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Diacylglycerol directly stimulates the insulin receptor tyrosine kinase

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Abstract Studies with detergent:lipid mixed micelles reveal that diacylglycerol directly stimulates the intrinsic tyrosine kinase activity of the insulin receptor. Kinetic analyses indicate that diacylglycerol activates the kinase by causing a marked increase in the affinity of the receptor for insulin. In contrast, diacylglycerol has no effect on the insulin receptor's catalytic activity or its affinity for ATP. Stimulation of the insulin receptor is not a result of protein kinase C activation. First, phorbol myristate acetate, a potent activator of protein kinase C, has no effect on insulin receptor activity. Second, the activation by diacylglycerol is not stereospecific, in marked contrast to the specificity for 1,2-diacyl-*sn*-glycerol in the activation of protein kinase C. Because circulating levels of insulin are below the K_d of the insulin receptor for insulin, the ability of diacylglycerol to modulate the affinity of the receptor for ligand suggests that increases in cellular levels of diacylglycerol directly sensitize the receptor to insulin.

Key words: Insulin receptor; Diacylglycerol; Tyrosine kinase; Protein kinase C

1. Introduction

Diacylglycerol is produced in response to the plethora of signals that activate phospholipase Cs. Such signals are now known to be processed both by G protein-coupled receptors [1], as originally discovered, and by receptor tyrosine kinases [2]. The former receptors couple to G proteins that activate members of the phospholipase Cβ family, whereas the latter receptors mediate activation of members of the phospholipase Cγ family via SH2 and SH3 domains [3]. Diacylglycerol is also produced by activation of phospholipase D to yield phosphatidic acid and then diacylglycerol [1]. The primary target of diacylglycerol is protein kinase C, a family of lipid-regulated enzymes that is allosterically regulated by diacylglycerol and phosphatidylserine [4,5]. Mounting evidence suggests that other proteins may also be sensitive to diacylglycerol. For example, the respiratory burst enzyme, NADPH oxidase, is stimulated by diacylglycerol and phosphatidic acid [6]. In addition, the activities of phospholipase A_2 [7,8] and CTP:phosphocholine cytidylyltransferase [9] are modulated by diacylglycerol.

The production of diacylglycerol in response to receptor tyrosine kinase activation led us to explore whether the insulin receptor may be regulated by diacylglycerol. This heterotetrameric protein comprises two extracellular α subunits disulfide-linked to two transmembrane β subunits [10]. Insulin binding promotes autophosphorylation, which activates the enzyme towards substrate phosphorylation. The activated receptor kinase phosphorylates its primary substrate, IRS-1, on YMXM motifs, thus converting IRS-1 into a docking protein for SH2 domain-containing proteins such as phosphatidylinositol 3-kinase or Grb2 [11]. Insulin stimulation has been reported to cause diacylglycerol levels to increase in several cell types [12,13]. The possibility that this increase is mediated by phospholipase Cγ is suggested by the finding that insulin stimulates the activity of this enzyme in plasma membrane preparations [14]. Insulin-stimulated increases in diacylglycerol concentrations have also been reported to result from de novo synthesis [12,13], from cleavage of a novel phosphatidylinositol glycan [15,16], and from cleavage of phosphatidylcholine [17].

Here we show that the lipid second messenger, diacylglycerol, directly stimulates the tyrosine kinase activity of the insulin receptor by increasing its affinity for insulin. Thus, generation of diacylglycerol in the plasma membrane may up-regulate insulin receptor activity by sensitizing the receptor to insulin.

2. Materials and methods

2.1. Materials

ATP, bovine serum albumin (BSA), 5-bromo-4-chloroindoyl phosphate, 1,2-dioleoyl-*sn*-glycerol, enzyme-free cell dissociation solution, HEPES, leupeptin, phorbol myristate acetate, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Company. 2,3-Dioleoyl-*sn*-glycerol was a generous gift from Dr. David Daleke. 1,3-Dioleoyl-*sn*-glycerol was purchased from Nu Chek Prep. All phospholipids were purchased from Avanti Polar Lipids. Alkaline phosphatase-conjugated goat anti-mouse IgG and peroxidase-conjugated goat anti-mouse IgG were supplied by Boehringer Mannheim Biochemicals and anti-phosphotyrosine antibody (PY20) was obtained from ICN Biomedicals, Inc. ^{125}I-labelled anti-mouse IgG was from Amersham and Renaissance Western blot chemiluminescence reagent was obtained from Dupont. Porcine insulin was purchased from Calbiochem and Triton X-100 (10% aqueous solution low in carbonyl and peroxide content) was supplied by Pierce Chemical Company. Nitrocellulose membranes were obtained from Schleicher and Schuell. Protein kinase C βII was purified to homogeneity from a baculovirus expression system, as described [18]. All other chemicals were reagent grade.

2.2. Methods

2.2.1. Insulin receptor. NIH 3T3 HIR 3.5 cells, which express on the order of 10^6 copies of the human insulin receptor per cell [19], served as the source of insulin receptor. Cells (approximately 5×10^6) were harvested in 10 ml enzyme-free cell dissociation solution, pelleted by centrifugation at $1300 \times g$ for 1 min at room temperature, then lysed in 4 ml solution containing 50 mM HEPES (pH 7.5), 1 mM EDTA and 1 mM PMSF. The lysate was centrifuged at $540{,}000 \times g$ for 10 min, 4°C, and the particulate fraction was resuspended in 50 mM HEPES (pH 7.5), 0.1% Triton X-100, and gently homogenized to solubilize membrane proteins. The solution was then centrifuged for 10 min at $630{,}000 \times g$, 4°C, to remove detergent-insoluble material. The supernatant, containing detergent-solubilized membrane proteins, was collected and used immediately for assays. Concentrations of endogenous lipid and protein in the detergent-soluble fraction were approximately 1 mM and 1 mg·ml^{-1}, respectively. Insulin receptor partially purified

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from rat liver by wheat germ agglutinin chromatography [20] was used in some assays.

2.2.2. Lipids. Lipids in chloroform solution were dried under a stream of N_2 and hydrated in 50 mM HEPES (pH 7.5) containing 0.1% Triton X-100 [20]. Lipid:detergent mixtures, containing 0–4 mM lipid, were diluted into an assay mix containing 0.1% Triton X-100 to yield a final detergent concentration of 0.1% and final lipid concentrations of 0–0.5 mM.

2.2.3. Insulin receptor assay. The detergent-solubilized fraction from NIH 3T3 HIR 3.5 cells (10 μl) was added to lipid:detergent mixtures (20 μl, 0–1.8 mM lipid and 0.1% Triton X-100) and vortexed. A solution (27 μl) containing 2.6 mM DTT, and 0 or 26 μM insulin in 50 mM HEPES (pH 7.5), 0.07% Triton X-100 was added and allowed to incubate for 5 min at 21°C or 13.5°C. Autophosphorylation was initiated by the addition of 14 μl of a solution containing 50 mM HEPES, 25 mM $MnCl_2$, 20 mM $MgCl_2$, 0.15% Triton X-100, and 250 μM ATP at 21°C or 13.5°C. The final concentrations of the species in reaction mixture were 10 nM insulin, 1 mM DTT, 0–0.5 mM lipid, 5 mM $MnCl_2$, 4 mM $MgCl_2$, 50 μM ATP, 0.1% Triton X-100, 50 mM HEPES (pH 7.5). The reaction was stopped after 2 min, unless otherwise indicated, by the addition of a quarter volume of SDS-polyacrylamide electrophoresis sample buffer (0.13 M Tris, 4.2% SDS, 21% glycerol, 0.004% bromophenol blue, 20% β-mercaptoethanol, pH 6.8). In some experiments, receptor sample was mixed with lipid and DTT, and then incubated with increasing concentrations of insulin (0–700 nM). In one experiment the ATP concentration was varied from 0 to 100 μM. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (7.5% acrylamide) followed by electrophoretic transfer of proteins to nitrocellulose membranes. Phosphotyrosine-labelled proteins were detected by Western blot analysis using primary antibodies specific for phosphotyrosine residues and secondary antibodies labelled with ^{125}I or coupled to alkaline phosphatase or peroxidase. Primary antibody labelling was detected by monitoring the formation of the insoluble product of 5-bromo-4-chloroindoyl phosphate hydrolysis [21], chemiluminescence, or autoradiography. Blots (chemiluminescent and ^{125}I-labelled) or photographic negatives of blots (stained by alkaline phosphatase reaction), were analyzed with a Molecular Dynamics Computing Densitometer or Phosphorimager; relative labelling of phosphotyrosine was quantified by integrating the area around the β subunit using the ImageQuant software. Analysis by all methods yielded qualitatively similar results.

2.2.4. Protein kinase C assay. The activity of protein kinase C towards phosphorylation of a peptide substrate [22] was measured in the presence of Ca^{2+} (1.2 mM) and Triton X-100 (0.1%) mixed micelles containing 15 mol% phosphatidylserine and 5 mol% either 1,2-dioleoyl-*sn*-glycerol, 1,3-dioleoyl-*sn*-glycerol, or 2,3-dioleoyl-*sn*-glycerol, as described [18].

3. Results and discussion

3.1. Diacylglycerol increases insulin receptor tyrosine kinase activity

The effect of diacylglycerol on the activity of human insulin receptor was measured by incubation of Triton X-100-solubilized membranes from NIH 3T3 HIR 3.5 cells with increasing concentrations of diacylglycerol[1]. Fig. 1 shows that dioleoylglycerol caused a 2.3-fold increase in the amount of phosphotyrosine associated with the β subunit of the receptor after 2 min incubation with ATP. Half-maximal stimulation was elicited by approximately 100 μM diacylglycerol, corresponding to 6 mol%. In contrast to its effects on insulin-stimulated activity, diacylglycerol had no significant effect on autophosphorylation in the absence of insulin. Autophosphorylation measured in the presence of a constant amount of diacylglycerol but increasing amounts of Triton X-100 revealed that stimulation was dependent on the mole fraction of diacylglycerol, rather than the absolute concentration of lipid (not shown). Protein kinase C is similarly dependent on the relative concentration of lipid in Triton X-100 mixed micelles [23]. Qualitatively similar results were obtained with insulin receptor partially purified from rat liver (not shown), indicating that membrane proteins present in the assay mixture were not involved in the diacylglycerol-mediated stimulation.

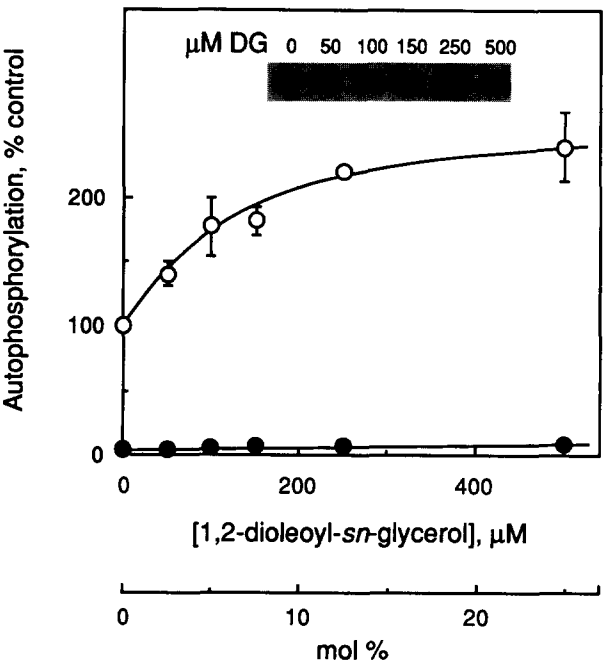

Fig. 1. Diacylglycerol stimulates insulin receptor autophosphorylation. Detergent-solubilized NIH 3T3 HIR 3.5 cell membranes were incubated in the presence of 0.1% Triton X-100 mixed micelles containing 0–500 μM (0–26 mol%) 1,2-dioleoyl-*sn*-glycerol and 50 μM ATP in the absence (●) or presence (○) of 10 nM insulin for 2 min at 21°C. Samples were analyzed by Western blot analysis and tyrosine phosphorylation of the β subunit detected by labelling with phosphotyrosine antibodies, as described in section 2. Data are presented as the intensity of phosphotyrosine staining relative to samples not incubated with diacylglycerol. Error bars represent the standard error of the mean for three sets of data. Inset: Western blot of samples probed with phosphotyrosine antibodies and detected with chemiluminescence, showing increasing tyrosine phosphorylation of the β subunit (95 kDa) of the insulin receptor with increasing diacylglycerol (DG) concentrations.

3.2. Diacylglycerol increases the affinity of the insulin receptor for insulin

To explore how diacylglycerol stimulates the insulin receptor, the effect of this lipid on kinetic parameters for regulation by insulin and ATP were examined. Table 1 shows that 250 μM dioleoylglycerol (15 mol%) decreased the EC_{50} for insulin 4-fold but had no significant effect on V_{max}. Dioleoylglycerol had no significant effect on the K_m or V_{max} for ATP, either at sub-saturating (10 nM) or saturating (700 nM) insulin concentrations. Thus, diacylglycerol stimulates the insulin receptor by increasing its affinity for insulin rather than by affecting catalysis.

One possible explanation for the diacylglycerol-mediated in-

[1]Assays contained 100 μM endogenous lipid (corresponding to 6 mol%), 0.1% Triton X-100 (corresponding to approximately 10 μM micelles based on an aggregation number of 140 at 25°C [24], 0–500 μM diacylglycerol, and approximately 100 $\mu g \cdot ml^{-1}$ (approximately 1 μM) protein. Thus, the ratio of micelles to total protein was on the order of 10:1.

Table 1
Effect of diacylglycerol on the insulin receptor's affinity for insulin or ATP

	Insulin		ATP			
	50 μM ATP		10 nM insulin		700 nM insulin	
	EC_{50} (nM)	V_{max}	K_m (μM)	V_{max}	K_m (μM)	V_{max}
Control	62 ± 14	7.9 ± 0.6	30 ± 5	3.2 ± 0.4	13.7 ± 0.3	6.6 ± 0.4
250 μM DODG	15 ± 1	6.4 ± 0.2	23 ± 6	3.8 ± 0.6	15 ± 4	7 ± 2

Insulin receptor autophosphorylation was measured in the presence of Triton X-100 mixed micelles (0.1%) with or without 250 μM 1,2-dioleoyl-*sn*-glycerol. Kinetic parameters for insulin were determined in the presence of 50 μM ATP. Kinetic parameters for ATP were determined in the presence of sub-saturating (10 nM) or saturating (700 nM) insulin concentrations. Autophosphorylation was measured as described in the legend of Fig. 1.

crease in affinity of the receptor for insulin is that diacylglycerol increases the partitioning of insulin into micelles. However, two results indicate that insulin-binding is not regulated by surface-partitioning. First, the measured EC_{50} is similar to the K_d for binding of insulin to NIH 3T3 HIR3.5 cells (1 nM for 38% of the receptor population and 100 nM for 63% of the receptor population [19]). Thus, the affinity for insulin is the same in detergent micelles or in the native plasma membrane. Second, the EC_{50} for insulin measured above was independent of detergent concentration (data not shown), indicating that partitioning into micelles (which would increase with increasing detergent) is unlikely to affect the accessibility of insulin to the insulin receptor.

Diacylglycerol-mediated stimulation was relatively insensitive to micelle structure as assessed by two criteria. First, inclusion of 6 mol% phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, phosphatidic acid, or sphingosine had no effect on the stimulation mediated by 6 mol% diacylglycerol (not shown). Thus, increasing micelle size or charge did not affect the ability of diacylglycerol to increase the insulin receptor's affinity for insulin. Second, the concentration of diacylglycerol mediating half-maximal stimulation was the same at 13.5°C and 21°C, even though micelles are significantly smaller at the lower temperature [24].

Although diacylglycerol had no effect on the catalytic activity of the insulin receptor, this lipid increased by approximately 20% the maximal amount of tyrosine phosphorylation of the receptor (Fig. 2, triangles) relative to controls (Fig. 2, circles). Thus, the lipid may be unmasking a phosphorylation site or unmasking a population of receptor.

Additional experiments revealed that the stimulation by diacylglycerol decreased with decreasing hydrophobicity: for example, the receptor was half-maximally stimulated by 250 μM dioctanoylglycerol (not shown) compared with 100 μM dioleoylglycerol. The hydroxyl group tethered to a hydrophobic moiety was insufficient for activation: oleyl alcohol had no significant effect on the receptor (not shown). Rather, stimulation required two acyl chains on the glycerol backbone: monooleoylglycerol (up to 500 μM) did not affect the activity of the insulin receptor (not shown). Thus, activation required the glycerol backbone and two acyl chains, with increasing hydrophobicity resulting in greater stimulation.

3.3. *Diacylglycerol-mediated stimulation of the insulin receptor is independent of protein kinase C*

One explanation for the diacylglycerol-mediated stimulation of the insulin receptor is that diacylglycerol modulates the function of another protein that regulates the insulin receptor. A protein that depends on diacylglycerol for activity [5] and whose activation affects the insulin receptor [25–27] is protein kinase C.

Four results revealed that the activation of the insulin receptor was independent of protein kinase C. First, phorbol myristate acetate, a potent activator of protein kinase C that substitutes for diacylglycerol [28], had no significant effect on the autophosphorylation of the insulin receptor (Fig. 2, squares). Second, the activation of the insulin receptor by diacylglycerol was not stereospecific; the enantiomer 2,3-diacyl-*sn*-glycerol and structural isomer 1,3 diacylglycerol activated the insulin receptor equally well (Fig. 3A). In marked contrast, activation of protein kinase C is specific for 1,2-diacyl-*sn*-glycerol (Fig. 3B) [29–32]. Third, Western blot analysis of samples revealed no detectable conventional isozymes of protein kinase C (not shown). Lastly, no protein kinase C activity towards a synthetic substrate was detected under the conditions of the assays. Thus, the stimulation of the insulin receptor by diacylglycerol was independent of protein kinase C.

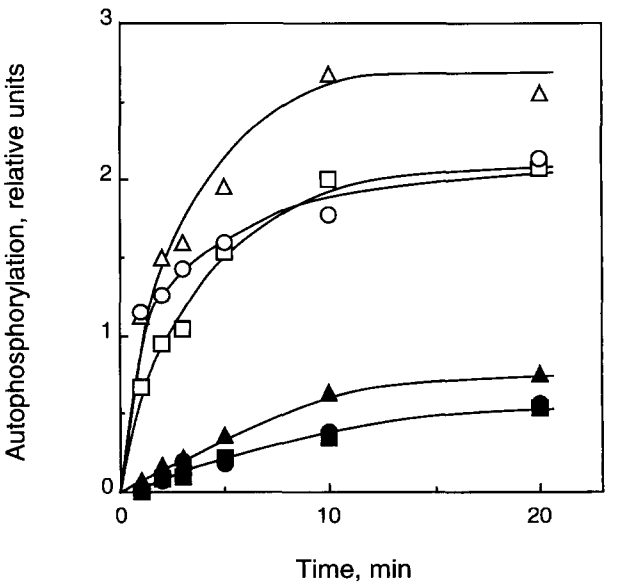

Fig. 2. Phorbol myristate acetate does not affect insulin receptor autophosphorylation. Time course of autophosphorylation of the insulin receptor was performed in the absence (closed symbols) or presence (open symbols) of 10 nM insulin and in the presence of 0.1% Triton X-100 micelles (○,●), or 0.1% Triton X-100 mixed micelles containing 100 μM dioleoylglycerol (△,▲) or 200 nM phorbol myristate acetate (□,■). Incorporation of phosphate onto tyrosine residues of the β subunit was determined as described in the legend of Fig. 1.

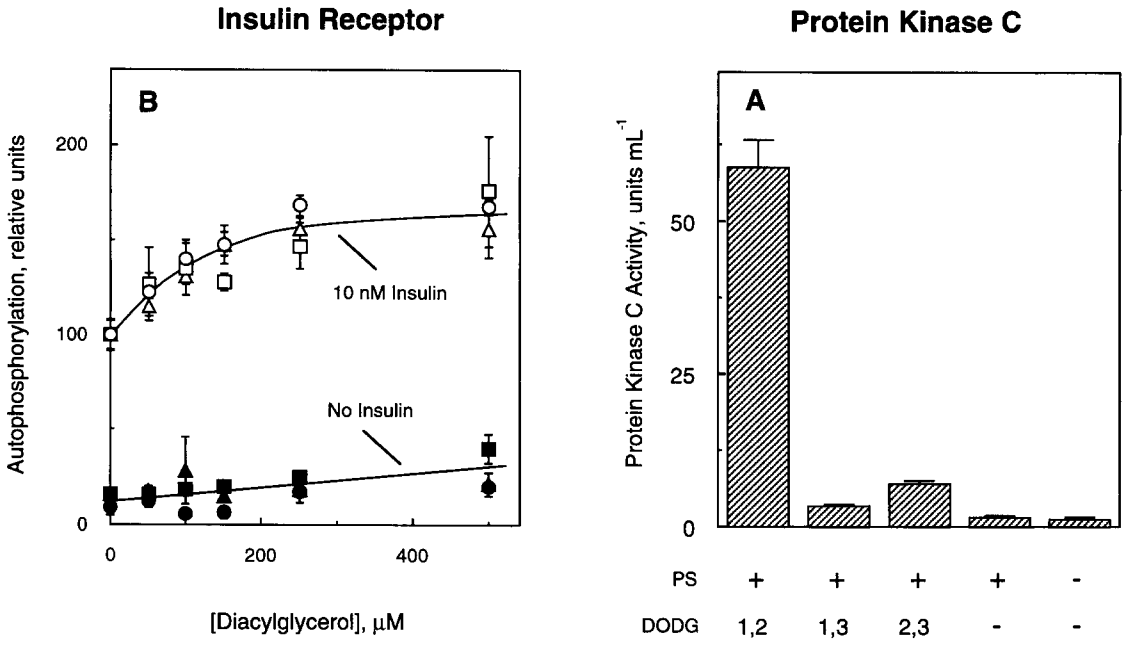

Fig. 3. Stimulation of insulin receptor autophosphorylation by diacylglycerol is not stereospecific. (A) Insulin receptor autophosphorylation was measured in the presence of 0.1% Triton X-100 mixed micelles containing increasing concentrations (0–500 μM) of 1,2-dioleoyl-*sn*-glycerol (○,●), 1,3-dioleoyl-*sn*-glycerol (△,▲) or 2,3-dioleoyl-*sn*-glycerol (□,■); assays included no insulin (closed symbols) or 10 nM insulin (open symbols). Other conditions are as described in the legend of Fig. 1. Error bars represent the standard error of the mean of three sets of data. (B) Phosphorylation of a peptide substrate by protein kinase C was measured in the presence of 1.2 mM Ca^{2+} and Triton X-100 mixed micelles containing 15 mol% PS and either 5 mol% 1,2-dioleoyl-*sn*-glycerol (DODG), 5 mol% 1,3-dioleoyl-*sn*-glycerol, 5 mol% 2,3-dioleoyl-*sn*-glycerol, or no diacylglycerol. One sample was measured in the absence of lipid and Ca^{2+}.

Grunberger and coworkers have also reported that diacylglycerol stimulates the tyrosine kinase activity of insulin receptor in human mononuclear cells [33]. However, in contrast to our findings, they reported that diacylglycerol stimulates the insulin receptor in the absence of insulin. The indirect involvement of protein kinase C in the mononuclear cell study may account for the difference. Indeed, earlier work by this group showed that treatment of human mononuclear cells by phorbol esters or diacylglycerols stereospecifically inhibited binding of insulin to the receptor; isomers that do not activate protein kinase C had no effect on insulin-binding [34].

3.4. Conclusions

The foregoing results reveal that diacylglycerol increases the tyrosine kinase activity of the insulin receptor by increasing its affinity for insulin. This stimulation is independent of protein kinase C and is likely mediated by a direct interaction of the lipid with the receptor: qualitatively similar data were obtained with receptor from two sources (NIH 3T3 HIR 3.5 cells and rat liver), either in crude extracts or partially purified.

Several mechanisms could account for the diacylglycerol-mediated stimulation of the insulin receptor. For example, diacylglycerol could alter the structure of lipids surrounding the insulin receptor thus affecting receptor function. The effect of diacylglycerol on the stability and structure of membranes is well-documented [35,36]. Most notably, high concentrations (>30 mol%) induce the formation of a nonlamellar phase [36]. For example, activation of phospholipase A_2 by diacylglycerol has been attributed to structural perturbations caused by this lipid [8,37–39]. With regards to the insulin receptor, compounds that alter membrane physical properties have been shown recently to affect the autophosphorylation of the insulin receptor in intact cells [40]. Although stimulation of the insulin receptor by diacylglycerol may result from structural perturbations induced by this lipid, it is noteworthy that diacylglycerol stimulated the insulin receptor in detergent:lipid mixed micelles, indicating that a specific membrane structure was not necessary for the stimulation. Furthermore, stimulation was relatively insensitive to micelle size (varied with temperature and added phospholipids) or charge.

A second possibility is that diacylglycerol interacts directly with the receptor, acting as an allosteric activator. Precedent for such an interaction exists in the specific binding of diacylglycerol to protein kinase C. This binding promotes a high affinity interaction with phosphatidylserine that results in an alteration in the structure of the enzyme [5]. The lack of stereospecificity in the interaction of the insulin receptor with diacylglycerol argues against the existence of a specific diacylglycerol binding site. Nonetheless, the requirement for two relatively hydrophobic acyl chains suggests that diacylglycerol may bind to the insulin receptor via a hydrophobic interaction. Such an interaction could increase the receptor's affinity for insulin by stabilizing the active conformation. Steric constraints may prevent other lipids, with much larger polar headgroups, from close apposition with the receptor.

The insulin-induced generation of diacylglycerol in the plasma membrane indicates that this second messenger may be a direct modulator of the receptor [12–14]. Because levels of insulin in the blood plasma (approximately 0.1 nM [41]) are well below the K_d for the insulin:receptor interaction, diacylglycerol would have a significant effect on insulin receptor activity under physiological conditions.

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