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Transport in isolated rat hepatocytes of the phospho-oligosaccharide that mimics insulin action

Effects of adrenalectomy and glucocorticoid treatment

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The addition to intact cells of an inositol phospho-oligosaccharide (POS), which is the polar head-group of an insulin-sensitive glycosylphosphatidylinositol, mimics and may mediate some of the biological effects of this hormone. Here we report the existence of a POS transport system in hepatocytes. This POS transport system is specific and time- and dose-dependent. Insulin-resistance caused by dexamethasone administration to rats was accompanied by a decrease in the hepatocyte POS transport system. In contrast, bilateral adrenalectomy provoked a significant increase in the transport of POS. Both the temporal uptake of POS and the regulation of this process by conditions known to modify the sensitivity to insulin suggest that this novel transport system might be involved in the insulin signalling mechanism.

INTRODUCTION

In the last few years, a glycosylphosphatidylinositol (glycosyl-PI) has been implicated in insulin action [1–4]. Insulin-dependent glycosyl-PI hydrolysis has been reported in BC3H1 myocytes [2], H35 hepatoma cells [3], rat hepatocytes [5] and T-lymphocytes [6]. The polar head-group of this glycosyl-PI, a phospho-oligosaccharide (POS) containing a glucosamine-inositol-phosphate moiety, galactose and several additional phosphates [7], has been reported to mimic some of the biological actions of insulin when added to intact adipocytes or hepatocytes. POS inhibits isoprenaline-stimulated phospholipid methyltransferase [8] and lipolysis [9], as well as stimulating lipogenesis [10] in adipocytes; furthermore, it antagonizes the effects of glucagon on glycogen phosphorylase *a* and pyruvate kinase activities and on cyclic AMP levels in isolated rat hepatocytes [11]. Moreover, POS has been found to evoke the phosphorylation and dephosphorylation of some of the same cellular phosphoproteins as does insulin [4, 12], and to stimulate amino acid uptake [13]. In addition, this molecule has been reported to modulate the activity of certain enzymes when added to cell extracts or to the purified enzymes. POS stimulates cyclic AMP phosphodiesterase [4] and pyruvate dehydrogenase [14–16], and inhibits adipocyte adenylate cyclase [14] and bovine heart cyclic AMP-dependent protein kinase [16,17].

A variety of experiments indicate that POS is generated extracellularly. The majority (about 80 %) of the insulin-sensitive glycosyl-PI in rat hepatocytes is localized at the outer cell surface [5,18], the extracellular levels of POS have been shown to increase in BC3H1 myocytes after the addition of insulin [19], and Reuber hepatoma cells release constitutively POS-like substances into the culture media [20]. Moreover, the addition to intact BC3H1 myocytes of an antibody raised against the oligosaccharide moiety of glycosyl-PI-anchored membrane proteins blocks the insulin-induced stimulation of pyruvate dehydrogenase and the effects of POS *in vitro* [21]. However, it is not known whether extracellular POS can be transported into the cells. In this paper we give evidence for the existence of a POS transport system in isolated rat hepatocytes.

EXPERIMENTAL

Materials

NaB^3H_4 (15 Ci/mmol) was from New England Nuclear, and [U-^{14}C]glucose (280 mCi/mmol) was purchased from Amersham. PI-specific phospholipase C purified from *Bacillus thuringiensis* [22] was generously supplied by Dr. S. Udenfriend, Roche Institute of Molecular Biology, Nutley, NJ, U.S.A. Galactose oxidase from *Doctylium dendroides*, β-galactosidase from *Escherichia coli* and lipid standards were purchased from Sigma. Silica gel G plates for t.l.c. were from Scharlau. Collagenase was from Boehringer Mannheim and human insulin (Actrapid HM) was obtained from Novo Industri A/S. Dexamethasone phosphate (Fortecortin) was obtained from Merck. All the remaining reagents were of analytical grade.

Labelling of purified glycosyl-PI with galactose oxidase/NaB^3H_4 and generation of [^{3}H]galactose-labelled POS

Glycosyl-PI was purified from rat liver membranes by sequential t.l.c. as previously described [3,7]. Purified glycosyl-PI from 20 rat livers was resuspended, using sonication, into 0.3 ml of 50 mM-phosphate buffer, pH 8.0, and treated with galactose oxidase (5 units) and 2 mCi of NaB^3H_4 for 1 h at 37 °C [23]. The mixture was then dried under vacuum, dissolved in chloroform/methanol (2:1, v/v) and purified by t.l.c. on silica gel G [3]. The plate was developed twice in chloroform/acetone/methanol/glacial acetic acid/water (10:4:2:2:1, by vol.). A 1 cm region around the origin was eluted with 2 × 2 ml of methanol at 37 °C, and this fraction was re-chromatographed in chloroform/methanol/NH_4OH/water (45:45:4:10, by vol.). Regions of 1 cm were then scraped off, and lipids were eluted with 2 × 2 ml of methanol at 37 °C and the radioactivity associated with each

Abbreviations used: PI, phosphatidylinositol; POS, phospho-oligosaccharide.
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fraction was determined by counting a sample for radioactivity. Labelled glycosyl-PI was then dissolved into 0.75 ml of chloroform/methanol (2:1, v/v) containing 0.03 M-HCl, and 0.2 ml of water was added to form two phases. After vigorous shaking the organic phase was removed and retained; the water phase was washed once with 0.3 ml of chloroform/methanol (2:1, v/v) and the organic phases were combined. The efficacy of the purification procedure was assessed by h.p.l.c. of the glycosyl-PI labelled with NaB^3H_4 using a silica column (Ultrasil-Si, 10 μm pore size, 4.6 mm $\times$ 250 mm; Beckman). A 20 min linear gradient from chloroform/methanol/glacial acetic acid (14:2:1, by vol.) to chloroform/methanol/glacial acetic acid/water (40:45:10:2, by vol.) was used. The flow rate was 1 ml/min and the elution of the labelled glycosyl-PI was detected by measuring the amount of radioactivity in each fraction. Purified labelled glycosyl-PI was evaporated to dryness under N_2, resuspended by sonication into 0.2 ml of 20 mM-sodium borate, pH 7.4, and treated with 1 unit of PI-specific phospholipase C (1 unit cleaves 0.8 nmol of PI/min at 37 °C [22]) for 12 h at 37 °C to generate labelled POS. The reaction was stopped by the addition of 0.75 ml of chloroform/methanol (2:1, v/v) containing 0.03 M-HCl to form two phases. After vigorous shaking, the water phase was removed and retained; the organic phase was washed once with 0.5 ml of 5 mM-NaCl in 50% methanol and the water phases were combined and lyophilized. After lyophilization, the sample was dissolved in water and the specific radioactivity of the labelled POS was adjusted to approx. 25000 d.p.m./nmol by the addition of non-labelled POS. The amount of POS was determined by measuring the concentration of free amino groups with fluorescamine [24]. Non-labelled POS was obtained by reaction of purified non-labelled glycosyl-PI with PI-specific phospholipase C as described above. The biological activity of POS thus generated was assessed by determining its ability to inhibit cyclic AMP-dependent protein kinase [17] and to stimulate amino acid transport in isolated rat hepatocytes [18]. This assay with intact cells was preferred to previous ones [9,11], since it measures a stimulatory rather than an inhibitory effect of POS.

Hepatocyte isolation and measurement of POS uptake

Fed male Wistar rats (180–200 g) from our inbred colony were used. Some of the animals were subjected to bilateral adrenalectomy, or to simulated operation, under Pentothal anaesthesia 5 days before they were used; adrenalectomized rats received 0.45% (w/v) NaCl solution as drinking water. Treatment of rats with glucocorticoids was carried out by administration of three doses of dexamethasone phosphate (0.2 mg/100 g body wt., subcutaneously) at 50, 26 and 2 h before the isolation of the hepatocytes [25]. Hepatocytes were isolated by perfusion of the liver with collagenase [26]. Cells were suspended in Krebs–Henseleit medium in the presence of 10 mM-glucose and incubated as reported elsewhere [11]. The viability of the isolated hepatocytes was evaluated by the Trypan Blue test; usually 90–95% of the cells excluded the stain. Unless otherwise stated, after 30 min preincubation [^{3}H]POS (10 μM final concentration, about 50000 d.p.m.) was added to cell suspensions (200 μl final volume) and incubated at 37 °C. At various times, reactions were stopped by the addition of 1 ml of ice-cold Krebs–Henseleit medium and without delay centrifuged for 10 s at 2000 rev./min in a Microfuge. The cell pellet was immediately resuspended and washed with 2 $\times$ 1 ml of ice-cold Krebs–Henseleit buffer. After the last centrifugation, the pellet was resuspended into 250 μl of water, and 250 μl of 20% (w/v) trichloroacetic acid was added. After standing for 20 min at 4 °C, samples were centrifuged and the radioactivity present in the supernatant was determined. Results are expressed as pmol of POS associated with the cell pellet/mg of protein. To determine glycogen synthesis, [U-^{14}C]glucose was added to the hepatocyte suspension to a final concentration of 20 mM. After 30 min, samples were taken for the measurement of [^{14}C]glucose incorporation into glycogen [25]. Protein was determined by the method of Bradford [27]. All assays were carried out in triplicate. The statistical significance of differences between values was calculated by Student's t test. The differences were considered to be statistically significant when the P value was less than 0.05.

RESULTS

Radiolabelling of glycosyl-PI with galactose oxidase and NaB^3H_4

Glycosyl-PI was purified from rat liver microsomes and labelled by treatment with galactose oxidase and NaB^3H_4 as described in the Experimental section. After labelling, radioactive glycosyl-PI was purified by sequential t.l.c. As shown in Fig. 1, one major radioactive peak, which comigrated with authentic

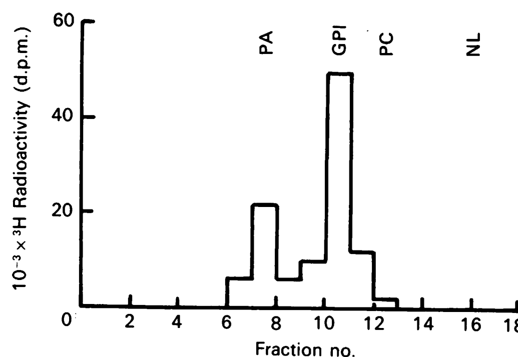

Fig. 1. Purification of glycosyl-PI after labelling with galactose oxidase and NaB^3H_4

Glycosyl-PI purified from rat liver microsomes was treated with galactose oxidase and NaB^3H_4, and the labelled lipid was purified by sequential t.l.c. as described in the Experimental section. The Figure shows the radioactivity associated with each fraction after the second chromatography in chloroform/methanol/NH_4OH/water (45:45:4:10, by vol.). PA, phosphatidic acid; PC, phosphatidylcholine; NL, neutral lipids; GPI, glycosyl-PI purified from rat liver membranes, as indicated in [7].

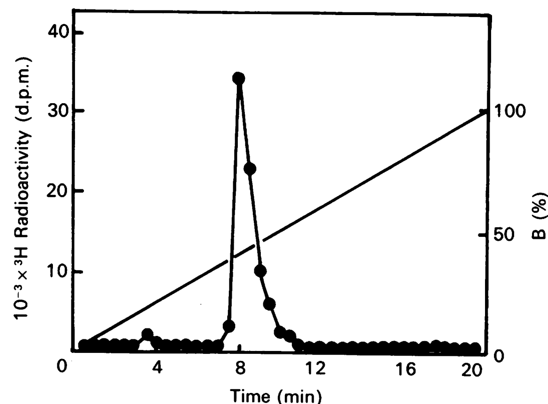

Fig. 2. Analysis of [*galactose*-^{3}H]glycosyl-PI by h.p.l.c.

The radioactive peak from Fig. 1 that comigrated with glycosyl-PI purified from rat liver membranes was analysed by h.p.l.c. using a silica-gel column (Ultrasil-Si, 10 μm pore size, 4.6 mm $\times$ 250 mm; Beckman). A 20 min linear gradient from chloroform/methanol/glacial acetic acid (14:2:1, by vol.) (100% A) to chloroform/methanol/glacial acetic acid/water (40:45:10:2, by vol.) (100% B) was used, as described in the Experimental section. The Figure shows the radioactivity associated with each fraction.

Table 1. Modification of [*galactose*-^{3}H]glycosyl-PI

[*galactose*-^{3}H]Glycosyl-PI was prepared by reaction with galactose oxidase and NaB^3H_4 and purified by sequential t.l.c. as described in the legend to Fig. 1. Samples of the purified labelled glycosyl-PI (containing at least 10000 d.p.m.) were dried under a stream of N_2, resuspended in 0.2 ml of the appropriate buffer by sonication and incubated with PI-specific phospholipase C from *B. thuringiensis* (1 unit in 20 mM-$NaBH_4$, pH 7.4, for 12 h at 37 °C), nitrous acid (0.16 M-$NaNO_2$ in 25 mM-sodium acetate, pH 3.5, for 5 h at 37 °C) [3,36] or β-galactosidase (1 unit in 50 mM-Tris/HCl, pH 7.4, for 4 h at 37 °C). Incubations were stopped by the addition of 0.75 ml of chloroform/methanol (2:1, v/v) containing 0.03 M-HCl, and 0.2 ml of water was added to form two phases. After vigorous shaking, the amount of radioactivity released into the aqueous phase was determined. The results were corrected for non-specific conversion to water-soluble metabolites by subtraction of values obtained from reactions carried out in the absence of enzymes or $NaNO_2$.

Treatment	Water-soluble metabolites formed (%)
PI-Specific phospholipase C	90
Nitrous acid	56
β-Galactosidase	82

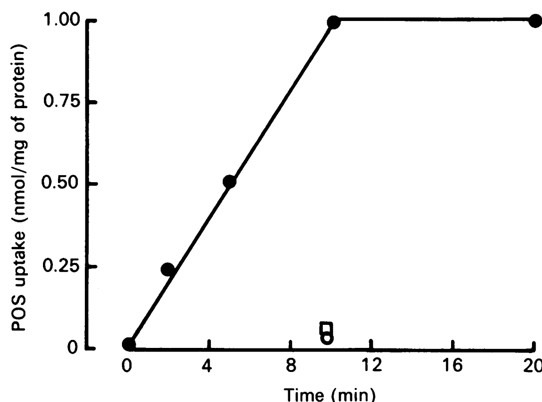

Fig. 3. Time course of POS uptake by hepatocytes

Hepatocytes were incubated in Krebs–Henseleit medium at the indicated temperature (○, 4 °C; ●, □, 37 °C) and at zero time [*galactose*-^{3}H]POS (300 μM) was added. At various times, reactions were stopped by the addition of 1 ml of ice-cold Krebs–Henseleit medium, centrifuged and washed, and the amount of radioactivity associated with the cell pellet was determined. Conditions were as indicated in the Experimental section. □, Hepatocytes preincubated for 90 min in the presence of 5 mM-KCN. Values are the means of triplicate determinations in one representative experiment. The amount of labelled POS which was taken up by the cells after 10 min incubation was 9.3 % of the total.

glycosyl-PI, was identified by this procedure. After elution of this peak from the silica gel plate with methanol, a portion was analysed by h.p.l.c. (to assess for the efficacy of the purification procedure) and the rest was concentrated to dryness under a stream of N_2. As shown in Fig. 2, only one radioactive peak, which contained about 80–90 % of the original radioactivity, was eluted from the h.p.l.c. column, with a retention time of about 8 min.

The major radioactive peak separated by t.l.c. (Fig. 1) had properties consistent with a [^{3}H]galactose-labelled glycosyl-PI structure, as follows. (1) After hydrolysis in 0.2 ml of 4 M-HCl at 110 °C for 5 h under vacuum, the majority of the initial radioactivity (80 %) detected by t.l.c. using pyridine/ethyl acetate/glacial acetic acid/water (5:5:1:3, by vol.) [3] migrated as galactose (results not shown), indicating that the radioactivity was not associated with another part of the molecule. (2) The labelled compound was susceptible (90 % cleavage) to a PI-specific phospholipase C from *Bacillus thuringiensis*, indicating the presence of a PI moiety (Table 1). (3) It was cleaved by nitrous acid deamination (56 % efficiency), indicating the presence of a non-*N*-acetylated glucosamine residue (Table 1). (4) It was efficiently cleaved by β-galactosidase (82 % cleavage), suggesting the presence of a terminal ^{3}H-labelled galactose residue (Table 1). These results are consistent with previous observations that rat liver glycosyl-PI contains a glucosamine-inositol-phosphate moiety, galactose and several additional phosphates [4,24], as well as having the properties of the insulin-sensitive glycosyl-PI isolated from BC3H1 myocytes [2] and H35 hepatoma cells [3]. Moreover, POS obtained after reaction with galactose oxidase and borohydride still retained insulin-like activity, as indicated by its ability to inhibit cyclic AMP-dependent protein kinase and to stimulate amino acid uptake by isolated rat hepatocytes (results not shown).

Time course of POS uptake by hepatocytes

[*galactose*-^{3}H]POS was generated by hydrolysis of the labelled glycosyl-PI with PI-specific phospholipase C as described in the Experimental section. Incubation of isolated rat hepatocytes with 300 μM-[^{3}H]POS at 37 °C caused a time-dependent increase in the amount of radioactivity associated with the cells (Fig. 3). The uptake of POS was linear for about 10 min after the addition of the labelled molecule (Fig. 3). When incubations were carried out at 4 °C, no significant radioactivity was associated with the cell pellet (Fig. 3). Metabolic poisoning of the cells with 5 mM-KCN abolished the uptake of POS at 37 °C in cells (Fig. 3). In one set of experiments, cells were incubated in the presence of 10 μM-[^{3}H]POS for 5 min at 37 °C, and at the end of this period the amount of radioactivity associated with the cells was measured (control hepatocytes), or the cells were washed and resuspended in 2 ml of Krebs–Henseleit medium (in the absence of POS) and incubated for another 15 min. After this period of time, the amount of radioactivity associated with the hepatocytes was determined, and was found to be 89 % ($n = 3$) of that measured in control hepatocytes, suggesting that the majority of the radioactivity associated with the cells was intracellular. In another set of experiments, cells were incubated in the presence of 10 μM-[^{3}H]POS for 5 min at 37 °C, and at the end of this period the radioactivity associated with the hepatocytes was determined (control hepatocytes) or cells were washed and resuspended in the presence of an excess (400 μM) of non-labelled POS. After a 15 min incubation, the radioactivity associated with the cells was determined, and was found to be 104 % ($n = 3$) of that measured in control hepatocytes, which further demonstrates that the radioactivity associated with the cells was intracellular. These results indicate the existence in rat hepatocytes of a POS uptake system which is time-, temperature- and, probably, energy-dependent.

To show that the labelled material taken up into cells is indeed POS and not a contaminant, cells were incubated in the presence of 10 μM-[^{3}H]POS for 10 min at 37 °C, and at the end of this period the radioactivity associated with he hepatocytes was analysed by h.p.l.c. on a SAX column. As shown in Fig. 4, the h.p.l.c. profile of the radioactivity associated with the cells was the same as that observed with a sample of purified [^{3}H]POS.

POS uptake by hepatocytes: effects of adrenalectomy and glucocorticoid treatment

Incubation of hepatocytes with increasing concentrations of POS resulted in a concentration-dependent cellular accumulation

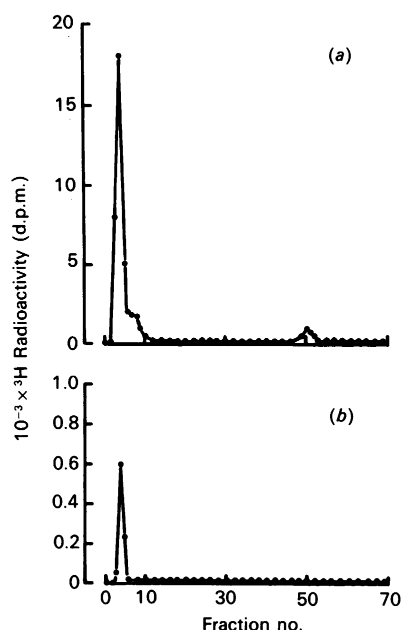

Fig. 4. H.p.l.c. profile of the labelled material taken up into cells

Cells were incubated in the presence of 10 μM-[^{3}H]POS for 10 min at 37 °C, and at the end of this period the reaction was stopped by the addition of 1 ml of ice-cold Krebs–Henseleit medium, washed twice and homogenized by freezing and thawing. The radioactivity associated with the hepatocytes was analysed by h.p.l.c. using a SAX column and the following gradient: 0–12 min, 100 % A; 12–17 min, 50 % A; 17–32 min, 0 % A. The flow rate was 3 ml/min; fractions contained 1.5 ml. Buffer A: KH_2PO_4, 7 mM; KCl, 7 mM (pH 4.0). Buffer B: KH_2PO_4, 250 mM, KCl, 500 mM (pH 5.0). (a) H.p.l.c. of a sample of purified [^{3}H]POS; (b) h.p.l.c. profile of the radioactivity associated with the cells.

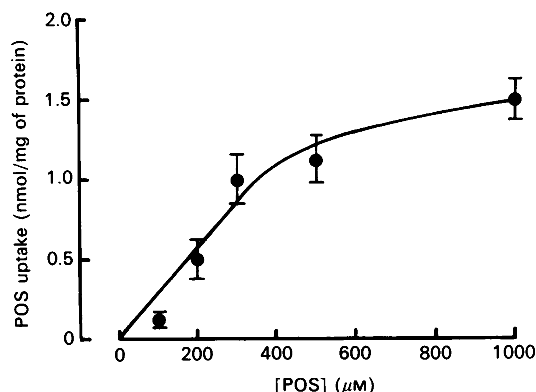

Fig. 5. Dose–response curve of POS uptake by hepatocytes

Hepatocytes were incubated in Krebs–Henseleit medium and at time zero the indicated concentrations of [*galactose*-^{3}H]POS were added. After a 10 min incubation the amount of radioactivity associated with the cells was determined as indicated in the Experimental section. Values are the means ± S.E.M. of four experiments. The fraction of labelled POS which was taken up by the cells after 10 min incubation was 7 % at 200 μM, 9 % at 300 μM, 6 % at 500 μM and 4 % at 1000 μM.

of this molecule (Fig. 5). As shown in Table 2, bilateral adrenalectomy caused a 40 % increase in the amount of POS accumulated in liver cells, as compared with that measured in hepatocytes isolated from sham-operated animals. In contrast, dexamethasone treatment decreased by about 30 % the amount

Table 2. Effects of adrenalectomy and dexamethasone treatment on the uptake of POS by hepatocytes

Hepatocytes from adrenalectomized or sham-operated rats, treated or not with dexamethasone, were suspended in Krebs–Henseleit medium in the presence of 10 mM-glucose and 150 μM-[*galactose*-^{3}H]POS. After a 5 min incubation, the amount of radioactivity associated with the cells was measured as described in the Experimental section. The 100 % value was 221 ± 43 pmol of [^{3}H]-POS/mg of protein. Values of POS uptake are the means ± S.E.M. of three to four different experiments. *$P < 0.02$ versus sham-operated rats; †$P < 0.05$ versus the corresponding animals not treated with dexamethasone.

	POS uptake (% of control)
Sham-operated rats	100
Adrenalectomized rats	142 ± 13*
Sham-operated rats + dexamethasone	70 ± 18†
Adrenalectomized rats + dexamethasone	66 ± 19†

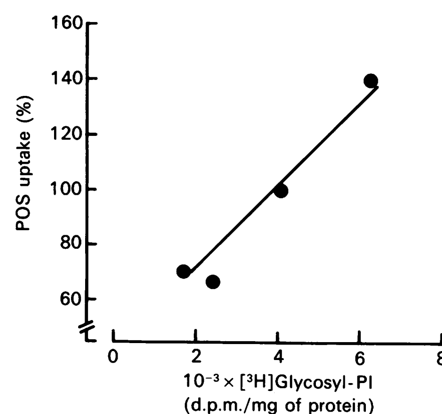

Fig. 6. Correlation between basal levels of glycosyl-PI and the rate of POS uptake

Values of POS uptake and of glycosyl-PI levels are from Table 2 and ref. [25] respectively. $r = 0.98$; $P < 0.05$.

of POS accumulated in hepatocytes isolated from either adrenalectomized or sham-operated animals. Previously we have reported that bilateral adrenalectomy caused an increase in the amount of glycosyl-PI present in liver cells, as compared with the levels measured in hepatocytes isolated from sham-operated animals, whereas dexamethasone treatment decreased the levels of glycosyl-PI [25]. As shown in Fig. 6, there was a close correlation ($r = 0.98$, $P < 0.05$) between basal levels of glycosyl-PI and POS uptake in hepatocytes isolated from adrenalectomized or sham-operated rats treated or not with dexamethasone.

As expected, treatment of the rats with dexamethasone almost completely blocked the stimulatory effect of insulin on [U-^{14}C]glucose incorporation into glycogen (Fig. 7). The consequence of dexamethasone treatment on the effect of POS on glycogen synthesis was also examined. As shown in Table 3, POS stimulated glycogen synthesis in hepatocytes isolated from either control or sham-operated animals. On the contrary, treatment with dexamethasone blocked the stimulatory effect of POS on [U-^{14}C]glucose incorporation into glycogen.

Specificity of POS uptake

The specificity of the uptake of POS was tested by incubating hepatocytes with 10 μM-[^{3}H]POS and 100 μM of a variety of

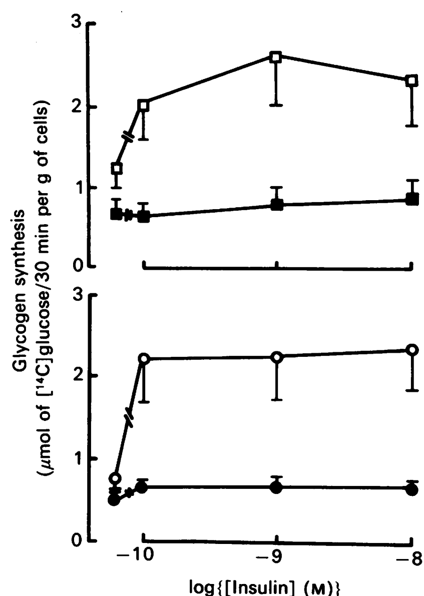

Fig. 7. Effect of insulin on the rate of glycogen synthesis

Glycogen synthesis was measured in hepatocytes isolated from sham-operated (□, ■) and adrenalectomized (○, ●) rats, treated (■, ●) or not (□, ○) with dexamethasone. Values are the means ± S.E.M. of six to nine experiments.

Table 3. Effects of dexamethasone treatment on insulin- and POS-stimulated glycogen synthesis in isolated rat hepatocytes

Glycogen synthesis was determined as described in the Experimental section. Values are the means ± S.E.M. of three experiments. The effect of dexamethasone treatment on insulin or POS-stimulated glycogen synthesis was statistically significant compared with that in sham-operated animals ($P < 0.05$).

	Glycogen synthesis (%)		
	Basal	POS (10 μM)	Insulin (10 nM)
Control rats	100	153±8	187±9
Sham-operated rats	100	168±4	172±20
Sham-operated rats + dexamethasone	100	86±5	105±9

Table 4. Specificity of the uptake of POS by hepatocytes

Hepatocytes were resuspended in Krebs–Henseleit medium in the presence of 10 μM-[*galactose*-^{3}H]POS and the various carbohydrates mentioned in the Table (100 μM final concentration). After a 5 min incubation, the amount of radioactivity associated with the cells was measured as described in the Experimental section. The 100% value was 2723 ± 298 d.p.m. of [^{3}H]POS/mg of protein. The means ± S.E.M. of three to four experiments are presented.

Additions (100 μM)	POS uptake (%)
None	100
myo-Inositol	90±8
Inositol 1-phosphate	81±6
Inositol 2-phosphate	82±12
Inositol hexakisphosphate	57±13
Mannose	67±11
Galactose	63±5
Glucosamine	45±6

compounds structurally related to POS. As shown in Table 4, *myo*-inositol, *myo*-inositol 1-phosphate and *myo*-inositol 2-phosphate had no significant effect on the uptake of POS. Mannose, galactose, *myo*-inositol hexakisphosphate and glucosamine inhibited POS uptake by only 34%, 37%, 43% and 55% respectively. It is important to note that, as mentioned in the Experimental section, all incubations were carried out in the presence of 10 mM-glucose. These results indicate that POS uptake is specific and cannot be efficiently blocked by a 10-fold excess of several simple sugars or inositols.

DISCUSSION

Insulin has been shown to promote the hydrolysis of a novel glycosyl-PI (reviewed in [28,29]). The polar head-group of this glycosyl-PI is a POS that contains a glucosamine-inositol-phosphate moiety, about four galactosyl residues and several additional phosphate groups which are probably associated with a galactosyl residue [7,24]. The presence of a cellular uptake process for POS has been suggested by the observation that the addition of this molecule to intact adipocytes or hepatocytes mimics certain biological effects of insulin [4,8–12], by the finding that POS is released into the incubation media of BC3H1 [19] and Reuber hepatoma cells [20], by the localization of the majority of the glycosyl-PI precursor at the outer surface of hepatocytes [5,18], and by the use of antibodies raised against the oligosaccharide moiety of glycosyl-PI-anchored proteins to block insulin action [21].

To investigate the mechanism by which POS acts in intact cells, we have developed a procedure to generate [*galactose*-^{3}H]POS by radiolabelling the glycosyl-PI precursor with NaB^3H_4 and galactose oxidase, followed by hydrolysis with a PI-specific phospholipase C. Using [*galactose*-^{3}H]POS thus prepared, we observed an efficient transport of POS by isolated rat hepatocytes. Evidence for this conclusion comes from the finding that POS uptake was detected at 37 °C but not at 4 °C, and from the observation that after being taken up by the cells radioactive POS was not released into the incubation medium either by incubation in the absence of POS or by the addition of non-labelled POS. Cellular accumulation of POS was abolished by metabolic poisoning with KCN. These results indicate that the uptake of POS is probably an energy-dependent process and cannot be explained by diffusion of the molecule through the cell membrane. The uptake of POS was time- and dose-dependent. Uptake was observed within 5 min of incubation, which conforms with the biological effects of POS in intact adipocytes or hepatocytes; these are observed within 5–10 min of its addition [4,11], and with the insulin-dependent hydrolysis of glycosyl-PI in myocytes [2], H35 hepatoma cells [3] and hepatocytes [5], which is maximal within 2–5 min of the addition of the hormone. The data of Fig. 5 do not permit us to estimate the affinity of the transport system. Since the uptake system is not yet saturated at 1 mM-POS, the affinity seems to be low relative to the likely physiological concentrations of POS. The concentration of POS generated in response to insulin is not known. Studies thus far suggest that the majority of the glycosyl-PI precursor of POS is present at the outer cell surface [4,18]. The organization and dynamic state of glycosyl-PI molecules is not known, although aggregation of certain glycolipids in lipid bilayers has been reported [30]. If glycosyl-PI molecules are concentrated in certain regions of the biomembrane and transport of POS occurs close to its site of production, the concentration of POS at this site might be higher than predicted for a molecule whose lipid precursor accounts for about 0.5% of the total plasma membrane phospholipids [5].

The data presented here indicate that inositol hexakisphosphate, mannose, galactose and glucosamine all inhibited

POS uptake to a similar extent (about 40–50%). It has been previously reported that, in adipocytes, inositol monophosphate, mannose and glucosamine do not alter the effects of insulin or POS on isoprenaline-stimulated cyclic AMP levels or lipolysis [31]. Similarly, in hepatocytes, mannose, glucosamine, galactose, *myo*-inositol and inositol hexakisphosphate have no effect on POS- or insulin-stimulated amino acid uptake [13]. In contrast, the stimulatory effect of POS on lipogenesis is blocked by mannose, glucosamine and inositol monophosphate [31]. Similarly, the effects of POS and insulin on protein phosphorylation in adipocytes are blocked by inositol phosphates and glucosamine [32]. These data have been interpreted as being suggestive of the existence of an uptake system for POS [31,32]. The mechanisms by which these sugars specifically affect only some of the biological effects of insulin or POS cannot yet be explained.

In man and animals, glucocorticoid excess is associated with insulin-resistance [33,34]. Conversely, adrenalectomized animals are abnormally sensitive to this hormone [35]. The mechanism by which glucocorticoids modulate insulin action is not well understood. There is experimental evidence suggesting that these steroids may influence insulin effects by acting at the insulin receptor level and at a post-receptor step [34]. Previously we have shown that insulin-resistance caused by dexamethasone administration is accompanied by a marked decrease in both the hepatic levels of the insulin-sensitive glycosyl-PI and the number of insulin receptors [25]. In contrast, bilateral adrenalectomy raised the cellular content of glycosyl-PI and the number of insulin receptors [25]. The present results indicate that dexamethasone administration is accompanied by a decrease in the hepatic uptake of POS, and that bilateral adrenalectomy enhances this process. Although the effect of dexamethasone on POS uptake was relatively small (about 30%), the inhibition of insulin- or POS-stimulated glycogen synthesis was almost complete. These results indicate that these steroids may influence insulin action by acting at more than one level and that the inhibition by dexamethasone of insulin- or POS-stimulated glycogen synthesis cannot be explained only by a decrease in the uptake of POS.

In conclusion, although there is no direct evidence for a role of this uptake system for POS in insulin action, the data described here on the temporal uptake of POS, and on the regulation of this process by conditions known to modify sensitivity to insulin, support the idea of such a transport system being functionally important in insulin signalling.

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