Evidence that the insulin receptor-associated protein kinase acts as a phosphatidylinositol kinase

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Insulin receptor preparations from human placenta at various states of purity were shown to catalyze insulin-stimulated phosphate incorporation from [γ-^{32}P]ATP into endogenous (membrane) and exogenous phosphatidylinositol. Our data suggest that the insulin receptor associated protein (tyrosine) kinase can act as a phosphatidylinsositol kinase, and that this mechanism may be of physiological importance in signal transduction of insulin.

Insulin receptor *Tyrosine kinase* *Signal transduction* *Phosphoinositide system* *Second messenger*

1. INTRODUCTION

The physiological significance of the insulin receptor-associated tyrosine protein kinase in the biological actions of insulin is not yet clear. Although various exogenous proteins like histone, casein and others were shown to be phosphorylated by the receptor kinase none of them appeared likely as a physiological substrate. The insulin-stimulated phosphorylation of actin by placental membranes [1] may perhaps be more relevant in this respect. These experiments further indicated that actin was phosphorylated at serine residues rather than at tyrosine residues when crude receptor preparation, not purified by lectin affinity chromatography, was used [2]. This suggested, in accordance with [3] the involvement of (a) serine-specific protein kinase(s) which in some way seem(s) to be activated by insulin through the receptor-associated tyrosine kinase. Our studies reported here may shed some new light on these regulatory interrelationships.

2. MATERIALS AND METHODS

Solubilization and purification of the insulin receptor from human placenta membranes, assay of insulin binding activity (IBA), protein determination, SDS–polyacrylamide gel electrophoresis was done as in [1,4]. Phosphatidylinositol (soybean, approx. 98%), phosphoinositides, phosphatidylserine, phosphatidylethanolamine were from Sigma (St Louis, MO); for other reagents and methods see [1,4].

2.1. *Standard phosphorylation assay*

The standard incubation medium consisted of 50 μM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 2.5 mM vanadate, 50 μM [γ-^{32}P]ATP (2.5–15 μCi), and the other components as specified in the figure and table legends. The final volume was 100 μl. Phospholipids were dried under a stream of nitrogen, evaporated in vacuo, and suspended by brief sonification in 50 mM Tris/maleate, pH 7.4. The samples were preincubated 25 min without or with insulin, 5×10^{-7} M, before the reaction was commenced by the addition of [γ-^{32}P]ATP. After 25 min at 23°C the reaction was terminated by addition of 300 μl 1 N HCl. Lipids were extracted with 300 μl chloroform/methanol, 2:1 (v/v) and then with 300 μl chloroform. The extracts were pooled and washed three times with 500 μl methanol/1 N HCl, 1:1 (v/v). Total lipid radioactivity of the

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chloroform extract was measured by liquid scintillation spectrometry.

2.2. *Separation of lipids by TLC*

Lipids were separated on silica gel 60 thin layer plates (Merck, Darmstadt) which were impregnated with 1% potassium oxalate in methanol/water, 2:3 (v/v), dried at room temperature and activated at 110°C for 15 min. 20 μl samples of the lipid extracts were applied to the plates and the chromatograms were developed in a paper lined chamber with chloroform/acetone/methanol/acetic acid/water, 40:15:13:12:8, by vol. [5]. The lipids were detected with iodine vapor, and the ^{32}P-labelled spots by autoradiography on Kodak X-Omat film. The spots were scraped off and counted for radioactivity in a liquid scintillation counter.

3. RESULTS

When a partially purified insulin receptor preparation from placenta membranes (wheat germ eluate [1]) was incubated with [γ-^{32}P]ATP and phosphatidylinositol (PtdIns) there was a rapid, time-dependent incorporation of ^{32}P-radioactivity into the lipid extracts which was clearly increased by insulin (fig.1). As shown in fig.2 separation of the lipids on TLC, and autoradiography yielded two labelled spots, a major one migrating with phosphatidylinositol 4-phosphate (PtdIns 4P) and a smaller one migrating with phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5 P_2). The spot remaining at the origin was not identified. The labelling of the phosphoinositides, particularly of PtdIns 4P was increased by insulin. Quantitative data on ^{32}P-incorporation into the corresponding fractions scraped from the plate are given in the lower panel of fig.2. It seems worth mentioning that insulin also clearly stimulated the phosphorylation of endogenous PtdIns of the membranes. It should be further noted that insulin receptor preparations after several purification

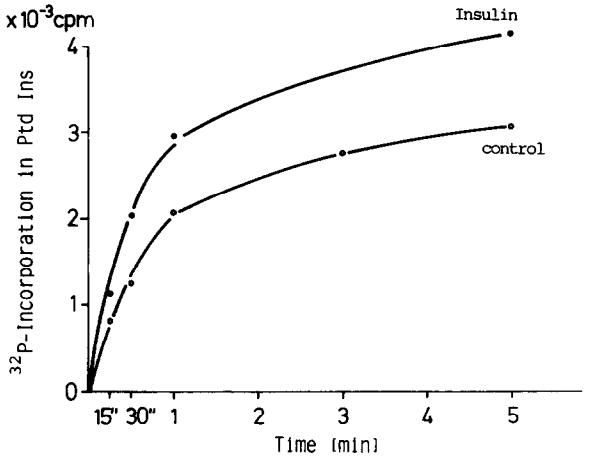

Fig.1. Phosphorylation of phosphatidylinositol by a partially purified insulin receptor preparation from human placenta. PtdIns, 1.4 mg was incubated with 'wheat germ eluate' (1) corresponding to 0.63 mU/IBA in the standard incubation mixture as in section 2. The final volume was 700 μl, and 100 μl samples were taken at the times indicated for lipid extraction and scintillation counting. There was essentially no ^{32}P-incorporation when the receptor preparation was boiled prior to incubation.

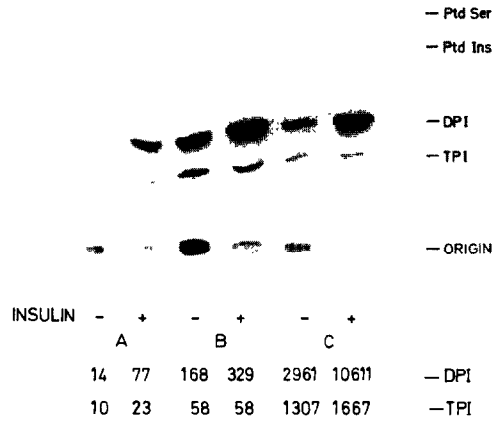

Fig.2. Thin layer chromatographic analysis of ^{32}P-labelled phosphoinositides. Phosphorylation, extraction, separation and autoradiography of lipids was done as in section 2. (A,B) Triton X-100 solubilized placenta membranes corresponding to 0.5 mg protein per assay without (A) or with (B) addition of PtdIns, 1.6 mg/ml. (C) Insulin receptor purified on Sepharose 6B-Cl and sucrose density gradient centrifugation (IBA 10 mU/mg [4]) corresponding to 9 μg protein per assay, and PtdIns (2 mg/ml). DPI, PtdIns 4P; TPI, PtdIns 4,5 P_2. Quantitative data on ^{32}P-labelling of the respective phosphoinositides calculated as cpm incorporated per μg protein are given at the bottom of the figure.

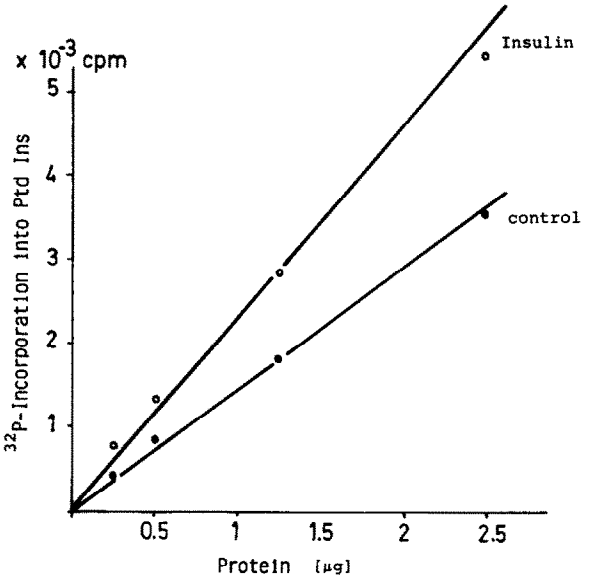

Fig.3. Phosphorylation of phosphatidylinositol as a function of protein concentration. Phosphorylation was carried out in the standard assay system containing PtdIns (1.6 mg/ml) and increasing amounts of highly purified insulin receptor (Sepharose 6B-Cl-sucrose gradient centrifugation – Sepharose 6B-Cl-hydroxyapatite [4], IBA > 50 mU/mg protein) corresponding to the protein concentrations indicated on the abscissa. Lipids were extracted as indicated in section 2 and counted for radioactivity.

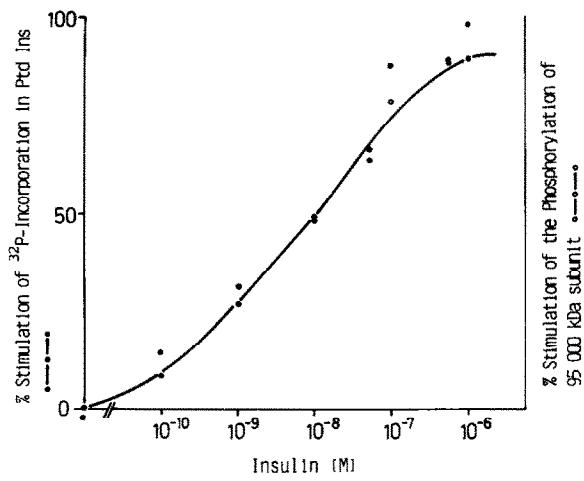

Fig.4. Insulin dose–response relationship of the phosphorylation of phosphatidylinositol and of the insulin receptor. Insulin receptor preparation ('wheat germ eluate' [1]) corresponding to 0.16 mU IBA/assay was incubated in the standard phosphorylation mixture containing PtdIns, 0.1 mg/assay in the presence of increasing insulin concentrations as indicated on the abscissa. Lipids were extracted and counted for radioactivity. Protein radioactivity was measured after TCA precipitation, washing the precipitate 4 times with 5% TCA/20 mM sodium pyrophosphate, and dissolving it in a solution containing 2% SDS, 20 mM Tris and 100 mM DTE. As judged from SDS–PAGE and autoradiography, over 90% of the radioactivity resided in the 95 kDa receptor subunit.

steps still effected insulin-stimulatable and protein concentration dependent PtdIns phosphorylation (figs.2,3) suggesting that this activity is related to the receptor-associated protein kinase. This was supported by the finding that the stimulation by insulin of the phosphorylation of the receptor β-subunit and of PtdIns displayed the same dose–response relationship (fig.4). Phosphorylation of PtdIns was dependent on substrate concentrations reaching near saturation at 2 mg/ml (data not shown).

4. DISCUSSION

Receptor-mediated hydrolysis of the phosphoinositides by phospholipase C plays a key role in signal transduction of many hormones and agonists that act through calcium as intracellular mediator (for review see [6]). The reaction products: inositol trisphosphate and diacylglycerol seem to function as important second messengers the former leading to mobilisation of intracellular calcium [7,8], and the latter stimulating protein phosphorylation by activation of protein kinase C [9]. Our studies presented here provide strong evidence that the insulin receptor-associated protein kinase, similar to the RSVpp $60^{v\text{-}src}$ tyrosine kinase [10], displays the activity of an inositol lipid kinase that catalyzes the phosphorylation of PtdIns. This may shed new light on the regulatory mechanisms following the binding of insulin to its receptor. It seems possible that the first target of the autophosphorylated protein kinase is membrane-bound PtdIns rather than some protein not yet detected. In our studies the major product of insulin-stimulated phosphorylation of PtdIns was PtdIns 4P while much smaller amounts of PtdIns 4,5 P_2 were present. This may be explained as being due to rapid cleavage or dephosphoryla-

tion of the latter compound by phosphodiesterase or monoesterase, respectively, that may be present, even in purified receptor preparations.

The release of inositol trisphosphate in our system has not yet been established. That inositol trisphosphate may in fact act as a second messenger of insulin is suggested from recent work of our laboratory showing that addition of micromolar concentrations of inositol trisphosphate to permeabilized adipocytes leads to an activation of pyruvate dehydrogenase quite similar to that observed with insulin [11]. Moreover, insulin was also found to activate phospholipase C in fat cells [12].

In conclusion, present available evidence opens the intriguing possibility that receptor-mediated regulation of the polyphosphoinositide system plays an important role in the signal transduction of insulin. The idea that this may involve changes in the level of intracellular calcium has been brought forward several times yet has remained controversial [13]. Tentatively, the sequence of events following formation of the insulin–receptor complex may be proposed roughly as follows: (i) Autophosphorylation and activation of the receptor-associated protein (tyrosine) kinase; (ii) phosphorylation of PtdIns; (iii) cleavage of PtdIns 4,5 P_2 by phospholipase C (insulin activated) yielding inositol trisphosphate and diacylglycerol; (iv) inositol trisphosphate as messenger for metabolic effects (e.g., PDH activation) probably via Ca^{2+} mobilisation; (v) diacylglycerol (plus Ca^{2+}) as second messenger for protein (serine) phosphorylation by protein kinase C. This latter mechanism has not yet received direct experimental support although protein kinase C is a widely distributed enzyme also shown to be present in human placenta (T. Urumow, unpublished).

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