstrate that membrane compositions can alter the signaling cascade by changing protein-protein interactions occurring predominantly in the hydrophobic region of the proteins, external to the lipid bilayer. These findings, if extended to other members of the superfamily of G protein-coupled receptors, suggest that a loss in efficiency of receptor-G protein binding is a contributing factor to the loss of cognitive skills, odor and spatial discrimination, and visual function associated with n-3 fatty acid deficiency.

The G protein-coupled motif is a fundamental mode of cell signaling, utilized in vision, taste, olfaction, and a variety of neurotransmitter systems. The receptors for these systems are integral membrane proteins, embedded in a lipid matrix. Neuronal and retinal tissues and the olfactory bulb contain high levels of the n-3 polyunsaturated acyl chain derived from docosahexaenoic acid (22:6n-3) in their cell membrane phospholipids (1, 2). Approximately 50% of the acyl chains in the phospholipids of the ROS disc membrane consist of 22:6n-3 (1). The physiological significance of 22:6n-3 is demonstrated by the impaired visual response (3), learning deficits (2), loss of odor discrimination (4), and reduced spatial learning (5) associated with n-3 fatty acid deficiency. In all cases where acyl chain analysis was carried out, the 22:6n-3 content of membrane phospholipids was dramatically reduced in the n-3 deficient animals where it was replaced by 22:5n-6 (5). These findings suggest that the high levels of 22:6n-3 in membrane phospholipids play a critical role in various membrane-associated signaling pathways. A common thread in several of these processes is the ubiquitous motif of G protein-coupled signaling systems. However, molecular mechanisms linking 22:6n-3 phospholipids with essential physiological functions remain to be clarified. The study described herein aims to elucidate such mechanisms by investigating the effect of membrane lipid composition on G protein-coupled signal transduction.

In G protein-coupled systems, the receptor activates an effector protein through the action of a G protein (6). Receptors in this superfamily are integral membrane proteins made up of seven transmembrane helices and their respective connecting loops. In contrast, the G protein and effector proteins are generally peripheral proteins, bound to the membrane by a combination of an isoprenoid chain-lipid bilayer interactions (7, 8) and electrostatic forces (9). The receptor-binding site for the ligand is formed by the transmembrane helices and lies near the midpoint of the membrane; hence, the conformational changes accompanying receptor activation would be expected to have a dependence on the membrane lipid composition. In contrast, the interaction of the G protein with the receptor occurs primarily external to the membrane bilayer (10, 11). How the lipid composition might affect the interaction between receptor and G protein external to membrane bilayer is not clear.

The visual transduction system is among the best characterized G protein-coupled signaling systems (12) and is used as a model in these studies (13, 14). Light absorption results in the generation of a rapid equilibrium between MI and MII (15), and the active conformation, MII, readily associates with G_t forming the MII-G_t complex, which is relatively stable in the absence of G_t (16). The interaction sites on MII involved in binding G_t are composed of three cytoplasmic loops formed by the peptide sequence connecting helices III and IV, V and VI, and a putative loop formed by amino acids 310–321, anchored in the bilayer by palmitate groups esterified to Cys-322 and Cys-323 (17–19). Recent structural studies of these loops indicate a level of secondary structure in the form of α-helices (20).
G, is a trimeric protein consisting of Ga, Gβ, and Gy subunits (17). Studies using various peptides representing the putative binding region of Gα implicate two regions in the carboxyl-terminal region of Ga and a segment of Gy as the interaction sites with MI (18, 19, 21). The interactions appear to be mainly hydrophilic in nature, because the interaction sites are located external to the membrane bilayer.

In this study, rhodopsin was reconstituted into large, unilamellar vesicles containing either di22:6PC, 18:0,22:6PC, or 18:0,18:1PC with and without cholesterol at a rhodopsin:phospholipid molar ratio of 1:100 as described elsewhere (32).

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—Rhodopsin was purified and reconstituted into vesicles consisting of di22:6PC, 18:0,22:6PC, and 18:0,18:1PC with and without cholesterol at a rhodopsin:phospholipid molar ratio of 1:100 as described elsewhere (32).

**Measurement of MII-Gt Binding**—The association constant of MII-Gt was determined spectrophotometrically, utilizing the fact that MII and MI-Gt have identical absorption spectra (22). MII-Gt complex formation is reflected as an intensity increase at the MI absorption band. The MI-MII equilibrium spectra in the absence and presence of Gt were collected and subsequently deconvolved to obtain the concentrations of MI, MII, MI-Gt, and Kₐ.

Rhodopsin-containing vesicles were pre-associated with Gt (0–2 μM) under isotonic conditions in dark for 4 h on ice. The sample mixtures were then used for spectroscopic measurements as described by Straume et al. (23). Briefly, a total of four sequential spectra were recorded for each sample: 1) following equilibration in isotonic buffer, pH 7.5 at 20 °C in the dark; 2) 3 s after partial bleaching (20–30%) by a flash lamp equipped with a 520-nm band pass filter; 3) 10 min after incubation with 30 mM hydroxylamine; and 4) following full bleaching. These spectra were used to calculate the MI-MII equilibrium difference spectra, which were subsequently deconvolved into concentrations of MI, MII, and MI-Gt, as described in the following section.

**Data Analysis**—The following equilibria represent the events subsequent to the light activation of rhodopsin in the absence and presence of Gt.

Equilibrium difference spectra, which had been corrected for the presence of unbleached rhodopsin, were deconvolved into two Gaussian peaks at 385 and 480 nm (23). The peak at 480 nm represents MI in the absence of Gt and — in the presence of Gt. The increase in absorbance at 385 nm in the presence of Gt indicates the formation of MI-Gt complex.

**FIG. 1. Lipid dependence of MII and MI-Gt complex formation.** Spectra were acquired from samples of reconstituted rhodopsin in 18:0,22:6PC vesicles (panel A) and 18:0,18:1PC with 30 mol% cholesterol vesicles (panel B) in TBS buffer at 20 °C, pH 7.5. Difference equilibrium spectra of MI and MI-Gt corrected for the presence of unbleached rhodopsin are shown: ○, in the absence of Gt; ●, in the presence of 1 μM Gt. Smooth curves are deconvolved spectra with two absorption peaks at 385 and 480 nm according to “Experimental Procedures.” — — — in the absence of Gt and — — — in the presence of Gt. The increase in absorbance at 385 nm in the presence of Gt indicates the formation of MI-Gt complex.

metrical distribution of rhodopsin in vesicles (24). The following equations were used to calculate [MI-Gt], [MI], and [G]free with consideration of this fact.

\[
[MII] = \frac{1}{2}[Rh^*] K_{eq}^{G} - K_{eq}^{G} + 1/(1 + K_{eq}^{G}) \quad (\text{Eq. 3})
\]

\[
[MII-Gt] = [Rh^*] K_{eq}^{G} - K_{eq}^{G} (1 + K_{eq}^{G}) \quad (\text{Eq. 4})
\]

\[
[G]_{free} = [G] - [Rh^*] K_{eq}^{G} - K_{eq}^{G} (1 + K_{eq}^{G}) \quad (\text{Eq. 5})
\]

Rh* is the amount of bleached rhodopsin. For each lipid composition, [MI-Gt]/[MI]_{initial} was analyzed as a function of [G]_{free} and Kₐ was determined according to Equation 2.

**RESULTS**

The MI-MII equilibrium and the association of MI with Gt can be readily monitored through changes in the absorption spectra of these photointermediates. Examples of the effect of two different lipid compositions on the MI-MII equilibrium and MI-Gt complex formation are shown in Fig. 1. The spectra for rhodopsin reconstituted in vesicles consisting of a highly unsaturated 18:0,22:6PC are shown in Fig. 1A, whereas those in a mono-unsaturated 18:0,18:1PC mixed with 30 mol% cholesterol are shown in Fig. 1B. In the absence of Gt, the spectra in Fig. 1A contained two absorption bands centered about 385 and 480 nm, associated with the MI and MI photointermediates, respectively. In Fig. 1A (open circles), the MI and MI peaks are approximately equal, whereas the MI peak was greatly reduced accompanied by a large increase in the MI peak in Fig. 1B (open circles). The presence of Gt caused an enhancement of...
the MII peaks in both bilayer systems (Fig. 1 A and B, filled circles). This is the result of MII-Gt complex formation and the fact that formation of this complex does not alter the spectral properties of MII.

The spectral contribution of the bands with absorption peaks centered at 480 nm and 385 nm in Fig. 1 (A and B) were deconvoluted into contributions as a result of MI and MII in the absence of Gt and MI and (MII + MII-Gt) in the presence of Gt, as shown by dashed and solid curves, respectively. It is clear that the amount of MII or (MII + MII-Gt) formed was greater in 18:0,22:6PC relative to 18:0,18:1PC with 30 mol% cholesterol, demonstrating the role of lipid composition in modulating the formation of MII and MII-Gt. The calculated values of $K_{eq}^{G}$ and $K_{eq}^{-G}$ in 18:0,22:6PC (Fig. 1A) were 1.01 ± 0.02 and 2.22 ± 0.14, respectively, whereas $K_{eq}^{G}$ and $K_{eq}^{-G}$ in 18:0,18:1PC with 30 mol% cholesterol (Fig. 1B) were 0.19 ± 0.04 and 0.35 ± 0.04, respectively.

Both $K_{eq}^{G}$ and $K_{eq}^{-G}$ varied by more than a factor of 5 over the range of bilayer compositions examined in this study, as shown in Fig. 2. In the absence of Gt, $K_{eq}^{G}$ followed the order of di22:6PC > 18:0,22:6PC > 18:0,18:1PC + 30 mol% cholesterol ~ 18:0,18:1PC > 18:0,18:1PC + 30 mol% cholesterol. This is consistent with previous findings (25–29) that showed that the reduced acyl chain unsaturation and the presence of cholesterol reduced the equilibrium concentration of MII. The presence of Gt increased the apparent amount of MII formed in all samples, as indicated by the values of $K_{eq}^{G}$. This results from the formation of the MII-Gt complex. The trend for $K_{eq}^{G}$ with bilayer composition followed that of $K_{eq}^{-G}$.

Values of $[G_{t}]_{free}$, [MII-Gt], and [MII] were calculated from $K_{eq}^{G}$ and $K_{eq}^{-G}$ according to Equations 3–5. A series of measurements made with increasing ratios of Gt to MII was used to produce the binding profiles of MII to Gt. Example plots of [MII-Gt]/[MII]$_{total}$ versus [Gt]$_{free}$ in two lipid compositions are shown in Fig. 3. Increased concentrations of Gt resulted in an increase amount of MII-Gt complex formation. However, the slopes in the binding plots were rather different, reflecting dissimilar binding constants in the two lipid bilayers. Analysis of the data according to Equation 2 gave $K_{eq}$ of $1.5 \times 10^{7}$ M$^{-1}$ for 18:0,22:6PC vesicles and $K_{eq}$ of $2.5 \times 10^{6}$ M$^{-1}$ for 18:0,18:1PC vesicles containing 30 mol% cholesterol.

Both acyl chain unsaturation and cholesterol content modulated the binding of MII to Gt, as shown in Fig. 4. Two key observations may be drawn from the values of $K_{eq}$: 1) The increase in acyl chain unsaturation going from 18:0,18:1PC to 18:0,22:6PC resulted in a 3-fold enhancement in $K_{eq}$, whereas further increase in unsaturation going to di22:6PC resulted in a slight reduction in $K_{eq}$ relative to 18:0,22:6PC. 2) Cholesterol reduced $K_{eq}$ in both monounsaturated 18:0,18:1PC and highly unsaturated 18:0,22:6PC. The $K_{eq}$ values in 18:0,18:1PC + 30 mol% cholesterol and 18:0,22:6PC + 30 mol% cholesterol are $2.5 \times 10^{6}$ M$^{-1}$ and $4.4 \times 10^{5}$ M$^{-1}$, respectively, showing a somewhat smaller effect in the polyunsaturated bilayer.

**DISCUSSION**

Previous studies have demonstrated that the formation of the active conformation of the G protein-coupled receptor rhodopsin, MII, is dependent on the membrane lipid composition (23,25–29), consistent with the present results regarding the lipid dependence of $K_{eq}^{G}$. A primary finding of this study is that MII-Gt complex formation, the initial amplification step in the visual cascade, is also modulated by the phospholipid acyl chain and cholesterol composition of the membrane. Increased acyl chain unsaturation and decreased level of cholesterol resulted in a higher affinity of MII to Gt. One characteristic of the native disc membrane is that ~50% of the total acyl chains are made of 22:6n-3, which is similar to that in 18:0,22:6PC. The reported $K_{eq}$ in native disc is on the order of $10^{7}$ M$^{-1}$ (30, 31), whereas $K_{eq}$ in 18:0,22:6PC reconstituted vesicles is $1.5 \times 10^{7}$ M$^{-1}$. The agreement between these values, despite the differences in phospholipid headgroup composition, indicates the important role of 22:6n-3 in modulating the coupling of rhodopsin to Gt.

Increased phospholipid acyl chain unsaturation was shown to increase the formation of MII (27, 29), whereas increased
cholereter concentration decreases MII (28). These findings
have been linked to the specific packing properties of poly-
saturated acyl chains and the effect of cholesterol on these
packing properties (29). Current evidence indicates that MII-Gt
interactions involve the three hydrophilic loops on the cytoplas-
mic surface of rhodopsin with regions in the carboxyl-terminal
region of Gt, placing the interaction surfaces external to the
bilayer. The dependence of the extent of MII-Gt complex for-

tomation in the phospholipid acyl chain composition demon-
strates that membrane lipid composition can not only play a
role in modulating the level of MII formation, but it has a
marked effect on the coupling of an integral membrane protein
receptor to a peripherally bound G protein. Hence, the acyl
chain packing in the hydrophobic region of the bilayer can
affect interactions thought to occur primarily in the hydrophilic
region of integral and peripheral membrane proteins.

Our results demonstrate that acyl chain composition and
cholesterol content modulate the coupling step of Gt to MII. To
understand how lipid composition may modulate MII-Gt inter-
actions, it is necessary to consider the molecular events asso-
ciated with MII-Gt binding. The formation of the MII-Gt com-
plex involves a diffusional search of MII and Gt for each other
changed. Gt is associated with the membrane through an iso-
lipid composition on the kinetics of MII and MII-Gt formation
were considered previously. In a separate study, the effect of
the rotation and diffusion of rhodopsin in the membrane. 2) The
a number of ways. 1) Acyl chain packing properties can affect
interactions thought to occur primarily in the hydrophilic
region of Gt, placing the interaction surfaces external to the
membrane surface. 3) Increased acyl chain saturation
have been linked to the specific packing properties of poly-
unsaturated acyl chains and the effect of cholesterol on these
packing properties (29). Current evidence indicates that MII-Gt
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mic surface of rhodopsin with regions in the carboxyl-terminal
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cholesterol content modulate the coupling step of Gt to MII. To
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ciated with MII-Gt binding. The formation of the MII-Gt com-
plex involves a diffusional search of MII and Gt for each other
on the membrane surface and subsequent productive collisions
leading to binding. Varying phospholipid acyl chain composi-
tion and cholesterol content can alter membrane properties in
a number of ways. 1) Acyl chain packing properties can affect
the rotation and diffusion of rhodopsin in the membrane. 2) The
lateral diffusion and association of Gt on the membrane can be
changed. Gt is associated with the membrane through an iso-
prenoid linkage. Acyl chain packing may affect the orientation
of Gt in the bilayer making MII-Gt collisions less productive in
terms of complex formation. 3) Increased acyl chain saturation
inhibits the formation of MII because the outward movement of
helices during MII formation may be hindered in a more rigid
lipid environment resulting in reduced MII-Gt complex forma-
tion. In addition, the sensitivity to acyl chain and cholesterol
content may indicate a greater role of protein-protein interac-
tions within the hydrophobic portion of the membrane than
were considered previously. In a separate study, the effect of
lipid composition on the kinetics of MII and MII-Gt formation
was studied using flash photolysis (32). We found that the
kinetics of MII formation, which is a unimolecular reaction, ex-
hibited relatively mild dependence on bilayer composition,
whereas the kinetics of MII-Gt formation was greatly dimin-
ished by the presence of cholesterol and more saturated lipids.
These findings support the role of lipid composition in modu-
ulating the diffusional coupling of MII to Gt on membrane
surface.

Visual signaling is initiated from rhodopsin and propagated
along the visual cascade through a series of coupled steps. In
this study we have demonstrated that the initial steps, which
are rhodopsin activation and MII-Gt coupling, are modulated
by lipid composition and cholesterol. The net effect of bilayer
composition on visual transduction can be evaluated in terms of
the yield of MII-Gt complex formation per bleached rhodopsin,
[MII-Gt]/[Rh+] . The following equation was used for such cal-

Equation 6 is derived from the following equations.

\[ K_m^{G} = \frac{[MII-Gt]}{[MII]} \]  

\[ K_m = \frac{[MII-Gt][Gt] - [MII-Gt]}{[MII] + [MII-Gt]} \]  

Under physiological conditions, the ratio of rhodopsin to Gt is
~10 to 1 in ROS membranes and in the range of 1 of 100,000
rhodopsins absorbs a photon, giving rise to a visual response.
The effect of bilayer composition on [MII-Gt]/[Rh+] is clearly
demonstrated in Fig. 5. Although ~90% of bleached rhodopsin
formed complex with Gt in 18:0:22:6PC and d122:6PC vesicles,
only 60% of such complex is formed in 18:0:18:1PC vesicles. The
presence of 30 mol% cholesterol in 18:0:22:6PC and 18:0:18:1
PC resulted in ~60 and 30% complex formation, respectiv-
ly. It was recently reported that ~10% of rhodopsin in the ROS
disc membrane is contained in a detergent-resistant membrane
fraction or lipid raft (33). If this raftlike phase exists under
physiological conditions, and like other lipid rafts, it is rich in
cholesterol and saturated acyl chains, the present results sug-
gest that II-MII-G binding strength and kinetics would be reduced for this population of rhodopsin. In addition to phosphatidylethanolamine (PE) (1), PS will add a negative surface charge to the membrane, whereas PE will contribute an increased level of acyl chain packing order because of its higher melting point relative to PC. Our current data would suggest that the presence of PE would be somewhat inhibitory relative to II-MII-G complex formation. The effect of PE is yet to be determined.

A model, relating the biochemical events in the visual transduction pathway with the neural response, as measured by the electroretinograms, was recently published (34). In this model, the response at any time after a light stimulus is directly proportional to the concentration of activated rhodopsin molecules, i.e. [MII]. Here, we have determined that, at very low light levels, the fraction of II-MII-G complex to bleached rhodopsin, [MII-G/Rh]n, depends on the lipid composition of the membrane, Fig. 5. If [MII-G/Rh]n is measured as the fraction of bleached rhodopsin that can participate in activating Gt, then the factor in the equation for the response time at physiological light exposures needs to be corrected for variation in membrane lipid composition. Both the 22:6n-3-containing PCs examined in this study support nearly full participation of the bleached rhodopsin in Gt activation, whereas the addition of 30 mol% of cholesterol reduces this to about 30%. Thus, the response time in the 22:6n-3-containing PC's would be expected to be faster relative to that observed in the 18:1n-9-containing PC by a factor of 1.67. In the case of n-3 deficiency, II-MII-G complex formation would also contribute to lower signal amplitude, because fewer Gt proteins would be activated. Although, it is not anticipated that the difference between 22:6n-3 and 22:5n-6 will produce as great a lag time as is indicated for 18:1n-9, the observed lag time and reduced signal amplitude in n-3 deficiency relative to n-3-sufficient subjects is consistent with the dependence of the level of II-MII-G complex formation on the membrane lipid composition.

The visual cascade, initiated by the light activation of rhodopsin, involves a series of protein-coupled reactions resulting in an amplified response. The first step in signal amplification in the visual pathway is the formation of the II-MII-G complex. The modulatory effect of bilayer acyl chain composition and cholesterol content on both the kinetics and extent of formation of the II-MII-G complex observed in this and the previous (32) study will have direct impact on the downstream steps of the visual cascade. Weakened II-MII-G interactions will result in reduced amplification and slower kinetics at the Gt activation step, which will propagate down the pathway to produce reduced activity of the effector protein, cGMP phosphodiesterase. These effects may well provide the molecular basis for the diminished amplitude and sensitivity (36, 37) and lag time in the electroretinogram a-wave (35) and the reduced visual acuity (38) associated with 22:6n-3 deficiency. Because of the similar signaling motif in other G protein-coupled signaling systems, the findings in this study should be generally applicable to other members in the G protein-coupled family, providing a molecular mechanism for the observed loss in cognitive skills (2), odor (4), and spatial discrimination (5) observed in n-3 fatty acid deficiency.

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