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Phosphatidic acid and phosphatidylinositol labelling in adipose tissue

Relationship to the metabolic effects of insulin and insulin-like agents

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Exposure to phospholipase C increased the incorporation of $[^{32}P]P_i$ into phosphatidate, CMP-phosphatidate and phosphatidylinositol in rat adipose tissue and isolated adipocytes. A similar effect was observed in response to insulin and oxytocin. Theophylline, 3-isobutyl-1-methylxanthine and adenosine deaminase decreased $[^{32}P]P_i$ incorporation, and adenosine and N^6-phenylisopropyladenosine reversed these effects. As with insulin, exposure of adipose tissue to phospholipase C stimulated oxidation of glucose, pyruvate and leucine and activated pyruvate dehydrogenase. Oxytocin and adenosine also mimicked the effects of insulin on leucine oxidation and pyruvate dehydrogenase. However, only insulin stimulated glycogen synthase activity, indicating that the regulation of synthase may be achieved by intracellular events distinct from those regulating changes in phospholipid metabolism, sugar transport and mitochondrial enzyme activities. It is postulated that exposure to phospholipase C forms diacylglycerol, which is phosphorylated to yield phosphatidate. The increased labelling of CMP-phosphatidate and phosphatidylinositol results from the conversion of phosphatidate into these lipids. The correlation between the effects of phospholipase C on phosphatidate synthesis and changes in adipose-tissue metabolism suggests the possibility that increased phosphatidate may directly or indirectly produce changes in membrane transport and enzyme activities. The pattern of phospholipid labelling produced by insulin, adenosine and oxytocin suggests that these stimuli may also increase phosphatidate synthesis, and, if so, changes in phospholipid metabolism could account for some of the metabolic actions of these stimuli.

A variety of neurotransmitters, hormones and other agents produce alterations in cellular phospholipid metabolism often characterized by an increase in the incorporation of radiolabelled phosphate into phosphatidylinositol and phosphatidate (Michell, 1975; Michell *et al.*, 1977; Berridge, 1981; Irvine *et al.*, 1982). The ubiquity of this 'phosphatidylinositol effect' has suggested the possibility that changes in membrane phospholipid composition may underlie some biological actions of these hormones (Michell, 1975). Although the biochemical mechanism that accounts for the selective stimulation of $[^{32}P]P_i$ incorporation has not been established in all cell types, it has been postulated that the changes reflect a stimulation of phosphatidylinositol 'turnover'. In this scheme, stimuli accelerate the breakdown of phosphatidylinositol to diacylglycerol, catalysed by a phospholipase C. The diacylglycerol is converted into phosphatidate, a reaction catalysed by diacylglycerol kinase using the terminal phosphate group of ATP, and, since the phosphorus atom of phosphatidic acid is retained in the synthesis of phosphatidylinositol, with CMP-phosphatidate (the term 'CMP-phosphatidate' rather than the more common 'CDP-diacylglycerol' will be used for the product of the reaction of phosphatidate and CTP to emphasize that the phosphorus atom of phosphatidate is retained in the product of this reaction) as the intermediate, an increase in phosphatidate labelling would lead to an accumulation of radiolabelled phosphatidylinositol.

Previous studies on the effects of hormones and other stimuli on $[^{32}P]P_i$ incorporation have largely

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focused on the association of such changes with biological responses, such as glandular secretion or contraction of smooth muscle, which utilize an increase in intracellular Ca^{2+} as a trigger for the biological responses. For instance, it has been postulated that a stimulus-dependent decrease in phosphatidylinositol of the plasma membrane may be linked to an opening of Ca^{2+} channels (Michell, 1975; Berridge & Fain, 1979; Fain & Berridge, 1979). Indeed it may be that the selective increase in $[^{32}P]P_i$ incorporation into phospholipids and the increase in Ca^{2+} conductance reflect a common biochemical event, the formation of phosphatidate from membrane diacylglycerol and the Ca^{2+}-ionophoretic properties of this phospholipid (Tyson *et al.*, 1976; Michell *et al.*, 1977; Gerrard *et al.*, 1979; Green *et al.*, 1980; Salmon & Honeyman, 1980; Weiss & Putney, 1981; Barritt *et al.*, 1981; Serhan *et al.*, 1982).

Relatively little attention has been paid to the role of changes in phospholipid metabolism in other significant hormone actions. For instance, de Torrentegui & Berthet (1966) documented that insulin increased $[^{32}P]P_i$ incorporation into adipose-tissue phospholipids, including phosphatidate and phosphatidylinositol. Although subsequent studies have confirmed this action of insulin (Stein & Hales, 1974; Garcia-Sainz & Fain, 1980), attention in adipose tissue has been focused primarily on the stimulation of $[^{32}P]P_i$ incorporation by α-adrenergic agents (Garcia-Sainz *et al.*, 1980). The present studies were initiated to examine the relationship of the effects of insulin on $[^{32}P]P_i$ incorporation into phospholipids and its effects on metabolism. To this end, we have compared the effects of insulin and three other stimuli (phospholipase C, oxytocin and adenosine), which mimic certain metabolic effects of insulin on $[^{32}P]P_i$ incorporation into phosphatidate and phosphatidylinositol.

Materials and methods

Male rats were obtained from Charles River Breeding Laboratories and maintained on standard laboratory chow for at least 2 weeks before study. The animals weighed 125–175 g at the time of study. The rats were killed by cervical dislocation, the epididymal fat-pads excised and the thin distal portion of each pad was dissected into segments weighing 50–100 mg. Segments were incubated in Krebs–Ringer bicarbonate buffer (De Luca & Cohen, 1964) supplemented with 5.5 mM-glucose and albumin (10 mg/ml; bovine plasma fraction V, obtained from Miles Laboratories) unless otherwise indicated. Segments were incubated at 37°C under an atmosphere of O_2/CO_2 (19:1). To preclude the influence of hormones present before excision of the tissue, the segments were preincubated for 30–45 min and then transferred to fresh incubation buffer.

Isolated fat-cells, prepared by the method of Rodbell (1964), were used in several experiments. After isolation, the fat-cell suspension was incubated for 30–40 min and then resuspended in fresh buffer before study.

Glucose oxidation was assessed from the liberation of $^{14}CO_2$ from [U-^{14}C]glucose during a 1 h incubation (Goodman, 1967; Honeyman & Goodman, 1980). The oxidations of pyruvate and leucine were assessed in a similar manner, by using [1-^{14}C]pyruvate and [1-^{14}C]leucine respectively. Pyruvate dehydrogenase activity was assayed in cell-free extracts of adipose-tissue segments by the procedure described by Taylor *et al.* (1973). After a 30 min preincubation period, the segments were transferred to fresh buffer and exposed to hormones or other agents for 15–20 min. The segments were homogenized in 4 vol. of ice-cold extraction buffer (10 mM-potassium phosphate, 1 mM-EDTA, 1 mM-dithiothreitol, 10 mg of bovine plasma albumin/ml, pH 7.4), and the extract was centrifuged at 4000 *g* for 5 min at 4°C. The resulting fat-poor infranatant was assayed within 20 min after homogenization. Pyruvate dehydrogenase activity was calculated from the liberation of $^{14}CO_2$ from [1-^{14}C]pyruvate during a 2 min incubation at 37°C.

Glycogen synthase activity was determined in cell-free extracts of adipose tissue by the method of Thomas *et al.* (1968) as modified previously (Honeyman & Goodman, 1980). Briefly, glycogen synthase activity of infranatant fractions of adipose-tissue extracts was determined by the incorporation of UDP-[U-^{14}C]glucose into glycogen. Assays were conducted in the absence and presence of glucose 6-phosphate, and the activities were termed I and D respectively. Changes in I activity as well as the ratio of I to D activities were used as indexes for the activation of the glycogen synthase.

$[^{32}P]P_i$ incorporation into cellular phospholipids was determined by transferring tissues or cells after a 30–45 min preincubation period to fresh buffer containing $[^{32}P]P_i$. The segments and cells were incubated for 45 min and exposed to stimuli for the last 15 min of incubation.

The phospholipids were extracted and purified by a modification (Salmon & Honeyman, 1980) of the method described by Abdel-Latif *et al.* (1977). Segments were quickly rinsed in warm tracer-free buffer, and homogenized in 3 ml of chloroform/methanol/12 M-HCl (400:400:3, by vol.). The extract was centrifuged at 500 *g* for 5 min, and the pellet was re-extracted with chloroform/methanol/12 M-HCl (800:400:1, by vol.) and the combined extracts were dried at 35°C under N_2 at reduced pressure with a Buchler Rotary Evapomix. The phospholipids were obtained by extraction with

methanol (3 × 1 ml) and the methanol extract was dried under reduced pressure. The phospholipid extract was dissolved in chloroform and the chloroform phase washed with 0.1 M-HCl. The phospholipids were purified by two-dimensional t.l.c. on silica-gel 60 plates (E. Merck, Darmstadt, West Germany). The plates were developed in the first dimension with a basic solvent [chloroform/methanol/conc. NH_3 (sp.gr. 0.880), 65:25:4, by vol.] and in the second dimension with an acidic solvent (butanol/acetic acid/water, 6:1:1, by vol.). Two-dimensional chromatography was found to be necessary to separate phosphatidylinositol, phosphatidylserine, CMP-phosphatidate and phosphatidate. The major phospholipids were identified by brief exposure to I_2 vapour, and the ^{32}P-labelled lipids were localized by autoradiography. The extent of $[^{32}P]P_i$ incorporation was quantified by liquid-scintillation counting of the radioactivity of appropriate spots with Liquiscint scintillation-counting solution.

Materials

Bovine insulin was obtained from Eli Lilly Co. and was stored in small portions at −20°C. Partially purified phospholipase C prepared from *Clostridium perfringens* (type I and type IX) was obtained from Sigma Chemical Co. Adenosine deaminase, theophylline, N^6-isopropyladenosine and the substrates for glycogen synthase and pyruvate dehydrogenase assays were obtained from Sigma Chemical Co. 3-Isobutyl-1-methylxanthine was obtained from Boehringer Mannheim. Radiolabelled substrates and $[^{32}P]P_i$ were obtained from New England Nuclear Corp. Liquiscint was obtained from National Diagnostics, Somerville, NJ, U.S.A.

Results

Table 1 summarizes the effects of insulin, oxytocin and phospholipase C on $[^{32}P]P_i$ incorporation into phosphatidate, phosphatidylinositol and CMP-phosphatidate. The last phospholipid was chosen for study because it is the metabolic intermediate between phosphatidate and phosphatidylinositol. In segments of adipose tissue incubated with $[^{32}P]P_i$ for a total of 45 min, exposure to insulin (7 nM) for the final 15 min of incubation increased $[^{32}P]P_i$ incorporation into phosphatidate, CMP-phosphatidate

Table 1. *Effects of insulin, oxytocin and phospholipase C on $[^{32}P]P_i$ incorporation into phosphatidate, CMP-phosphatidate and phosphatidylinositol*

Segments of adipose tissue or samples of cell suspension were incubated in the presence of $[^{32}P]P_i$ (20–60 μCi/ml) for a total of 45 min. Except where indicated, stimuli were added for the final 15 min of incubation. Each value represents the mean ± S.E.M. calculated from the number of experiments indicated in parentheses. To correct for differences in the amount of $[^{32}P]P_i$ added, the data are normalized to the average for all experiments (tissue experiments, 41 μCi/ml; cell experiments, 60 μCi/ml). In experiments on tissues, duplicate determinations were made on pooled segments obtained from four rats. In experiments on isolated adipocytes, duplicate determinations were made on replicate samples of cell suspension (150–200 mg of triacylglycerol/ml). In all cases, the ratio of treated to control value was significantly greater than 1.0, as assessed by a paired *t* test (for insulin or phospholipase on tissue segments, $P < 0.05$; for all other conditions, $P < 0.02$).

	$[^{32}P]P_i$ incorporated (c.p.m./mg of tissue or c.p.m./sample of cell suspension)		
	Phosphatidate	CMP-phosphatidate	Phosphatidylinositol
Tissue segments			
Control ($n = 7$)	25 ± 5.3	5.5 ± 1.6	18 ± 5.5
Insulin (7 nM)	49 ± 11	8.7 ± 2.7	23 ± 8.5
Ratio of insulin to control	1.9 ± 0.14	1.7 ± 0.19	1.2 ± 0.08
Control ($n = 5$)	24 ± 5.4	6.0 ± 2.6	19 ± 5.4
Phospholipase C (0.1 unit/ml)	79 ± 24	15 ± 5.4	28 ± 9.6
Ratio of phospholipase C to control	3.1 ± 0.3	2.9 ± 0.5	1.4 ± 0.2
Control ($n = 7$)	19 ± 2.7	4.8 ± 1.2	9.6 ± 1.4
Oxytocin (10 μM)	72 ± 18	14 ± 3.1	46 ± 7.2
Ratio of oxytocin to control	3.5 ± 0.5	3.2 ± 0.4	4.8 ± 0.5
Isolated cells			
Control ($n = 7$)	7.7 ± 1.8	1.3 ± 0.61	3.2 ± 0.8
Insulin (7 nM)	12 ± 2.4	2.5 ± 0.90	5.8 ± 1.5
Ratio of insulin to control	1.7 ± 0.21	2.3 ± 0.91	1.9 ± 0.15
Control ($n = 1$)	7.3	1.0	1.7
Phospholipase C (0.1 unit/ml for 3 min)	22	3.1	3.2
Ratio of phospholipase C to control	3.0	3.3	1.8

and phosphatidylinositol. On average, the increments in incorporation into phosphatidate and CMP-phosphatidate were nearly equal, but the increase into phosphatidylinositol was somewhat smaller. A nearly identical pattern of $[^{32}P]P_i$ incorporation was noted when suspensions of isolated fat-cells were exposed to insulin.

The peptide hormone oxytocin (1 μM) also increased $[^{32}P]P_i$ incorporation into phosphatidate, CMP-phosphatidate and phosphatidylinositol. The magnitude of the change in $[^{32}P]P_i$ incorporation was much greater than with insulin, but, like insulin, oxytocin produced parallel increases in the labelling of phosphatidate and CMP-phosphatidate.

Exposure of adipose-tissue segments to 0.1 unit of phospholipase C/ml for 15 min produced a parallel increase in $[^{32}P]P_i$ incorporation into phosphatidate and CMP-phosphatidate averaging nearly 3-fold. Exposure of isolated cells to phospholipase C produced a similar change in $[^{32}P]P_i$ labelling of these phospholipids.

Our other studies documented that adenosine stimulated $[^{32}P]P_i$ incorporation into phosphatidate and phosphatidylinositol in adipocytes from hamsters (Schimmel *et al.*, 1983). A similar effect was noted in the present studies on rat adipose tissue and isolated cells (Table 2). As was noted in hamster adipocytes, the effects of adenosine are somewhat obscured unless the influence of endogenously formed adenosine was diminished by inclusion of methylxanthines or adenosine deaminase. Under these conditions a stimulatory effect of the adenosine analogue N^6-isopropyladenosine was apparent.

Exposure of adipose-tissue segments to phospholipase C increased the rate of oxidation of glucose, pyruvate and leucine to a similar extent as insulin (Table 3). Oxytocin and adenosine also stimulated leucine oxidation (results not presented).

The oxidation of leucine is largely determined by the activity of the mitochondrial enzyme complex branched-chain amino acid dehydrogenase (Frick & Goodman, 1980). Thus the ability of oxytocin, adenosine and phospholipase C to stimulate leucine oxidation suggested that they may affect the mitochondrial enzyme activities. To obtain more direct evidence in this regard, effects on another mitochondrial enzyme, pyruvate dehydrogenase, were determined. In one experimental series insulin produced an 8-fold increase in pyruvate dehydrogenase (Table 4). In this series phospholipase C activated the enzyme to a similar extent. In other experiments the effects of insulin were less marked,

Table 2. *Effects of adenosine on $[^{32}P]P_i$ incorporation into phosphatidate, CMP-phosphatidate and phosphatidylinositol*
In each experiment, tissue segments or samples of fat-cell suspensions were incubated in the presence of $[^{32}P]P_i$ for a total of 45 min. Theophylline, 3-isobutyl-1-methylxanthine (IBMX) or adenosine deaminase was present during the entire incubation time. Exposure to adenosine or N^6-phenylisopropyladenosine (PIA) was for the times indicated before termination of the incubation. Each value represents the average of duplicate determinations. To correct for differences in the amount of $[^{32}P]P_i$ added, the data are normalized to the average for all experiments (tissue experiments, 32 μCi/ml; cell experiments, 57 μCi/ml). In experiments with tissues, each determination was made on pooled segments obtained from four rats. In experiments on isolated adipocytes, determinations were made on replicate samples of a single cell suspension (150–200 mg of triacylglycerol/ml).

	$[^{32}P]P_i$ incorporated (c.p.m./mg of tissue or 10^{-3} × c.p.m./sample of cell suspension)		
	Phosphatidate	CMP-phosphatidate	Phosphatidylinositol
Tissue segments			
Control	24.1	7.8	6.4
Adenosine (0.1 mM, 10 min)	32.2	8.4	7.4
Control	24.8	4.9	14.0
IBMX (0.1 mM)	12.3	5.7	2.3
IBMX + PIA (0.1 mM, 15 min)	17.8	6.5	4.8
Control	17.4	2.9	12.1
Theophylline (1 mM)	10.6	2.5	5.4
Theophylline + PIA (10 μM, 15 min)	28.8	2.5	14.5
Isolated cells			
Control	27.5	2.4	14.1
IBMX (0.1 mM)	17.5	1.2	9.2
IBMX + PIA (0.1 mM, 10 min)	24.4	2.5	8.9
Control	39.7	7.8	9.1
IBMX (0.1 mM)	28.5	7.8	4.1
IBMX + PIA (0.1 mM, 15 min)	43.5	8.5	8.3
Adenosine deaminase (1 unit/ml)	10.1	3.9	2.2
Deaminase + PIA (0.1 mM, 15 min)	18.1	3.8	3.9

Table 3. *Effects of insulin and phospholipase C on oxidation of glucose, pyruvate and leucine by adipose tissue*
Tissue segments were preincubated for 45 min, transferred to fresh medium containing [U-^{14}C]glucose, [1-^{14}C]-pyruvate or [1-^{14}C]leucine and the incubation was continued for 1 h (glucose) or 30 min (pyruvate and leucine). Tissues were exposed to insulin (7 nM) or phospholipase C (0.1 unit/ml) during the last 15 min of the preincubation period. Insulin was also present during the subsequent incubation period. Each group of values represents the mean ± S.E.M. calculated from data obtained from tissue segments of the numbers of rats indicated in parentheses.

	Control	Insulin	Phospholipase C
Glucose oxidation ($^{14}CO_2$ released as c.p.m./h per mg of tissue)	9.5 ± 1.0 (12)	35 ± 5.0	38 ± 4.1
Pyruvate oxidation ($^{14}CO_2$ released as c.p.m./30 min per mg of tissue)			
Incubation without fructose	91 ± 12 (8)	130 ± 9.6	138 ± 13
Incubation with fructose	104 ± 3.8 (8)	129 ± 11.6	146 ± 10.5
Leucine oxidation ($^{14}CO_2$ released as c.p.m./30 min per mg of tissue)	10.0 ± 0.8 (8)	26.0 ± 1.5	24.2 ± 2.3

Table 4. *Effects of insulin, phospholipase C, adenosine and oxytocin on pyruvate dehydrogenase activity of extracts of adipose tissue*
Segments were preincubated for 40–60 min, and transferred to fresh buffer 15 min before homogenization. In Expts. I and III tissue segments were incubated and assayed separately, and each value represents the mean ± S.E.M. calculated from the number of rats indicated in parentheses. In Expt. II tissue segments were incubated separately and pooled before homogenization. These values represent the mean ± S.E.M. calculated from five experiments. Rats used in Expt. I were smaller than those used in Expts. II and III (125 rather than 175 g), which may account for differences in control values for enzyme activity.

Expt.		Pyruvate dehydrogenase activity (pmol of $^{14}CO_2$ released/2 min per mg of tissue)
I	Control (n = 9)	13.3 ± 2.6
	Insulin (7 nM)	111.0 ± 8.4
	Phospholipase C (0.1 unit/ml)	102.0 ± 9.5
II	Control (n = 5)	48.2 ± 12
	Insulin (7 nM)	90.4 ± 19
	Adenosine (0.1 mM)	90.0 ± 16
III	Control (n = 8)	49.0 ± 12
	Oxytocin (1 μM)	103.0 ± 16

but adenosine and oxytocin produced similar activations.

It has been proposed that factor(s) which account for the effects of insulin on pyruvate dehydrogenase might also account for activation of the cytosolic enzyme, glycogen synthase (Kuo *et al.*, 1979; Seals & Jarrett, 1980; Seals & Czech, 1980). It was therefore decided to determine the effects of these agents on glycogen synthase. Exposure to insulin increased glycogen synthase nearly 3-fold, but

Table 5. *Effects of insulin and phospholipase C on glycogen synthase activity of adipose-tissue extracts*
Segments of adipose tissue were exposed to insulin (7 nM) or phospholipase C (0.1 unit/ml) for 15 min before homogenization. Extracts were assayed in the absence (I) or presence (D) of glucose 6-phosphate. Each value represents the mean ± S.E.M. calculated from data obtained from tissues of 32 rats incubated and assayed separately.

	Glycogen synthase (nmol of UDP-[U-^{14}C]glucose incorporated/h per g of tissue)		
	I	D	Ratio I/D
Control	8.93 ± 1.8	97.8 ± 10.2	0.088
Insulin	21.2 ± 5.2	102 ± 15	0.21
Phospholipase C	8.5 ± 1.7	97 ± 16	0.085

Table 6. *Effects of adenosine, oxytocin and insulin on glycogen synthase activity of adipose-tissue extracts*
Segments of adipose tissue were exposed to insulin (7 nM), adenosine (0.1 mM) or oxytocin (3.3 μM) for 15 min before homogenization. Extracts were assayed in the absence (I) or presence (D) of glucose 6-phosphate. Each value represents the mean ± S.E.M. calculated from tissues obtained from eight rats incubated and assayed individually. Although the enzyme activity in these experiments was less than that observed in studies shown in Table 5, the effects of insulin were somewhat greater (3-fold activation versus 2-fold).

	Glycogen synthase (nmol of UDP-[U-^{14}C]-glucose incorporated/h per g of tissue)		
	I	D	Ratio I/D
Control	0.70 ± 0.12	50.1 ± 4.3	0.014
Insulin	2.10 ± 0.35	50.2 ± 3.3	0.042
Adenosine	0.73 ± 0.11	47.1 ± 1.4	0.016
Oxytocin	0.93 ± 0.16	58.2 ± 3.0	0.016

phospholipase C failed to affect enzyme activity (Table 5). Adenosine and oxytocin were also unable to activate the synthase, although the effects of insulin were readily apparent (Table 6).

Discussion

Numerous studies have documented that hormones and other stimuli can effect changes in adipose-tissue metabolism that are similar to those produced by insulin. Several previous studies have shown that exposure of adipocytes to phospholipase C mimics the stimulatory effects of insulin on glucose oxidation and conversion into fat as well as the inhibitory effects on lipolysis and cyclic AMP accumulation (Blecher, 1965; Rodbell, 1966; Rodbell & Jones, 1966; Elsbach & Rizack, 1970; Rosenthal & Fain, 1971; Fredholm *et al.*, 1978). The present studies extend these observations to intact adipose-tissue segments and demonstrate that phospholipase C shares with insulin the ability to activate pyruvate dehydrogenase and to stimulate leucine oxidation, presumably by the activation of another mitochondrial enzyme, branched-chain amino acid dehydrogenase (Goodman, 1977; Frick & Goodman, 1980).

The original studies by Blecher (1965), Rodbell (1966) and Rodbell & Jones (1966) indicated that phospholipase C produces a biphasic effect; at low amounts of enzyme, glucose utilization is stimulated, whereas greater amounts of enzyme or prolonged exposure produced inhibition owing to cell lysis. Rodbell (1966) demonstrated that these effects could largely be reversed by inclusion of egg lipoprotein (which contains phosphatidylcholine and provides an alternative substrate for the enzyme) or removal of Ca^{2+} from the medium (the enzyme requires Ca^{2+} for activity), and concluded that the catalytic activity was responsible for the metabolic effects of the phospholipase C. In the present studies, partially purified phospholipase C was studied. Therefore it is possible that some of the metabolic effects could have been produced by contaminating agents. For instance, a sterol-complexing protein, theta toxin, may be present in some commercial preparations of phospholipase C (Mollby *et al.*, 1973; Fredholm *et al.*, 1978), and it is well documented that sterol-complexing agents such as the theta toxin, filipin or saponins can produce insulin-like changes in adipose-tissue metabolism (Kuo, 1968; Rosenthal & Fain, 1971; Fredholm *et al.*, 1978). The metabolic effects of the phospholipase-C preparations used in the present studies would not appear to reflect contamination with theta toxin, for several reasons. The effects of the phospholipase were not abolished by heating the preparation at 60°C for 10–30min, conditions that destroy the theta toxin (Sabban *et al.*, 1972; Smyth *et al.*, 1975), whereas conditions that decrease the catalytic action of the lipase (removal of Ca^{2+} from the incubation medium or inclusion of aqueous dispersions of phosphatidylcholine) markedly decreased its effects. Finally, exposure of adipose-tissue segments to another sterol-complexing agent, saponin, produced the expected metabolic changes (stimulation of glucose oxidation, activation of pyruvate dehydrogenase), but failed to stimulate $[^{32}P]P_i$ incorporation into phospholipids. Therefore the effects of the phospholipase-C preparation are largely due to the catalytic activity of the lipase.

Several insulin-like metabolic effects of oxytocin have been documented (Mirsky & Perisutti, 1962; Bonne *et al.*, 1978; Muchmore *et al.*, 1981). The present studies confirm the demonstration that oxytocin can activate pyruvate dehydrogenase (Mukerjee & Mukerjee, 1982; Hanif *et al.*, 1982), and demonstrate that it can stimulate the oxidation of leucine. Although the present studies failed to observe a significant effect on synthase activity, Muchmore *et al.* (1981) observed that oxytocin produced a small increase in the rate of glycogen deposition. However, this increase in glycogen deposition was only one-tenth that produced by insulin, and it is unlikely that changes of this small magnitude would be detected in assays of enzyme activity.

Previous studies have also demonstrated that adenosine can mimic insulin effects on glucose transport and oxidation, cyclic AMP accumulation, phosphodiesterase activity and lipolysis (Schwabe *et al.*, 1974; Londos *et al.*, 1978; Green & Newsholme, 1979; Schimmel *et al.*, 1980; Teo *et al.*, 1981; Trost & Schwabe, 1981). The findings that adenosine can activate pyruvate dehydrogenase and stimulate leucine oxidation indicate that adenosine mimics insulin actions on two mitochondrial enzymes.

The observation that stimuli can mimic some but not all of the effects of insulin on adipose-tissue metabolism is not unique to this study. Other studies have also identified agents that mimic certain metabolic effects of insulin, but not the activation of glycogen synthase (Lawrence & Larner, 1978; Kuo *et al.*, 1979). Conversely, somatotropin shares with insulin the ability to activate the synthase (Honeyman & Goodman, 1980). Since phospholipase C, oxytocin and adenosine do not activate the synthase, they may exert their effects by intracellular mechanisms distinct from those of insulin. Alternatively glycogen synthase, pyruvate dehydrogenase and other metabolic processes may be regulated by independent intracellular signals, and phospholipase C, oxytocin and adenosine generate some but not all of the same signals as insulin. The latter possibility is seemingly at odds with the postulate that insulin regulates both pyruvate dehydrogenase and glycogen synthase by

the production of a 'peptide signal' (Kuo *et al.*, 1979; Seals & Jarrett, 1980; Seals & Czech, 1980). Thus it will be of interest to establish whether phospholipase C, oxytocin and adenosine can also generate this 'peptide signal'.

The present studies demonstrate that phospholipase C stimulates the incorporation of [^{32}P]P_i into phosphatidate, CMP-phosphatidate and phosphatidylinositol. This effect is not unexpected, as others have noted that phospholipase C can stimulate [^{32}P]P_i incorporation into phosphatidate in erythrocytes, platelets or cultured neuroblastoma cells (Allan *et al.*, 1975, 1978; Mauco *et al.*, 1978; Chap *et al.*, 1979; Ohsako & Deguchi, 1981). In erythrocytes the increase in phosphatidate labelling coincided with an increase in the formation of diacylglycerol as a result of the hydrolysis of phospholipids of the membrane (Allan *et al.*, 1978). Indeed exposure of intact cells to large amounts of phospholipase C can lead to the formation of intramembrane 'droplets' of diacylglycerol (Smyth *et al.*, 1975). It is not unreasonable to propose that the increase in [^{32}P]phosphatidate accumulation observed in adipose tissue arises in a similar manner (i.e. from the phosphorylation of diacylglycerol catalysed by diacylglycerol kinase). The observed increase in [^{32}P]P_i incorporation into CMP-phosphatidate and phosphatidylinositol follow from the increased labelling of phosphatidate. However, as these reactions are thought to occur in the endoplasmic reticulum, the phosphatidate must be rapidly transferred to the internal membranes of the cell. Although much of the diacylglycerol is produced as a direct consequence of the lipase, studies by Hirasawa *et al.* (1981) have demonstrated that in cell-free systems the hydrolysis of phosphatidylinositol can be promoted by phosphatidate. Therefore it is conceivable that at least part of the diacylglycerol is produced by phosphatidate activation of endogenous phospholipases.

The present studies are the first report of an effect of oxytocin on [^{32}P]P_i incorporation into adipose-tissue phospholipids. This finding is also not unexpected, as peptide hormones and other stimuli that elicit contraction of smooth muscle have been shown to stimulate [^{32}P]P_i incorporation into phosphatidylinositol in smooth muscle and liver (Takhar & Kirk, 1981; Kirk *et al.*, 1981). The effects of oxytocin were nearly equal in magnitude to those observed in response to phospholipase C and somewhat greater than those observed in response to insulin or adenosine. In other experiments we have found significant changes in phosphatidate labelling with concentrations of oxytocin as low as 10nM.

The findings that adenosine can stimulate [^{32}P]P_i incorporation and that conditions that diminish the influence of endogenously formed adenosine (methylxanthines or adenosine deaminase) decrease [^{32}P]P_i incorporation are in agreement with our previous observations in hamster adipocytes (Schimmel *et al.*, 1983). The results of these studies suggest the possibility that adenosine synthesized endogenously may affect phospholipid labelling in adipocytes not exposed to exogenous stimuli. Furthermore, changes in adenosine formation may well contribute to changes in phospholipid labelling.

Several previous studies have observed insulin-dependent increases in [^{32}P]P_i labelling of phospholipids, including phosphatidate and phosphatidylinositol. de Torrentegui & Berthet (1966) noted that insulin increased [^{32}P]P_i incorporation into phosphatidate and phosphatidylinositol nearly 2-fold. Stein & Hales (1974) reported that insulin increased [^{32}P]P_i incorporation into a number of phospholipid classes, including phosphatidate and phosphatidylinositol. More recently Garcia-Sainz & Fain (1980) found that insulin increased phosphatidate and phosphatidylinositol labelling in adipocytes and that the effects were abolished by inclusion of EGTA. Farese *et al.* (1982) observed that insulin increased phosphatidylinositol content, but, curiously, did not change its [^{32}P]P_i incorporation.

The manner by which insulin, oxytocin and adenosine elicit an increase in [^{32}P]P_i incorporation in phosphatidate is yet to be established, although various possibilities are excluded by the data. The stimuli produced nearly parallel increases in [^{32}P]P_i incorporation into phosphatidate, CMP-phosphatidate and phosphatidylinositol, so the increase in labelling is not caused by changes in the reactions that convert phosphatidate into phosphatidylinositol (Hokin-Neaverson, 1977). It is also not likely that the change in labelling reflects an inhibition of phosphatidate degradation, as such a change would tend to increase the labelling of CMP-phosphatidate relative to phosphatidate (Hauser & Eichberg, 1975). Under conditions used for their study (simultaneous exposure to [^{32}P]P_i and insulin for 1h, incubation in the absence of exogenous substrate), Stein & Hales (1974) observed that insulin increased [^{32}P]P_i incorporation into ATP and suggested that the changes in lipid labelling may result from changes in the specific radioactivity of ATP. This would not seem to be the case for the present studies, since labelling of ATP was nearly maximal during the 30min preincubation period and was unaffected by subsequent exposure to insulin ([^{32}P]P_i incorporation into ATP was 2442 ± 343c.p.m./mg of tissue at 30min, 2543 ± 565c.p.m./mg at 45min and 2297 ± 241c.p.m./mg for tissues exposed to insulin for final 15min; values represent the means $\pm$ S.E.M. for six tissues).

Although insulin, oxytocin and adenosine may all increase the synthesis of phosphatidate, they may do so by different means. For instance, oxytocin may increase phosphatidate synthesis as a consequence

of increasing the hydrolysis of phosphatidylinositol, as has been postulated for the actions of α-adrenergic agents in adipose tissue. Alternatively insulin may increase phosphatidate synthesis by accelerating its synthesis *de novo*.

A surprising finding in these studies was the extent to which [^{32}P]P_i was incorporated into CMP-phosphatidate. Studies in liver and other cell types have shown that CMP-phosphatidate is present in much smaller amounts (50–100-fold) than its precursor phosphatidate (Thompson & MacDonald, 1975, 1976). The ratio of [^{32}P]P_i incorporation between these two phospholipids was observed to be approx. 5, a value far greater than predicted from the presumed ratio of their contents. It may be that adipose tissue contains a much higher CMP-phosphatidate content than liver and other cell types. Alternatively it may be that [^{32}P]CMP-phosphatidate is synthesized from a pool of phosphatidate of high specific radioactivity. The latter possibility is consistent with the observation that phospholipase C produced a parallel increase in labelling of phosphatidate and CMP-phosphatidate, i.e., phosphatidate synthesized from diacylglycerol in the plasma membrane is a separate pool that can rapidly be converted into CMP-phosphatidate. Moreover, the observation that insulin and oxytocin also increased [^{32}P]P_i incorporation in parallel must mean either that there are other pools of phosphatidate or that these stimuli affect the same pool of phosphatidate as phospholipase C.

The present studies indicate a correlation between changes in adipose-tissue metabolism and increased phosphatidate synthesis. Since the generation of phosphatidate may be a primary action of phospholipase C, it follows that the changes in phospholipid could be responsible for the metabolic effects of the enzyme. Several possible mechanisms that could link these events come to mind. For one, the accumulation of membrane phosphatidate may be a key change in the actions of neurotransmitters and hormones that elevate intracellular Ca^{2+}, owing to the Ca^{2+}-ionophoretic properties of phosphatidate. For example, exposure of neuroblastoma cells to exogenous phosphatidate, or stimulating endogenous phosphatidate formation by exposure to phospholipase C, markedly increased intracellular $^{45}Ca^{2+}$ accumulation (Ohsako & Deguchi, 1981). Since the activation state of pyruvate dehydrogenase can be affected by changes in mitochondrial Ca^{2+} (McCormack & Denton, 1980), such a mechanism could account for the activation of pyruvate dehydrogenase by phospholipase C. A similar mechanism might account for the stimulation of leucine oxidation.

Indirect evidence indicates that changes in membrane lipid organization can affect the rate of glucose transport (Melchior & Czech, 1979; Czech, 1980; Pilch *et al.*, 1980), and it is well documented that acidic phospholipids, such as phosphatidate, can provoke changes in lipid organization such as lateral segregation and non-bilayer configurations (Shimshick & McConnell, 1973; Cullis & De Kruijff, 1979; Krebecek *et al.*, 1979). Thus changes in phosphatidate content could alter membrane lipid organization such that glucose transport is facilitated. In fact a similar hypothesis was put forward by Rodbell (1966) '...that insulin and phospholipase C act on the plasma membrane to alter configuration of its lipoprotein from a laminated to micellar or globular form. The latter configuration might have interstices that permit the carrier mediated passage of solutes into the cell.'

It may be that changes in lipids metabolically linked to phosphatidate are of importance. The demonstration of a membrane-associated Ca^{2+}-dependent protein kinase activated by diacylglycerol suggests the possibility that changes in diacylglycerol are linked to cellular metabolism (Kaibuchi *et al.*, 1981). In this regard, the demonstration that phorbol esters, which are known to produce various changes in membrane properties, including glucose transport (Dicker & Rozengurt, 1980), can substitute for diacylglycerol in the activation of this kinase is particularly noteworthy (Castagna *et al.*, 1982).

The above studies indicate that exposure of adipose tissue to phospholipase C stimulates phosphatidate synthesis and elicits various metabolic effects similar to those produced by insulin. It is postulated that an increased synthesis of phosphatidate might mediate the effects of the enzyme. Furthermore, since oxytocin, insulin and adenosine may also increase phosphatidate synthesis, this lipid may play some role in the metabolic actions of these hormones.

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