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Raman Spectroscopic Imaging of Cholesterol and Docosahexaenoic Acid Distribution in the Retinal Rod Outer Segment

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Abstract

Raman vibrational spectroscopic imaging was performed on retinal rod cells isolated from bullfrogs (*Rana catesbeiana*). The Raman spectra enable determination of the lipid and protein rich rod outer segment (ROS) from the nucleus and inner segment of the cell. Peak fitting analysis of spectra obtained from individual rod photoreceptor cells show characteristic vibrational modes that can be associated with cholesterol and docosahexaenoic acid containing lipids. These results provide direct observations of biomolecular gradients in the rod photoreceptor cells, which, thus far, have been based on indirect detergent extracts and histochemical analysis with indicators such as filipin. The detected biomolecules are associated with regulation of the integral membrane protein rhodopsin, and methods capable direct observation of these biomolecules offer new routes to exploring their role in the regulation of cellular processes.

Introduction

Cellular membranes are comprised of lipids and proteins that act to contain and control biological processes. While proteins are generally regarded as the work-horse machinery, the lipid environment plays an important role in regulating the activity and function of the embedded proteins. For example, it is also well known that reconstitution of membrane proteins in bilayer assemblies is dependent on the lipid composition of the model membrane. (1) Whether this represents specific physical-chemical interactions or a more general mechanical effect often must be determined for individual systems. The composition of the lipids that make up cellular membranes have profound effects on the mechanical properties, such as the area stretch modulus(2–4) and the related compressibility,(5–9) as well as the thickness,(10) and fluidity of the membrane.(7, 11, 12) Indeed hydrophobic matching has been shown to alter the conformational mobility of transmembrane proteins.(13, 14) Additionally, specific chemical interactions, such as hydrogen bonding, can alter the stability of membrane proteins.(15–17) While model systems have been useful in understanding the role of lipids in regulating protein activity, translation of these results to real systems requires detection and characterization of the lipid environments in actual cellular systems.

The rod photoreceptor provides a useful cellular system for studying the impact of the lipid environment on the function of embedded proteins. The outer segment of the rod cell (ROS) contains the principal photoreceptor rhodopsin and the retinal chromophore. When light is incident on the rod assembly, the retinal undergoes photo-isomerization and the rhodopsin protein changes conformation initiating the signaling cascade that results in closing ion channels in the plasma membrane, hyperpolarizing the cell, and thereby transmitting the detected photon to the brain, where the signal is perceived as light in the vision process.(18) Rhodopsin, an archetypal G-protein coupled receptor, and the photo-transducer in retinal

rod cells, shows a strong structural dependence to its lipid environment. When activated by a photon, the isomerization of the 11-cis retinal chromophore to the all-transretinal, the metarhodopsin I to metarhodopsin II transition, results in an expansion of the protein, the rate of which is modulated by the mechanical properties of the lipid matrix (19–21). Additionally hydrogen bonds between cholesterol and rhodopsin have also been shown to alter the protein's stability.(15, 17) It is hypothesized on the basis of detergent extractions that a cholesterol gradient exists in the ROS. (22) Both the role played by this cholesterol and the mechanism by which the gradient forms is uncertain. Additionally, on the basis of the cholesterol gradient, an opposing gradient in unsaturated lipid acyl chains has also been suggested.(23) Maintaining the appropriate balance of cholesterol and unsaturated acyl chains has been shown to be important for proper function of rod photoreceptor cells.(24)

An important challenge is clarifying the important roles of these lipid microenvironments in modulating proteins within cellular membranes.(8) Cholesterol has been shown to have a condensation effect on lipid bilayers, which alters numerous characteristics of cellular membranes.(25–27) Unsaturated lipids are characterized by increased fluidity; however they typically phase separate from cholesterol rich regions.(28–30). The ability of the lipid matrix to modulate protein behavior reflects the lipids' ability to compensate for changes in protein conformation, as for example, in the case of rhodopsin adjusting to the volume change accompanying the photo-isomerization of the retinal chromophore. Membranes containing both cholesterol and the poly-unsaturated docosahexaenoic acid containing lipids have been shown to impact the stability of embedded rhodopsin molecules.(31) Additionally, phenotypes that are deficient in polyunsaturated acids and cholesterol have been linked to visual disfunction.(32) (22) Given the importance of these species in regulating rhodopsin, increased understanding of the distribution of these lipids in intact cells may provide further insight into role of lipid environments that regulate protein function.

Significant effort has gone into the detection of lipid environments,(29) such as microdomains and lipid rafts.(33, 34) These characterizations have been performed using mass spectrometry,(35) atomic force microscopy,(36–38) and also optical spectroscopic imaging.(38–42) Lipids are particularly adept to study by Raman spectroscopic methods.(43–51) Recent nonlinear Raman imaging work has taken advantage of the strong Raman cross-section of lipids for studying lipid droplets in cells.(52, 53) Of import, Raman allows analysis in a label free manner, avoiding artifacts associated with the incorporation of reporter molecules. Raman imaging provides a direct method to detect the spatial heterogeneity of these biomolecules, which will be key to understanding these processes. While cholesterol has a low Raman cross-section making it difficult to detect, deconvolution of the spatially resolved spectra enable mapping the distribution of this and other bioanalytes.

The results presented here demonstrate Raman spectroscopic imaging as a label-free method for detecting lipid distributions in the retinal rod cell, particularly in the outer segment.

Results and Discussion

Rod photoreceptor cells isolated from bullfrogs were isolated and examined by Raman spectroscopic imaging. As shown in figure 1, cells can be obtained that have the cell body containing the nucleus, the inner segment, and ROS still intact. In figure 1, the Raman spectra of the respective regions of the cell are plotted. To generate the representative spectra, the entire cell was imaged and the pixels in the region of interest (ROI) were binned to obtain an average spectrum for the ROI. The measured Raman spectra enable discrimination of different regions of the rod cell. The principal bands observed in the spectra corresponding to the nucleus and inner segment correspond to phosphate (1000–

1100 cm^{-1}) and C-H deformations (1450 cm^{-1}). A broad feature located near 1350 cm^{-1} is observed in all spectra, including spectra obtained away from the cell and is attributed to the substrate. Other low intensity bands observed in the nucleus and inner segment, such as the phenylalanine band (1000 cm^{-1}) are consistent with proteins and nucleic acid molecules expected to be observed in cells.(45, 54) The ROS shows a much richer Raman spectrum as seen in Figure 1D. This is expected given the high density of lipids and proteins that comprise the disk membranes that are contained within the outer segment. The outer segment is composed of 100's of individual lipid disk membranes embedded with rhodopsin that are stacked along the length of the cell. The approximate ratio of lipids to protein in these disks is reported to be between 100:1 and 50:1. (32, 55, 56) A Raman spectrum of a lipid standard containing saturated acyl chains, cholesterol, docosahexaenoyl acyl groups, and phosphatidylcholine (PC) headgroups can qualitatively reproduce many of the bands observed in the average Raman spectrum of the outer segment (Fig 1E). The greater abundance and higher Raman cross section of lipids relative to proteins allows us to assign prominent bands observed in the ROS spectrum. The prominent peak at 1655 cm^{-1} is attributed to the large proportion of docosahexanoic acid containing lipids found in the disc membranes. Similarly, the distinct stretch at 715 cm^{-1} corresponds to the tetrahedral nitrogen present in the PC headgroup. In the lipid model, this choline stretch appears more intense relative to the other lipid bands observed in the ROS. This is due to the lipids in the ROS containing only 45% PC headgroups. The other dominant headgroup found in the ROS is phosphatidyl ethanolamine (42%), (57) which has no spectral feature in the spectral region associated with the choline moiety.

Figure 2 shows the Raman image of an isolated rod cell where the heat map corresponds to the intensity at 1655 cm^{-1} . The spectra obtained from the ROIs depicted are shown in Figure 2b. As evidenced in the map, the raw intensity at a single frequency can be misleading regarding the relative composition of a peak in the vibrational spectrum. Autofluorescence variations generate a variable background that necessitates higher-level spectral analysis to discriminate the vibrational contribution to the observed intensity. The 785 nm laser excitation minimizes the fluorescent background arising from cofactor and flavin molecules. As shown in Figure 2b, the Raman scattering is still detected in the presence of the autofluorescence. By binning over regions of the cell, the spectra can be analyzed with peak fitting to determine the contribution of specific Raman modes to the observed signal. The vibrational mode at 1655 cm^{-1} being characteristic of the docosahexanoic acyl chains, 1450 cm^{-1} representing methyl and methylene deformations (a common approximation of total organic content), 1000 cm^{-1} characteristic of phenylalanine found in proteins, and the aforementioned choline stretch at 715 cm^{-1} .

Polyunsaturated lipids show distinct Raman modes at 1655 cm^{-1} and 1265 cm^{-1} . The trend in the polyunsaturated lipid bands, shown in figure 3, supports an increased concentration at the terminus farthest from the cell body. In figure 3 the peak areas of the reported bands, at various points along the rod cell, were determined by fitting a Lorentz lineshape to the average spectrum of the ROIs plotted in figure 2. The area under the Lorentzian curve associated with the individual vibrational modes was then ratioed. To further compensate for possible variation in the focal volume, the band area was compared to the area of the 1450 cm^{-1} band and to the 715 cm^{-1} choline stretch. The 715 cm^{-1} represents C-N vibrations unique to the choline headgroup. This tetrahedral coordination increases the Raman activity of the mode, enabling determination of choline independently of C-H content. Control experiments involving ethanolamine headgroups, which has N-H bonds in the place of N-CH₃, show no intensity at 715 cm^{-1} due to the absence of the tetrahedral symmetry around the nitrogen atom. In the ROS, an increase in polyunsaturated lipids is in fact observed. Interestingly, the magnitude of the increase is dependent upon the reference vibration band. The choline stretch at 715 cm^{-1} corresponds to phosphatidylcholine lipids, which are

reported to have a uniform distribution along the ROS;(23) however, the 1450 cm^{-1} band is characteristic of both lipids and proteins suggesting an increase in protein content along the ROS. An interesting observation, the relative concentration of docosahexanoic acid in the inner segment is quite high, although direct inspection of the spectra in this region suggests this represents lower total concentrations of the reference bands, which is consistent with decreased chemical concentrations in these segments.

Figure 2 clearly demonstrates that Raman microspectroscopy is capable of detection of biomolecule gradients along the rod photoreceptor. The Raman results provide direct evidence of the polyunsaturated lipid gradient. Previously this gradient was derived from the assumption of a cholesterol gradient in the ROS.(17, 22) The micrometer spatial resolution of Raman spectroscopy can enhance the discrimination of cell compartmentalization, but identification of specific biomolecules may be complicated by the low Raman cross section of, for example, cholesterol. However, close inspection reveals a distinct Raman active cholesterol band at 695 cm^{-1} can be deconvolved from other competing modes in the vibrational spectrum. In figure 4, Raman spectra obtained from different spatial locations along the cell show a decreasing cholesterol contribution as a shoulder on the 715 cm^{-1} choline peak. A peak fitting analysis of the cholesterol and choline vibrational features, indeed, shows decreasing cholesterol concentration as one scans from the proximal to the apical end of the ROS.

To quantitate the relative concentration of cholesterol in the ROS, a model system comprised of aqueous multilamellar vesicles consisting of phosphocholine lipids with varying cholesterol concentration was examined, as shown in figure 5. Increasing cholesterol concentration clearly increased the intensity of the 695 cm^{-1} peak evident as a shoulder on the choline band. At 10 % cholesterol the shoulder becomes visible on the choline stretch, and at 50% cholesterol the peak is distinctly resolved. This model suggests the relative concentration of cholesterol in the ROS varies for approximately 20–30% to less than 10% consistent with previous results indicating a change from 30% to 5%.(22) The ability to detect this cholesterol gradient confirms the signals are arising from the disk membranes as opposed to the plasma membrane, where the cholesterol concentration is reported to remain high and constant along the length of the ROS.(22)

It has been well documented that the lipid bilayer matrix affects the function of membrane proteins.(58) More recently, the concentration of cholesterol and docosahexanoic acid (DHA) containing lipids have been shown specifically to affect the properties of rhodopsin from retinal rod cells.(31) In this recent study, rhodopsin reconstituted into artificial lipid assemblies, containing varying concentrations of these two biomolecules, exhibited changes in its denaturation temperature dependent upon the bilayer composition. Increased cholesterol appeared to stabilize the protein. Additionally, increased cholesterol decreased the kinetics of rhodopsin activation in the presence of DHA.(31) The lipid composition of the bilayer also affects the lateral mobility of embedded proteins that has also been reported to impact rod cell activity.(56) Our Raman results thus allow determination of phospholipid composition in intact cells, which will facilitate studies of rhodopsin activity in intact cellular membranes.

The gradients detected provide direct measurements of previous results that indicated lipids are trafficked between disc membranes in the ROS. Docosahexanoic acid, and omega-3 fatty acid, cannot be synthesized by most animals and is commonly obtained from dietary sources. The synthesis and renewal of disc membranes is reported to efficiently conserve the fatty acids in the ROS and recycle them back to the inner segment to produce new disc membranes.(22) Similarly, the amount of cholesterol found in these cells also seems to take advantage of these lipid-recycling mechanisms. Although cholesterol can be synthesized

within the body, the levels necessary for maintaining the disc membranes are substantial. Since the higher-level organisms are known to be able to regulate cholesterol, the cholesterol gradient may be responsible for establishing the gradient detected in docosahexaenoyl containing lipids. The relative insolubility of cholesterol in poly-unsaturated lipids may, thus, promote localization of the poly-unsaturated lipids toward the apical terminus of the ROS. Thus, the label-free, non-invasive, nature of Raman spectroscopy may offer a new route to characterizing these phenomena.

Conclusions

Raman vibrational spectroscopic measurements provide direct observation of biomolecular gradients, which thus far has been based on indirect detergent extracts and histochemical analysis with indicators such as filipin. Confirmation of these gradients raises questions about the regulation of the cholesterol, the origin of the increased poly-unsaturated lipids, the mechanism controlling these gradients, and their role in the vision cascade. Increased understanding of the lipid functions in retinal rod cells will be beneficial for alleviating visual impairment associated with rod cell dysfunction. Raman spectroscopic imaging results presented here demonstrate the potential for Raman to study the processes by which cholesterol and unsaturated lipids are sorted in the ROS as well as for the study of biomolecule distributions in other cellular systems.

Experimental

Isolation of Rod Outer Segments

Rod cells are isolated from eyes of bullfrogs (*Rana catesbiana*). The eyes are excised from frogs, which have been euthanized immediately prior to dissection in accordance with approved methods.⁽⁵⁹⁾ Live frogs are necessary to minimize retinal decomposition. The eyeball is cut along the limbus to remove the lens and vitreous body to permit access to the retina. The optic nerve is severed, and the retina is peeled away from the pigment epithelium. To aid in the identification of the orientation of the ROS, thin slices of retina are isolated. Filter paper is used to fix one side of the retina, and a thin slice is then made. The retinal tissue slices are dispersed into a small volume of frog ringers solution on freshly cleaned microscope slides. Gentle suction and flow of buffer solution over the isolated samples can be used to dislodge intact rod photoreceptor cells for imaging. Cells isolated in this manner have been shown to remain functional. Cells are examined *in situ* via a water immersion objective. Examining cells *in situ*, prevents rupture of the cell membrane upon drying. Cells maintained in buffer solution exhibit the same chemical gradients observed in dried samples. Aluminized quartz slides are used as substrates, enabling both infrared and Raman vibrational imaging of the same samples. Several individual cells were examined showing consistent gradients.

Model Liposome Preparation

The multilamellar vesicles used to model the Raman spectral response were prepared from cholesterol and 1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (PDhPC). The lipids were purchased from Avanti Polar Lipids and used without further purification. The PDhPC was received in chloroform, while a stock solution of cholesterol in chloroform was prepared. The lipids were combined volumetrically and evaporated to dryness under reduced pressure. The lipid film was hydrated in H₂O to produce a 33% (wgt.) lipid suspension. To insure hydration of the lipids and the formation of fluid liposomes, the suspension was subjected to 10 freeze-melt cycles by cooling in liquid nitrogen and then warming to room temperature. The liposomes were then encapsulated in a quartz tube for Raman measurements.

Raman Spectroscopy and Imaging

Raman vibrational mapping is performed on isolated ROSs utilizing commercial Raman microscope (Renishaw) retrofitted with a single mode diode laser (Torsana Laser Technologies AB) with an output of up to 500 mW of 785 nm radiation. The power incident on the sample is adjusted in tandem with collection times to maximize signal to noise ratios and to prevent damage to the sample. Line focus imaging expedites imaging experiments compared to point mapping. The laser intensity is shaped by a Powell lens into a line focus approximately 1 μm wide x 50 μm long. The intensity along the line focus is corrected using the image of the 520 cm^{-1} phonon measured from a silicon wafer. The scattering from the line is imaged onto a CCD camera and recorded across the region of interest. An attenuated power of 30 mW at the sample and a collection time of 30 s/line are typical for imaging the ROS.

Data Analysis

Data analysis was performed using ENVI 4.5 (ITT, Corp). ROIs were identified and statistics evaluated on the selected regions of the image. Representative spectra were fit using a partial least squares regression model and Grams/AI software (Thermo Corp.). Background subtraction was performed by fitting a high order, quintic, polynomial to the entire spectrum. Care must be taken when subtracting background to avoid artifacts. By treating the entire spectrum simultaneously, broad features attributable to fluorescence can be minimized with minimal perturbation to the narrow vibrational features. Lorentzian lineshapes were fit to the average spectrum in each region to establish the number of peaks. The frequency and peak-width for each peaks were then fixed within error and only the intensity was allowed to modulate to account for changes in the observed spectral lineshape.

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Figure 1.

The optical image of an isolated rod photoreceptor cell is shown (A). The dotted lines denote the boundary of the nucleus & inner segment as well as the boundary of the inner and outer segment of the cell. Raman spectra averaged over the different cell regions are shown (B-D). A reference spectrum comprised of 1-palmitoyl-2-docosaheptaenoyl-sn-glycero-3-phosphocholine and cholesterol is shown for comparison.

Figure 2.

The Raman map of a rod cell is shown depicting the intensity as a heat map of the 1655 cm^{-1} band (A). ROIs are depicted and numbered to correspond the mean spectrum obtained as plotted in B. The spectra are offset for clarity and the dotted lines are guides for the eye.

Figure 3.

The band area ratios comparing the intensity of the 1655 cm^{-1} band representative of docosahexaenoyl acyl chains relative to the 715 cm^{-1} choline mode (red) and C-H deformations (white) are plotted for the ROIs depicted in figure 2. The lines are guides for the eye.

Figure 4.

An isolated rod photoreceptor is depicted as viewed under white illumination in the Raman microscope (A). The numbered regions indicate the location from which Raman spectra are collected and analyzed. Deconvolution of the Raman spectra (B) from the indicated regions show decreasing cholesterol contribution along the Rod cell. The Raman spectrum of cholesterol is provided to indicate the expected location of a cholesterol band. The relative cholesterol gradient is graphed against the normalized length of the cell showing decreasing cholesterol content in the ROS (C).

Figure 5.

The Raman spectra obtained from aqueous multilamellar vesicles suspensions consisting of the indicated mixtures of cholesterol with 1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine are plotted to demonstrate the relative Raman response of cholesterol relative to the choline vibrational mode at 715 cm⁻¹. The line denotes the peak observed in the cholesterol standard shown in Figure 4.