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The Role of Docosahexaenoic Acid Containing Phospholipids in Modulating G Protein-Coupled Signaling Pathways

Visual Transduction

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Abstract

In order to understand the role of the high levels of docosahexaenoic acid (DHA) in neuronal and retinal tissue, a study of the effect of membrane lipid composition on the visual pathway, a G protein-coupled system, was undertaken. The level of metarhodopsin II (MII) formation was determined to be a function of phospholipid acyl-chain unsaturation, with the highest levels seen in DHA-containing bilayers. Similarly, the rate of coupling of MII to the retinal G protein, G_t, to form a MII-G_t complex, was enhanced in DHA bilayers relative to less unsaturated phospholipids. Complex formation initiates the first stage of amplification in the visual pathway. The activation of the cGMP phosphodiesterase (PDE), the effector enzyme, represents the integrated pathway function. DHA-containing bilayers were found to support PDE levels comparable to those of the rod outer segment (ROS) disk membranes. Inclusion of 30 mol% cholesterol in the reconstituted bilayers had an inhibitory effect on each step in the visual pathway studied. Inclusion of cholesterol reduced MII formation and PDE activity and increased the lag time between the appearance of MII and the formation of the MII-G_t complex. However, signaling in DHA bilayers was far less affected by the addition of cholesterol than in bilayers containing less unsaturated phospholipids. These studies point up the importance of DHA acyl chains in promoting optimal function in G protein-coupled signaling pathways. The results reported here suggest that visual and cognitive deficits observed in n-3 deficiency may result from decreased efficiency in related neurotransmitter and visual signaling pathways in the absence of DHA.

Index Entries: Docosahexaenoic acid; DHA; G protein-coupled signaling; visual transduction; microdomains; receptor activation.

Introduction

Docosahexaenoic acid (DHA) is a major constituent of the membranes of neuronal and retinal tissue, representing about 50% of the acyl chain composition of the retinal rod outer segment (ROS) disk membrane (Salem, 1989; Stinson et al., 1991a). The high levels of this fatty acid suggest that DHA plays a critical structural role in modulating various membrane functions. Efforts to deplete the retina of DHA suggest a mechanism to retain this fatty acid in ROS disk membrane phospholipids (Stinson, 1991b; Bazan et al., 1993). There is a great deal of speculation in the literature as to the role of PUFAs in brain and retina function. Visual and cognitive deficits observed in animals maintained on an n-3 deficient diet are well-documented (for reviews, see Hamosh and Salem, 1998; Gibson, 1998). Studies carried out on nonhuman primates demonstrate that infants born to mothers raised on n-3 deficient diets have

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impaired visual function (Neuringer et al., 1984). This data is in good agreement with the results of studies on preterm infants, which have been raised on formula, formula supplemented with DHA, or breast-fed (Birch et al., 2000). The latter two groups performed significantly better than the formula-fed group in both cognitive and visual testing.

Recent studies suggest that there is a direct relationship between serotonin levels and membrane DHA content, whereby low DHA yields low serotonin, resulting in an individual being susceptible to depression or other affective diseases (Hibbeln and Salem, 1995). It is speculated that this relationship could arise through changes in membrane physical properties, induced by the depletion of DHA, which in turn influences the function of either serotonergic receptors or serotonin-reuptake systems. Studies of the light activation of rhodopsin, in a variety of bilayers of defined lipid composition, support this speculation (Mitchell and Litman, 1996, 1998). Maximal levels of metarhodopsin II, the conformation of light-activated rhodopsin, which binds and activates the ROS G protein, were observed in DHA containing bilayers.

The literature contains conflicting data with respect to the role of cholesterol in depression and suicide. It is reported that there is an inverse relationship between the ratio of total cholesterol to high-density lipoprotein cholesterol (TC/HDLC) and the level of plasma PUFA, suggesting that a high TC/HDLC ratio is a potential marker for low plasma PUFA and an indicator of low levels of tissue PUFA in the body (Siguel, 1996). Thus, correlations generally made with variations in cholesterol ratio might better be correlated with plasma PUFA levels. There is an ongoing discussion relative to the association of total serum cholesterol levels and the rate of suicide and depression. Several reports have cited a correlation between low serum cholesterol and both suicide and violent behavior (Muldoon et al., 1990; Engelberg, 1992), while other investigators have reported a positive correlation between serum cholesterol and suicide and violent behavior. A recent report indicates that the ratio of violent to nonviolent suicide rates correlate directly with total serum cholesterol, so this suicide ratio may be a better correlative parameter with cholesterol levels than the overall suicide rate (Tanskanen et al., 2000).

The controversy over the effects of cholesterol on behavior, the linkage between cholesterol and PUFA levels in the serum, and the nature of the functional deficits associated with DHA-deficient diets, highlight the importance of understanding how acyl-chain and cholesterol composition affect membrane physical properties and how these changes influence signaling processes localized in these membranes. In this manuscript, the effect of varying phospholipid acyl-chain composition and cholesterol on various steps in the visual transduction-signaling pathway is discussed. This system is a prototypical G protein-coupled receptor pathway and rhodopsin is the best-characterized member of this superfamily. Because many neurotransmitter receptors, as well as the olfactory and taste receptors, are members of this superfamily, the observations made for the vision system should form a basis for understanding these related signaling systems.

Materials and Methods

Sample Preparation

Rhodopsin from frozen bovine retinas was solubilized in octylglucoside and purified using concanavalin affinity chromatography (Litman, 1982). All phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL), and their purity was verified by HPLC. Purified rhodopsin was reconstituted into large unilamellar vesicles using a dilution reconstitution procedure at a lipid to protein ratio of 100 : 1 (Jackson and Litman, 1985). All procedures were carried out under argon in a glove box to minimize oxidation of the polyunsaturated acyl chains and using either red light or dark conditions.

PDE Activity Measurements

The activity of the cGMP-specific phosphodiesterase (PDE) of the ROS was assayed using the continuous pH method of Yee and Liebman (1978) with the following modifications. A semi-micro electrode equipped with temperature compensation was used to monitor the pH change resulting from the hydrolysis of cGMP by PDE. Data was collected using a high-speed acquisition board installed in a personal computer. Samples were preincubated with the pH electrode in a thermo-regulated 1-mL quartz cuvet and bleached by a light pulse generated from a high-output flash lamp attenuated with neutral density filters to obtain the desired bleaching level of rhodopsin. The data acquisition and flash were synchronized in such a way that a pre-flash baseline as well as post-flash data was collected for each flash. Measurements were carried out at 25°C.

Measurements of MII and MII•G_t Kinetics

Flash photolysis samples were matched aliquots of rhodopsin-containing vesicles with and without

G_t. Absorbance changes, induced by a photolyzing flash, were monitored using a flash photolysis instrument constructed in our laboratory. The intensity of a very dim light beam at 380 nm, the wavelength of maximum absorbance of both MII and MII•G_t, was monitored with a cooled photomultiplier tube. Signals from this photomultiplier tube were digitized at a rate of 3–5 μs per point by a high-speed data acquisition board installed in a personal computer. Up to 500,000 points were acquired in a single trace and 5–15 traces were averaged to improve the signal-to-noise ratio. Included in each file was the preflash baseline, which was used to convert the flash-induced change in transmission into the change in absorbance. The kinetics of meta II formation were determined by analyzing the absorbance change at 380 nm in G_t-free samples in terms of a specific photoreaction model for rhodopsin (Thorgeirsson et al., 1993). The kinetics of MII•G_t complex formation was modeled as the sum of two exponential processes. The rates and associated amplitudes of these processes were derived using a nonlinear least-squares routine, after fixing the rate constants for MII formation at the values determined in a matched, G_t-free sample.

Results

The initial step in the visual pathway is the light-induced formation of MII, the form of rhodopsin that binds and activates G_t. This receptor-conformation change was found to be sensitive to both membrane acyl-chain composition and cholesterol content. The level of MII formation increased with increasing acyl-chain unsaturation (Litman and Mitchell, 1996). In the case of both mixed-chain and symmetrically substituted PC's, the greatest level MII was formed in DHA-containing systems (Fig. 1). In the presence of 30 mol% cholesterol, MII formation was diminished in all lipid systems (Fig. 2) (Mitchell and Litman, 1998). However, DHA-containing PC species once again supported the highest levels of MII formation, demonstrating the ability of these phospholipids to partially buffer the inhibitory effects of cholesterol.

The next step in the visual pathway is the formation of a MII-G_t complex; this leads to the first stage of signal amplification in the pathway. In the absence of guanosine triphosphate (GTP), this complex is long-lived. In this study, the kinetics of the appearance of both MII and the MII-G_t complex were measured. A characterizing feature of the coupling of MII to G_t is the ratio of the rates of formation of MII and MII-G_t complex. The ratio of these rates is indicative of the delay time from the formation of MII to its having a fruitful interaction with G_t, i.e., the subsequent formation of a MII-G_t complex. The ratio of the rates varied from 1.39 in native disk membranes to 4.95 in 18:0, 18:1 PC bilayers, while in 18:0,22:6 PC bilayers, it had an intermediate value of 3.46 (Table 1). The addition of 30mol% cholesterol to these bilayers had a dramatic effect in 18:0,18:1 PC bilayers, increasing the ratio of the rates by 83% to 9.07,

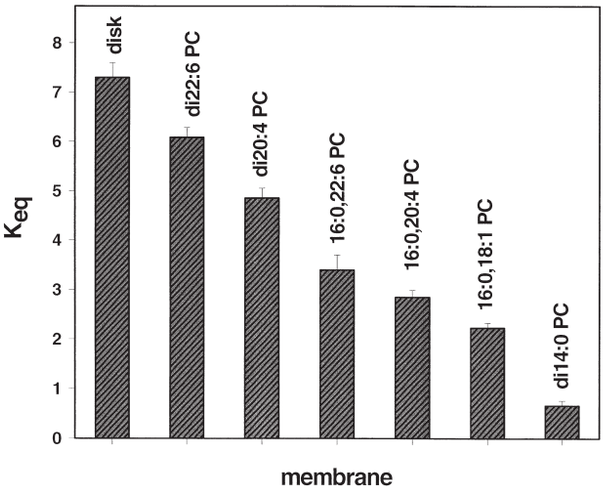

Fig. 1. The compositional dependence of the MI↔MII equilibrium constant, K_{eq} determined for rhodopsin in a compositionally defined bilayers varying in degree of unsaturation at 37°C. Adapted with permission from Litman and Mitchell, 1996.

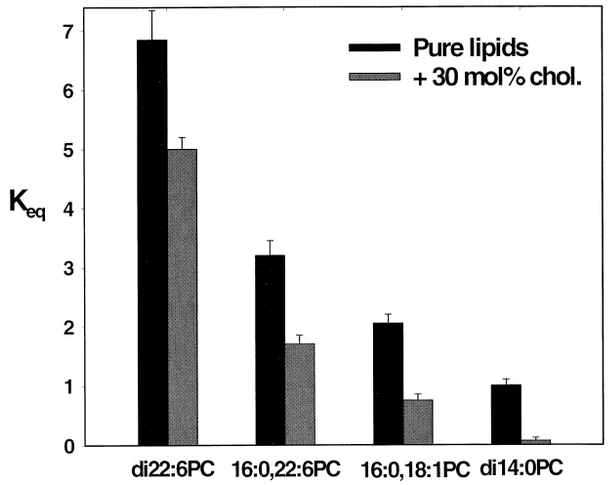

Fig. 2. The effect of adding 30 mol% cholesterol to several compositionally defined bilayers on the MI↔MII equilibrium constant, K_{eq} at 37°C. Adapted with permission from Mitchell and Litman, 1998.

Table 1
Ratio of the Formation Rate of MII-G Complex to MIII[a]

Membrane	$k_{MII\text{-}Gt}/k_{MII}$
Disks	1.4
18:0,22:6 PC	3.5
18:0,22:6 PC + 30 MOL% CH	2.8
18:0,18:1 PC	4.9
18:0,18:1 PC + 30 MOL% CH	9.1

[a]Measurements made at 37°C.

Table 2
PDE Activity Dependence on ACYL-Chain Composition[a]

	Percent Bleached	
Membrane	0.10	100
ROS Disks	87%	100%
16:0,22:6 PC	59%	97%
16:0,18:1 PC	26%	50%

[a]PDE activity is measured in as the percent of maximal activity obtained with native disk membranes. Measurements made at 25°C.

whereas the ratio was reduced by about 20% in 18:0, 22:6 PC bilayers.

The integrated signal in the visual pathway is represented by the activity of the PDE. In these studies, the light-stimulated activity of the PDE was measured in reconstituted system formed from G_t, PDE, and rhodopsin containing unilamellar vesicles of defined lipid composition. Dose-response curves were obtained by measuring the level of PDE activity as a function of the concentration of light activated rhodopsin. Each rhodopsin absorbing a photon is analogous to an agonist-activated receptor. Rhodopsin, at a level where only 1 in 1000 molecules are light-activated, in 16:0, 22:6 PC bilayers produce 59% of the activity obtained in disks, whereas in 16:0,18:1 PC bilayers this level of rhodopsin activation yielded only 26% of the disk activity (Table 2). Under saturation conditions, yielding maximum response, rhodopsin in 16:0, 22:6 PC vesicles yielded 97% of the activity of native disk membranes, whereas in 16:0,18:1 PC vesicles rhodopsin produced only 50% of the disk activity. Once again the superiority of the DHA-containing bilayers in supporting activity in the visual pathway was demonstrated.

Studies were also undertaken to determine if DHA-containing bilayers were associated with the formation of lateral domains. This was accomplished by the use of acyl chain-specific fluorescent probes in the form of di22:6 PE-pyrene and di16:0 PE-pyrene. The overlap of pyrene fluorescence emission and the absorption spectrum of rhodopsin make this an efficient energy-transfer pair. In these experiments, a mixture of rhodopsin reconstituted into bilayers of di22:6 PC, di16:0 PC, and cholesterol was employed to examine the affinity of rhodopsin for polyunsaturated acyl chains, relative to saturated acyl chains. The lateral organization of di16:0 PC or di22:6 PC relative to rhodopsin can be determined by measuring the efficiency of the energy transfer of the

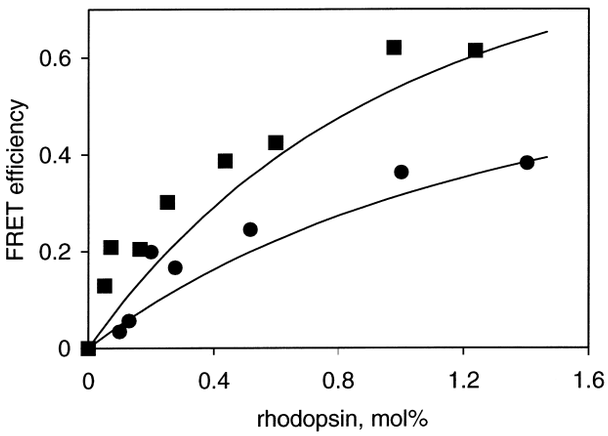

Fig. 3. PE-pyrene-rhodopsin fluorescence resonance energy-transfer (FRET) efficiency. Dark-adapted rhodopsin in bilayers formed of 3:7:3 di22:6 PC/di16:0 PC/cholesterol with 1% of either di22:6 PE-pyrene (■) or di16:0 PE-pyrene (•). Measurements were made at 50°C, which is above the phase transition of di16:0 PC. Solid curves are derived from a theoretical calculation for the FRET efficiency for a cluster model. Adapted with permission from Polozova and Litman, 2000.

pyrene probes to rhodopsin. Enhanced energy-transfer efficiency means that the probe has a high probability of being close to rhodopsin. A higher energy transfer efficiency was observed for the di22:6 PE-pyrene probe than for the di16:0 PE-pyrene probe (Fig. 3) (Polozova and Litman, 2000). This finding demonstrates that there is a much higher probability of the di22:6 PC phospholipid being in close proximity to rhodopsin than the di16:0 PC. In the system represented in Figure 3, there is a sixfold enhancement of di22:6 PC around rhodopsin, relative to its bulk concentration in the bilayer. The formation of this microdomain of di22:6 PC around

rhodopsin required the presence of both cholesterol and rhodopsin, indicating that rhodopsin had a specific preference for the DHA containing PC.

Discussion

The essentiality of DHA for the development of the nervous system and retina is clear from a variety of investigations. In the studies reported here, several steps in the visual pathway, which is a prototypical G protein-coupled receptor system, were shown to be optimized in bilayers containing DHA phospholipids. Our previous findings demonstrated that the highest levels of MII formation in reconstituted systems occurred in DHA-containing bilayers (Litman and Mitchell, 1996; Mitchell and Litman, 1998). Subsequent measurements show that both the kinetics and the level of MII-G_t complex formation are most favorable in DHA-containing bilayers. In particular, the ratio of the rates of MII and MII-G_t complex formation show that in the native disks, MII and G_t interact to form a complex rapidly upon MII formation, making the transfer of the signal along the pathway quite efficient, the ratio of the rates being 1.39. As the bilayer acyl chains become less unsaturated this process becomes delayed. Thus in 18:0,18:1 PC, the ratio of the rates of MII an MII-G_t complex formation increases to 4.95.

An important aspect of DHA-containing bilayers is their ability to buffer the effects of cholesterol. Thus the ratio of rates in the 18:0,22:6 PC bilayer is decreased by about 20% in the presence of 30 mol% cholesterol, whereas this level of cholesterol increased the ratio by about 83% in the 18:0,18:1 PC bilayers, resulting in about a ninefold increase in lag time in complex formation relative to native disk membranes. Complex formation requires that MII and G_t find each other through lateral diffusion in the surface of the membrane. Our findings suggest that the diffusion process is dramatically slowed in the 18:0,18:1 PC plus 30 mol% cholesterol bilayers, whereas there is relatively little effect of cholesterol in a 18:0,22:6 PC plus 30 mol% cholesterol bilayer. This delay in the coupling of MII with the G_t will decrease the response time of signaling along the pathway. In other experiments, we have observed a reduction in the binding affinity of MII to G_t (Niu, Mitchell and Litman, unpublished results). This will greatly reduce signal amplification along the pathway. Although there is a large literature showing that cholesterol affects the order of phospholipid acyl chains, the data presented here represents the first observation to explicitly show that DHA-containing phospholipids can buffer the inhibitory effects of cholesterol in a signaling pathway.

The aforementioned findings are in good agreement with measurements of the PDE activity, which measures the integrated pathway function. Here again, the system properties are optimized in DHA-containing bilayers. The presence of lateral domains in di22:6 PC bilayers demonstrates an additional mechanism whereby DHA-containing bilayers can enhance signaling processes. If, in addition to rhodopsin, G_t and PDE also show preferential partitioning into regions rich in DHA, then lateral domain formation will increase the efficiency of association of these proteins by reducing the diffusion pathway for their interaction and increasing their effective concentration in the region of the microdomains.

Our earlier studies showed that the enhanced formation of MII correlated very well with acyl chain packing free volume, as measured by time-resolved fluorescence anisotropy decay of a fluorescent probe (Mitchell et al., 1992). Given the requirement of diffusion for association of both MII to G_t and for the alpha subunit of G_t to PDE, these packing properties are likely responsible for the more efficient kinetics of MII-G_t complex formation in DHA-containing bilayers. In addition, these studies demonstrate that in the presence of DHA acyl chains, the inhibitory effects of cholesterol on significant biological functions are greatly reduced. These findings may help explain some of the observations in psychological disorders that appear to be correlated with variable levels of cholesterol. In the presence of reduced levels of DHA, associated with n-3 deficiency, and an increased cholesterol level, our results would predict a reduced sensitivity in G protein-coupled receptor signaling. This would reduce the effectiveness of serotonin or other associated neurotransmitters.

In summary, the formation of the active receptor conformation MII and its coupling to the G_t, are greatly enhanced in DHA-containing lipid bilayers, as is the overall pathway function, as measured by the PDE activity. These findings are in excellent agreement with observations in retinal electroretinogram (ERG) measurements made on n-3 deficient animals, where a reduced signal amplitude and a lag time in signal development are observed. These results are predicted by our findings that there is an increased lag time in the MII-G_t coupling and reduced formation of MII and its association with G_t protein in less unsaturated lipids. The former can

explain the lag time in the ERG, while the latter can explain the reduced signal amplitude. Generalization of the findings in the visual system to other G protein-coupled receptor pathways is supported by studies evaluating olfactory discrimination of rats raised on either an n-3 deficient or n-3 adequate diet. The n-3 deficient group made more errors in odor-discrimination tests than the n-3 adequate group (Greiner et al., 1999). Acyl-chain analysis of the olfactory bulb showed an 82% decrease in DHA relative to rats raised on an n-3 adequate diet. The olfactory-signaling system is also a G protein-coupled receptor pathway and its function appears to be less sensitive in DHA deficient animals, as is the visual signaling pathway.

The studies reported here provide insight into the functional role played by DHA-containing phospholipids in optimizing membrane-associated signaling systems. Given the similarities between the visual pathway and other G protein-coupled systems, as well as the results obtained in studies of odor discrimination in n-3 deficient rats (Greiner et al., 1999), the sensitivity of G protein-coupled receptor systems to levels of DHA in membrane phospholipids should be generally observed and likely contributes to an explanation of the deficiencies in cognitive processes observed in n-3 deficiency.

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