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Different phosphorylated forms of an insulin-sensitive glycosylphosphatidylinositol from rat hepatocytes

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Labeling with [^{3}H]galactose was employed to isolate a glycosylphosphatidylinositol from rat hepatocytes which might be involved in the action of insulin. The polar head group of this glycosylphosphatidylinositol was generated by phosphodiesterase hydrolysis with a phosphatidylinositol-specific phospholipase C from *Bacillus cereus*. By Dowex AG1 × 8 chromatography the polar head group could be separated into three radioactive peaks eluting at 100 mM (peak I), 200 mM (peak II) and 500 mM (peak III) ammonium formate, respectively. Peak III was the most active as an inhibitor of the cAMP-dependent protein kinase. Treatment of peak III with alkaline phosphatase markedly reduced its activity on cAMP-dependent protein kinase. When peaks I, II or III were treated with alkaline phosphatase and analyzed again by Dowex AG1 × 8 chromatography, the radioactivity eluted with the aqueous fraction. The above results indicate that the polar head group of the insulin-sensitive glycosylphosphatidylinositol from rat hepatocytes exists in three different phosphorylated forms and that the biological activity of this molecule depends on its phosphorylation state.

Insulin action; Glycosylphosphatidylinositol; (Rat hepatocyte)

1. INTRODUCTION

A glycosylphosphatidylinositol (glycosyl-PI) has been implicated in the mechanism of action of insulin [1–4]. Insulin was shown to stimulate the hydrolysis of a glycosyl-PI in a variety of cells including BC3H1 myocytes [2,5], H35 hepatoma cells [3] and rat hepatocytes [6]. The polar head group of this glycosyl-PI has been reported to act similarly to insulin when added to intact cells. Thus, this head group has insulin-like effects on phospholipid methyltransferase [7], lipolysis [8], lipogenesis [9] and pyruvate dehydrogenase [10] in adipocytes, and glycogen phosphorylase, pyruvate kinase and cAMP levels in hepatocytes [11]. Furthermore, this head group was shown to copy the insulin-directed effects on phosphorylation and dephosphorylation of target proteins of the hormone [4]. In rat liver, this head group that modulates protein phosphorylation consists of inositol phosphate linked to non-*N*-acetylated glucosamine and four residues of galactose with an average of two additional phosphates that are not associated to the inositol moiety [12]. We report here the separation and biological activity of three different forms of the polar head group of glycosyl-PI isolated from rat hepatocytes.

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2. MATERIALS AND METHODS

2.1. *Materials*

D-[G-^{3}H]Galactose (3.9 Ci/mmol), [^{32}P]phosphate (10 mCi/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from New England Nuclear; insulin from Lilly; histone IIA and lipid standards from Sigma; bovine kidney alkaline phosphatase from Millipore; silica gel G plates for TLC from Merck; collagenase from Boehringer Mannheim and Dowex AG1 × 8 from BioRad.

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2.2. *Cell labeling, isolation of glycosyl-PI and generation of the head group*

Hepatocytes from normally fed Wistar rats (200 g) were prepared by the collagenase method described in [13]. Isolated cells (about 5×10^6 cells/ml) were incubated for 90 min in the presence of [^{3}H]galactose (20 μCi/ml) or 0.1 mM [^{32}P]phosphate (0.2 mCi/ml). At the end of incubation, cells were lysed by addition of 1 ml ice-cold 10% trichloroacetic acid and, after standing for 10 min at 4°C, phospholipids were extracted and glycosyl-PI isolated as in [12]. To generate the polar head group, purified glycosyl-PI was incubated with phosphatidylinositol-specific phospholipase C (PI-PlC) from *Bacillus cereus* (25 mM Hepes-NaOH, pH 7.4, containing 1.6 μg enzyme, for 2 h at 37°C) in a final volume of 0.2 ml. At the end of incubation, lipids were extracted according to [12] and radioactivity associated with aqueous and organic phases determined by scintillation counting.

2.3. *Measurement of cAMP-dependent protein kinase activity*

Protein kinase activity was determined as described [14] using the catalytic subunit of cAMP-dependent protein kinase purified from bovine heart and histone IIA as substrate. Reactions were carried out for 5 min at 37°C. After incubation, the reaction was stopped by precipitation with trichloroacetic acid and incorporation of [^{32}P]phosphate into histone determined as in [14].

2.4. *Purification of PI-PlC from* B. cereus

PI-PlC from *B. cereus T* was purified using the procedure of Kominami et al. [15]. The specific activity of the purified enzyme was 200 U/mg protein. As described in [15], the purified enzyme exerted phospholipase C activity specific for phosphatidylinositol but not for other phospholipids, including phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine. Phospholipase C activity was determined according to Low [16]. Organic phosphate and protein were respectively determined as described by Barlett [17] and Bradford [18].

2.5. *Determination of non-*N*-acetylated glucosamine*

Non-*N*-acetylated glucosamine was determined in the sample of polar head group by reacting its non-*N*-acetylated amino group with fluorescamine [19] as described by Nakai et al. [20] with some modifications. The sample of head group and known concentrations of glucosamine were dissolved in 0.1 ml distilled water. After addition of 1.4 ml of 0.2 M sodium borate (pH 9.0), 0.5 ml fluorescamine (0.3 mg/ml) was added with vigorous shaking. Fluorescence detection was then measured with a Kontron spectrofluorometer (excitation 390 nm, emission 475 nm).

3. RESULTS AND DISCUSSION

Rat hepatocytes were labeled with [^{3}H]galactose for 90 min and after this period lipids were extracted and glycosyl-PI purified by TLC (fig.1). This glycosyl-PI comigrated with the insulin-sensitive form purified from H35 hepatoma cells [3] and could also be labeled with [^{32}P]phosphate or [^{3}H]glucosamine (not shown). The turnover of

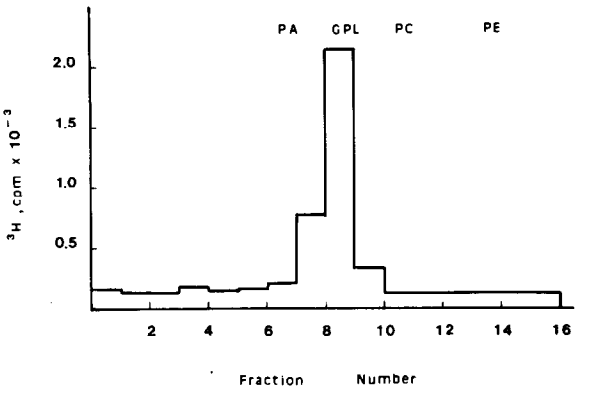

Fig.1. Purification of [^{3}H]galactose-labeled glycosyl-PI from rat hepatocytes. Rat hepatocytes were labeled with [^{3}H]galactose for 90 min as described in the text. After this period, cells were lysed with trichloroacetic acid and glycosyl-PI extracted and purified by sequential TLC as described in section 2. After chromatography, 1-cm regions were scraped off and the radioactivity associated with each fraction determined by scintillation counting. PA, phosphatidic acid; GPL, glycosyl-PI purified from rat liver membranes as in [12]; PC, phosphatidylcholine; PE, phosphatidylethanolamine. Migration positions of the known phospholipids were determined with markers.

glycosyl-PI was rapidly stimulated in response to insulin. Within 1 min insulin (100 μU/ml) increased (177 ± 4%, $n = 4$) the content in radioactivity of this glycosyl-PI. This effect of insulin was independent of whether glycosyl-PI was labeled with [^{3}H]galactose or [^{32}P]phosphate. The [^{3}H]-galactose-labeled polar head group was released from its diacylglycerol moiety by treatment with *B. cereus* PI-PlC for 2 h at 37°C. About 70% of the radioactivity was recovered in the aqueous fraction irrespective of whether the glycosyl-PI sample was labeled with [^{3}H]galactose or [^{32}P]phosphate. As previously reported, the head group released by PI-PlC treatment inhibited cAMP-dependent protein kinase activity [14] and copied the antilipolytic effect of insulin in rat adipocytes [8]. The ratio of organic phosphate/free amino groups (the latter as a measurement of the amount of non-*N*-acetylated glucosamine) in the sample of polar head group generated by PI-PlC treatment was 2.9. This value agrees with our previous observation (based on results obtained by radiolabeling H35 hepatoma cells with [^{32}P]phosphate), indicating that the insulin-sensitive glycosyl-PI contains an average of three phosphate groups [12].

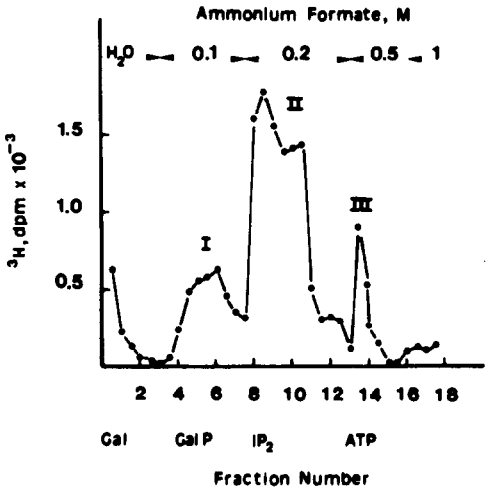

Fig.2. Analysis by Dowex AG1 × 8 chromatography of the aqueous products after treatment of [^{3}H]galactose-labeled glycosyl-PI with PI-PlC. A sample of [^{3}H]galactose-labeled glycosyl-PI was treated with PI-PlC (see text). After hydrolysis, the aqueous fraction was applied to a Dowex AG1 × 8 column (2 ml bed volume) and, after washing the column with water, eluted with various concentrations of ammonium formate. After elution, the amount of radioactivity in each fraction (2 ml) was determined by counting an aliquot. Gal, galactose; GalP, galactose 1-phosphate; IP_2, inositol 1,4-bisphosphate; ATP, adenosine trisphosphate. Migration positions of the known substances were determined with markers.

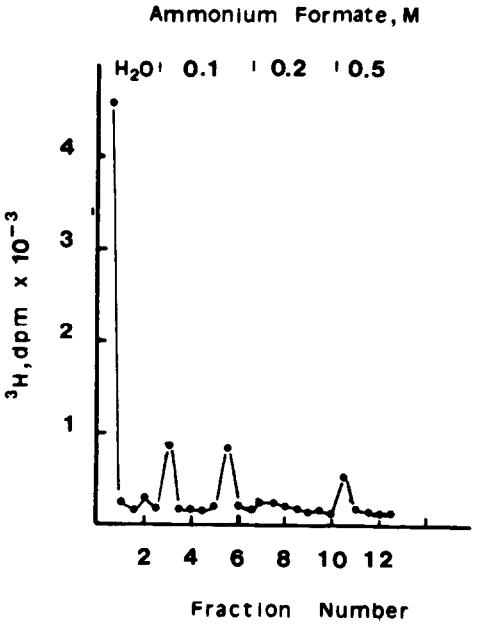

Fig.3. Analysis by Dowex AG1 × 8 chromatography of peak III from fig.2 after treatment with alkaline phosphatase. Peak III from fig.2 was lyophilized, treated with alkaline phosphatase (25 mM Tris-HCl, pH 8.3, containing 5 U/ml, for 2 h at 37°C), applied to a Dowex AG1 × 8 column (2 ml bed volume), and after washing with water, eluted with various concentrations of ammonium formate. After elution, the amount of radioactivity in each fraction (2 ml) was determined by counting an aliquot.

After hydrolysis, the polar head group was purified by passage through a column of Dowex AG1 × 8 equilibrated in water. The column was eluted with three different concentrations of ammonium formate (100, 200 and 500 mM, respectively) and a sample of each fraction was taken for scintillation counting. As shown in fig.2, with this procedure the polar head group was resolved into three different peaks. The same chromatographic profile was obtained by using a polar head group generated from a sample of glycosyl-PI labeled with [^{32}P]phosphate (not shown). When peak III from fig.2 was incubated with alkaline phosphatase and analyzed again by passage through a column of Dowex AG1 × 8 equilibrated in water, most of the radioactivity eluted with the aqueous fraction (fig.3). Peaks I and II in fig.2 were also sensitive to alkaline phosphatase treatment (not shown). These results indicate that elution of peaks in the Dowex column was determined by the number of phosphates and that these phosphates formed monoester bonds. The interpretation of these results is that insulin-sensitive glycosyl-PI exists in three forms that differ in phosphate content. Whereas one phosphate binds the inositol residue of the head group to the diacylglycerol molecule, the position of the other phosphates remains to be determined. Presumably, peaks I–III of fig.2 might contain 2–4 phosphate groups, respectively. This would agree with the observed average value of three phosphate groups per residue of glucosamine. To exclude the possibility that heterogeneity in the number of phosphate residues is the result of unusual phosphatase activity during extraction of glycosyl-PI, a number of control experiments were carried out. Thus, we have not detected phosphatase activity in our preparation of PI-PlC. Furthermore, we have added purified ^{32}P-labeled glycosyl-PI to a trichloroacetic acid precipitate of cells which was extracted in the same way as the present glycosyl-PI. About 80–90% of the [^{32}P]phosphate was re-

covered by this procedure. Finally, we have extracted cells immediately after trichloroacetic acid precipitation and after standing at 4°C for 4 h. Under both conditions, the amount of [^{32}P]phosphate-labeled glycosyl-PI recovered was the same.

Table 1 details the biological activity on cAMP-dependent protein kinase of each peak in fig.3. This table shows that no activity was detected with samples eluting with water or 100 mM (peak I) ammonium formate. Fractions eluting with 200 mM (peak II) and 500 mM (peak III) ammonium formate were active. Since the amount of radioactivity recovered in the 200 mM fraction was greater than that for the 500 mM fraction, the results in table 1 indicate that peak III was the most active as an inhibitor of cAMP-dependent protein kinase. The effect of alkaline phosphatase treatment on the activity of the fraction eluting with 500 mM ammonium formate was also assayed. As shown in table 1, after alkaline phosphatase treatment, the activity of the fraction eluting with 500 mM ammonium formate was markedly reduced. Furthermore, no activity was observed in the aqueous fraction, where most of the radioactive sample eluted after alkaline phosphatase treatment (see table 1 and fig.3).

In conclusion, the present evidence suggests that rat liver membranes contain three forms of an insulin-sensitive glycosyl-PI that differ in phosphate content. Presumably, these forms are interconnected by the action of specific kinases and phosphatases. Interestingly, the biological activity of the head group of this glycosyl-PI depends on its phosphorylation state. This scheme is akin to receptor-induced phosphatidylinositol hydrolysis where the phospholipid exists in different phosphorylated forms.

Table 1

Activity of the various phosphorylated forms of the head group of insulin-sensitive glycosyl-phosphatidylinositol

Fraction	Percent inhibition of cAMP--dependent protein kinase
Aqueous	0 (9)
Ammonium formate	
100 mM	2.7 ± 1.2 (9)
200 mM	38.7 ± 6.2 (9)
500 mM	75.6 ± 2.3 (9)
500 mM (AP)	17.3 ± 2.3 (3)
Aqueous (AP)	0 (3)

The polar head group of insulin-sensitive glycosyl-PI was purified from 4 rat livers as in [14]. After purification the sample was applied to a Dowex AG1 × 8 column in the presence of trace amounts of purified [^{3}H]galactose-labeled glycosyl-PI. The column was eluted as described in the legend to fig.2. The various fractions were concentrated by extensive lyophilization and assayed for ability to inhibit cAMP-dependent protein kinase as described in section 2. Results were compared to those obtained with controls treated in the same way in the absence of head group. (AP) Results obtained with a sample of head group which had been previously treated with alkaline phosphatase as described in the legend to fig.3. Results are the average ± SE of the number of experiments indicated between parentheses

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