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Insulin provokes co-ordinated increases in the synthesis of phosphatidylinositol, phosphatidylinositol phosphates and the phosphatidylinositol–glycan in BC3H-1 myocytes

Robert V. FARESE,* Denise R. COOPER, T. Suman KONDA, Govindan NAIR, Mary L. STANDAERT and Robert J. POLLET

James A. Haley Veterans Hospital and Departments of Internal Medicine and Biochemistry, University of South Florida College of Medicine, Tampa, FL 33612, U.S.A.

BC3H-1 myocytes were cultured in the presence of [^{3}H]inositol or [^{3}H]glucosamine during their entire growth cycle to ensure that all lipids containing inositol and glucosamine were labelled to isotopic equilibrium or maximal specific radioactivity. After such labelling, a lipid (or group of lipids), which was labelled with both inositol and glucosamine, was observed to migrate between phosphatidylinositol 4-phosphate and phosphatidylinositol (PI) in two different t.l.c. systems. Insulin provoked rapid, sizeable, increases in the inositol-labelling of this lipid (presumably a PI–glycan), and these increases were similar to those observed in PI and PI phosphates. Our results indicate that insulin provokes co-ordinated increases in the net synthesis *de novo* of PI and its derivatives, PI phosphates and the PI–glycan, in BC3H-1 myocytes. This increase in synthesis of PI may serve as the mechanism for replenishing the PI–glycan during stimulation of its hydrolysis by insulin. Moreover, increases in the content of the PI–glycan may contribute to increases in the generation of head-group 'mediators' during insulin action.

INTRODUCTION

Insulin is known to increase the net synthesis of phosphatidylinositol (PI) and its mono- and bis-phosphates (PIP and PIP_2) in BC3H-1 myocytes [1,2] and rat adipocytes [2–4]. Saltiel *et al.* [5] have reported that insulin activates a phospholipase C which hydrolyses a PI–glucosamine complex (PI–glycan) in BC3H-1 myocytes, and the head group of this, or other related glycolipids, is thought to account for certain actions of insulin in several tissues [5–9]. In [^{3}H]inositol- and [^{3}H]glucosamine-labelling experiments in BC3H-1 myocytes, Saltiel *et al.* [5] have also observed that the hydrolysis of the PI–glycan is accompanied by increases in labelling by both precursors. However, in these relatively short-term labelling experiments, it was unclear whether the increase in [^{3}H]inositol and [^{3}H]glucosamine incorporation reflected an increase in specific radioactivity of the PI-glycan, as would occur with hydrolytic turnover and simple compensatory resynthesis, or an increase in net synthesis of the PI–glycan *de novo*. To address this issue, we used long-term labelling with [^{3}H]inositol or [^{3}H]glucosamine in BC3H-1 myocytes.

EXPERIMENTAL

BC3H-1 myocytes were cultured in 35 mm-diam. plates as described previously [1,2] except that 10 μCi of [^{3}H]inositol (American Radiolabeled Chemicals; sp. radioactivity 15 Ci/mmol) or 2 or 10 μCi of [^{3}H]-glucosamine (New England Nuclear; sp. radioactivity 30 Ci/mmol) was added to 3 ml of Dulbecco's Modified Eagle's Medium, containing 20% (v/v) fetal-calf serum, at the time of subculturing. The cells were grown to confluence over 10 days without changing the media. (This medium contains approx. 40–50 μM-inositol.) On the day of experiment, the media were removed, and the cells were washed and incubated in inositol-free Dulbecco's phosphate-buffered saline (DPBSGA), containing 0.1 mM-$CaCl_2$, 1 mg of glucose/ml and 1 mg of radioimmunoassay-grade bovine serum albumin/ml. After 90 min of equilibration at 37 °C, insulin (200 nM, a maximally effective dose [1,2]), was added as indicated and incubation was continued for various times. After incubation, media were removed and 2 ml of cold 5% (w/v) trichloroacetic acid was added. Precipitates were transferred to tubes and extracted with chloroform/methanol/0.01 M-HCl (3:1:1, by vol.). Lipid extracts were washed with 3 × 3 ml of 0.01 M-HCl, concentrated, and chromatographed on (system A) silica-gel t.l.c. plates impregnated with 10% (w/v) magnesium acetate, developed with chloroform/methanol/4.3 M-NH_3 (19:13:4, by vol.) (see [1,2]). In some experiments, the lipid extracts were chromatographed (system B) on silica-gel plates impregnated with 1% potassium oxalate, developed with chloroform/acetone/methanol/acetic acid/water (5:2:2:1:1, by vol.) (see [5]). In some experiments, the acid-soluble fractions were analysed for [^{3}H]inositol labelling of total inositol phosphates (mono- plus poly-phosphates) as described previously [2].

Abbreviations used: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PA, phosphatidic acid.

* To whom reprint requests should be sent.

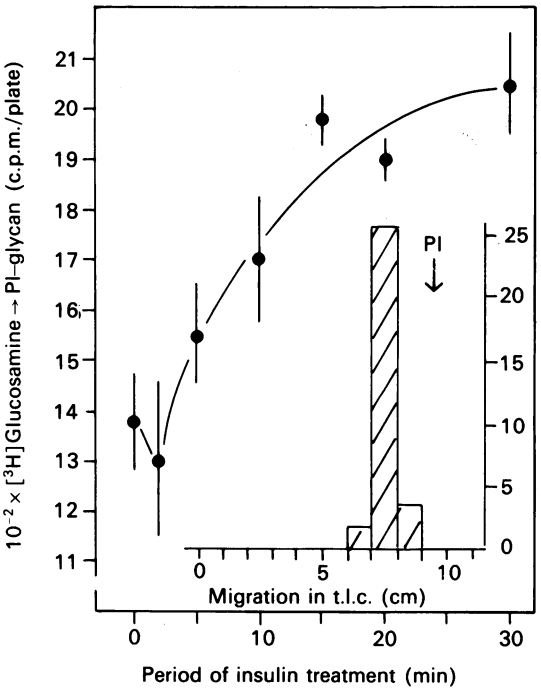

Fig. 1. Time course of insulin effects on [^{3}H]glucosamine-labelled lipids in BC3H-1 myocytes

[^{3}H]Glucosamine (2 μCi) was added to the culture medium at the time of subculturing. After 10 days of cellular growth to confluence, media were replaced with DPBSGA, and after 90 min equilibration, cells were exposed to 200 nM-insulin for 2–30 min, as indicated. (All cells, including controls, were incubated for identical times, and only the duration of treatment with insulin was varied.) The inset shows the analysis of glucosamine-labelled lipids over the lower two thirds (starting at the origin) of a thin-layer chromatogram (system A) of a representative sample, expressed as c.p.m. ($\times 10^{-2}$)/cm of migration in the t.l.c. system. (A smaller labelled peak was also seen above PI in the phosphatidylcholine plus phosphatidylethanolamine area, but this did not correlate with [^{3}H]inositol labelling.) Insulin-induced changes in labelling, expressed as c.p.m. ($\times 10^{-2}$)/culture plate of myocytes, of the lipid in the peak migrating behind PI, i.e. the 'PI–glycan', are shown by ●. Values are means ± S.E.M.. for four determinations.

RESULTS

As shown in Fig. 1, after 10 days of labelling with [^{3}H]glucosamine, a major peak of radioactivity was found to migrate several centimetres behind PI in t.l.c. system A. In this experiment, upon stimulation with insulin, there was a small decrease in this labelled lipid over the first few minutes, followed by a subsequent increase (in other experiments, the initial decrease was much more substantial; see below). In these experimental conditions, i.e. after 10 days of labelling, little or no radioactivity was observed in PI or other more rapidly migrating lipids (e.g. phosphatidylcholine, phosphatidylethanolamine, neutral lipids), and it is therefore clear that [^{3}H]-glucosamine labelling of the slower-migrating lipid did not reflect conversion of [^{3}H]glucosamine into glycerol or fatty acids.

After 10 days of labelling with [^{3}H]inositol, three peaks of inositol-labelled lipids were found after purification by t.l.c. in system A (Fig. 2): (*a*) one containing PIP and PIP_2, which were unresolved at the origin; (*b*) PI, the most mobile [^{3}H]inositol-labelled lipid; and (*c*) a third inositol-containing lipid of intermediate chromatographic mobility, which co-migrated with the [^{3}H]-glucosamine-labelled lipid, i.e. several centimetres behind PI (see Fig. 1). Moreover, insulin provoked sizeable increases in the labelling of all inositol lipids,

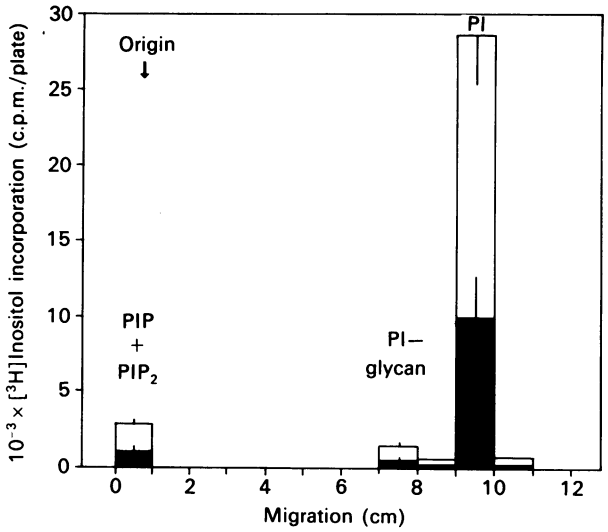

Fig. 2. Thin-layer chromatograms of [^{3}H]inositol-labelled lipids in control (black areas) and insulin-treated (total bars) BC3H-1 myocytes

[^{3}H]inositol (10 μCi) was added to the culture medium at the time of subculturing. After 10 days of cellular growth to confluence, media were replaced with DPBSGA, and after 90 min of equilibration cells were treated with or without 200 nM-insulin for 20 min, as indicated. Shown here are 1 cm fractions of the lower two-thirds of thin-layer chromatograms in system A (there was little or no radioactivity in the upper third). Values are means ± S.E.M. for four determinations.

Table 1. Simultaneous comparison of rapid insulin-induced changes in [^{3}H]glucosamine- or [^{3}H]inositol-labelled phosphatidylinositol–glycan

Myocytes in 35 mm-diam. plates were labelled simultaneously for 10 days by adding either 10 μCi of [^{3}H]glucosamine or 10 μCi of [^{3}H]inositol to the medium at the time of subculturing. After 10 days of cellular growth to confluence, media were replaced with DPBSGA, and, after 90 min of equilibration at 37 °C, cells were treated with 200 nM-insulin for the indicated times. PI–glycan areas were identified by scanning of the thin-layer chromatograms, scraped into vials, and counted for radioactivity. Results are means ± S.E.M. for four determinations. The mean percentage changes are shown in parentheses.

	Labelled PI–glycan (c.p.m./plate)	
Treatment	[^{3}H]Glucosamine	[^{3}H]Inositol
Control	32997 ± 1047	1233 ± 158
Insulin, 1 min	29036 ± 11794 (−12%)	1082 ± 220 (−12%)
Insulin, 2 min	20809 ± 3360 (−37%)	716 ± 93 (−42%)

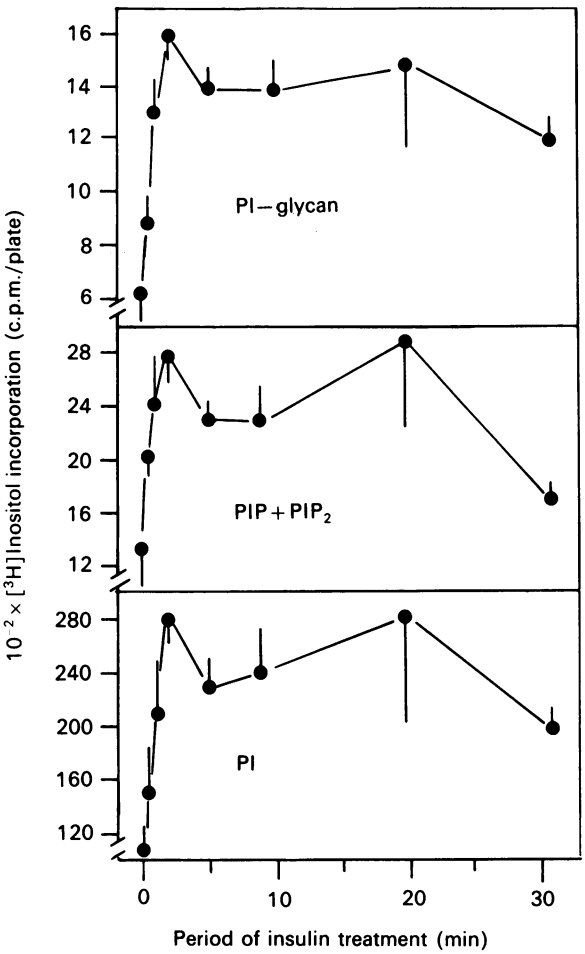

Fig. 3. Time course of insulin-induced increases in [^{3}H]inositol-labelled lipids in BC3H-1 myocytes

Cells were labelled with 10 μCi of [^{3}H]inositol for 10 days as described in Fig. 2 and in the Experimental section. Media were replaced with DPBSGA, and after 90 min of equilibration cells were exposed to 200 nM-insulin for the times indicated over the ensuing 30 min. The three peaks of [^{3}H]inositol-labelled lipids were resolved by t.l.c. in system A as shown in Fig. 2, and counted for radioactivity. Values are means $\pm$ S.E.M. for four determinations.

and, in the experiment portrayed in Fig. 3, these increases were maximal within 2 min, persisted for 20 min and diminished somewhat thereafter. In other experiments, the increases in [^{3}H]inositol labelling of the PI–glycan were preceded by short initial declines during the first 1–2 min of insulin treatment, comparable with that seen with [^{3}H]glucosamine labelling (see above). A striking example of the latter is shown in Table 1, and it should be noted that initial decreases were similar, as percentages, for the PI–glycan, as labelled with either radioisotope. This variability in observing an initial decrease in the PI–glycan may reflect experiment-to-experiment variability in the rate of degradation of the PI–glycan, or variability in the rapidity of PI synthesis and/or its conversion into the PI–glycan.

We found similar [^{3}H]inositol- or [^{3}H]glucosamine-labelled lipids after purification by t.l.c. in system B. As reported by Saltiel *et al.* [5], who used this chromatography system, a glycolipid containing [^{3}H]inositol and [^{3}H]glucosamine was found to migrate between PIP and PI, and addition of insulin to [^{3}H]inositol-labelled cells provoked similar changes (i.e. variable initial decreases, followed by increases) in the labelling of this lipid (results not shown).

As reported previously [2], we failed to observe significant insulin-induced increases in labelling of inositol phosphates after 10 days of prelabelling with [^{3}H]inositol. In the experiment shown in Fig. 3, total inositol phosphates were 14678$\pm$916, 16625$\pm$429, 15100$\pm$583, 14901$\pm$953, 14979$\pm$934, 17062$\pm$717, 15400$\pm$288 and 14362$\pm$238 c.p.m./plate, in control myocytes and after 0.5, 1, 2, 5, 10, 20 and 30 min of insulin treatment respectively.

DISCUSSION

In accordance with the reports by Saltiel *et al.* [5–7], we found that BC3H-1 myocytes contain a lipid (or group of lipids), which migrates between PIP and PI in two different t.l.c. systems, and appears to contain both glucosamine and inositol. Furthermore, insulin treatment provoked changes (i.e. variable initial decreases, followed by increases) in this lipid, and, in accordance with evidence presented by Saltiel *et al.* [5–7], this lipid appears to be the insulin-sensitive PI–glycan.

Under the present experimental conditions, there was little question that inositol-containing lipids were labelled with [^{3}H]inositol to isotopic equilibrium (or to a specific radioactivity that would be maximal at the beginning of the experimental treatment period), as the cells were exposed to labelled inositol during their entire growth cycle. Thus it may be estimated that, in the BC3H-1 myocyte, the mass of the PI–glycan is approx. 5% of that of PI, and this is similar to that of PIP and PIP_2, which combined are equal to 10% of PI.

As it is likely that inositol lipids were labelled to isotopic equilibrium or maximal specific radioactivity, the insulin-induced increases in PI, PIP, PIP_2 and the PI–glycan can be taken as strong evidence that insulin increases the net synthesis and mass of these substances. (It is possible that [^{3}H]inositol could be diluted from metabolism of glucose to inositol, but this metabolism would be expected to be increased by insulin and would therefore only result in an underestimation of the insulin effect.) Moreover, the fact that all of these inositol-containing lipids changed in parallel suggests that these effects of insulin are co-ordinated. The simplest explanation for these co-ordinated increases is that insulin increases net synthesis *de novo* of PI, which then serves as substrate for subsequent synthesis of its derivatives, i.e. PIP, PIP_2 and the PI–glycan.

It may be noted that, although initial transient insulin-induced decreases in the PI–glycan were comparable in inositol- and glucosamine-labelled cells (see Table 1), subsequent increases in [^{3}H]glucosamine labelling of the PI–glycan were substantially less than increases in [^{3}H]inositol labelling of this lipid (cf. Figs. 1 and 3). This was regularly observed and probably reflects that there may be relatively little inositol synthesis or metabolic losses in the myocyte culture system (save for incorporation into inositol lipids and their inositol phosphate derivatives), whereas glucosamine is undoubtedly continually being synthesized from glucose over the 10-day labelling period. (Note that the culture medium contains inositol and glucose, but not glucosamine). Thus the

specific radioactivity of glucosamine may have decreased much more than that of inositol over the 10-day culture period, and during subsequent insulin treatment the newly synthesized PI–glycan may be labelled with glucosamine of much lower specific radioactivity than that synthesized much earlier, particularly during the exponential growth phase, which occurs soon after subculturing. For these reasons, [^{3}H]inositol would be expected to be a better indicator of net synthesis of the PI–glycan than [^{3}H]glucosamine during insulin stimulation. Nevertheless, even though [^{3}H]glucosamine labelling may underestimate the real insulin-induced increases in the PI–glycan, increases (after initial decreases) were regularly observed, and this clearly reflects net synthesis rather than simple turnover.

It is uncertain whether insulin-induced increases in the content of some of the inositol lipids may be due to decreased hydrolytic turnover, rather than increased synthesis. Along these lines, despite remarkable increases in the mass of all inositol lipids in the experiment of Fig. 3, inositol phosphates did not change appreciably, and this suggests that there is a decreased fractional rate of turnover, but an unchanged total turnover, of inositol lipids through this pathway. Although we did not measure the release of PI–glycan head groups, Saltiel *et al.* [5] have documented that this is stimulated by insulin in BC3H-1 myocytes, probably through stimulated hydrolysis of the PI–glycan by a relatively specific phospholipase C, which does not hydrolyse PI directly. Thus, although phospholipase C-mediated hydrolysis of PIP_2, PIP and PI does not appear to be increased by insulin, conversion of PI into the PI–glycan is apparently stimulated, and this argues against the possibility that increases in PI content are due to decreased hydrolytic turnover. It is possible, however, that hydrolytic turnover of PIP and PIP_2 may be diminished during insulin treatment.

As reported previously [10] the increase in PI synthesis *de novo* which is observed between 2 and 20 min of insulin treatment in BC3H-1 myocytes appears to occur largely at the expense of pre-existing phospholipids, rather than from phosphatidic acid (PA) synthesized *de novo* from glycerol 3-phosphate and fatty acyl-CoA. Phospholipids, such as phosphatidylcholine and phosphatidylethanolamine, may be converted into PI through diacylglycerol and PA, by a stimulation of either phospholipase C or phospholipase D. In either case, the increase in PA would lead to an increase in CDP-diacylglycerol, and ultimately to PI and its derivatives.

It is of interest that net synthesis of PI, PIP and PIP_2 has been observed, not only with insulin treatment in several tissues [1–3,10], but also with corticotropin and angiotensin II treatment in adrenal tissue [11,12]. A PI–glycan has now been found in adrenal tissue [13], and this raises the possibility that a number of hormones may use synthesis of PI *de novo* as the back-up mechanism to resynthesize the PI–glycan during hormone-induced hydrolysis of the latter by a specific phospholipase C. Moreover, increases in the synthesis of the PI–glycan through these mechanisms could serve to amplify the hydrolysis response and increase the generation of head-group 'mediators' for insulin and perhaps other hormones.

In summary, our findings suggest that the mechanism for replenishing the PI–glycan, after its hydrolysis during insulin treatment in BC3H-1 myocytes, is through synthesis of PI *de novo* (from PA derived both from hydrolysis of pre-existing non-inositol phospholipids and from PA synthesis *de novo* from glycerol 3-phosphate and fatty acyl-CoA), and subsequent rapid conversion of this PI into the PI–glycan. Resultant increases in the synthesis of the PI–glycan could enhance the production of head-group 'mediators' and contribute importantly to mediator-dependent biological effects of insulin.

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