Isolation of insulin-sensitive phosphatidylinositol-glycan from rat adipocytes

Its impaired breakdown in the streptozotocin-diabetic rat

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In this study an insulin-sensitive glycophospholipid from rat adipocytes was isolated and partially characterized. A material that activated pyruvate dehydrogenase was extracted from rat adipocyte membrane supernatants. Its release was stimulated by insulin and phosphatidylinositol-specific-phospholipase C and its activity was destroyed by nitrous acid deamination. These findings suggested that insulin might stimulate breakdown of a glycophospholipid containing inositol and glucosamine, as previously reported for some other cell types [Low & Saltiel (1988) *Science* **239**, 268–275]. A lipid that incorporated [^3H]glucosamine, [^3H]galactose, [^3H]inositol, and [^3H]myristate and whose turnover was stimulated by insulin was subsequently isolated from intact adipocytes by sequential t.l.c. using an acidic solvent system followed by a basic solvent system. The effects of insulin on turnover of the lipid in these cells were transient, with maximal effects at 1 min, and there was a typical concentration–response curve to insulin (0.07 nM–7 nM), with effects being detected over the physiological range of insulin concentrations. In contrast with studies in other cells, there was appreciable turnover of the sugar labels. The majority of the [^3H]glucosamine and [^3H]galactose labels were cycled through to triacylglycerol in the adipocyte. However, of that recovered in the glycophospholipid band, a major proportion (> 40%) was recovered as the native label. Digestion of the purified molecule with phosphatidylinositol-specific phospholipase C generated a material that activated both pyruvate dehydrogenase and low- K_m cyclic AMP phosphodiesterase. Impairment in insulin-stimulated breakdown of the molecule in adipocytes of streptozotocin-diabetic rats was found, consistent with the impaired insulin activation of pyruvate dehydrogenase and glucose utilization seen in this model. These findings suggest that insulin stimulates breakdown of this glycophospholipid by stimulating an insulin-sensitive phospholipase in adipocytes. This compound may serve a function as a precursor for intracellular insulin mediators.

INTRODUCTION

Insulin is the key anabolic hormone regulating metabolism of carbohydrates, lipids and proteins, but many events involved in its mechanism of action are still unclear. In particular, molecular events occurring subsequent to hormone–receptor interaction which signal intracellular metabolic changes are only beginning to be defined. An immediate consequence of insulin binding has been shown to be the activation of the insulin receptor tyrosine kinase [1]. Recent studies largely using cell lines also suggest that the binding of insulin to its receptor may activate a specific phospholipase C or D that acts on a phosphatidylinositol-glycan (PI-gly), resulting in the intracellular accumulation of the phosphoinositol-glycan head-group (IP-gly) within the cell [2–11]. Several laboratories have now demonstrated the presence of this insulin-sensitive PI-gly in various cell lines, including BC3H-1 myocytes [3–5,9] and H35 hepatoma cells [6,10], as well as splenic T cells [7] and liver cells or membrane fractions [2,8,11]. Liver has been used as the primary source of the IP-gly for investigation of its actions following release by phospholipase action on the PI-gly. This IP-gly added to intact cells and cell fractions has been shown to mimic insulin action in a wide variety, although not all, of insulin-sensitive processes. These include insulin action on pyruvate dehydrogenase (PDH), adenylate cyclase, cyclic AMP phosphodiesterase, cyclic AMP-

dependent protein kinase, pyruvate kinase and phospholipid methyltransferase, as well as general cellular phospho–dephospho control [2–4,6,7,12–14]. The majority of these studies have been performed using adipocytes or adipocyte subfractions as the target tissue, despite the fact that the presence of PI-gly in the adipocyte has not previously been demonstrated. Difficulties in extraction of the PI-gly from adipocytes due to cycling of the sugar labels via the glycolytic pathway is the most likely reason for the lack of evidence supporting the presence of PI-gly in adipocytes. Furthermore, one study was recently published that suggested that insulin does not activate a PI-specific phospholipase C (PI-PLC) in adipocytes [15].

Our past studies [16] provided preliminary evidence to suggest the presence of PI-gly in adipocytes by the demonstration that PI-PLC mimicked insulin action on PDH and generation of a potential mediator for the effects of insulin on the enzyme. Preliminary data to support insulin stimulation of PI-gly breakdown were also provided. In the present study, despite difficulties encountered in cycling of the sugar labels via the glycolytic pathway, more extensive evidence is provided to support the presence of an insulin-sensitive PI-gly in this major insulin target tissue. Impaired breakdown of this PI-gly by insulin in an animal model of type 1 diabetes, the streptozotocin-induced diabetic rat, is also demonstrated, further supporting a role for this compound in insulin action.

Abbreviations used: PI-gly, phosphatidylinositol-glycan; IP-gly, phosphoinositol-glycan head-group; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; PDH, pyruvate dehydrogenase.

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MATERIALS AND METHODS

Materials

D-[6-³H]Glucosamine hydrochloride, D-[G-³H]galactose, D-[5-³H]glucose and [1-¹⁴C]pyruvic acid were obtained through du Pont from New England Nuclear, Boston, MA, U.S.A. *myo*-[2-³H]Inositol, [9,10(*n*)-³H]myristic acid, [9,10(*n*)-³H]palmitic acid and 2-deoxy-D-[U-¹⁴C]glucose were from Amersham International, Amersham, Bucks., U.K. Purified PI-PLC from *Bacillus thuringiensis* was generously provided by Dr. M. Low, Columbia University, New York, NY, U.S.A., and was used at an activity dilution of 2.5–5 μ mol/min per ml, estimated using [³H]PI as substrate at pH 7 in the presence of 0.1 % deoxycholate. Preparations tested for proteinase activity were below detectable limits (< 2.5 ng/25 units of PI-PLC; M. Low, personal communication). Materials for cell culture were obtained from Flow Laboratories, McLean, VA, U.S.A., except for Medium 199 (Earls Salts), which was obtained from the Commonwealth Serum Laboratories, Parkville, Vic., Australia. Silica gel G plates for t.l.c. were from Whatman, Clifton, NJ, U.S.A. Porcine insulin and most other chemicals were from Sigma, St. Louis, MO, U.S.A.

Animals

Male Sprague–Dawley rats (120–150 g) were obtained from the colonies maintained at Monash University, Clayton, Vic., Australia, and were allowed free access to food and water. Diabetes, when studied, was induced in rats weighing less than 100 g by a single intraperitoneal injection of 80 mg of streptozotocin/kg in 100 mM-citrate buffer, pH 4.0. Diabetes was confirmed by the presence of glycosuria and elevated blood glucose concentrations (> 15 mM). Animals were killed 3 weeks after induction of diabetes.

Epididymal rat adipocyte isolation and culture

Adipocytes were isolated by the collagenase digestion method of Rodbell [17] in Krebs–Ringer bicarbonate/Hepes buffer, pH 7.4, containing half the usual amount of Ca²⁺ (1.15 mM), 3 % BSA and 5 mM-glucose. The number of cells in each preparation was determined using a haemocytometer.

Adipocytes were cultured, as required, using slight modifications of the method described in [18], in Medium 199, containing 3 % BSA, 5 % fetal bovine serum, 100 units of penicillin/ml, 100 μ g of streptomycin/ml and 1 mg of Fungizone/ml (Squibb), and radioisotopes as required. Cell incubations [(3–5) $\times$ 10⁵ cells/ml] for stimulation with insulin were carried out as detailed previously [16] in either Krebs–Ringer/bicarbonate buffer containing 5 mM-glucose and 3 % BSA serum albumin, pH 7.4, or Medium 199 containing 3 % BSA after three washes of the cells in fresh medium and a 30 min preincubation before insulin stimulation.

Adipocyte fractionation

Adipocytes were fractionated to prepare plasma membranes (for mediator generation *in vitro*), microsomes (for determination of effects on insulin-sensitive low-*K_m* cyclic AMP phosphodiesterase) and mitochondria (for determination of the effects of mediator on PDH activity *in vitro*) using modifications [19] of the method described by Jarrett [20]. Membranes and mitochondria were resuspended for use in 50 mM-potassium phosphate buffer, pH 7.4.

Enzyme assays *in vitro*

Insulin mediator activity was assessed using adipocyte sub-fractions as the enzyme source. Effects on PDH were measured using adipocyte mitochondria as described in

[16,19,21]. ‘Active’ PDH was determined in the presence of 50 μ M-MgCl₂ and 50 μ M-CaCl₂. ‘Total’ PDH was determined following preincubation with 20 mM-MgCl₂ and 0.5 mM-CaCl₂ for 30 min. Effects of mediator fractions on low-*K_m* cyclic AMP phosphodiesterase were measured at 0.1 μ M-cyclic AMP as described previously [22] after addition of mediator fractions to adipocyte microsomal fraction used as the enzyme source.

Insulin-mediator PI-gly breakdown

Adipocytes were cultured for 16–24 h in medium containing [³H]glucosamine and extracted for glucosyl-lipids using the scheme employed previously for H35 cells and splenic T cells [6,7], modified for fat cells by the development of a method for removal of the adipocyte neutral lipid fraction before t.l.c. of membrane glucosyl phospholipid. This modification was developed from methods described for the extraction of the PI-gly from hepatocytes [11]. PI-gly breakdown by insulin was determined after overnight labelling of cells with [³H]glucosamine (12.5 μ Ci/ml), unless otherwise stated, as we have previously described [16]. Briefly, cells were taken out into fresh medium containing 3 % BSA and samples were placed in Microfuge tubes 30 min before stimulation with insulin for the appropriate time (1 min, unless otherwise stated). Control incubations were performed for the same period of time. After incubation, cells were centrifuged in a Microfuge with a 1 s pulse, and the medium under the cells was removed. Acidified chloroform/methanol (1:2 v/v) (4 ml) was added to each tube, followed by 1 ml of chloroform and 2 ml of 0.1 M-KCl, which induced phase separation. The organic phase was collected and the aqueous phase was washed with a further 1 ml of chloroform. Silica gel 60 (Merck, Darmstadt, Germany) (1 g) in 3 ml of chloroform, was then added to the pooled organic extracts and stored at –20 °C overnight. The PI-gly and phospholipids were adsorbed to the silica under these conditions and were separated from neutral lipid (which did not adsorb) by four washes with 3 ml of chloroform/methanol/0.05 M-HCl (300:50:3, by vol). The PI-gly and phospholipid were then eluted from silica with three washes of 2 ml of methanol, followed by one wash with 2 ml of chloroform/methanol/25 % ammonia (2:1:0.075, by vol.). The PI-gly was then purified in two solvent systems, as in [6,7]. It remained at or near the origin after t.l.c. in chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, by vol.) (solvent system I) and after elution (as in the silica step above) and t.l.c. in chloroform/methanol/ammonia/water (45:45:3.5:10, by vol.) (solvent system II) on oxalate-impregnated plates, run with an *R_F* of approx. 0.5. Radiolabelled lipid and standard bands were located by autoradiography of t.l.c. plates sprayed with Enhance (New England Nuclear), and were quantified, after scraping of the bands, by liquid scintillation counting.

Nitrous acid deamination

Samples of PI-gly labelled with [³H]glucosamine were dried and resuspended in 0.1 ml of 50 mM-sodium acetate, pH 3.5. To half of the sample was added 0.1 ml of 0.33 M-NaNO₂ (nitrous acid deamination), and to the other half was added 0.1 ml of 0.33 M-NaCl (control). Samples were then incubated for 5 h at room temperature and the lipids were separated as above. Each sample was then purified by t.l.c. in solvent system II above. The extent of deamination was determined as the loss of PI-gly label compared with the control. For the determination of deamination of the PDH activator obtained in plasma membrane supernatants, 0.5 ml samples were lyophilized and then treated as above. Samples were then resuspended in 10 ml and desalted by concentrating to 0.5 ml using ultrafiltration on YC05 membranes (Amicon Corp., Danvers, MA, U.S.A.). They were prepared for assay of PDH stimulatory activity after lyophi-

lization by resuspension to 100 μ l. PDH stimulatory activity of 25 μ l aliquots was determined as described above.

PI-PLC digestion of PI-gly

PI-PLC digestion of PI-gly was carried out as in [6]. Samples of purified [^{3} H]glucosamine-labelled PI-gly were incubated with *Bacillus thuringiensis* PI-PLC (gift from Dr. M. Low) for 60 min at 37 °C at an activity dilution of 2.5 μ mol/min per ml after sonication of the lipid in a mixture containing 50 μ g of phosphatidylcholine and 50 μ g of phosphatidylethanolamine in 25 mM-Hepes, pH 7.4. Reaction was stopped by extraction of the aqueous mixtures on Baker C₁₈ Sep-pak columns. The columns were washed with 3 $\times$ 1 ml of water. Unreacted lipid was eluted with 3 $\times$ 1 ml washes of methanol, and was analysed by t.l.c. Radioactivity released into the aqueous fraction was used to indicate sensitivity to PI-PLC and was expressed as a percentage of the unreacted lipid. In some experiments the lipids were suspended in ether (1 ml), and PI-PLC was incubated as an aqueous layer (0.5 ml) under the organic phase. For experiments in which the ability of the PI-PLC digestion products to modify the activity of insulin-sensitive enzymes was measured, the aqueous fractions were collected and separated from PI-PLC by ultrafiltration in 1 mM-formic acid through YM2 membranes (Amicon; 2000- M_r cut-off). The eluates were collected, lyophilized and resuspended in 150 μ l of 1 mM-formic acid for measurement of effects on the PDH activity and the low- K_m cyclic AMP phosphodiesterase activity of adipocyte subfractions as detailed above.

Assays of insulin action

Glucose utilization was determined as the conversion of [^{3} H]glucose into ^{3} H₂O [16,21]. Cells [(2–5) $\times 10^5$] in 0.5 ml were incubated in the presence or absence of insulin for 10 min followed by 4 μ Ci of [^{3} H]glucose for an additional 30 min. ^{3} H₂O in the medium was then measured after absorption into CaCl₂. PDH activity was measured in the same cells after removal of the medium and extraction of the cells with 0.2% Triton X-100/2 mM-dithiothreitol/2 mM-EDTA/2 mM-EGTA/50 mM-potassium phosphate buffer (pH 7.4) at 0 °C as described in [16,19,21]. Cell suspensions were vortex-mixed for 3 $\times 10$ s at 0 °C, and centrifuged for 5 s at 5 °C and 10000 g. PDH activity was determined in the supernatant fractions as described above for the measurement *in vitro*.

2-Deoxyglucose was used as the index of glucose transport and was measured as in [16,21]. Briefly, adipocytes were suspended in Krebs–Ringer bicarbonate/Hepes buffer, pH 7.4, containing 3% BSA, 0.1 mM-glucose and 2 mM-pyruvate. Approx. 1×10^5 cells were incubated in the presence or absence of insulin for 30 min. The assay was then initiated by the addition of 0.1 mM-2-deoxy-[U- ^{14} C]glucose at 0.25 μ Ci/tube for a further 5 min. The assay was terminated by spinning a portion of the cells through oil.

Statistical analyses

Statistical analyses were performed using Student's *t* test. Results are expressed as the means $\pm$ S.E.M. Statistical significance was determined at the *P* < 0.05 level and is indicated in the Results section.

RESULTS

Insulin and PI-PLC stimulate generation of a mediator of PDH activation from adipocyte plasma membranes

The ability of insulin and of PI-PLC to stimulate insulin-mediator generation from adipocyte plasma membranes was assessed (Table 1). The addition of 7 nM-insulin or of *B. thuringiensis* PI-PLC to rat adipocyte plasma membranes caused

Table 1. Effects on PDH activity of supernatants from insulin- and PI-PLC-treated adipocyte plasma membranes: inhibition of activity by nitrous acid deamination

Insulin (7 nM), PI-PLC (2.5 units/ml) or diluent (0.1% BSA in 0.9% NaCl; control) was incubated with 1 ml portions of adipocyte plasma membrane suspension in 50 mM-KH₂PO₄, pH 7.4, at 2 mg of protein/ml. After 5 min at 37 °C (in the case of insulin incubations) or 30 min (in the case of PI-PLC or control incubations), samples were Microfuged for 1 min at 10000 g. [Data from 5 min incubations of control membranes were not different from those from 30 min incubations (results not shown).] The supernatants obtained were lyophilized and the protocol for nitrous acid deamination carried out as described in the Materials and methods section. Half of each sample (0.5 ml) was treated with sodium nitrite (nitrous acid deamination) whereas the other half was treated with NaCl (control). The PDH stimulatory activity of each mediator preparation was determined in three independent incubations of the samples with mitochondria. Basal PDH activity was determined in the presence of buffer samples treated in the same manner as the samples. Total PDH activity, determined after preincubation with high Mg²⁺ (20 mM) and Ca²⁺ (0.5 mM) concentrations to stimulate the phosphatase, is given in the second line. Data are the means $\pm$ S.E.M. of results obtained from three or more independent membrane incubations within the same experiment. Similar findings were obtained with two different membrane preparations. * Denotes significant difference from appropriate basal PDH activity (*P* < 0.05); † denotes significant difference from control mediator (*P* < 0.05).

Incubation condition	PDH activity (nmol/min per mg)	
	Control	Nitrous acid treatment
Basal	2.88 $\pm$ 0.95	1.98 $\pm$ 0.18
High Ca ²⁺ and Mg ²⁺	24.51 $\pm$ 2.20*	22.56 $\pm$ 1.10
Control mediator	5.62 $\pm$ 0.72*	4.48 $\pm$ 1.18
Insulin mediator	8.12 $\pm$ 0.44*†	3.62 $\pm$ 0.20*
PI-PLC mediator	8.59 $\pm$ 0.84*†	3.38 $\pm$ 0.84

the release from the membranes of a material that activated PDH of rat adipocyte mitochondria *in vitro*, consistent with it being a mediator of insulin action on this enzyme (Table 1). Material extracted from the supernatant obtained from membranes treated with PI-PLC activated PDH to a similar extent to that extracted from insulin-treated membranes, i.e. approx. 2-fold over that extracted from untreated membranes after subtraction of basal activity, and almost 2.5-fold over basal enzyme activity, confirming our previous study [16]. Each fraction was then treated with sodium nitrite at pH 3.5, a treatment which has been shown to cause deamination of glycosidically linked glucosamine within active mediator fractions [3,6,7,12] and PI-anchored proteins [23]. Buffer fractions taken through this protocol had no significant effect on either basal activity or the ability of Ca²⁺ and Mg²⁺ to stimulate PDH, indicative that ultrafiltration effectively removed the inhibitory potential of the acid treatment. By subtracting basal activity from the data given in Table 1, it is apparent that approx. 70% inactivation of the PDH stimulatory activity generated by the PI-PLC and insulin treatment was achieved by treatment of the mediator preparations with sodium nitrite at pH 3.5. This criterion has been used in past mediator studies to indicate the presence of glycosidically linked glucosamine within an active mediator preparation [3,6,7,12]. Sodium nitrite has also been shown to cause deamination of glycosidically linked glucosamine within PI-anchored proteins (for review, see [21]). Thus these data suggest that both insulin and PI-PLC stimulate generation of a similar material from rat adipocyte plasma membranes, potentially containing glucosamine and inositol, that activates adipocyte mitochondrial PDH.

Table 2. Effect of insulin on 2-deoxyglucose uptake before and after primary culture of rat adipocytes

Insulin stimulation of 2-deoxyglucose transport before and after primary culture of rat adipocytes. Cells were prepared and the effect of 7 nM-insulin on 2-deoxyglucose transport was determined either immediately or after overnight culture, as detailed in the Materials and methods section. Results are taken from five separate experiments, in which each condition was tested in three independent cell incubations within the same experiment. Significant differences compared with the non-insulin-stimulated condition are indicated by *.

Incubation condition	2-Deoxyglucose uptake (pmol/min per 10 ⁶ cells)	
	Preculture	Postculture
– Insulin	132 ± 13	245 ± 23
+ Insulin	494 ± 75*	458 ± 54*

Further studies examined the nature of the PI-gly that generated this material in adipocytes more directly by employing a series of labelling experiments.

Insulin responsiveness of adipocyte primary cultures

Maintenance of adipocytes in culture for up to 24 h as described in the Materials and methods section resulted in no significant decrease in cell number (results not shown). Moreover these cells maintained insulin responsiveness after overnight culture, as reflected by insulin stimulation of glucose transport (Table 2). Treatment of freshly prepared cells with insulin for 30 min caused an approx. 4-fold stimulation of 2-deoxyglucose uptake. Treatment of cells that had previously been maintained in culture for 24 h with insulin elicited a 2-fold stimulation of 2-deoxyglucose uptake. The decreased stimulation of glucose uptake by insulin was a consequence of increased basal transport activity. This increased basal activity was not due to increased cellular adenosine levels, since preincubation of cells with adenosine deaminase failed to lower the basal transport activity of the overnight-cultured cells significantly (results not shown). Some incubation medium modifications (such as culture in Dulbecco's minimum essential medium containing 5 mM-glucose) did not appreciably alter the percentage stimulation of glucose transport by insulin, whereas others (such as decreasing the fetal bovine serum concentration or BSA concentration) led to decreased insulin responsiveness. Therefore the conditions used to generate these data (Table 2) were used throughout the study.

Isolation of PI-gly from adipocytes

Adipocytes prelabelled to equilibrium with [³H]glucosamine (overnight) were extracted for glucosyl-lipids as described in the Materials and methods section. In contrast with studies in other cell types [3,4,6,7] there was marked incorporation of the label into the neutral lipid fraction. Neutral lipid incorporation of [³H]glucosamine label accounted for approx. 99% of the label associated with lipids, indicating significant turnover of the glucosamine label via the glycolytic pathway. Interestingly, the incorporation was almost exclusively into triacylglycerol and very little incorporation into diacylglycerol was detected by t.l.c. of the neutral lipid fraction in light petroleum (b.p. 60–80°C)/diethyl ether/acetic acid (70:30:1, by vol.) (results not shown). This neutral lipid fraction was discarded before analysis of membrane phospholipid and glucosyl phospholipid by t.l.c.

Fig. 1 shows the t.l.c. profile of [³H]glucosamine-labelled

Fig. 1. Incorporation of [³H]glucosamine into cell lipids and purification of PI-gly

In (a), adipocytes cultured overnight with [³H]glucosamine as described in the Materials and methods section were separated by t.l.c. in chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, by vol.). After t.l.c., peaks of radioactivity were located by autoradiography; 1 cm fractions were taken and associated radioactivity determined. Peaks of radioactivity are identified as I–V. In (b), peak I from the previous chromatogram was eluted and further purified by t.l.c. in chloroform/methanol/ammonia/water (45:45:3.5:10, by vol.). After t.l.c. 1 cm fractions were scraped off and radioactivity was determined. The migration patterns of phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylethanolamine (PE) markers were determined.

adipocyte extracts after removal of the neutral lipid fraction. As shown in Fig. 1(a), a major peak was detected near the origin (peak I) in the acidic solvent system, consistent with the position of PI-gly in other cells. In addition, similar to the report on the isolation of PI-gly from H35 hepatoma cells [6], several other peaks were obtained. Of the material applied to the t.l.c. run in the acidic solvent system (Fig. 1a), excluding the neutral lipid fraction [the remainder of which ran with the solvent front (peak V)], 27% of the label ran with phosphatidylcholine (peak III), 10% ran with phosphatidylinositol (peak II) and 32% ran as PI-gly (peak I). The finding that a high proportion of the [³H]glucosamine label was incorporated into phospholipids other than PI-gly indicates significant turnover of the glucosamine label through the glycolytic pathway, consistent with the above finding that the majority of the label was incorporated into the neutral triacylglycerol lipid fraction. The PI-gly region (peak I) was purified further, after elution, by t.l.c. in a second basic solvent system (Fig. 1b). The major peak separated between phosphatidylinositol and phosphatidic acid standards, consistent with previous reports of PI-gly separation from other cell types.

Fig. 2. Determination of distribution of label in [³H]glucosamine-labelled PI-gly after acid hydrolysis

Samples of PI-gly band were dried under N₂ and resuspended in 0.2 ml of 4 M-HCl. Following incubation at 110 °C for 5 h, samples were concentrated by lyophilization, applied to a t.l.c. plate and developed twice in pyridine/ethyl acetate/acetic acid/water (5:5:1:3, by vol.). Fractions corresponding to the bar sizes were taken and radioactivity was determined after identification of the markers choline (Cho), inositol (Ins), glucosamine (GluNH₂), galactose (Gal), glucose (Glu) and glycerol (Glyc) by autoradiography. Results are pooled for four separate preparations of PI-gly which were analysed independently.

This second t.l.c. separation was necessary to separate the PI-gly from phosphatidylinositol; the latter phospholipid represents a potential contaminant after t.l.c. in the first solvent. The first t.l.c. separation was necessary to separate phosphatidylcholine from the PI-gly, as it runs very close to the PI-gly in the second solvent. The present data contrast with studies in other cell types in which the bulk of the label associates with the PI-gly band [3,4,6,7]. Thus, for fat cell studies, quantification of PI-gly with [³H]glucosamine is not possible and can only be used as a marker together with other indicators of PI-gly changes. Clearly, interpretation of PI-gly changes in fat cells requires that the PI-gly band is separated from the major phospholipids, especially phosphatidylcholine.

Confirmation that the PI-gly purified through the two solvent systems was labelled with authentic [³H]glucosamine, and not only with label cycled through the glycolytic pathway, was obtained by acid hydrolysis of several preparations of the PI-gly band obtained after t.l.c. in solvent II and t.l.c. of the component sugars (Fig. 2). Analysis of the PI-gly band in this way showed that 44% of the label ran on t.l.c., with an *R_f* similar to that of a glucosamine standard, whereas 24% ran near the top of the t.l.c. plate where glycerol and *N*-acetylglucosamine would be expected to run, and 7% migrated with an *R_f* similar to that of an inositol standard. These findings indicate that authentic [³H]glucosamine labelling of the PI-gly band accounts for a significant proportion of associated label after equilibrium labelling of the cells with [³H]glucosamine. These findings further emphasize the need for care in interpretation of data obtained using [³H]glucosamine-labelled adipocytes.

The clear demonstration of PI-gly within the PI-gly band relies in part on its sensitivity to PI-PLC and sodium nitrite. Sodium nitrite treatment or nitrous acid deamination, as indicated earlier, cleaves glucosamine with free amino groups from glycosidic linkage (in the case of PI-gly, from a glycosidic linkage to inositol). PI-PLC cleaves the phosphodiester linkage of

Table 3. Effects on enzyme activity of a PI-PLC digest of the PI-gly band

A PI-gly sample extracted from pooled adipocytes from 10 rats and purified by t.l.c. in the acidic and basic solvent systems (Fig. 1) was treated with *B. thuringiensis* PI-PLC for 60 min at 37 °C, as described in the Materials and methods section in parallel with a control fraction (Control) taken from lanes to which no sample had been applied. The PI-gly digest and control fractions were then separated from unreacted lipid and PI-PLC by chromatography on Baker Sep-paks and ultrafiltration through YM2 membranes (Amicon, 2000-*M_w* cut-off). The ultrafiltered eluates were collected, lyophilized and resuspended to 150 µl for assay of the effects on PDH activity of rat adipocyte mitochondria (at a 1:4 dilution) and for effects on low-*K_m* cyclic AMP phosphodiesterase (at a 1:10 dilution) in three separate incubations. Basal enzyme activities are given and compared with the PI-PLC digests of the control fraction and of the PI-gly band. Total PDH activity was 11.68 ± 0.25 nmol/min per mg of protein. The statistical significance of PI-gly digest stimulation of enzyme activity versus that of control extracts is indicated by *.

Enzyme	Basal	Control	PI-gly
PDH (nmol/min/per mg)	2.92 ± 0.39	3.58 ± 0.31	4.45 ± 0.17*
Low- <i>K_m</i> cyclic AMP phosphodiesterase (pmol/min per mg)	34.36 ± 1.49	32.16 ± 1.96	39.02 ± 0.74*

phosphatidylinositol and to varying extents the PI-glucosamine-anchored proteins (for review, see [23]) and the insulin-sensitive PI-gly from various cells types [2-4,6-9]. Both treatments were applied to the PI-gly band. Each resulted in loss of glucosamine associated with this band, albeit to different extents. Treatment of the eluted PI-gly band with PI-PLC as detailed in the Materials and methods section resulted in a 29 ± 6% (*n* = 3) loss of glucosamine label associated with the band, and this was recovered in the aqueous phase on organic extraction of the samples. Nitrous acid treatment resulted in a loss of 40 ± 6% (*n* = 3) of the label associated with the PI-gly band, indicated by rechromatography of the band in the second basic solvent system. The finding that the PI-gly band was sensitive to both treatments provides strong supportive evidence that PI-gly is contained within this band.

Further supportive evidence that the PI-gly is contained within the PI-gly band in the adipocyte is the demonstration that PI-PLC digestion of the band generates a potential mediator for insulin activation of some of its intracellular actions. Therefore, after purification, the aqueous product of PI-PLC digestion of a sample of the PI-gly band was assayed for its effects *in vitro* on two insulin-sensitive enzymes, rat adipocyte mitochondrial PDH and microsomal low-*K_m* cyclic AMP phosphodiesterase (Table 3). PDH activity measured after incubation of adipocyte mitochondria with the PI-PLC digest of PI-gly was 52% higher than basal activity. Control extracts assayed in parallel had no significant effect on the enzyme. The PI-PLC digest of the PI-gly band also significantly stimulated the low-*K_m* cyclic AMP phosphodiesterase activity of an adipocyte microsomal fraction, whereas the control extracts had no effect on the enzyme.

Effect of insulin on PI-gly breakdown

A condition for identifying the presence of PI-gly within the PI-gly band is sensitivity to insulin. Cells were incubated with [³H]glucosamine for 16-24 h as described above to achieve steady-state PI-gly labelling. Cells were then incubated in serum-free medium for 30 min, after which time they were stimulated with 7 nM-insulin and the effects on [³H]glucosamine label

Fig. 3. Time course of the effect of insulin on PI-gly turnover

Cells were prelabelled with [^3H]glucosamine as described in the Materials and methods section and taken into fresh serum and [^3H]glucosamine-free medium for the final 30 min. Cell stimulations with 7 nM-insulin were carried out for the time course indicated. Incubations were staggered so that each cell suspension was incubated for a similar length of time. Results represent the mean of six or more experiments, and within each experiment each point was determined in three independent cell incubations. Significance differences compared with PI-gly from cells not stimulated with insulin are indicated by *.

associated with the PI-gly band were determined (Fig. 3). Insulin caused a rapid decrease of 29 % in the amount of label associated with the PI-gly band at 1 min, which returned towards basal by 5 min. The time course of PI-gly turnover was variable from experiment to experiment. In some experiments the label associated with the PI-gly band was still significantly less than basal at 5 min, whereas in others the label associated with the band was similar to basal by this time. The time at which insulin stimulated maximal loss of label associated with the PI-gly band also varied. In a separate series of experiments (results not shown), it was found that maximal loss of label associated with the PI-gly band on insulin treatment varied in its time of occurrence from 30 s to 2 min. The rapid turnover of label associated with the PI-gly band is consistent with rapid break-

Fig. 4. Dose-response to insulin of PI-gly breakdown

Adipocytes prelabelled with [^3H]glucosamine were incubated with insulin at the concentrations indicated in the legend to Fig. 3, except that incubations were carried out for 1 min. Results given are the means $\pm$ S.E.M. of four or more experiments, and within each experiment values for each point were determined in three independent cell incubations. Significant differences compared with PI-gly extracted from cells incubated in the absence of insulin are indicated by *.

down of the molecule and resynthesis, as would be expected for a compound with a potential second messenger precursor role.

Concentration-response of PI-gly to insulin

The concentration-response to insulin of PI-gly breakdown was determined, since the concentration used for the time course studies could be considered to act potentially via stimulation of insulin-like growth factor-I receptors (Fig. 4). Insulin caused a loss of label associated with the PI-gly band that was significant and maximal (25 %) at 0.7 nM-insulin. The dose at which significant stimulation of breakdown was detected varied from experiment to experiment (0.07–0.7 nM), as did the dose at which maximal breakdown was achieved (0.7–7 nM). This variability probably reflects slight differences in cell preparation or culture, which were unavoidable. The concentration-response curve of PI-gly breakdown caused by insulin (Fig. 4) is similar to the concentration-response curve obtained from insulin stimulation of PDH that we reported in the past [21,24], and indicates that this action of insulin occurs via activation of its own receptor.

Characterization of the adipocyte PI-gly

Previous labelling studies have shown differences in the component fatty acids forming the membrane-anchoring domains in the BC3H-1 myocyte and H35 hepatoma cell lines [3,6]. The nature of the fatty acids present in the adipocyte PI-gly were investigated together with the associated sugars in a series of cell labelling studies (Table 4). Since the isolation studies indicated potential for contamination of the PI-gly with label cycled via the glycolytic pathway when using glucosamine as the label, insulin stimulation of PI-gly breakdown was used as a criterion for association of the labels with PI-gly. Further, label present in PI-gly after overnight labelling of adipocytes with the sugars galactose and inositol was analysed by t.l.c. in a similar manner to that in glucosamine-labelled cells (see Fig. 2) after acid hydrolysis of the appropriate eluted band in order to determine the potential contribution of sugar cycled via the glycolytic pathway to the band. Table 4 shows that insulin treatment of adipocytes resulted in 35 %, 36 %, 30 % and 40 % losses of label associated with the PI-gly band with glucosamine, galactose, inositol and myristate labelling respectively of cells.

Table 4. Effect of insulin on association of labelled precursors with the PI-gly band

Adipocytes were prelabelled with the indicated PI-gly precursors: [^3H]glucosamine (12.5 $\mu\text{Ci/ml}$), [^3H]galactose (12.5 $\mu\text{Ci/ml}$), [^3H]inositol (5 $\mu\text{Ci/ml}$), [^3H]myristate (12.5 $\mu\text{Ci/ml}$) or [^3H]palmitate (12.5 $\mu\text{Ci/ml}$). Cell incubations with 7 nM-insulin and extraction of PI-gly were carried out as in the legend to Fig. 3. Values for each precursor represent the mean $\pm$ S.E.M. of the percentage loss of label associated with the PI-gly band after insulin stimulation for the number of experiments given in parentheses. The label associated with the PI-gly band was determined from three independent cell incubations carried out for 1 min in the absence or presence of 7 nM-insulin for each experiment. The significance of associated PI-gly label loss after insulin stimulation indicated by *.

Labelled precursor	Insulin effect on precursor label associated with PI-gly (% of basal)
[^3H]Glucosamine	65.5 $\pm$ 3.4 (16)*
[^3H]Galactose	63.7 $\pm$ 5.3 (7)*
[^3H]Inositol	70.2 $\pm$ 1.1 (5)*
[^3H]Myristate	59.5 $\pm$ 3.6 (9)*
[^3H]Palmitate	85.7 $\pm$ 9.6 (7)

Table 5. Lipid labelling profile of adipocytes after overnight incubation with [³H]inositol, [³H]glucosamine or [³H]galactose

The labelling profiles of portions of cell extracts prepared in a similar manner to those in Table 4 were analysed by silica extraction and t.l.c. as in Fig. 1. Results are from representative t.l.c. profiles and are expressed as percentages of total cell-associated lipid for inositol- (total cell-associated lipid = 14472 d.p.m.), glucosamine- (total cell-associated lipid = 511378 d.p.m.) and galactose- (total cell-associated lipid = 994035 d.p.m.) labelled cells. Neutral lipid was that fraction that did not bind to silica. PI-gly was the band isolated by sequential t.l.c. in solvent systems I and II. Phosphatidylinositol (PI), lysophosphatidylinositol (lyso-PI) and phosphatidylcholine (PC) were isolated in solvent system I.

Lipid fraction	Label (% of cell-associated lipid)		
	[³ H]Inositol	[³ H]Glucosamine	[³ H]Galactose
Neutral lipid	16.38	96.65	95.57
PI-gly	1.46	0.18	0.41
PI plus lyso-PI	83.37	0.11	0.27
PC	0.44	0.20	0.56

Although palmitate labelling of the band was detected, it was not consistently affected by insulin. The lack of consistent effect of insulin on palmitate labelling of the PI-gly band suggests that the band interpreted as containing the insulin-sensitive PI-gly is not homogeneous and includes other compounds that incorporate palmitate. However, the finding of loss of associated palmitate label in some experiments makes it difficult to exclude palmitate as a component of PI-gly structure in the adipocyte.

Analysis of the labelling profiles for [³H]galactose- and [³H]inositol-labelled cells (Table 5), like that for [³H]glucosamine-labelled cells, indicated significant turnover of the labels through the glycolytic pathway, albeit to different extents. The majority of [³H]galactose label, 96% of the total cellular lipid-associated label, as for the [³H]glucosamine label, appeared in the neutral lipid fraction as triacylglycerol. [³H]Inositol was turned over to a much lesser extent. Only 24% of the total cell-associated label appeared in the neutral lipid fraction. Analysis of the sugar components of the PI-gly band from [³H]galactose- and [³H]inositol-labelled cells, like the analysis of the sugar components of [³H]glucosamine-labelled cells (Fig. 2), also indicated turnover of the labels though the glycolytic pathway (results not shown). The t.l.c. profile of component sugars for [³H]galactose-labelled cells showed a pattern similar to that for [³H]glucosamine-labelled cells. Approx. 39% of the [³H]galactose label associated with the PI-gly band migrated with an R_f similar to that of a galactose standard. Over 80% of the [³H]inositol label associated with the PI-gly band migrated with an R_f similar to that of an inositol standard after acid hydrolysis of the band.

Effect of insulin on breakdown of PI-gly in an animal model of diabetes

If the PI-gly is a potential precursor for a mediator of insulin action, then an impairment in its breakdown or amount may be a potential site of post-receptor insulin resistance in diabetes. Initial assessment of this possibility was made using adipocytes extracted from streptozotocin-diabetic and non-diabetic rats and cultured in the same manner. Table 6 shows that adipocytes from streptozotocin-diabetic rats remain insulin-unresponsive after overnight culture, with respect both to insulin stimulation of glucose utilization and to insulin activation of PDH. Insulin at 7 nM stimulated glucose utilization in adipocytes from age-matched control rats after overnight culture by approx. 2-fold and PDH activity by approx. 3-fold.

Table 6. Insulin responsiveness of adipocytes from control and streptozotocin-diabetic rats after overnight culture

Adipocytes from control or streptozotocin-diabetic rats were prepared and cultured overnight. Cells were transferred into fresh serum-free medium containing 3% BSA and incubated in the absence or presence of 7 nM-insulin for 10 min. [⁵⁻³H]Glucose was then added for the next 30 min, after which the medium was removed for determination of glucose utilized. A 'cocktail' for determination of PDH activity was added to the cell pellet. Glucose utilization and 'active' PDH of the cells were determined as described in the Materials and methods section. Total cell PDH activity did not alter significantly with insulin treatment and was 4.97 ± 0.58 nmol/min per 10^6 cells (control) and 4.73 ± 0.62 nmol/min per 10^6 cells (diabetic). Values were pooled from three or more experiments in which three independent cell incubations were performed within each experiment. * Denotes significant difference from non-insulin-treated samples.

Assay	Control		Diabetic	
	- Insulin	+ Insulin	- Insulin	+ Insulin
Glucose utilization (pmol/min per 10^6 cells)	506 ± 67	$1058 \pm 12^*$	263 ± 4	182 ± 27
PDH (pmol/min per 10^6 cells)	265 ± 29	$813 \pm 100^*$	297 ± 32	268 ± 46

The effect of insulin on breakdown of the PI-gly band was investigated after overnight labelling of cells with [³H]glucosamine. Insulin at 7 nM caused a significant loss of $46 \pm 3\%$ ($n = 6$) in the label associated with the PI-gly of control adipocytes, consistent with the findings in Figs. 3 and 4. It caused only a $13 \pm 6\%$ ($n = 6$) loss of label associated with the PI-gly of adipocytes extracted from streptozotocin-diabetic rats. This was not significantly different from label recovered in the PI-gly band of adipocytes from streptozotocin-diabetic rats not treated with insulin. There was also less [³H]glucosamine label incorporated into the PI-gly of cells from streptozotocin-diabetic rats [809 ± 74 d.p.m./ 10^6 cells ($n = 6$)] than into adipocytes from control rats [1333 ± 120 d.p.m./ 10^6 cells ($n = 6$)]. This does not necessarily indicate a decreased amount of the PI-gly in adipocytes from these animals, but may merely reflect a lower specific activity of labelling.

DISCUSSION

In the present report we provide data obtained with a major insulin target cell, the adipocyte, to support the presence of a PI-gly that is turned over on stimulation of cells with insulin. PI-PLC treatment of adipocyte plasma membranes, like insulin treatment, stimulated release from the membranes of a material that activated PDH, confirming our recent report [16] and indicating the likelihood that the PDH activator is anchored to the membrane by phosphatidylinositol, as in other cell types [8,12]. The activity of this component released from membranes was sensitive to nitrous acid treatment, which has been used in the past to suggest the presence of glucosamine within the PI-gly structure [6-8,12]. More direct analysis of adipocyte PI-gly structure in a series of cell-labelling studies demonstrated an insulin-sensitive band labelled with [³H]glucosamine, [³H]inositol, [³H]galactose and [³H]myristate that ran on t.l.c. with an R_f similar to that of PI-gly from other cell types [6-8]. This band was sensitive to PI-PLC and to nitrous acid treatment, again consistent with it being a phospholipid containing inositol and glucosamine.

Interpretation of the labelling studies was complicated by cycling of the added labels through the glycolytic pathway. Adipocytes labelled to equilibrium with [^3H]glucosamine or [^3H]galactose showed marked turnover of added label, in contrast with previous studies using splenic T cells [7], H35 hepatoma cells [6] or BC3H-1 myocytes [3–5]. Most of this label was recovered in triacylglycerol. Label was also recovered in the major phospholipid species, as well as in the band we tentatively identified as PI-gly. Of label recovered in phospholipid, that in the PI-gly band accounted for a major fraction, even though PI-gly represents only a minor proportion of the total cellular phospholipid. This indicates that the PI-gly was labelled predominantly with authentic [^3H]glucosamine and [^3H]galactose and not only with label cycled through the glycolytic pathway. This contention was confirmed by sugar analysis of acid hydrolysates of the PI-gly band. Studies in other cell types also show that the PI-gly contains glucosamine and galactose [3–12,25]. [^3H]inositol was not turned over to the same extent as [^3H]glucosamine and [^3H]galactose. Of the organic extracted [^3H]inositol label, 1.5 % was found in the PI-gly band, which was marginally higher than that reported for the PI-gly from splenic T cells [7]. This is consistent with the possibility of slight contamination of the band with lysophosphatidylinositol, which runs close to the PI-gly in the purification scheme employed in the present study. PI-PLC sensitivity and nitrous acid deamination of the PI-gly band further supported the presence of glucosamine and inositol within this band, although each treatment only resulted in partial release of the label. Partial resistance to PI-PLC hydrolysis has been observed in previous PI-gly studies [6,7] as well as in studies with several PI-gly-linked proteins (for review see [23]). This partial resistance to PI-PLC may thus be a property of the PI-gly, or it may indicate the additional potential contamination of the band with other compounds.

The effect of insulin in stimulating breakdown of the PI-gly band (Figs. 3 and 4; Table 4) substantiates the contention that the insulin-sensitive PI-gly is contained within the PI-gly band. Insulin caused a rapid transient stimulation of PI-gly breakdown, with maximal effects being achieved between 30 s and 2 min, consistent with the proposed role of the molecule in cellular signalling [2,3,6,11,26]. The insulin concentration–response curve was also similar to that for insulin stimulation of PDH activation that we have previously reported [22,24], and demonstrates that the action of insulin to stimulate breakdown of this molecule occurs via activation of its own receptor. This is important, since it has become increasingly clear that this PI-gly or a related molecule in other cell types is sensitive to breakdown by serum [27], as well as by some growth factors, including nerve growth factor [28], epidermal growth factor and insulin-like growth factor-I [29].

The insulin-mimetic effect of PI-PLC digests of the adipocyte PI-gly in the present study confirms previous studies in other cell types [6,7,11,13,14] and supports the idea that insulin stimulates a phospholipase that acts on the PI-gly. Our previous studies, and those of others, had already provided some circumstantial support for this contention in the adipocyte. PI-PLC or PLC incubation with adipocytes had been shown to mimic some actions of insulin, including insulin stimulation of PDH, generation of the mediator and breakdown of the PI-gly [16]. Another study provided evidence to support the presence of an insulin-sensitive phospholipase activity in cell extracts of adipocytes, although the phospholipid specificity of this activity was unclear [30]. One group demonstrated an effect of insulin [31], whereas another group found no effect of insulin on turnover of inositol phospholipid [15], but they did not directly assess the effects of insulin on turnover of the insulin-sensitive PI-gly. We

found no consistent effect of insulin on turnover of inositol phospholipids other than the PI-gly in the present study (results not shown).

The dependence of PI-gly breakdown on insulin receptor kinase activation, generally accepted to be the initiating step in signalling insulin action (see [1] for review), is uncertain, since our initial membrane studies and several other studies [12,16,32] have shown insulin to stimulate release of the PI-gly headgroup or an activator of PDH from plasma membranes incubated *in vitro* in the absence of ATP. These studies indicate the possibility that, in part, the action of insulin to stimulate PLC breakdown of PI-gly is receptor-kinase-independent. This does not preclude a major role of the receptor kinase in mediating PI-gly breakdown. Indeed, Larner and co-workers [32] showed that incubation of liver membranes with ATP markedly augmented release of an activator of PDH by insulin, consistent with a major role for the kinase. Furthermore, a recent study using cells transfected with normal or kinase-deficient insulin receptors demonstrated a correlation between expression of receptors having functional kinase activity and PI-gly breakdown [33]. Thus it is likely that the receptor kinase plays a pivotal role in mediating PI-gly breakdown, although a portion of the activity appears to be kinase-independent.

Further correlative support for a role of the PI-gly in insulin action was obtained from investigation of adipocytes from streptozotocin-diabetic rats. In this model of insulin deficiency, impaired insulin action in glucose-utilizing tissues, including the adipocyte, is apparent. It is likely that this is due in part to impaired receptor kinase activity [34]. Consistent with the impaired receptor activity in this model was our finding of an impairment in insulin stimulation of breakdown of the PI-gly. These findings complement a recent study [35] that demonstrated a similar impairment in PI-gly breakdown in hepatocytes from glucocorticoid-treated rats (a model of type II diabetes). The development of antibodies to the PI-gly [36] may aid in the determination of possible defects in the absolute level of PI-gly in tissues.

In conclusion, the present study confirms reports [2–11] supporting the concept that insulin stimulates turnover of a PI-gly containing glucosamine, galactose, inositol and myristate that plays a significant role in insulin signal transduction. It further demonstrates that defects in breakdown of this compound are a part of the impaired insulin action in at least one model of diabetes, i.e. the streptozotocin-diabetic rat. Further work on the chemical nature of this compound and the mechanism of its turnover should lead to a better understanding of insulin action.

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