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EFFECT OF CALCIUM, INSULIN AND GROWTH HORMONE ON MEMBRANE FLUIDITY

A SPIN LABEL STUDY OF RAT ADIPOCYTE AND HUMAN ERYTHROCYTE GHOSTS

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Summary

ESR spectra were recorded from rat epididymal adipocyte ghosts labeled with the 5-nitroxide stearic acid spin probe, I(12,3). Polarity-corrected and approximate order parameters, that are sensitive to the flexibility of the incorporated label, were used to evaluate the membrane lipid fluidity. Addition of $CaCl_2$ at 37°C decreased the fluidity, as indicated by positive increases in the order parameters. The ordering effect of Ca^{2+} was concentration-dependent, reached saturation at approx. 3—4 mM, and was completely reversed by excess EGTA. Previous studies indicated that low- and high-affinity sites on adipocyte plasma membranes are able to bind $^{45}Ca^{2+}$, and our results suggest that Ca^{2+}-induced alterations in the lipid fluidity involve cation binding to low-affinity sites.

The cellular movements of Ca^{2+} and, in particular, the binding of Ca^{2+} to the plasma membrane may play important roles in insulin's action on fat cell function. The possibility that insulin directly alters the membrane fluidity was tested by adding hormone to freshly-prepared I(12,3)-labeled adipocyte ghosts. Insulin, at concentrations (10^{-6} M) that enhance glucose uptake into intact adipocytes, did not affect the fluidity of ghosts suspended in buffers with or without Ca^{2+}. The fluidities of I(12,3)-labeled rat adipocyte ghosts or human

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Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

erythrocyte ghosts were also unaffected by various forms of human growth hormone.

Introduction

The adipocyte plasma membrane provides a selective permeability barrier to various substrates and ions and contains receptors for numerous agents that influence fat cell activities. The effects induced by growth hormone and insulin on the metabolism of carbohydrates and lipids in adipose tissue [1,2], and the action of extracellular calcium on fat cell functions [3,4], have been particularly well studied. The surface membrane undoubtedly plays an important role in mediating the effects of these agents. For example, Batchelor et al. [5] suggested that in vitro addition of growth hormone may increase glucose metabolism in adipose tissue by initially perturbing the cell surface, presumably through the interaction of growth hormone with specific receptors on the plasma membrane [6]. Subsequent to the binding of insulin to fat cell surface receptors, the membrane exhibits alterations in glucose and ion transport and in enzymatic activities [7]. Lastly, Ca^{2+} has generally been found to influence a wide variety of functional properties of biological membranes [8,9]. Previous studies indicate that Ca^{2+} modulates the response of fat cell functions to insulin [3,4,10], and Bonne et al. [4] suggested that Ca^{2+} stabilizes the conformation of the adipocyte membrane to insure an optimal response of the cell to this hormone. Several investigators [7,11,12] have also proposed that Ca^{2+} may act as a 'second messenger' for insulin.

The molecular details of the structural alterations underlying the effects of hormones and divalent cations on adipocyte activities remain largely uncharacterized. An intriguing possibility that has been experimentally investigated in various tissues is that insulin [13,14], growth hormone [15] and Ca^{2+} [9,16] may change the lipid fluidity, thereby altering cellular functions. If hormones and/or divalent cations were to perturb the plasma membrane fluidity, then these agents might also exert pleiotypic (multiple) effects on cell activities since the fluid state of lipids regulates such membrane-associated properties as glucose [17,18] and passive ion transport [19—21], and hormone-stimulated adenylate cyclase [22] and $(Na^+ + K^+)$-ATPase [23] activities. In the case of adipocyte plasma membrane, conflicting evidence has been obtained with fluorescence probe techniques concerning the ability of insulin to induce widespread structural alterations in hydrophobic regions [14,24]. Moreover, the effects of Ca^{2+} and growth hormone on the fluidity of adipocyte membranes have not been previously investigated.

The spin-label method was employed here as a tool to examine the effects that insulin, human growth hormone and Ca^{2+} additions exert on the lipid structure of rat adipocyte ghost membranes. The electron spin resonance (ESR) spectra of rat adipocyte ghosts labeled with 5-nitroxide stearate, I(12,3), were used to monitor the fluidity of the lipid environment of the probe. Here, we report that millimolar concentrations of Ca^{2+} decreased the fluidity of I(12,3)-labeled adipocyte ghosts. Since changes in Ca^{2+} binding to membranes can be triggered by certain hormones such as insulin (for reviews see Refs. 7, 8, 11),

we tested the effects of insulin and various forms of human growth hormone on the fluidity of I(12,3)-labeled adipocyte ghosts suspended in buffers with (and without) mM Ca^{2+}. In addition, the perturbations induced by human growth hormone forms on the fluidity of I(12,3)-labeled human erythrocyte ghosts were examined.

Methods

Preparation of cells

Erythrocytes. Human blood was collected by venipuncture from normal male donors at Scripps Clinic. Erythrocytes were isolated by low speed centrifugation and ghosts were prepared according to Dodge et al. [25]. The ghosts were washed three times by centrifuging at 20 000 × *g* for 40 min and then suspended (10 mg ghost protein/ml) in either 50 mM Tris/8% sucrose or Krebs-Ringer bicarbonate buffer, pH 7.4. For the spin label experiments, only freshly-preparated erythrocyte ghosts were employed.

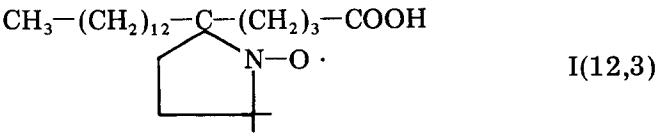
Adipocytes. Adipocytes were isolated from epididymal adipose tissue of male Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA) as described by Rodbell [26]. For each batch of intact cells, tissue was collected from 10 rats, individually weighing 130—160 g. The tissue was washed in saline and digested (in 4-g portions) with 3.2 mg collagenase (Lot No. 4194CLS475239 from Worthington Chemicals, Freehold, NJ) and 2.1 ml Krebs-Ringer bicarbonate/3% albumin (Fraction V from Sigma Chemical Co., St. Louis, MO) per g fat for approx. 30 min. The cells were expressed through polyester multifilament cloth (mesh 0.0056 inch) (McLogan Supply Co., Inc., San Diego, CA) with the aid of a plastic syringe, collected from the filtrate and washed in Krebs-Ringer bicarbonate/3% albumin by repeated centrifugation at 400 × *g* for 15 s. Ghosts of adipocytes were prepared by lysing batches of approx. 4—6 ml of packed cells in hypotonic buffer essentially as described in [27]. The ghosts were washed by repeated centrifugation at 900 × *g* for 15 s and finally suspended in either 50 mM Tris/8% sucrose, pH 7.4, or in Krebs-Ringer bicarbonate without albumin. Protein was determined by the method of Hartree [28].

Glucose uptake measurements

Following the procedure of Olefsky [29], adipocytes ($2 \cdot 10^6$ cells, assuming that a 5 ml packed cell volume contains approx. $39 \cdot 10^6$ cells [30]) were incubated in four vials, each containing 2 ml Krebs-Ringer bicarbonate/3% albumin, pH 7.4, and 1 mM glucose (Mallinckrodt, St. Louis, MO). Dinonyl phthalate oil was added to the vials, and the cell suspensions were centrifuged at 900 × *g* for 15 s to separate the cells from the buffer. The glucose concentrations of the infranatants were determined in quadruplicate from each vial by the glucose oxidase-peroxidase method (kits were purchased from Sigma Chemical Co. and were used as described previously [31]). Glucose uptake was defined as the difference between the average glucose concentration in the zero time control vials and additional vials incubated for 2 h at 37 °C. To examine the effects insulin exerts on glucose uptake, additional vials of cells were incubated at 37 °C for 2 h after addition of porcine insulin (kindly supplied by Eli Lilly Co., Indianapolis, IN).

Spin labeling and spectral measurements

The spin probe 5-nitroxide stearate (I(12,3)), where nitroxide refers to the 4′,4′-dimethyl-*N*-oxyloxazolidine ring, came from Syva Co., Palo Alto, CA.



I(12,3)

The label was dissolved in absolute ethanol (10^{-3} M) and stored at −70°C in liquid nitrogen storage tubes (Microbiological Assoc., Los Angeles, CA). Freshly-prepared adipocyte ghosts, or adipocyte ghosts stored at −70°C, were labeled with I(12,3) at room temperature as in Ref. 9. The ratio of spin label to adipocyte ghost protein was varied from 1.0 to 9.5 μg I(12,3)/330 μg protein. Erythrocyte ghosts were similarly labeled with a probe/ghost protein ratio of 4.3 μg I(12,3)/mg. ESR spectra were recorded, after a 4–5 min period for temperature equilibration of the sample, with a Varian E-104A Century Series ESR spectrometer equipped with a variable temperature accessory [9].

The fluidity of the membrane-incorporated label was quantitated by first measuring the outer and inner hyperfine splittings $2T_{\parallel}$ and $2T_{\perp}$ (shown in Fig. 1) and then employing the following order parameter expressions [32]:

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

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

$$S = \frac{(T_{\parallel} - T_{\perp})(a_N)}{(T_{zz} - T_{xx})(a_{N'})} \tag{3}$$

T_{zz} and T_{xx} were previously determined by incorporating nitroxide derivatives into host crystals as substitutional impurities (T_{xx},T_{zz}) = (6.1, 32.4)G [33].

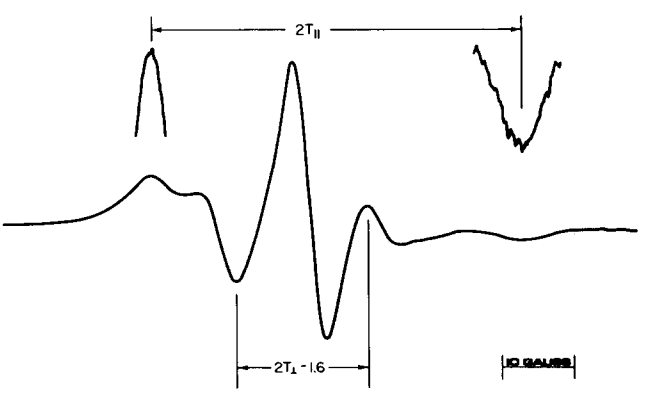


Fig. 1. ESR spectrum of rat epididymal adipocyte ghosts labeled with I(12,3) (1 μg I(12,3)/330 μg ghost protein) at 37°C. The complete unexpanded spectrum was recorded at a 100 G field sweep, a receiver gain of $5 \cdot 10^3$, 4 min scan time, $0.63 \cdot 10^1$ G modulation amplitude at 10 mW microwave power at 37°C. The outer wings were also magnified by recording at a $5 \cdot 10^4$ receiver gain with a 16-min scan time. $2T_{\perp}$ was corrected by the addition of 1.6 G [34].

$a_{N'}$ and a_N are the isotropic hyperfine coupling constants for the probe in the membrane and crystal state, respectively [$a_{N'} = 1/3(T_\parallel + 2T_\perp)$ and $a_N = 1/3(T_{zz} + 2T_{xx})$]. $a_{N'}$ is sensitive to the polarity of the membrane environment of the probe [32,34].

The order parameters S, $S(T_\parallel)$ and $S(T_\perp)$ are a reflection of the membrane fluidity (or, more accurately, the flexibility of the membrane-incorporated probe). S, $S(T_\parallel)$ and $S(T_\perp)$ may each assume values between 0 and 1; these extreme order parameters indicate that the probe samples fluid and immobilized environments, respectively. The order parameter S, which requires both hyperfine splittings, corrects for small polarity differences between the membrane and reference crystal. Although $S(T_\parallel)$ and $S(T_\perp)$ do not include corrections for polarity contributions, these expressions have been found to be useful approximate measures of the fluidity in those cases where only one hyperfine splitting is useable [32]. We here continue to assess the relative abilities of S, $S(T_\parallel)$, and $S(T_\perp)$ to monitor changes in the flexibility of the probe with I(12,3)-labeled adipocyte ghosts.

Addition of agents

Clinical grade human growth hormone, a 191 amino acid monomeric growth-hormone form (intact monomeric human growth hormone), and a naturally-occurring diabetogenic substance (i.e., a peptide isolated from human pituitaries that induces glucose intolerance and hyperinsulinemia in the dog [35] and causes insulin release from perifused rat pancreatic islets [36]), were prepared in this laboratory [35].

The effects of such agents as $CaCl_2$, NaCl, EGTA (all from Sigma Chemical Co.), various forms of human growth hormone, and insulin on the membrane fluidity were examined as follows. Duplicate ESR spectra of adipocyte (or erythrocyte) ghosts, labeled with a given I(12,3) probe concentration, were initially recorded at 37°C. The agent was then added to the sample, and spectra were measured after an 8 min period. We used this protocol since the binding of $^{45}Ca^{2+}$ to isolated adipocyte membranes [37] and adipocyte ghosts [38] reached equilibrium within 10 min.

Results

Isolated adipocytes in six separate experiments showed a basal net uptake of glucose that averaged 683 ± 157 nmol per 2-h period. Insulin (5 μg/ml) enhanced the uptake of glucose by 45 ± 20% in six preparations. The basal uptake of glucose may be slightly higher while the insulin-induced increase may be somewhat lower than data previously reported by Olefsky [29].

An ESR spectrum of I(12,3)-labeled rat adipocyte ghosts (shown in Fig. 1) is similar to that obtained for other I(12,3)-labeled model [32,34] and biological [9,16,39,40] membranes. Fig. 1 indicates that the label undergoes rapid, anisotropic motion about its long molecular axis in the membrane. Moreover, flexing or bending motions of the probe (i.e., the angular deviation of the hydrocarbon chain away from the preferred orientation perpendicular to the membrane surface) are relatively restricted. Alternatively, the spectrum recorded from I(12,3)-labeled adipocytes was essentially isotropic, suggesting

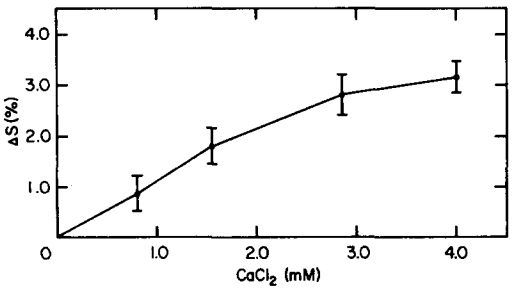


Fig. 2. Effects of Ca^{2+} on the fluidity of I(12,3)-labeled adipocyte ghosts. Three separately-prepared batches of ghosts, stored at −70°C, were pooled for these experiments. Each point represents an average of four separate additions of $CaCl_2$. $\Delta S(\%)$ is the percentage difference between order parameters measured from samples with and without $CaCl_2$. Error bars indicate ± S.D. The probe/ghost protein ratio was 1 μg I(12,3)/330 μg.

that the probe primarily distributes into intracellular lipids. We were therefore unable to assess the direct effects of agents on the membrane fluidity of intact cells.

Similar to results observed with I(12,3)-labeled rat liver and heart plasma membranes [9,39] and lymphocytes [41], spectral alterations characteristic of probe-probe interactions occurred when fat-cell ghosts were labeled with experimentally-determined 'high' I(12,3) probe concentrations. Accordingly, all order parameters presented in this report were measured from adipocyte ghosts labeled with 'magnetically-dilute' probe concentrations (i.e., less than 3.1 μg I(12,3)/330 μg protein). The use of experimentally-determined low I(12,3) concentrations enabled us to evaluate intrinsic properties of adipocyte ghost membranes as defined by Sauerheber et al. [39].

The effect of calcium on the adipocyte ghost fluidity was tested by adding $CaCl_2$ to I(12,3)-labeled ghosts at 37°C. Millimolar $CaCl_2$ decreased the membrane fluidity of ghosts that had been stored at −70°C, as indicated by positive increases in ΔS (Fig 2). The calcium effect on S was apparently a concentration-dependent saturable process reaching a plateau at about 3 mM. The use of saturating concentrations of $CaCl_2$ increased S by approximately 3%; a decrease in the adipocyte ghost membrane temperature from 37 to 34°C [42] would be necessary to increase S by a similar amount. 2.8 mM $CaCl_2$ elevated $\Delta S(T_\parallel)$, $\Delta S(T_\perp)$ and ΔS (Table I) to the same degree and also had no effect on the isotropic hyperfine coupling constant $a_{N'}$ (data not shown). This indicates that the Ca^{2+} effects were due only to changes in the flexibility of the membrane-incorporated probe and were not the result of alterations in the polarity of the environment of the probe. Thus, $S(T_\parallel)$, $S(T_\perp)$ and S were equally efficacious in detecting Ca^{2+}-induced changes in the flexing of I(12,3) in adipocyte ghost membranes.

The nature of the Ca^{2+}-induced rigidization of the I(12,3)-labeled adipocyte ghosts was characterized in additional experiments. The ordering effect of 2.8 mM Ca^{2+} was readily reversed by adding the divalent cation-chelating agent EGTA (25 mM) (Table I). Further, a premixed solution of $CaCl_2$ and EGTA did not increase the order parameters of the labeled fat cell ghosts, and 9.1 mM NaCl had no significant effect. These data suggest that the Ca^{2+}-induced order-

TABLE I

THE EFFECTS OF $CaCl_2$, EGTA, AND NaCl ON THE ORDER PARAMETERS OF I(12,3)-LABELED ADIPOCYTE GHOSTS AT 37°C

The relative effects of additions of $CaCl_2$ (I), NaCl (IV), and a premixed solution of $CaCl_2$ and EGTA (III) on the order parameters of I(12,3)-labeled adipocyte ghosts. The reversibility of the effect of $CaCl_2$ was demonstrated in II by adding EGTA to the samples with 2.8 mM $CaCl_2$ used in I. The premixed EGTA/$CaCl_2$ solution used in III contained these agents in a 8.9:1 molar ratio so that the final concentrations after addition to the ghost samples were 25 and 2.8 mM, respectively. Δ(order parameters)% is the percentage difference in the order parameters measured in the presence and absence of each agent. The numbers in parentheses indicate the standard deviations from 4(I), 3(II), 3(III), and 4(IV) separate determinations. The probe/ghost protein ratio was 1 μg I(12,3)/330 μg. Adipocyte ghosts were stored at −70°C before use.

Agent additions		$\Delta S(T_\parallel)\%$	$\Delta S(T_\perp)\%$	$\Delta S\%$
2.8 mM $CaCl_2$	(I)	+2.7 (1.0)	+3.1 (1.0)	+2.8 (0.4)
2.8 mM $CaCl_2$ plus 25 mM EGTA	(II)	−2.0 (0.3)	−0.7 (0.6)	−1.2 (0.7)
Premixed $CaCl_2$ (2.8 mM) and EGTA (25 mM)	(III)	−3.0 (1.3)	−1.3 (0.6)	−2.1 (0.3)
9.1 mM NaCl	(IV)	+0.5 (1.0)	+0.5 (1.1)	+0.6 (0.65)

ing of the ghost membrane is due to the reversible binding of Ca^{2+} to discrete membrane sites and is not simply the result of an increase in ionic strength.

Next we examined the direct effects of insulin, clinical grade human growth hormone, intact monomeric human growth hormone, and the naturally-occurring diabetogenic substance on the fluidity of freshly-prepared adipocyte ghosts and found that none of these agents appreciably changed the order parameters at 37°C (Table II). The increase in the order parameters of fresh adipocyte ghosts induced by 2 mM Ca^{2+} is shown in Table II and compares favorably with the corresponding effects found with adipocyte ghosts stored frozen at −70°C (Fig. 2). In additional experiments, insulin did not significantly alter the membrane fluidity when ghosts were incubated in Tris buffer without $CaCl_2$ or in Krebs Ringer bicarbonate buffer, pH 7.4, from which

TABLE II

EFFECTS OF INSULIN, HUMAN GROWTH HORMONE FORMS AND $CaCl_2$ ON THE ORDER PARAMETERS OF I(12,3)-LABELED ADIPOCYTE GHOSTS AT 37°C

The effects of each agent on the order parameters were tested with fresh adipocyte ghost samples within 4—6 h after preparation. The labeled ghosts were suspended in 50 mM Tris/8% sucrose, pH 7.4, but for experiments II through V, 2 mM $CaCl_2$ was also present in the buffer. Δ(order parameter)% is the percentage difference between initial order parameters and those calculated after addition of the agent. The numbers in parentheses indicate the standard deviations for three separate determinations. The probe/ghost protein ratio was 2.5 μg I(12,3)/330 μg.

Agent additions		$\Delta S(T_\parallel)\%$	$\Delta S(T_\perp)\%$	$\Delta S\%$
2.0 mM $CaCl_2$	(I)	+3.3 (0.7)	+2.1 (0.7)	+2.5 (0.6)
Clinical grade human growth hormone (29 μg/ml)	(II)	+0.2 (0.7)	+0.5 (1.0)	+0.3 (0.5)
Naturally-occurring diabetogenic substance (15 μg/ml)	(III)	−1.1 (1.0)	−0.9 (1.0)	−0.5 (1.2)
Intact monomeric human growth hormone (15 μg/ml)	(IV)	+0.3 (0.7)	+0.2 (1.0)	+0.2 (0.5)
Insulin (7 μg/ml = $1.2 \cdot 10^{-6}$ M)	(V)	−0.3 (0.5)	+0.0 (0.5)	+0.5 (0.6)

TABLE III

EFFECT OF HUMAN GROWTH HORMONE ON THE ORDER PARAMETERS OF I(12,3)-LABELED HUMAN ERYTHROCYTE GHOSTS

Fresh erythrocyte ghosts, suspended in Krebs-Ringer bicarbonate buffer, were labeled with 4.3 μg I(12,3)/mg protein. Order parameters were calculated from spectra of the labeled ghosts and then from the same sample after addition of each agent at 37°C. Two separate batches of ghosts were employed and the errors represent the standard deviations of three determinations for each substance.

Agent additions	$S(T_{\parallel})$	S	$S(T_{\perp})$
None	0.693 (0.012)	0.640 (0.009)	0.599 (0.010)
40 μg/ml intact monomeric human growth hormone	0.695 (0.012)	0.637 (0.010)	0.596 (0.009)
21 μg/ml naturally occurring diabetogenic substance	0.698 (0.004)	0.642 (0.007)	0.604 (0.010)
27 μg/ml clinical grade human growth hormone	0.692 (0.011)	0.634 (0.008)	0.592 (0.011)

albumin was omitted (data not shown). Bailey et al. [14] recently reported that 150 nM insulin altered the viscosity of rat adipocyte plasma membranes labeled with the fluorescence probe 12-(9-anthroyl)stearate only if the membranes were incubated with hormone before labeling. Therefore, we preincubated adipocyte ghosts (suspended in buffers with or without $CaCl_2$) with insulin at room temperature for approx. 10 min and then incorporated the I(12,3) probe. The order parameters, however, were the same as those determined from I(12,3)-labeled samples not treated with insulin.

Because human growth hormone preparations reportedly alter structural properties of human erythrocyte ghosts [15,43,44], we tested clinical grade human growth hormone, intact monomeric human growth hormone and naturally-occurring diabetogenic substance with fresh I(12,3)-labeled human erythrocyte ghosts. The experimental conditions described by Sonenberg [43], in which erythrocyte ghosts were suspended in Krebs-Ringer bicarbonate buffer, were closely followed; ghosts incubated in Tris buffer were also examined (data not shown). The order parameters calculated before and after the addition of each agent were not significantly different (Table III).

Discussion

Ca^{2+} decreased the lipid fluidity of I(12,3)-labeled rat adipocyte ghosts in a concentration-dependent, saturable and reversible manner, probably by binding to specific membrane sites. In previous studies of isolated rat adipocyte plasma membranes, two distinct classes of binding sites for $^{45}Ca^{2+}$ were detected: (1) low-affinity sites with an association constant of $2 \cdot 10^3\ M^{-1}$ and a maximum binding capacity of 13.7 nmol/mg membrane protein, and (2) high-affinity sites (association constant = $4.5 \cdot 10^4\ M^{-1}$) having a maximum binding capacity of 1.8 nmol/mg protein [37]. The fact that Ca^{2+} induces alterations in the fluidity of the I(12,3)-labeled adipocyte ghosts that are half-saturated at approx. 1.5 mM suggests that these effects are due to Ca^{2+} binding to low-affinity plasma membrane sites. Conceivably, Ca^{2+}-binding constituents of membranes such as acidic phospholipids, protein, or sialic acid residues are involved in this process. One possibility that should be explored is whether

other divalent cations might similarly lower the fluidity of labeled ghosts by binding to these low-affinity sites.

It is of interest that the order parameters of I(12,3)-labeled adipocyte ghosts treated with excess EGTA are significantly decreased below those values measured in the absence of any agent (Table I), inasmuch as similar EGTA treatments of I(12,3)-labeled rat liver (Sauerheber, R. and Gordon, L., unpublished data) or heart [9] plasma membranes did not increase the membrane fluidity. The protocol for obtaining adipocyte ghosts involves the use of Ca^{2+}- and Mg^{2+}-containing media, and residual divalent cations may remain bound to the membranes despite repeated washings with Tris buffer. Although the EGTA-dependent fluidization of adipocyte ghosts may reflect the removal of Ca^{2+} from membrane sites of relatively high affinity, additional experiments will be necessary to test this possibility.

The effects that Ca^{2+} exerts on the fluidity of adipocyte ghost lipids may be physiologically significant. Basal and insulin-stimulated incorporation of [^{3}H]histidine into adipocyte protein require millimolar concentrations of extracellular Ca^{2+} [10]. Moreover, millimolar Ca^{2+} is also necessary for insulin to exert optimal effects on glucose oxidation, the membrane transport of 2-deoxyglucose, and lipogenesis in adipocytes [3,4]. Lastly, millimolar Ca^{2+} decreases the activity of adenylate cyclase in adipocyte ghosts [45]. Since the lipid fluidity may regulate such properties of membranes as glucose and ion transport and the activities of adenylate cyclase and $(Na^+ + K^+)$-ATPase [17—23], Ca^{2+} might exert its effects on adipocytes by mediating decreases in the membrane lipid fluidity. Thus, the rigidizing effect of Ca^{2+} may be an important structural feature of the plasma membrane of adipocytes that are optimally responsive to insulin. Although changes in the lipid fluidity may be one mechanism by which Ca^{2+} alters adipocyte membrane activities, other regulating models involving direct cation-protein interactions cannot be excluded.

A number of hormones interact with receptors on cells without penetrating the surface, yet nevertheless influence the cell's physiological activity [15, 46,47]. In the case of hormones such as glucagon and epinephrine that stimulate adenylate cyclase activity, it is useful to define a transducer as being a membrane component that propagates signals from the surface receptor to a membrane effector (i.e., adenylate cyclase on the inner face of the membrane) [47]. However, the precise mechanism by which information is transferred through the membrane for hormones that act through adenylate cyclase is largely undefined. There is even more uncertainty concerning the nature of the tranducer(s) for insulin and growth hormone, since the primary action of these hormones is believed to be independent of cyclic AMP production. Undoubtedly, plasma membrane components must undergo physicochemical alterations after a hormone binds to its receptor. These alterations might be integral to hormone action, and could conceivably be present on a large scale, particularly for hormones that exert pleiotypic effects on target tissues. For example, insulin influences the transport of glucose, amino acids and ions, and the activities of membrane-bound $(Na^+ + K^+)$- and Mg^{2+}-ATPases [7]. In this regard, insulin also induces gross alterations in certain structural properties of membranes. Elevated numbers of microvilli have been observed on the sur-

face of insulin-treated fibroblasts [15], and insulin increases the invaginations and intramembranous particles of fat cell plasma membranes as detected by freeze-fracture electron microscopy [48]. The interrelationships of these insulin-dependent structural and functional perturbations remain indeterminate, at present.

One hypothesis that has recently been investigated with various spectroscopic probes is that changes in the lipid structure and/or fluidity of the membrane may serve as a transducer of hormonal information. Sonenberg [43] suggested that human growth hormone-induced alterations in the fluorescence properties of human erythrocytes reflect a wide-spread perturbation in the membrane structure; these effects were attributed to changes in either the protein conformation or the lipid viscosity. Postel-Vinay et al. [49] also noted bovine growth hormone modified the emission spectra of the hydrophobic (lipid) fluorescence probe 7,12-dimethylbenzanthracene in rat liver plasma membrane, and suggested that bovine growth hormone produces a conformational change in membrane protein and lipid. Luly and Shinitzky [13] reported that direct in vitro addition of insulin to rat liver plasma membranes labeled with the fluorescence probe 1,6-diphenyl-1,3,5-hexatriene increased the lipid viscosity. On the other hand, Bailey and coworkers [14] observed no alterations in the lipid viscosity when insulin was directly added to rat adipocyte plasma membranes labeled with the fluorescence probe 12-(9-anthroyl)stearate; however, incubation of a membrane suspension with insulin prior to labeling effected a decrease in the microviscosity of 12-(9-anthroyl)stearate. The findings of Bailey et al. [14] are in contrast to an earlier structural and functional study by Avruch and coworkers [24] of plasma membranes from adipocytes pre-treated with insulin. Although membranes from such insulin-treated cells exhibit increased glucose uptake [24] and Ca^{2+} binding capacities [50], no changes occurred in the native membrane infrared spectra, native membrane protein fluorescence, or the fluorescence of 8-anilino-1-naphthalene sulfonate added to the membrane. Avruch et al. [24] concluded from these results that insulin action is not associated with large-scale alterations in adipocyte membrane hydrophobic regions. All of the above studies must, however, be viewed as preliminary inasmuch as none have been confirmed by independent investigators.

The above uncertainties in the existing literature prompted us to investigate whether insulin or human growth hormone affects the fluidity of spin-labeled biological membranes. We report here that the direct addition of insulin or human growth hormone does not alter the order parameter of I(12,3)-labeled adipocyte ghosts and that human growth hormone also does not affect the lipid ordering of I(12,3)-labeled erythrocytes ghosts. Furthermore, no changes were detected in the fluidity of adipocyte ghost membranes that were pretreated with insulin following the procedure of Bailey et al. [14]. Earlier studies (Sauerheber, R. and Gordon, L., unpublished data) employing fresh rat liver plasma membranes (prepared as in Ref. 40) indicated that insulin had no effect on the fluidity of I(12,3)-labeled membranes, suspended in buffers with or without Ca^{2+}.

The present observations that in vitro additions of either insulin or human growth hormone do not alter the fluidity of several I(12,3)-labeled biological

membranes may be viewed as supporting the findings of Avruch et al. [24] and as contradicting the fluorescence studies of Sonenberg [43], Luly and Shinitzky [13] and Bailey et al. [14]. It is worthwhile to consider in more detail the nature of these conflicts. There is evidence which indicates that spin labels [39,51,52] and fluorescence probes [53] may not uniformly distribute in mixed membrane systems. I(*m*,*n*) probes have been found in several binary-phase model lipid systems to partition preferentially into the more fluid phase [51,52]; moreover, the fluorescence probe 12-(9-anthroyl)stearate primarily samples the more fluid lipid (dimyristoyl phosphatidyl choline) in a binary mixture of dimyristoyl phosphatidyl choline/dipalmitoyl phosphatidyl choline containing equimolar proportions of these lipids [53]. Since lipid domains of differing structure and/or fluidity may coexist in biological membranes [20,21], it is possible that fluorescence and spin probes do not similarly distribute in complex membrane systems such as liver or adipocyte plasma membranes. Thus, the apparent disparate effects of insulin on the fluidity of rat liver plasma membrane reported by ourselves (see above) and Luly and Shinitzky [13] may be due to I(12,3) sampling membrane regions that are not only distinct from those probed by 1,6-diphenyl-1,3,5-hexatriene but are also unaffected by insulin binding to its receptor. This explanation could also be invoked to account for why insulin increased the fluidity of 12-(9-anthroyl)-stearate labeled adipocyte plasma membranes [14], while we observed no effect when I(12,3)-labeled fat cell ghosts were similarly treated. Lastly, the absence of a perturbation by human growth hormone on the fluidity of I(12,3)-labeled human erythrocyte ghosts does not necessarily conflict with the results of Sonenberg [43], if human growth hormone were to induce widespread changes in protein conformation without affecting the lipid viscosity.

Another explanation as to why insulin and human growth hormone do not affect the lipid fluidity of I(12,3)-labeled adipocyte ghosts might be that the spin probe primarily distributes into intracellular membranes that are unresponsive to hormone. Although adipocyte ghosts are right-side-out saccules of plasma membrane that are essentially devoid of fat and nuclei, the I(12,3) label may partially incorporate into the intracellular membranous material (e.g., mitochondria and endoplasmic reticulum) that contaminate these preparations. However, some evidence suggests that a substantial proportion of probe resides in the surface membrane of adipocyte ghosts. For example, Fig. 2 indicates that the affinity of those sites involved in the Ca^{2+}-dependent ordering of the membrane is comparable to that reported for low-affinity $^{45}Ca^{2+}$ binding sites on isolated adipocyte plasma membranes (see above). Moreover, the addition of 1 mM $LaCl_3$ to I(12,3)-labeled adipocyte ghosts dramatically decreased the lipid fluidity (data not shown). If La^{3+} were to be barred from the interior of ghosts, as it is effectively excluded in a wide variety of whole cells [54—56], then the La^{3+} effect is probably associated with the plasma membrane *. In

* The decrease in the bilayer fluidity effected by lanthanum is probably due to its interaction with the same low-affinity sites that bind calcium. The strong binding of La^{3+} to negatively-charged, Ca^{2+}-membrane sites might be expected since the hydrated radius of La^{3+} (3 Å) is similar to that of Ca^{2+} (2.8 Å). Furthermore, La^{3+} reportedly displaces bound Ca^{2+} from the surface membranes of a variety of tissues [55,56].

view of the above discussion, we find it unlikely that the probe principally resides in the cytosol membranes of adipocyte ghosts.

The results presented here do not preclude the possibility that the lipid structure or fluidity of plasma membranes is influenced by hormones in vivo. Liver plasma membrane isolated from rats pretreated with ethinyl estradiol exhibit increased levels of cholesterol, and the I(12,3)- or I(5,10)-labeled membranes were less fluid than those from untreated rats [57]. Furthermore, streptozotocin-diabetic rats with chronic insulin deficiency have decreased cholesterol contents in liver plasma membranes and erythrocyte ghosts [58]; these changes may be associated with alterations in the lipid fluidity, inasmuch as cholesterol additions to model [59] and biological membranes [60] either increase or decrease the membrane fluidity, depending on the initial lipid state [59]. It is also possible that the amount of Ca^{2+} bound to surface membrane sites in vivo is dependent on circulating levels of this cation. Thus, the fluidity of adipocyte (or other) plasma membranes could be altered by fluctuations in blood concentrations of Ca^{2+}, such as those that occur after oral glucose ingestion [61] or injection of glucagon [62].

Addendum

In agreement with the present study, Amatruda and Finch [63] recently reported that direct addition of insulin did not affect the fluidities of either I(12,3)-labeled rat adipocyte ghosts or plasma membranes. However, these investigators did present evidence to indicate that the physical state of membrane lipids modulates the action of insulin on hexose uptake into intact adipocytes.

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