Biochem. J. (1985) 231, 269–278 (Printed in Great Britain)

The *de novo* phospholipid effect of insulin is associated with increases in diacylglycerol, but not inositol phosphates or cytosolic Ca^{2+}

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We have previously reported that insulin increases the synthesis *de novo* of phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP_2) and diacylglycerol (DAG) in BC3H-1 myocytes and/or rat adipose tissue. Here we have further characterized these effects of insulin and examined whether there are concomitant changes in inositol phosphate generation and Ca^{2+} mobilization. We found that insulin provoked very rapid increases in PI content (20% within 15 s in myocytes) and, after a slight lag, PIP and PIP_2 content in both BC3H-1 myocytes and rat fat pads (measured by increases in ^{32}P or ^{3}H content after prelabelling phospholipids to constant specific radioactivity by prior incubation with $^{32}P_i$ or [^{3}H]inositol). Insulin also increased $^{32}P_i$ incorporation into these phospholipids when $^{32}P_i$ was added either simultaneously with insulin or 1 h after insulin. Thus, the insulin-induced increase in phospholipid content appeared to be due to an increase in phospholipid synthesis, which was maintained for at least 2 h. Insulin increased DAG content in BC3H-1 myocytes and adipose tissue, but failed to increase the levels of inositol monophosphate (IP), inositol bisphosphate (IP_2) or inositol trisphosphate (IP_3). The failure to observe an increase in IP_3 (a postulated 'second messenger' which mobilizes intracellular Ca^{2+}) was paralleled by a failure to observe an insulin-induced increase in the cytosolic concentration of Ca^{2+} in BC3H-1 myocytes as measured by Quin 2 fluorescence. Like insulin, the phorbol diester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) increased the transport of 2-deoxyglucose and aminoisobutyric acid in BC3H-1 myocytes. These effects of insulin and TPA appeared to be independent of extracellular Ca^{2+}. We conclude that the phospholipid synthesis *de novo* effect of insulin is provoked very rapidly, and is attended by increases in DAG but not IP_3 or Ca^{2+} mobilization. The insulin-induced increase in DAG does not appear to be a consequence of phospholipase C acting upon the expanded PI + PIP + PIP_2 pool, but may be derived directly from PA. Our findings suggest the possibility that DAG (through protein kinase C activation) may function as an important intracellular 'messenger' for controlling metabolic processes during insulin action.

INTRODUCTION

We have recently reported that insulin increases the contents of PA and inositol phospholipids in rat adipose tissue (Farese *et al.*, 1982, 1984*b*) and BC3H-1 myocytes (Farese *et al.*, 1984*a*). In the latter tissue, DAG was found to increase concomitantly, and this was of particular interest since (*a*) DAG activates protein kinase C and, via changes in protein phosphorylation, appears to function as a 'second messenger' in the control of various metabolic processes (Nishizuka, 1984*a*, *b*), and (*b*) we have recently found (Farese *et al.*, 1985) that the phorbol diester TPA and 1-oleoyl-2-acetyl-s*n*-glycerol, which mimic DAG and/or activate protein kinase C (Nishizuka, 1984*a*, *b*; Niedel *et al.*, 1983), provoke insulin-like effects on 2-deoxyglucose transport, aminoisobutyric acid transport and activation of pyruvate dehydrogenase.

A presently unanswered question is whether insulin-induced increases in PA and PI, PIP and PIP_2 are attended by increases in the inositol phosphates (IP, IP_2 and IP_3) which are derived from the inositol phospholipids by phospholipase C action. This question seems particularly important, since IP_3 appears to function as a 'second messenger' to mobilize intracellular Ca^{2+} (Downes & Michell, 1982; Berridge, 1982, 1984; Streb *et al.*, 1983; Joseph *et al.*, 1984; Prentki *et al.*, 1984; Berridge & Irvine, 1984), and the role of Ca^{2+} in the action of insulin has remained enigmatic (see Czech, 1977, 1981). In the present paper, we have addressed this question, further characterized the effects of insulin on phospholipid metabolism, and further evaluated the potential roles of DAG, IP_3 and Ca^{2+} as 'second messengers' in the action of insulin.

EXPERIMENTAL

The conditions for culturing BC3H-1 myocytes and incubating rat epididymal fat pads have been described

Abbreviations used: PI, PIP, PIP_2, phosphatidylinositol and its 4-phosphate and 4,5-bisphosphate; IP, IP_2, IP_3, the mono, bis and tris-phosphates of inositol; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; DAG, diacylglycerol; PA, phosphatidic acid; DMEM, Dulbecco's modified essential medium; DPBS, Dulbecco's phosphate-buffered saline; BSA, bovine serum albumin; 2-DOG, 2-deoxyglucose; AIB, aminoisobutyrate.

in detail previously (Farese *et al.*, 1982, 1984*a*, *b*; Standaert & Pollet, 1984; Standaert *et al.*, 1984). In brief, BC3H-1 myocytes were used for experiments 10–14 days after subculturing when the cells were confluent and maximally responsive to insulin. The myocytes were grown in 35 mm plastic Petri dishes [except in a few experiments, where 24-well cluster plates (Costar) were used, as indicated in the text] at 37 °C in 3 ml of DMEM, supplemented with 20% (v/v) fetal calf serum, under a humidified CO_2/air (1:9) atmosphere. Where indicated, 10 μCi of $^{32}P_i$ or 5 μCi of [^{3}H]inositol were present in the medium for the last 3 days of culture; phospholipids were thus labelled to constant specific radioactivity, and consequently, as shown previously (Farese *et al.*, 1984*a*), radioactivity reflected phospholipid content rather than turnover. The fat pads were preincubated and incubated at 37 °C under O_2/CO_2 (19:1) in Krebs–Ringer bicarbonate buffer containing 11.2 mM-glucose, 0.2% BSA and 1.25 mM-$CaCl_2$. Where indicated, 25–50 μCi of $^{32}P_i$ or 5 μCi of [^{3}H]inositol were added to 2.5 or 5 ml of buffer. In incubations of BC3H-1 myocytes and rat fat pads, insulin or TPA was added in small volumes of incubation media or dimethyl sulphoxide, respectively, and control vessels received equal volumes of medium or dimethyl sulphoxide alone, which were without effect. Incubations were terminated rapidly by addition of chloroform/methanol/HCl (2:1:0.075, by vol.) or cold 5% (w/v) trichoroacetic acid.

Lipid extracts were analysed for phospholipids as described previously (Farese *et al.*, 1982, 1983, 1984*a*, *b*). In some experiments, trichloroacetic acid precipitates were extracted for phospholipids and subsequently analysed; these results [as reported previously (Farese *et al.*, 1983)] were similar to those obtained with direct extraction of the whole tissue with chloroform/methanol/HCl. Phospholipids were purified by t.l.c. on silica gel plates (Supelco) which contained 10% (w/v) magnesium acetate; the chromatograms were developed one-dimensionally with two successive solvents, chloroform/methanol/4.3 M-NH_4OH (19:13:4, by vol.) followed by propan-1-ol/4.3 M-NH_4OH (3:2, v/v) containing 10 mM-cyclohexanediaminetetraacetic acid. After localization by autoradiography and visualization by acid-charring or iodine staining, phospholipids were scraped into vials and counted for ^{32}P and/or ^3H radioactivity.

DAG was determined from lipid extracts as described previously (Farese *et al.*, 1984*a*; Banschbach *et al.*, 1974). In brief, chromatographically purified DAG was acetylated with [^{3}H]acetic anhydride, purified again by t.l.c. and quantified by counting [^{3}H]acetyl-DAG (this is directly proportional to DAG mass).

Trichloroacetic acid-soluble substances, after removal of trichloroacetic acid by extraction with diethyl ether, were analysed for contents of ^3H-labelled glycerophosphoinositol, IP, IP_2 and IP_3 by anion-exchange chromatography (on Dowex-1 columns; formate form) as described by Berridge *et al.* (1982, 1983). In these experiments, ^3H in $IP + IP_1 + IP_2$ was found to range from 10 to 25% of the ^3H found in $PI + PIP + PIP_2$.

The methods for uptake of 2-DOG and AIB in BC3H-1 myocytes have been reported in detail previously (Standaert & Pollet, 1984; Standaert *et al.*, 1984). In brief, for 2-DOG uptake, the cells were grown in 24-well cluster plates for 13 days, supplemented with 25 mM-glucose for the last 24 h, then washed two or three times with DPBS containing 0.1% BSA but no $CaCl_2$, and preincubated for 60 min in DPBS containing 25 mM-glucose, with or without 0.2 mM-$CaCl_2$, and with or without 1 mM-EGTA. The cells were rinsed twice with buffer and 460 μl of the same preincubation buffer minus glucose was added to the cells. The cells were preincubated for another 10 min at 37 °C and then incubated for 20 min without (controls) or with insulin or TPA. 2-DOG uptake was monitored by addition of 40 μl of buffer containing 5 nmol (0.25 μCi) of [^{3}H]2-DOG and subsequent incubation for 6 min. For determination of AIB uptake, the cells were grown on 35 mm plates, rinsed with DPBS containing 0.1% BSA but no $CaCl_2$, preincubated for 60 min at 37 °C in 1 ml of DPBS containing 0.1% BSA with or without 0.9 mM-$CaCl_2$, and then incubated for an additional 4 h without (control) or with insulin or TPA. AIB uptake was measured by the addition of 10 nmol of [*methyl*-^{3}H]AIB (50 μCi/μmol) and subsequent incubation for 12 min. After rinsing the cells three times with cold DPBS, the cells were lysed with 1% sodium dodecyl sulphate. Protein concentrations were generally within 10% of the mean.

Cytoplasmic free Ca^{2+} concentrations were measured by the method of Tsien *et al.* (1982). Monolayers of BC3H-1 cells were loaded with Quin 2 by incubation for 30 min at 37 °C in DMEM containing 25 μM-Quin 2/AM (added in dimethyl sulphoxide). Intracellular hydrolysis of the Quin 2 ester was virtually complete after this period (as determined fluorometrically). Removal of unhydrolysed ester was achieved by two washes in DMEM, followed by gentle scraping to suspend the cells (10^6/ml) in buffer containing 140 mM-NaCl, 5 mM-KCl, 1 mM-$MgCl_2$, 5 mM-glucose, 10 mM-Hepes buffer (pH 7.40) and 1 mM-$CaCl_2$. The cells were then washed twice, resuspended in the same buffer and kept in the dark at room temperature. Control cells were similarly treated, except that dimethyl sulphoxide without Quin 2/AM was present during the loading period. Cellular fluorescence signals derived from the Quin 2–Ca^{2+} complex and reduced nicotinamide nucleotides were measured at an excitation wavelength of 339 nm (slit 5 nm) and emission wavelengths of 495 nm and 440 nm, respectively (slit 10 nm). The observed nicotinamide nucleotide changes (at 440 nm) were very small and much slower than Quin 2–Ca^{2+} changes (at 495 nm), and no correction of the fluorescence signal was required to determine the Quin 2–Ca^{2+} component. All measurements were made with a Perkin–Elmer MPF-44A spectrofluorometer equipped with a magnetic stirrer and a thermostated (at 37 °C) cell holder. Maximum ($F_{max.}$) and minimum ($F_{min.}$) fluorescence were measured by rapidly saturating intracellular Quin 2 with Ca^{2+} ($F_{max.}$) by permeabilizing the cells with 50 μM-digitonin or 0.1% Triton X-100, and by adding 5 mM-EGTA in Tris buffer, pH 8.5, to determine the basal fluorescence ($F_{min.}$) where virtually no Ca^{2+} was bound to Quin 2. Measurements were corrected for the intrinsic fluorescence of the cells and detergents. Cell viability (Trypan Blue exclusion or ^{86}Rb content) was determined at the end of each set of determinations. Only experiments in which the viability was above 80% were considered as being acceptable for the calculation of $[Ca^{2+}]_i$.

Cytoplasmic free Ca^{2+} concentration $[Ca^{2+}]_i$ was calculated from the observed fluorescence (F) according to the equation:

$$[Ca^{2+}]_i = 115 \cdot \frac{F - F_{min.}}{F_{max.} - F}$$

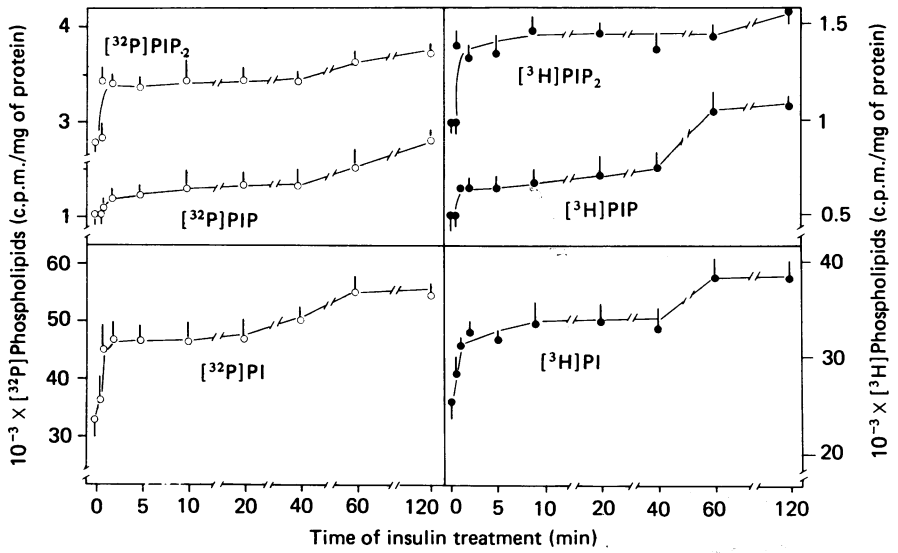

Fig. 1. Time course of insulin effects on PI, PIP and PIP_2 content in BC3H-1 myocytes

After prelabelling cells in culture for 3 days with $^{32}P_i$ and [^{3}H]inositol (see under 'Experimental'), 200 nM-insulin was added where indicated and incubation was continued for the indicated times (30 s–120 min). Control levels of ^{32}P and ^3H in these phospholipids were monitored at multiple time points along the 120 min experimental period and did not change significantly (for simplicity only the zero-time control level is shown). $^{32}P_i$ (left, ○) and ^3H (right, ●) were determined by simultaneous double isotope counting of the phospholipid samples. Mean values ± S.E.M. for four plates are shown.

where 115 nM represents the apparent K_d for Quin 2–Ca^{2+} at cytoplasmic ionic conditions.

Carrier-free $H_2{}^{32}PO_4$ ($^{32}P_i$) was purchased from ICN Biomedical. [^{3}H]Inositol (sp. radio activity 15.8 mCi/mmol) and [^{3}H]acetic anhydride (100 mCi/mmol) were purchased from New England Nuclear. Male rats (250 g) were obtained from Holtzman. We purchased culture media from Gibco, Quin 2/AM from Calbiochem and other biochemicals from Sigma.

RESULTS

Effects of insulin on phosphoinositide levels in prelabelled BC3H-1 myocytes

After prelabelling for 3 days in culture with $^{32}P_i$ or [^{3}H]inositol, phospholipids of BC3H-1 myocytes were labelled to constant specific radioactivity and changes therein could be equated with changes in phospholipid mass (see Farese *et al.*, 1984*a*). The time course of very rapid, intermediate and later effects of insulin on PI, PIP and PIP_2 levels is shown in Fig. 1. As is apparent, insulin provoked a very rapid increase in PI content, as evidenced by increases in both ^{32}P and ^3H radioactivity therein. PI content increased by approx. 30% within 2 min, and 60% within 60 min of insulin treatment; thereafter, PI content remained constant. PIP content, after a 30–60 s lag, increased rapidly at first, and then more gradually. PIP_2 content, also after a short lag, increased rapidly to elevated levels which were maintained throughout the entire 120 min treatment period. As is also apparent, there was good correlation between ^{32}P and ^3H radioactivity in PI, PIP, and PIP_2 in double-labelling experiments.

Increases in PI content were also evident within 15 s of insulin treatment (Table 1); on the other hand, there were no demonstrable changes in PIP or PIP_2 content at this early time point.

Effects of insulin on acute $^{32}P_i$ incorporation into phospholipids of BC3H-1 myocytes

With acute exposure of BC3H-1 cells to $^{32}P_i$, labelling of PA, PI and PIP_2 increased progressively over 2 h of incubation (Fig. 2); PIP labelling, on the other hand, levelled off after 1 h (this was observed regularly). With simultaneous addition of insulin and $^{32}P_i$, there were sizeable insulin-induced increases in the acute labelling of these phospholipids; after a 30–60 min delay, insulin provoked increases in other phospholipids as well [i.e. the combined phosphatidylcholine + phosphatidylethanolamine + phosphatidylglycerol chromatographic area (results not shown)], but to a lesser extent.

To determine whether the insulin-induced increase in phospholipid synthesis was maintained throughout the 2 h incubation period, $^{32}P_i$ was added 1 h after insulin, i.e., when the insulin effect on phospholipid content was

Table 1. Rapid changes in phosphatidylinositol content during 15 s of insulin treatment in BC3H-1 myocytes

Myocytes were prelabelled with $^{32}P_i$ for 3 days and then treated with 200 nM-insulin for 15 s. Mean (± S.E.M.) results for four separate experiments (with four to eight replicates of control and insulin-treated cells in each experiment) are shown here. In each experiment the mean control and mean insulin results were used to calculate the percentage change. *P* was determined by the paired *t*-test; NS, not significant.

Substance	Change (%) due to insulin	*P* (insulin versus control)
PI	+20±3	< 0.01
PIP	+5±4	NS
PIP_2	+4±3	NS

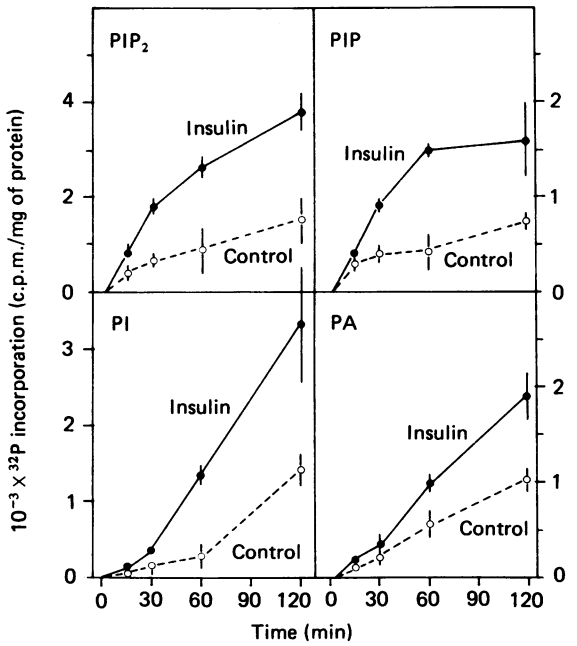

Fig. 2. Effects of insulin on acute $^{32}P_i$ incorporation into phospholipids in BC3H-1 cells

Cells were incubated for the indicated times with 50 μCi of $^{32}P_i$ with (●) or without (○) simultaneous addition of 200 nM-insulin. Mean values ± S.E.M. for four plates are shown.

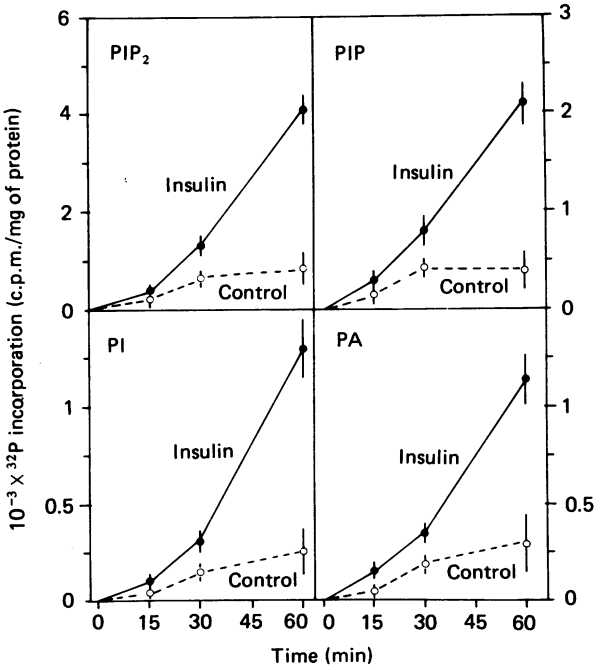

Fig. 3. Continued effects of insulin on phospholipid synthesis in BC3H-1 cells

Cells were incubated for 1 h with (●) or without (○) 200 nM-insulin; 50 μCi of $^{32}P_i$ was then added and the incubation was continued for the indicated times. Shown here are mean values ± S.E.M. for four plates.

maximal or nearly maximal and being maintained in a steady state. As shown in Fig. 3, the insulin-induced increases in $^{32}P_i$ incorporation into PA, PI, PIP and PIP_2 were as rapid and as great during the second hour of insulin treatment as that observed during the first hour (compare Figs. 3 and 2). It thus appears that insulin continues to increase phospholipid synthesis even after maximal increases in phospholipid content have reached a steady state, as opposed to simply maintaining increases in phospholipid content by decreasing degradation. The latter finding is important, since the increased rate of PI + PIP + PIP_2 synthesis must be balanced by a comparably increased rate of PI + PIP + PIP_2 degradation during the steady-state condition observed in the last hour of insulin treatment. This inference is important in the evaluation of insulin effects on the generation of IP, IP_2 and IP_3, as the latter are products of phospholipase C-mediated degradation of PI, PIP and PIP_2.

Effects of insulin on $^{32}P_i$ labelling of phospholipids in rat epididymal fat pads

Upon addition of insulin 30 min after $^{32}P_i$ to incubations of rat epididymal fat pads, there were 2–4-fold increases in the rates of $^{32}P_i$ incorporation into PA, PI, PIP, PIP_2 and other phospholipids, such as phosphatidylserine and the combined chromatographic area containing phosphatidylcholine + phosphatidylethanolamine +

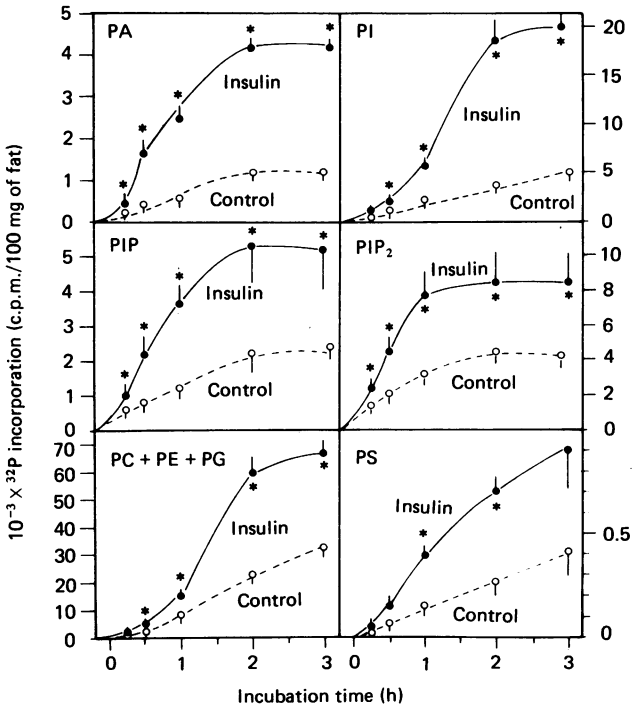

Fig. 4. Time course of insulin effects on acute $^{32}P_i$ incorporation into phospholipids in rat fat pads

After preincubation for 30 min with 25 μCi of $^{32}P_i$ in 5 ml of Krebs–Ringer bicarbonate buffer, fat pads were incubated for the indicated times with (●) or without (○) 1 munit of insulin/ml. Shown here are mean results ± S.E.M. for six determinations. Additional abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine, PG, phosphatidylglycerol; PS, phosphatidylserine.

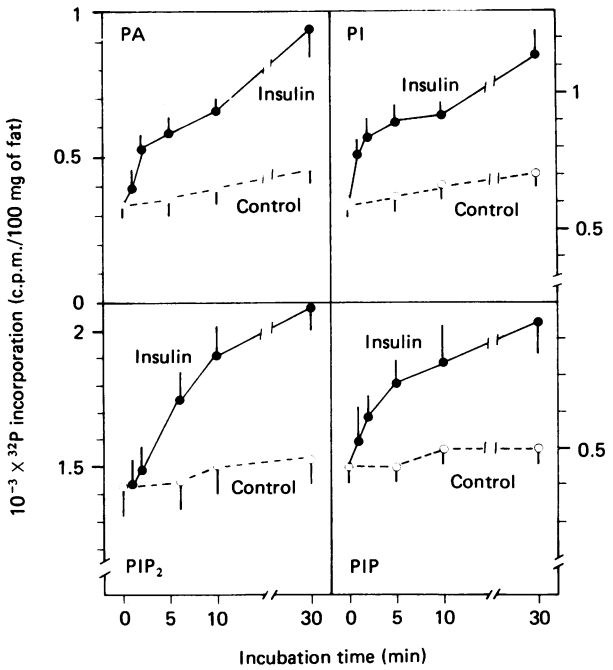

Fig. 5. Time course of insulin effects on ^{32}P content of prelabelled phospholipids in rat fat pads

Fat pads were preincubated for 120 min with 50 μCi of $^{32}P_i$ in 5 ml of medium, and then treated with (●) or without (○) 1 munit of insulin/ml for the indicated times. Shown here are mean values ± S.E.M. for 16 determinations.

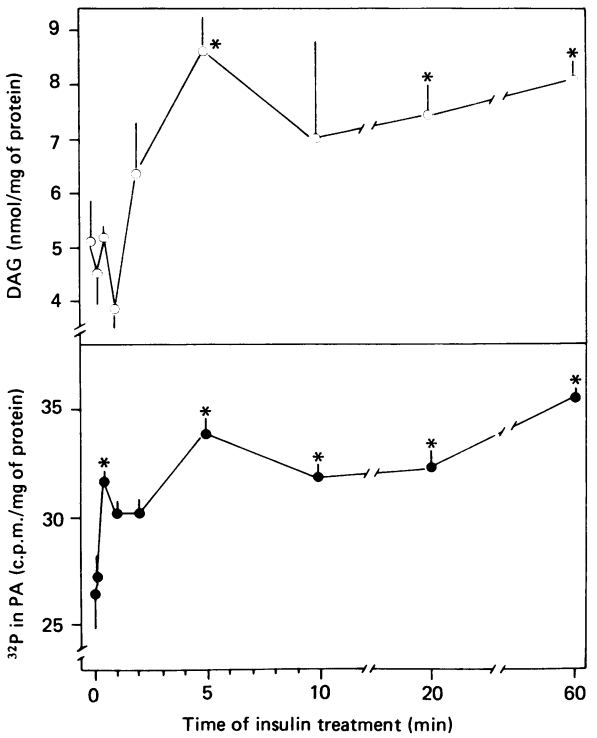

Fig. 6. Comparison of time course of insulin effects on DAG (○) and PA (●) contents in BC3H-1 myocytes

Myocytes were prelabelled with $^{32}P_i$ for 3 days. After treatment with 200 nM-insulin for the time periods depicted (15 s–60 min), the lipid extracts were divided and analysed for PA and DAG contents. Shown here are mean values ± S.E.M. for four determinations; $*P < 0.05$ (as determined by comparison with control values by standard *t*-testing).

phosphatidylglycerol (Fig. 4). [Note: we have previously shown that insulin does not increase ATP specific radioactivity (Farese *et al.*, 1983), and the increased labelling of phospholipids which are extrinsic to the phosphoinositide cycle is in keeping with the contention that insulin increases synthesis *de novo* of PA, which is the source, not only of PI, PIP and PIP_2, but also of DAG and a variety of other phospholipids and lipids which are derived from DAG.] As is apparent, the $^{32}P_i$ labelling of PA, PI, PIP and PIP_2 was approaching a plateau after 2 h of incubation, and it would thus appear that insulin-induced increases in ^{32}P labelling reflected increases in the mass of these substances as observed previously (Farese *et al.*, 1982, 1984*b*). This was evaluated further in experiments in which fat pad phospholipids were prelabelled by incubation with $^{32}P_i$ for 2 h (Fig. 5); upon addition of insulin, there were prompt increases in ^{32}P-labelled PA, PI and PIP, and, after a slight lag, PIP_2; control tissues, on the other hand, showed only slight (if any) increases in ^{32}P labelling of these phospholipids in the 30 min experimental period.

Effects of insulin on diacylglycerol levels in BC3H-1 myocytes and rat fat pads

The rapidity of insulin-induced increases in PA and DAG were directly compared in BC3H-1 myocytes. Within 30 s of insulin treatment, PA content had increased significantly by 20% and remained elevated thereafter; on the other hand, DAG, as determined from the same lipid extracts, showed no increase during the first minute, but increased rapidly thereafter (Fig. 6). From these results, it appears that PA synthesis precedes, and thus may be the source of, the increase in DAG during insulin action.

Insulin also provoked increases in DAG content in rat epididymal fat pads (Fig. 7). Similar to the BC3H-1 myocyte, insulin effects on DAG were evident at 2 min and maximal within 5 min in the fat pad.

Effects of insulin on inositol phosphate levels in BC3H-1 myocytes and rat fat pads

After 3 days of prelabelling with [^{3}H]inositol, PI, PIP and PIP_2 had been labelled to constant specific radioactivity, and ^{3}H radioactivity in IP, IP_2 and IP_3 remained constant in control BC3H-1 myocytes (^{3}H in inositol phosphates was approx. 10–25% of the ^{3}H in these phospholipids). Upon addition of insulin, there were documented increases in PI, PIP and PIP_2 (see above and Table 2), but no significant changes in the levels of IP, IP_2 and IP_3 at any time during the insulin treatment period (30 s–40 min) (Fig. 8). Li^+ (10 mM, which was found to provoke maximal increases in IP), which inhibits IP phosphatase, failed to unmask any insulin-induced increases in IP, IP_2 or IP_3. In other experiments (not shown), 10^{-5} M-phenylephrine increased [^{3}H]inositol phosphate generation approx. 2-fold in BC3H-1 myocytes which were incubated under similar experimental conditions. [Phenylephrine-induced increases in IP, IP_2 and IP_3 in BC3H-1 myocytes have also been observed by Ambler *et al.* (1984).] In addition to IP, IP_2 and IP_3, insulin did not influence the rate of generation of glycerophospho[^{3}H]inositol.

Insulin also failed to influence ^{3}H labelling of IP, IP_2

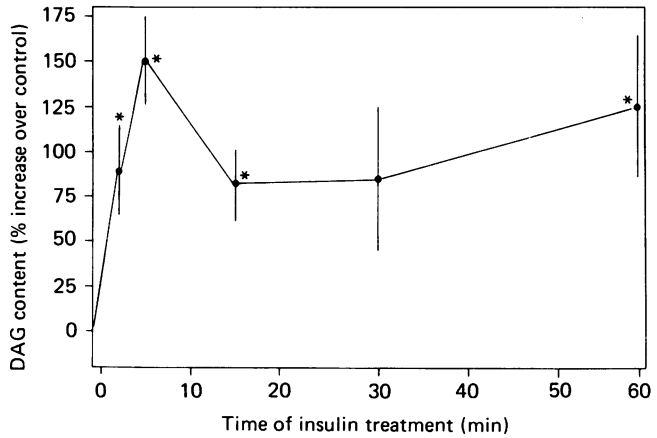

Fig. 7. Time course of insulin-induced increases in DAG content in rat epididymal fat pads

After preincubation for 30 min, insulin (1 munit/ml) was added to the medium, and incubations were continued for the indicated times. The DAG content of control tissues ranged from 125 to 190 nmol/100 mg wet wt. of tissue. Shown here are results from five experiments conducted in quadruplicate. To facilitate comparisons between experiments, results have been expressed as percentage increase over the mean control zero-time value (which did not change during the course of the 60 min incubation). $*P < 0.05$ (as determined by paired t-test evaluation of the mean difference).

and IP_3 in rat fat pads which were incubated in the presence or absence of 10 mM-LiCl (results not shown).

Effects of insulin and TPA on the cytosolic Ca^{2+} concentrations in BC3H-1 myocytes

Cytosolic free [Ca^{2+}] was determined from the fluorescence emitted by the Ca^{2+} indicator, Quin 2, which was trapped inside detached, resuspended myocytes [we have previously shown (Standaert & Pollet, 1984; Standaert *et al.*, 1984) that BC3H-1 cells suspended by gentle scraping retain the characteristics of insulin binding and hexose uptake of the monolayer cultures.] The concentration of Ca^{2+} in the cytosol (i.e. $[Ca^{2+}]_i$) of control BC3H-1 myocytes was found to be, on average, 175 ± 20 nM (range 150–220). Upon addition of 200 nM-insulin there were no significant changes in $[Ca^{2+}]_i$ as shown by a representative experiment in Fig. 9. This failure to observe an effect of insulin on $[Ca^{2+}]_i$ contrasts with increases observed with 10 μM-phenylephrine in the BC3H-1 myocyte (Fig. 9). TPA (500 nM), like insulin, failed to influence $[Ca^{2+}]_i$ appreciably (results not shown).

Table 2. Effects of insulin on phosphoinositides in BC3H-1 myocytes

After labelling with [^{3}H]inositol (5 μCi/3 ml of medium) for 3 days, BC3H-1 myocytes were treated with insulin (200 nM) or vehicle alone (controls) for 40 min. Inositol phosphates and inositol phospholipids were obtained from trichloroacetic acid extracts and precipitates, respectively, purified (see under 'Experimental') and counted for ^{3}H radioactivity. Shown here are means ± S.E.M. for four separate experiments in which there were three to five control and treatment dishes. In each experiment, the mean control and mean treatment values were used to determine the percentage change due to insulin. P was determined by paired t-test analysis of the mean difference; NS, not significant.

Substance	Change (%) due to insulin ($n = 4$)	P
PI	+69±10	< 0.01
PIP	+85±8	< 0.005
PIP_2	+95±21	< 0.025
IP	−2±1	NS
IP_2	+1±3	NS
IP_3	+3±10	NS

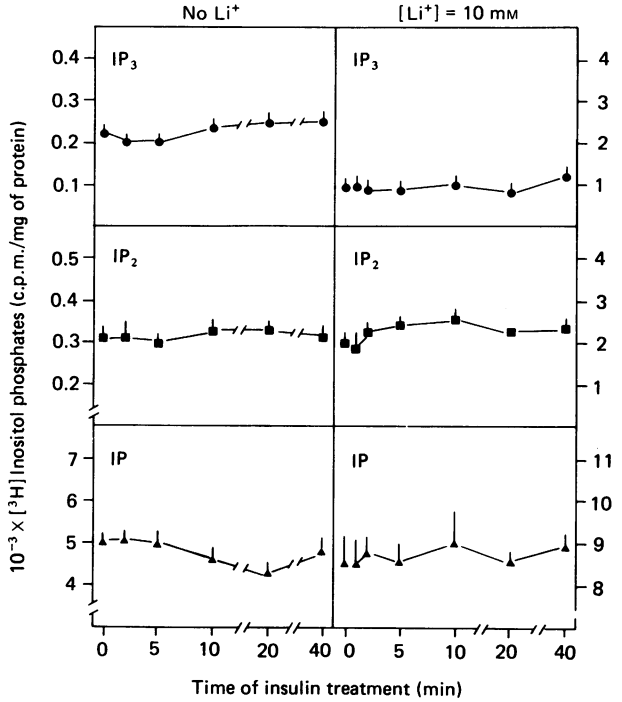

Fig. 8. Failure of insulin to alter levels of inositol phosphates in BC3H-1 myocytes

Cells were prelabelled by incubation for 3 days with [^{3}H]inositol. Where indicated (at right), 10 mM-LiCl was added 15 min before the experimental period. Insulin (200 nM) was then added and the incubation was continued for the indicated times. Control values were monitored throughout the experimental period and these did not change significantly (for simplicity only the zero-time control values are shown). Shown here are mean results ± S.E.M. for four plates.

Effects of extracellular Ca^{2+} on insulin- or TPA-stimulated uptake of 2-DOG or AIB

The time-courses and dose–responses for insulin and TPA effects on 2-DOG and AIB uptake in BC3H-1 myocytes have been determined previously (Farese *et al.*, 1985). These effects of insulin and TPA were previously shown (Farese *et al.*, 1985) to have similar time courses, to be non-additive at maximally effective concentrations of TPA, and were not observed with other phorbol esters which do not activate protein kinase C. As shown in Tables 3 and 4, insulin- and TPA-stimulated 2-DOG and AIB uptake were found to be largely independent of extracellular Ca^{2+} in BC3H-1 myocytes. Although in

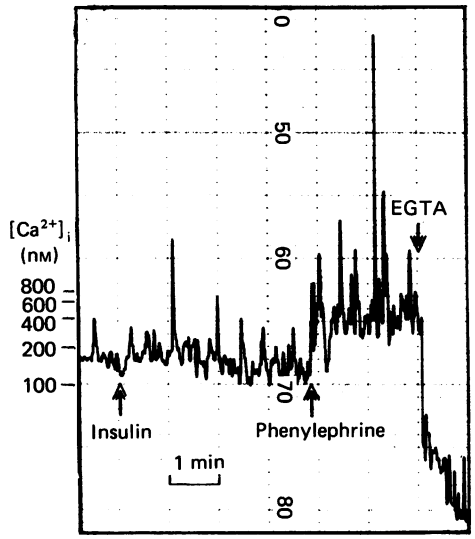

Fig. 9. Effects of insulin and phenylephrine on $[Ca^{2+}]_i$ in BC3H-1 cells

BC3H-1 myocytes were loaded with Quin 2/AM and Ca^{2+}-dependent fluorescence was continuously measured (see under 'Experimental'). Insulin (200 nM) and phenylephrine (10 μM) were added after 10 min of preequilibration at 37 °C. The calibration of the $[Ca^{2+}]_i$ scale was determined by additions of digitonin and EGTA (see under 'Experimental') Shown here is a representative experiment in which insulin and phenylephrine were added successively to the same myocyte suspension.

some experimental groups, there appeared to be an inhibitory effect of Ca^{2+} itself on 2-DOG uptake in control cells, the stimulatory effects of both insulin and TPA on both 2-DOG and AIB uptake remained clearly evident both in the presence of Ca^{2+} in the incubation medium and when the latter was decreased to low levels.

Effect of TPA on phospholipid metabolism in BC3H-1 myocytes

We have now observed (Farese *et al.*, 1985; R. V. Farese, unpublished work) insulin-like effects of TPA on glucose transport, AIB transport, pyruvate dehydrogenase activation, and $(Na^+ + K^+)$-ATPase activation. It was therefore of importance to determine whether TPA was capable of provoking the phospholipid synthesis *de novo* effect of insulin. As shown in Table 5, TPA, in concentrations of 10–1000 nM [the ED_{50} values for AIB and 2-DOG uptake are 10 and 30 nM-TPA, respectively (Farese *et al.*, 1985)], failed to influence $^{32}P_i$ content in PI, PIP or PIP_2 in BC3H-1 myocytes which had been prelabelled to constant specific radio activity by culturing for 24 h in the presence of $^{32}P_i$; on the other hand, insulin provoked increases in phospholipids in cells incubated in parallel.

DISCUSSION

The present results provide additional evidence that insulin increases PI, PIP and PIP_2 content in both cultured BC3H-1 myocytes and rat epididymal adipose tissue. More importantly, these increases, particularly in PA and PI, have now been shown to occur very rapidly.

Table 3. Effects of extracellular Ca^{2+} on insulin- and TPA-stimulated 2-DOG uptake in BC3H-1 myocytes

The medium for preincubation and incubation (see under 'Experimental') contained the indicated concentrations of $CaCl_2$ and EGTA. Data shown are means ± S.E.M. of [^{3}H]2-DOG uptake. *P* values (in parentheses) were determined by the *t*-test.

	Uptake (c.p.m./plate)		
Treatment	0.2 mM-$CaCl_2$	No $CaCl_2$	No $CaCl_2$ 1 mM-EGTA
Control	1340 ± 97	1812 ± 116	3102 ± 132
Insulin, 200 nM	3746 ± 175	5162 ± 326	6312 ± 392
(*P*, insulin versus control)	(< 0.001)	(< 0.001)	< 0.001)
Control	3236 ± 460	4036 ± 640	3088 ± 246
TPA, 1000 nM	10170 ± 290	11458 ± 716	11120 ± 374
(*P*, TPA versus control)	(< 0.001)	(< 0.01)	(< 0.001)

Table 4. Effects of extracellular Ca^{2+} on insulin- and TPA-stimulated AIB uptake in BC3H-1 myocytes

Data shown are means ± S.E.M. of [^{3}H]AIB uptake. *P* was determined by *t*-test comparison of the insulin or TPA group versus the control group.

	Uptake (c.p.m./0.5 plate)	
Treatment	No $CaCl_2$	0.9 mM-$CaCl_2$
Control	13223 ± 673	15252 ± 2079
Insulin, 200 nM	25925 ± 868 ($P < 0.01$)	36195 ± 2055 ($P < 0.01$)
TPA, 1000 nM	26108 ± 1286 ($P < 0.01$)	23655 ± 770 ($P < 0.02$)

Table 5. Failure of TPA to mimic the insulin effect on inositol phospholipid synthesis *de novo* in BC3H-1 myocytes

BC3H-1 myocytes (approx. 150 μg of protein/well at the time of experiment) were grown to confluence in 24-well cluster plates and prelabelled with $^{32}P_i$ (5 μCi/ml of medium) for 24 h to label phosphoinositides to constant specific radio activity. Insulin or TPA were added, and incubation was continued for 60 min. Mean values ± S.E.M. for six to eight determinations are shown: $*P < 0.05$, $**P < 0.005$ (*t*-test versus control)

Addition	$^{32}P_i$ incorporation (c.p.m./sample)		
	PI	PIP	PIP_2
None (control)	2597 ± 263	165 ± 5	480 ± 52
Insulin, 200 nM	3604 ± 303*	248 ± 32*	754 ± 55**
TPA, 10 nM	2270 ± 222	113 ± 13	470 ± 47
TPA, 100 nM	2086 ± 180	115 ± 10	409 ± 28
TPA, 1000 nM	2022 ± 224	135 ± 30	475 ± 86

Increases in PIP and PIP_2 were observed after a slight lag and may therefore derive from the prior increase in PI. However, insulin, possibly via its receptor (Machicao & Wieland, 1984), may simultaneously increase PI and PIP kinase activities to shuttle newly synthesized PI to PIP and PIP_2.

It is important to note that PI, PIP and PIP_2 contents did not decrease at very early time points during insulin treatment. Thus, we can find no evidence to suggest that insulin initially activates phospholipase C prior to increasing phospholipid synthesis.

We previously showed that the insulin-induced increases in PI, PIP and PIP_2 in BC3H-1 myocytes were not due to decreases in the degradation rate of pre-existing phospholipids (Farese *et al.*, 1984*a*). That information, and the present findings in acute $^{32}P_i$ incorporation experiments, clearly indicate that insulin increases the synthesis *per se* of PI, PIP and PIP_2; furthermore, this increase in synthesis appears to be sustained throughout the first 2 h of insulin treatment.

The failure to observe increases in IP, IP_2 and IP_3 during insulin treatment was surprising, since there were concomitant increases in the contents of their precursors, PI, PIP and PIP_2. However, to explain this dissociation, the following should be considered: (*a*) insulin-induced inositol phospholipids may be degraded by phospholipase C at a reduced fractional rate, thus keeping the absolute rate of degradation via this pathway constant; (*b*) PIP_2 and PIP may be degraded by phosphomonoesterases to PI; (*c*) PI may be degraded directly to PA by phospholipase D, or to CDP-DAG by reversal of the PI synthesis reaction between inositol and CDP-DAG; (*d*) CDP-DAG may be converted to phosphatidylglycerol and possibly to other phospholipids and lipids; and (*e*) the rate of degradation of IP, IP_2 and IP_3 may be concomitantly increased during insulin action.

Whatever the cause, the present failure to observe insulin-induced increases in inositol phosphates is important for several reasons. First, it may be surmised that a simple increase in PI, PIP and PIP_2 does not necessarily imply that IP, IP_2 and IP_3 levels will be increased; stated differently, the latter may require a concomitant activation of phospholipase C. Second, insulin apparently does not employ IP_3 as a 'second messenger' to mobilize intracellular Ca^{2+} in BC3H-1 myocytes. In accord with the latter, insulin did not increase cytosolic Ca^{2+} concentrations significantly (this does not rule out the possibility that small increases may have been buffered and masked by Quin 2, or that insulin may alter the state of Ca^{2+} in other subcellular compartments).

The observed increase in DAG, but not IP_3, has several important implications. First, the insulin-induced increase in DAG is probably not derived from phospholipase C acting upon PI, PIP or PIP_2, but may be derived directly from PA (via PA phosphatase action), which, in turn may be synthesized *de novo* and/or derived from degradative turnover of phospholipids via enzymes other than phospholipase C. Second, the insulin-induced increase in DAG may activate protein kinase C and result in the phosphorylation of specific cellular proteins (Nishizuka, 1984*a*, *b*). Third, those proteins whose phosphorylation is dependent on IP_3 generation and a consequent increase in cytosolic Ca^{2+} should not be influenced by insulin treatment.

In keeping with the possibility that the insulin-induced increases in DAG, and the consequent activation of protein kinase C, may be important in the control of metabolic processes during insulin action, we have recently demonstrated the presence of protein kinase C in BC3H-1 myocytes, and have found that the phorbol diester, TPA, and/or the synthetic diacylglycerol, 1-oleoyl-2-acetyl-*sn*-glycerol, mimic the effects of insulin in BC3H-1 myocytes, and provoke similar time-related increases in the transport of 2-DOG and AIB and pyruvate dehydrogenase activity (Farese *et al.*, 1985). As shown in the present paper the effects of TPA and insulin on 2-DOG and AIB transport were not influenced appreciably by changes in extracellular Ca^{2+}. Additionally, neither insulin nor TPA increased $[Ca^{2+}]_i$, as determined by Quin 2. It therefore seems more likely that these effects are mediated by activation of protein kinase C, rather than by increases in Ca^{2+}. Thus, our findings fit well with the possibility that DAG, but not IP_3 and IP_3-induced Ca^{2+} mobilization, is a 'second messenger' or intracellular mediator during insulin action.

In evaluating the potential importance of observing insulin-like effects of TPA, it was important to determine whether TPA was acting at a step which is prior to the phospholipid effect in insulin action, e.g. by activating the insulin receptor directly or via protein kinase C-mediated phosphorylation [see Jacobs *et al.* (1983)]. As shown in the present paper, TPA did not provoke the *de novo* phospholipid effect of insulin, and this is an important negative finding, since, if we are to postulate that insulin activates cellular processes [such as glucose transport,

amino acid transport, pyruvate dehydrogenase activation and $(Na^+ + K^+)$-ATPase activation] via increases in phospholipid and associated DAG synthesis, TPA (which mimics DAG) should act on processes which are distal, but not proximal, to the phospholipid–DAG effect.

Whether or not the observed insulin-induced increase in DAG activates protein kinase C in intact tissue is presently uncertain. To gain insight into this question, we have, in initial experiments, examined protein phosphorylation patterns in BC3H-1 myocytes, which were analysed by one-dimensional polyacrylamide-gel electrophoresis. Unfortunately, neither insulin nor TPA provoked a characteristic phosphorylation pattern. Recently, however, Trevillyan *et al.* (1985) have shown that both insulin and TPA provoke identical phosphorylation of highly purified peptides derived from tryptic digestion of ribosomal protein S6 in a Reuber H35 hepatoma cell line. The authors concluded that TPA provoked this effect through activation of protein kinase C (rather than by activation of the insulin receptor), but it was not certain whether the specific phosphorylation of S6 was mediated directly by protein kinase C or indirectly via activation of another proteinase-activated kinase II. In any event, this phosphorylation of S6 is another notable effect that is commonly provoked by insulin and TPA. Obviously, further studies are needed to determine whether other peptides and proteins are commonly phosphorylated by insulin, TPA and/or protein kinase C.

In addition to the possibility that insulin-induced increases in DAG may activate protein kinase C and alter protein phosphorylation, DAG may activate some cellular processes through increases in membrane fusogenicity. Allan *et al.* (1976) have suggested that DAG is responsible for increased fusogenicity and vesiculation of erythrocyte membranes after A23187-induced activation of phospholipase C. Perhaps the insulin-induced increase in DAG promotes the translocation of intracellular glucose transporters to functional sites in the plasma membrane by alterations in membrane fusogenicity.

While the present and previous [Farese *et al.* (1985)] findings are in keeping with the possibility that phospholipid synthesis and DAG generation may function as an intracellular regulatory system during insulin action, several recent reports have raised some uncertainty in this regard. First, Roach & Goldman (1983) have found that TPA inhibits glycogen synthase in rat hepatocytes, and, furthermore, Takayama *et al.* (1984) have reported that TPA antagonized insulin-induced increases in both glycogen synthase and tyrosine aminotransferase activity in cultured rat Fao hepatoma cells. These findings suggest that these effects of insulin are mediated by a mechanism which does not involve protein kinase C activation. However, it should be realized that some TPA effects, e.g. most notably net glycogenolysis in hepatocytes (see Kimura *et al.*, 1984), appear to be mediated by increases in Ca^{2+}, rather than by protein kinase C activation, and increases in Ca^{2+} may inhibit insulin effects on glycogen synthase and other enzymes. Second, Kirk & Michell (1981) and Taylor *et al.* (1985) have failed to observe effects of insulin on acute $^{32}P_i$ and [^{3}H]inositol (respectively) incorporation into phosphoinositides in rat hepatocytes. These observations raise the possibility that insulin may not stimulate phospholipid and DAG synthesis in this tissue. If this is the case, the hepatic effects of insulin would have to be mediated by intracellular substances other than DAG and phospholipids. Along the latter lines, insulin effects on glucose transport are seen only in extrahepatic tissues, and, in the case of fat and muscle, insulin is known to increase both phospholipid and DAG synthesis and glucose transport. Accordingly, it is possible that DAG is important in mediating certain insulin effects, e.g. glucose uptake in extra-hepatic tissue, but is not important as a mediator of insulin effects in the liver. However, before any conclusions can be drawn, more definitive phospholipid and DAG synthesis studies will need to be conducted in the liver, particularly since acute labelling studies may be misleading, and, furthermore, since acute insulin treatment of rat liver preparations *in vitro* results in small but significant increases in glycerol-phosphate acyltransferase activity (for PA synthesis *de novo*) (Bates *et al.*, 1977), glycerol 3-phosphate availability (Beynen *et al.*, 1980), and incorporation of [1-^{14}C]acetate (Geelen *et al.*, 1978) and [9,10-^{3}H]palmitate and [1-^{14}C]oleate (Beynen *et al.*, 1980) into triacylglycerol, phosphatidylcholine and phosphatidylethanolamine. Since the latter three substances derive from DAG, it remains a distinct possibility that insulin may increase DAG in the liver.

Finally, the rapidity of increase in PA and PI synthesis demonstrates that the phospholipid synthesis *de novo* effects occurs very early in the action of insulin. The precise subcellular site for initiation of this phospholipid effect and the mechanism whereby it is provoked are presently unknown. Nevertheless, the *de novo* effect apparently initiates a number of rapid changes in the phospholipid and lipid composition of cellular membranes. Through associated increases in DAG and possible activation of protein kinase C, insulin may regulate a variety of cellular processes. Further studies are needed to evaluate more fully the role of DAG and other lipids or phospholipids as 'second messengers' in the action of insulin.

This work was supported by NIH Grants HL-28290 (R.V.F.), HD-17939 (J.S.D.) and AM-18608 (R.J.P.) and the Research Service of the Veterans Administration.

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Received 7 February 1985/4 June 1985; accepted 14 June 1985