Insulin-stimulated Diacylglycerol Production Results from the Hydrolysis of a Novel Phosphatidylinositol Glycan

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The molecular mechanisms of transmembrane signaling in insulin action are poorly understood. It has been suggested that some of the metabolic effects of the hormone may be explained by the generation from the plasma membrane of a unique substance or group of substances which acutely regulate certain insulin-sensitive enzymes. Moreover, the selective action of this insulin-sensitive phosphodiesterase can be distinguished from the arachidonate-containing diacylglycerol derived from the hormone-stimulated hydrolysis of the phosphoinositides in these cells.

EXPERIMENTAL PROCEDURES

Materials. All reagents were from Sigma, with the exception of tissue culture medium (Flow Laboratories), Nu Serum (Collaborative Research), collagenase (Millipore), Dowex AG 1-X8 (Bio-Rad), bovine insulin (Burroughs Wellcome), [2,8-3H]cAMP, [2,6-3H]inositol, [1,6-3H]glucosamine, [9,10-3H]myristic acid, and [5,6,8,9,11]2,14,15-3H]arachidonic acid (New England Nuclear). The analytical SAX HPLC column was from Whatman, LC-Silica HPLC column was from Supelco, and Silica Gel G plates were from Fisher. Male rats (100-125 g) were from Sprague-Dawley. Purified PI-phospholipase C was the generous gift of Dr. Martin Low of the Oklahoma Medical Research Foundation. This enzyme was free of protease activity and was inactive in the hydrolysis of polyphosphoinositides and other phospholipids.

Cell Culture—BC3H1 cells were cultured on collagen-coated multiwell plates or Petri dishes in Dulbecco’s minimal essential medium supplemented with 20% Nu Serum or 10% fetal calf serum. Cells were grown to maximal density and confluency, at which time they become responsive to insulin (14). Radiolabeled precursors were

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added for 20 h in serum-free medium. [3H]Myristic and arachidonic acids were complexed 1:1 to bovine serum albumin prior to addition.

Extraction and Analysis of Lipids—Following preincubations with the appropriate isotopes, cells were resuspended in Dulbecco's minimal essential medium without serum and treated with 10 nM insulin at the designated intervals. Reactions were terminated by the addition of 1 ml of chloroform, methanol, 1 N HCl (200:100:1), followed by 0.5 ml of 10 mM formic acid. Following centrifugation at 500 x g for 5 min, the upper phase was discarded. For phospholipid analyses, the lower organic phase was dried under N2, resuspended in chloroform/methanol/H2O (9:7:2) and spotted on oxalate-impregnated Silica Gel G plates. These were sequentially developed in chloroform/acetonitrile/ methanol/glacial acetic acid/H2O (10:4:2:2:1) and chloroform, methanol, 4 N HNO3 (45:35:10). One-cm regions were scraped and eluted with chloroform/methanol (2:1) and counted. Phosphoinositide standards were identified by iodine staining.

For analysis of neutral lipids, the chloroform/methanol organic phases were dried under N2 and resuspended in 1 ml of diethyl ether followed by addition of 1 ml of 50 mM formic acid. The upper ether phase was aspirated, and the lower aqueous phase was re-extracted with 1 ml of diethyl ether. The pooled ether phases were dried under N2, resuspended in chloroform, and spotted on Silica Gel G plates which were preactivated at 60°C for 1 h. Plates were twice developed in petroleum ether/diethyl ether/glacial acetic acid (70:30:2). Five µg of unlabeled dimyristoyl glycerol, myristic acid, and monomyristoyl glycerol were added as carriers to each sample.

HPLC—The phospholipid precursor was further chromatographed on a silica HPLC column, eluted with a linear 20-min gradient of chloroform/methanol/glacial acetic acid/H2O (65:25:10:1 to 40: 45:10:5) at 1 ml/min. The aqueous products of the hydrolysis reactions were identified on an analytical SAX HPLC column, eluted with a linear 15-min gradient of 80% methanol to 0.5 M triethylamine-formate, pH 4.5, at 1 ml/min, as described previously (3).

Bioassay of Water-soluble Hydrolysis Products—The water-soluble products of the hydrolysis reactions were also identified by the ability to stimulate cAMP phosphodiesterase in adipocyte particulate fraction (8). The high affinity enzyme was assayed as described previously (15).

RESULTS

Diacylglycerol Production in BC3H1 Cells—The time course of production of diacylglycerol was evaluated in response to hormones. Cells were labeled with [3H]myristic acid (Fig. 1) or [3H]arachidonic acid (Fig. 2) and exposed to insulin or epinephrine, known to act as an α-1 adrenergic agonist in these cells (13). In [3H]myristate-labeled cells (Fig. 1) insulin caused a rapid increase in labeled diacylglycerol, which was maximal at 1 min, declined by 2 min, and slowly increased thereafter. This biphasic pattern was observed in several experiments. In contrast, exposure to epinephrine resulted in no significant change in [3H]myristoyl diacylglycerol above basal levels. In cells incubated with [3H]arachidonic acid (Fig. 2), insulin treatment caused a slow increase in labeled diacylglycerol. In contrast, epinephrine produced a rapid increase in [3H]arachidonoyl diacylglycerol which was maximal at 1 min, declined by 5 min, and then slowly increased. Insulin had no detectable effect on the generation of [3H]stearyl diacylglycerol (not shown).

Identification of a Phosphatidylinositol-Glycan Precursor—

In a previous report (3, 4) a novel PI-glycan was identified which served as a precursor for the insulin-dependent generation of phosphodiesterase-modulating substances in liver and muscle cells. Cultured BC3H1 cells were separately incubated with [3H]inositol, [3H]glucosamine, or [3H]myristic acid for 20 h. Lipids were extracted, treated with or without PI-phospholipase C purified from Staphylococcus aureus, and chromatographed on thin layer plates (Fig. 3). Extraction of phospholipids from inositol-labeled cells revealed two tritiated spots which were diminished by incubation with PI-phospholipase C (Fig. 3a). The PI-phospholipase C-sensitive spot migrating toward the solvent front coeluted with PI. The remaining PI-phospholipase C-sensitive spot, which migrated...
between phosphatidylinositol 4-phosphate and phosphatidyl-
inositol, exhibited chromatographic behavior identical to the
glycolipid identified as precursor for the PI-phospholipase C-
generated phosphodiesterase modulators (3). This spot was
ventitatively identified as the PI-glycan. Extraction of cells
preincubated with \[^{3}H\]glucosamine revealed one major PI-
phospholipase C-sensitive spot on TLC which comigrated
with the PI-glycan (Fig. 3b). \[^{3}H\]Myristic acid was incorpo-
rated into several lipid species, although only the radioactivity
residing in spots corresponding to PI and the PI-glycan was
diminished by treatment with PI-phospholipase C (Fig. 3c).

HPLC of the PI-Glycan—The radiolabeled PI-glycan could
be purified further by an HPLC silica column. The region of
the thin layer plate which contained the PI-glycan was
scraped and eluted. The resulting substance was a poor sub-
strate for hydrolysis by PI-phospholipase C. However, when
reconstituted by sonication with lipid vesicles containing 1:1
mixtures of phosphatidylethanolamine:phosphatidylcholine,
complete hydrolysis was observed. The TLC-eluted lipid was
reconstituted and incubated with or without PI-phospholipase C
for 2 h and then extracted with chloroform/methanol and
phase separated. The organic phases were chromatographed
on an HPLC silica column (Fig. 4). A predominant peak
containing labeled inositol (Fig. 4a) or myristic acid (Fig. 4b)
was detected at 22 min which was diminished in PI-phos-
pholipase C-treated extracts. Twenty-four-h incubation of this
fraction with PI-phospholipase C resulted in its complete
hydrolysis (data not shown).

Product Analysis of PI-Phospholipase C Hydrolysis of the
PI-Glycan—After PI-phospholipase C digestion of the PI-
glycan, both the aqueous and organic products were analyzed.
The labeled PI-glycan was purified by HPLC and treated with
PI-phospholipase C as described above. For glycolipid ex-
tracted from cells labeled with \[^{3}H\]inositol the aqueous prod-
ucts were resolved by phosphatidylcholine standards were visualized by iodine staining. PIP, phosphatidylinositol phosphate.

![Fig. 3. Thin layer chromatography of the labeled glycolipid precursor. Cells (1 x 10⁷/plate) were preincubated with (a) 2 µCi of
[^{3}H]inositol; (b) 2 µCi of \[^{3}H\]glucosamine; or (c) 5 µCi of \[^{3}H\]myristic acid, extracted with chloroform/methanol/HCl (200:100:1) and resus-
pended in 1 ml of 50 mM ammonium bicarbonate, pH 6.0. Following
2-h incubation with or without 1 µg/ml PI-phospholipase C (PLC),
solutions were re-extracted and organic phases were spotted on silica
plates, developed as described under "Experimental Procedures." Phosphoinositide standards were visualized by iodine staining. PIP, phosphatidylinositol phosphate.](Fig. 3)

![Fig. 4. Silica HPLC of the PI-glycan (PI-G). Following thin
layer chromatography, the PI-phospholipase C (PLC)-sensitive spot (as
detected in Fig. 3) was scraped and eluted, and resuspended with
100 µg of a phosphatidylethanolamine:phosphatidylcholine into 1 ml of 50
mM ammonium bicarbonate, pH 6.0, by sonication. These solutions
were treated with (■) or without (○) 1 µg of PI-phospholipase C for
2 h. Following extraction the organic phase was injected into a silica
HPLC column, eluted as described under "Experimental Procedures." One-mL fractions were counted. Phosphoinositide standards were
identified by TLC of eluted fractions. PIP, phosphatidylinositol phosphate.](Fig. 4)
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FIG. 5. SAX HPLC of the aqueous PI-phospholipase C (PLC) hydrolysis products. Following silica HPLC, the [3H]inositol-labeled PI-glycan was reconstituted with phospholipids as described in the legend to Fig. 4 and treated with (O) or without (0) 1 μg/ml PI-phospholipase C for 2 h. Following extraction the aqueous phase was injected directly onto an analytical SAX HPLC column, eluted as described under "Experimental Procedures." One-ml fractions were counted. Cyclic 1,2-inositol monophosphate (IP) and inositol 2-monophosphate were detected by TLC (30) of collected fractions.

were extracted and the PI-glycan was identified on HPLC by coelution with trace amounts of the labeled purified glycolipid. The resulting silica HPLC-purified PI-glycan was treated with or without PI-phospholipase C, aqueous products were injected into an HPLC SAX column, and fractions were assayed for phosphodiesterase-modulating activity (Fig. 6). A single phosphodiesterase-modulating substance was identified with a retention time (15 min) identical to that of the radiolabeled product. Further analysis of elution behavior on P-2 gel filtration columns and high voltage thin layer electrophoresis (3) revealed that the radioactive and bioactive substances displayed identical chromatographic and electrophoretic properties (data not shown).

To analyze the nonaqueous product of the PI-phospholipase C-catalyzed hydrolysis, the purified [3H]myristic acid-labeled PI-glycan was treated with or without PI-phospholipase C and subsequently extracted with ether. TLC of this neutral lipid fraction (Fig. 7) indicated that PI-phospholipase C treatment caused the generation of diacylglycerol which contained [3H]myristic acid.

Insulin Stimulates the Hydrolysis of the PI-Glycan—The effect of insulin on the turnover of the PI-glycan was evaluated (Fig. 8). Cells were prelabeled with [3H]inositol or [3H]myristate, and radioactivity in the PI-glycan spot identified by TLC was determined. As previously reported, a small (20%) but significant decrease in [3H]inositol labeling was observed after 30 s of insulin exposure, followed by a gradual (40%) increase in counts, perhaps reflecting resynthesis (Fig. 8a). In [3H]myristate-labeled cells (Fig. 8b) insulin caused a 60% decrease in the labeling of the PI-glycan by 1 min, which did not return to basal levels until 20–30 min. These differences in the rate of relabeling by these two precursors were consistently observed in several experiments and may be due to inability to attain equilibrium during the labeling period.

**DISCUSSION**

We recently reported (3–5) that insulin stimulated the production of two related complex carbohydrate-phosphate substances in B3H11 cells containing inositol and glucosamine. The generation of these substances was accompanied by the production of diacylglycerol, followed by phosphatidic acid, and appeared to result from the phosphodiesteratic cleavage of a novel PI-glycan. In this report we characterize this process further by examining the rapid insulin-dependent production of [3H]myristate-labeled diacylglycerol, which appeared to result from the hydrolysis of a novel precursor, rather than from de novo synthesis as suggested previously (12, 13). This rapid generation of [3H]myristoyl diacylglycerol could be distinguished from a second slower phase which was also observed in [3H]arachidonic acid-labeled cells. The significance of the biphasic pattern of diacylglycerol production is unclear, although it may result from a phosphorylation and subsequent dephosphorylation reactions, as reflected by a
The possible role of diacylglycerol in insulin action has been considered (4, 16–19). Diacylglycerol and the structurally related phorbol esters are known to specifically activate a Ca\(^{2+}\)- and phospholipid-dependent protein kinase C (20). Phorbol esters or protein kinase C mimic certain actions of insulin (16–19), yet antagonize other effects of the hormone (21–23). Furthermore, direct activation of protein kinase C or intracellular translocation of the enzyme by insulin have not been observed (24). The discrepancies between the actions of insulin and phorbol esters may be explained in part by the unique pathway of diacylglycerol formation described here. The myristoyl diacylglycerol may produce only a limited activation of protein kinase C or is perhaps directed to an enzyme which is similar but not identical to the kinase. Such an enzyme, which was phospholipid dependent but Ca\(^{2+}\)-independent, has recently been described (25). Moreover, the recent identification of a new family of protein kinase C-related genes provides further evidence for multiple kinase C proteins, perhaps with distinct regulatory domains (26). Alternatively, the production of diacylglycerol in the absence of insulin Tris phosphate-induced calcium mobilization may result in the selective regulation of a kinase, perhaps causing the activation in situ of the membrane-associated form of the enzyme, but ineffective in facilitating the translocation of a cytoplasmic component of the kinase to the plasma membrane. Although we have detected increased labeling in fractions of diacylglycerol in response to insulin, it is possible that the minor changes in mass which occur are insufficient to produce stimulation or translocation of protein kinase C, or that these observed changes in diacylglycerol levels are transient increase in phosphatidic acid in response to insulin (4). In contrast to insulin, epinephrine, acting through \(\alpha\)-1 adrenergic receptors in these cells, caused the rapid generation of [\(^{3}\)H]arachidonyl diacylglycerol, but had no discernible effect on generation of [\(^{3}\)H]myristoyl diacylglycerol. These results indicate that insulin and epinephrine stimulate the activities of distinct phospholipases C which hydrolyze different substrates. The putative insulin-sensitive phospholipase C appears to catalyze the phosphodiesteratic cleavage of a novel glycolipid which contains PI, glucosamine, and other monosaccharides and is labeled with myristic acid. This action of insulin causes the generation of two potential second messengers: 1) the inositol phosphate-glycan which modulates several insulin-sensitive enzymes including cAMP phosphodiesterase, pyruvate dehydrogenase, and adenylate cyclase (3, 5); and 2) myristoyl diacylglycerol. Significantly, the insulin-stimulated hydrolysis of this glycolipid does not produce inositol Tris phosphate (11, 13), whereas epinephrine stimulates a phosphoinositide-specific phospholipase C, resulting in the production of inositol phosphates (15) and arachidonyl diacylglycerol. This proposed substrate specificity for hormonally regulated phosphodiesterase hydrolysis reactions is further supported by the recent identification of a phospholipase C activity with a distinct substrate specificity for the PI-glycan and a related phosphoinositide glycoside.\(^2\)

\(^2\)J. A. Fox, N. M. Soliz, and A. R. Saltiel, manuscript in preparation.

**Fig. 7.** Thin layer chromatography of the nonaqueous PI-phospholipase C (PLC) hydrolysis products. Following silica HPLC, the [\(^{3}\)H]myristate-labeled PI-glycan was reconstituted with phospholipids as described in the legend to Fig. 4 and treated with or without 1 \(\mu\)g/ml PI-phospholipase C for 1 h. Following extraction, the lower organic phase was spotted on Silica Gel G plates for analysis of neutral lipids as detailed under “Experimental Procedures.” Diacylglycerol (DAG) was identified by iodine staining.

**Fig. 8.** Time course of the hydrolysis of the PI-glycan. BC3H1 cells (10\(^6\)/well) were labeled with 1 \(\mu\)Ci of [\(^{3}\)H]inositol or 2 \(\mu\)Ci of [\(^{3}\)H]myristate as described in Fig. 1 and exposed to 10 nM insulin at the designated intervals. Labeling of the PI-glycan was determined by scraping and counting of a spot on silica gel thin layer chromatography corresponding to an HPLC-purified [\(^{3}\)H]inositol-labeled PI-glycan standard. All reactions were performed in triplicate, and results were reproduced in several experiments.
significant only in certain membrane compartments. In this regard, we are unable to assess changes in mass in this specific diacylglycerol, and thus the precise chemical magnitude of the change is impossible to determine.

The novel PI-glycan described here shares some homology with the glycolipid responsible for the anchoring of several proteins to the plasma membrane (27). The insulin-sensitive hydrolysis of the PI-glycan and the enzymatic release of the variant surface glycoprotein in Trypanosoma brucei exhibit several similarities (28). The trypanosome protein is covalently linked to dimyristoyl PI via a glucosamine-containing glycan, which can be hydrolyzed by the S. aureus PI-phospholipase C (28) and an endogenous phospholipase C (29). The endogenous enzyme cleaves this PI-glycan anchor but is inactive in hydrolyzing phosphoinositides or other phospholipids (29). In a separate report, we describe the purification and characterization of a phospholipase C in liver plasma membranes with similar specificity. The activation of this enzyme by the insulin receptor complex may be a critical transduction mechanism in the action of the hormone.

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