DHA-RICH PHOSPHOLIPIDS OPTIMIZE G-PROTEIN–COUPLED SIGNALING

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Objective To assess the effects of n–3 polyunsaturated phospholipid acyl chains on the initial steps in G-protein–coupled signaling.

Study design Isolated components of the visual signal transduction system, rhodopsin, G protein (G_t), and phosphodiesterase (PDE), were reconstituted in membranes containing various levels of n–3 polyunsaturated phospholipid acyl chains. In addition, rod outer segment disk membranes containing these components were purified from rats raised on n–3-deficient and n–3-adequate diets. The conformation change of rhodopsin, coupling of rhodopsin to G_t, and PDE activity were each measured separately.

Results The ability of rhodopsin to form the active metarhodopsin II conformation and bind G_t were both compromised in membranes with reduced levels of n–3 polyunsaturated acyl chains. The activity of PDE, directly related to the integrated cellular response, was reduced in all membranes lacking or deficient in n–3 polyunsaturated acyl chains. PDE activity in membranes containing 22:5n–6 PC was 50% lower than in membranes containing either 22:6n–3 PC or 22:5n–3 PC.

Conclusions The earliest events in G-protein–coupled signaling; receptor conformation change, receptor–G-protein binding, and PDE activity are reduced in membranes lacking n–3 polyunsaturated acyl chains. Efficient and rapid propagation of G-protein–coupled signaling requires polyunsaturated n–3 phospholipid acyl chains. *(J Pediatr 2003;143:S80-S86)*

One of the principle goals of membrane biochemistry is an understanding of the molecular basis for the requirement for highly unsaturated phospholipid acyl chains in many biological membranes. A large body of research demonstrates the need for high levels of polyunsaturated fats, particularly n–3 polyunsaturates, for optimum infant development and performance in later life.[1-3] The conclusions drawn from decades of research on nutrition and infant development strongly suggest that specific but unidentified biochemical processes are optimized by the presence of n–3 polyunsaturates and/or compromised by their absence. In this report, we demonstrate that multiple aspects of an important and ubiquitous signaling motif, G-protein–coupled signal transduction, require docosahexaenoic (22:6n–3) phospholipid acyl chains for optimum functional efficacy.

The G-protein–coupled motif is a fundamental mode of intracellular signaling and includes the sensory pathways for vision, olfaction, taste, touch, and the neurotransmitters dopamine, serotonin, GABA, and histamine.[4] Each of these chemical and physical agents acts on a unique integral membrane protein that is embedded in a lipid membrane. Many of the membranes that contain significant amounts of G-protein–coupled activity, such as neuronal and retinal tissues and the olfactory bulb, contain high levels of the n–3 polyunsaturated acyl chain derived from docosahexaenoic acid.[5,6] Dietary n–3 deficiency leads to the replacement of 22:6n–3 phospholipid acyl chains with 22:5n–6 acyl chains in these membranes.[7] The functional significance of 22:6n–3 is demonstrated by the impaired visual response,[8] learning deficits,[6] loss of odor

MI, MII	Metarhodopsin I, II	ROS	Rod outer segment
PDE	Phosphodiesterase		

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discrimination,[9] and reduced spatial learning[7] associated with n–3 fatty acid deficiency. These findings suggest that a high level of 22:6n–3 in membrane phospholipids is required for optimum function of a number of diverse signaling pathways. A common feature of these pathways is the central role of G-protein–coupled signaling.

Receptors in the G-protein–coupled superfamily are integral membrane proteins made up of 7 transmembrane helixes 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 interaction[10,11] and electrostatic forces.[12] The ligand binding site on the receptor is formed by transmembrane helixes and generally lies near the midpoint of the membrane; hence the conformational changes accompanying receptor activation would be expected to have a dependence on the physical properties of the membrane lipid bilayer. The interaction of the G protein with the receptor occurs in the hydrophobic region of the protein, external to the membrane bilayer.[13,14] How the composition and physical properties of the membrane might affect the interaction between receptor and G protein external to the membrane bilayer is a question that remains largely unexamined.

Most of the basic information about G-protein–coupled signaling pathways was obtained from work on the visual system,[15,16] and it is the only G-protein–coupled pathway for which there are 3-dimensional structures for both the receptor, rhodopsin,[17] and the G protein, transducin (G_t).[18] In these studies, we used the visual transduction system as a model system for investigating the effects of membrane composition on G-protein–coupled signal transduction. After absorption of light by all-trans retinal, rhodopsin exists as an equilibrium mixture of an active conformation, metarhodopsin II (MII), and an inactive conformation, metarhodopsin I (MI). Each MII activates several hundred G_t molecules, which then activate the effector enzyme, a cGMP-specific phosphodiesterase (PDE). The activated PDE catalyzes the hydrolysis of cGMP, triggering closure of cGMP-gated Na^+/Ca^{2+} channels, leading to hyperpolarization of the rod outer segment (ROS) plasma membrane and the visual response (Fig 1).

In this study, we reconstituted rhodopsin, G_t, and PDE into large, unilamellar vesicles containing a range of n–3 polyunsaturated acyl chains and cholesterol. In addition, we purified ROS disk membranes from rats that had been raised on an n–3 fatty acid–deficient or n–3 fatty acid–adequate diet. Our results demonstrate that the degree of unsaturation in the acyl chain and the level of cholesterol in the membrane significantly affect MII formation, MII-G_t coupling efficiency and speed and PDE activity. Since the visual signaling system is the prototype member in the superfamily of G-protein–coupled signaling systems, our findings regarding the effect of lipid composition and cholesterol on receptor–G-protein coupling may well serve as a general demonstration of the modulation of cell signaling by membrane composition.

EXPERIMENTAL PROCEDURES

Sample Preparation

Bovine ROS were isolated from frozen retinas (James and Wanda Lawson, Lincoln, Neb),[19] and intact rod disk membranes were isolated from ROS by centrifugation on a Ficol step gradient.[20] For reconstitution in vesicles with defined lipid composition, rhodopsin was solubized in octylglucoside and purified on a Concanavilin A affinity column.[21] Phospholipids were purchased from Avanti Polar Lipids Inc (Alabaster, Ala), and their purity was ascertained by HPLC. Cholesterol was purchased from Calbiochem (La Jolla, Calif). All preparation of phospholipids was carried out in an argon-filled glove box and in thoroughly degassed buffers due to the susceptibility to oxidation of polyunsaturated phospholipids. Large unilamellar vesicles containing rhodopsin at a ratio of 1 rhodopsin to 100 phospholipids were prepared by means of the rapid dilution technique.[22] After dialysis to remove detergent, all vesicle preparations were suspended in pH 7.5 Tris basal salt (TBS) buffer consisting of 10 mmol/L TRIS, 60 mmol/L NaCl, 30 mmol/L KCl, 50 μmol/L DTPA. G_t was prepared from ROS as a hypotonic extract[23] and stored in pH 7.5 TBS buffer with 30% glycerol at −20°C for no longer than 2 weeks. The phospholipid, cholesterol, and rhodopsin content of each reconstituted vesicle preparation were determined by independent phosphate, cholesterol (Waco Chemicals USA, Inc, Richmond, Va), and ΔA_{500} assays, respectively.

Measurements

The MI-MII equilibrium in the absence and presence of G_t was measured in isotonic buffer at pH 7.5, using a series of equilibrium absorption spectra.[24] The amount of additional MII formed in the presence of G_t is proportional to the amount of MII·G_t complex formed, thus the MII·G_t binding constant was determined by a series of measurements with varying ratios of rhodopsin to G_t. Kinetics of both MII and MII·G_t formation were assessed by measuring the transient absorption at 380 nm, using a flash photolysis system constructed in the laboratory. The kinetic data were analyzed in terms of a microscopic photoreaction model, as previously described.[25] PDE activity was assayed by a continuous pH method[26] with minor modifications. Samples were preincubated with the pH electrode in a thermo-regulated 1-mL quartz cuvette and bleached by a light pulse that was attenuated with neutral density filters to obtain the desired bleaching level of rhodopsin. Data acquisition and flash were synchronized in such a way that a set of preflash data points were collected to serve as baseline.

RESULTS

The MI-MII equilibrium and MII association with G_t can be readily monitored through changes in the absorption spectra of these photointermediates. A summary of the effects of 22:6n–3 acyl chains and cholesterol on the MI-MII equilibrium and binding of G_t to MII is shown in Figure 2. In

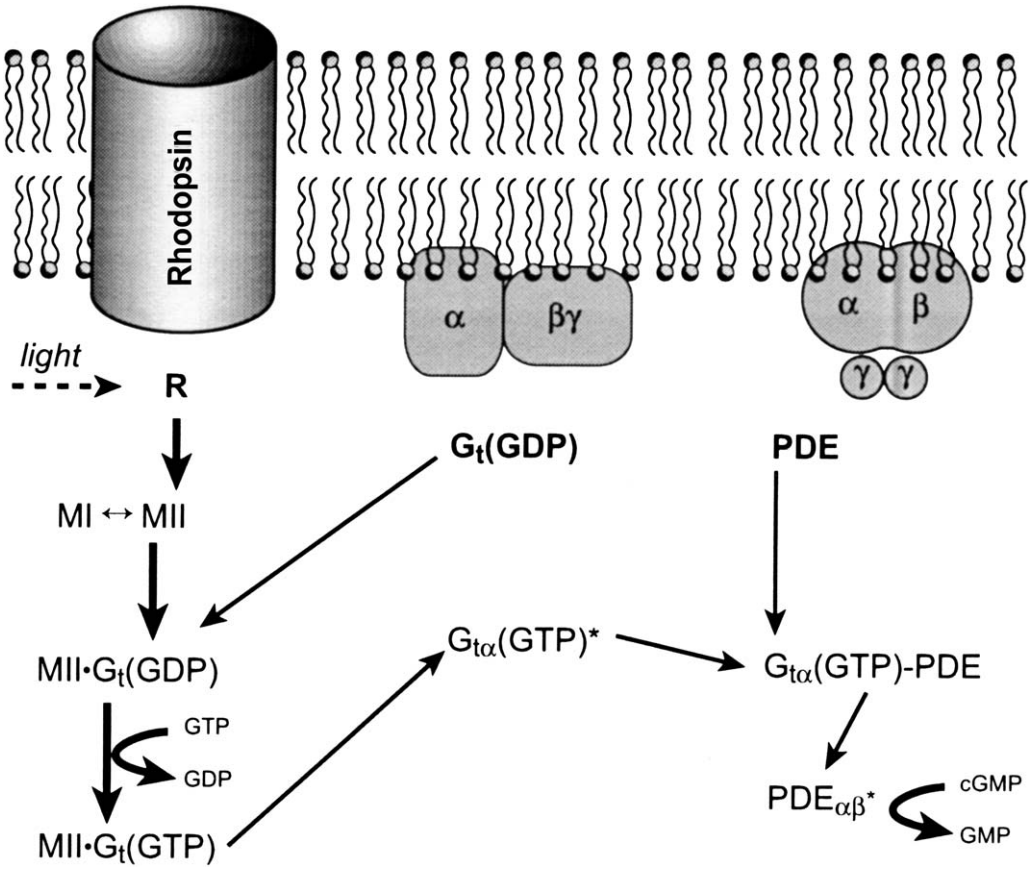

Fig 1. Visual signal transduction: The three proteins involved in the G-protein–coupled signal transduction of the visual system are shown in relation to the rod outer segment disk membrane. Rhodopsin is a transmembrane protein; the G protein, transducin or G_t, and the effector, cGMP phosphodiesterase or PDE, are bound to the membrane surface. Light converts rhodopsin to an equilibrium mixture of an inactive form, metarhodopsin I or MI, and an active form, metarhodopsin II or MII. MII binds and activates G_t (GDP) by catalyzing the exchange of bound GDP for GTP. G_t (GTP)* then dissociates and Gt_α(GTP)* binds to the inactive form of PDE. This complex dissociates to yield the active subunit complex $PDE_{\alpha\beta}$*, which hydrolysis cGMP. The lowered concentration of cGMP induces closure of cGMP-gated sodium channels in the plasma membrane, hyperpolarizing the cell.

the absence of G_t, K_{eq} followed the order di22:6PC > 18:0, 22:6PC > 18:0, 22:6PC + 30 mol% cholesterol ≈18:0, 18:1PC > 18:0, 18:1PC + 30 mol% cholesterol.[27] This is consistent with previous findings[28,29] that reduced acyl chain polyunsaturation and the presence of cholesterol reduce the amount of MII formation.

Membrane lipid composition and cholesterol also modulate the binding of MII to G_t, as shown by the data summarized in Figure 2, *B*.[27] Increasing acyl chain unsaturation from 18:0, 18:1PC to 18:0, 22:6PC resulted in a 3-fold enhancement in K_a. Further increase in unsaturation to di22:6PC resulted in a reduction in K_a relative to 18:0, 22:6PC. Cholesterol reduced K_a in both monounsaturated 18:0, 18:1PC, and highly unsaturated 18:0, 22:6PC. The K_a values in 18:0, 18:1PC + 30 mol% cholesterol, and 18:0, 22:6PC + 30 mol% cholesterol are 2.5×10^6 M^{-1} and 4.4×10^6 M^{-1}, respectively.

The time course of the formation of both MII and the MII·G complex after an activating flash was measured at physiological temperature for rhodopsin in membranes consisting of 18:0,22:6PC, and 18:0,18:1PC with and without 30 mol% cholesterol. Complete analysis of the kinetic data acquired at 37°C is summarized in Figure 3 and shows that cholesterol has very little effect on the kinetics of either MII or MII·G_t formation in a bilayer consisting of 18:0,22:6PC.[25] This is consistent with a number of measurements that show that cholesterol has its smallest effect on bilayer properties for bilayers containing 22:6n–3 acyl chains.[30] In contrast, in an 18:0,18:1PC bilayer, cholesterol increases the time required for MII formation by 50% and quadruples the time required for MII·G_t formation. Reducing *sn-2* acyl chain unsaturation from 22:6 to 18:1 doubles the time required for both MII formation and MII·G_t formation. In 18:0,22:6PC at 37°C, MII formation and MII·G_t complex formation are nearly coincident. Since MII cannot react with G_t any faster than it is formed from MI, the rate of formation of MII·G_t complex in 18:0,22:6PC appears to be maximal. In 18:0,22:6PC, the rate of MII·G_t complex formation, which is limited by lateral diffusion, is only slightly altered by the presence of 30 mol% cholesterol, further suggesting that 18:0,22:6PC has the optimum acyl chain composition for rapid formation of receptor–G-protein complex.

The results summarized in Figures 2 and 3 show the effects of 22:6n–3 phospholipid acyl chains and cholesterol on the two earliest events in G-protein–coupled signal transduction; formation of activated receptor and receptor–G-protein binding. The implications of these effects for the final, integrated cellular response can be illustrated by using the molecular model for the leading edge, or a-wave, of the retinal ERG response developed by Lamb and Pugh[31] and recently demonstrated to be accurate for in vitro assays of the type performed in this study.[32] The two dashed curves in Figure 4 show the results of applying the relative amounts of activated receptor and receptor–G-protein complex for rhodopsin in 18:0,22:6PC and 18:0,18:1PC/30 mol% cholesterol to the molecular model of the ERG response. An ERG trace obtained from a juvenile rhesus monkey (courtesy of Dr Brett Jeffrey and Dr Martha Neuringer) is also shown in Figure 4. The level of flash intensity used for the two calculated curves was matched to the flash intensity of the experimental ERG response. The response calculated for the 18:0,22:6PC membrane (circles) is remarkably similar to the a-wave of the experimental ERG trace, suggesting that the cellular response that would result from the flash intensity used in the in vitro system is similar to the timing of the response measured in vivo. In contrast, the response calculated for 18:0,18:1 PC/30 mol% cholesterol is significantly delayed. This delay is similar to the delay observed in the a-wave of the ERGs of n–3 deficient rhesus monkeys,[33] and the reduced amplitude of the response in the 18:0,18:1 PC/30 mol% cholesterol membrane is similar to the reduced a-wave amplitude observed in n–3–deficient guinea pigs.[34]

It is informative to directly measure the integrated response of the G-protein–coupled signaling pathway within the cell by measuring the activity of the PDE. In visual signal transduction, each MII that is formed activates several hundred G_t molecules, each of which activates a PDE. Thus, PDE activity is highly amplified relative to the initial stimulus and would be expected to be sensitive to any alteration in functional efficiency of the receptor and/or G protein. For this reason, we examined PDE activity in large unilamellar vesicles containing rhodopsin, G_t, and PDE reconstituted in three different PCs with small but significant variation in the composition of the polyunsaturated *sn-2* acyl chain. The three PCs all had an 18:0 acyl chain at the *sn-1* position and 22:6n–3, 22:5n–6, or 22:5n–3 acyl chains at the *sn-2* position. At a light stimulus level of 1 bleached rhodopsin per 20,000 rhodopsins, the PDE activity in the two n–3 containing membranes was twice as high as in the 22:5n–6 membrane, as shown in Figure 5.

The results presented above show in detail that G-protein–coupled signal transduction is sensitive to both extensive and subtle changes in membrane composition in reconstituted membranes. To determine the effects of changes in membrane composition resulting from in vivo processes, we examined MII formation and PDE activity in ROS disk membranes purified from Long-Evans rats fed diets that were either adequate or deficient in n–3 fatty acids (Niu, Mitchell, Lim, Wen, Kim, Salem, and Litman; in preparation). The

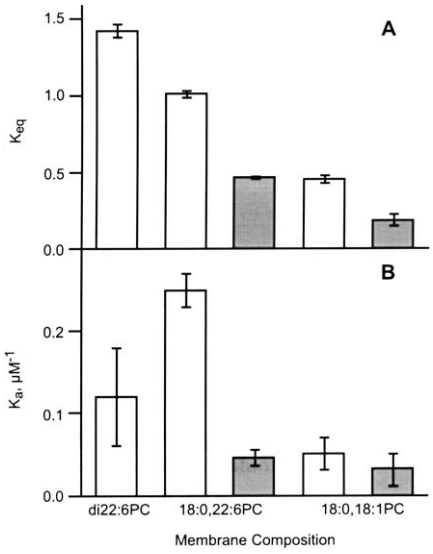

Fig 2. **A**, Effect of lipid composition on the MI-MII equilibrium constant, K_{eq}, at 20°C, pH 7.5. K_{eq} is defined as the ratio [MII]/[MI]. **B**, Effect of lipid composition on K_a of G_t binding to MII at 20°C, pH 7.5. Ka is defined as $[MII{\cdot}G_t]/[MII] \times [G_t]_{free}$. *Open bars* correspond to rhodopsin in vesicles consisting of the designated pure phospholipid; *crosshatched bars* correspond to rhodopsin in vesicles consisting of the designated phospholipid plus 30 mol% cholesterol.[27]

MI-MII equilibrium constant was about 15% higher in the membranes from the n–3–adequate animals, but the difference was not statistically significant. However, the membranes from the adequate animals produced a level of PDE activity that was nearly 3-fold higher than in membranes from the n–3–deficient animals, and the difference was statistically significant ($P < .001$; paired t test). A detailed analysis of the phospholipid species present in these two ROS disk membranes is underway, but a preliminary fatty acid analysis shows that about 80% of the 22:6n–3 in the ROS disk membranes of the n–3–adequate animals was replaced with 22:5n–6 in the n–3–deficient animals.

DISCUSSION

Previous studies showed that both decreased phospholipid acyl chain unsaturation and increased cholesterol concentration reduce the formation of MII through a mechanism linked to the specific packing properties of polyunsaturated acyl chains and the effect of cholesterol on these packing properties.[27,28,35] The results presented here demonstrate that the functional ramifications of polyunsaturated acyl chains extend beyond the unimolecular transition from MI to MII to the rapid and efficient coupling of receptor and G protein and to the functional efficacy of the effector. Current evidence indicates that $MII{\cdot}G_t$ interactions involve the three hydrophilic loops on the surface of rhodopsin with G_t, which means that the protein-protein interaction surfaces are external to the bilayer.[36] The sensitivity of the MII –G_t

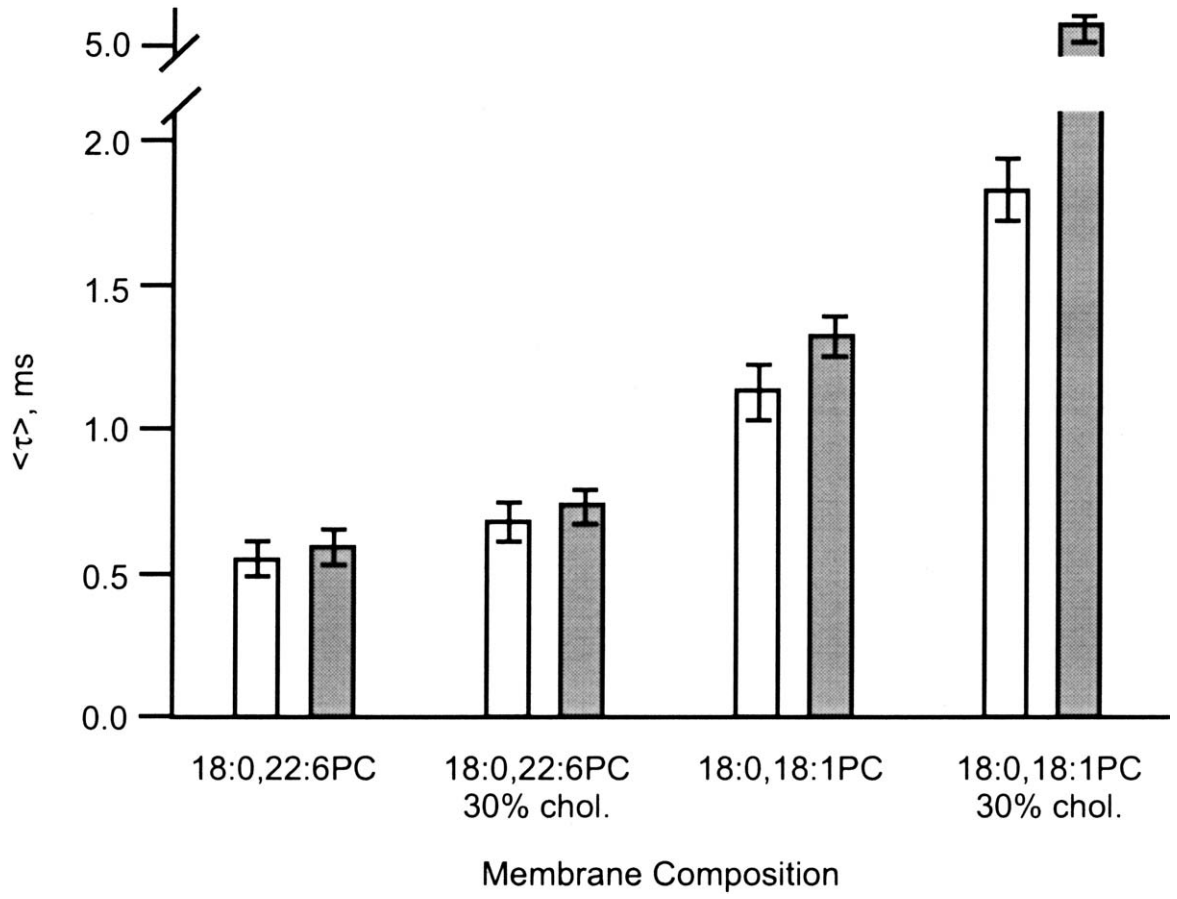

Fig 3. Summary of the effects of phospholipid acyl chain composition and bilayer cholesterol content on the average time constant (<τ>) for formation of MII and MII·G_t complex at 37°C. *Open bars* correspond to pure phospholipid membranes; *diagonal stripped bars* correspond to phospholipid/30 mol% cholesterol membranes. Processes that lead to formation of both the MII conformational state and the MII·G_t complex consist of multiple kinetic steps.[25] The average time constant is presented to facilitate comparison between the different membrane compositions of the overall time course of signal transduction.

binding interaction to membrane composition, Figure 2, *B*, and Figure 3 demonstrate that protein-protein interactions that occur on the hydrophilic surfaces of membrane proteins are affected by changes in membrane composition in the hydrophobic core of the membrane. This finding suggests for the first time that the protein-protein interactions, which occur on membrane surfaces in all forms of signal transduction, may be modulated by changes in the acyl chain composition of the membrane.

The alterations in receptor conformation change, receptor–G-protein–binding strength, and rate of receptor–G-protein complex formation summarized in Figures 2 and 3 probably underlie the changes in PDE activity shown in Figure 5. This might reasonably be expected, since the events measured in Figures 2 and 3 precede PDE activity in the G protein–mediated signaling cascade. In addition, the simulated cellular responses (Figure 4) demonstrate that a membrane with a level of 22:6n–3 or 22:5n–3 phospholipid acyl chain equivalent to that found in a normal, healthy rod cell produces a response similar to that recorded in vivo, whereas a membrane in which the n–3 polyunsaturate has been replaced by 22:5n–6 produces a much slower response.

The dependence of PDE activity on the presence of a polyunsaturated n–3 acyl chain at the *sn-2* position (Fig 4) clearly demonstrates that G-protein–coupled signaling is exquisitely sensitive to phospholipid acyl chain unsaturation. The reduced activity of PDE in 18:0,22:5n–6 PC compared with 18:0,22:6n–3 PC is a quantitative measure of the functional inequivalence of these two polyunsaturated phospholipid acyl chains. This comparison is crucial to our understanding of the biochemical basis for the effects of dietary n–3 deficiency because such deficiency generally leads to the replacement of 22:6n–3 with 22:5n–6.[1] The changes in MII formation and PDE activity in rod outer segments obtained from rats raised on n–3–adequate or n–3–deficient diet are essentially identical to the results obtained with reconstituted vesicle systems and isolated ROS disk membranes. Taken together, these results form a body of information that provides an understanding at the molecular level of the changes in ERG associated with dietary n–3 fatty acid deficiency in animals and nonhuman primates.

The results presented here suggest that the delays in ERG response observed in dietary n–3 deficiency[8,33,37] are at least partially due to reduced MII-G_t coupling efficiency and

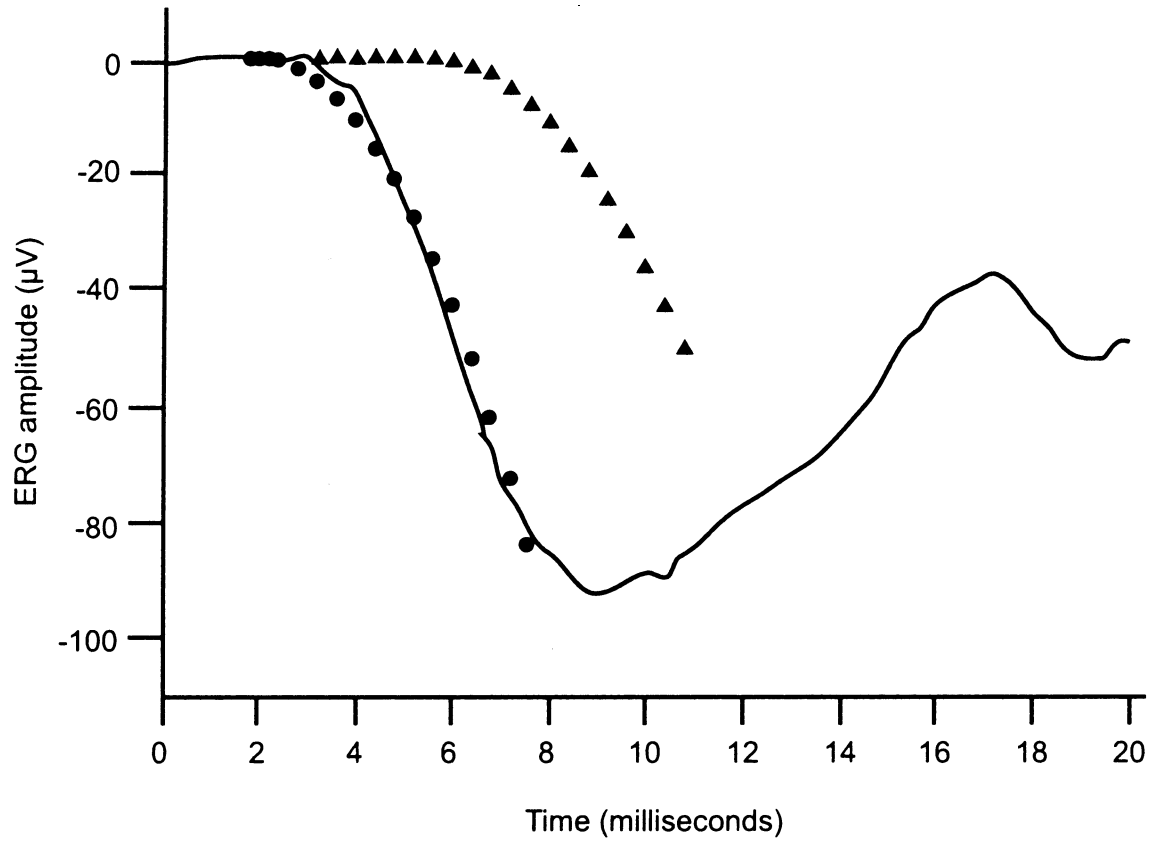

Fig 4. Implications of in vitro findings for in vivo retinal response. *Solid curve*, ERG measured in a rhesus monkey; *circles*, integrated cellular response calculated by using the molecular model of visual transduction and parameters for MII and MII·G_t complex formation determined for rhodopsin in 18:0,22:6 PC membrane; *Triangles*: integrated cellular response calculated using the molecular model of visual transduction and parameters for MII and MII·G_t complex formation determined for rhodopsin in 18:0,18:1 PC/30 mol% cholesterol membrane.

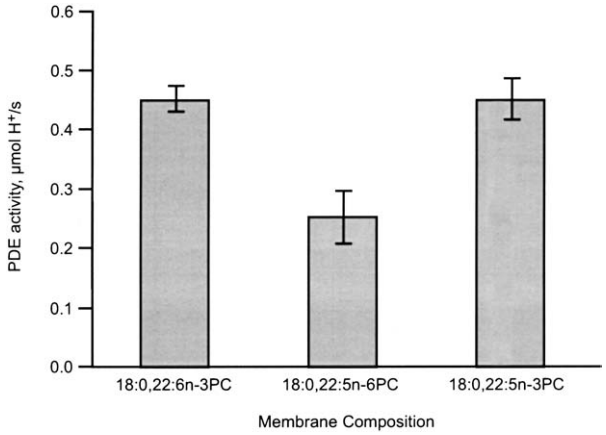

Fig 5. Comparison of activities of the effector enzyme PDE for rhodopsin, G_t, and PDE reconstituted in three membranes containing PCs with a 22-crabon, polyunsaturated *sn-2* acyl chain. Light intensity for this comparison was sufficient to bleach 1 rhodopsin per 20,000 rhodopsins, which is within the range of stimulus conditions associated with normal rod cell vision.

slower rate of MII·G_t formation when 22:6n–3 phospholipid acyl chains are replaced by 22:5n–6. Biochemical analysis shows that the rates of G_t activation and PDE catalytic subunit activation are approximately equal.[32] Assuming that the rates of G_t binding and G_t activation are similar, this means that a reduction in the rate of MII·G_t complex formation by 10% will delay the rod cell photoresponse by 5%. The results presented here indicate that the effects of membrane composition on the rate and efficiency of receptor–G-protein coupling lateral diffusion would be sufficient to account for the delays in photoreceptor activity observed in dietary n–3 deficiency.[8,33,37] Because of the similar signaling motif in other G-protein–coupled signaling systems, the findings presented here should be generally applicable to other members in the G-protein–coupled family, providing a molecular mechanism for the observed loss in cognitive skills,[6] odor,[9] and spatial discrimination[7] observed in n–3 fatty acid deficiency.

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