Mechanisms whereby insulin increases diacylglycerol in BC3H-1 myocytes

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We previously suggested that insulin increases diacylglycerol (DAG) in BC3H-1 myocytes, both by increases in synthesis *de novo* of phosphatidic acid (PA) and by hydrolysis of non-inositol-containing phospholipids, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE). We have now evaluated these insulin effects more thoroughly, and several potential mechanisms for their induction. In studies of the effect on PA synthesis *de novo*, insulin stimulated [2-^{3}H]glycerol incorporation into PA, DAG, PC/PE and total glycerolipids of BC3H-1 myocytes, regardless of whether insulin was added simultaneously with, or after 2 h or 3 or 10 days of prelabelling with, [2-^{3}H]glycerol. In prelabelled cells, time-related changes in [2-^{3}H]glycerol labelling of DAG correlated well with increases in DAG content: both were maximal in 30–60 s and persisted for 20–30 min. [2-^{3}H]Glycerol labelling of glycerol 3-phosphate, on the other hand, was decreased by insulin, presumably reflecting increased utilization for PA synthesis. Glycerol 3-phosphate concentrations were 0.36 and 0.38 mM before and 1 min after insulin treatment, and insulin effects could not be explained by increases in glycerol 3-phosphate specific radioactivity. In addition to that of [2-^{3}H]glycerol, insulin increased [U-^{14}C]glucose and [1,2,3-^{3}H]glycerol incorporation into DAG and other glycerolipids. Effects of insulin on [2-^{3}H]glycerol incorporation into DAG and other glycerolipids were half-maximal and maximal at 2 nM- and 20 nM-insulin respectively, and were not dependent on glucose concentration in the medium, extracellular Ca^{2+} or protein synthesis. Despite good correlation between [^{3}H]DAG and DAG content, calculated increases in DAG content from glycerol 3-phosphate specific radioactivity (i.e. via the pathway of PA synthesis *de novo*) could account for only 15–30% of the observed increases in DAG content. In addition to increases in [^{3}H]glycerol labelling of PC/PE, insulin rapidly (within 30 s) increased PC/PE labelling by [^{3}H]arachidonic acid, [^{3}H]myristic acid, and [^{14}C]choline. Phenylephrine, ionophore A23187 and phorbol esters did not increase [2-^{3}H]glycerol incorporation into DAG or other glycerolipids in 2 h-prelabelling experiments; thus activation of the phospholipase C which hydrolyses phosphatidylinositol, its mono- and bis-phosphate, Ca^{2+} mobilization, and protein kinase C activation, appear to be ruled out as mechanisms to explain the insulin effect on synthesis *de novo* of PA, DAG and PC. Exogenously added non-specific phospholipase C (from *Clostridium perfringens*) increased [^{3}H]DAG in [^{3}H]glycerol-prelabelled cells, but apparently at the expense of pre-existing [^{3}H]PC/PE, rather than via synthesis of PA *de novo*. Thus, PC/PE hydrolysis may contribute to increases in DAG, but does not initiate the effect of insulin on synthesis of PA, DAG and PC *de novo*. The present findings provide further evidence that insulin increases synthesis *de novo* of PA, DAG and PC, but activation of this pathway can only partly account for observed increases in DAG content; and other mechanisms, such as hydrolysis of pre-existing lipids, also appear to be involved.

INTRODUCTION

Insulin is known to increase the synthesis of phosphatidic acid (PA), inositol phospholipids and other phospholipids in BC3H-1 myocytes [1,2] and rat adipose tissue [2–4]. This increase in general phospholipid synthesis occurs rapidly and is associated with increases in diacylglycerol (DAG) [1,2,5] and cytosolic and membrane-bound protein kinase C activity [6,7]. Moreover, activation of the DAG–protein kinase C signalling system is associated with increases in glucose transport, amino acid transport and/or pyruvate dehydrogenase activity in the BC3H-1 myocyte [8] and rat adipose [9,10] and cardiac [11] tissues. More recently, we have reported that insulin increases [^{3}H]glycerol incorporation into PA within seconds, and this PA is primarily converted directly into DAG and phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [5]. To explain concurrent increases in PI content, we have also postulated [5] that insulin increases DAG and PA by stimulating the hydrolysis of PC or other lipids in BC3H-1 myocytes. In the present paper, we present further evidence that

Abbreviations used: PA, phosphatidic acid: DAG, diacylglycerol; MAG, monoacylglycerol; TAG, triacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4′-phosphate; PIP_2, phosphatidylinositol 4,5′-bisphosphate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; DPBSGA, Dulbecco's phosphate-buffered saline containing $CaCl_2$, glucose and bovine serum albumin.

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insulin increases DAG by two mechanisms, i.e. synthesis *de novo* of PA, DAG and PC, and hydrolysis of preformed lipids; we also examined several potential mechanisms and metabolic requirements for these effects of insulin.

EXPERIMENTAL

BC3H-1 myocytes were cultured for 10 days to confluence, cellular maturity and maximal responsiveness to insulin as described previously [1,2,5]. On the day of the experiment, Dulbecco's Modified Eagle's Medium containing 20% fetal-calf serum was removed and replaced with Dulbecco's Phosphate-Buffered Saline (DPBSGA) containing 0.1 mM-$CaCl_2$, 5.6 mM-glucose and 1 mg of bovine serum albumin/ml (see [1,2]), unless indicated otherwise. In most experiments, cells were grown on 35 mm-diam. plates (0.5 mg of cellular protein) and 1 or 2 ml of DPBSGA buffer was used during the experimental period. In some experiments, the cells were grown on 60 mm or 100 mm-diam. plates, and 3 or 5 ml, respectively, of DPBSGA buffer was used. As specified in the text, the cells were incubated with [2-^{3}H]glycerol for 2 h or 3–10 days in the absence of treatments, and then insulin or other treatments were added and reactions were followed for various periods (1–30 min). In some experiments, [1,2,3-^{3}H]glycerol, [U-^{14}C]glucose, [9,10-^{3}H]-myristic acid, or [5,6,7,11,12,14,15-^{3}H]arachidonic acid was used instead of [2-^{3}H]glycerol during a 2 h or 20 h prelabelling period. In some experiments, insulin and [^{3}H]glycerol were added simultaneously. Reactions were usually stopped by addition of ice-cold 50% (v/v) methanol. Contents of plates were scraped off, transferred to glass tubes, and extracted with chloroform/methanol/water (or 0.01 M-HCl instead of water in experiments in which the PI–glycan was assessed) (2:1:1, by vol.), and centrifuged (2000 *g* for 10 min) to separate lipid and aqueous extracts.

Lipid extracts were washed three times with water (or 0.01 M-HCl) and analysed by t.l.c. Neutral lipids, including DAG, triacylglycerol (TAG) and monoacylglycerol (MAG), along with total phospholipids, were purified on silica-gel plates, with benzene/diethyl ether/ethanol/acetic acid (25:20:1:1, by vol.), followed by hexane/diethyl ether (47:3, v/v) as described previously [1,2,5]. For chromatographic separation of PA, PI, combined phosphatidylcholine and phosphatidylethanolamine (PC/PE) and total neutral lipids, we used thin-layer silica-gel plates impregnated with 10% (w/v) magnesium acetate and the solvent system chloroform/methanol/4.3 M-NH_3 (9:6:2, by vol.), as described previously [1,2]. In some experiments, PA was purified by t.l.c. with chloroform/pyridine/formic acid (50:30:7, by vol.), and PC and PE were separated by chromatography on Whatman LKSD silica-gel plates, developed with chloroform/methanol/water (75:25:3, by vol.) For separation of the PI–glycan, PI, PC and PE, the t.l.c. system of Saltiel *et al.* [12] was used, i.e. plates with 1% potassium oxalate impregnated developed with chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, by vol.). Phospholipids and lipids were identified by I_2-vapour staining, and R_F values were determined by comparison with standards which were chromatographed in parallel. Phospholipids and lipids were scraped into counting vials, and radioactivity was determined after addition of scintillation medium.

Glycerol 3-phosphate labelling and mass were determined as follows. After incubation, media were removed by aspiration and cells were extracted with chloroform/methanol/water (3:1:1, by vol.). After vortex-mixing and centrifugation, aqueous phases were removed and samples were chromatographed on Dowex-1 columns (0.5 cm × 2 cm; formate form, Bio-Rad). Free glycerol and glycerol 3-phosphate were quantitatively eluted with water and 0.2 M-ammonium formate/0.1 M-formic acid respectively (see [13]). It should be noted that [^{3}H]-glycerol labelled in the 2-position cannot be converted into dihydroxyacetone phosphate or other glycolytic intermediates without loss of radioactivity. Thus all radioactivity in the aqueous glycerol 3-phosphate column fraction can only be attributed to this substance. This conclusion is supported by the fact that aqueous extracts from cells labelled with [2-^{3}H]glycerol for 2 h or 3 days yielded only a single peak of radioactivity (in addition to free glycerol, which did not bind to the column) during elution of the columns with a linear ammonium formate gradient in 0.1 M-formic acid, and the elution pattern was identical with that of [U-^{14}C]-glycerol 3-phosphate standard. The mass of glycerol 3-phosphate in the column fraction was determined by spectrophotometric determination of the NADH generated after addition of glycerol-3-phosphate dehydrogenase, NAD^+, glycine buffer (pH 9.6) and hydrazine as a trapping agent [14].

DAG mass in lipid extracts was determined by the method of Preiss *et al.* [15], which uses DAG kinase and [γ-^{32}P]ATP to convert DAG into [^{32}P]PA. The latter was purified by t.l.c. (see above), and DAG mass was estimated by comparison of samples with diolein standards.

[2-^{3}H]Glycerol (sp. radioactivity 5 Ci/mmol) and D-[U-^{14}C]glucose (sp. radioactivity 8 mCi/mmol) were obtained from ICN. [1,2,3-^{3}H]Glycerol (sp. radioactivity 34 Ci/mmol), [9,10-^{3}H]myristic acid (sp. radioactivity 22.4 Ci/mmol), [5,6,7,11,12,14,15-^{3}H]arachidonic acid (sp. radioactivity 238 Ci/mol), [1,2-^{14}C]choline (sp. radioactivity 7.2 mCi/mmol) and [U-^{14}C]glycerol 3-phosphate (sp. radioactivity 120 mCi/mmol) were obtained from Dupont/New England Nuclear. Highly purified pig insulin (28 units/mg) was obtained from Elanco. Culture media and fetal-calf serum were obtained from Gibco. Ionophore A23187, cycloheximide, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), bovine serum albumin (radioimmunoassay grade), phospholipid and lipid standards, glycerol-3-phosphate dehydrogenase, NAD^+, hydrazine, type IX phospholipase C (from *Clostridium perfringens*) and other biochemicals were obtained from Sigma.

RESULTS

In the absence of insulin, [^{3}H]glycerol was incorporated into total glycerolipids (including phospholipids and neutral lipids) at a relatively slow but steady rate over a 150 min experimental period (Fig. 1). Incorporation into DAG, on the other hand, reached a steady state by 120 min, and incorporation into glycerol 3-phosphate was beginning to level off at this time. After addition of 200 nM-insulin at 120 min of incubation, incorporation of [^{3}H]glycerol into total glycerolipids increased briskly and was usually maximal within 1–2 min (Figs. 1 and 2). Thereafter, the rate of total glycerolipid labelling

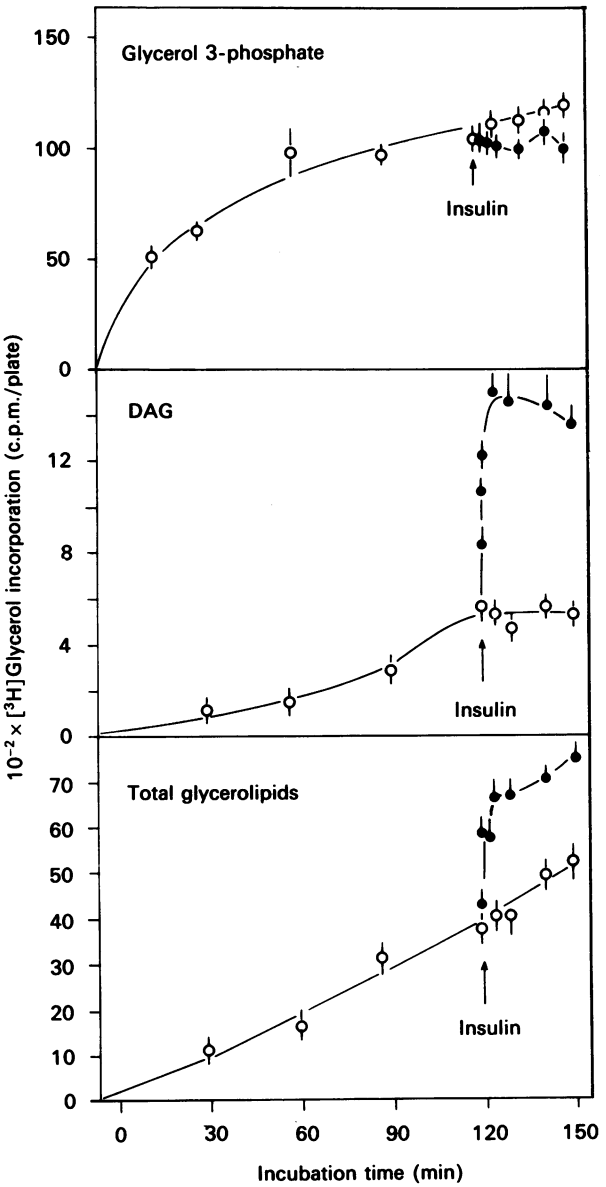

Fig. 1. [2-^{3}H]Glycerol incorporation into glycerol 3-phosphate, DAG and total glycerolipids in control and insulin-stimulated myocytes

Myocytes in 35 mm-diam. plates were incubated with 10 μCi of [2-^{3}H]glycerol in 1 ml of DPBSGA for the indicated times. Where indicated (●), insulin (200 nM) was added at 120 min; ○, controls. Results are means ± S.E.M. for four determinations.

diminished somewhat, but remained sufficiently high to maintain the insulin stimulatory effect over the next 20–30 min.

Increases in total glycerolipid labelling by [^{3}H]glycerol were accompanied by prompt 4-fold (on average) increases in the labelling of DAG, these usually being maximal within 1–2 min, and sustained for 20–30 min (Figs. 1 and 2). These increases in DAG labelling were associated with concomitant, but lesser, relative increases in the labelling of TAG, MAG, PC, PE (Table 1) and other phospholipids such as PA and PI (see [5]). Insulin-induced increases in [^{3}H]glycerol labelling of both DAG and total glycerolipids correlated well temporally with increases in DAG mass, which was measured simultaneously in the same lipid extracts (Fig. 2).

In contrast with increases in [^{3}H]glycerol incorporation into PA, DAG and other neutral lipids and phospholipids, [^{3}H]glycerol incorporation into glycerol 3-phosphate, the precursor for PA and all subsequently synthesized glycerolipids, was decreased by insulin treatment (Figs. 1, 2 and 5), probably reflecting increased utilization of glycerol 3-phosphate for PA synthesis. These results rule out the possibility that insulin effects on [^{3}H]glycerol incorporation into glycerolipids are due to an increase in glycerokinase activity, or other factors which may result in an increase of the labelling of glycerol 3-phosphate, e.g. a decrease in glycerol-3-phosphate dehydrogenase activity.

As shown in Table 2, tissue concentrations of glycerol 3-phosphate in control and 1 min-insulin-treated myocytes were 0.34 and 0.38 mM respectively. Also, the specific radioactivity of total glycerol 3-phosphate was unchanged by 1 min of insulin treatment. Although not examined here (since insulin effects were usually maximal at 1 min), longer times of insulin treatment would be expected to increase glucose uptake, glycolytic flux and amounts of unlabelled glycerol 3-phosphate [13].

The above results indicated that insulin abruptly increased the net rate of synthesis *de novo* of PA and total glycerolipid. Since the control rate of [^{3}H]glycerol labelling of total glycerolipids was relatively constant during the 150 min experimental period, it was possible to calculate this rate (or velocity) as c.p.m. incorporated into total glycerolipids/min of incubation. In a series of eight separate experiments, the control rate of labelling (i.e. the resultant of synthesis and degradation) was determined and compared with that observed during the first 1 min of treatment with 200 nM-insulin: thus the net rate of total glycerolipid labelling increased approx. 60-fold (from 167 ± 47 to 10893 + 3428 c.p.m./min; mean ± S.E.M.) during this 1 min treatment period (also see Fig. 3).

The effect of insulin on total glycerolipid labelling was maximal at insulin concentrations of 20–200 nM, and half-maximal at approx. 2 nM (Fig. 3). These doses of insulin are comparable with those previously reported to provoke maximal and half-maximal increases in glucose transport [16].

In 2 h prelabelling experiments, the effects of insulin on [^{3}H]glycerol incorporation into DAG and other neutral lipids and phospholipids were apparent regardless of whether glucose was present in the preincubation and incubation medium (results not shown). Also, insulin effects on [^{3}H]glycerol incorporation were not impaired by addition of 10 μM-cycloheximide (which inhibits protein synthesis [1]), or omission of Ca^{2+} from the preincubation and incubation media and addition of 1 mM-EGTA (results not shown).

[^{14}C]Glucose incorporation into DAG and other glycerolipids was considerably less than that of [^{3}H]-glycerol; nevertheless, rapid stimulatory effects of insulin on [^{14}C]glucose incorporation were apparent (Table 3). (There is a lag of several minutes before insulin-induced increases in 2-deoxyglucose uptake are apparent in BC3H-1 myocytes [16].) Incorporation of [1,2,3-^{3}H]glycerol into DAG and other glycerolipids of control and insulin-stimulated myocytes was similar to that observed with [2-^{3}H]glycerol (Table 3). The [1,2,3-

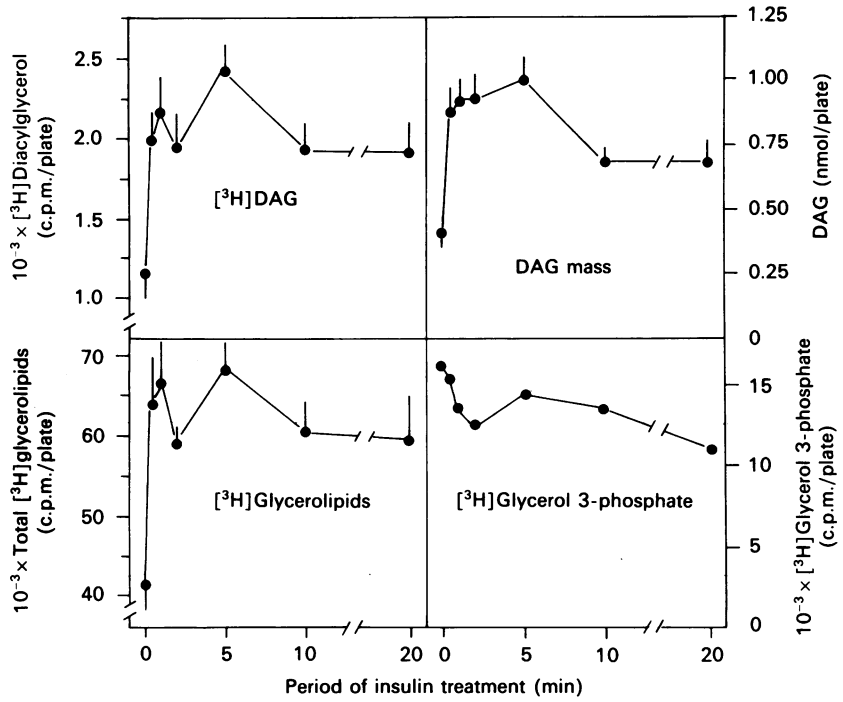

Fig. 2. Simultaneous measurement of insulin-induced changes in DAG mass and [^{3}H]glycerol incorporation into DAG, total glycerolipids and glycerol 3-phosphate

Myocytes in 35 mm-diam. plates were incubated for 140 min with 10 μCi of [2-^{3}H]glycerol in 1 ml of DPBSGA. During the last 20 min, insulin (200 nM) was present for the indicated times. After incubation, samples of the lipid extracts were used to determine DAG mass and [^{3}H]glycerol incorporation into DAG and total glycerolipids. Samples of the aqueous extracts were used to measure glycerol 3-phosphate labelling by [2-^{3}H]glycerol. Results are mean ± S.E.M. for four determinations.

Table 1. Effects of insulin on [^{3}H]glycerol incorporation into neutral lipids, PC and PE

Myocytes in 60 mm-diam. plates were incubated for 120 min with 3 ml of DPBSGA containing 20 μCi of [^{3}H]glycerol. Insulin (200 nM) was then added for the indicated times and cells were incubated for another 2 min. Lipid extracts were divided and chromatographed both for neutral lipids [diacylglycerol (DAG), monacylglycerol (MAG) and triacylglycerol (TAG)] and for separation of phospholipids, PC and PE. Results are means ± S.E.M. for four to nine determinations.

Duration of insulin treatment (min)	[^{3}H]Glycerol incorporation (c.p.m./plate)				
	DAG	MAG	TAG	PC	PE
0 (control)	2770 ± 414	2246 ± 122	9310 ± 942	19930 ± 2416	1510 ± 114
0.5	6866 ± 868	5496 ± 142	12342 ± 1714	32862 ± 1464	2320 ± 323
1	6912 ± 1650	4354 ± 896	10002 ± 1494	28770 ± 5966	2154 ± 380
2	8832 ± 520	5654 ± 142	11834 ± 1680	28548 ± 674	2334 ± 160

^{3}H]glycerol 3-phosphate, unlike the 2-^{3}H substance, retains two-thirds of its radioactivity upon conversion into dihydroxyacetone phosphate, which may also be incorporated into lipids through synthesis *de novo*; nevertheless, this difference did not appear to change the lipid-labelling pattern from that observed with [2-^{3}H]-glycerol. The fact that insulin effects on [^{14}C]glucose incorporation were similar to those observed with [^{3}H]-glycerol serves to rule out the possibility that a sudden decrease in the flow of unlabelled glycolytic intermediates into the pool of glycerol 3-phosphate which is used for PA synthesis could explain the observed insulin effects on [^{3}H]glycerol incorporation.

Although insulin effects on [^{3}H]glycerol incorporation were best observed after prelabelling of the glycerol 3-phosphate pool, it was also possible to observe stimulatory effects when insulin and [^{3}H]glycerol were added simultaneously (Fig. 4). Under these conditions, [^{3}H]-glycerol labelling was greatest in PA, intermediate in DAG, and least in PC during a 5–10 min period (TAG and PI were very poorly labelled; these results not shown). These results further confirm that the pathway for synthesis *de novo* flows primarily from PA to DAG to PC. Furthermore, the insulin-induced increase in [^{3}H]-DAG observed in these acute labelling experiments is more likely to be derived from PA than from PC, PI or other phospholipids.

In cells which were prelabelled with [2-^{3}H]glycerol for

Table 2. Glycerol 3-phosphate amounts and specific radioactivity before and after 1 min of insulin treatment of BC3H-1 myocytes

Myocytes in 100 mm-diam. plates (6 mg of protein) were incubated for 120 min in 5 ml of DPBSGA containing 10 μCi of [^{3}H]glycerol. Insulin (200 nM) was added during the last 1 min, where indicated. Glycerol 3-phosphate concentrations and radioactivity were determined in pooled contents of three plates as described in the Experimental section. Results are means ± S.E.M. for four determinations.

	Glycerol 3-phosphate			
Treatment	(nmol/g wet wt. of tissue)	(nmol/mg of protein)	(c.p.m./mg of protein)	Sp. radioactivity (c.p.m./nmol)
None (control)	338 ± 70	1.88 ± 0.39	2385 ± 248	1240 ± 160
Insulin	381 ± 27	2.12 ± 0.15	2350 ± 105	1135 ± 123

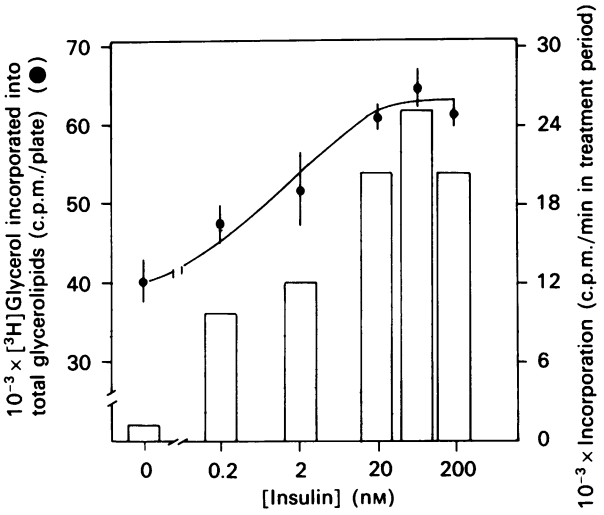

Fig. 3. Dose-related effects of insulin on total glycerolipid labelling with [^{3}H]glycerol

Myocytes in 35 mm-diam. plates were incubated for 121 min with 10 μCi of [^{3}H]glycerol in 1 ml of DPBSGA. Insulin was added in the indicated amounts 1 min before the end of the 121 min incubation period. [^{3}H]Glycerol incorporation into glycerolipids is expressed both as total ^{3}H found at the end of incubation (●) and as net velocity (□) during the last 1 min (see the text). Results are means ± S.E.M. for four determinations.

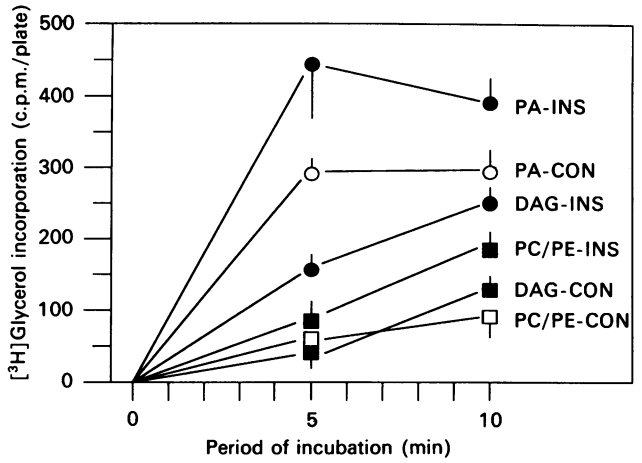

Fig. 4. Effects of insulin on incorporation of simultaneously added [^{3}H]glycerol into PA, PC/PE and DAG

Myocytes in 35 mm-diam. plates were incubated in 1 ml of DPBSGA, and 10 μCi of [2-^{3}H]glycerol was added in the absence or presence of 200 nM-insulin (INS); CON, control. Results are means ± S.E.M. for four determinations.

3 days, insulin effects were particularly striking. In the experiment shown in Fig. 5, rapid initial increases in labelling of PA, DAG, PC/PE, PI, MAG and TAG were followed by transient declines (more in some lipids than others) and subsequent increases (this biphasic pattern was frequently observed, and the short-lived nadir provides some insight into the extremely rapid turnover of most newly synthesized lipids). As in 2 h-prelabelling experiments, labelling of glycerol 3-phosphate was reciprocally related to PA labelling, presumably reflecting increased utilization of glycerol 3-phosphate for PA synthesis, and temporal changes in DAG mass correlated well with changes in [^{3}H]glycerol incorporation into DAG. It should be noted that the secondary increases in ^{3}H-labelled DAG and DAG mass were more rapid than the secondary increase in labelling of PA, possibly suggesting that some of this DAG may have been derived from sources other than newly synthesized PA, e.g. [^{3}H]-PC, which was heavily labelled. In addition, after 3 days of prelabelling, insulin-induced increases in [^{3}H]PI were more steady than those observed in 2 h-prelabelling experiments (see [5]), also possibly owing to better labelling of lipids such as PC/PE, which may be hydrolysed and converted into PA, CDP-DAG and PI [17]. Finally, the mean increase in [^{3}H]PC/PE was quite large, and, based on calculations from the specific radioactivity of glycerol 3-phosphate, this was equivalent to approx. 2 nmol per 35 mm plate, which is 4% of the total PC/PE content. {In cells which were prelabelled with [^{3}H]glycerol for 10 days, i.e. during their entire growth cycles, insulin effects on labelling of PA, DAG, PC/PE, TAG, MAG and PI were essentially the same as those shown in Fig. 5 (results not shown).}

Experiments with [^{3}H]myristate and [^{3}H]arachidonate labelling also supported the possibility (see above and [5]) that insulin increases the synthesis of PC. In 2 h labelling experiments, [^{3}H]myristate and [^{3}H]-arachidonate labelled PC to the greatest extent, and did not label the PI–glycan appreciably (results not shown). {Myristate is slowly incorporated into PC, whereas arachidonate is very rapidly incorporated (> 75% in 2 h), probably largely via transacylation. At 24 h of labelling, in accordance with results of Saltiel *et al.* [12,18], incorporation of [^{3}H]myristate, but not [^{3}H]arachidonate, into the PI–glycan could be detected.} After 2 h of [^{3}H]myristate prelabelling, insulin provoked small but consistent increases in PC labelling during the first 0.5–2 min (Fig. 6) (24 ± 5% increase; mean ± S.E.M.; $P < 0.05$, paired t test from three separate experiments). Over the next few minutes, consistent transient decreases

Table 3. Effects of insulin on incorporation of [2-^{3}H]glycerol, [1,2,3-^{3}H]glycerol and [U-^{14}C]glucose into diacylglycerol and other glycerolipids in BC3H-1 myocytes

Myocytes in 35 mm-diam. plates were incubated in parallel for 120 min in 2 ml of DPBSGA containing 1 mM-glucose and either 10 μCi of [2-^{3}H]glycerol or [1,2,3-^{3}H]glycerol or 20 μCi of [U-^{14}C]glucose. Insulin was added 2 min before the end of incubation, as indicated. Abbreviation: Total GL, total glycerolipids. Results are means ± S.E.M. for four determinations.

	[2-^{3}H]Glycerol (c.p.m./plate)		[1,2,3-^{3}H]Glycerol (c.p.m./plate)		[U-^{14}C]Glucose (c.p.m./plate)	
Treatment	DAG	Total GL	DAG	Total GL	DAG	Total GL
None	63 ± 11	764 ± 75	109 ± 14	928 ± 89	40 ± 4	463 ± 42
Insulin	655 ± 114	3835 ± 484	654 ± 135	3659 ± 42	202 ± 35	2081 ± 249

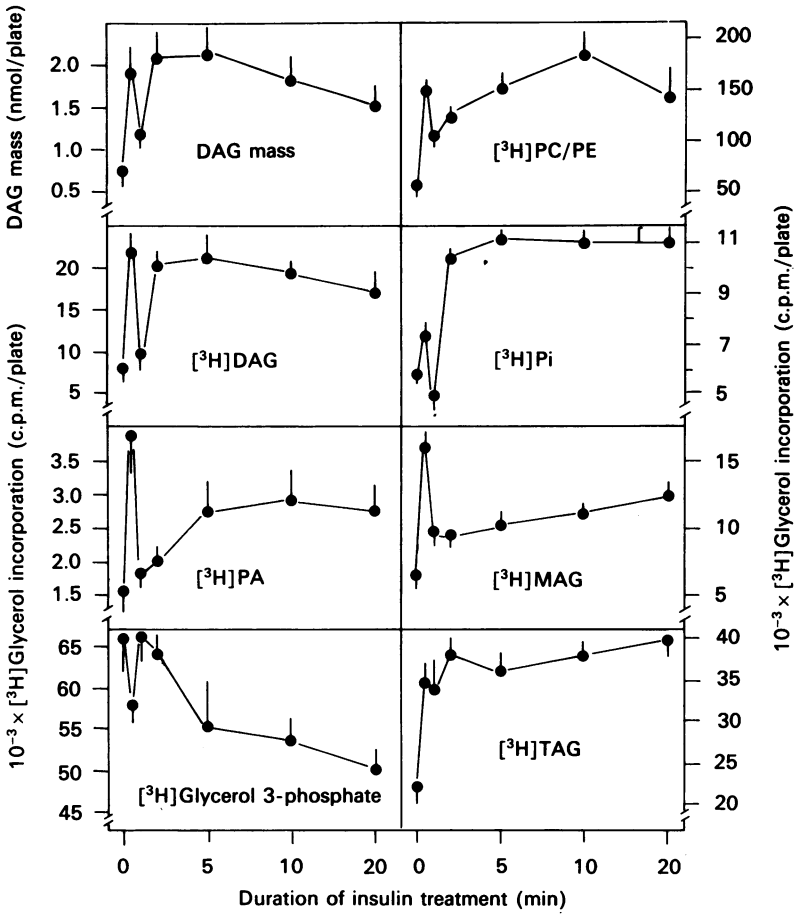

Fig. 5. Time course of insulin effects on DAG mass and [^{3}H]glycerol labelling of DAG, PA, glycerol 3-phosphate, PI, PC/PE, MAG and TAG

Myocytes in 35 mm-diam. plates were prelabelled for 3 days with 10 μCi of [2-^{3}H]glycerol in 3 ml of serum-free Dulbecco's Minimum Essential Medium containing 1 mg of bovine serum albumin/ml. On the day of the experiment, the medium was changed to DPBSGA, and after a 2 h equilibration period cells were treated with 20 nM-insulin for the incubated times. Results are means ± S.E.M. for four determinations.

in [^{3}H]myristate-labelled PC were observed, and these were accompanied by continued increases in the labelling of DAG and PA, which subsequently declined after 10 min. Findings with cells prelabelled with [^{3}H]-arachidonate for 2 h were similar to those observed with [^{3}H]myristate, i.e. initial transient increases in PC/PE labelling of 23 ± 9 % (mean ± S.E.M.; $n = 7$; $P < 0.05$, paired t test), followed by consistently observed transient decreases over the next few minutes (Fig. 7) (see [5] for other details of [^{3}H]arachidonate labelling of DAG and other lipids). In experiments in which cells were labelled with [^{3}H]arachidonate for 20 h, insulin provoked virtually the same changes in DAG and other lipids as those observed in 2 h-labelled cells (see [5]), i.e. changes in

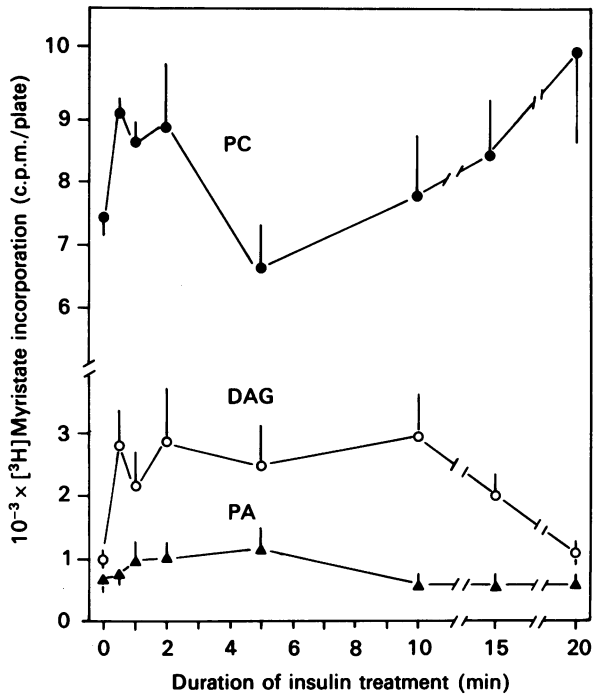

Fig. 6. Effects of insulin on [^{3}H]myristic acid labelling of PC, DAG and PA

Myocytes in 35 mm-diam. plates were incubated for 2 h in 1 ml of DPBSGA with 1 μCi of [^{3}H]myristic acid. Over the next 20 min, cells were treated with insulin for the indicated times. Results are means ± S.E.M. for four determinations.

PC/PE as described above, and 1.5–2-fold increases in [^{3}H]DAG at 0.5–20 min of insulin treatment (Table 4). Arachidonate-labelled DAG was approx. 1% of arachidonate-labelled PC/PE.

Experiments with [^{14}C]choline provided further evidence that insulin increases the synthesis of PC/PE. After 3 days of [^{14}C]choline labelling, there was substantial labelling of PC/PE, very slight labelling of sphingomyelin, and no labelling of other lipids (results not shown). Within 30 s of insulin addition to [^{14}C]-choline-labelled myocytes, PC/PE labelling increased by nearly 50% (Fig. 7). Over the next 1 min the amount of labelled PC/PE decreased to baseline, only to increase again during the next several minutes.

We have reported that insulin provokes a small transient increase in inositol-phospholipid hydrolysis in rat adipose tissue [19], but apparently not in BC3H-1 myocytes [2]. Insulin is also known to increase the hydrolysis of a PI–glycan by a specific phospholipase C [12,18], and we have suggested that insulin may activate a phospholipase C which hydrolyses lipids such as PC/PE [5]. We therefore questioned whether the increase in PA–DAG synthesis *de novo* observed during insulin action may be triggered by increases in phospholipase C activity, Ca^{2+} mobilization, DAG generation from sources other than newly synthesized PA, and/or protein kinase C activation. To evaluate these possibilities, we used phenylephrine {which increases inositol-phospholipid hydrolysis, inositol phosphates, cytosolic Ca^{2+} and protein kinase C activity [20,21] and DAG mass (R. V. Farese, D. R. Cooper, G. P. Nair, C. Sierra, M. L. Standaert & R. J. Pollet, unpublished work) in BC3H-1

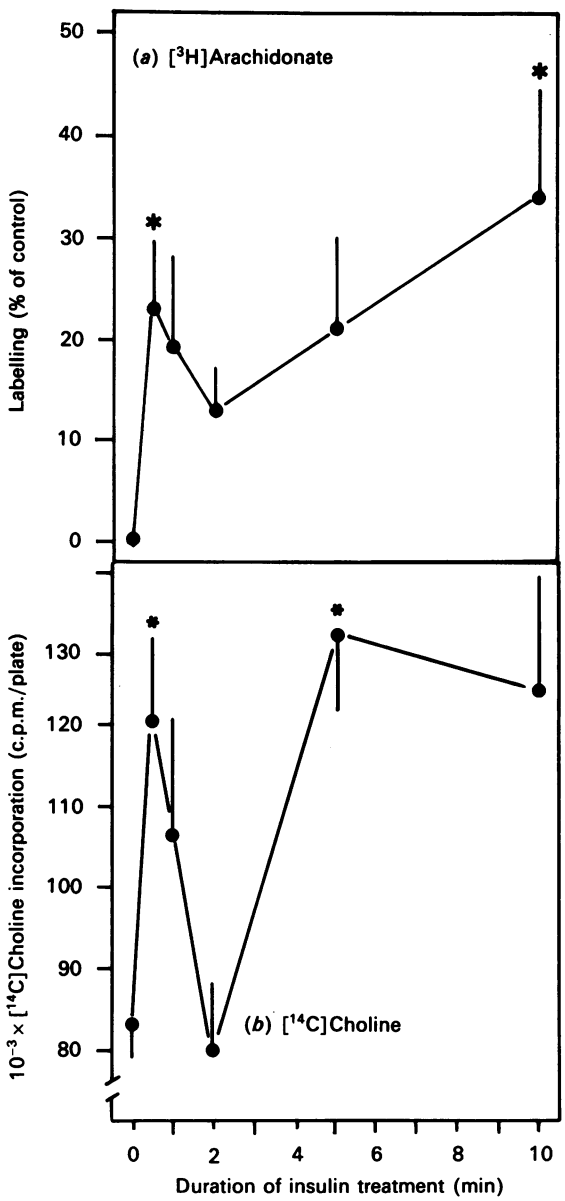

Fig. 7. Effects of insulin on [^{3}H]arachidonate and [^{14}C]choline labelling of PC/PE

In [^{3}H]arachidonate-labelling experiments (*a*), myocytes in 35 mm-diam. plates were incubated for 2 h in 1 ml of DPBSGA with 0.1–1 μCi of [^{3}H]arachidonic acid. Over the next 10 min, cells were treated with 200 nM-insulin for the indicated times. Mean results of five separate experiments, each with four determinations, are summarized here as a percentage of control. In [^{14}C]choline-labelling experiments (*b*), myocytes in 35 mm-diam. plates were incubated for 3 days in 3 ml of serum-free Dulbecco's Minimum Essential Medium containing 1 mg of bovine serum albumin/ml and 0.5 μCi of [^{14}C]choline. On the day of the experiment, the medium was changed to 2 ml of DPBSGA, and after 2 h of equilibration cells were treated with 20 nM-insulin for the indicated times. Results are means ± S.E.M. for four determinations: $^*P < 0.05$.

myocytes}, A23187 (which increases intracellular Ca^{2+}), TPA (which directly activates protein kinase C in BC3H-1 myocytes [6]), and an exogenously added phospholipase C preparation (which increases DAG and release of

Table 4. Effects of insulin on labelling of PC/PE and DAG in cells prelabelled for 20 h with [^{3}H]arachidonate

Myocytes in 35 mm-diam. plates were labelled with 0.8 mCi of [^{3}H]arachidonic acid for 20 h. Cells were washed and media were changed to DBPSGA. After 2 h of equilibration, the incubation was continued without or with insulin (200 nM) being present for the times indicated. Results are means ± S.E.M. for four determinations.

Treatment	Incorporation (c.p.m.)	
	[^{3}H]PC/PE	[^{3}H]DAG
None	71752 ± 1750	624 ± 26
Insulin, 0.5 min	89376 ± 3946	910 ± 42
Insulin, 1 min	81002 ± 4286	830 ± 18
Insulin, 2 min	72530 ± 6538	822 ± 62
Insulin, 5 min	89592 ± 2964	1330 ± 226
Insulin, 10 min	82218 ± 2010	1088 ± 118
Insulin, 20 min	89332 ± 2200	952 ± 76

Table 5. Effects of phenylephrine, A23187 and TPA on [^{3}H]glycerol incorporation into DAG in BC3H-1 myocytes

Myocytes in 35 mm-diam. plates were incubated for 120 min with 10 μCi of [^{3}H]glycerol in 1 ml of DPBSGA. The indicated treatments were then done, and incubation was continued for another 2 min. TPA and A23187 were dissolved in dimethyl sulphoxide (final concn. 0.025%), which was also added to the controls and insulin-treated samples in Expt. B. Phenylephrine, A23187 and TPA also had little or no effect on [^{3}H]glycerol incorporation into DAG or other lipids or phospholipids at other times of incubation, i.e. 0.5, 5, 10 and 20 min (results not shown). Results are means ± S.E.M. for the numbers of experiments in parentheses: * $P < 0.001$ versus control (t test).

Expt.	Treatment (2 min)	[^{3}H]Glycerol incorporation into DAG (c.p.m./plate)
A	None (control)	1385 ± 207 (7)
	Phenylephrine, 1 nM	1390 ± 294 (6)
	Insulin, 200 nM	4416 ± 260 (4)*
B	None (control)	3797 ± 159 (4)
	A23187, 1 μM	3962 ± 604 (5)
	TPA, 1 μM	4175 ± 388 (5)
	Insulin, 200 nM	7017 ± 525 (5)*

phospholipid head groups). After 2 h of [^{3}H]glycerol prelabelling, phenylephrine, A23187 and TPA (Table 5) did not provoke insulin-like effects on [^{3}H]glycerol incorporation into DAG (and other lipids and phospholipids; these results not shown). These results appear to exclude the possibility that activation of a phospholipase C which hydrolyses inositol-phospholipids, or Ca^{2+} mobilization, or protein kinase C activation, is responsible for insulin-induced increases in PA–DAG synthesis *de novo*. With addition of a non-specific phospholipase C (obtained from *Clostridium perfringens*), there was a 3-fold increase in [^{3}H]DAG (Fig. 8),

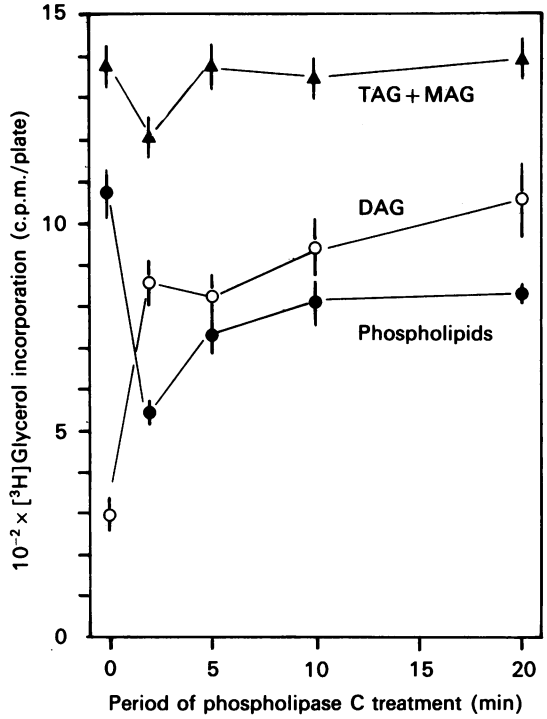

Fig. 8. Effects of exogenously added phospholipase C on [^{3}H]glycerol incorporation into DAG, phospholipids and TAG plus MAG

Myocytes in 35 mm-diam. plates were incubated for 140 min with 10 μCi of [2-^{3}H]glycerol in 1 ml of DPBSGA. Phospholipase C (0.5 unit) was added 2–20 min before the end of incubation. Results are means ± S.E.M. for four determinations.

but this was associated with a decrease in labelled phospholipids (chiefly PC) and, moreover, total glycerolipid labelling (the sum of phospholipids, DAG, TAG and MAG), and, therefore, presumably PA–DAG synthesis *de novo*, was not increased.

DISCUSSION

The present findings provide further evidence that insulin rapidly increases DAG in BC3H-1 myocytes by stimulating both synthesis *de novo* of PA and hydrolysis of pre-existing phospholipids. With respect to the mechanism of PA synthesis *de novo*, insulin, if anything, provoked decreases in glycerol 3-phosphate labelling, and increases in [^{3}H]glycerol incorporation into PA, DAG, PC and other glycerolipids cannot be explained by an increase in the labelling or specific radioactivity of glycerol 3-phosphate. Further evidence that increased incorporation of [^{3}H]glycerol into glycerolipids reflects increased PA synthesis *de novo* is provided by the following: (*a*) insulin also increased [^{14}C]glucose incorporation into glycerolipids at early time points, when insulin effects on glucose transport would not be expected [16]; (*b*) PA, other phospholipids [1,2], and DAG mass are increased by insulin, and increases in the mass and [^{3}H]glycerol labelling of DAG were temporally well correlated; (*c*) increases in labelling of PC/PE were observed with several precursors, including [^{3}H]glycerol, [^{3}H]myristic acid, [^{3}H]arachidonic acid and [^{14}C]choline,

suggesting that PC/PE synthesis itself is stimulated; (*d*) increases in lipid labelling were observed regardless of whether insulin and [^{3}H]glycerol were added simultaneously, or whether insulin was added to cells which had been prelabelled for 2 h or 3 or 10 days; and (*e*) in all labelling protocols, total glycerolipid labelling was increased.

The present and previous [5] findings indicate that increases in synthesis *de novo* of PA, DAG and PC/PE occur within seconds of addition of insulin to BC3H-1 myocytes. The effect on this synthesis seems to be biphasic, with a nadir at 1–5 min, followed by a secondary increase. For PC/PE labelling, it is at present uncertain how much of this nadir reflects a transient decrease in PC/PE synthesis, or an increase in PC/PE hydrolysis. With respect to the latter possibility, previous data [5] from 2 h glycerol-labelling experiments had indirectly suggested the existence of concurrent hydrolysis of non-inositol phospholipids, as there was no other reasonable explanation for increases in PI mass, which were observed at times when [^{3}H]glycerol incorporation into PI was not commensurately increased. The present findings provide further evidence for existence of a hydrolytic component in that: (*a*) persistent and commensurate increases in [^{3}H]glycerol labelling of PI were observed, but only in experiments in which cellular phospholipids were extensively prelabelled for 3–10 days, before insulin treatment; and (*b*) on the basis of the specific radioactivity of glycerol 3-phosphate, the pathway of PA–DAG–PC synthesis *de novo* could account for no more than 15–30% of the observed increases in DAG content.

The present findings illustrate some of the difficulties encountered in trying to estimate the relative contributions of PA synthesis *de novo* and phospholipid hydrolysis to observed increases in either the [^{3}H]glycerol labelling or content of DAG during insulin action. Although there were reasonably good correlations between time-related increases in mass and [^{3}H]glycerol labelling of DAG in 2 h, 3-day and 10-day prelabelling experiments, this may reflect either stimulation of the pathway of PA synthesis *de novo* or hydrolysis of pre-existing phospholipids, or both, since, as shown above, (*a*) the glycerol 3-phosphate pool may be heavily labelled even after 10 days of prelabelling (in fact, we have not been successful in attempts to deplete radioactivity in this pool sufficiently in prolonged 'chase' experiments), and (*b*) [^{3}H]glycerol-labelled PC/PE may also provide substrate for phospholipase C even in 2 h prelabelling experiments. Nevertheless, increased early labelling of PA and DAG in experiments in which insulin was added simultaneously with [^{3}H]glycerol, and increases in PC and total glycerolipid labelling, coupled with knowledge of the fact that most newly synthesized PA is directly converted into DAG and thence into PC, make it abundantly clear that PA synthesis *de novo* must contribute substantially to, and phospholipid hydrolysis cannot be the sole mechanism for, insulin-induced increases in DAG. Similarily, the estimation of the contribution of PA synthesis *de novo* (based on glycerol 3-phosphate specific radioactivity), indicates that hydrolysis of pre-existing phospholipids must also contribute significantly.

We have now evaluated several possible mechanisms whereby insulin may activate PA–DAG synthesis *de novo*. Clearly, hydrolysis of inositol phospholipids (as provoked by phenylephrine), Ca^{2+} mobilization and protein kinase C activation do not appear to be responsible for initiating the insulin effect on glycerolipid synthesis *de novo*. Similarly, non-specific hydrolysis of phospholipids such as PC/PE can not explain the insulin effect on the '*de novo*' pathway. Other mechanisms that need to be evaluated include fatty acyl-CoA availability and stimulation of glycerol-3-phosphate acyltransferase activity by PI–glycan head groups (see [22]).

In summary, our previous [1,2,5] and present findings in BC3H-1 myocytes suggest that there are at least two mechanisms for increasing DAG during insulin action, i.e. PA–DAG–PC/PE synthesis *de novo* and phospholipid hydrolysis. With respect to the latter, DAG may be increased by hydrolysis of the PI–glycan, but it is difficult to determine how much DAG is derived from this source, since, as shown here, myristate labelling of DAG is not specific and may derive from hydrolysis of PC as well as the PI–glycan. Increases in PI synthesis may result from increases in PA, derived either from 'true' synthesis *de novo* from glycerol 3-phosphate and fatty acyl-CoA, or from hydrolysis of phospholipids by phospholipase C or D. The metabolic consequences of increases in DAG by these mechanisms remain to be determined, but protein kinase C activation seems to occur [6,23,24].

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