Inhibition of the Insulin Receptor Tyrosine Kinase by Sphingosine†

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ABSTRACT: Sphingosine inhibits autophosphorylation of the insulin receptor tyrosine kinase in vitro and in situ. This lysosphingolipid has been shown previously to inhibit the Ca^{2+}/lipid-dependent protein kinase C. Here we show that insulin-dependent autophosphorylation of partially purified insulin receptor is half-maximally inhibited by 145 μM sphingosine (9 mol %) in Triton X-100 micelles. Half-maximal inhibition of protein kinase C autophosphorylation occurs with 60 μM sphingosine (3.4 mol %) in Triton X-100 mixed micelles containing phosphatidylserine and diacylglycerol. Sphingomyelin does not inhibit significantly the insulin receptor, suggesting that, as with protein kinase C, the free amino group may be essential for inhibition. Similar to the effects observed for protein kinase C, inhibition of the insulin receptor kinase by sphingosine is reduced in the presence of other lipids. However, the reduction displays a marked dependence on the lipid species: phosphatidylserine, but not a mixture of lipids compositionally similar to the cell membrane, markedly reduces the potency of sphingosine inhibition. The inhibition occurs at the level of the protein-/membrane interaction: a soluble form of the insulin receptor comprising the cytoplasmic kinase domain is resistant to sphingosine inhibition. Lastly, sphingosine inhibits the insulin-stimulated rate of tyrosine phosphorylation of the insulin receptor in NIH 3T3 cells expressing the human insulin receptor. These results suggest that sphingosine alters membrane function independently of protein kinase C.

Binding of insulin to the cell surface results in a multitude of cellular responses, from short-term effects (e.g., alterations in the permeability of the plasma membrane and in intracellular enzyme activity) to long-term changes (e.g., synthesis of protein, lipid, and nucleic acids) (Rosen, 1987). Phosphorylation plays a central role in transduction of the insulin signal. Insulin stimulates the autophosphorylation of the β subunit of the $\alpha_2\beta_2$ heterotetrameric insulin receptor, and considerable evidence suggests that this initial phosphorylation is required for the intracellular processing of the insulin signal (Rosen, 1987; Ellis et al., 1986; Chou et al., 1987; Morgan & Roth, 1987; Steele-Perkins & Roth, 1990).

Sphingosine and other lysosphingolipids are potent inhibitors of the Ca^{2+}/lipid-dependent protein kinase C, both in vitro and in situ (Hannun & Bell, 1987, 1989). Protein kinase C is activated by receptor-mediated hydrolysis of phosphatidylinositol bisphosphate to yield diacylglycerol and inositol trisphosphate (Nishizuka, 1986, 1988). The kinase is also activated by phorbol esters, potent tumor promoters, which compete with diacylglycerol. Pretreatment with sphingosine prevents many of the cellular effects mediated by protein kinase C upon phorbol ester stimulation (Merrill et al., 1986; Wilson et al., 1986; Hannun et al., 1987). Mechanistic studies by Bell and co-workers suggest that sphingosine competes with diacylglycerol and phorbol esters, that the inhibition is subject to surface dilution, and that the inhibition occurs by an interaction with the lipid-interacting domain of protein kinase C (Hannun et al., 1986). Structural investigations revealed that the free amine group of sphingosine is required for inhibition (Merrill et al., 1989).

Sphingosine has been shown recently to inhibit several insulin-stimulated cellular events. Pretreatment of rat adipocytes with sphingosine inhibits insulin-stimulated hexose transport and glucose oxidation, without affecting insulin binding (Robertson et al., 1989), and inhibits insulin-stimulated lipogenesis (Smal & De Meyts, 1989). In addition, sphinganine and sphingosine inhibit glucose transport in 3T3-L1 fibroblasts (Nelson & Murray, 1986). The involvement of protein kinase C in processing the insulin signal has been suggested based, in part, on these observations.

Here we report that sphingosine is a potent inhibitor of the autophosphorylation and substrate phosphorylation catalyzed by purified insulin receptor. Thus, the involvement of protein kinase C is not necessary to account for the inhibition of insulin-stimulated events by this lipid. While protein kinase C may mediate some of the effects of insulin, our results indicate that sphingosine inhibits transduction of the insulin signal at the level of the receptor.

Materials and Methods

Materials

Porcine insulin was purchased from Calbiochem, [γ-^{32}P]-ATP (3000 Ci mmol^{-1}; 10 mCi mL^{-1}) from Dupont–New England Nuclear, and Triton X-100 (10% aqueous solution low in carbonyl and peroxide content) from Pierce Chemical Co. Anti-phosphotyrosine antibody (PY20) was obtained from ICN Biomedicals, Inc. Alkaline phosphatase conjugated goat anti-mouse was from Boehringer Mannheim Biochemicals and nitrocellulose membrane from Schleicher & Schuell. Aprotinin, ATP, benzamidine, bovine serum albumin (BSA),[1] 5-bromo-4-chloroindolyl phosphate, cholesterol, gentamicin sulfate, HEPES, histone type VII-S, leupeptin, pepstatin A, phenylmethanesulfonyl fluoride (PMSF), phenyl-Sepharose CL-4B, poly(lysine)–agarose, sphingosine, Tris base, and wheat germ agglutinin–agarose (WGA–agarose) were supplied by

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[1] Abbreviations: BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride; WGA, wheat germ agglutinin; R-BIRK, soluble baculovirus insulin receptor kinase (includes residues 941–1343 of the human insulin receptor).

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Sigma Chemical Co. Fetal bovine serum was from Biocell Laboratories. L-α-Phosphatidylserine (bovine brain), 1,2-dioleoylglycerol, phosphatidylcholine (egg), phosphatidylethanolamine (bovine liver), and sphingomyelin (bovine brain) were obtained from Avanti Polar Lipids. Dulbeco's modified Eagle's medium was purchased from Fisher Scientific. Sephacryl S-400 resin and the Mono Q HR 5/5 column were supplied by Pharmacia, DEAE-cellulose by Whatman, and hydroxylapatite (Bio-Gel HT) by Bio-Rad. Centricon-30 microconcentrators were from Amicon. All other chemicals were reagent grade.

Methods

Insulin Receptor. Livers from 20 male Long Evans or Sprague Dawley rats (approximately 150 g) were homogenized in buffer containing 2 mM EDTA, 10 mM EGTA, 0.25 mM sucrose, and 50 mM HEPES, pH 7.5 at 4 °C. Insulin receptor was partially purified from the Triton X-100 (2%)-solubilized membrane fraction by sequential gel filtration (Sephacryl S-400) and WGA–agarose chromatography, as described (O'Hare & Pilch, 1989). Insulin receptor was stored at −20 °C in buffer containing 0.15 M *N*-acetylglucosamine, 50% glycerol, 0.1% Triton X-100, 0.5 mM EDTA, 0.5 mM EGTA, and 25 mM HEPES, pH 7.5. In some cases, WGA–agarose-purified receptor was purified by Mono Q chromatography, using a modification of the procedure of O'Hare and Pilch (1989). Briefly, sample was applied at 1.0 mL min^{-1}, the column was washed with buffer A (0.1% Triton X-100, 0.02% NaN_3, and 20 mM Tris, pH 7.6), and insulin receptor was eluted with a gradient of 0–0.23 M Na_2SO_4 in buffer A, at 2.0 mL min^{-1} over a 10-min period. The concentration of Na_2SO_4 was reduced 100-fold by concentration of pooled insulin receptor fractions using a Centricon-30 microconcentrator (Amicon) followed by dilution into 50 mM HEPES, pH 7.5. The sample was stored in 0.1% Triton X-100, 50% glycerol, and 25 mM HEPES, pH 7.5 at −20 °C. WGA–agarose-purified receptor or Mono Q purified receptor yielded similar results in phosphorylation assays.

Protein Kinase C. Protein kinase C was isolated from the cytosolic extract of homogenized rat brain (Sprague Dawley) by sequential DEAE, phenyl-sepharose, poly(lysine)–agarose, and hydroxylapatite chromatography (Huang et al., 1986a,b). Hydroxylapatite isozyme I was employed in these studies.

Lipids. Triton X-100 mixed micelles containing the indicated lipids were prepared by drying a chloroform solution of lipid with a stream of N_2 and hydrating in buffer containing either 0.1% Triton X-100 in 50 mM HEPES, pH 7.5 (insulin receptor assays), or 1.0% Triton X-100 in 20 mM Tris, pH 7.5 (protein kinase C assays). For insulin receptor assays, micelles were diluted 2.8-fold into the assay mix which contained 0.1% Triton X-100; thus, the lipid was diluted 2.8-fold, but the detergent concentration remained constant. For protein kinase C assays, micelles were diluted 10-fold into the assay mixture. The final Triton X-100 concentration was 0.1% for both insulin receptor kinase and protein kinase C assays. On the basis of a molecular weight of 650 for Triton X-100, this corresponds to 1.5 mM detergent. The mole percent of lipid was calculated relative to this detergent concentration.

Insulin Receptor Assay. Autophosphorylation was assayed by measuring the incorporation of phosphate from [γ-^{32}P]ATP into the β subunit of the insulin receptor. Insulin receptor (21 μL of receptor in 50 mM HEPES, pH 7.5, and 0.1% Triton X-100), micelles (25 μL of 0.1% Triton X-100 with or without lipid), insulin (7 μM in 50 mM HEPES, pH 7.5), and DTT (18 mM in 0.1% Triton X-100 and 50 mM HEPES, pH 7.5) were combined and incubated for 5 min at 25 °C. The reaction was initiated by the addition of 14 μL of a solution containing 50 mM HEPES, 0.15% Triton X-100, 25 mM $MnCl_2$, 20 mM $MgCl_2$, and 250 μM [γ-^{32}P]ATP (approximately 850 mCi $mmol^{-1}$). The final concentrations of species in the reaction mixture were 0.7 μM insulin, 5 mM $MnCl_2$, 4 mM $MgCl_2$, 50 μM ATP, 1 mM DTT, 0–630 μM lipid, and 0.1% Triton X-100. The reaction was stopped after 60 s by the addition of 25 μL of SDS–PAGE sample buffer (0.13 M Tris, 4.2% SDS, 21% glycerol, 0.004% bromophenol blue, and 20% β-mercaptoethanol, pH 6.8) containing 0.05 M ATP and 0.05 M EDTA. In some cases, autophosphorylation reactions were performed varying the insulin or ATP concentrations. Histone phosphorylation assays were performed similarly, except that sphingosine and partially purified receptor were preincubated[2] for 5 min, 25 °C, before the addition of insulin, DTT, and histone (2 mg mL^{-1}). Reactions were initiated by addition of [^{32}P]ATP, as described above, and terminated after 20 min, at 25 °C, by adding SDS–PAGE sample buffer. Samples were analyzed by SDS–polyacrylamide gel electrophoresis (7.5% polyacrylamide for autophosphorylation experiments; 15% for histone experiments) (Ames, 1974) followed by autoradiography. Incorporation of ^{32}P into the β subunit of the insulin receptor or histone was determined by liquid scintillation counting of bands excised from gels. The rates of autophosphorylation and histone phosphorylation were constant during the course of the assays. Sphingosine did not affect the pH of the reaction mixture.

Autophosphorylation of the soluble baculovirus insulin receptor kinase (R-BIRK) (Villalba et al., 1989) was assayed by measuring the incorporation of phosphate from [γ-^{32}P]ATP in the absence or in the presence of manganese. R-BIRK (5 μL of stock enzyme in 50 mM Tris, pH 7.4, 100 mM NaCl, and 1 mM DTT) was combined with buffer (32 μL of 22 mM $MgCl_2$, 2 mM DTT, and 0.15% Triton X-100, in 50 mM HEPES, pH 7.5, with or without 11 mM $MnCl_2$) and micelles (25 μL of 0.1% Triton X-100 with or without 0–30 mol % sphingosine) and then incubated for 5 min at 25 °C. The reaction was initiated by the addition of 14 μL of a solution containing 50 mM HEPES, pH 7.5 and 250 μM [γ-^{32}P]ATP (850 mCi $mmol^{-1}$). The final concentrations of species in the reaction mixture were 7 mM NaCl, 9 mM $MgCl_2$, 0 or 5 mM $MnCl_2$, 50 μM ATP, 1 mM DTT, 0–630 μM lipid, and 0.1% Triton X-100. The reaction was stopped after 2 min by the addition of 25 μL of SDS–PAGE sample buffer, and samples were analyzed by SDS–polyacrylamide gel electrophoresis (10% polyacrylamide) and autoradiography, as described above.

Sphingosine Treatment of Cell Membranes. NIH mouse 3T3 cells expressing human insulin receptor [NIH 3T3 HIR3.5 cells (Whittaker et al., 1987)] were grown to confluency at 37 °C in Dulbeco's modified Eagle's medium containing 10% fetal bovine serum and 50 μg mL^{-1} gentamicin sulfate. Trypsin-digested cells (approximately 4×10^6 cells) were harvested by centrifugation (2000*g*, 10 min, 4 °C) and resuspended in 1 mL of 50 mM HEPES, pH 7.5. Cells were lysed by homogenization in 50 mM HEPES, pH 7.5, and membranes were isolated by centrifugation of the lysate at 500000*g* for 15 min, 4 °C. The membrane pellet was resuspended in 1 mL of 50 mM HEPES, pH 7.5. Insulin receptor tyrosine kinase activity was assayed by measuring the incor-

[2] Preincubation of receptor with sphingosine was essential for sphingosine-mediated inhibition of histone phosphorylation. Incubation first with histone may result in binding of this basic protein to the receptor, thus masking potential sites on the receptor that interact with the positively charged lysosphingolipid.

poration of phosphate from [γ-^{32}P]ATP into the β subunit of the insulin receptor. Membrane fraction (10 μL) was preincubated with 25 μL of 50 mM HEPES, pH 7.5, containing 0–1 mM sphingosine for 1.5 min at 25 °C, followed by addition of a solution (22 μL) containing 2.2 μM insulin, 3.3 mM DTT, and 484 mM KCl in 50 mM HEPES, pH 7.5. The reaction was initiated by the addition of 14 μL of a solution containing 25 mM $MnCl_2$, 20 mM $MgCl_2$, 250 μM [γ-^{32}P]-ATP (approximately 200 mCi mmol^{-1}), and 50 mM HEPES, pH 7.5. The final concentrations of species in the reaction mixture were 0.7 μM insulin, 150 mM KCl, 5 mM $MnCl_2$, 4 mM $MgCl_2$, 50 μM ATP, 1 mM DTT, and 0–350 μM sphingosine. The reaction was stopped after 2 min at 25 °C by the addition of 25 μL of SDS–PAGE sample buffer, and samples were analyzed by SDS–polyacrylamide gel electrophoresis (7.5%) followed by autoradiography. Relative ^{32}P incorporation into the β-subunit band (approximately 100 kDa) was determined by scanning autoradiograms using a Hoefer densitometer.

Sphingosine Treatment of Intact Cells. NIH 3T3 HIR3.5 cells were harvested and resuspended at a concentration of approximately 4 × 10^6 cells mL^{-1} in phosphate-buffered saline containing 137 mM NaCl, 13 mM KCl, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 16 mM Na_2HPO_4, and 0.9 mM $CaCl_2$ (PBS). Sphingosine (1.5 μL of a 0.06 or 0.6 M solution in ethanol) was added to 100 μL of PBS containing BSA at a concentration yielding a ratio of sphingosine to BSA of 10:1. BSA solution (55 μL), with or without sphingosine, was added to 0.45 mL of cell suspension. Thus, the concentration of sphingosine in the cell suspension was 0, 0.1, or 1 mM. After incubation for 30 min (0 and 1 mM sphingosine) or 60 min (0 and 0.1 mM sphingosine) at 25 °C, insulin (17 μM in PBS) was added to yield a final concentration of 80 nM. Aliquots of cell suspension (56 μL) were added to sample buffer after 15–300 s. The viability of control and sphingosine-treated cells was similar as judged by exclusion of trypan blue. Samples were analyzed by SDS–polyacrylamide electrophoresis followed by electrophoretic transfer to a nitrocellulose membrane (Towbin et al., 1979), and tyrosine-phosphorylated insulin receptor was labeled with a monoclonal antibody specific for phosphotyrosine (PY20, ICN). Blots were incubated with alkaline phosphatase conjugated IgG, and primary antibody labeling was detected by monitoring the formation of the insoluble product of 5-bromo-4-chloroindolyl phosphate hydrolysis (Blake et al., 1984). Relative phosphotyrosine labeling was quantified by scanning densitometry of blots.

Protein Kinase C Assay. Protein kinase C autophosphorylation was assayed in the presence of Triton X-100/phosphatidylserine/diacylglycerol [(75–85):10:5; 0.1% Triton X-100]-mixed micelles containing 0–10 mol % (0–196 μM) sphingosine, as described (Newton & Koshland, 1989). Autophosphorylation reactions were allowed to proceed for 4 min at 30 °C before being quenched in SDS–PAGE sample buffer. Samples were analyzed by SDS–polyacrylamide gel electrophoresis (10%), and incorporated radioactivity was determined by liquid scintillation counting of excised gel bands.

Results

Insulin Receptor Autophosphorylation in Mixed Micelles. Incubation of WGA–agarose-purified insulin receptor with increasing concentrations of sphingosine resulted in decreased incorporation of [^{32}P]phosphate into the β subunit of the receptor (Figure 1A). Triton X-100 mixed micelles containing 0–30 mol % sphingosine, corresponding to 0–630 μM sphingosine, were incubated with insulin receptor under conditions where the rate of phosphate incorporation was linear with time

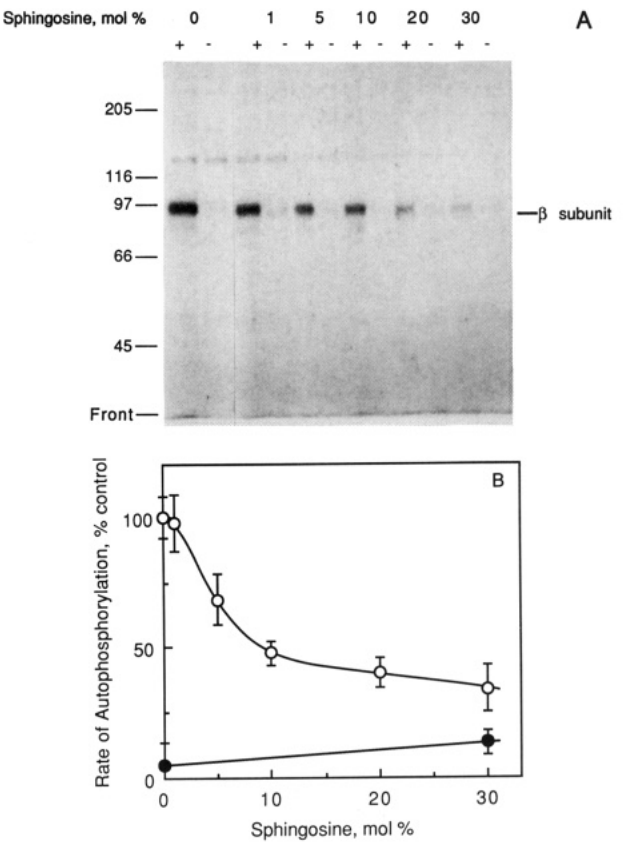

FIGURE 1: Sphingosine inhibits autophosphorylation of the insulin receptor tyrosine kinase. (A) Autoradiogram of an SDS–polyacrylamide gel (7.5%) showing [^{32}P]phosphate incorporated into the insulin receptor β subunit, in 1 min at 25 °C, in the presence of Triton X-100 (0.1%) mixed micelles containing 0–30 mol % sphingosine. Autophosphorylation of WGA–agarose-purified receptor was measured in the presence (+) or absence (−) of 0.7 μM insulin. 30 mol % corresponds to 630 μM sphingosine. Exposure time was 48 h at −70 °C. (B) Rate of autophosphorylation, expressed relative to control (pure Triton X-100 micelles, no lipid), of the insulin receptor β subunit measured in the presence of Triton X-100 mixed micelles containing the indicated sphingosine concentrations. Autophosphorylation of Mono Q purified insulin receptor was measured in the presence (○) or absence (●) of 0.7 μM insulin. Rate represents the amount of [^{32}P]phosphate incorporated into the insulin receptor β subunit in 1 min at 25 °C. Error bars represent standard error of the mean of triplicate assays. Under the conditions of these assays, the rate of autophosphorylation was constant for the first minute of the reaction.

(1 min, 25 °C). Mono Q purified insulin receptor was inhibited similarly by sphingosine. Results from 10 separate experiments revealed that 9 ± 1 mol % sphingosine, corresponding to 145 μM lipid, half-maximally inhibited insulin-stimulated autophosphorylation. Insulin-independent activity was <10% of control activity (no sphingosine) and increased slightly with sphingosine. Inhibition of insulin receptor autophosphorylation was biphasic: in the experiment presented in Figure 1B, 65% of the activity was inhibited half-maximally by 4 mol % sphingosine (61 μM), while the remaining 35% was inhibited to a significantly lower degree (78% inhibition of insulin-dependent autophosphorylation was observed at 30 mol % sphingosine). The degree of inhibition with increasing concentration of lipid varied slightly with different sphingosine preparations. Similar results were obtained when proteins from phosphorylation reactions were transferred to nitrocellulose and probed with anti-phosphotyrosine antibody (not shown). This indicates that sphingosine inhibits directly the autophosphorylation of the insulin receptor.

Sphingosine also inhibited the insulin-stimulated phosphorylation of the substrate histone (Figure 2) and a synthetic

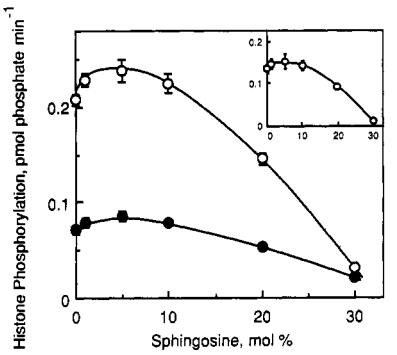

FIGURE 2: Sphingosine inhibits substrate phosphorylation catalyzed by the insulin receptor. Rate of histone phosphorylation measured in the presence of Triton X-100 (0.1%) mixed micelles containing 0 or 30 mol % sphingosine in the presence (○) or absence (●) of 0.7 μM insulin. Phosphorylation was measured in the presence of 2 mg mL^{-1} histone, for 20 min at 25 °C; incorporation of phosphate was linear for the first 20 min of the assay. Error bars represent standard error of the mean of triplicate assays. Inset: Insulin-dependent histone phosphorylation, obtained from the data in the main graph, expressed as picomoles of [^{32}P]phosphate incorporated per minute.

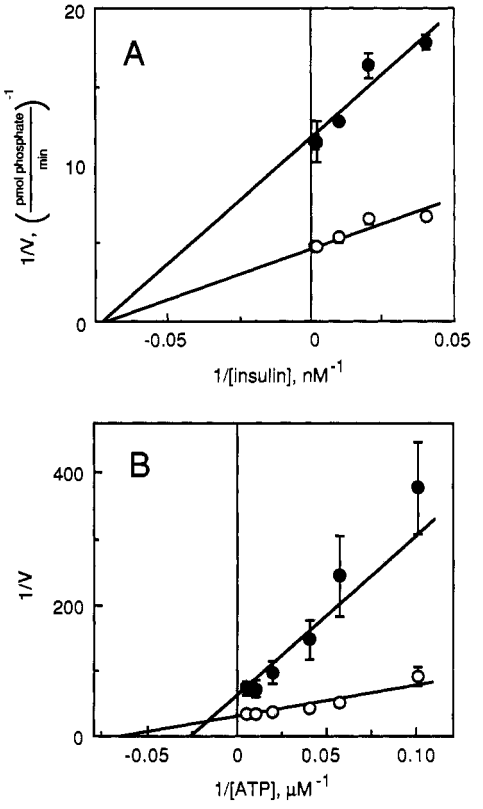

FIGURE 3: Effect of sphingosine on the interaction of the insulin receptor with insulin and ATP. (A) Double-reciprocal plot of the activity of the insulin receptor and insulin concentration at 0 mol % (○) and 20 mol % sphingosine (●). Activity represents [^{32}P]phosphate incorporated into the receptor in 1 min, 25 °C, in the presence of 50 μM [^{32}P]ATP. (B) Double-reciprocal plot of the activity of the insulin receptor and ATP concentration at 0 mol % (○) and 20 mol % sphingosine (●). Activity (relative units) represents the amount of phosphotyrosine-labeled receptor after incubation for 1 min, 25 °C, in the presence of 0.7 μM insulin, determined by densitometric analysis of Western blots.

peptide substrate of the receptor (not shown). The inset in Figure 2 shows that 23 mol % sphingosine half-maximally inhibited the rate of insulin-dependent histone phosphorylation.

Sphingosine had no significant effect on the K_m for insulin, which was 13 ± 3 nM insulin in the absence of sphingosine and 18 ± 6 nM insulin in the presence of 20 mol % sphingosine

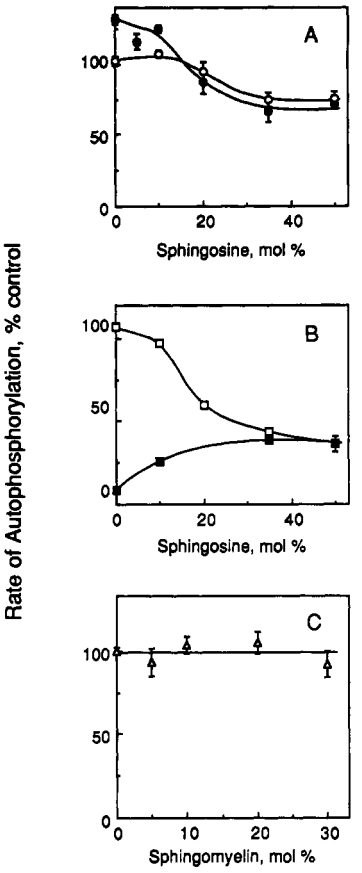

FIGURE 4: Effect of various lipid/sphingosine combinations and sphingomyelin on the inhibition of the insulin receptor kinase. (A) Rate of autophosphorylation of the insulin receptor, expressed relative to pure Triton X-100 (0.1%) micelles (no lipid), measured in the presence of Triton X-100 (0.1%) mixed micelles containing 0–50 mol % (1.8 mM) sphingosine and 10 mol % phosphatidylcholine (●) or 10 mol % phosphatidylserine (○). (B) Rate of autophosphorylation of the insulin receptor measured in the presence of Triton X-100 (0.1%) mixed micelles containing 10 mol % of a mixture of lipids compositionally similar to those found in the inner monolayer of erythrocytes (phosphatidylserine:phosphatidylethanolamine:phosphatidylcholine:sphingomyelin:cholesterol 15:24:8:6:47 mole (ratio) and 0–50 mol % sphingosine. Assay was performed in the presence (□) or absence (■) of 0.7 μM insulin. (C) Rate of autophosphorylation of the insulin receptor, expressed as percent of a control (no lipid), measured in the presence of Triton X-100 (0.1%) mixed micelles containing 0–50 mol % sphingomyelin (1.5 mM) (△). All assays were analyzed by measuring [^{32}P]phosphate incorporation into the β subunit of the insulin receptor in the presence of 0.7 μM insulin for 1 min at 25 °C, except as indicated.

(Figure 3A). In contrast, sphingosine reduced the V_{max} to 42% of the rate observed in the absence of the lipid. Thus, sphingosine does not affect the affinity of the insulin receptor for insulin. Separate experiments revealed that sphingosine does not inhibit the binding of ^{125}I-insulin to the receptor (data not shown), consistent with the results of Robertson et al. (1989).

Both the K_m and V_{max} for ATP were affected by sphingosine (Figure 3B). Sphingosine (20 mol %) resulted in an approximately 3-fold increase in the K_m (from 15 ± 5 μM ATP to 40 ± 14 μM ATP) and a 46% reduction in the V_{max}. These results suggest that sphingosine reduces the affinity of the kinase for ATP.

The presence of the neutral lipid phosphatidylcholine resulted in a reduction in the ability of sphingosine to inhibit the β-subunit autophosphorylation. Figure 4A (filled circles) shows that half-maximal inhibition of the initial rate of insulin-stimulated autophosphorylation occurred with 17 mol

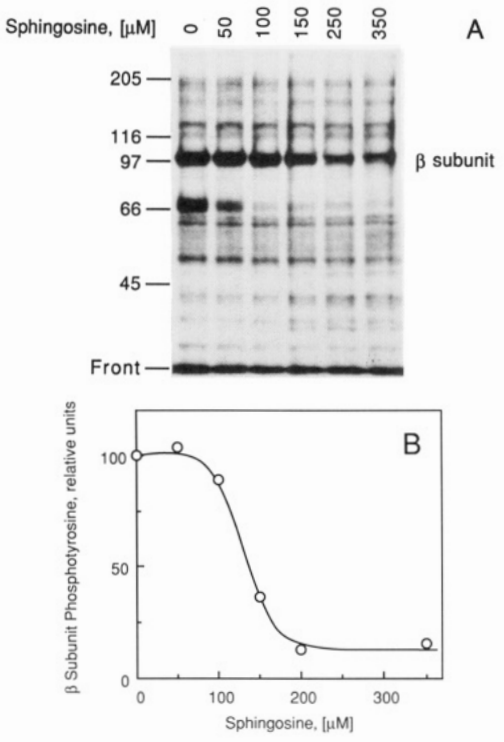

FIGURE 5: Sphingosine inhibits the phosphorylation of plasma membrane bound insulin receptor. Membranes from NIH 3T3 HIR 3.5 cells were incubated with 0–350 μM sphingosine. [^{32}P]Phosphate incorporation into membrane proteins was measured in the presence of 150 mM KCl and 0.7 μM insulin for 2 min at 25 °C, as described under Methods. (A) Autoradiogram of phosphorylated membrane proteins; the position of the β subunit is indicated. Exposure time was 15 h at −70 °C. (B) Densitometric analysis of the autoradiogram in (A) showing the intensity of the phosphorylated β-subunit band relative to control (no sphingosine).

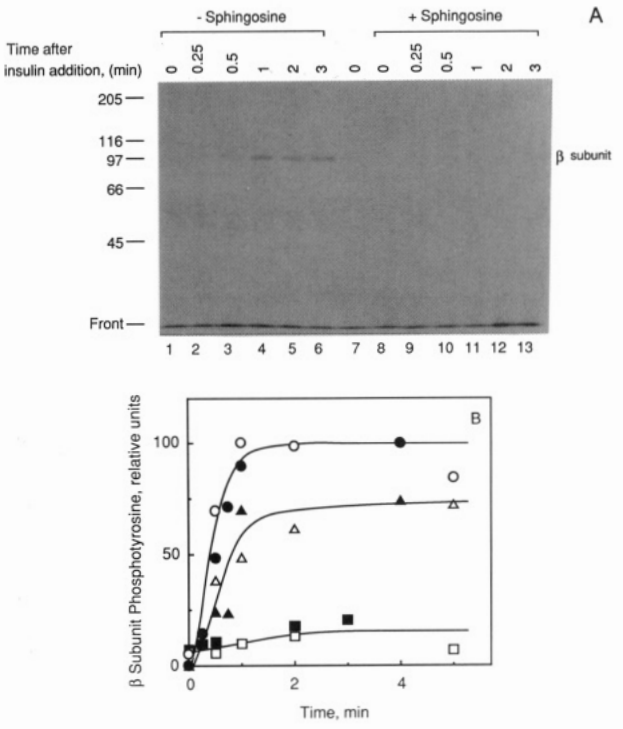

FIGURE 6: Sphingosine inhibits insulin receptor autophosphorylation in situ. (A) Western blot showing phosphotyrosine in NIH 3T3 HIR 3.5 cells after addition of 80 nM insulin to cells preincubated in medium containing BSA and 0 or 1 mM sphingosine, as described under Methods. Lane 7 shows cells incubated in the absence of BSA. (B) Insulin-stimulated β-subunit phosphotyrosine, determined from densitometric analysis of Western blots, in cells preincubated with 0 (○, ●), 100 μM (▲, △), or 1 mM (□, ■) sphingosine. Open and filled symbols represent data from two separate experiments.

% sphingosine (342 μM) when micelles contained 10 mol % phosphatidylcholine. Furthermore, approximately half the activity appeared to be resistant to sphingosine inhibition. This resistance may have resulted from stimulation of the receptor kinase activity by phosphatidylcholine, since phosphatidylcholine alone increased the rate of autophosphorylation 31% relative to lipid-free Triton X-100 micelles.

Inclusion of phosphatidylserine in mixed micelles resulted in a marked diminution of sphingosine potency. Figure 4A (open circles) shows that the rate of autophosphorylation of the insulin receptor was relatively insensitive to sphingosine (0–50 mol %) if micelles contained 10 mol % phosphatidylserine. For example, only 26% inhibition was observed in the presence of 50 mol % (1.8 mM) sphingosine.

The potency of sphingosine in the presence of lipids representative of those in the inner monolayer of the plasma membrane was investigated. Mixed micelles contained 10 mol % of a mixture of lipids compositionally similar to that found in the inner monolayer of erythrocytes (phosphatidylserine:phosphatidylethanolamine:phosphatidylcholine:sphingomyelin:cholesterol 15:24:8:6:47 mole ratio) (Verkleij et al., 1973). Figure 4B shows that the presence of a constant mole percent of inner monolayer lipid reduced only slightly the degree of sphingosine-mediated inhibition of [^{32}P]phosphate incorporation into the β subunit relative to no inner monolayer lipid (see Figure 1). Half-maximal inhibition was observed at 18 mol % sphingosine (338 μM). The insulin-dependent phosphorylation was completely inhibited by 50 mol % sphingosine. In the presence of cell lipids, sphingosine caused a slight increase in the insulin-independent phosphorylation of the receptor.

Figure 4C shows that sphingomyelin did not inhibit autophosphorylation of the insulin receptor tyrosine kinase. The rate of β-subunit autophosphorylation was unaffected by up to 30 mol % (630 μM) sphingomyelin in Triton X-100 micelles.

Insulin Receptor Autophosphorylation in Cell Membranes. The ability of sphingosine to inhibit plasma membrane bound insulin receptor was investigated in NIH 3T3 cell transfected with the cDNA for the human insulin receptor (NIH 3T3 HIR3.5 cells) (Whittaker et al., 1987). These cells express on the order of 10^6 copies of human insulin receptor per cell. Figure 5 reveals that addition of sphingosine to membranes isolated from NIH 3T3 HIR3.5 cells inhibited the insulin-stimulated phosphorylation of the β subunit, measured in the presence of 150 mM KCl. Moreover, sphingosine affected the phosphorylation state of several other membrane proteins. The autoradiogram in Figure 5A shows that sphingosine inhibited the phosphorylation of a protein with an apparent molecular weight of 70K, in addition to the β-subunit phosphorylation. In contrast, the phosphorylation of other proteins (apparent molecular weights of 41K and 35K; Figure 5A) was stimulated by sphingosine. Because sphingosine is relatively water-soluble, the final concentration of this lipid in the membrane was not established. However, insulin-stimulated phosphorylation of the β subunit was half-maximally inhibited by 133 μM sphingosine (Figure 5B).

Insulin Receptor Autophosphorylation in Situ. Pretreatment of NIH 3T3 HIR3.5 cells with sphingosine inhibited the insulin-stimulated tyrosine phosphorylation of the insulin receptor. Figure 6 shows that 1 mM sphingosine reduced by >90% the insulin-stimulated tyrosine phosphorylation of the β subunit, detected on Western blots of whole cell extracts using phosphotyrosine antibodies. Cells pretreated with 100

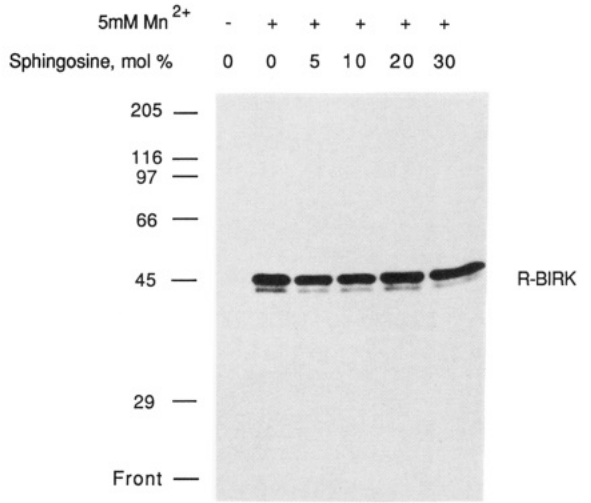

FIGURE 7: Sphingosine does not inhibit the soluble insulin receptor kinase domain. Autoradiogram of an SDS–polyacrylamide gel (10%) showing [^{32}P]phosphate incorporation into baculovirus-expressed cytosolic domain (R-BIRK) in the presence of 0–30 mol % (630 μM) sphingosine. Assays were performed in the absence (–) or presence (+) of 5 mM $MnCl_2$ as described under Methods. Exposure time was 19 h at –70 °C.

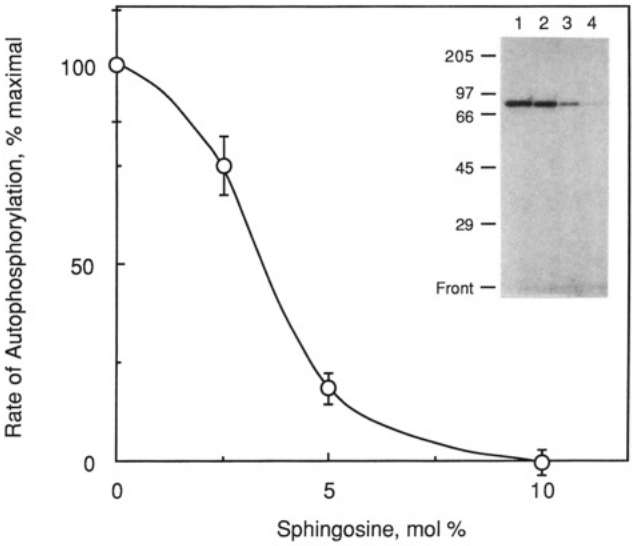

FIGURE 8: Sphingosine inhibits autophosphorylation of protein kinase C. Protein kinase C autophosphorylation was measured in the presence of Triton X-100 (0.1%) mixed micelles containing 10 mol % phosphatidylserine, 5 mol % diacylglycerol, and 0–10 mol % sphingosine. Assays were performed in the presence of 0.63 mM $CaCl_2$, at 30 °C for 4 min, as described under Methods. Inset: Autoradiogram of an SDS–polyacrylamide gel (10%) showing ^{32}P-labeled protein kinase from the experiment described above: 0 mol % sphingosine (lane 1); 2.5 mol % sphingosine (lane 2); 5 mol % sphingosine (lane 3); 10 mol % sphingosine (lane 4). Absolute lipid concentrations in micelles containing 10 mol % sphingosine were as follows: sphingosine, 196 μM; phosphatidylserine, 196 μM; diacylglycerol, 98 μM.

μM sphingosine also incorporated significantly less phosphate on β-subunit tyrosines in response to insulin (Figure 6B). Inhibition of the receptor in intact cells is consistent with sphingosine inhibiting insulin-stimulated events by inhibiting the tyrosine kinase activity of the insulin receptor.

R-BIRK Autophosphorylation. The autophosphorylation of the soluble kinase domain of the insulin receptor, R-BIRK, was not affected by sphingosine (Figure 7). This recombinant protein comprises residues 941–1343 inclusive, of the human insulin receptor, and autophosphorylates in an insulin-independent, but Mn^{2+}-dependent manner (Villalba et al., 1989). The inability of sphingosine to affect the activity of the soluble kinase domain indicates that sphingosine inhibition occurs at the level of the receptor/membrane interaction.

Protein Kinase C Autophosphorylation. Figure 8 shows that sphingosine also inhibited the autophosphorylation of protein kinase C. The rate of autophosphorylation was half-maximally inhibited by micelles containing 3.4 mol % sphingosine (60 μM) in Triton X-100/phosphatidylserine/diacylglycerol-mixed micelles (10 mol % phosphatidylserine; 5 mol % diacylglycerol). This degree of inhibition agrees with recent work by Hannun and Bell (1990) showing that autophosphorylation is half-maximally inhibited by 1 mol % sphingosine in Triton X-100 mixed micelles containing 8 mol % phosphatidylserine and 2 mol % diacylglycerol.

Discussion

In this report, we have shown that sphingosine directly inhibits the rate of autophosphorylation of the insulin receptor in mixed lipid/detergent micelles, in the cell membrane, and in intact cells. The rate of substrate phosphorylation is inhibited similarly. Sphingosine does not alter the affinity of insulin for the receptor. This is consistent with previous studies showing that insulin binding to the surface of adipocytes is unaffected by sphingosine (Robertson et al., 1989; Smal & De Meyts, 1989). Therefore, sphingosine inhibits transduction of the insulin signal by reducing the intrinsic tyrosine kinase activity of the insulin receptor. The affinity of the kinase for ATP is reduced by sphingosine. Inhibition occurs at the level of the receptor/membrane interaction, since the soluble kinase domain is not sensitive to sphingosine. Similar to its effect on protein kinase C, sphingomyelin did not inhibit the intrinsic tyrosine kinase activity of the insulin receptor, suggesting that the free amine group may be essential for inhibition of both kinases. Indeed, the positively charged stearylamine also inhibits the receptor (data not shown). Electrostatic interactions likely play a role in the sphingosine-mediated inhibition of the receptor since ongoing work reveals that altering the surface charge of the membrane or ionic strength of the medium affects the potency of sphingosine (data not shown). Thus, an interaction of the positively charged sphingoid base with a lipid-interacting domain of the receptor is likely responsible for the altered catalytic efficiency of the receptor.

The inhibition of purified insulin receptor by sphingosine was biphasic: approximately half the activity was inhibited by <10 mol % sphingosine, while the remainder of the activity was relatively insensitive to the lysolipid. Different phosphorylation sites on the insulin receptor may be more or less sensitive to inhibition by sphingosine. If this is the case, sphingosine may not act on the catalytic activity of the β subunit, but rather may mask potential phosphorylation sites. Alternatively, a population of receptor may be less susceptible to inhibition by sphingosine. Evidence for different species of insulin receptor in rat liver has been reported (Koch et al., 1986).

The inhibitory potency of sphingosine was sensitive to the presence of specific phospholipids, consistent with the lysolipid affecting the receptor/membrane interaction. The acidic phospholipid phosphatidylserine was the most effective lipid in reducing the inhibitory effect of sphingosine, while a mixture of lipids similar to that found in the inner leaflet of the plasma membrane (only 15 mol % phosphatidylserine) had very little effect on the potency of sphingosine inhibition. Ongoing experiments have revealed that the tyrosine kinase activity of the insulin receptor is sensitive to the surrounding lipid environment (Arnold and Newton, unpublished results). Thus, a high affinity for phosphatidylserine may protect the receptor from interacting with the amine group of sphingosine. Several

reports indicate that the activity of the insulin receptor is sensitive to phospholipid headgroup (Sweet et al., 1987; Lewis & Czech, 1987) and acyl chain composition (Ginsberg et al., 1981). The ability of specific lipids to modulate receptor function adds complexity to the intracellular regulation of membrane function.

Sphingosine stimulated the phosphorylation of some proteins in membranes from NIH 3T3 cells, while inhibiting the phosphorylation of others. Curiously, sphingosine treatment activates phosphorylation of membrane-bound EGF receptor, but inhibits phosphorylation of Triton X-100 solubilized receptor (Davis et al., 1988). While a slight increase in insulin receptor autophosphorylation in cell membranes was observed at low (≤ 50 μM) sphingosine concentrations, sphingosine was an effective inhibitor of plasma membrane bound as well as solubilized insulin receptor.

We have also shown that sphingosine inhibits protein kinase C autophosphorylation, consistent with a recent report by Hannun and Bell (1990). Bazzi and Nelsestuen (1987) have proposed that sphingosine inhibits protein kinase C activity (previously measured with histone as a substrate) by neutralizing the charge of the acidic phosphatidylserine, thus preventing binding of the basic substrate to the membrane or micelle surface. Because protein kinase C autophosphorylates by an intrapeptide reaction (Newton & Koshland, 1987), this reaction depends on the micelle/kinase interaction and is independent of protein/protein interactions. The positively charged sphingosine does not alter the binding of protein kinase C to micelles (Hannun et al., 1986). This is consistent with sphingosine inhibiting the intrinsic catalytic activity of protein kinase C, rather than by affecting the binding of protein to the membrane. While sphingosine may decrease substrate/membrane or substrate/kinase interactions, it is a potent inhibitor of the intrinsic activity of protein kinase C.

Sphingosine inhibits autophosphorylation of both the insulin receptor tyrosine kinase and the serine/threonine kinase, protein kinase C. The first is a transmembrane receptor, while the latter is an amphipathic protein that can bind reversibly to membranes. Inhibition of two structurally and functionally distinct kinases by sphingosine suggests that the lysolipid may inhibit membrane-interacting kinases by a similar mechanism. Consistent with this, sphingosine also inhibits *src* kinase (Igarashi et al., 1989) and rhodopsin kinase (Arnold and Newton, unpublished data). Sphingosine has also been shown recently to inhibit the membrane-interacting enzymes phosphocholine cytidylyltransferase (Sohal & Cornell, 1990) and phosphatidate phosphohydrolase (Mullman et al., 1991). Thus, the cellular effects observed after treatment of cells with sphingosine are not solely a result of protein kinase C inhibition. In support of this, several recent reports indicate that sphingosine alters cell function (e.g., lipid hydrolysis, membrane permeability, cell proliferation) independently of protein kinase C (Lavie & Liscovitch, 1990; Oishi et al., 1990; Zhang et al., 1990).

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Bilayer/Cytoskeleton Interactions in Lipid-Symmetric Erythrocytes Assessed by a Photoactivable Phospholipid Analogue†

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ABSTRACT: Two mechanisms have been proposed for maintenance of transbilayer phospholipid asymmetry in the erythrocyte plasma membrane, one involving specific interactions between the aminophospholipids of the inner leaflet of the bilayer and the cytoskeleton, particularly spectrin, and the other involving the aminophospholipid translocase. If the former mechanism is correct, then erythrocytes which have lost their asymmetric distribution of phospholipids should display altered bilayer/cytoskeleton interactions. To test this possibility, normal erythrocytes, erythrocytes from patients with chronic myelogenous leukemia or sickle disease, and lipid-symmetric and -asymmetric erythrocyte ghosts were labeled with the radioactive photoactivable analogue of phosphatidylethanolamine, 2-(2-azido-4-nitrobenzoyl)-1-acyl-*sn*-glycero-3-phospho[^{14}C]ethanolamine ([^{14}C]AzPE), previously shown to label cytoskeletal proteins from the bilayer. The labeling pattern of cytoskeletal proteins in pathologic erythrocytes and lipid-asymmetric erythrocyte ghosts was indistinguishable from normal erythrocytes, indicating that the probe detects no differences in bilayer/cytoskeleton interactions in these cells. In contrast, in lipid-symmetric erythrocyte ghosts, labeling of bands 4.1 and 4.2 and actin, and to a lesser extent ankyrin, by [^{14}C]AzPE was considerably reduced. Significantly, however, labeling of spectrin was unaltered in the lipid-symmetric ghosts, suggesting that its relationship with the bilayer is normal in these lipid-symmetric cells. These results do not support a model in which spectrin is involved in the maintenance of an asymmetric distribution of phospholipids in erythrocytes.

The phospholipids of the erythrocyte plasma membrane are nonrandomly distributed between the inner and outer leaflets of the bilayer, with the aminophospholipids phosphatidylserine (PS)[1] and phosphatidylethanolamine (PE) concentrated in the inner leaflet and the choline phospholipids phosphatidylcholine (PC) and sphingomyelin (Sph) concentrated in the outer leaflet (Op den Kamp, 1979). Since the rate of transbilayer diffusion of the phospholipids is fast in comparison to the lifetime of the cell (Middlekoop et al., 1986), some mechanism must maintain lipid asymmetry, and two have been proposed. One postulates that the aminophospholipids are concentrated in the inner leaflet by the action of an ATP-dependent aminophospholipid translocase (Seigneuret & Devaux, 1984; Morrot et al., 1990). The other proposes that the aminophospholipids are trapped in the inner leaflet by interactions with the proteins of the cytoskeleton (Haest & Deuticke, 1976; Haest et al., 1978; Williamson et al., 1982). Several authors have suggested that both mechanisms operate cooperatively to maintain the normal lipid distribution (Middlekoop et al., 1988; Connor & Schroit, 1990; Kumar et al., 1990).

If cytoskeleton/lipid interactions maintain lipid asymmetry, no single protein, binding at a 1:1 molar ratio, is present in sufficient quantity to bind all of the aminophospholipids in the inner leaflet, since the inner leaflet contains about 3×10^7 molecules of PS and 6×10^7 molecules of PE, only 1×10^5 molecules each of the spectrin chains, band 4.1, band 2.1, and perhaps 5×10^5 molecules of actin (Goodman & Shiffer, 1983). However, the large size of the spectrin molecules and

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[1] Abbreviations: BSA, bovine serum albumin; CML, chronic myelogenous leukemia; [^{14}C]AzPE, 2-(2-azido-4-nitrobenzoyl)-1-acyl-*sn*-glycero-3-phospho[^{14}C]ethanolamine; PC, phosphatidylcholine; PDA, pyridyldithioethylamine; PE, phosphatidylethanolamine; PMSF, phenylmethanesulfonyl fluoride; PS, phosphatidylserine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Sph, sphingomyelin.

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