Insulin-induced phospho-oligosaccharide stimulates amino acid transport in isolated rat hepatocytes

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The ability of the insulin-induced phospho-oligosaccharide to stimulate amino acid transport was studied in isolated rat hepatocytes. At low α -aminoisobutyric acid concentrations (0.1 mM), both 100 nM-insulin and 10 μ M-phospho-oligosaccharide doubled amino acid uptake after 2 h of incubation. This stimulation was prevented by 0.1 mM-cycloheximide or 5 μ g of actinomycin D/ml, indicating that the phospho-oligosaccharide, like insulin, was acting via the synthesis of a high-affinity transport component. The effects of the phospho-oligosaccharide and of insulin were blocked by Ins2P (2.5 mM), but not by *myo*-inositol, inositol hexaphosphoric acid or several monosaccharides such as mannose, glucosamine and galactose. Both the temporal effect on amino acid entry and the extent of stimulation of this process by the phospho-oligosaccharide indicate that this molecule mimics, and may mediate, some of the long-term actions of insulin. However, the effects of phospho-oligosaccharide and insulin were not exactly the same, since the effect of insulin, but not of the phospho-oligosaccharide, was additive with that of glucagon.

INTRODUCTION

The mechanism of insulin action has been extensively studied, but our understanding of the molecular events linking the occupation of the insulin receptor to the biological effects of the hormone is still limited. Insulin has been reported to stimulate the hydrolysis of a novel glycosyl-phosphatidylinositol (glycosyl-PtdIns) in a variety of cells [1–3]. The resulting phospho-oligosaccharide contains phosphate, inositol, glucosamine and galactose [4] and is referred to as insulin-induced phospho-oligosaccharide (POS). The insulin-sensitive glycosyl-PtdIns from rat liver and the glycosyl-PtdIns anchor of a number of membrane proteins [5,6] share several properties, such as sensitivity to hydrolysis by the PtdIns-specific phospholipase C (PtdIns-PLC) and loss of biological activity after treatment with nitrous acid [7,8].

Several lines of evidence indicate that POS can mimic some of the short-term effects of insulin. It shows insulin-like effects on lipolysis [9] and lipogenesis [10] in adipocytes; it decreases cyclic AMP concentration, inactivates glycogen phosphorylase and activates pyruvate kinase in hepatocytes [11]. Moreover, POS has been found to induce a phosphorylation–dephosphorylation pattern of cellular proteins [12] similar to that caused by insulin [12] in intact adipocytes. Whether POS can mimic some of the long-term effects of insulin is not known. One of these effects is the stimulation of neutral amino acid transport in isolated rat hepatocytes. Insulin and glucagon have been shown to stimulate this transport through the synthesis of a high-affinity component of the saturable A (alanine) transport system [13–15]. In the present work we have studied whether POS can modulate amino acid transport in hepatocytes. We show that POS, like insulin, stimulates α -aminoisobutyric acid (AIB) transport specifically and that this stimulation depends on protein synthesis.

EXPERIMENTAL

Materials

α -Amino[1-¹⁴C]isobutyric acid (60 mCi/mmol) and 1-amino-cyclopentane[1-¹⁴C]carboxylic acid (60 mCi/mmol) were from Amersham. The unlabelled non-metabolizable amino acids AIB, α -(methylamino)isobutyric acid and 1-aminocyclopentane-1-carboxylic acid (cycloleucine) were from Sigma. Pig insulin and bovine glucagon were from Novo Industri A/S (Copenhagen, Denmark). Collagenase was purchased from Boehringer. PtdIns-PLC from *Bacillus thuringiensis* (EC 3.1.4.3.) was generously provided by Dr. S. Udenfriend (Roche Institute of Molecular Biology, NJ, U.S.A.). All other reagents were of the highest grade commercially available.

Purification of POS

This compound was prepared by treating a purified liver glycolipid fraction with the bacterial PtdIns-PLC, as described elsewhere [2]. Briefly, rat liver membranes were recovered in the pellet obtained by centrifugation (100 000 *g* for 1 h) of a liver extract (supernatant after 10 000 *g* for 5 min). Phospholipids were extracted from the membrane fraction and the glyco-phospholipids were purified by sequential t.l.c. as described [2]. The purified glycolipids were then resuspended in 20 mM-sodium borate buffer, pH 7.4, and treated with 5 units of PtdIns-PLC/ml for 10 h at 37 °C. (One unit of PtdIns-PLC activity is defined as the amount of enzyme that hydrolyses 0.8 nmol of PtdIns in 1 min at 37 °C.) The treated glycolipids were then extracted with chloroform/methanol/1 M-HCl (2:1:0.03, by vol.), the organic phase was re-extracted with 5 mM-NaCl in 50% methanol, and the upper aqueous phases were pooled, evaporated to remove methanol, dissolved in distilled water and lyophilized. The concentration of POS was calculated by measuring free amino

Abbreviations used: POS, phospho-oligosaccharide; PLC, phospholipase C; AIB, α -aminoisobutyric acid.

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groups, assuming that each molecule of POS contains one amino group [16]. Nitrous acid deamination of POS was performed by incubation of the sample with 0.2 M-sodium nitrite in 50 mM-ammonium acetate buffer, pH 4.0, for 5 h as described [2]. The biological activity of POS was assessed *in vitro* by testing its capacity to inhibit the phosphorylation of histone IIA by the cyclic AMP-dependent protein kinase [8].

Isolation of hepatocytes and incubation procedure

Hepatocytes were isolated from fed male Wistar rats (150–200 g) by collagenase dissociation of the liver as previously described [17]. Hepatocytes were incubated at 37 °C in Krebs–Henseleit bicarbonate buffer, pH 7.4, containing 1 % (w/v) BSA (Fraction V) and bacitracin (0.8 mg/ml). The gas phase contained 5 % CO₂ in O₂. Bacitracin did not influence the basal rate of amino acid uptake. Hormones were added to the suspension of hepatocytes after 30 min and the incubation was continued for the indicated periods of time. At the beginning of the experiment, each cell preparation was checked for viability by Trypan Blue exclusion, and only preparations containing at least 90 % viable cells were used. After 2 h incubation periods, cell viability was 91 ± 2 % (control), 86 ± 2 % (insulin-treated) and 89 ± 1 % (POS-treated) (means $\pm$ S.E.M., $n = 6$).

AIB uptake

After incubation for the indicated periods of time, hepatocytes were collected by centrifugation (600 *g* for 10 s) and resuspended in Krebs–Henseleit bicarbonate buffer without albumin and bacitracin. AIB transport assays were carried out in 1.5 ml microfuge tubes as described previously [13,15]. The assay was initiated by the addition of 0.2 ml of the cell suspension (about 2 mg of protein, determined on the cell pellets using the Bradford method [18]) to 50 μ l of Krebs–Henseleit bicarbonate buffer containing 0.5 mM-[¹⁴C]AIB (0.1 μ Ci) to give a final concentration of 0.1 mM-AIB. At this concentration, amino acid uptake occurs through a high-affinity component of the transport system [15]. The tubes were incubated at 37 °C for various periods of time and the reaction was terminated by the addition of 1 ml of cold buffer. Hepatocytes were collected (2000 *g*, 5 s), resuspended in 1 ml of cold buffer and re-centrifuged. The cell pellet was then lysed in 250 μ l of water, deproteinized by adding 250 μ l of 10 % trichloroacetic acid, and the radioactivity was determined by liquid scintillation counting. Trapping and non-specific uptake did not exceed 5 % of total uptake. Increasing the number of washings (from two to three) did not modify the uptake or the non-specific trapping of AIB. The uptake was linear with time for at least 6 min (results not shown). Therefore in all subsequent studies transport was measured over a 5 min period.

RESULTS AND DISCUSSION

Time course of the stimulation of AIB uptake

Incubation of hepatocytes with insulin is known to result in a time-dependent increase in AIB uptake which is about doubled after 2 h of incubation [15]. The time courses of the effects of 10 μ M-POS and 100 nM-insulin on the uptake of 0.1 mM-AIB were compared and are illustrated in Fig. 1. In these experiments, hepatocytes were incubated for the indicated periods of time, collected by centrifugation and then incubated with radioactive AIB to measure the uptake over a 5 min period (see the Experimental section). Fig. 1 shows that incubation with either POS or insulin for 2 h resulted in a linear increase in AIB uptake. After 2 h of incubation, POS and insulin caused 2- and 2.5-fold increases respectively. Longer incubation periods were not tested since, after a 3 h-incubation period, cell viability decreased considerably, to as low as 60 % in some cases.

Fig. 1. Time courses of the stimulation of AIB transport by POS and insulin

Cells were incubated for the indicated periods of time without addition (○) or with 100 nM-insulin (□) or 10 μ M-POS (●). Hepatocytes were then harvested by centrifugation, and 0.1 mM-[¹⁴C]AIB uptake was measured over 5 min periods as described in the Experimental section. The values are means $\pm$ S.E.M. for at least four different cell preparations. * Denotes values that are statistically different from the controls ($P < 0.05$).

When hepatocytes were incubated for 2 h with POS that had been hydrolysed by treatment with nitrite at pH 4 for 5 h (see the Experimental section), the stimulatory effect was lost. Similarly, AIB transport remained unaffected when hepatocytes were treated for 2 h with the lyophilized aqueous phase of the PtdIns-PLC reaction mixture without glycolipid (results not shown).

Unlike POS, an autocrine factor obtained from Reuber hepatoma cells and containing an inositol-carbohydrate structure failed to stimulate AIB uptake, although it displayed several insulin-like actions [19]. Differences in carbohydrate composition, as reported for modulators of pyruvate dehydrogenase [20], may explain the discrepancy. Furthermore, the putative

Fig. 2. Effect of inhibitors of RNA and protein synthesis on the stimulation of AIB uptake by insulin or POS

AIB uptake was determined over 5 min periods in hepatocytes which had been incubated for 2 h at 37 °C without (□) or with 100 nM-insulin (▨) or 10 μ M-POS (▩) with or without 0.1 mM-cycloheximide (CHX) or 0.5 μ g of actinomycin D/ml (AMD). The values are means $\pm$ S.E.M. for the number of different cell preparations given in each column. * Denotes values that are statistically different from the respective controls ($P < 0.05$).

Table 1. Nature of the transport component regulated by insulin and the phospho-oligosaccharide

Amino acid uptake was measured in hepatocytes that had been incubated with or without 100 nM-insulin or 10 mM-POS for 2 h at 37 °C. The influx of [¹⁴C]AIB (0.1 mM) was measured in the absence or presence of 10 mM-methyl-AIB. The influx of 0.2 mM-[¹⁴C]cycloleucine was measured in Na⁺-free medium in which Na⁺ was replaced by choline. The values are means ± S.E.M. for at least five different preparations.

Amino acid present during transport assay	Na ⁺ in medium	Amino acid influx (nmol/5 min per mg of protein)		
		Basal	Insulin	POS
0.1 mM-AIB	Yes	0.59 ± 0.06	1.48 ± 0.06	1.18 ± 0.03
0.1 mM-AIB + 10 mM-methyl-AIB	Yes	0.34 ± 0.03	0.38 ± 0.03	0.35 ± 0.06
0.1 mM-AIB	No	0.24 ± 0.02	0.16 ± 0.02	0.23 ± 0.03
0.2 mM-Cycloleucine	No	0.74 ± 0.07	0.99 ± 0.07	0.88 ± 0.03

glycosyl-PtdIns precursor of the autocrine factor has not been described.

Dependence on protein synthesis

The stimulation of amino acid transport by POS and insulin showed an absolute dependence on protein synthesis [15]. Fig. 2 shows that preincubation for 2 h in the presence of 0.1 mM-cycloheximide completely blocked the stimulation of AIB transport by POS and insulin. Under the same conditions, actinomycin D (5 µg/ml) also inhibited the effects of both insulin and POS. The inhibitory effect of actinomycin D was however not as complete as that of cycloheximide. None of these inhibitors affected the basal rate of AIB uptake or cell viability. The dose of cycloheximide used is known to inhibit protein synthesis by about 90 %, and that of actinomycin D inhibits RNA synthesis and protein synthesis by 95 % and 45 % respectively in isolated hepatocytes [14,21]. Thus it is concluded that, as for insulin, the effect of POS was completely blocked under conditions where protein synthesis was abolished. Therefore the data indicate that POS enhances amino acid uptake by inducing synthesis of high-affinity transport proteins *de novo*.

Specificity of the effect

The stimulatory action of POS was restricted to the A (alanine) transport system (Table 1). POS did not stimulate uptake of 0.1 mM-AIB in the presence of an excess of 10 mM-*N*-methyl-AIB. Under these conditions, the *N*-methyl-AIB-insensitive part of AIB uptake is believed to represent transport through the ASC (alanine, serine, cysteine) system [22]. This was not affected by POS or insulin. Similarly, neither POS nor insulin influenced the non-saturable, sodium-independent, AIB transport, nor did they influence the L (leucine) transport system as measured by the uptake of 0.2 mM-cycloleucine in a sodium-free medium in which choline replaced sodium (Table 1). Under the latter conditions, amino acid transport occurs only through the L system [13].

Available information on the structure and properties of POS suggests that it might require a transport system in order to enter the cells [10,12,16,23]. Therefore we tested the ability of several substances, such as *myo*-inositol and inositol phosphates, to block the effects of insulin and POS on AIB uptake. Mannose, glucosamine, galactose and inositol hexaphosphoric acid up to 15 mM were without effect, whereas they abolished insulin action unspecifically when used at higher doses (results not shown). Glucagon, like insulin, is known to stimulate AIB uptake, but the effects of these hormones are additive [15], suggesting that they control AIB uptake through different mechanisms. Fig. 3 shows that 2.5 mM-*myo*-inositol had no effect on insulin-, POS- or glucagon-stimulated AIB uptake. In contrast, 2.5 mM-Ins2P

Fig. 3. Effect of *myo*-inositol and Ins2P on the stimulation of AIB transport by insulin or POS

AIB uptake was measured over 5 min periods in cells that had been incubated for 2 h without (□) or with 100 nM-insulin (▨), 10 µM-POS (▩), or 100 nM-glucagon (■) with 2.5 mM-Ins2P or 2.5 mM-*myo*-inositol. The values are means ± S.E.M. for the number of different cell preparations given in each column. * Denotes values that are statistically different from the respective controls (*P* < 0.05).

Table 2. Additivity of the effects of insulin, glucagon and POS on AIB transport

AIB uptake was measured in hepatocytes that had been incubated for 2 h with 100 nM-insulin, 100 nM-glucagon or 10 µM-POS used alone or in combination. The values are means ± S.E.M. for the number of different cell preparations given in parentheses. * Denotes values that are statistically different from the values of the controls (*P* < 0.05).

Addition	Amino acid uptake (nmol/5 min per mg of protein)
None	0.56 ± 0.08 (17)
100 nM-Glucagon	2.17 ± 0.09 (15)*
100 nM-Insulin	1.41 ± 0.06 (17)*
10 µM-POS	1.13 ± 0.03 (6)*
100 nM-Glucagon + 100 nM-insulin	2.98 ± 0.11 (6)*
100 nM-Glucagon + 10 µM-POS	0.78 ± 0.04 (5)*

was found to decrease both insulin- and POS-stimulated AIB transport, whereas it was without effect on glucagon-dependent AIB uptake (Fig. 3). These results confirm further that POS might act as a second messenger for insulin induction of amino acid transport. They also indicate that the uptake of POS may occur through a specific transport system.

Additivity of the effects of POS, insulin and glucagon

Both insulin and glucagon at 100 nM enhanced AIB transport after 2 h of cell exposure, and their effects were partially additive (Table 2). By contrast, the effect of POS (10 μ M) was not additive with that of glucagon (100 nM), and an inhibition of the glucagon effect was even observed in the presence of POS (Table 2). These data suggest that POS does not mimic all insulin effects. However, one should consider that the stimulation by glucagon of AIB transport is mediated by cyclic AMP [15], and that POS has been reported to stimulate cyclic AMP phosphodiesterase in liver plasma membrane preparations [7] and to decrease cyclic AMP levels in isolated rat hepatocytes [11]. Thus a 2 h incubation period with POS may antagonize the glucagon-stimulated AIB uptake by decreasing the cellular cyclic AMP content. On the other hand, the effect of glucagon is partially additive with that of dibutyl cyclic AMP [15]. This suggests that glucagon is acting through two different, cyclic AMP-dependent and -independent, pathways. The same conclusion can be drawn from the data presented here, since a significant 1.4-fold stimulation of AIB entry was observed when cells were incubated in the presence of both glucagon and POS.

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