Biochimica et Biophysica Acta, 776 (1984) 267–278
Elsevier

BBA 72223

EFFECTS OF INSULIN ON THE LIPID STRUCTURE OF LIVER PLASMA MEMBRANE MEASURED WITH FLUORESCENCE AND ESR SPECTROSCOPIC METHODS

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(Received March 6th, 1984)

Key words: Membrane structure; Lipid order; Insulin; Fluorescent label; ESR; (Liver plasma membrane)

Insulin increased the lipid order of rat and mouse liver plasma membrane domains sampled by the hydrophobic fluorescent probe 1,6-diphenyl-1,3,5-hexatriene in a concentration-dependent saturable manner. The ordering is half maximal at $5.1 \cdot 10^{-11}$ M and fully saturated at $1.7 \cdot 10^{-10}$ M insulin. Membranes prepared from obese hyperglycemic (ob/ob) mice demonstrated a right-shift in the dose-dependent ordering induced by insulin, such that ordering was half maximal at $1.2 \cdot 10^{-10}$ M and fully saturated at $2.0 \cdot 10^{-10}$ M. Insulin also increased the order of rat liver plasma membranes labeled with the *cis*- and *trans*-parinaric acid methyl esters. The ordering caused by insulin as detected with *cis* methyl parinarate was complete within approx. 15 min. after hormone addition at 37°C, and the ordering was approximately double that observed with the *trans* isomer. Additional ESR experiments demonstrated that the addition of insulin increased the outer hyperfine splittings of spectra recorded from membranes labeled with the steroid-like spin labels, nitroxide cholestane and nitroxide androstane, but not the fatty acid spin probe, 5-nitroxide stearate. Studies utilizing model membrane systems strongly suggest that the 5-nitroxide stearate samples a cholesterol-poor domain of the membrane, while the steroid-like probes preferentially sample cholesterol-rich regions of the membrane. Finally, insulin-induced membrane ordering was dose-dependently inhibited by cytochalasin B in the range 1–50 μM. From these results, we conclude that (1) the ordering effect of insulin addition to isolated liver plasma membrane fractions occurs within the physiological range of hormone concentration, and the dose-response is right-shifted in membranes from 'insulin resistant' animals; (2) the relative responses of the fluorescent and spin probes suggest that the effects of insulin are confined to specific domains within the membrane matrix; and (3) the direct effects of insulin on the membranes may involve protein components having cytochalasin B binding sites.

Introduction

The metabolic effects that insulin exerts in responsive peripheral tissues occur subsequent to the interaction of the hormone with the cell surface membrane [1]. It is usually found that most membrane (or other) effects of the hormone require the presence of an intact cell structure, although insulin treatment of intact cells appears to induce functional alterations that are retained in subsequently isolated membrane fragments [2]. In some instances, however, direct effects of insulin addition on functional activities of purified plasma membrane preparations [3–5] have been reported. Studies with isolated membrane systems that are responsive to the addition of insulin in vitro are useful for probing the early events that take place at the cell surface that give rise to the transduction of information from the ligand-receptor complex to the effector systems that modulate cell function.

One possibility that has been considered is that insulin mediates widespread alterations in membrane functions by causing relatively gross changes in the structure of the membrane itself [6]. Studies employing several biophysical techniques, such as IR, ESR, fluorescence spectroscopy, and electron microscopy have been conducted to determine whether insulin exerts experimentally detectable structural alterations on molecular components of isolated plasma membranes [2,6–13].

The studies of Luly and Shinitzky [6] and Schroeder [13] have demonstrated that the addition of insulin to isolated liver plasma membranes induces changes in the motional properties of the lipid phase, as reported by extrinsic fluorescent probes. Also, Luly et al. [12] correlated similar insulin-induced lipid phase motional changes observed in erythrocyte membranes with alterations in the erythrocyte plasma membrane $(Na^+ + K^+)$-ATPase activity. As yet, nothing is known concerning the mechanism by which the binding of insulin to its receptor could ultimately influence the motional properties of the membrane bilayer. Furthermore, it is not known whether an alteration of the physical properties of the plasma membrane is involved in the metabolic actions of the hormone or whether it is a secondary effect of intrinsic protein reorganization within the membrane matrix.

We have attempted to further evaluate the direct ordering effect of the hormone on liver plasma membranes by utilizing both fluorescence and spin lable membrane probes and also the cytotoxic agent, cytochalasin B. Finally, we more closely characterize the relationship between insulin binding and the observed structural effects of insulin by analyzing the dose-responses of membranes isolated from normal and 'insulin-resistant' animals.

Materials and Methods

Liver plasma membrane purification. Liver plasma membranes were isolated from male Sprague-Dawley rats (ranging in weight from 120 to 220 g) and from normal C57B1J (ob/?) and obese (ob/ob) mice (aged 6–7 weeks), essentially according to Ray [14]. The final $5000 \times g$ membrane pellet was diluted into 25 mM 3-(*N*-morpholino)propanesulphonic acid (Mops) buffer containing 100 mM NaCl and 100 μM $CaCl_2$ (pH 7.4) at 4°C. Protein was estimated by the method of Lowry et al. [15], and membranes were suspended at 100 μg/ml protein for fluorescence spectroscopy and 2–4 mg/ml for ESR studies. The degree of membrane purification was determined by measuring the fold purification of the plasma membrane marker enzyme 5′-nucleotidase [19,22] and the membrane fractions were routinely examined by phase contrast microscopy. The highest specific activity of 5′-nucleotidase occurs at the interface between the 41 and 37% sucrose layers after centrifuging samples up the discontinuous gradient as described by Ray [14]. The presence of membrane fragments was confirmed by examination with phase contrast microscopy, and mitochondria were not detectable in significant amounts in this gradient band. All spectroscopic experiments were conducted within 8 h after killing the animals.

Membrane labeling conditions. Solutions of DPH (Sigma Chemical Co.) in tetrahydrofuran (1 mM) (Fig. 2) and the *cis* and *trans* methyl parinarate esters (Molecular Probes, Inc. Plano, TX) in ethanol (1 mM) were stored at −70°C under nitrogen. Membrane suspensions were mixed with small aliquots of probe (8.3 nmoles probe/μmole membrane phospholipid) and incubated at 37°C

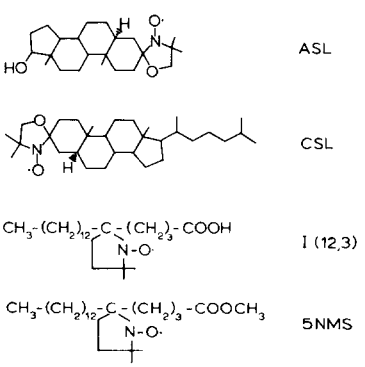

Fig. 1. Spin labels used in the present study. CSL is the cholestane spin label, 4′,4′-dimethylspiro[5α-cholestane-3, 2-oxazolidin]-3′-yloxy; ASL is the androstane spin label 17β-hydroxy-4′,4′-dimethylspiro[5α-androstane-3,2-oxazolidin]-3′-yloxy; I(12,3) is the *N*-oxyl-4′,4′-dimethyloxazolidine nitroxide derivative of 5-ketostearic acid, and 5-NMS is the methyl ester of I(12,3).

for 15 min for equilibration of label. Cytochalasin B (Calbiochem-Behring, La Jolla, CA), insulin (Sigma), or control additions of ethanol/buffer were added to separate samples in duplicate at 37 °C. Fluorescence measurements of the membranes were conducted with an SLM series 4800 spectrophotofluorimeter, equipped with a circulation bath to regulate the sample temperature at 37 + 0.1 °C.

Liver membranes were labeled with the 5-nitroxide stearate spin label I(12,3) (Syva Co., Palo Alto, CA) (see Fig. 1) at room temperature as described elsewhere [7]. Aliquots of nitroxide androstane (Syva) were added to the sample in small aliquots (1–2 μl) ethanol. Nitroxide cholestane (Syva) was incorporated into the membrane by the Percoll method [24]. Membranes were labeled with experimentally determined low probe/membrane protein concentrations to avoid radical interaction effects on the hyperfine splittings. In the case of steroid labeled liver plasma membranes, the probe concentration can be increased to high levels without any detectable effects on the outer hyperfine splitting magnitude [16], and this was confirmed in our plasma membrane samples. ESR spectra were recorded at 37 °C with a Varian E-104A Century Series ESR spectrometer, equipped with a Varian variable temperature accessory, after 5 min waits for temperature equilibration. In all cases, duplicate spectra were recorded both before and after addition of insulin (Sigma).

Membrane vesicle preparation and labeling. Egg phosphatidylcholine (PC) and mixed PC/cholesterol vesicles were prepared by sonication of the lipids (from Sigma) in 150 mM NaCl, 40 mM Mops buffer (pH 7.4) at room temperature. Vesicles were stored frozen at −70 °C prior to use. Titrations of vesicles were conducted by adding various (dried) aliquots of 5-nitroxide stearate to several plastic microcentrifuge tubes. Aliquots of vesicles were added so that equal amounts of total lipid were used in each sample. Probe-probe interaction effects on ESR spectra recorded in duplicate at 37 °C were determined in this manner.

ESR spectral measurements. The order parameters S, $S(T_{\parallel})$ and $S(T_{\perp})$ are sensitive to the flexibility of membrane-incorporated spin labels undergoing rapid anisotropic rotational motion about a unique symmetry axis [17]. Each may be measured from the outer and inner hyperfine splittings observed in the ESR spectra of the I(12,3)-labeled membranes as described previously [7,18,19].

$$S(T_{\parallel}) = 1/2\left[\frac{3(T_{\parallel} - T_{xx})}{(T_{zz} - T_{xx})} - 1\right]$$

$$S(T_{\perp}) = 1/2\left[\frac{3[(T_{zz} + T_{xx}) - 2T_{\perp}]}{(T_{zz} - T_{xx})} - 1\right]$$

$$S = \frac{(T_{\parallel} - T_{\perp})(a_N)}{(T_{zz} - T_{xx})(a_{N'})}$$

where T_{zz} and T_{xx} are the hyperfine splitting elements parallel to the static principal nuclear hyperfine axes x, y, and z, respectively. The x axis is parallel to the N-O bond direction and the z axis is parallel to the nitrogen $2p\pi$ orbital. T_{zz} and T_{xx} determined from single crystal studies were $(T_{xx}, T_{zz}) = (6.1, 32.4)$G [20]. $a_{N'}$ and a_N are the isotropic hyperfine coupling constants for the probe in the membrane and crystal states, respectively ($a_{N'} = 1/3(T_{\parallel} + T_{\perp})$ and $a_N = 1/3\ (T_{zz} + 2T_{xx})$). $a_{N'}$ is sensitive to the polarity of the membrane environment of the probe [21]. The order parameter $S(T_{\perp})$ is particularly sensitive to radical interaction effects that occur at high I(12,3)/membrane ratios [22].

As indicated earlier by Hubbell and McConnell [23], biological membrane dispersions labeled with steroid spin probes exhibit ESR spectra that indicate the labels undergo rapid rotational motion about an axis essentially parallel to the y principal axis and the fused ring of the steroid (the long molecular axis). Thus, for steroid spin labels, the outer (maximum) splitting in the ESR spectra of labeled membrane dispersions ($2T'_{\perp}$) corresponds roughly to the time-average splitting ($2T_{\perp}$) observed when the membrane surface is aligned parallel to the applied magnetic field $\vec{H}$ [16]. For most conditions, the observed splitting can be approximated by $2T' = T_{zz} + T_{xx}$, since these principal values are averaged as a result of rapid rotational motion about y.

In view of the above discussion, we here employ the polarity-uncorrected order parameter expression $S(T_{\perp})$ as an index of the ordering of the

steroid molecular z axis about the normal to the membrane surface, by measuring the outer splitting value ($2T'_{\perp}$) of the steroid labeled membrane [24].

Fluorescence measurements. Membrane suspensions were pre-incubated with or without insulin and/or cytochalasin B at 37 °C for 20 min, under a nitrogen atmosphere in sealed polypropylene sample tubes prior to fluorescence anisotropy measurements. Fluorescence assays were performed in stirred water-jacketed quartz cuvettes (3 ml) in a nitrogen atmosphere at 37 °C. Cuvettes were siliconized utilizing Prosil-28 (PRC Research Chemicals, Inc.) after acid washing.

Measurements of intensity of polarized fluorescence were performed on samples containing 100 $\mu g \cdot ml^{-1}$ membrane protein. Excitation wavelength was 360 nm for diphenylhexatriene studies, 315 nm and 323 nm for *trans* and *cis* methyl parinatrate studies, respectively. Excitation bandpass was 1 nm, and emission intensities were observed through Corning cut-off filters (No. 373 for diphenylhexatriene emission; No. 375 for methyl parinarate emission). Polarized intensities were delivered and analyzed through glan prism polarizers interposed in the excitation and emission light paths.

Instrumental corrected fluorescence anisotropy was calculated from the following parameters:

$$r_S = \frac{(I_{VV} - L_{VV}) - \left[(I_{VH} - L_{VH}) \cdot \frac{I_{HH} - L_{HH}}{I_{HV} - L_{HV}}\right]}{(I_{VV} - L_{VV}) + 2\left[(I_{VH} - L_{VH}) \cdot \frac{I_{HH} - L_{HH}}{I_{HV} - L_{HV}}\right]}$$

where I = total fluorescence intensities, L = light scatter intensity + background membrane fluorescence, and the first subscript (H of V) indicates the orientation (horizontal or vertical) of the polarizer, and the second subscript (H or V) indicates the orientation of the analyzer.

For diphenylhexatriene anisotropy measurements, Van Blitterswijk et al. [25] noted that r_s can be resolved into both static (slow decaying, r^{∞}) and dynamic (fast decaying, r_f) components by ns lifetime measurements. These parameters contain information about the molecular motion of the fluorophore, where r_{∞} reflects the orientational distribution of the elongated diphenylhexatriene probe that is axially symmetric about the normal to the membrane surface (i.e., 'wobble'), and r_f is sensitive to the rotational relaxation time. These authors demonstrated that for diphenylhexatriene embedded in biological membranes, it is r^{∞} that largely determines the value of r_s. Thus, within limits, r_s is proportional to a lipid order parmeter which describes the wobbling motion of the probe.

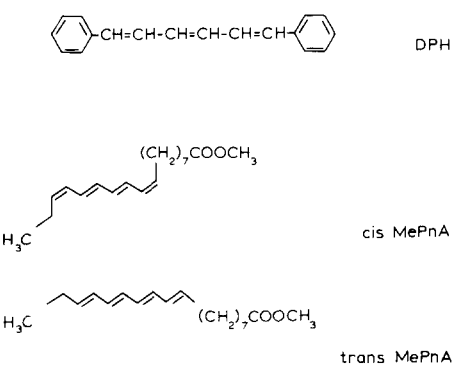

Fig. 2. Fluorescence labels used in the present study. DPH is the 1,6-diphenyl-1,3,5-hexatriene probe. *cis* and *trans* MePnA refer to the *cis* and *trans* isomers of parinaric acid methyl ester. The *cis* is 9,11,13,15,*cis,trans,trans,cis*-octadecatetraenoic acid methyl ester, and the *trans* isomer is the 9,11,13,15,*all trans*-octadecatetraenoic acid methyl ester.

Fluorescence anisotropy measurements of labeled liposomes containing *cis* and *trans* isomers of parinaric acid and its methyl esters have been discussed by Sklar, et al. [26]. The relative partitioning behavior of the *cis* and *trans* isomers of parinaric acid have been utilized to detect formation of gel phase lipid within biological membranes [27], since these probes preferentially partition into co-existing fluid and solid domains, respectively. Formation of gel phase lipid is detected by a relative increase in both fluorescence anisotropy and quantum yield of the *trans* probe relative to the *cis* [26].

Results

A total of 56 plasma membrane preparations were utilized for the series of experiments reported in this study. All preparations were labeled with diphenylhexatriene, and the fluorescence anisotropy in the absence and presence of insulin was measured, as a postive control for all investiga-

tions with other labels. It was found that 14 of the 56 preparations gave no response to the hormone, while a further ten preparations responded significantly, but to a low degree (5–10% change in r_s). The largest group (25 preparations) responded to insulin with a 10–15% increase in r_s, while seven preparations responded with a 15–20% increase. The mean ($\pm$S.D., $n = 56$) anisotropy values for diphenylhexatriene-labeled membranes were $r_s = 0.170 \pm 0.005$ in the absence and 0.185 ± 0.0102 ($P < 0.001$) in the presence of insulin for the 56 preparations. If the non-responsive membranes are excluded from the statistical analysis of data, then the anisotropy value ($\pm$S.D., $n = 42$) in the presence of insulin becomes 0.190 ± 0.006 ($P < 0.001$). Differences in average diphenylhexatriene fluorescence anisotropy values (measured at 37°C) calculated from rat liver plasma membrane fractions exhibited similar values to those obtained by Luly and Shinitzky [6] ($\bar{r}_s \pm$ S.E. $= 0.188 \pm 0.005$, $n = 7$ in the absence, and $\bar{r}_s \pm$ S.E. $= 0.201 \pm 0.007$, $n = 7$ in the presence of insulin), when the polarization data is converted to anisotropy units. The polarity-corrected order parameter S for I(12,3)-labeled liver membranes prepared according to Ray [14] was $S = 0.572 \pm 0.18$ and is somewhat greater than values reported earlier for membranes prepared according to Evans ($S = 0.558$; as reported in Refs. 22, 28), and less than values reported for liver plasma membrane prepared according to Pilkis ($S = 0.58 - 0.59$) [29].

The above differences in membrane order may reflect a combination of differences in aninal strain, nutrition, age, variable plasma membrane purity, and relative purification of the different plasma membrane regions of the hepatocyte.

The relative purity of the plasma membrane fractions were assessed by measuring the enrichment of the specific activity of 5′-nucleotidase. The plasma membrane activity typically was approx. 2.7 units (μmoles phosphate (P_i produced per min)/mg protein, and the observed purification of the enzyme was approx. 9-fold over the specific activity in the homogenate. These data are in good agreement with that reported earlier by Ray [14], where the specific activity of the enzyme in the plasma membrane fraction was approx 1.3 units/mg. We did not, however, determine whether the activity of 5′-nucleotidase quantitatively corresponded with insulin sensitivity of the membrane preparations; there is no a priori reason to suspect that any insulin-induced alterations would occur in the domain in which the enzyme resides or that alterations in membrane purity sufficient to change the insulin response observed would be sensed by changes in enzyme activity.

Insulin dose-response curves

The dose-response relationship of the insulin effect on diphenylhexatriene-labeled mouse liver membranes is shown in Fig. 3. Detectable effects were noted at 0.2 ng · ml^{-1} ($3.4 \cdot 10^{-11}$ M) and the response was saturated at 1 ng · ml^{-1} ($1.7 \cdot 10^{-10}$ M). No further change in anisotropy could be detected at 10 or 100 ng · ml^{-1} insulin.

Insulin titrations of lean (ob/?) and obese (ob/ob) mouse liver plasma membrane labeled with diphenylhexatriene were compared in additional experiments. A clearly evident rightward shift in the dose-response of the hormone-induced changes in anisotropy values were seen in the obese mouse liver membrane (Fig. 3). For example, half-maximal changes due to insulin for membranes from the normal and obese mice occurred at hormone concentrations of 0.3 and 0.7 ng/ml, respectively. The magnitude of diphenylhexatriene anisotropy was also lower in liver plasma membranes from obese mice, in agreement with the findings of York et al., [30].

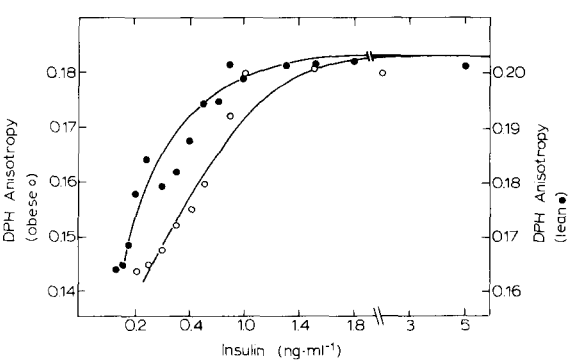

Fig. 3. Insulin dose-response of the increase in fluorescence anisotropy of diphenylhexatriene (DPH)-labeled mouse liver plasma membranes. Values are averages of quadruplicate incubations at each concentration from a single experiment. Data are reported for membranes prepared from four lean (ob/?) and four obese hyperglycemic (ob/ob) mice.

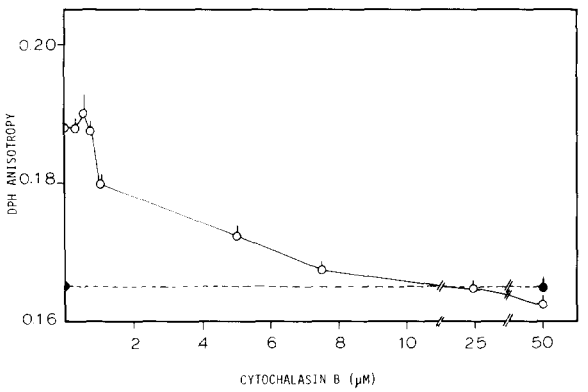

Fig. 4. Effects of cytochalasin B (○) on insulin (15 ng/ml)-induced changes in fluorescence anisotropy of diphenylhexatriene (DPH)-labeled rat liver plasma membranes. Values are averages of quadruplicate incubations from a representative experiment at 37 °C. Control samples (●) were incubated under identical conditions but in the absence of insulin. All measurements were conducted at least 15 min after addition of insulin to the experimental incubations.

Cytochalasin B was found to modulate the insulin-induced diphenylhexatriene anisotropy changes in rat liver plasma membranes. Addition of this drug to the plasma membranes prior to incubation with insulin progressively reduced the ability of the hormone to decrease probe motion, inhibition being complete at 25 μM (Fig. 4). Addition of cytochalasin B (50 μM) to membranes in the absence of hormone had no detectable effects on diphenylhexatriene anisotropy.

Studies with isomers of methyl parinarate

Rat liver plasma membranes were labeled with *cis*- and *trans*-parinarate methyl esters, and insulin effects on the steady-state fluorescence anisotropy of these probes were investigated. The results of this study are reported in Table I. Four different membrane preparations were utilized in this study, that had responded to insulin in diphenylhexatriene studies with a 10–15% increase in anisotropy. The methyl esters rather than the free acids were used in order to abolish any possible interaction of the probes with divalent cations. Insulin incubations with the membranes caused a 10.2% increase in *cis* isomer anisotropy, and a 3.9% change of *trans* anisotropy. A time-course of the effects of the hormone on *cis* methyl parinarate fluorescence anisotropy demonstrated that the hormone exerts its effects on the membrane rapidly after exposure, and the effects are largely completed by 10–15 min (fig. 5).

TABLE I

EFFECTS OF INSULIN ON FLUORESCENCE ANISOTROPY

Values of Parinaric Acid methyl Ester Labeled Rat Liver Plasma Membrane at 37 °C. Rat liver plasma membranes were purified and labeled with either *cis*- or *trans*-parinaric acid methyl ester labels as described in Materials and Methods. Fluorescence anisotropy values were measured as described in text. Each value represents an average of quadruplicate determinations for each membrane preparation. Percentage differences with and without insulin were calculated as the average % change from four separate membrane preparations. MePnA, parinaric acid methyl ester.

cis MePnA	*trans* MePnA	Insulin (10 ng/ml)
0.138	0.170	–
0.153	0.175	+
0.144	0.182	–
0.159	0.192	+
0.162	0.191	–
0.177	0.196	+
0.144	0.184	–
0.160	0.191	+
$\bar{X} = 0.147 \pm 0.010$	$\bar{X} = 0.182 \pm 0.009$	–
$\Delta\% + 10.2$	$\Delta\% + 3.9$	
$\bar{X} = 0.162 \pm 0.010$	$\bar{X} = 1.189 \pm 0.009$	+

Experiments were conducted to determine the relative sensitivity of the *cis* and *trans* methyl parinarate ester labels to increasing the cholesterol content of mixed PC/cholesterol vesicles, and the results of this study are presented in Fig. 6. Increasing the cholesterol content of liposomes increased the fluorescence anisotropy of both probes monotonically, although the recorded values of r_s were always higher for the *trans* isomer, due to the shorter fluorescent lifetime of this molecule [26].

ESR studies

Insulin elicited detectable alterations in the outer hyperfine splittings of ESR spectra recorded from membrane labeled with androstane and cholestane spin labels. Membrane preparations were used that gave 10–15% increase in diphenylhexatriene anisotropy on exposure to insulin. In each case, duplicate spectra were recorded both

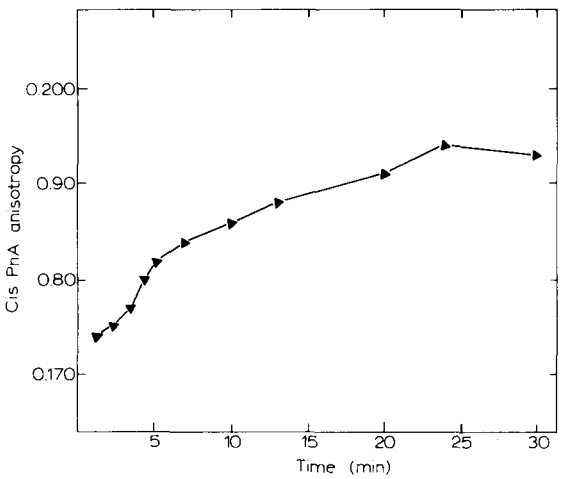

Fig. 5. Time-course of the insulin-induced changes in fluorescence anisotropy of the *cis*-parinarate-labeled rat liver plasma membrane. Values are from a representative experiment and illustrate the magnitude changes after addition of insulin (15 ng/ml) at 37 °C. cis PnA, *cis*-parinaric acid methyl ester.

before and after addition of hormone; insulin-treated membrane spectra were usually recorded between 15 to 20 min after hormone addition at 37 °C. In eleven experiments conducted on four separate liver plasma membrane preparations, 67 ng/ml insulin caused a 3% change in the calculated $S(T_\perp)$ value for the cholestane-labeled liver membrane. Similar changes were noted when expanded spectra were recorded with the expansion-superposition method described previously [17,22] to improve the precision of calculating the outer hyperfine splittings. Androstane-labeled membranes were similarly responsive to insulin at this hormone concentration and yielded a $5.6 \pm 1.4\%$ change in the order parameter in five experiments (Table II). These spectral effects correspond to approx. a 0.3–0.5 G shift in the outer hyperfine splitting values.

Earlier studies clearly indicated that insulin was without detectable effect on liver or fat cell plasma membranes labeled with I(12,3) [7,8]. We here investigated with I(12,3) the liver plasma membrane preparations employed in the fluorescence label experiments. No effects of insulin were noted on the order parameters calculated from the I(12,3)-labeled membranes (Table III). The order parameters S, $S(T_\parallel)$, and $S(T_\perp)$ were unchanged within experimental error in three separate experiments on freshly-prepared batches of liver mem-

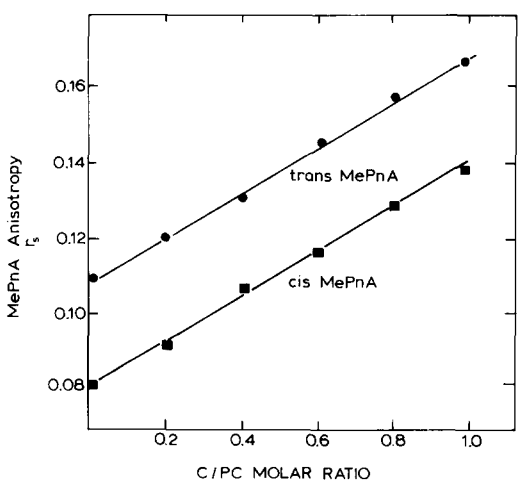

Fig. 6. Fluorescence anisotropy of *cis* and *trans* methyl parinaric acid ester (MePnA) labels in cholesterol/phospholipid mixed vesicles. Liposomes of pure egg phosphatidylcholine (PC) and of PC/cholesterol were prepared and labeled with *cis* and *trans* MePnA as described in Materials and Methods. The fluorescence anisotropy was measured at 37 °C as indicated in text. Values are averages of duplicate determinations from a representative experiment.

TABLE II

INSULIN EFFECTS ON ANDROSTANE-LABELED RAT LIVER PLASMA MEMBRANE AT 37 °C

Rat liver plasma membranes were purified and labeled with androstane as described in Materials and Methods. Each value represents an average of duplicate spectra recorded before and after insulin addition. $S(T_\perp)$ was calculated from the outer hyperfine splittings as indicated in text. The probe/membrane protein ratio was less than 7 nmoles label/300 μg membrane protein in each study. Differences between insulin-treated and control samples were evaluated statistically with a t-test of significance where $P < 0.01$ [51].

$T'_\perp$ (G)	$S(T_\perp)$	Insulin (ng/ml)
17.26	−0.288	–
17.58	−0.325	67
17.30	−0.292	–
17.48	−0.313	67
17.29	−0.291	–
17.5	−0.315	67
17.4	−0.304	–
17.62	−0.329	67
16.94	−0.251	–
17.17	−0.277	67

TABLE III

EFFECTS OF INSULIN OF I(12,3)-LABELED RAT LIVER PLASMA MEMBRANE AT 37 °C

Rat liver plasma membranes were prepared and labeled with I(12,3) as described in Materials and Methods. S, $S(T_{\parallel})$, and $S(T_{\perp})$ were calculated from duplicate spectra both before and after hormone addition as indicated in text. Experimentally determined low probe/membrane protein ratios were employed in each experiment (Sauerheber et al. (1977) [19]). Each value represents an average ±1 S.D. of three separate experiments.

Additions	Order parameter		
	$S(T_{\parallel})$	S	$S(T_{\perp})$
None	0.608 ± 0.033	0.572 ± 0.018	0.545 ± 0.009
Insulin (10–44 ng/ml)	0.605 ± 0.035	0.569 ± 0.018	0.537 ± 0.003

brane. Here, special care was taken to examine the effects of insulin on I(12,3)-labeled liver plasma membrane batches that responded to insulin in diphenylhexatriene label studies. Furthermore, control experiments were performed in order to ensure that the presence of the I(12,3) label in the membranes was not itself inhibiting the ordering effect of insulin. Isolated membranes labeled with the diphenylhexatriene probe responded to insulin addition with the same fold increase in diphenylhexatriene anisotropy in the presence and absence of the I(12,3) label (not shown). Also, studies were conducted with membranes labeled with widely differing I(12,3) concentrations, both with and without insulin. The results also indicated that no significant effects of insulin occurred at any label concentration employed (data not shown).

Titrations of PC and PC/cholesterol vesicles with I(12,3) were conducted over a wide probe/total lipid ratio range at 37 °C. Plots of $S(T_{\perp})$ vs. the probe/total lipid or probe/PC ratio are shown in Figs. 7A and B. Increasing the probe/lipid ratio leads to progressive decreases in $S(T_{\perp})$ for both methods of data presentation. We earlier presented arguments that radical interaction effects noted at high probe/membrane ratios cause selective broadening of $T_{\perp}$ in I(12,3)-labeled membranes so that, in the absence of polarity or motional changes, $S(T_{\perp})$ decreases dramatically with increasing probe/lipid ratios at a given temperature [22]. Least-squares lines were drawn through the experimental point for each PC/cholesterol vesicle titrated. Each line had high correlation coefficients (r^2 greater than 0.98). It is apparent that the slopes of the titration lines, when reported as a function of probe/total lipid, increase in magnitude as the cholesterol content of the liposome increases. However, this effect is dramatically reduced when the data are replotted specifically as a function of the probe/PC ratio (Fig.

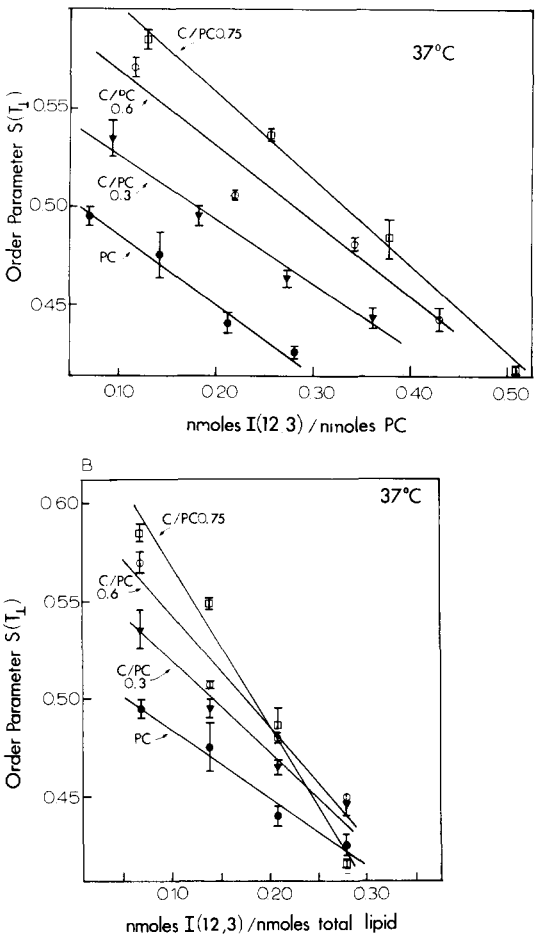

Fig. 7. The effects of increasing I(12,3) probe concentration on the order parameter $S(T_{\perp})$ of egg phosphatidylcholine/cholesterol mixtures. (A) $S(T_{\perp})$ plotted as a function on nmoles I(12,3)/nmoles of egg phosphatidylcholine. (B) $S(T_{\perp})$ plotted as a function of nmoles I(12,3)/nmoles total lipid. $S(T_{\perp})$ was calculated at 37 °C as indicated in Materials and Methods. Least-squares lines were drawn through the points for each vesicle, having cholesterol/phospholipid molar ratios of 0, 0.75, 0.6, and 0.3, respectively.

7A). The x axis becomes extended in the latter plot because PC contributes a lesser percentage of the total lipid in the mixed vesicles and a consequent higher probe/lipid (as PC) ratio occurs for each given label amount added. $S(T_\perp)$ values, for vesicles labeled with the least amount of probe, progressively increase with increases in cholesterol content, reflecting the well-known 'condensing' effect of cholesterol incorporation into model and biological membranes [29].

Discussion

The time-course for the insulin-induced ordering effects observed utilizing *cis* methyl parinarate demonstrates that the effect is largely complete after 15 min of incubation at 37 °C. The saturable insulin dose-response curve for diphenylhexatriene-labeled mouse liver membranes closely parallels the dose-dependence of insulin-stimulated hexose transport into isolated adipocytes from the same colony of mice [31], and suggests that the doses of insulin utilized in this study are physiologically relevant. Furthermore, the above time and hormone concentration dependence of the ordering effect may be correlated with the response of a variety of insulin sensitive functions in a number of cell types, such as stimulation of glucose transport, protein synthesis, intracellular enzyme activity, and inhibition of lipolysis [32].

The concept that insulin can modify the ordering of the lipid matrix is consistent with the detected effects of insulin on membranes labeled with the steroid spin labels cholestane and androstane. Although $S(T_\perp)$ is a polarity-uncorrected approximate order parameter, it is likely that the noted decreases in absolute magnitude due to addition of insulin are due to a more ordered membrane lipid environment.

Although the insulin-induced ordering within the membrane must be relatively substantial to be detected with extrinsic probe molecules equilibrated into the membrane, our data suggest that the effect is limited to certain lipid domains in the liver membrane. The fluorescent and steroid spin probe derivatives are capable of sampling relatively ordered lipid regions, whereas the I(12,3) spin label appears to preferentially reside in fluid lipid domains [35,36]. The absence of any effects of insulin on I(12,3)-labeled liver plasma membrane spectra suggests that the hormone does not influence the lipid structure of widespread ('bulk fluid' lipid) regions in the bilayer. Our experiments with PC/cholesterol vesicles support this interpretation. For example, the vesicle titration data (Fig. 7) suggests that I(12,3) preferentially samples cholesterol-poor regions of lipid in the mixed vesicles for the following reasons: Probe-probe interaction effects that cause spectral broadening and decreases in $S(T_\perp)$ at high probe/total lipid ratios are greater in vesicles containing increasing amounts of cholesterol. That this is due to exclusion of I(12, 3) from cholesterol-rich regions in the vesicles is supported by the finding that the broadening effects may be 'normalized' by replotting the $S(T_\perp)$ values as a function of nmol probe/nmol PC. The relative spectral broadening ($\Delta S(T_\perp)\%$) is then comparable for all the vesicles examined. This would be expected if I(12, 3) preferentially incorporated into PC molecules in the mixed system.

We also were unable to detect any insulin effects on order parameters calculated from liver membranes with the 5-nitroxide stearate methyl ester (5-NMS) probe (data not shown). Earlier studies with the I(12,3) and 5-NMS labels suggests that in biological membranes these spin probes sample distinct lipid environments themselves [33,34]. On the basis of our observations of differential effects of the spin probes to insulin, we suggest that the hormone causes structural changes in specific domains within the membrane matrix, which may be cholesterol-rich with respect to the bulk lipid phase.

In order to investigate the cause of these changes in the membrane, it is first necessary to consider how the motion of lipid domains may be influenced by structural reorganization of membrane components. Our studies indicating that insulin treatment induces a larger change in the anisotropy of the *cis* compared to the *trans* methyl ester isomer of parinaric acid suggest that gel phase domains within the membrane are not increased by insulin treatment, since the *trans* isomer would be expected to partition preferentially into such a domain [26]. In this case, a larger change in anisotropy would be expected for the *trans* probe compared to the *cis* [26].

Studies with methyl esters of *cis*- and *trans*-parinaric acid embedded in liposomes of PC with differing molar ratios of cholesterol to PC, demonstrated that, at least in this model system, the two isomers yielded identical changes in anisotropy as a function of cholesterol content. This suggests that both probes were capable of sensing cholesterol rich domains formed within the PC liposomes, and are equally sensitive to their formation. In liver plasma membranes, the change in anisotropy of the *trans* probe due to insulin binding was on the order of about 50% of that of the *cis* isomer. This observation is not consistent with the concept that insulin induces a redistribution of cholesterol to membrane domains sensed by these probes, assuming that the relative partitioning behavior of the parinaric acid isomers is similar in the membrane to that of PC liposomes. The observations of Shlatz and Marinetti [39] are not consistent with the view that insulin induces ordering of the lipid matrix by increasing calcium binding, as these authors demonstrated that insulin actually causes a reduction in the total calcium bound to the liver plasma membrane at 10^{-8} M insulin.

There is some evidence, however, to suggest that insulin can modify the organization of intrinsic membrane proteins. Luly and Shinitzky [6] observed that insulin binding to liver plasma membranes causes an increase in tryptophan fluorescence quenching by aqueous quenchers. These authors interpreted these results as being due to membrane proteins becoming more exposed to the aqueous environment, presumably because they become partially 'squeezed out' of the membrane by the effect of increased lipid ordering (i.e., that it was a secondary phenomenon to increased order within the membrane matrix). The possibility must also be considered, however, that protein reorganization due to insulin binding may itself mediate the observed effects on the lipid phase.

The significance of the cytochalasin B inhibition of insulin-induced ordering is difficult to interpret. Binding sites for this drug have been established as the glucose transporter [40], the barbed end of actin filaments [41], and actin-actin interfilament cross-linking sites [41]. Binding of cytochalasin B to the liver plasma membrane glucose transporter does not seem to be the basis of action of the drug in this study, as in additional experiments we found that 50 μM dihydrocytochalasin B was equally effective at inhibiting insulin-induced ordering in membranes as cytochalasin B. Dihydrocytochalasin B does not bind to the glucose transporter [42]. Binding of cytochalasin B to the barbed end of actin filaments of the liver plasma membrane cytoskeleton (preventing further polymerization) is also an unlikely explanation for the observed effects of insulin, since actin polymerization cannot occur in in vitro preparations in the absence of G-actin monomer. However, binding of cytochalasin B to actin interfilament junctions may alter the strength of the cytoskeletal gel, as postulated by MacLean-Fletcher and Pollard [41]. Although other mechanisms cannot be ruled out, proteins of the cytoskeletal matrix may be involved in the observed direct effects of insulin on the plasma membrane. It is of interest that a number of receptor systems become associated with the plasma membrane cytoskeleton after ligand occupancy, such as platelet surface glycoproteins [43], lymphocyte surface Ig receptor [44], and the neutrophil *N*-formyl peptide receptor [45]. Furthermore, both the association of the human neutrophil *N*-formyl peptide receptor with the plasma membrane cytoskeleton and the internalization process of the ligand/receptor complex are inhibited by incubation of the cells with cytochalasin B [45,46]. The concept that the occupied insulin receptor can modify the cytoskeletal organization is supported by the studies of Goshima et al. [47]. These authors demonstrated that insulin binding to KB cells induces a 'ruffling' of the plasma membrane, with actin accumulating in the ruffled regions. That this phenomena is linked to the mechanism of action of the hormone is supported by the observation that cytochalasins inhibit both the ruffling phenomena and insulin induced acceleration of amino acid transport in these cells. In an earlier study, Jarett and Smith [48] observed that cytochalasins were without effect on insulin-induced acceleration of hexose uptake into rat adipocytes although the drugs did inhibit insulin receptor clustering. Therefore, the mechanisms of activation of these different transport processes may be distinct.

The observed rightward shift in the insulin dose-response curve of DPH labeled plasma mem-

branes from ob/ob mice observed in this study is consistent with the reduced specific insulin binding to hepatocytes from this 'insulin resistant' animal [49]. It is also of interest that the insulin concentrations required for both half-maximal effects in this study closely parallel those required for insulin activation of hexose transport into adipocytes from normal (ob/?) and obese (ob/ob) mice from the same colony [31]. These observations indicate that the saturable insulin ordering effects on liver plasma membrane occurs at physiologically relevant hormone concentrations and is modified by insulin resistance at the membrane level.

Membrane structural alterations elicited by insulin may provide an explanation for some of the pleiotropic effects of the hormone at the membrane level as postulated by Luly and Shinitzky [6]. Indeed many studies suggest that many membrane bound enzyme activities are modulated by the motional properties of membrane lipids [50,51]. With respect to insulin action, for example, it is possible that the hormone may mediate decreases in basal and glucagon-stimulated adenylate cyclase in intact hepatocytes [52,53] by increasing the 'ordering' of membrane lipid surrounding the enzymes [54]. Such structural alterations may be involved in the ability of insulin to antagonize effects of catabolic hormones on cell function. It is of interest that the insulin antagonist norepinephrine was reported to 'disorder' liver plasma membran lipids sampled by diphenylhexatriene [55]. However, additional studies are necessary to determine whether such direct hormone structural/functional relationships exist.

Acknowledgements

We gratefully acknowledge Drs. L.A. Sklar and A.G. Lee for helpful comments with the preparation of the manuscript, Dr. Arne N. Wick for advice, Christopher E. Kuhn for excellent technical assistance, and the California Metabolic Research Foundation for support. This work was supported in part by a grant from the NIH (AM28431).

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