Biochimica et Biophysica Acta, 986 (1989) 161–166
Elsevier

BBAMEM 74622

Infrared spectroscopic study of ethanol-induced changes in rat liver plasma membrane

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(Received 15 May 1989)

Key words: Infrared spectroscopy; Liver plasma membrane; Ethanol; Membrane order; Interdigitated lipid chain

Vibrational infrared spectroscopy, a noninvasive method for probing the structural and dynamic properties of biological membranes, is used to characterize the in vivo and in vitro perturbations of ethanol on various liver plasma membrane preparations derived from alcohol-treated rats. Spectral frequency shifts of the bilayer lipid chain methylene carbon-hydrogen symmetric stretching modes indicate that the adaptive response of the liver plasma membranes of the alcohol-treated animals results in an increase in membrane order on the vibrational time scale. Additional in vitro ethanol treatment of these membrane preparations leads to further significant increases in bilayer order. The observed membrane ordering effects are consistent with a bilayer model of partial interdigitation, or chain overlap, of the opposing membrane monolayers near the bilayer center.

Introduction

Ethanol interactions with biological membranes differ depending upon whether there is chronic or acute in vivo exposure to the agent [1–3]. Acute exposures generally increase membrane disorder through a physical interaction defined by the partitioning of ethanol into the lipid matrix of the membrane bilayer. Since this disordering effect is directly related to lipid solubility [4], membrane spectroscopic order parameters exhibit a dependence on both alcohol and bilayer depth [5,6]. In contrast, chronic ethanol exposure gives rise to membranes that are resistent to the in vitro disordering effect of ethanol, a behavior which suggests an adaptive response to the presence of the agent [7,8]. This membrane resistance to disordering, or membrane tolerance, is not limited to ethanol alone, but to alcohol-treated membranes interacting with a variety of membrane-active agents [9]. Although structural rearrangements are expected to occur in tolerant membranes, baseline order parameters determined by electron paramagnetic resonance (EPR) techniques are equivalent in both control and ethanol-treated systems [7]. After alcohol withdrawal, however, this tolerance in quickly lost [10]. Reflecting metabolic adjustments, the promotion of membrane tolerance has been related to the cholesterol [5,11,12] and phospholipid content of the bilayer [13,14]. In recent studies involving reconstituted vesicle assemblies derived from ethanol-fed animals, tolerance has been linked, for example, solely to the presence of either phosphatidylinositol [15] or cardiolipin [16].

Most of the studies designed to monitor changes in the physical properties of tolerant membranes have used either EPR or fluorescence polarization techniques. These methods, which require the insertion of either a spin label or a bulky fluorophore into the membrane preparation, report changes in the localized environment surrounding the probe molecule. In comparison, vibrational infrared spectroscopy, which has not been used previously to characterize tolerant membranes, can provide a sensitive, but noninvasive, means of monitoring the structural and dynamic properties of both the lipid and the protein fractions in either intact or model membranes. In addition to distinguishing between the various components of the membrane assembly, the observed spectral features can also be related to vibrational modes describing the conformational behavior of specific molecular moieties of individual bilayer constituents [17]. For example, as shown both experimentally and theoretically, the frequencies of the lipid chain methylene carbon-hydrogen (C–H) symmetric stretching modes, centered at approx. 2850 cm^{-1} are extremely sensitive to the presence of *gauche* conformers within the hydrocarbon region of the bilayer

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[18]. Since disorder on the vibrational time scale is defined as an increase in the relative numbers of *gauche* to *trans* conformers along the lipid chains, infrared spectroscopic measurements provide a straightforward manner in which to probe and to compare the order/disorder properties of biological membranes. Model multilamellar liposomes clearly illustrate the use of vibrational spectroscopy in monitoring bilayer disorder. Specifically, at low temperatures the lipid chains of the model systems are in the nearly all-*trans* conformation [19]; however, as either the temperature is increased or various perturbants are introduced into the bilayer, the intramolecular disorder, governed by the formation of *gauche* conformers, increases. An observed increase in the frequency of the 2850 cm^{-1} feature, which can be measured to $\pm 0.02 - \pm 0.06$ cm^{-1}, reflects this induced disorder. Frequency shifts of these vibrational modes to higher wavenumbers of about 4 cm^{-1} are observed for model membrane systems, with saturated lipid chains, when the bilayer passes from the ordered gel phase to the disordered liquid-crystalline phase [20]. This is a reversible phenomenon; and, for the methylene stretching modes, shifts to lower frequency occur when the number of *gauche* conformers decreases and the bilayer reverts to the ordered gel phase. Intact membranes, such as, for example, the rat liver plasma membranes examined in this study, are liquid crystalline at physiological temperatures and exhibit greater vibrational disorder compared to model bilayer systems composed of saturated chains. In particular, we observe the methylene symmetric stretching modes at about 2852.5 cm^{-1} in the liver plasma membrane assembly at 35°C in comparison to approximately 2849 cm^{-1} for the more ordered dipalmitoylphosphatidylcholine (DPPC) bilayer, also incubated at 35°C.

Recently, new, interdigitated bilayer phases, in which the hydrocarbon chains of the opposing monolayers interpenetrate across the bilayer center, have been observed for a variety of model systems, including those exposed to ethanol [21–24]. The interdigitated phase gives rise to a decrease in the overall bilayer thickness, an increase in the lateral chain-chain interactions and fewer *gauche* conformers per hydrocarbon chain. Using vibrational spectroscopic techniques, one observes the induction of the interdigitated gel phase as a further decrease in the frequency of the methylene symmetric stretching vibrational modes relative to those of the non-interdigitated gel phase. Fourier-transform Raman spectra recorded of model bilayer systems of dipalmitoylphosphatidylcholine (DPPC), for example, indicate a decrease in the methylene symmetric stretching mode of 1.0 cm^{-1} on passing from the non-interdigitated to the interdigitated gel phase (unpublished data). Also, lipid dispersions of model bilayer systems exhibit biphasic phase-transition behavior in the presence of various concentrations of ethanol [25]. Gel-phase dipalmitoylphosphatidylcholine (DPPC) liposomes, for example, only exist in the interdigitated form at concentrations of ethanol in excess of 50 mg/ml [24].

In the present study infrared spectra are recorded of various liver plasma membrane preparations derived from ethanol-treated rats. The relative frequencies of the 2850 cm^{-1} methylene C–H stretching mode features are precisely determined and are used to assess the degree and type of perturbation induced when these membranes are subjected to both in vivo and in vitro treatment with ethanol. Our data indicate that the adaptive response of liver plasma membranes of ethanol-treated animals leads to a significant decrease in the lipid chain symmetric stretching mode frequencies; that is, membrane order increases on the vibrational time scale. In vitro ethanol treatment of membrane preparations from animals exposed to alcohol lead to further substantial decreases in the frequencies of the methylene stretching modes. This observed increase in average lipid chain order is consistent with a model of partial interdigitation, or chain overlap, of the opposing membrane monolayers near the bilayer center.

Experimental procedures

Materials. Sucrose (ultrapure) was purchased from Bethesda Research Laboratories, Rockville, MD. All chemicals were reagent-grade. Male Sprague Dawley rats weighing 200–250 g were obtained from Taconic Farms, Germantown, NY. According to established methods, physical dependence was induced by repeated intragastric administration of 20% (w/v) aqueous ethanol 10 times a day for 4 days [11,26]. Control animals received equal volumes of water. Food and water were freely available to all animals throughout the study.

Rat liver plasma membrane (LPM) isolation. Rats were killed by decapitation and their livers were immediately perfused with ice-cold normal saline. Liver plasma membranes were prepared by the method of Hubbard et al. [27]. Membranes were suspended in 10 mM Tris-HCl, pH 7.4, at 2.5 mg/ml and stored at −80°C for no longer than 8 weeks. No changes in lipid composition occurred during that period of time as determined by protein and lipid analyses. Typically four rat livers from each group of animals were pooled for LPM isolation.

Protein and lipid analysis. Protein was determined according to the method of Lowry et al. [28] or that of Bradford [29] using bovine serum albumin as a standard.

Total cholesterol content was determined using a colorimetric assay (Sigma Assay Kit, No. 350, Sigma Chemical Company) of the enzymatic oxidation of cholesterol to cholest-4-en-3-one. Sample was added

directly to the assay mix and absorbance was recorded at 500 nm. Turbidity from the sample (< 3% of the total absorbance) was subtracted after remeasuring the absorbance in the presence of ascorbic acid. The phospholipid content was determined by the method of Fiske and SubbaRow [30] after extraction of the lipid according to the procedure of Bligh and Dyer [31].

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed according to Laemmli [32] using a 5–15% gradient separating gel system. The gels were stained with either Coomassie blue R-250 or silver according to the manufacturer's instructions.

Electron microscopy. Thin-section electron microscopy was performed as previously reported [33]. In short, an aliquot of plasma membrane was fixed in 1.5% glutaraldehyde (EM Sciences, Fort Washington, PA) 0.1 M sodium cacodylate, pH 7.4, and postfixed in 1% osmium tetroxide for 2 h. After dehydration in ethanol and propylene oxide, the pellets were embedded in Poly-Bed 812/Araldite 502 resin. Ultrathin sections were cut with a Sorvall microtome MT-2 and stained with lead citrate and uranyl acetate. The sections were examined in a Phillips EM300 electron microscope.

Infrared spectroscopic measurements. Infrared spectra were recorded with a Perkin-Elmer 580B dispersive spectrometer controlled by a Perkin-Elmer data station. Spectra were recorded at 0.85 cm^{-1} resolution and data points were encoded every 0.1 cm^{-1}. Spectra were transmitted as ASCII files into our laboratory PDP 11-84 minicomputer for storage, manipulation and analysis. (Fourier-transform infrared spectrometers are equally applicable for studies of this nature.)

A jacketed, variable-path-length cell equipped with CaF_2 windows was used to contain the various liver plasma membrane preparations for the recording of spectra. A small amount of the hydrated, pelleted membrane preparation was placed in the center of one window of the cell and the opposing window was lowered until the sample formed a uniform bubble-free film. The assembled unit was then thermostatted at various temperatures using a Lauda RM6 water bath.

Spectra were collected by signal-averaging five scans in the 2825–2880 cm^{-1} range while continually purging the instrument of water vapor using dry nitrogen gas. Relative frequency shifts of the lipid chain methylene symmetric stretching modes at 2850 cm^{-1} were accurately determined by fitting unsmoothed data files to a single three-parameter gaussian function using a nonlinear minimization technique. Data points from 2843 cm^{-1} to 2857 cm^{-1} were used, since this is a region which clearly defines the lipid methylene stretching vibration without any significant signal contribution from other sources. 141 data points were used to determine frequency shifts to an uncertainty of less than 0.06 cm^{-1}.

Results and Discussion

All membranes were obtained from animals complying with the definition of tolerance that was outlined in the model of Majchrowicz [26,27]. Purity of the isolated rat liver plasma membranes (LPM) was assessed by thin-section electron microscopy. Morphological examination of the LPM preparation revealed the presence of extended sheets of membrane with many features reminiscent of the hepatocyte plasma membrane in situ (data not shown). Careful examination of the thin-section electron micrographs, as well as sodium dodecyl sulfate–polyacrylamide gels and asialoglycoprotein receptor activity, revealed no detectable difference between plasma membranes isolated from either control, tolerant or withdrawn animals. No statistically significant differences were noted in phospholipid or cholesterol content of the respective membranes.

Fig. 1 shows a typical spectrum obtained for a liver plasma membrane in the 2880–2825 cm^{-1} region. The main feature centered at about 2852 cm^{-1} is assigned to the methylene symmetric stretching modes of the hydrocarbon chains of the lipid component of the membrane [17]; secondary spectral features are assigned to various other lipid chain C–H stretching vibrations, and to spectral contributions from water and ethanol. The dotted line indicates the curve calculated from the parameters of the least-squares fit described in the Experimental procedures section. The calculated curve is evaluated between 2825 cm^{-1} and 2880 cm^{-1}, while the fit, as previously stated, is only carried out using data points between 2843 cm^{-1} and 2857 cm^{-1}. This function provides a systematic and statistically robust means of precisely determining the peak maximum, the

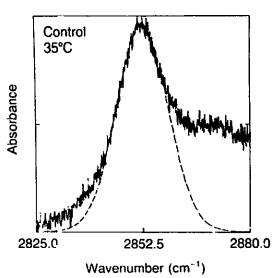

Fig. 1. Infrared absorbance spectrum of a control rat liver plasma membrane preparation at 35°C in the frequency range 2825–2880 cm^{-1}. The dashed line indicates the gaussian curve calculated from the parameters of the least-squares fit.

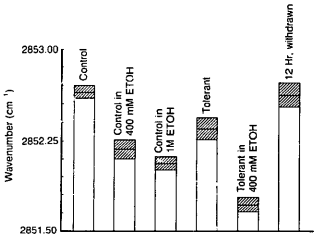

Fig. 2. Histogram representing the effect of ethanol on the frequency of the methylene symmetric stretching mode of the lipid fraction of the various membrane preparations studied. The error bars indicate the uncertainty derived from the spectral least-squares fitting procedure.

spectral property for determining the direction and degree of any frequency displacement occurring as a result of a membrane perturbation.

Fig. 2 provides a graphical presentation of the relative frequency shifts observed for the various membrane preparations. Except for the frequencies of the control and withdrawn membrane preparations, there is no overlap in the error bars, calculated at the 95% confidence interval of the least-squares spectral fit, for the control and alcohol-perturbed preparations. The first bar represents the vibrational frequency calculated for a control liver plasma membrane incubated at 35°C, while the second and third bars show the frequencies calculated for the same membrane preparation incubated at 35°C in 0.4 M and 1.0 M ethanol for 1 h. For the three systems, the frequency drops from 2852.45 cm^{-1} to 2852.17 cm^{-1} and 2852.13, cm^{-1}, respectively. The fourth bar represents the frequency determined for a plasma membrane isolated from an animal after 4 days of ethanol treatment and incubated at 35°C. The value of 2852.28 cm^{-1} is significantly lower than the result obtained for the control animal. This value is not as low, however, as that observed for the control animals under conditions of in vitro ethanol incubation (bars two and three). Bar five shows the effect of incubating the membranes of the ethanol-treated animal in 0.4 M ethanol at 35°C, also for 1 h, and indicates that after in vitro alcohol exposure the vibrational mode has shifted even lower, to a frequency of 2851.90 cm^{-1}. This relative perturbation is greater than the corresponding changes observed when a control membrane was incubated in 0.4 M ethanol (bar two) The final bar gives the stretching mode frequency observed at 35°C for the plasma membrane isolated from an animal after a 12-h withdrawal from the ethanol diet. The frequency of 2852.43 cm^{-1} is very close to the value of 2852.45 cm^{-1} observed for the control animal and suggests a complete reversal of the adaptive changes leading to the increased membrane order. Table I summarizes the observed frequencies for all six membrane preparations, the associated errors and the $\Delta\nu$ values, the changes in observed frequencies compared to the control. To verify the sensitivity of this vibrational mode to changes in the lipid bilayer packing characteristics, we also recorded spectra of control membranes as a function of decreasing temperature. We observe decreases, as expected, in frequency from 2852.45 cm^{-1} to 2852.04 cm^{-1} and 2851.04 cm^{-1} for control membrane preparations at 35°C, 21°C and 4°C, respectively (Table I). These frequency shifts to lower values provide a sense of the magnitude of the ethanol-induced perturbation in that an ethanol-treated membrane also incubated with 0.4 M ethanol at 35°C has a lower frequency than the corresponding control membrane incubated at 21°C.

Decreases in the frequency of the methylene symmetric stretching mode for these systems in the presence of ethanol indicate a reorganization of the membrane to an average bilayer structure that is inherently more ordered in terms of fewer *gauche* conformers along the lipid hydrocarbon chains. An increase in the vibrational order parameters of the liver plasma membranes may arise from either a partial ordering of the membrane as a whole or as domains of highly ordered lipid chains interspersed throughout the lipid matrix. Specifically, the ordering behavior may be related to the biphasic disorder/order characteristics noted for the effect of ethanol on model liposomal assemblies [25]. That is, in model bilayer systems, disordering may be observed after incubation with low ethanol concentrations followed by ordering at higher concentrations as the bilayer enters an interdigitated phase. Interdigitated hydrocarbon chains are only observed in the gel phase of model systems; however, as the model bilayer reverts to its usual non-interdigitated morphology in the

TABLE I

Summary of the methylene symmetric stretching mode frequencies (cm^{-1}) for liver plasma membrane-ethanol assemblies

Membrane assembly	Frequency (cm^{-1})	$\Delta\nu$ [b]
Control 35°C	2852.45 ± 0.03 [a]	
Control 35°C + 400 mM ethanol	2852.17 ± 0.05	−0.28
Control 35°C + 1 M ethanol	2852.10 ± 0.03	−0.35
Ethanol-tolerant 35°C (4 days)	2852.27 ± 0.05	−0.18
Ethanol-tolerant 35°C + 400 mM ethanol in vitro	2851.89 ± 0.04	−0.56
Ethanol withdrawn 35°C (12 h)	2852.43 ± 0.06	−0.02
Control 21°C	2852.04 ± 0.04	−0.41
Control 4°C	2851.04 ± 0.02	−1.41
Control 4°C + 1 M ethanol in vitro	2851.18 ± 0.02	−1.27

[a] Uncertainty is twice the spectral least-squares fit error.
[b] Difference in frequency observed from that of the control (35°C).

liquid-crystalline phase, we have observed a significant increase in bilayer order at low ethanol concentrations (unpublished observations). This observation is relevant to intact membranes at physiological temperatures. Further, our studies on model systems suggest that the biphasic behavior is highly dependent on lipid type and temperature and may proceed as a dehydration-driven phenomenon as the ethanol displaces bound water within the lipid headgroup and interface regions. It is interesting to note that the frequency of 2851.18 cm^{-1} measured for a control liver plasma membrane incubated with 1 M ethanol at 4°C is greater (more disordered) than that of 2851.04 cm^{-1} for the control membrane at 4°C in the absence of ethanol. These two frequencies, different within the statistical errors quoted in Table I, signify that the response of membranes to ethanol at 4°C is different from that at 35°C. That is, the membranes indicate a greater degree of disorder when ethanol is added in vitro at a reduced temperature. This, in turn, suggests the possibility of a complicated biphasic behavior for ethanol in intact membranes.

The interdigitation process in model systems is relatively straightforward to measure, since the uniformity of the systems gives rise to large, concerted changes that are easily monitored by the vibrational technique. However, the relatively smaller, though still significant, changes in the vibrational spectra of intact liver plasma membranes is consistent with a hypothesis of partial hydrocarbon chain interdigitation. Also consistent with a partial overlap or interdigitation of lipid chains is the EPR study of mouse synaptosomal membranes by Lyon and Goldstein [6]. These authors measured an increase in chain order for an EPR spin label monitoring the carbon-twelve position of the lipid hydrocarbon chain, near the bilayer center, but not for a spin label at the carbon-five position, which is closer to the membrane surface. Other workers have also measured a decrease in the partitioning of ethanol and other hydrophobic agents into membranes of ethanol-exposed animals [9], observations which are also consistent with increased lipid packing densities, and perhaps resulting from a limited degree of chain interdigitation. Our results have also shown that the membrane structural rearrangements are rapid and that no changes were discerned between control animals and ethanol-treated animals 12 h after withdrawal. This could be interpreted in terms of ethanol metabolization and the return of the membranes to a fully hydrated condition, results which are comparable with reports from other workers that membrane tolerance was quickly lost after alcohol withdrawal [10].

In summary, these results suggest that intact membranes may exhibit biphasic behavior when subjected to both in vivo and in vitro treatment with ethanol and that the membrane ordering effects may be a consequence of partial bilayer interdigitation. The results observed in this study are for membranes obtained within a 4-day alcohol exposure period and are probably not directly comparable to longer-term studies where the induced membrane behavior is associated with changes in membrane lipid composition. Further, behavior in intact membranes under various conditions of ethanol treatment, which is analogous to the biphasic phenomenon observed in model systems, will depend not only on ethanol concentrations, but also on other factors. That is, changes in membrane composition and temperature could lead to different spectral trends for the same membrane preparation under somewhat different experimental conditions. Our observations suggest that no clear distinctions can be drawn between membranes chronically treated with ethanol in vivo over a 4-day period and control membranes treated with ethanol in vitro; that is, both intact membrane systems are characterized by an increase in order.

The definition of tolerance has been based on the inability of ethanol to induce disorder in membranes isolated from animals chronically treated with ethanol. In addition, baseline order parameters of both the control and the tolerant membrane preparations are equivalent. However, implicit in the definition of tolerance is the animal's associated physical dependence on ethanol. In our model, we show that although a physical dependence on ethanol is demonstrated for the animal, the liver plasma membranes do not exhibit tolerance, as previously defined. In the light of these findings we suggest that the strict definition of membrane tolerance by reevaluated.

Acknowledgement

The authors would like to thank Dr. Edward Majchrowicz for providing assistance with the animal protocol outlined in this manuscript.

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