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Inhibition of the Insulin Receptor Tyrosine Kinase by Phosphatidic Acid

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Abstract The lipid second messenger, phosphatidic acid, inhibits the intrinsic tyrosine kinase activity of the insulin receptor in detergent–lipid mixed micelles or in reconstituted membranes. Enzymatic studies revealed that this lipid second messenger inhibits the catalytic activity of partially purified insulin receptor without affecting the affinity of *the receptor for insulin*. Selectivity in the protein–lipid interaction is suggested by the inability of several other acidic lipids to affect the kinase activity of the receptor and by the relative insensitivity of the inhibition to increasing ionic strength and, in some cases, micelle surface charge. Lysophosphatidic acid and phosphatidic acids with short acyl chains do not affect significantly the receptor's kinase activity, suggesting that hydrophobic interactions are involved in the inhibition. Thus, both a high affinity interaction of the insulin receptor with the phosphate headgroup and a stabilizing hydrophobic interaction with the acyl chains contribute to the inhibitory protein–lipid interaction. The selective sensitivity of the insulin receptor to phosphatidic acid suggests that the receptor-mediated generation of this lipid in the plasma membrane could negatively modulate insulin receptor function.

Key words: phosphatidic acid, tyrosine kinase activity, insulin receptor, lipid second messengers, hydrophobic interactions

Specificity in the interaction with lipid regulates the structure and function of a wide variety of integral and extrinsic membrane proteins. Sensitivity to bulk properties of the lipid bilayer, such as fluidity, as well as to interactions with specific lipids modulates the activity of transporters, receptors, cytoskeletal proteins, and a growing number of peripheral membrane enzymes involved in signal transduction [Newton, 1993]. Thus, interaction of proteins with specific lipids provides a mechanism for fine-tuning membrane function. A particularly sensitive regulation is afforded by minor lipid species whose levels increase in response to extracellular signals: an example of such a lipid is phosphatidic acid, which is present as 1 mol% of plasma membrane lipid [Zambrano et al., 1975] and whose levels increase in response to a number of extracellular signals [Bocckino et al., 1987; Billah and Anthes, 1990].

The insulin receptor is a heterotetramer comprised of two extracellular, insulin-binding α-subunits and two transmembrane β-subunits that are tyrosine kinases. Binding of insulin at the cell surface activates the β-subunit to autophosphorylate, a phosphorylation required for transduction of the insulin signal to its intracellular targets [Rosen, 1987; Myers and White, 1993]. The transmembrane topography of the insulin receptor would allow physical and chemical properties of lipids to affect its function. In this regard, treatment of adipocytes with phospholipase C was shown to decrease insulin-stimulated glucose oxidation over 20 years ago [Cuatrecasas, 1971]. Several reports indicate that alterations in membrane fluidity modulate insulin sensitivity in situ [Hubert et al., 1991; Field et al., 1990; Ginsberg et al., 1981]. In addition, the kinase activity of partially purified insulin

Abbreviations: DLPA, dilauroylphosphatidic acid; DMPA, dimyristoylphosphatidic acid; DOPA, dioleoylphosphatidic acid; DPPA, dipalmitoylphosphatidic acid; DSPA, distearoylphosphatidic acid; lysoPA, lysophosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP, phosphatidylinositol monophosphate; PIP_2, phosphatidylinositol bisphosphate; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SM, sphingomyelin; WGA, wheat germ agglutinin.

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receptor has been shown to be sensitive to lipid composition [Gould et al., 1982; Lewis and Czech, 1987; Sweet et al., 1987; Arnold and Newton, 1996] and to be inhibited by the lysolipid sphingosine [Arnold and Newton, 1991]. The function of a related receptor kinase, the epidermal growth factor receptor, is modulated by glycosphingolipids [Hakomori, 1990].

Insulin stimulates lipid synthesis as well as lipid hydrolysis, raising the possibility that insulin-mediated alterations in lipid composition may regulate the function of the insulin receptor. In particular, several reports indicate that insulin stimulates the rapid de novo synthesis of phosphatidic acid [Farese et al., 1987, 1988; Vila et al., 1990], the hydrolysis of a phosphatidylinositol-glycan to yield diacyglycerol [Saltiel et al., 1987; Suzuki et al., 1991] and the phospholipase D-catalyzed hydrolysis of phosphatidylcholine [Standaert et al., 1994].

This contribution examines the role of lipid structure in the regulation of insulin receptor function. Of the lipids tested, phosphatidic acid was a potent inhibitor of the tyrosine kinase activity of the insulin receptor. Kinetic studies with partially purified insulin receptor revealed that phosphatidic acid inhibits catalysis without altering the affinity of the receptor for insulin. Inhibition by this lipid and another inhibitory lipid, sphingosine, was studied as a function of lipid composition, surface charge, ionic strength, and pH. The data suggest that phosphatidic acid selectively inhibits the receptor, presenting the possibility that alterations in the level of this lipid may regulate insulin receptor function.

METHODS AND MATERIALS

L-α-Phosphatidylserine (bovine brain) (PS), L-α-phosphatidylcholine (egg) (PC), L-α-phosphatidylethanolamine (bovine liver) (PE), L-α-1,2-dioleoyl-*sn*-phosphatidylglycerol (PG), L-α-1,2-dioleoyl-*sn*-phosphatidic acid (PA), L-α-phosphatidylinositol (PI), and sphingomyelin (bovine brain) (SM) were obtained from Avanti Polar Lipids. Aprotinin, ATP, benzamidine, 5-bromo-4-chloroindoyl phosphate, cholesterol, HEPES, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride (PMSF), dilauroylphosphatidic acid (DLPA), dimyristoylphosphatidic acid (DMPA), dipalmitoylphosphatidic acid (DPPA), distearoylphosphatidic acid (DSPA), L-α-phosphatidylinositol 4-monophosphate (PIP), monooleoyl lysophosphatidic acid (lysoPA) and stearylamine were purchased from Sigma Chemical Company. L-α-Phosphatidic acid (semisynthetic from egg) was obtained from Matreya (Pleasant Gap, PA). Alkaline phosphatase-conjugated goat anti-mouse IgG and L-α-phosphatidyl-D-myo-inositol 5-bisphosphate (PIP_2) were supplied by Boehringer Mannheim Biochemicals (Indianapolis, IN) and phosphotyrosine antibody (PY20) was from ICN Biomedicals (Costa Mesa, CA). Porcine insulin was from Calbiochem, [γ-^{32}P]ATP (3,000 Ci mmol^{-1}; 10 mCi ml^{-1}) from Dupont-New England Nuclear, and wheat germ agglutinin agarose (WGA agarose) from Pharmacia. Triton X-100 (10% aqueous solution low in carbonyl and peroxide content) was supplied by Pierce Chemical Company (Rockford, IL). Nitrocellulose membrane were obtained from Schleicher and Schuell (Keene, NH). The peptide substrate comprising residues 1142–1153 of the human insulin receptor (peptide 1142–1153) [Shoelson et al., 1988] was synthesized by the Biochemistry Biotechnology Facility, Indiana University School of Medicine. All other chemicals were reagent grade.

Insulin Receptor

Insulin receptor was partially purified from the livers of 50 male Sprague Dawley rats (approximately 150 g) by WGA chromatography, as described previously [Arnold and Newton, 1991]. Insulin receptor was stored at −20°C in buffer containing 0.2 M N-acetylglucosamine, 40% glycerol, 0.1% Triton X-100, 0.5 mM EDTA, 0.5 mM EGTA, 25 mM HEPES, pH 7.5 (storage buffer). For the experiments in Figures 3B and 4, insulin receptor was partially purified from National Institutes of Health (NIH) 3T3 HIR 3.5 cells, which express on the order of 10^6 copies of the human insulin receptor per cell [Whittaker et al., 1987]. Confluent cells were digested with enzyme-free solution. The cells were pelleted by centrifugation at 1,300*g* for 1 min at room temperature, lysed in 50 mM HEPES, pH 7.5 containing 1 mM EDTA, and 1 mM PMSF followed by homogenization. Membranes were pelleted at 543,000*g* for 10 min at 4°C and resuspended in 50 mM HEPES, pH 7.5, containing 0.1% Triton X-100. The resuspended membranes were further homogenized and then centrifuged at 627,000*g* at 4°C for 10 min. Insulin receptor kinase activity in the supernatant (containing solubilized membrane proteins) was assayed directly or after reconstitution of proteins with exogenous lipids (described below). Samples from NIH 3T3 HIR 3.5 cells contained approximately 100 μM endogenous lipid in assay mixtures. Results from experiments using receptor from rat liver or from NIH 3T3 HIR 3.5 cells were

qualitatively similar; more PA was required to inhibit the human receptor, likely as a result of dilution by endogenous lipids.

Lipids

Triton X-100:lipid mixed micelles were prepared by drying a chloroform solution of the indicated lipid under a stream of N_2 and hydrating in 50 mM HEPES, pH 7.5, containing 0.1% Triton X-100. Mixed micelles, containing 0–9 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 0–0.9 mM. Lipids used in reconstitution experiments were prepared as above, except that Triton X-100 was omitted from the hydration buffer.

Reconstitution

One vol of WGA-purified receptor in storage buffer was mixed with 3 vol of 50 mM HEPES, pH 7.5, containing 2-mM lipid vesicles (compositions noted in legend of Fig. 2); insulin was added to some samples (+ insulin sample) to yield a final concentration of 700 nM. The solution was vortexed vigorously for 3 sec and then diluted fivefold in 50 mM HEPES, pH 7.5 with (+ insulin sample) or without (− insulin sample) 700 nM insulin. The final concentrations of species in the mixture were 10 mM N-acetylglucosamine, 2% glycerol, 0.005% Triton X-100, 25 μM EDTA, 25 μM EGTA, 300 μM lipid, and 0 or 700 nM insulin. Membranes were pelleted at 627,000*g*, 4°C for 30 min and resuspended in a solution (57 μl) containing 0-870 nM insulin and 1.2 mM DTT. Solutions were incubated for 5 min at 21°C and then assayed as described below.

Insulin Receptor Assay

Autophosphorylation of the insulin receptor was assayed by measuring the incorporation of phosphate from [γ-^{32}P]-ATP into the β-subunit, as described [Arnold and Newton, 1991]. For mixed micelle studies, WGA-purified insulin receptor, DTT, insulin, and lipid were preincubated in 1 vol of 57 μl (containing 0.09% Triton X-100) for 5 min at 25°C before initiation of the phosphorylation reaction 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 [γ-^{32}P]-ATP (approximately 850 mCi mmol^{-1}). The final reaction mixture contained 0–0.7 μM insulin, 5 mM $MnCl_2$, 4 mM $MgCl_2$, 50 μM ATP, 1 mM DTT, 0–900 μM lipid, 0.1% Triton X-100, and 10–20 μg protein. For assays with reconstituted receptor, detergent was omitted from the assays. The ionic strength was varied from 53 to 553 mM by the addition of 0–500 mM NaCl. In some cases, the pH of the assay solution was adjusted from 6 to 9 by addition of appropriately adjusted HEPES buffer; the pH of the assay mixture was measured using a Physitemp pH probe. The reaction was stopped after 60 sec (micelle studies) or 10 min by the addition of SDS–PAGE sample buffer (0.13 M Tris, 4.2% SDS, 21% glycerol, 0.004% bromophenol blue, 20% β-mercaptoethanol, pH 6.8) containing 0.05 M ATP, and 0.05 M EDTA. Samples were analyzed by SDS–PAGE (7.5% acrylamide) followed by autoradiography. Incorporation of [^{32}P] into the β-subunit of the insulin receptor was determined by liquid scintillation counting of bands excised from gels or by analysis of gels with a Phosphorimager (Molecular Dynamics [Sunnyvale, CA]). In some cases, electrophoresis was followed by transfer of the proteins to nitrocellulose membranes. Phosphotyrosine-labeled receptor was detected by Western blot analysis with primary antibodies specific for phosphotyrosine (PY20, ICN) and alkaline phosphatase-conjugated secondary antibody (IgG); primary antibody labelling was detected by monitoring the formation of the insoluble product of 5-bromo-4-chloroindoyl phosphate hydrolysis [Blake et al., 1984]. Labeling of phosphotyrosine was quantified by scanning densitometry of blots. Substrate phosphorylation assays were performed by including peptide 1142–1153 (0.5 mM) in the reaction mixture. The reaction was allowed to proceed for 1 min at 24°C, before being quenched by addition of 25 μl of a solution containing 0.1 M ATP and 0.1 M EDTA, pH 7. Aliquots (75 μl) were spotted on Whatman P81 ion-exchange papers, washed in 0.4% (v/v) phosphoric acid, and [^{32}P]-phosphate incorporation determined by scintillation counting, as described [Orr and Newton, 1992]. No significant changes in light scattering of assay mixtures containing increasing concentrations of phospholipid were observed.

Data Analysis

The dependence of insulin receptor activity on lipid or substrate concentration was analyzed by a nonlinear least-squares fit to a modified Hill

equation, as described [Orr and Newton, 1992], or to the Michaelis Menton equation, using the program Grafit.

RESULTS

Inhibition of Insulin Receptor Activity by Phosphatidic Acid in Mixed Micelles

Figure 1 shows the amount of autophosphorylation of the insulin receptor β-subunit, catalyzed in 1 min, measured in the presence of Triton X-100 (0.1%) micelles containing increasing concentrations of dioleoylphosphatidic acid (DOPA) (0–500 μM, corresponding to 0–25 mol% relative to Triton X-100). DOPA caused a marked inhibition of the insulin-stimulated autophosphorylation, but had no significant effect on basal insulin-independent activity of the insulin receptor. The PA-mediated inhibition of activity displayed sigmoidal kinetics (Fig. 1, inset). Nonlinear least squares analysis of the data to the Hill equation revealed that half maximal inhibition occurred at 155 ± 5 μM DOPA, with a Hill coefficient of 4.7 ± 0.7. Inhibition of the receptor by PA was reversible because inhibition was relieved by dilution of the PA with additional Triton X-100 (data not shown).

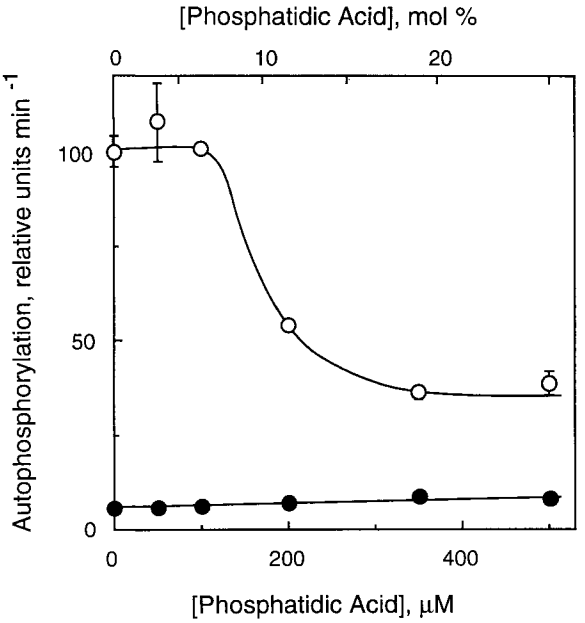

Fig. 1. Inhibition of insulin receptor auto- and substrate phosphorylation in mixed micelles. Partially purified rat liver insulin receptor was incubated with ^{32}P-ATP in the presence of Triton X-100 (0.1%) mixed micelles containing 0–500 μM DOPA, in the presence (○) or absence (●) of 700 nM insulin for 1 min at 25°C, as described in Methods. Autophosphorylation is expressed relative to the control (no lipid) and reflects the amount of phosphate incorporated into the β-subunit in 1 min. *Inset,* data from four separate experiments plotted as relative inhibition. Curve is that predicated from the Hill equation.

PA also inhibited insulin-stimulated phosphorylation of a synthetic peptide substrate by the insulin receptor (data not shown). Half-maximal inhibition of substrate phosphorylation was mediated by 185 ± 12 μM DOPA and displayed sigmoidal kinetics, as observed for autophosphorylation. The extent of maximal inhibition (approximately 70%) was similar to that observed for autophosphorylation.

Kinetic analysis revealed that PA decreased the maximal extent of autophosphorylation of the receptor without affecting the half-time for autophosphorylation (1.4 min) (data not shown). Thus, the rate constant for autophosphorylation was unaffected by PA, however effectively fewer phosphorylation sites were available in the presence of PA. Because the insulin receptor autophosphorylates on multiple residues, this could arise because some sites on all receptors are masked or because a population of receptors is inactive and not phosphorylated on any sites. The similar inhibition of substrate phosphorylation is consistent with a population of catalytically inactive receptors.

Inhibition of Insulin Receptor Activity by Phosphatidic Acid in Reconstituted Membranes

The insulin receptor tyrosine kinase was also inhibited by PA when reconstituted in membranes composed primarily of egg PC. The rate of autophosphorylation of the insulin receptor was reduced 2.4-fold in membranes containing 20 mol% DSPA compared with membranes containing 20 mol% PE, 20 mol% PS, or composed entirely of PC (data not shown). For these experiments, insulin-dependent activity accounted for approximately one-half the observed phosphorylation.

Acyl Chain Dependence of Phosphatidic Acid-Mediated Inhibition

The effect of varying acyl chain length on PA-mediated inhibition of the insulin receptor in Triton X-100 mixed micelles was examined in Figure 2. The concentration of lipid resulting in half-maximal inhibition of the receptor (IC_{50}) was inversely proportional to the number of methylene groups on the acyl chain from C_{12} to C_{18}. To test whether the decreased potency of the shorter-chained lipids resulted from their decreased partitioning in the micelles, PA-mediated inhibition was compared for receptor

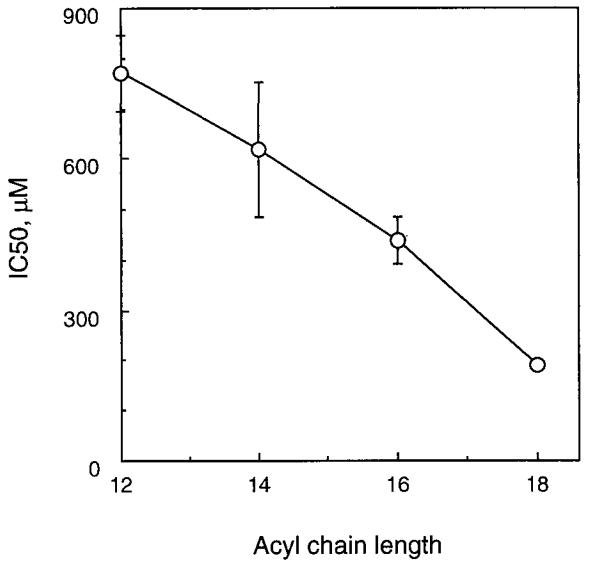

Fig. 2. Inhibition of the insulin receptor by phosphatidic acid is acyl chain dependent. Human insulin receptor from NIH 3T3 HIR 3.5 cells was autophosphorylated for 2 min in the presence of 700 nM insulin and increasing concentrations of PA with different acyl chain lengths (DLPA, C_{12}; DMPA, C_{14}; DPPA, C_{16}; DSPA, C_{18}). Incorporation of phosphate into the β-subunit was analyzed using phosphotyrosine antibodies, as described in Methods. The IC_{50} values for each PA species are plotted with respect to acyl chain length. Error bars represent the standard error of the mean of triplicate assays.

reconstituted in egg PC membranes containing 20 mol% DLPA or DSPA. TLC analysis confirmed that both membranes contained approximately the same proportion of short- or long-chained PA relative to PC. As observed using micelles, DLPA was less effective than DSPA in inhibiting the insulin receptor tyrosine kinase in membranes. LysoPA, with one acyl chain, had no significant effect on insulin receptor activity (data not shown). The increased inhibition mediated by increasing hydrophobicity of the nonpolar component of the lipids suggests that hydrophobic forces are involved in the interaction of the insulin receptor with PA. In addition, lipids with saturated acyl chains were more inhibitory than lipids with unsaturated acyl chains (not shown). For comparison, approximately 60% of acyl chains in lipids from rat liver membranes are 18 carbons long [Bergelson et al., 1970], thus the inhibitory potency by DSPA and DOPA would be representative of potential inhibition by PA in vivo.

Effect of Phosphatidic Acid on Insulin Receptor's Affinity for Insulin and ATP

PA did not alter the affinity of the insulin receptor for insulin; the concentration of insulin eliciting half-maximal tyrosine kinase activity was the same in the absence or presence of PA. Analysis of the data in Figure 3 revealed that approximately 50 nM insulin resulted in half-maximal stimulation of the insulin receptor tyrosine kinase from NIH 3T3 HIR cells, whether or not 200 μM DOPA was present. This value is consistent with the reported K_d values for insulin binding to receptor expressed in these cells, that is, 1 nM for approximately 40% of the receptor population and 100 nM for approximately 60% of the receptor population [Whittaker et al., 1987]. Qualitatively similar results were obtained for insulin receptor from rat liver, except that one order of magnitude less insulin was required for half-maximal activity (not shown). For both receptor sources, PA caused a twofold decrease in the V_{max}, indicating that the lipid inhibits catalysis and follows noncompetitive kinetics. PA had no significant effect on the receptor's affinity for ATP: in the absence or presence of 200 μM PA the K_m for ATP was approximately 20 μM (results not shown). Rather, PA reduced the maximal rate of autophosphorylation.

Effects of Other Lipids on Insulin Receptor Activity

The effects of PC, PE, PG, PS, PI, PIP, and PIP_2 on insulin-stimulated autophosphoryla-

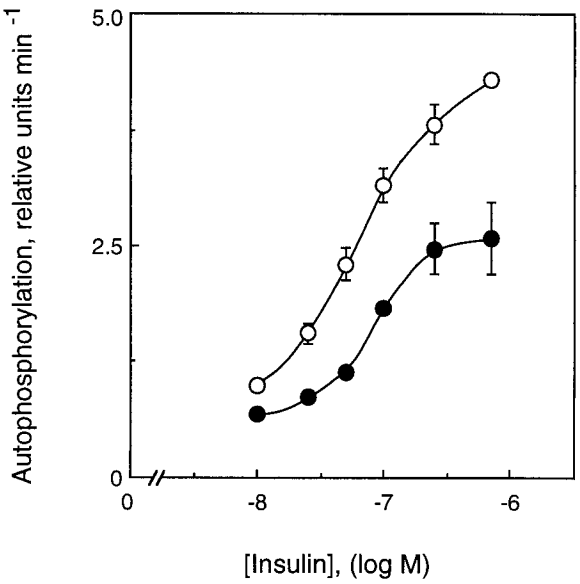

Fig. 3. Phosphatidic acid does not affect the insulin receptor's affinity for insulin. Autophosphorylation of human insulin receptor was measured in the presence of 0–700 nM insulin in Triton X-100 (0.1%) mixed micelles containing no lipid (○) or 200 μM DOPA (●) for 2 min at 21°C. Phosphorylation of the β subunit was detected using phosphotyrosine antibodies. Error bars represent the standard error of the mean of nine sets of data.

tion of the insulin receptor are presented in Figure 4. PE, PG, PS, and PI had no significant effect on receptor activity, while PC caused a small (15%) but reproducible increase in activity (Fig. 4A,B). In contrast, PIP (charge between −2 and −3 at pH 7.5) and PIP_2 (charge between −3 to −5 at pH 7.5) inhibited the receptor, with the degree of inhibition increasing with number of phosphates on the headgroup (Fig. 4B). Unlike the sigmoidal kinetics observed for the inhibition by PA, inhibition was linearly related to inositide concentration at the lipid concentrations tested. Half-maximal inhibition of insulin receptor occurred in the presence of 270 μM PIP_2, whereas >500 μM PIP was necessary for half-maximal inhibition of the receptor. On the basis of a pKa of 8 for the second ionizable group [Tocanne and Teissié, 1990], the charge of PA at neutral pH would be approximately −1.3. Thus, lipids with more than double the charge of PA are much less effective inhibitors of the kinase, suggesting that the negative headgroup charge is insufficient for inhibition.

Effects of Other Lipids on Phosphatidic Acid-Mediated Inhibition of the Insulin Receptor

The effects of various charged and neutral lipids on the PA-mediated inhibition of the insulin receptor are presented in Figure 5. Autophosphorylation of the insulin receptor was measured in the presence of Triton X-100 (0.1%) micelles containing 200 μM PA and increasing concentrations of SM, PS, PE, PC, PG, cholesterol, PI, PIP, or PIP_2. PA alone inhibited insulin receptor autophosphorylation approximately 50% relative to the control (no lipid). Lipids fell into three classes: (1) lipids that restored activity, (2) lipids that had no significant effect on activity, and (3) lipids that resulted in further inhibition of the insulin receptor tyrosine kinase. PA-mediated inhibition was reversed by inclusion of PE, PC, PG, cholesterol, or PI in mixed micelles. PE and PC completely relieved the PA-mediated inhibition (Fig. 5A). PE was the most effective of the two, with full enzymatic activity restored in the presence of 300 μM PE compared with 450 μM PC. At low concentrations (≤200 μM), PG was more effective than PC at restoring activity. However, this lipid was unable to restore activity to above 85% of the control value. Cholesterol and PI were as effective as PC at low concentrations (≤200 μM) but were only able to restore activity to 75% of the control value. Neither SM nor PS had any significant effect on the ability of PA to inhibit the insulin receptor tyrosine kinase. In contrast, the presence of PIP and PIP_2 in mixed micelles resulted in further inhibition of the insulin recep-

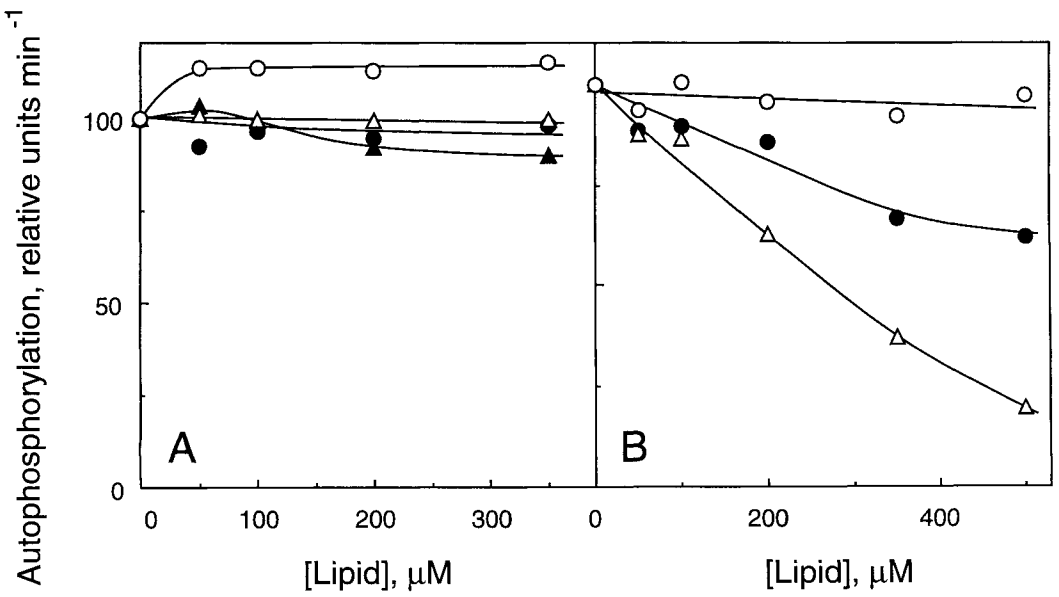

Fig. 4. Effects of different lipids on insulin receptor autophosphorylation. Insulin-dependent autophosphorylation of the insulin receptor from rat liver was measured in the presence of Triton X-100 (0.1%) mixed micelles containing: **A:** 0–350 μM PC (○), PE (●), PG (△), or PS (▲). **B:** 0–500 μM PI (○), PIP (●), or PIP_2 (△). Error bars represent standard error of the mean of three sets of data. Autophosphorylation was measured as described in the legend of Figure 1 and is expressed relative to ^{32}P-incorporation in the absence of lipid.

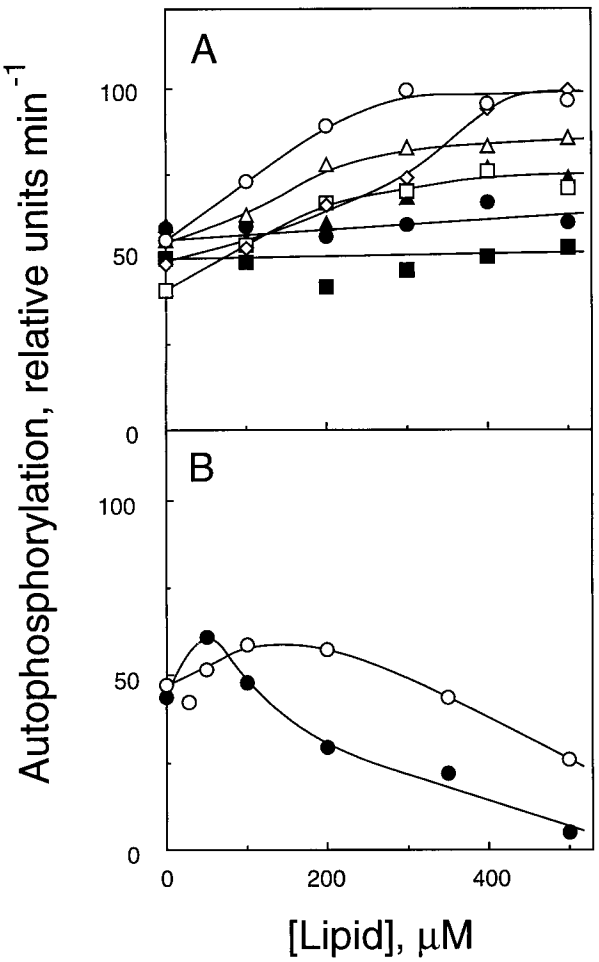

Fig. 5. Effects of other lipids on phosphatidic acid-mediated inhibition of the insulin receptor. Insulin-dependent autophosphorylation of rat liver insulin receptor was measured in the presence of Triton X-100 (0.1%) mixed micelles containing 200 μM DOPA and 0–500 μM of the following lipids. **A:** PE (○), PC (◇), PG (△), cholesterol (□), PI (▲), PS (●) or SM (■). **B:** PIP (○) or PIP_2 (●). Autophosphorylation was measured as described in the legend of Figure 1 and is expressed relative to ^{32}P-incorporation in the absence of lipid. Data in A represent the average of three separate experiments.

tor tyrosine kinase (Fig. 5B). While lower concentrations of these lipids ($\leq$50 μM PIP_2; $\leq$200 μM PIP) resulted in a slight increase in activity, significant inhibition was observed as the concentration of the inositides was increased to 500 μM. At this concentration, PIP_2 abolished insulin receptor tyrosine kinase activity, while PIP lowered the activity to 25% of the control value. Thus, the order in which the lipids tested affected PA-mediated inhibition was as follows: (1) lipids that restored activity, PE > PC > PG > cholesterol ≈ PI; (2) lipids that had no significant effect, PS = SM; and (3) lipids that resulted in further inhibition, PIP_2 > PIP.

The possibility that the inhibition mediated by the negatively charged PA could be reversed by neutralizing the charge of the micelles was explored by including the positively charged lipid sphingosine, itself an inhibitor [Arnold and Newton, 1991], in mixed micelles. Sphingosine has been shown to neutralize the effective charge of another acidic lipid, PS, by forming a 2:1 PS–sphingosine complex [López-Garciá et al., 1993]. Figure 6 shows the insulin-stimulated autophosphorylation of the insulin receptor in the presence of Triton X-100 micelles containing 1] 200 μM PA and increasing sphingosine concentrations (open circles) or 2] 200 μM sphingosine and increasing PA concentrations (closed circles). PA alone resulted in 60% activity relative to that in the absence of the lipid. This inhibition was relieved by sphingosine, with 100 μM to 400 μM abolishing the inhibition. Higher concentrations of sphingosine resulted in inhibition of the receptor, presumably because of the inhibitory effect of sphingosine. Similarly, PA (100 μM)

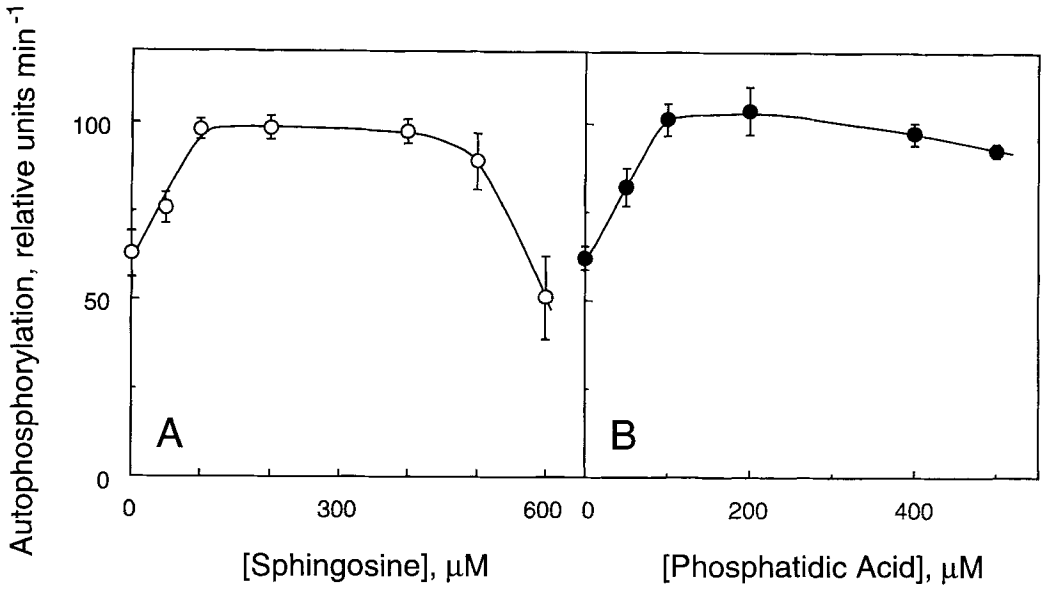

Fig. 6. Effect of a combination of phosphatidic acid and sphingosine on insulin receptor autophosphorylation. Autophosphorylation of rat liver insulin receptor was measured in the presence of Triton X-100 (0.1%) mixed micelles containing the following: **A:** 200 μM DOPA and increasing concentrations of sphingosine (0–600 μM). **B:** 200 μM sphingosine and 0–500 μM DOPA. Points in A are the mean ± standard error of four experiments performed in triplicate, with the exception of the last point which is the result of one triplicate assay; mean ± standard error of triplicates are shown in B. Autophosphorylation conditions were as described in the legend of Figure 1.

relieved the inhibition mediated by 200 μM sphingosine. Thus, neutralizing the charge of the micelles reversed the inhibition by PA, suggesting that the charge on the phosphate headgroup, although not sufficient for inhibition (see above), contributes to a productive interaction with the receptor.

Effects of Ionic Strength on Phosphatidic Acid- and Sphingosine-Mediated Inhibition of Insulin Receptor Activity

To test the role of electrostatic interactions in mediated the inhibition of the insulin receptor by PA, the effect of increasing ionic strength on the lipid-mediated inhibition of the receptor was examined in Figure 7. Insulin-stimulated autophosphorylation was measured in Triton X-100 micelles in the absence or presence of PA or sphingosine, and in the presence of 0 to 500 mM NaCl (53- to 553-mM ionic strength). Sphingosine-mediated inhibition was reduced with increasing ionic strength: in the absence of NaCl, sphingosine inhibited the receptor by 50%, whereas inhibition was reduced to 15% in the presence of 200 mM NaCl (open triangles). In contrast, PA-mediated inhibition was considerably less sensitive to ionic strength: relative to activity in the absence of lipid, the receptor was inhibited by 50% in the absence of NaCl and by 30% in the presence of 500 mM NaCl (closed circles). However, PA-mediated inhibition was reversed by $CaCl_2$ (>100 μM), suggesting that the phosphate headgroup was not inhibitory when complexed to Ca^{2+} (data not shown). The intrinsic catalytic activity of the insulin receptor was sensitive to ionic strength, with 40% inhibition observed in the presence of 500 mM NaCl (open circles).

The effect of increased ionic strength on the kinetics of lipid-mediated inhibition is shown in Figure 7B. Insulin-stimulated autophosphorylation was measured in the presence of Triton X-100 (0.1%) mixed micelles containing increasing concentrations of PA in the absence (53 mM ionic strength; open symbols) or presence (353 mM ionic strength; closed symbols) of 300 mM NaCl. In the absence of lipid, NaCl resulted in 25% inhibition of the intrinsic kinase activity of the receptor. NaCl had no significant effect on the sigmoidal kinetics of PA-mediated inhibition: half-maximal inhibition was observed at 170 μM PA, with a Hill coefficient of 5, in the presence or absence of NaCl. The relative insensitivity of the PA-mediated inhibition to increasing ionic strength suggests a high-affinity interaction between the receptor and acidic lipid that may involve forces in addition to electrostatic. For example, hydrophobic interactions sug-

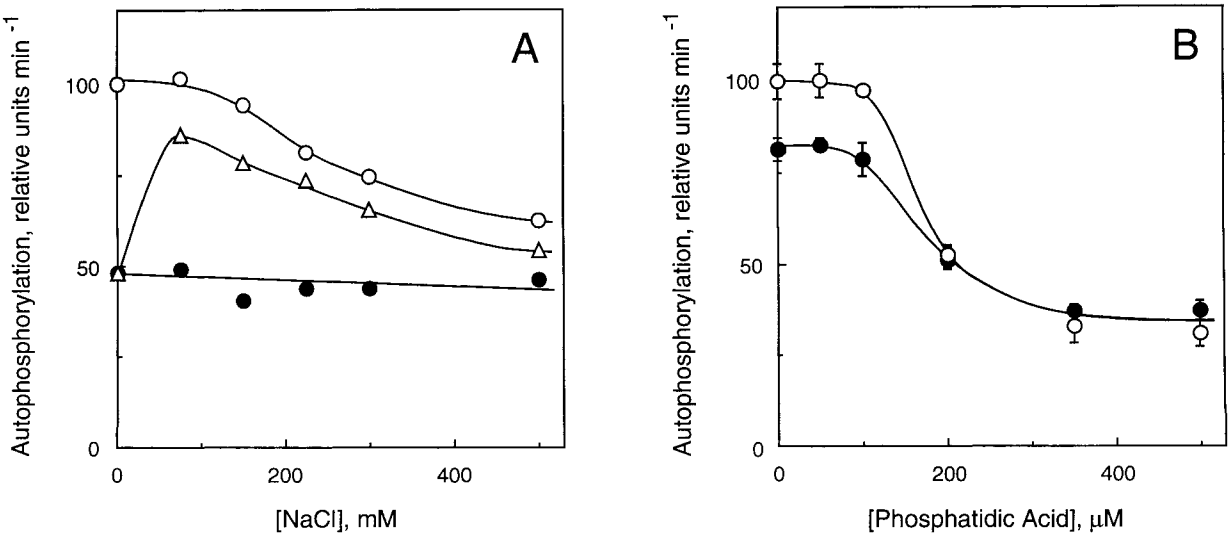

Fig. 7. Effect of NaCl on phosphatidic acid- or sphingosine-mediated inhibition of insulin receptor autophosphorylation. **A:** Insulin receptor (rat liver) was autophosphorylated in the presence of Triton X-100 (0.1%) mixed micelles containing no lipid (○), 366 μM sphingosine (△), or 200 μM DOPA (●) at the indicated NaCl concentrations (0–500 mM). **B:** Insulin receptor was autophosphorylated in the presence of Triton X-100 (0.1%) mixed micelles containing 0–500 μM DOPA in the absence (○) or presence (●) of 300 mM NaCl. Assays were performed under the conditions described in the legend of Figure 1. Error bars in B represent the standard error of the mean for three sets of data.

gested from the results of varying the hydrophobicity of the lipid (Fig. 2) would be strengthened by increasing ionic strength. The interaction with PA contrasts with the lower affinity electrostatic interaction with sphingosine, which is effectively screened with increasing ionic strength.

Effect of pH on Phosphatidic Acid- and Sphingosine-Mediated Inhibition of Insulin Receptor Activity

Figure 8 shows that the concentration of PA resulting in half-maximal inhibition of insulin receptor autophosphorylation was the same at pH 6.5 and pH 7.5.[1] The reported pKa values for the second ionizable oxygen on PA are around 8 [Tocanne and Teissié, 1990], indicating that a charge of −1.0 (expected at pH 6.5) or −1.3 (expected at pH 7.5) does not change appreciably the IC_{50} for inhibition by PA. Thus, a single negative charge on the phosphate headgroup is sufficient to mediate inhibition by this lipid. The degree of inhibition, however, was decreased at the lower pH (approximately 50%, compared with 80% at neutral pH). Thus, the insulin receptor may bind PA with the same affinity at the more acidic pH, but the effect of the lipid interaction is less inhibitory at the lower pH.

In contrast to PA, the inhibitory potency of sphingosine was absolutely dependent on pH (Fig. 8B). The inhibitory potency of sphingosine decreased with increasing pH (closed circles), with no inhibition observed for pH ≥ 8. The inhibitory ability of sphingosine correlated with the pK_a of its amine group (reported values of 7–8 in the presence of Triton X-100) [Merrill, 1991], indicating that only the charged form of the molecule inhibits the receptor.

DISCUSSION

Studies in detergent:lipid mixed micelles and reconstituted membranes have revealed that the lipid second-messenger, PA, inhibits the intrinsic tyrosine kinase activity of the insulin receptor. Kinetic measurements indicate that PA does not affect insulin binding, but rather inhibits catalytic activity. Consistent with our results, phospholipase C treatment of intact cells (to generate diacylglycerol and then PA) has been shown to inhibit insulin receptor tyrosine kinase activity without affecting insulin binding [Zoppini and Kahn, 1992].

Inhibition of the insulin receptor by PA displays selectivity for the phosphate headgroup: other acidic lipids of similar charge had little or

[1]The inhibition by DPPA was greater in this experiment than that described in Figure 2 because Figure 2 employed human insulin receptor from NIH 3T3 HIR3.5 cells rather than from rat liver; assays with recombinant human receptor may have been less sensitive to PA because endogenous lipid was not removed (see Methods).

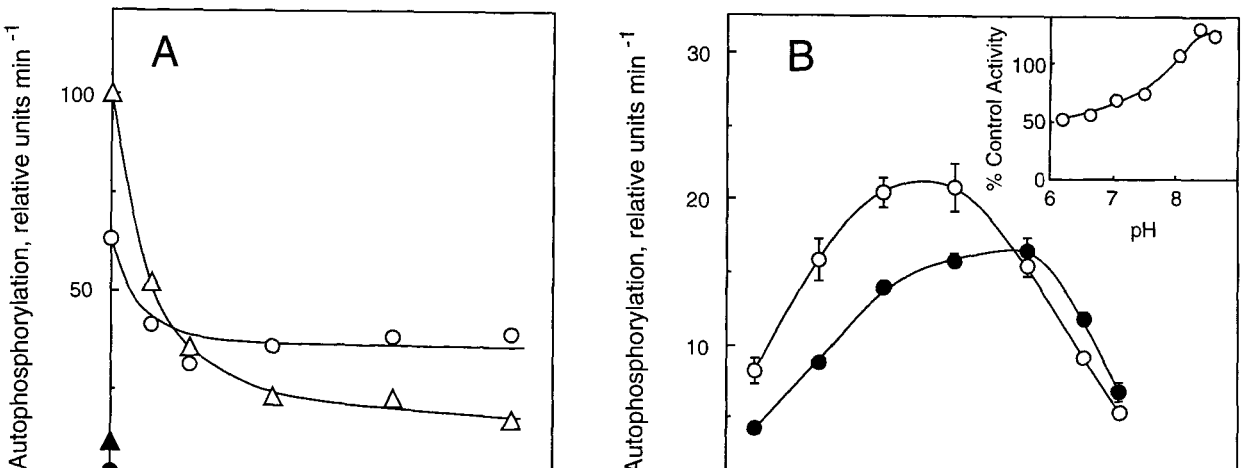

Fig. 8. Effect of pH on PA- and sphingosine-mediated inhibition. **A:** Insulin receptor (rat liver) was autophosphorylated in the presence of Triton X-100 (0.1%) mixed micelles containing 0–580 μM DPPA at pH 6.5 (△) or pH 7.5 (○); other conditions are as described in the legend of Figure 1. Closed symbols represent autophosphorylation in the absence of insulin. **B:** Autophosphorylation was measured in the presence of 0 (○) or 200 μM (●) sphingosine at the indicated pH. The pH of the assay mixture was measured with a Physitemp pH probe. Autophosphorylation conditions are described in the legend of Figure 1. *Inset,* activity of sphingosine-treated samples expressed relative to activity in the absence of lipid.

no effect on the activity of the receptor. At the pH of the experiments (6.5 or 7.5), PA would be expected to have a charge of −1 or −1.3, respectively (or less in the presence of detergent) [Tocanne and Teissié, 1990]. Lipids with a charge of −1 (PG, PS, PI) had no effect on the tyrosine kinase activity of the receptor, suggesting that negative charge was not sufficient to effect inhibition. Indeed, the negatively charged lipids PG and PI were able to relieve PA-mediated inhibition. Nonetheless, negative charge may play some role in the PA-mediated inhibition because the multiply charged acidic lipids PIP (2–3 negative charges) and PIP_2 (3–5 negative charges) inhibited the insulin receptor in proportion to their charge. However, the kinetics of inhibition by the inositides were distinct from those describing the inhibition by PA, and the multiply charged acidic lipids were less potent than PA at inhibiting the receptor. Inhibition was relatively insensitive to ionic strength and pH, suggesting that electrostatic interactions and lipid charge are not the primary driving force in the interaction of PA with the receptor. However, positive charge in the plane of the membrane reversed the inhibitory effect of PA, indicating that electrostatic interactions play some role in the binding of PA to the receptor. Inhibition is sensitive to the hydrophobicity of the lipid: lysophosphatidic acid, with a single acyl chain, did not inhibit the receptor, and shorter-chained lipids such as DLPA were relatively poor inhibitors of the receptor in micelles or reconstituted membranes. Our results are consistent with a high-affinity interaction of the insulin receptor with the phosphate headgroup stabilized by hydrophobic interactions with the acyl chains. Interaction with multiple PA molecules is suggested by the kinetic cooperativity observed.

In contrast to the inhibition mediated by PA, the effects of sphingosine on insulin receptor function were completely reversed at high ionic strength and at alkaline pH. Thus, charge screening by ionic strength or surface charge (not shown) eliminated the inhibitory potency of sphingosine, as did protonation of the amine group. These data indicate that the primary driving force in the sphingosine:insulin receptor interaction is electrostatic in nature and contrasts with the inhibition by PA, which is sensitive to hydrophobic as well as electrostatic interactions.

Because the insulin receptor is active in detergent, it was difficult to assess the ability of lipids to "activate" the receptor. However, the ability of other lipids to relieve PA-mediated inhibition provided an indication of the affinity of the receptor for various lipids which, alone, had no

significant effects on insulin receptor function. Of the lipids tested, PE was the most effective at competing with either PA or sphingosine (not shown), suggesting a preferential interaction of this lipid with the receptor. Even though PC alone caused a slight increase in insulin receptor activity, this lipid was less effective at relieving lipid-mediated inhibition compared with PE. Of the negatively charged lipids, PG was more effective than PI in relieving PA-mediated inhibition of the receptor, suggesting that the receptor has a higher affinity for PG compared with PI. At the concentrations tested, PS was unable to relieve PA-mediated inhibition, suggesting that *its affinity* for the receptor is lower than that for PA. This lipid was, however, effective at relieving sphingosine-mediated inhibition, indicating that the lipid does not form a microdomain that is inaccessible to the receptor (not shown). In summary, the insulin receptor likely has different affinities for particular phospholipids, but only the interaction with PA causes a marked effect on the function of the receptor. It is noteworthy that PA has been shown to form domains in membranes [Haverstick and Glaser, 1988], so that increased local concentrations of this lipid may modulate the activity of the receptor even though the bulk concentration of PA in membranes is low.

CONCLUSIONS

Our data reveal that protein:lipid interactions regulate insulin receptor function. The finding that PA is a potent inhibitor of the insulin receptor presents the intriguing possibility that this lipid is a physiological regulator of insulin receptor function. Mounting evidence suggests that PA is a "second messenger in its own right" [Knauss et al., 1990]. The lipid is not only a product in the protein kinase C signaling pathway [Merrill and Liotta, 1991] but is also produced by phospholipase D activation [Exton, 1990]. It is a potent mitogen, stimulating DNA synthesis and inducing the expression of c-*fos* and c-*myc* [Moolenaar et al., 1986]. Considerable evidence reveals that PA is produced in direct response to insulin stimulation: insulin-stimulated increases in PA levels have been reported to arise from both de novo synthesis [Farese et al., 1987, 1988; Vila et al., 1990] and via hydrolysis of a phosphatidylinositol glycan to yield diacylglycerol which is then metabolized to PA [Suzuki et al., 1991]. This lipid has been reported to regulate several membrane enzymes, including regulatory proteins such as kinases [Bocckino et al., 1991] and a GTPase activating protein [Tsai et al., 1989]. Sphingosine has also been reported to increase PA levels, both by stimulating a phospholipase D and by inhibiting PA phosphohydrolase [Zhang et al., 1990; Lavie et al., 1990; Lavie and Liscovitch, 1990]. Increases in PA could act as negative regulators of insulin receptor activity, perhaps contributing to downregulation of insulin transduction. A similar role for sphingosine has been proposed for the protein kinase C signaling pathway and the insulin pathway [Arnold and Newton, 1991; Hannun and Bell, 1989].

It is most interesting that another lipid second messenger, diaylglycerol, activates the insulin receptor by increasing its affinity for insulin [Arnold and Newton, 1996]. Thus, lipid second messengers may fine tune insulin signaling by direct interactions with the insulin receptor. Such regulation may be particularly relevant in diabetes where increased lipid metabolism affects membrane lipid composition [Whiting et al., 1977; Woods et al., 1981; Keelan et al., 1985; Baldini et al., 1989].

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