Differential Effects of Pertussis Toxin on Insulin-Stimulated Phosphatidylcholine Hydrolysis and Glycerolipid Synthesis de Novo. Studies in BC3H-1 Myocytes and Rat Adipocytes†

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ABSTRACT: Insulin-induced increases in diacylglycerol (DAG) have been suggested to result from stimulation of de novo phosphatidic acid (PA) synthesis and phosphatidylcholine (PC) hydrolysis. Presently, we found that insulin decreased PC levels of BC3H-1 myocytes and rat adipocytes by approximately 10–25% within 30 s. These decreases were rapidly reversed in both cell types, apparently because of increased PC synthesis de novo. In BC3H-1 myocytes, pertussis toxin inhibited PC resynthesis and insulin effects on the pathway of de novo PA–DAG–PC synthesis, as evidenced by changes in [^{3}H]glycerol incorporation, but did not inhibit insulin-stimulated PC hydrolysis. Pertussis toxin also blocked the later, but not the initial, increase in DAG production in the myocytes. Phorbol esters activated PC hydrolysis in both myocytes and adipocytes, but insulin-induced stimulation of PC hydrolysis was not dependent upon activation of PKC, since this hydrolysis was not inhibited by 500 μM sangivamycin, an effective PKC inhibitor. Our results indicate that insulin increases DAG by pertussis toxin sensitive (PA synthesis de novo) and insensitive (PC hydrolysis) mechanisms, which are mechanistically separate, but functionally interdependent and integrated. PC hydrolysis may contribute importantly to initial increases in DAG, but later sustained increases are apparently largely dependent on insulin-induced stimulation of the pathway of de novo phospholipid synthesis.

Insulin increases the synthesis of diacylglycerol (DAG)[1] and other lipids in BC3H-1 myocytes (Farese et al., 1984, 1985, 1987, 1988a; Luttrell et al., 1988), rat adipocytes (Augert & Exton, 1988; Casals et al., 1986), rat skeletal muscle (Ishizuka et al., 1990), and other tissues (Hesketh & Campbell, 1987; Chiarugi et al., 1989). The source(s) of this DAG remain(s) unclear, but three possible mechanisms have been suggested: (a) stimulation of de novo synthesis of phosphatidic acid (PA) and DAG via the activation of glycerol-3-phosphate acyltransferase (Farese et al., 1987, 1988a; Augert & Exton, 1988; Ishizuka et al., 1990; Chiarugi et al., 1989; Stevens & Husbands, 1987); (b) hydrolysis of a phosphatidylinositol (PI)–glycan via activation of specific phospholipase (PLase) C (Saltiel et al., 1987); and (c) hydrolysis of phosphatidylcholine (PC), as evidenced by release of phosphocholine (Nair et al., 1988).

Because PC constitutes more than 50% of the membrane phospholipids in mammalian cells (White, 1973), stimulation of PC hydrolysis could readily provide significant amounts of not only DAG, but also other lipids, such as PA and phosphatidylinositol (PI), which increase during insulin action. However, because insulin also stimulates PC synthesis (Farese et al., 1987, 1988a; Nair et al., 1988; Kiechle et al., 1986; Kelly et al., 1988), it has been difficult to separate the hydrolysis of this lipid from its synthesis by isotopic turnover studies. Presently, we found that insulin rapidly decreased PC levels in both BC3H-1 myocytes and isolated rat adipocytes. These decreases in PC were followed by rapid resynthesis of PC in both cell types, and, in BC3H-1 myocytes, resynthesis was blocked by pertussis toxin, which also inhibited insulin effects on PA synthesis de novo. We also found that, although phorbol ester induced protein kinase C (PKC) activation resulted in PC hydrolysis in both cell types, insulin-stimulated PC hydrolysis was not dependent upon PKC.

Materials and Methods

Culturing of BC3H-1 Myocytes. BC3H-1 myocytes were subcultured in 35-mm petri dishes in Dulbecco's modified Eagle's medium (DMEM) plus 15% controlled process serum replacement I for 10–12 days as described previously (Farese et al., 1984, 1985, 1987, 1988a). Where indicated, pertussis toxin (100 ng/mL) was added in fresh serum-free DMEM containing 0.1% BSA, 24 h prior to experimental use. In some studies, [^{3}H]myristate (2 μCi/plate) was added to the cells 24 h prior to experimental use. In some studies, [^{3}H]choline (1 μCi/plate) or [^{3}H]inositol (5 μCi/plate) was added at the time of subculturing. On the day of the experiment, the cells were washed 3 times with Dulbecco's phosphate-buffered saline, supplemented with 0.1% BSA and 5 mM glucose (DPBSGA). One milliliter of DPBSGA was then added, and the cells were equilibrated for 1–2 h at 37 °C (with 100 ng/mL pertussis toxin, where indicated) prior to addition of agonists. In some experiments, [2-^{3}H]glycerol (10 μCi/plate) or [^{3}H]myristate (2 μCi/plate) was added to DPBSGA on the day of the experiment.

Preparation of Isolated Rat Adipocytes. Rat adipocytes were prepared from fed male Sprage-Dawley rats (150–300 g, Holtzman) by collagenase digestion over 30 min at 37 °C

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[1] Abbreviations: BSA, bovine serum albumin; DAG, 1,2-*sn*-diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; KRP, Krebs–Ringer phosphate; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PKC, Ca^{2+}–phospholipid-dependent protein kinase (protein kinase C); PLase, phospholipase; TAG, triacylglycerol; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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in Krebs–Ringer phosphate (KRP) buffer (pH 7.4), supplemented with 2.5 mM glucose, 1 mg/mL collagenase, and 3% bovine serum albumin (BSA). Cells were washed twice in glucose-free KRP containing 1% BSA, suspended (6%, by volume of packed cells) in this buffer, and equilibrated for 30 min at 37 °C, and 0.5-mL aliquots of suspension were placed in polypropylene tubes for direct incubation as described. When adipocytes were cultured for overnight labeling with [^{3}H]choline, the cells were prepared under sterile conditions and washed and suspended (10% v/v) in DMEM, supplemented with 0.5% BSA, 10 mM HEPES (pH 7.4), and penicillin–streptomycin (25 units/mL and 25 mg/mL, respectively). These adipocytes were cultured for 24 h in the presence of [^{3}H]choline (0.2 μCi/mL). The medium was then removed, and the cells were washed 3 times with, and resuspended in, isotope- and glucose-free KRP containing 1% BSA as described above. All adipocyte preparations were tested for insulin responsiveness before and after culturing by assaying the uptake of 2-[^{3}H]deoxyglucose as previously described (Olefsky, 1978), and only preparations showing greater than 2-fold insulin-induced increases in hexose uptake (usually 4–8-fold in fresh adipocytes and 2–3-fold in cultured adipocytes, in which there is an increase in basal uptake but no change in insulin-stimulated uptake) were used for lipid analysis.

Adipocyte and Myocyte Treatments. Following equilibration in the appropriate medium, cells were treated with either 200 nM (BC3H-1 myocytes) or 3 nM (adipocytes) insulin, or 500 nM (both cell types) TPA. In some experiments, where indicated, treatments were added in a "retrograde sequence", so that total incubation times were equal for all groups, and only the duration of treatment was varied, for 0.25–20 min. When the effects of PKC inhibition were examined, 500 μM sangivamycin was added to the cells 15 min prior to insulin treatment. For [^{3}H]myristate "chase" experiments in BC3H-1 myocytes, after 24 h of labeling (see above), 10 μM unlabeled myristate was added to the DPBS used for washing and equilibration procedures, and insulin was added after 2 h of incubation of myocytes in the myristate-containing buffer. Treatments were terminated by adding 0.5–1 mL of cold methanol (final concentration = 50%) to the myocytes or adipocytes.

Extraction and Analysis of Cellular Lipids. Cells, in 4 mL of 50% methanol, were transferred to borosilicate glass tubes, 4 mL of chloroform was added, and the lipids were extracted into the lower chloroform phase. In [^{3}H]inositol-labeled cells, 0.01 N HCl was substituted for water. The aqueous phase was aspirated, and the lower chloroform phase was washed 3 times with water. In [^{3}H]choline-labeled samples, an aliquot of the chloroform phase was dried under air, and the radioactivity was determined by liquid scintillation counting. Thin-layer chromatography (see below) of lipid extracts revealed that [^{3}H]choline was incorporated exclusively into PC in adipocytes and BC3H-1 myocytes (data not shown).

Measurement of PC Lipid–Phosphorus. Chloroform extracts (80–100% of total sample) of BC3H-1 myocytes were dried under air, and PC and phosphatidylethanolamine (PE) were isolated by thin-layer chromatography (TLC) on LK5D (Whatman)-prescored silica gel 150A plates, developed in a solvent system of chloroform/methanol/water (75:25:3, by volume). PC and PE spots were visualized by iodine staining and scraped into glass tubes containing 0.2 mL of perchloric acid. The tubes were heated for 3 h at 200 °C in a sand bath, 0.55 mL of water was added, and an alqiquot (0.25 mL) was assayed for phosphate content, using 0.7 mL of malachite green (0.16%, w/v) reagent (Hallberg, 1984) containing 1.3 N HCl, 9.3 mM ammonium molybdate, and 0.065% (v/v) Tween-20. After standing at room temperature for 1 h, the absorbance at 660 nm (A_{660}) relative to a phosphate-free blank was determined. The amount of phosphate in the samples was determined by comparison with a standard curve, and corrected for the size of the aliquot taken from the original sample. In this method, there is linear color development (approximately 0–1 absorbance unit) with phosphate standards of 0–12.5 nmol/sample. Recovery of phosphate in TLC-purified PC and PE standards was approximately 85–90%, and readings of replicate PC samples were within a 10% range.

Other Lipid Assay Procedures. Incorporation of radiolabel into different classes of neutral glycerolipids and phospholipids was determined following thin-layer chromatography as described previously (Farese et al., 1987, 1988a,b). DAG mass was assayed by the DAG kinase method (Preiss et al., 1987). [^{3}H]Phosphorylcholine in the aqueous phase of lipid extractions was determined as described previously (Nair et al., 1988).

Materials. TPA, HEPES, BSA (RIA grade), controlled process serum replacement I, glucose, dimethyl sulfoxide, malachite green, 2-deoxyglucose, cardiolipin, ATP, diethylenetriaminepentaacetic acid, imidazole, and Tween-20 were obtained from Sigma Chemical Co. (St. Louis, MO). Collagenase type 1 from *Clostridium histolyticum* was from Worthington Biochemical (Freehold, NJ). DAG kinase was purchased from Lipidex (Westfield, NJ). DMEM and penicillin–streptomycin were from GIBCO (Grand Island, NY). Insulin was obtained from Elanco (Indianapolis, IN). Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA). [^{3}H]Choline (86.7 Ci/mmol), [^{3}H]myristate (22.4 Ci/mmol), and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from New England Nuclear (Boston, MA). [^{3}H]Inositol (10–20 Ci/mmol) was from American Radiolabel Corp. (St. Louis, MO). 2-[^{3}H]Deoxyglucose (50 Ci/mmol) and [2-^{3}H]glycerol (5 Ci/mmol) were from ICN Radiochemical (Irvine, CA). Sangivamycin was supplied by Dr. Matthew Suffness of the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD), and was prepared as a 10 mM stock solution in 0.02 N HCl. All other materials were reagent grade or better.

Results

Effects of Insulin on PC Mass in BC3H-1 Myocytes. During the first 30 s, insulin provoked a 20% (approximately 3–4 mol/35-mm plate) decrease in total cellular PC content (Figure 1). Thereafter, in the absence of pertussis toxin treatment (see below), PC content rapidly returned to control or slightly elevated levels, which were maintained over the next 20 min. In contrast to PC, PE mass did not change appreciably during insulin treatment (data not shown).

Effects of Insulin on [^{3}H]Glycerol Incorporation into PC in BC3H-1 Myocytes. Synthesis of PC de novo was evaluated as described previously (Farese et al., 1987, 1988a) using [2-^{3}H]glycerol. After an initial lag (Figure 2), PC labeling increased steadily over 120 min and then tended to level off, and did not change appreciably in control myocytes over the next 20 min [note, as reported previously (Farese et al., 1988a), this does not reflect full equilibrium labeling, as incorporation gradually increases to higher levels over 24 h]. Upon addition of insulin after 120 min of prelabeling, the rate of PC labeling increased markedly (at least 60-fold, since the increase in labeling within 2 min was twice that achieved after 120 min in control cells), and total labeling was increased by approximately 50%, 100%, and 170% within 0.5, 2, and 20 min, respectively. We also measured glycerol 3-phosphate labeling

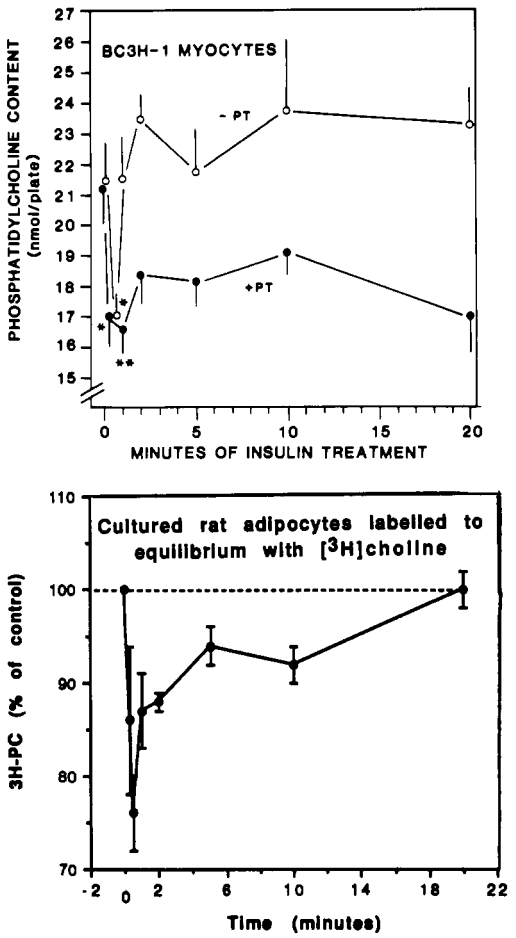

FIGURE 1: Time-dependent effects of insulin on phosphatidylcholine levels in BC3H-1 myocytes and rat adipocytes. (Upper panel) Myocytes were treated with (closed circles) or without (open circles) 100 ng/mL pertussis toxin (PT) for 24 h. During the last 140 min, the cells were incubated in 2 mL of DPBSGA, and PC hydrolysis was examined after retrograde addition of 200 nM insulin as described under Materials and Methods. Mean ± SE of 15–25 determinations in pertussis toxin treated myocytes and 10–24 determinations in myocytes not treated with pertussis toxin. One asterisk, $P < 0.025$ (t test); two asterisks $P < 0.01$ (t test). (Lower panel) Rat adipocytes were cultured for 24 h in the presence of 0.2 μCi/mL [^{3}H]choline/1 mL of medium. Unincorporated radiolabel was washed away, and cells were resuspended in glucose-free KRP. After equilibration, vehicle (controls) or 3 nM insulin was added, and incubation was continued for the times indicated. Control cells contained approximately 40 000 cpm/tube incorporated into phosphatidylcholine. All results are means ± SEM of 4–5 experiments, each conducted in quadruplicate, and are expressed as percent of control. Differences were significant ($P < 0.05$; paired t test) for all values at 0.5, 1, 2, 5, and 10 min of insulin treatment vs pooled experimental control values, which did not change appreciably, during the final incubation.

simultaneously as described previously (Farese et al., 1988a), and the specific activity of this precursor for PA–DAG–PC synthesis after 120 min of labeling was approximately 10 000 cpm/nmol. With this specific activity, it may be estimated (Farese et al., 1988a) that the de novo pathway could account for increases of 0.5, 1.0, and 1.7 nmol/plate during 0.5, 2, and 10 min of insulin treatment. Although this does not fully account for absolute changes in PC mass noted above (approximately 3 nmol/plate), these calculations assume that glycerol 3-phosphate is uniformly labeled throughout the cell. Obviously, this may not be the case, particularly during insulin treatment, which rapidly increases glycolytic flux and glucose uptake.

Effects of Pertussis Toxin on Insulin-Induced PC Hydrolysis, PA Synthesis de Novo, and DAG Production in BC3H-1 Myocytes. When myocytes were incubated with 100 ng/mL pertussis toxin for 24 h [as reported by Luttrell et al. (1988), this pretreatment in BC3H-1 myocytes is optimal for inhibition of (a) subsequent ADP-ribosylation of a 40-kDa substrate in vitro, (b) insulin effects on PI-glycan hydrolysis, and (c) generation of [^{3}H]myristate- and [^{3}H]arachidonate-labeled DAG], the basal PC content was not altered, and the initial insulin-induced decrease in PC at 30 s was comparable (approximately 20%) to that observed in the absence of pertussis toxin pretreatment (Figure 1). On the other hand, pertussis toxin pretreatment largely inhibited the return of PC to starting levels (i.e., PC resynthesis), and PC levels remained low during the ensuing 20 min of insulin treatment.

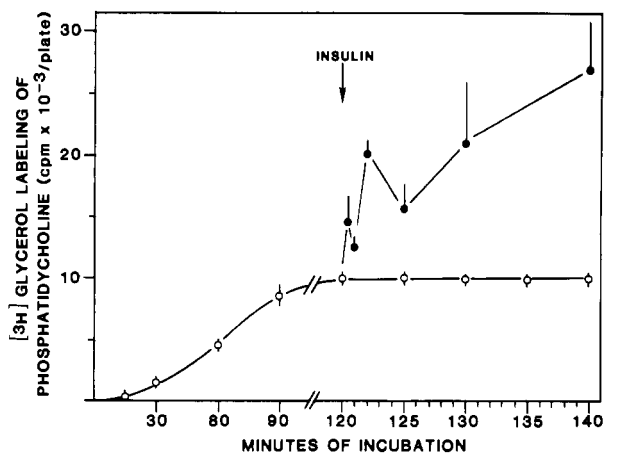

FIGURE 2: Effects of insulin on synthesis of phosphatidylcholine de novo in BC3H-1 myocytes. Cells were incubated for 120 min in 2 mL of DPBSGA containing 10 μCi of [2-^{3}H]glycerol. Insulin (200 nM; closed circles) or vehicle (controls; open circles) was added at 120 min, and incorporation into TLC-purified PC was measured at time points depicted (note the change in abscissa scale after 120 min). Mean ± SE of 4 determinations.

The failure to resynthesize PC during insulin treatment in pertussis toxin pretreated myocytes suggested that this toxin may inhibit the de novo phospholipid synthesis pathway. This was evaluated by examining [^{3}H]glycerol incorporation into glycerolipids (Figure 3): pertussis toxin did not alter [^{3}H]-glycerol incorporation into total phospholipids or neutral lipids in control myocytes, but the effects of insulin on [^{3}H]glycerol incorporation were largely inhibited. This correlates well with other findings (Vila et al., 1990) indicating that pertussis toxin inhibits insulin-induced increases in, but not the basal activity of, glycerol-3-phosphate acyltransferase, which catalyzes PA synthesis de novo.

The above-described results indicated that pertussis toxin could be used to study consequences of insulin effects on pertussis toxin resistant PC hydrolysis separately from pertussis toxin sensitive effects of insulin on both the de novo pathway, as shown presently, and PI–glycan hydrolysis, as shown by Luttrell et al. (1988). As shown in Figure 4, insulin provoked rapid, 60–80% sustained increases in DAG content in the absence of pertussis toxin pretreatment. With pertussis toxin treatment, basal DAG content was unaltered, and insulin provoked a transient (but significant, $P < 0.005$) increase in DAG at 30 s, followed by a rapid return to the starting level, and a later small, but significant ($P < 0.05$), increase. Note that the increase in DAG at 30 s in the presence of pertussis toxin was only slightly less than that observed in the absence of pertussis toxin treatment. Also note that the frequently observed biphasic nature of changes in DAG may reflect that DAG arises from multiple sources during insulin action and the initial increase may be largely due to hydrolysis of

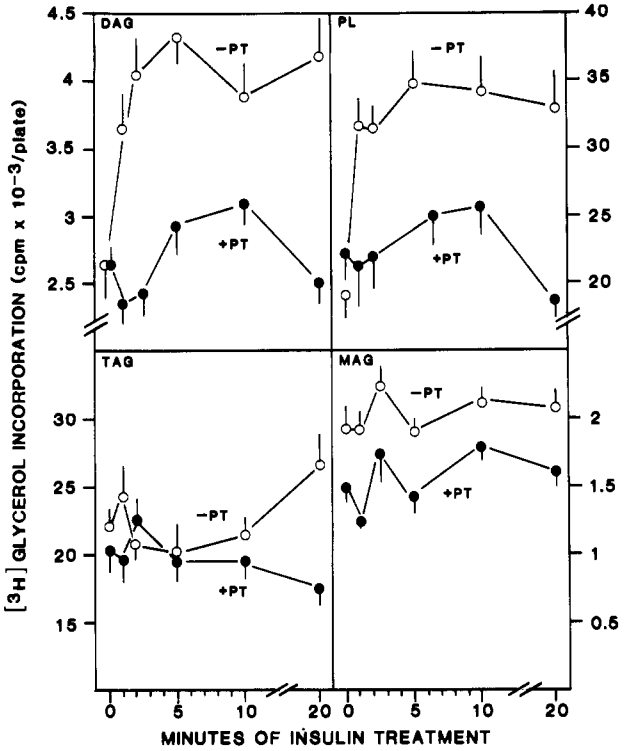

FIGURE 3: Effects of pertussis toxin on insulin-stimulated, glycerolipid synthesis de novo in BC3H-1 myocytes. Myocytes were treated with (closed circles) or without (open circles) 100 ng/mL pertussis toxin for 24 h. During the last 140 min, the myocytes were labeled with 10 μCi of [2-^{3}H]glycerol added to 1 mL of DPBSGA. During the final 20 min of incubation, 200 nM insulin was added to the medium by "retrograde addition" (see Materials and Methods), and the duration of treatment was for the indicated periods of time. Vehicle alone was added to controls at 1 or 20 min prior to the end of incubation, and this did not alter incorporation. Incorporation of [^{3}H]glycerol into diacylglycerol (DAG), total phospholipids (PL), triacylglycerol (TAG) and monoacylglycerol (MAG) was measured after purification of these lipid classes by TLC. Mean ± SE of 8–14 determinations.

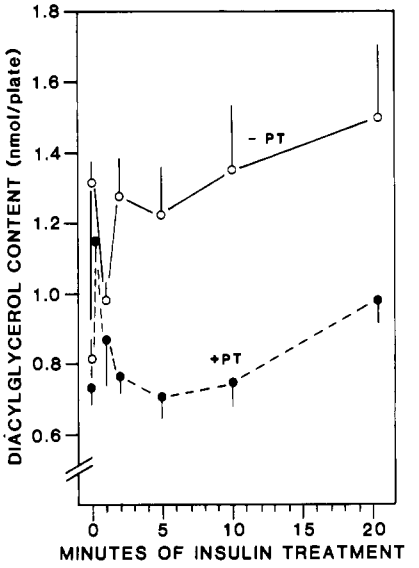

FIGURE 4: Effects of pertussis toxin on diacylglycerol (DAG) content of BC3H-1 myocytes. Myocytes were treated with (closed circles) or without (open circles) 100 ng/mL pertussis toxin for 24 h. During the last 140 min, myocytes were incubated in 2 mL of DPBSGA, and during the last 20 min, insulin (200 nM) was added in a "retrograde" manner (as in Figures 1, 3, and 4), so that only the duration of exposure to insulin was varied, as depicted on the abscissa. DAG was measured as described under Materials and Methods. Mean ± SE of 10–21 determinations. In the pertussis toxin treated groups, insulin effects (vs control) were statistically significant (as per t test) at 0.5 min ($P < 0.005$) and 20 min ($P < 0.05$). In the myocytes not treated with pertussis toxin, insulin effects were significant ($P < 0.05$) at all time points, except for the transient nadir at 1 min.

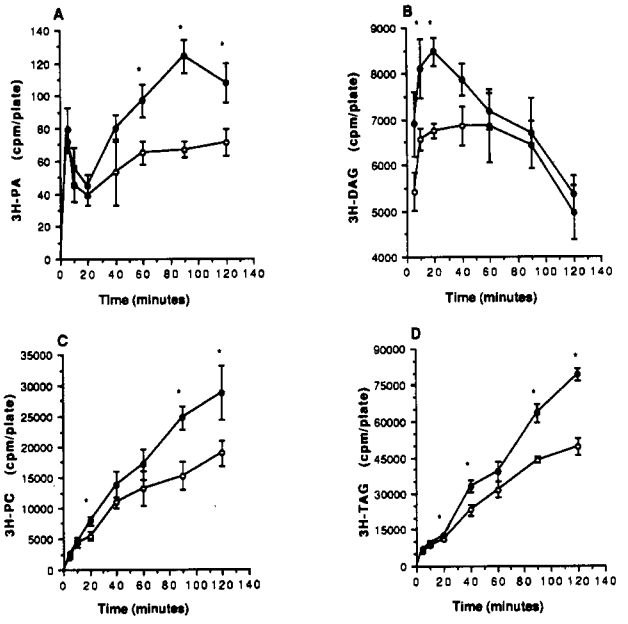

FIGURE 5: Effects of insulin on acute incorporation of [^{3}H]myristate into lipids of BC3H-1 myocytes. Unlabeled myocytes were treated with insulin (200 nM, closed circles) or buffer (open circles) for 30 min, and then [^{3}H]myristate (2 μCi/plate) was added, and incubation was continued for the times indicated. Means ± SEM of 3–4 determinations are depicted. (A) Labeling of PA. (B) Labeling of DAG. (C) Labeling of PC. (D) Labeling of TAG. (Other lipids were poorly, if at all, labeled.)

preexisting phospholipids whereas the secondary increase may be greatly dependent upon the de novo pathway and PI-glycan hydrolysis.

Effects of Insulin on [^{3}H]Myristate Incorporation into Lipids of BC3H-1 Myocytes. Although [^{3}H]myristate has been used to label the PI-glycan to follow its hydrolysis during insulin action (Luttrell et al., 1988; Saltiel et al., 1987), we have previously reported (Farese et al., 1988a) that, with 2 h of prelabeling with this isotope, there is no appreciable labeling of the PI-glycan but there is heavy labeling of PC and small labeling of PI. Moreover, with 2-h prelabeling, we also reported (Farese et al., 1988a) that insulin rapidly increases myristate labeling of both DAG and PC. As myristate-labeled DAG in 2-h prelabeling experiments did not appear to be derived from the PI-glycan, we presently evaluated the possibility that myristate labeling may also reflect de novo PA-DAG-PC synthesis and/or PC hydrolysis. To study the de novo pathway more critically, we added [^{3}H]myristate 30 min after insulin. As shown in Figure 5, incorporation of [^{3}H]myristate into PA and DAG was rapid, and this incorporation was stimulated by ongoing insulin treatment (although not statistically significantly at all time points). Levels of [^{3}H]PA were very low and rapidly approached equilibrium. Levels of [^{3}H]DAG were more substantial but also rapidly approached equilibrium with 10 min and (for uncertain reasons) decreased after 60 min. [^{3}H]PC and [^{3}H]TAG, on the other hand, continued to increase steadily over 2 h, and insulin increased this labeling at multiple time points. These results provided further evidence suggesting that (a) insulin stimulates de novo synthesis of PA, followed by its conversion to DAG and PC (or TAG), (b) [^{3}H]myristate labeling may reflect increases in de novo PA-DAG-PC synthesis, and (c) enhanced

PC turnover persists for hours during insulin treatment.

We also presently evaluated the possibility that PC hydrolysis could be a source of myristate-labeled DAG. To enhance our ability to discern an initial hydrolytic decrease in [^{3}H]myristate-labeled PC during insulin stimulation, we labeled myocytes for 24 h and then washed the cells and incubated them for 2 h in medium containing 10 μM unlabeled myristate to diminish new PC labeling. During the first 30 s, insulin provoked a 25 ± 4% (mean ± SE; $n = 4$; $P < 0.025$, paired t test) decrease in [^{3}H]PC. At later time points, [^{3}H]PC increased in insulin-treated myocytes (but not in control myocytes, wherein labeling, if anything, decreased), presumably reflecting increased synthesis and utilization of residual available label. Although residual label precluded our ability to observe sustained or continued decreases in myristate-labeled PC in these "chase" experiments, it seems clear that insulin causes an immediate decrease in labeled PC and the latter may therefore be a source of myristate-labeled DAG.

Effects of Insulin on [^{3}H]Choline Incorporation into Adipocyte PC. To test the possibility that insulin stimulates the hydrolysis and synthesis of PC in another insulin-responsive cell type, we first examined the incorporation of [^{3}H]choline into PC in rat adipocytes which were prelabeled for 1 h with [^{3}H]choline. In these acutely labeled cells (data not shown), insulin provoked a rapid, small, but significant, decrease (13 ± 5%, mean ± SE, $P < 0.05$, at 30 s) in radiolabel incorporation into PC, which remained apparent for up to 5 min. Afterward, insulin stimulated [^{3}H]choline incorporation into PC, and at 20 min, a relatively small increase (approximately 10%) was apparent.

Culturing adipocytes for 24 h in the presence of [^{3}H]choline resulted in the labeling of PC pools to apparent isotopic equilibrium. After equilibrium labeling, insulin caused [^{3}H]PC to decrease to 76 ± 4% (mean ± SE) within 30 s ($P < 0.05$, paired t test) (Figure 1), and [^{3}H]PC gradually returned to control levels over 20 min. We did not evaluate changes in PC mass in rat adipocytes because of difficulties in chromatographing large amounts of lipids to recover sufficient amounts of PC–phosphorus for measurement in the presently used assay.

In addition to insulin, we examined the effects of 500 nM TPA and 20 mM glucose on [^{3}H]PC in cultured adipocytes, since both agents (Ishizuka et al., 1989b; Draznin et al., 1988), like insulin (Draznin et al., 1988; Ishizuka et al., 1989b; Pershadsingh et al., 1987), activate PKC in these cells. TPA and glucose each provoked decreases in [^{3}H]PC after 20 min [TPA or glucose, 79 ± 4% (mean ± SE) of control]. However, the glucose-induced decreases occurred more slowly than those provoked by TPA, which were slower than those provoked by insulin (data not shown).

Role of PKC in Insulin-Stimulated PC Hydrolysis. Previous (Nair et al., 1988) and the present studies indicated that PC hydrolysis may result from activation of PKC in both myocytes and adipocytes. To determine whether the insulin effect on PC hydrolysis may be mediated by PKC activation, we used the PKC inhibitor sangivamycin or, in a few experiments, staurosporine. As shown in Figure 6, 500 μM sangivamycin completely blocked TPA-induced increases in DAG content in BC3H-1 myocytes but did not affect insulin-induced DAG increases. [This dose of sangivamycin alone had no effects on DAG, PC, or any other parameters presently studied, but fully blocked TPA-stimulated glucose transport in both cell types (Standaert et al., 1990).] In contrast to inhibition of TPA-induced PC hydrolysis (not shown), 500 μM sangivamycin (or 16 μM staurosporine) had no effect upon insulin-stimulated decreases (approximately 10–15%) in myocyte PC content over the first 30–120 s of treatment (Figure 6). (The PC nadir was more persistent in these experiments than in the experiments shown in Figure 1: this may reflect subtle variations in myocyte characteristics during repeated subculturing.) However, sangivamycin did inhibit the return of PC to control levels, and PC was persistently decreased for

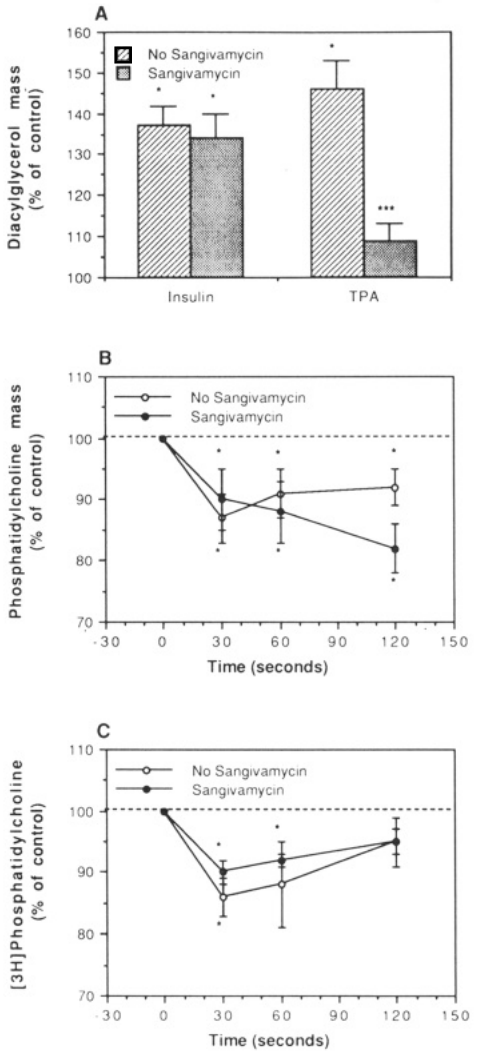

FIGURE 6: Effects of sangivamycin on insulin- and TPA-induced changes in phosphatidylcholine and diacylglycerol. (A) Effect of sangivamycin on DAG content in BC3H-1 myocytes. Myocytes were preincubated for 15 min in the presence or absence of 500 μM sangivamycin and then treated with vehicle (control), 200 nM insulin, or 500 nM TPA for 10 min. Values are means ± SEM of the percent of the appropriate experimental control (i.e., ±sangivamycin) for 17 (insulin) or 9 (TPA) determinations. One asterisk, $P < 0.05$, insulin or TPA vs. control; three asterisks, $P < 0.05$, TPA plus sangivamycin vs. TPA alone (unpaired t test). DAG mass in the control was approximately 0.7–1 nmol/plate. (B) Effect of sangivamycin on PC mass in BC3H-1 myocytes. Myocytes were preincubated for 15 min with or without 500 μM sangivamycin and then treated with 200 nM insulin for the times indicated, or with vehicle (controls) for 30–60 s. Mean ± SEM of 6–8 determinations (results expressed as percent of mean control of each experiment). One asterisk, $P < 0.05$, insulin vs control (unpaired t test). PC levels in controls (±sangivamycin) were approximately 23 nmol/plate. (C) Effect of sangivamycin on [^{3}H]PC in cultured rat adipocytes. Rat adipocytes were cultured for 24 h in the presence of 0.2 μCi of [^{3}H]choline/mL of medium, washed, and resuspended in glucose-free KRP. Cells were incubated for 15 min with or without 500 μM sangivamycin and then treated with 3 nM insulin for the times indicated, or with vehicle (control) for 30–60 s. Mean ± SEM for 3 experiments, each conducted in quadruplicate. One asterisk, $P < 0.05$, paired t test.

up to 20 min of insulin stimulation (data not shown). The latter may reflect the fact that PKC activation is important for PC synthesis from DAG and CDP-choline in some tissues, particularly during hormonal stimulation (Pelech & Vance, 1989).

As insulin increases phosphorylcholine release in BC3H-1 myocytes (Nair et al., 1988), we questioned whether sangivamycin would inhibit this release. Using myocytes prelabeled for 10 days with [^{3}H]choline, [^{3}H]phosphorylcholine was increased by 30-s insulin treatment from a control level of 13 239 ± 908 (mean ± SE; n = 15) to 19 369 ± 2596 cpm/plate (n = 6; $P < 0.01$, unpaired t test). This insulin-induced increase remained apparent, despite pretreatment with sangivamycin (17 448 ± 2378 cpm/plate; n = 6; $P < 0.05$, vs control, unpaired t test).

The effect of sangivamycin on PC hydrolysis was also studied in adipocytes labeled to equilibrium with [^{3}H]choline, and insulin-stimulated decreases in [^{3}H]PC (10–15%) remained apparent despite pretreatment with 500 μM angivamycin (Figure 6). (These insulin effects on [^{3}H]PC were quantitatively different from those in Figure 1, possibly reflecting differences in metabolic activities of adipocytes cultured at widely spaced intervals.) In contrast, in other experiments, 500 nM TPA induced a 20% decrease in [^{3}H]PC over 20 min (control = 36 648 ± 575 cpm/tube; TPA = 30 342 ± 1656 spm/tube; mean ± SE; n = 4; $P < 0.005$, unpaired t test), and this decrease was completely blocked by a 15-min pretreatment with 500 μM sangivamycin (36 535 ± 418 cpm/tube; mean ± SE; n = 4).

Discussion

Although insulin increases DAG labeling and/or content in BC3H-1 myocytes (Farese et al., 1984, 1985, 1987, 1988a; Luttrell et al., 1988), rat adipocytes (Augert & Exton, 1988; Casals et al., 1986), and other tissues (Ishizuka et al., 1990; Hesketh & Campbell, 1987; Chiarugi et al. 1989), the sources of this DAG are uncertain. Insulin stimulates de novo synthesis of PA and DAG (Farese et al., 1984, 1985, 1987, 1988a; Augert & Exton, 1988; Casals et al., 1986; Ishizuka et al., 1990; Chiarugi et al., 1989) and activates a specific PLase C to generate DAG from a PI–glycan (Saltiel et al., 1987). Initial studies in BC3H-1 myocytes (Farese et al., 1987, 1988a; Nair et al., 1988) also suggested that insulin-induced increases in DAG may derive from stimulation of PC hydrolysis, by PLase C or PLase D, and that this PC effect is mimicked by the PKC activator TPA. The present findings provided more convincing evidence that insulin increases PC hydrolysis, not only in BC3H-1 myocytes but also in isolated rat adipocytes. Moreover, the PC hydrolysis effects of insulin appeared to be independent of PKC activation.

The findings (a) that insulin provoked 10–25% decreases in PC levels in BC3H-1 myocytes and rat adipocytes and (b) that these decreases were rapidly reversed are noteworthy for several reasons. First, although this may seem to be an extraordinary amount of PC which is hydrolyzed and resynthesized during the early moments of insulin action, combined increases in DAG and PI contents in BC3H-1 myocytes (Farese et al., 1984, 1985, 1987, 1988a,b) are of the same order of magnitude, i.e., several nanomoles per 35-mm plate. Second, increases in PC synthesis through the de novo pathway are sizable, as estimated from [^{3}H]glycerol labeling studies, i.e., 0.5–1.7 nmol/plate, and these may be low estimates, if unlabeled precursors are used more efficiently than labeled precursors. (Alternatively, the de novo pathway may not fully account for restoration of PC mass.) Third, when activation of the de novo pathway was blocked by pertussis toxin in BC3H-1 myocytes, the initial hydrolytic effect of insulin on PC was readily apparent, but PC levels remained low at multiple time points during the ensuing 20-min period. The latter findings confirmed the magnitude of insulin-induced decreases in PC content and further indicated that insulin-induced activation of de novo PA–DAG–PC synthesis is required for PC resynthesis but not PC hydrolysis.

As discussed above, insulin increased the rate of PC synthesis via the de novo pathway by at least 60-fold. In addition to the de novo pathway, insulin also activates phospholipid methyltransferase (Kiechle et al., 1986) and CDP:phosphocholine cytidylyltransferase (Kelly et al., 1988), which stimulate PC synthesis from PE and DAG, respectively. These enzyme activations may contribute to PC synthesis, but the importance of the de novo PA synthesis pathway seems clear from the finding that inhibition of this pathway by pertussis toxin largely prevented PC resynthesis during insulin action.

It may seem surprising that pertussis toxin inhibits both PI–glycan hydrolysis (Luttrell et al., 1988) and de novo PA synthesis in BC3H-1 myocytes. However, note that (A) Hesketh et al. (1987) reported that insulin-induced increases in [^{3}H]glycerol-labeled DAG in 3T3 cells are inhibited by pertussis toxin, (b) Stevens and Husbands (1987) suggested that insulin "mediators" activate skeletal muscle glycerol-3-phosphate acyltransferase, and (c) we reported (Vila et al., 1990) that insulin- and PI-specific PLase C directly stimulate the release of a soluble activator (M_r <5000) of glycerol-3-phosphate acyltransferase from membranes of the BC3H-1 myocyte, and this effect is blocked by pertussis toxin pretreatment, and by antisera which specifically inhibit PI–glycan PLase C or $G_i\alpha$. Thus, insulin apparently stimulates de novo PA synthesis by activation of a $G_i\alpha$-dependent, pertussis toxin sensitive PLase C, which hydrolyzes a PI–glycan, releases a soluble factor, and thereby activates glycerol-3-phosphate acyltransferase. In contrast, insulin stimulates PC hydrolysis by a decidedly different mechanism, which is pertussis toxin insensitive and independent of PI–glycan hydrolysis and PA synthesis de novo.

The finding that pertussis toxin blocked insulin-induced increases in DAG only after the first minute of insulin action suggests that PC hydrolysis contributes significantly to the initial increase in DAG but, at later times, PI–glycan hydrolysis and de novo PA synthesis are required to sustain full insulin effects on DAG. This could explain why later (after 1 min) insulin-induced increases in [^{3}H]arachidonate-labeled DAG were inhibited more effectively by pertussis toxin than initial increases in this labeled DAG species in BC3H-1 myocytes (Luttrell et al., 1988). Also, pertussis toxin resistant, PC hydrolysis-dependent increases in DAG may explain why pertussis toxin only partially inhibits insulin effects on glucose transport in BC3H-1 myocytes (Luttrell et al., 1988).

Previously, we reported (Farese, 1987, 1988a) that insulin stimulates incorporation of [^{3}H]glycerol, [^{3}H]arachidonate, [^{3}H]choline, and [^{3}H]myristate into PC in BC3H-1 myocytes. Because of interest in the use of [^{3}H]myristate as an indicator of PI–glycan hydrolysis (Luttrell et al., 1988; Saltiel et al., 1987), we questioned whether insulin increases [^{3}H]myristate-labeled DAG through PC hydrolysis and/or de novo PA synthesis. Indeed, we observed transient decreases in [^{3}H]-myristate-labeled PC in BC3H-1 myocytes "chase" with unlabeled myristate, and when [^{3}H]myristate was added to myocytes previously stimulated by insulin, enhanced labeling could be followed sequentially into PA, DAG, and PC, but not the PI–glycan, over a 2-h period. Thus, (a) [^{3}H]myristate-labeled DAG reflects PC hydrolysis and de novo PA

synthesis, as well as PI-glycan hydrolysis, (b) [^{3}H]myristate is incorporated into PC in BC3H-1 myocytes largely via the de novo pathway, in which DAG is an obligatory intermediate between PA and PC (Kennedy & Weiss, 1956), rather than via deacylation–reacylation (Chakravarthy et al., 1986), and (c) even when PC levels seem stationary during ongoing insulin action, rapid, stimulated turnover of PC is occurring, presumably reflecting balanced hydrolysis and resynthesis.

That insulin stimulates PC hydrolysis in rat adipocytes, to an extent comparable to that observed in the BC3H-1 myocytes, is important for several reasons. First, since insulin increases de novo PA–DAG synthesis in these (Farese et al., 1987, 1988a; Augert & Exton, 1988; Casals et al., 1986; Draznin et al., 1988) and other (Ishizuka et al., 1990; Hesketh & Campbell, 1987; Chiarugi et al., 1989; Cooper et al., 1990a) cells, at least some effects of insulin on phospholipid metabolism are similar in a variety of tissues. Second, PC hydrolysis may contribute to the apparent activation (Cooper et al., 1987; Draznin et al., 1988; Pershadsingh et al., 1987) and translocation of PKC (Ishizuka et al., 1989a; Egan et al., 1990; Cooper et al., 1990b) in adipocytes, myocytes, and perhaps other cell types.

As in the BC3H-1 myocyte (Nair et al., 1988), TPA stimulated PC hydrolysis in the rat adipocyte, as did glucose, which activates PKC in these cells (Ishizuka et al., 1989b; Draznin et al., 1988). However, the PKC inhibitor, sangivamycin, had no effect on insulin-stimulated PC hydrolysis in adipocytes and myocytes, nor upon insulin-stimulated increases in DAG in the myocytes. Since sangivamycin effectively inhibits PKC-dependent processes in both the myocyte and adipocyte [e.g., insulin- or TPA-stimulated glucose transport (Standaert et al., 1990) and TPA-stimulated PC hydrolysis], it seems clear that PKC is not essential for activation of PC hydrolysis by insulin.

In summary, insulin rapidly stimulated the hydrolysis and resynthesis of PC in BC3H-1 myocytes and rat adipocytes. PC hydrolysis was not blocked by pertussis toxin pretreatment in myocytes and did not appear to be dependent upon PKC activation in either tissue. PC resynthesis in the myocytes, on the other hand, was largely blocked by pertussis toxin, which also inhibited the pathway of de novo PA synthesis. PC hydrolysis appeared to be an important mechanism for increasing production of DAG during the initial moments of insulin action, but after 1 min, pertussis toxin sensitive mechanisms (e.g., PI-glycan hydrolysis and de novo PA synthesis) became rate-limiting for DAG production. Our findings indicate that insulin perturbs phospholipid metabolism by separable and pertussis toxin sensitive and insensitive mechanisms. Moreover, these mechanisms seem to be interdependent and integrated, such that de novo PA–DAG–PC synthesis is triggered by PI-glycan hydrolysis, and serves to replenish PC, which is hydrolyzed to augment production of DAG and possibly other phospholipids.

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Pressure Effects on Dipalmitoylphosphatidylcholine Bilayers Measured by ^2H Nuclear Magnetic Resonance†

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ABSTRACT: The effects of pressure, up to 5 kbar, on multilamellar vesicles of 1,2-dipalmitoyl-*sn*-phosphatidylcholine perdeuterated in the acyl chains (DPPC-d_{62}) were examined by using high-pressure NMR techniques. A deuterium probe was built, and the quadrupole splitting was measured against pressure at various temperatures. The experiments were performed on pure lipid bilayers in the liquid-crystalline state and on bilayers in the liquid-crystalline state containing the local anesthetic tetracaine. The results show that the order parameter of all segments of the acyl chains increases with pressure in the liquid-crystalline state. The more highly ordered regions of the chains are affected slightly more than the regions near the methyl ends. The addition of tetracaine increases the disorder of the chains, and pressure reverses the effect of anesthetic on the lipid as seen by the reversal of the changes in line shape and the measured order parameter.

The thermodynamic understanding of any system depends on the measurement of changes caused by the variation in the classic parameters of temperature and pressure. Temperature studies of biochemical systems are easy to perform and have been the mainstay of the thermodynamic measurements in the past. Pressure studies of protein and membrane systems, while technically more difficult, have been initiated in the last decade or so in the interest of a more complete physicochemical understanding of these systems.

Of particular relevance to this study are the high-pressure investigations of membranes and their model systems that have been performed with a variety of techniques including IR and Raman spectroscopy (Wong, 1987a,b), NMR[1] spectroscopy (Jonas et al., 1988, 1990), ESR (Trudell et al., 1974), X-ray diffraction (Stamatoff et al., 1978), neutron scattering (Braganza & Worcester, 1986a,b; Winter & Pilgrim, 1989), light transmission (Prasad et al., 1987), fluorescence spectroscopy (Chong & Weber, 1983; Chong, 1988), volumetric measurements (Tosh & Collings, 1986), and others.

Of particular physiological interest has been the effect of pressure on anesthetic action both in vivo (Lever et al., 1971) and in vitro (Mountcastle et al., 1978). In most cases pressure reverses anesthetic action. The mechanism of this action is still uncertain; however, the site of action is generally presumed to be the cellular membrane of the neuron. Whether anesthetics have a direct action on the membrane proteins, membrane lipids, or both is uncertain, but the correlation of anesthetic potency with membrane solubility is well-known. The reversal and antagonism of anesthetic action by pressure have been regarded as a possible key to the mechanism of action of anesthetics. Anesthetics increase the volume of the bilayer (Miller et al., 1973) and its fluidity (Trudell et al., 1973), and pressure is presumed to reverse both of these effects. It should be noted, however, that there are several reported cases (Halsey & Wardley-Smith, 1975; Kending & Cohen, 1977; Smith et al., 1984; Lodge, 1985) where pressure does not reverse the anesthetic effect.

To further investigate the effects of pressure on a model membrane system, we decided to use ^2H NMR to probe the pressure effects on DPPC multilamellar vesicles. Previously, we showed the feasibility of using high-pressure ^{13}C NMR as a method of studying pressure-induced phase changes in sonicated DPPC vesicles (Jonas et al., 1988). We have also used high-pressure one- and two-dimensional ^1H NMR to study pressure-induced changes in the spectra of DMPC and POPC sonicated vesicles (Jonas et al., 1990). In the latter paper, our results were consistent with an increase in the order parameter of the acyl chains in the liquid-crystalline state as pressure was increased.

In this study, ^2H NMR is used to study the effects of pressure on multilamellar vesicles of chain-perdeuterated DPPC. ^2H NMR has proven to be a very useful nonperturbing method for quantitatively measuring order and dynamics in lipid bilayer systems in both the gel and liquid-crystalline states

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[1] Abbreviations: NMR, nuclear magnetic resonance; DPPC 1,2-dipalmitoyl-*sn*-phosphatidylcholine; DPPC-d_{62}, chain-perdeuterated DPPC; DMPC, 1,2-dimyristoyl-*sn*-phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine.

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