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Phosphorylation control by insulin in adipocytes is interfered with at a post-receptor step by phosphoinositol and glucosamine

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Inositol-phosphates, glucosamine and glucose-6-phosphate blocked the effects of insulin on target protein phosphorylation in adipocytes, but the unsubstituted or sulphated derivatives of inositol or of glucose, or *N*-acetyl-glucosamine were without effect. The insulin stimulated tyrosine phosphorylation of the insulin receptor was not affected. The sugar-phospates inositol-phosphate and glucose-6-phosphate did not enter into the cells. They also blocked the insulin-like effects of a potential second messenger of insulin, a phosphooligosaccharide (POS), which has previously been shown to mimick the effects of insulin on protein phosphorylation in intact cells.

Protein phosphorylation; Insulin; Adipocyte; Phosphoinositol; Glucosamine; Insulin receptor

1. INTRODUCTION

The molecular mechanisms whereby insulin controls metabolism of cells in target tissues have remained elusive and are partly understood only. Receptor autophosphorylation is known to be necessary for signal generation by insulin [1,2] and the function of target cellular proteins is acutely controlled by changes in their state of phosphorylation at serine or threonine residues in response to the hormone [3,4]. Recently, phospholipase C-catalyzed hydrolysis of a glycophospholipid (or a group of structurally similar glycophospholipids) has been suggested to take part in signalling by insulin [5-12]. Thus insulin will generate diacylglycerol and a polar head-group, which is a phosphooligosaccharide that seems to consist of inositol-monophosphate linked to non-acetylated glucosamine and several residues of galactose (5-8). When added to intact cells diacylglycerol increased glucose transport [10,13] while the phosphooligosaccharide (POS) closely mimicked the effects of insulin on target protein phosphorylation [9,14,15] as well as on activities of enzymes in cells [12,16,17] and as isolated [5,18,19]

2. EXPERIMENTAL PROCEDURES

2.1. *Materials*

Carrier-free $^{32}P_i$, D-*myo*-[2-^{3}H]inositol-1-monophosphate, D-[U-^{14}C]glucose-6-phosphate, and [^{3}H]sucrose were from Amersham. Insulin (porcine) and mono-[^{125}I]-(Tyr-A14)insulin was from Novo, Copenhagen. ^{125}I-labelled protein G was a kind gift from Bo Åkerström, University of Lund, and antibodies against phosphotyrosine [20] were a kind gift from James Woodgett, Ludwig Cancer Institute, London. Isoproterenol, D-*myo*-inositol-hexakisphosphate, D-*myo*-inositol-1-monophosphate, *myo*-inositol-hexakissulphate, glucose-6-sulphate and D-glucosamine were from Sigma and D-glucose-6-phosphate from Boehringer. POS was purified from rat liver membranes as described [7,9].

2.2. *Preparation and incubation of adipocytes*

Adipocytes were isolated by collagenase digestion of rat epididymal fat and incubated with [^{32}P]phosphate as described [21]. ^{32}P-Labelled cells were than added to tubes containing the indicated final concentrations of inositol-hexakisphosphate (or other sugar derivatives as indicated) and incubated for 5 min when insulin (or POS) was added and the incubations continued for a further 20 min. Reactions were terminated and processed for SDS-PAGE as detailed in [21]. SDS-PAGE was performed according to Laemmli [22] in 8% polyacrylamide gels. Dried gels were autoradiographed against Hyperfilm β-max (Amersham). The reported experiments have been performed at least twice with similar results.

2.3. *Determination of insulin receptor autophosphorylation*

Isolated adipocytes at a concentration of 90 μl packed-cell volume per ml were prepared and incubated as described above, but without $^{32}P_i$. After SDS-PAGE (7% polyacrylamide) of total-cell protein from ca 1.5×10^6 cells the gel was electrophoretically blotted against a nitrocellulose filter [23]. Immunoblotting analysis with antibodies against phospho-tyrosine [20] was performed exactly as described [20]. The bound antibody was detected as in [20], but using ^{125}I-labelled protein G [24] and autoradiography against Kodak X-Omat film with intensifying screen (Dupont).

2.4. *Determination of ^{125}I-labelled insulin binding*

Mono [^{125}I]-(Tyr-A14)insulin, at 100 pM, binding to the adipocytes was determined as described [25]. Adipocytes were incubated as described above: after 5 min incubation in the presence of the different sugar derivatives the labelled hormone was added for 20 min, non-specific binding was measured in the presence of an excess of unlabelled insulin.

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3. RESULTS

When added to intact adipocytes insulin induced an increased phosphorylation of phosphoproteins with apparent molecular masses 116 kDa and 40 kDa [9]. In the presence of *myo*-inositol-hexakisphosphate this effect of insulin was to a large extent blocked (Fig. 1A). Inositol-phosphate alone was seen also to slightly increase phosphorylation of the 65-kDa phosphoprotein, but this was not so for glucose derivatives that blocked the insulin effect (see below). As previously noted the effect of insulin on phosphorylation of the 40-kDa protein was variable and not always apparent [9]. Inositol-hexakisphosphate, similarly, blocked the ability of insulin to counteract the protein phosphorylations increased by cAMP elevation in response to isoproterenol (Fig. 1B), so that in the presence of the inositol-phosphate the effect of isoproterenol on protein phosphorylation predominates over that of insulin.

The insulin mimicker POS enhances the phosphorylation of the same 116-kDa and 40-kDa phosphoproteins as does insulin [9]. And, as was found for insulin, inositol-hexakisphosphate, and also glucose-6-phosphate (data not shown), blocked the increased phosphorylation of these phosphoproteins in response to POS (Fig. 2A). Inositol-phosphate seemed to block the effects of POS and insulin on protein phosphorylation through the same mechanism, because further addition of POS could overcome the inhibition of insulin due to inositol-phosphate (Fig. 2B).

myo-Inositol-1-monophosphate (Fig. 3A), glucosamine (Fig. 3B) or glucose-6-phosphate (data not shown), compounds that have structural similarities to POS, also blocked the effect of insulin on protein phosphorylation. Glucose, glucose-6-sulphate, N-acetyl-glucosamine, at 80 mM, *myo*-inositol or *myo*-inositol-hexakissulphate, at 20 mM, had no effect (data not shown).

The insulin blocking effect of inositol-1-monophosphate and glucose-6-phosphate appeared to be exerted at the outside of the cells because these radiolabelled compounds were not taken up by the cells during the course of incubation: $0.053 \pm 0.004\%$ (mean $\pm$ SD, $n = 5$) and $0.047 \pm 0.003\%$ of D-*myo*-[2-^{3}H]inositol-1-monophosphate (Amersham) at 10 mM, or $0.055 \pm 0.003\%$ and $0.056 \pm 0.002\%$ of D-[U-^{14}C]glucose-6-

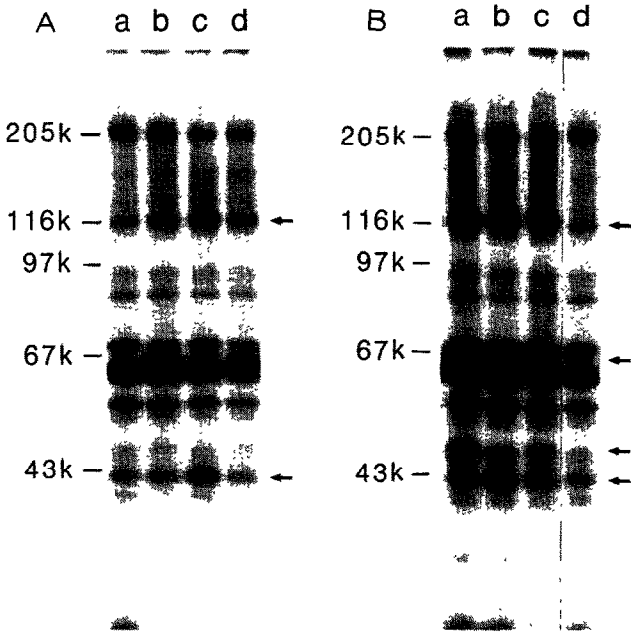

Fig. 1. Effect of inositol-hexakisphosphate on phosphorylation control by insulin. Adipocytes were prepared and incubated as described in section 2. Shown are autoradiographs after SDS-PAGE of total cell-protein. (A) (Lane a) control with no additions; (b) 20 mM inositol-hexakisphosphate; (c) 150 pM insulin; (d) 150 pM insulin + 20 mM inositol-hexakisphosphate. (B) (a) 100 nM isoproterenol; (b) 100 nM isoproterenol + 150 pM insulin; (c) 100 nM isoproterenol + 150 pM insulin + 20 mM inositol-hexakisphosphate; (d) control with no additions. The positions of the following marker proteins are indicated: myosin (205 kDa), β-galactosidase (116 kDa), glycogen phosphorylase (97 kDa), bovine serum albumin (67 kDa), and ovalbumin (43 kDa). Also indicated are 116-kDa, 65-kDa, 47-kDa and 40-kDa phosphoproteins.

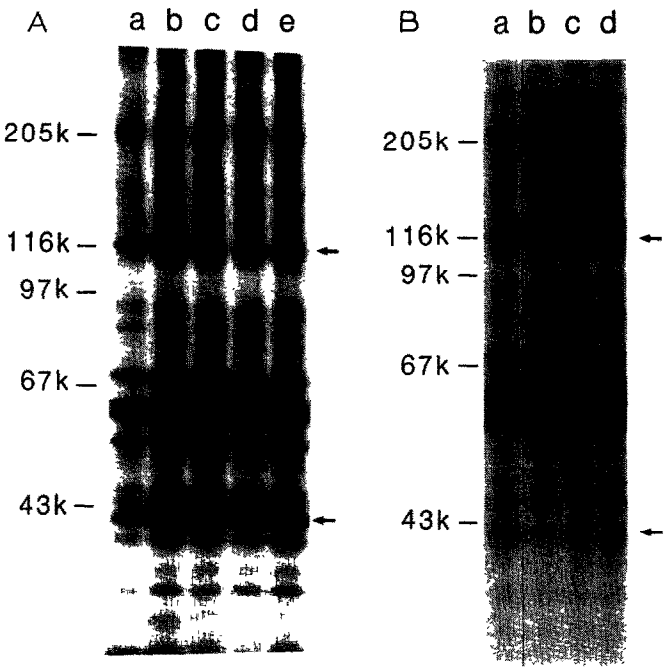

Fig. 2. Effect of inositol-hexakisphosphate on phosphorylation control by POS. Adipocytes were prepared and incubated as described in section 2. Shown are autoradiographs after SDS-PAGE of total-cell protein. (A) (Lane a) control with no additions; (b) 1 nM insulin; (c) 25 mM inositol-hexakisphosphate; (d) 15 μM POS + 25 mM inositol-hexakisphosphate; (e) 15 μM POS. (B) (a) control with no additions; (b) 200 pM insulin + 10 mM inositol-hexakisphosphate; (c) 200 pM insulin + 10 mM inositol-hexakisphosphate + 25 μM POS; (d) 200 pM insulin. The positions of the marker proteins described in the legend to Fig. 1 are indicated. Also indicated are 116-kDa and 40-kDa phosphoproteins.

phosphate (Amersham) at 50 mM was associated with the cells after <0.5 and 25 min incubation, respectively, and centrifugation through dinonylphthalate [26]. This corresponded to the trapped medium around the cells as determined with [^{3}H]sucrose [26].

These insulin-blockers did not obstruct the interaction between insulin and its receptor, since insulin stimulated tyrosine phosphorylation of a 95 kDa protein, most likely the insulin receptor, was not inhibited by the insulin-blockers (Fig. 4). The insulin stimulated increase in tyrosine phosphorylation demonstrates that insulin can activate its receptor tyrosine protein kinase also in the presence of the insulin-blockers. Moreover, ^{125}I-labelled insulin binding to cells was not impaired by the insulin-blockers (data not shown).

4. DISCUSSION

Intracellular phosphorylation controlled by insulin was blocked without inhibiting insulin stimulation of its receptor tyrosine protein kinase activity, or insulin binding to its receptor, the earliest events in insulin signalling. Hence, the blocking effect was likely exerted at a post-receptor step. The findings, furthermore, suggest that this post-receptor step is extracellular, because the intracellular phosphorylation control by insulin seemed to be blocked at the outside of the cell. Our data can not exclude that small amounts of the sugar phosphates entered the cells, but this is not likely to explain the observed inhibitions since: (i) inositol-phosphates are known not to enter cells, without prior permeabilization of the cells, in order to have effects on cellular metabolism; and (ii) glucose-6-phosphate is a natural constituent of the cytosol where its steady state concentration increases from 0.24 mM to 0.57 mM in response to insulin [27]. During preparation of this article it was reported that inositol phosphate and glucosamine blocked the effect of insulin also on lipogenesis [28].

POS exerts its insulin-like effects when added to intact cells [9]. It thus seems possible that a mechanism for cellular uptake of POS exists in adipocytes (cf [29]) and that this is inhibited by the, with POS structurally related, insulin-blockers. Phosphoinositolglycans that are structurally similar to POS anchor a number of proteins to the plasma membrane at the cell surface [30–34]. Of these alkaline phosphatase [32], lipoprotein lipase [34] and a proteoglycan ([31], L.A. Fransson, personal communication) has been shown to be released at the outside of cells in response to insulin stimulation. The proteoglycan, attached to the inositolglycan, was shown to be taken up by cells in a receptor mediated way and this uptake was blocked by inositol-phosphates and glucose-6-phosphate [31]. It is possible that similar mechanisms operate in the generation and action of POS in response to insulin. In support of such an idea the parent glycophospholipid of POS has been shown to be largely located to the outside of the plasma membrane [35,36] and substances similar or identical to POS to be released in the incubation medium of insulin stimulated cells [12,32]. Furthermore, POS and insulin exhibited similar time-courses of protein phosphorylation when added to intact cells [9].

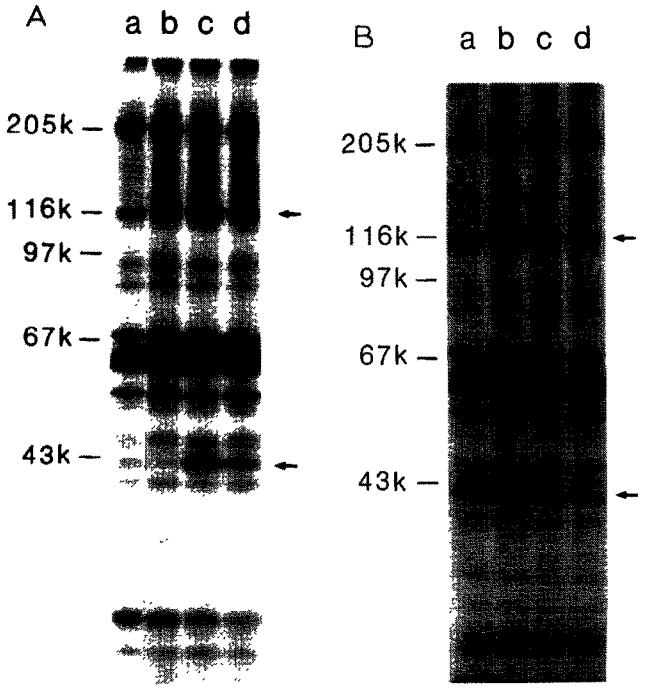

Fig. 3. Effects of D-*myo*-inositol-1-monophosphate and D-glucosamine on phosphorylation control by insulin. Adipocytes were prepared and incubated as described in section 2. Shown are autoradiographs after SDS-PAGE of total-cell protein. (A) (Lane a) control with no additions; (b) 20 mM inositol-1-monophosphate; (c) 150 pM insulin; (d) 150 pM insulin + 20 mM inositol-1-monophosphate. (B) (a) control with no additions; (b) 150 pM insulin; (c) 150 pM insulin + 80 mM glucosamine; (d) 80 mM glucosamine. The positions of the marker proteins described in the legend to Fig. 1 are indicated. Also incidated are 116-kDa and 40-kDa phosphoproteins.

Fig. 4. Effect of insulin-blockers on autophosphorylation of the insulin receptor on tyrosine. Adipocytes were prepared and incubated as described in section 2. After SDS-PAGE the gel was electroblotted against a nitrocellulose filter for immunoblotting analysis with antibodies against phospho-tyrosine. ^{125}I-Labelled protein G was used for detection of bound antibodies. Shown is an autoradiographic replica of the filter. (Lane a) control with no additions; (b) 9 nM insulin; 9 nM insulin + 20 mM inositol-hexakisphosphate; (d) 9 nM insulin + 80 mM glucose-6-phosphate; (e) 9 nM insulin + 80 mM glucosamine. The β-subunit of the insulin receptor was identified by its apparent molecular mass and by its enhanced tyrosine phosphorylation in response to insulin. Indicated is the position of glycogen phosphorylase (97 kDa) run on the same gel. The region containing the β-subunit is shown.

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