The phosphatidylinositol 3-kinase inhibitor, wortmannin, inhibits insulin-induced activation of phosphatidylcholine hydrolysis and associated protein kinase C translocation in rat adipocytes

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We questioned whether phosphatidylinositol 3-kinase (PI 3-kinase) and protein kinase C (PKC) function as interrelated signalling mechanisms during insulin action in rat adipocytes. Insulin rapidly activated a phospholipase D that hydrolyses phosphatidylcholine (PC), and this activation was accompanied by increases in diacylglycerol and translocative activation of PKC- α and PKC- β in the plasma membrane. Wortmannin, an apparently specific PI 3-kinase inhibitor, inhibited insulin-stimulated, phospholipase D-dependent PC hydrolysis and subsequent translocation of PKC- α and PKC- β to the plasma

membrane. Wortmannin did not inhibit PKC directly *in vitro*, or the PKC-dependent effects of phorbol esters on glucose transport in intact adipocytes. The PKC inhibitor RO 31-8220 did not inhibit PI 3-kinase directly or its activation *in situ* by insulin, but inhibited both insulin-stimulated and phorbol ester-stimulated glucose transport. Our findings suggest that insulin acts through PI 3-kinase to activate a PC-specific phospholipase D and causes the translocative activation of PKC- α and PKC- β in plasma membranes of rat adipocytes.

INTRODUCTION

Phosphatidylcholine (PC) hydrolysis is an important widespread mechanism for receptor-mediated activation of protein kinase C (PKC) [1]. Other than for antecedent phosphatidylinositol 4,5-bisphosphate (PIP₂) hydrolysis, which stimulates PC hydrolysis through increases in cytosolic Ca²⁺ and/or PKC activation, there is little insight into other receptor-dependent mechanisms that lead to PC hydrolysis, except that G-proteins are probably involved [2]. In the case of insulin action, PC is rapidly hydrolysed independently of PIP₂ hydrolysis, Ca²⁺ mobilization, or antecedent PKC activation [3–5]. In rat hepatocytes and BC3H-1 myocytes, a PC-specific phospholipase D (PC-PLD) is rapidly activated by insulin and this activation is followed by, or associated with, increases in diacylglycerol (DAG) and increases in membrane PKC activity [3–5]. In rat adipocytes, insulin-induced PC hydrolysis is accompanied by rapid increases in DAG mass and PKC enzyme activity in the plasma membrane [6–8]. Concomitantly, insulin activates phosphatidylinositol 3-kinase (PI 3-kinase) in the plasma membrane and other membranes of rat adipocytes [9,10].

In view of the fact that inhibitors of PI 3-kinase [11–14], and inhibitors [15–18] or depletors [19–21] of PKC, inhibit insulin effects on glucose transport in rat adipocytes, we evaluated the possibility that PI 3-kinase, PC hydrolysis and PC hydrolysis-dependent PKC activation may be interrelated events in adipocytes of normal non-diabetic (ND) rats and type-II diabetic Goto-Kakizaki (GK) rats. GK rat adipocytes were particularly useful in these studies, since, as reported here, they selectively retain insulin effects on phospholipase D-dependent PC hydrolysis and on associated DAG production and PKC activation in the plasma membrane. However, as previously reported [22],

they lack confounding effects on glycosylphosphatidylinositol hydrolysis and *de novo* phosphatidic acid (PA) synthesis. We found that wortmannin, the PI 3-kinase inhibitor, blocked the effects of insulin on PLD-dependent PC hydrolysis and PKC translocation in plasma membranes of both ND and GK rats. Our findings suggest that activation of PC-PLD and associated PKC is downstream of PI 3-kinase in the action of insulin.

EXPERIMENTAL

ND and GK male rats weighing approx. 200 g (approx. 2 months old) were fed standard rat chow *ad libitum*. ND rats were obtained from Holtzman. GK diabetic rats were obtained from Drs. S. Suzuki and T. Toyota of Tohoku University (Sendai, Japan) and were housed in a colony established at the Tampa VA Hospital as described in [22]. Serum glucose levels of ND and GK rats were approx. 150 and 275 mg/dl respectively. Except for increases in serum glucose and moderate 2-fold increases in fasting serum insulin levels [23], GK rats have relatively normal body weights (as per age), appear outwardly healthy, reproduce readily, and develop diabetic complications only much later in life. GK rats have hepatic and extrahepatic insulin resistance, as measured in euglycaemic-hyperinsulinaemic clamp studies [22,24], but retain normal glucose transport responses to insulin in their isolated adipocytes [22] and certain skeletal muscles [22,24].

Adipocytes were prepared from epididymal fat pads of ND and GK rats by collagenase digestion, and were suspended in glucose-free Krebs–Ringer phosphate (KRP) buffer containing 1% (w/v) BSA for acute studies, or in Dulbecco's modified Eagle's medium (DMEM) containing 0.5% BSA and 10 mM

Abbreviations used: DAG, diacylglycerol; 2-DOG, 2-deoxyglucose; DMEM, Dulbecco's modified Eagle's medium; GK, Goto-Kakizaki; G3PAT, glycerol-3-phosphate acyltransferase; KRP, Krebs–Ringer phosphate; MAP kinase, mitogen-activated protein kinase; MARCKS, myristoylated alanine-rich C-kinase substrate; ND, non-diabetic; PA, phosphatidic acid; PC, phosphatidylcholine; PC-PLD, PC-specific phospholipase D; PET, phosphatidylethanol; PI 3-kinase, phosphatidylinositol 3-kinase; PIP₂, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

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Figure 1 Effects of wortmannin (A) and RO 31-8220 (B) on insulin- and PMA-stimulated [3 H]2-DOG uptake in ND or GK rat adipocytes

Wortmannin and RO 31-8220, at the indicated concentrations, were added 15 min before addition of 10 nM insulin (INS), 500 nM PMA, or vehicle (CON, controls). ND rat adipocytes were used in RO 31-8220 experiments. After treatment for 30 min, [3 H]2-DOG uptake was measured over a 1 min period. Values are means $\pm$ S.E.M. of four experiments, each with quadruplicate determinations. Shown in the inset in (B) are the effects of RO 31-8220 on MARCKS phosphorylation in intact insulin-stimulated ND rat adipocytes; similar results were observed in duplicate incubations.

Hepes for studies that involved overnight labelling of lipid pools prior to acute insulin treatment, as described previously [3,6,7,22].

Insulin-induced PC hydrolysis was studied as described previously [3]. Adipocytes were incubated batchwise for 20–24 h in the presence of [3 H]choline to label PC to virtually constant specific radioactivity [3]. Medium was removed and adipocytes were washed. They were then suspended in glucose-free KRP medium containing 1% BSA, divided into various treatment groups, equilibrated for 20 min, and then treated with or without 10 nM insulin for the indicated times. Reactions were stopped with 50% (v/v) methanol; chloroform was added and washed extracts were analysed for labelled [3 H]PC in the lipid phase and release of [3 H]choline and [3 H]phosphocholine into the aqueous phase, as described [3]. In some experiments plasma membranes and microsomes were isolated at the end of incubation, as described previously [6,7], in order to measure changes in [3 H]PC in the lipid extracts of these specific membrane fractions. In other experiments, PLD activation was assessed by production of [3 H]phosphatidylethanol ([3 H]PET), as described previously [4], in adipocytes which were incubated with [3 H]-glycerol or [3 H]oleic acid for 20–24 h to prelabel PC and other lipids, then washed and incubated in glucose-free KRP medium containing 1% (w/v) BSA and 1.5 or 2% (v/v) ethanol, and treated with or without 10 nM insulin for the indicated times. In all experiments, insulin was added in a reverse sequence (i.e. longer treatments before shorter), so that the incubation time for all samples was kept constant, and the only variable was the duration of exposure to insulin.

Insulin-induced activation of *de novo* PA/PC synthesis was assessed in two ways. First, glycerol-3-phosphate acyltransferase (G3PAT) enzyme activity was measured (as [14 C]glycerol 3-phosphate incorporation into PA) in cell-free homogenates, as described elsewhere [24,25], following a 2 min treatment of intact adipocytes in the presence or absence of 10 nM insulin. Secondly, acute incorporation of [3 H]glycerol into microsomal PC (via PA

and DAG; see ref. [3]) in intact cells was assessed after a 30 min incubation of adipocytes in the presence of [3 H]glycerol, followed by treatment for 0.5 or 1 min with or without 10 nM insulin as described previously [26], and subsequent isolation of microsomes (see above).

To study PKC translocation, adipocytes in glucose-free KRP medium containing 1% (w/v) BSA were treated with or without 10 nM insulin for the indicated times. PKC- α , PKC- β and PKC- ϵ levels were then analysed in subcellular fractions by Western blotting, as described in [6], except for the use of ECL (Amersham) to improve immunodetection. Polyclonal antisera used for measurement of PKC- α and PKC- ϵ were obtained from Life Technologies, Inc., and that used for measurement of PKC- β was kindly provided by Drs. J. Mehegan, B. Roth and M. Iadarola [27]. As previously described [6], PKC- α and PKC- β migrated as 80 kDa proteins, and PKC- ϵ migrated as a 90–95 kDa protein, on SDS/PAGE. Epitope specificity was verified by showing that immunoreactivity of samples was lost when assays were conducted in the presence of the immunizing peptide. Specificities of anti-PKC sera were further verified with recombinant PKCs (α , β , γ , δ , ϵ and ζ) obtained from NIH-3T3 cells stably transfected with various PKCs, and/or from baculovirus-infected insect cells, as described previously [6]. Samples that were compared from each experiment were analysed on the same immunoblot, and relative changes were quantified by scanning laser densitometry as described in [6].

In a few experiments, inhibition of PKC in intact cells was assessed by phosphorylation of myristoylated alanine-rich C-kinase substrate (MARCKS), as described previously [7]. In brief, adipocytes were prelabelled for 2 h with [32 P]P $_i$, then treated with or without 10 nM insulin for 10 min; cell extracts were then prepared using 1% Triton X-100 and examined for immunoprecipitable MARCKS. Direct effects of inhibitors on PKC enzyme activity were measured by phosphatidylserine/dioleiln/Ca $^{2+}$ -dependent histone IIIs phosphorylation, using PKC

purified from rat brain cytosol by Mono-Q column chromatography [28].

PI 3-kinase enzyme activity was measured in rat adipocyte membrane fractions, or in anti-phosphotyrosine immunoprecipitates, as described by Okada et al. [12]. Mitogen-activated protein kinase (MAP kinase; myelin basic protein phosphorylation) was measured in rat adipocyte cytosol fractions as described previously [22].

Methods for measurement of DAG and 2-deoxyglucose (2-DOG) uptake have been described previously [3,20].

The following materials and their sources, given in parentheses, were used: insulin (Elanco); phorbol 12-myristate 13-acetate (PMA) (Sigma); wortmannin (Sigma); RO 31-8220, a relatively specific bisindolemaleimide-type PKC inhibitor [29] (kindly provided by Dr. Geoff Lawton, Roche Research Centre, Welwyn Garden City, U.K.); calphostin C (Calbiochem and LC Laboratories); GF109203X, another bisindolemaleimide-type PKC inhibitor [29] (Calbiochem); [*methyl*- ^3H]choline (NEN); $\text{H}_3^{32}\text{PO}_4$ (NEN); [$2\text{-}^3\text{H}$]glycerol (NEN); [$\text{U-}^{14}\text{C}$]glycerol 3-phosphate (NEN); [^3H]2-DOG (NEN); [$9,10\text{-}^3\text{H}$]oleic acid (NEN); DMEM (Life Technologies); BSA (Sigma); collagenase (Worthington); anti-phosphotyrosine monoclonal antibodies [Transduction Labs or Upstate Biotechnology Incorporated (UBI)]. Anti-MARCKS serum was kindly provided by Drs. Ivar Walaas and Paul Greengard. All other biochemical substances were obtained from Sigma.

RESULTS

Wortmannin, the PI 3-kinase inhibitor, inhibited insulin-stimulated 2-DOG uptake in both ND and GK rat adipocytes (Figure 1A): the dose-related inhibitory effects of wortmannin on 2-DOG uptake observed here were identical to those reported previously, with maximal inhibition observed at 0.1–1 μM [11–14]. These results indicated that PI 3-kinase functioned in a similar way in ND and GK rat adipocytes.

In contrast to insulin effects, wortmannin did not inhibit PMA-stimulated 2-DOG uptake, which was approx. 30 % of insulin-stimulated 2-DOG uptake in ND and GK adipocytes (Figure 1A). In addition, wortmannin (1–1000 nM) had no effect on rat brain or rat adipocyte PKC enzyme activity, as assayed *in vitro*, or upon PMA-induced activation of MAP kinase (results not shown). These results suggest that: (a) wortmannin does not inhibit at least certain kinases (e.g. PKC and MAP kinase) that may operate downstream of PI 3-kinase; and (b) either PMA activates glucose transport by a signalling pathway that is different from that used by insulin, or PKC may operate downstream of PI 3-kinase during insulin action.

Different in some respects from wortmannin, the PKC inhibitor RO 31-8220 inhibited the stimulatory effects of both insulin and PMA on 2-DOG uptake (Figure 1B). Moreover, the dose-dependent inhibition of insulin-stimulated 2-DOG uptake correlated reasonably well with the inhibitory effects of RO 31-8220 on PKC-dependent MARCKS protein phosphorylation in intact adipocytes (inset, Figure 1B). (As reported previously [7], insulin, like PMA, provokes a 2-fold increase in adipocyte MARCKS phosphorylation.) In contrast to inhibition of PKC, RO 31-8220 (0.2–20 μM) did not inhibit insulin-stimulated PI 3-kinase either directly in assays *in vitro* of crude membranes (Figure 2D) and anti-phosphotyrosine immunoprecipitates (results not shown), or in intact adipocytes, as measured both in crude membranes (Figure 2C) and, perhaps more definitively, in anti-phosphotyrosine immunoprecipitates (Figures 2A and 2B) isolated from these adipocytes. (Also note in Figures 2C and 2D

Figure 2 Effects of RO 31-8220 on insulin-stimulated PI 3-kinase in ND rat adipocytes

Shown here are representative autoradiograms of the PI 3-kinase-dependent phosphorylation of TLC-purified phosphatidylinositol 3-phosphate (arrowhead), which migrated just below authentic phosphatidylinositol 4-phosphate standard (asterisk) (also see [12]). Similar results were obtained in three or more experiments. PI 3-kinase was assayed in anti-phosphotyrosine (anti-PY) immunoprecipitates in (A) (using UBI antibodies) and (B) (using Transduction Laboratories antibodies), and in whole membrane fractions (300 000 g for 45 min) in (C) and (D). In (A), (B) and (C) intact adipocytes were incubated for 20 min in the absence or presence of 300 nM insulin (I) with or without 20 μM RO 31-8220 (Ro), as indicated above the panels by the *in situ* designation, and whole membrane fractions (C) or anti-PY immunoprecipitates of total cell lysates (A and B) were then isolated and assayed for PI 3-kinase activity. In (D), increasing concentrations of RO 31-8220, as indicated at the bottom of the panel by the *in vitro* designation, were added directly to assays of PI 3-kinase in insulin-treated membranes (note that the TLC developing system used in this experiment was slightly different from that used in A, B and C). In (C), as indicated at the bottom of the panel, the second lane depicts inhibitory effects of 100 nM wortmannin (W) added directly to the assay of insulin-treated membranes *in vitro*; also note that, to facilitate side-by-side comparisons, there are duplicate assays of membranes obtained from insulin-treated and insulin + RO 31-8220-treated adipocytes in this panel.

that phosphatidylinositol 3-phosphate migrated below the level of the phosphatidylinositol 4-phosphate standard, and that 100 nM wortmannin inhibited phosphatidylinositol 3-phosphate labelling in insulin-treated membranes.) These findings suggested that: (a) RO 31-8220 does not inhibit PI 3-kinase or more proximal kinases in the insulin cascade; and (b) PI 3-kinase may function upstream, but apparently not downstream, of an RO 31-8220-sensitive PKC during insulin action.

In addition to RO 31-8220, other PKC inhibitors, i.e.

Figure 3 Time-dependent effects of insulin on [^3H]PC hydrolysis (A) and PKC- α and PKC- β translocation (B) in GK rat adipocytes

In experiments shown in (A), adipocytes were labelled for 20–24 h with [*methyl*- ^3H]choline, washed, equilibrated in glucose-free KRP medium, and aliquots containing approx. 25 000 c.p.m. in [^3H]PC and 20 000 c.p.m. in aqueous phase in 0.5 ml of a 6% cell suspension were then treated with or without 10 nM insulin for the indicated times. After stopping reactions with methanol/chloroform (1:2, v/v), [^3H]choline and [^3H]phosphocholine in the aqueous phase were measured after purification by TLC (note, the ratio of [^3H]choline/[^3H]phosphocholine was approx. 1:4, and these substances accounted for over 80% of total aqueous radioactivity). Values are means $\pm$ S.E.M. of five separate experiments, each conducted in quadruplicate. In experiments shown in (B), freshly prepared GK rat adipocytes were equilibrated in glucose-free KRP medium and then treated with or without 10 nM insulin for the indicated times during a 20 min treatment period. The total incubation time was equal for all samples. Cytosol (Cyt) and total membrane (Mem) fractions were prepared from 2 to 4 ml of adipocytes. Mean values $\pm$ S.E.M. of (*n*) experiments are depicted as percentages of the control. Asterisks indicate $P < 0.05$ (paired *t*-test).

calphostin C and GF109203X, inhibited both insulin- and PMA-stimulated 2-DOG uptake (results not shown). (Note that calphostin C inhibits the regulatory PMA- or DAG-binding domain of PKC, whereas RO 31-8220 and GF109203X, both staurosporine-like indolemaleimide derivatives, inhibit the catalytic domain.) The concentrations of RO 31-8220 and GF109203X required to fully inhibit both insulin- and PMA-stimulated 2-DOG uptake in adipocytes were greater (10–20 μM) than those required to inhibit PKC-dependent processes in certain other cells (e.g. approx. 3 μM in [29]): this may reflect involvement of different PKC isoforms or other protein kinases that vary in inhibitor sensitivity, or the partitioning of lipid-soluble inhibitors into large inert lipid storage depots of adipocytes. With calphostin C, maximal and half-maximal inhibition of both insulin- and PMA-stimulated 2-DOG uptake were observed at approx. 10 and 3 μM respectively. With GF109203X, maximal and half-maximal inhibition of 2-DOG uptake were observed at 10 and 20 μM for insulin treatment, and at 5 and 10 μM for PMA treatment.

To evaluate further the possibility that PKC operates downstream of PI 3-kinase, we used both ND and GK rat adipocytes, since the latter were particularly helpful for selective studies of PC hydrolysis in the absence of confounding effects of insulin on glycosylphosphatidylinositol hydrolysis and *de novo* PA synthesis. After overnight incubation for 20–24 h in the presence of [*methyl*- ^3H]choline, PC pools of GK and ND rat adipocytes were labelled almost equally. After washing and equilibration in glucose-free KRP medium, insulin provoked rapid, sustained,

mean decreases of 10–15% in total levels of [^3H]PC in GK rat adipocytes, and this was accompanied by rapid, biphasic, mean increases of 70% in the release of [^3H]choline into the aqueous phase, along with smaller changes in [^3H]phosphocholine release (Figure 3). In ND rat adipocytes, the increases in [^3H]choline release into the aqueous phase were virtually identical in timing and magnitude (see below), although decreases in [^3H]PC levels were smaller and more transient (results not shown here, but see ref. [3], and see below in [^3H]oleic acid labelling experiments). As discussed below, this difference in the observed magnitude of the [^3H]PC nadir may reflect *de novo* synthesis of PC in ND, but not GK, rat adipocytes.

In conjunction with insulin-induced PC hydrolysis in GK rat adipocytes, there were concomitant increases in total cellular membrane levels of PKC- α and PKC- β (Figure 3). Relative increases in membrane PKC- α and PKC- β were slightly greater in ND rat adipocytes (results not shown), particularly at later time points, presumably reflecting the presence of additional insulin effects on glycosylphosphatidylinositol hydrolysis and *de novo* PA synthesis in these cells.

Rapid, sizeable decreases in [^3H]PC content, increases in DAG mass and translocation of PKC (PKC- α in particular, PKC- β to a slightly lesser extent, and PKC- ϵ to only a small extent) were readily apparent in plasma membranes, although not in microsomal membranes, of GK rat adipocytes (Figure 4). As expected, relative changes in the plasma membrane were greater than those observed in total cellular membranes (cf. Figures 3 and 4). The observed decreases in plasma membrane [^3H]PC were particularly

Figure 4 Effects of insulin on [3 H]PC hydrolysis, DAG production and levels of PKC- α , PKC- β and PKC- ϵ in plasma membranes (upper panels) and microsomes (lower panels) of GK rats

In each experiment, adipocytes from the same suspension were incubated in glucose-free KRP medium with or without 10 nM insulin for 0, 0.5 or 1 min, as indicated. Plasma membranes and microsomes were isolated from approx. 5 ml of adipocytes in each sample. In experiments in which [3 H]PC hydrolysis was measured (left-hand panels), adipocytes were labelled with [3 H]choline during a prior incubation for 20–24 h, and then washed and equilibrated in glucose-free KRP medium as described in Figure 3. In all experiments in which DAG and PKC were measured (other panels), freshly prepared adipocytes were used. Shown on the right are representative immunoblots of changes in PKC- α , PKC- β and PKC- ϵ in plasma membranes and microsomal membranes. Results shown in line drawings are means $\pm$ S.E.M. values of (n) experiments. Note that changes in [3 H]PC (left-hand panels) are expressed as percentages of the control, whereas changes in DAG and PKC (two centre panels) are expressed as percentage increase over the control. Asterisks indicate $P < 0.05$ (paired t -test).

prominent in GK rat adipocytes, presumably reflecting PC hydrolysis in the face of an impaired *de novo* PA/PC synthesis response. In support of the latter postulation, *de novo* synthesis of PC from [3 H]glycerol occurred very rapidly in microsomes (the site of insulin-sensitive G3PAT [25]) of ND, but not GK, rat adipocytes (Figures 5G and 5C respectively). In further support of this hypothesis, microsomal G3PAT is activated by insulin in ND, but not GK, rat adipocytes [22], and, with respect to G3PAT, it should be noted that insulin-induced activation of G3PAT was not inhibited by wortmannin in ND rat adipocytes (Table 1). Along these lines, it should also be noted that insulin activates microsomal DAG/PKC signalling in ND rat adipocytes [7,8], but, as shown here (see above), not in GK rat adipocytes.

Of particular interest is the finding that the effect of insulin on hydrolytic decreases in [3 H]PC in the plasma membrane of GK rat adipocytes was inhibited by wortmannin (Figure 5B). Moreover, wortmannin inhibited the effects of insulin on [3 H]choline release, presumably via PC-PLD, in both GK and ND rat adipocytes (Figures 5A and 5E). Similarly, insulin-induced increases in [3 H]PET production, as another indicator of phospholipase D activation (albeit less specific than [3 H]choline release with respect to the phospholipid source), were inhibited by wortmannin (Figure 5F; also see below).

In association with blockade of phospholipase D-dependent PC hydrolysis, rapid insulin-induced increases in the trans-

location of both PKC- α and PKC- β to plasma membranes were largely or fully inhibited by wortmannin in both GK and ND rat adipocytes (Figures 5D and 5H, and Figure 6). In other experiments using GK rat adipocytes (results not shown) insulin effects on both PKC- α and PKC- β at 10 min of treatment were also fully inhibited by wortmannin. The small residual effect of insulin on plasma membrane PKC that persisted in ND rat adipocytes (Figure 5H) in the presence of wortmannin may reflect glycosylphosphatidylinositol hydrolysis, which does not appear to be inhibited by wortmannin. In keeping with the latter suggestion, G3PAT activation was not wortmannin-sensitive (Table 1), and G3PAT activation appears to be a consequence of glycosylphosphatidylinositol hydrolysis [3,22,25,30] during insulin action.

In the above-described experiments, we focused upon initial events that were occurring primarily in the plasma membrane, and we used 1 μ M wortmannin to be certain that PI 3-kinase was fully inhibited in this compartment. However, we also found that 0.1 μ M wortmannin fully inhibited initial and subsequent insulin-induced effects: namely (a) increases in [3 H]PET production in ND rat adipocytes (Figure 7); (b) [3 H]choline release in GK rat adipocytes (Figure 8); and (c) decreases in [3 H]PC in ND rat adipocytes (Figure 7) and GK rat adipocytes (Figure 8). Again, it may be noted that PC hydrolysis was biphasic and PC nadirs were more pronounced in GK rat adipocytes.

Figure 5 Effects of wortmannin (W) on insulin-stimulated PC hydrolysis and PKC translocation in plasma membranes of GK (left-hand panels) and ND (right-hand panels) rats

Release of [3 H]choline into the aqueous phase (A and E) was measured as in Figure 3. Formation of [3 H]PET (F) was measured as described in the Materials and methods section using [3 H]glycerol to label phospholipids. [3 H]PC levels in plasma membranes (B) were measured as in Figure 4. [3 H]Glycerol-labelling of microsomal PC, as a measure of acute *de novo* PA/PC synthesis (C and G), was determined as described in the Materials and methods section. PKC- α levels in plasma membranes (D and H) were determined as in Figure 4. For simplicity, changes in plasma membrane PKC- β are not shown here, but insulin-induced increases were approx. 100% over those in controls (see Figure 4), and, like PKC- α , were largely or completely inhibited by wortmannin. In all experiments, insulin effects were simultaneously measured in the absence (—W, solid lines) or presence (+W, broken lines) of 1 μ M wortmannin. Wortmannin alone did not affect measured parameters. Where indicated by parentheses, values are means $\pm$ S.E.M. of (*n*) experiments. Results in (B), (C) and (G) are from representative experiments, repeated at least twice with similar results. Asterisks indicate $P < 0.05$ (*t*-test).

Table 1 Effects of wortmannin on insulin-induced activation of G3PAT in rat adipocytes

Values are means $\pm$ S.E.M. of six determinations.

Treatment	$[^{14}\text{C}]$ Glycerol incorporation into PA (c.p.m./mg of protein per min)	
	Without wortmannin	Plus 1 μ M wortmannin
None (control)	4896 $\pm$ 291	4687 $\pm$ 418
Insulin (3 nM)	8781 $\pm$ 256	9562 $\pm$ 675

DISCUSSION

The present findings suggest that insulin provokes rapid increases in PC hydrolysis in the plasma membrane of rat adipocytes, and this is accompanied by increases in DAG content and trans-

Figure 6 Effects of wortmannin on insulin-stimulated translocation of PKC to plasma membranes of ND rat adipocytes

PKC- α and PKC- β were assayed in plasma membranes (50 μ g of protein) of adipocytes that were treated with or without 1 μ M wortmannin (W) for 15 min, followed by 10 nM insulin (I) for 1 min, as indicated. The control (C) is shown in the centre lane. Shown here is a representative immunoblot. See Figures 4 and 5 for results of multiple experiments in which PKC- α and PKC- β were measured in plasma membranes of ND and GK rat adipocytes.

Figure 7 Effects of wortmannin on insulin-induced increases in [3 H]PET (upper panel) production and [3 H]PC hydrolysis (lower panel) in ND rat adipocytes

Adipocytes were labelled overnight with [3 H]oleic acid, washed, resuspended in glucose-free KRP containing 1% BSA, equilibrated with (+W) or without (—W) 0.1 μ M wortmannin and 1.5% ethanol for 15 min, and then treated with or without 10 nM insulin for the indicated times. Note that the total incubation time for all samples was identical; also wortmannin did not influence initial levels of label in phospholipids (PL). Values depicted are means $\pm$ S.E.M. of three or four determinations.

location of PKC- α and PKC- β to these membranes. These findings therefore complement previous findings of rapid insulin-induced increases in DAG content and PKC enzyme activity of rat adipocyte plasma membranes [6–8]. Moreover, it is clear

Figure 8 Effects of wortmannin on insulin-induced increases in [³H]choline release and [³H]PC hydrolysis in GK rat adipocytes

Adipocytes were labelled overnight with [³H]choline as in Figure 3 and then, as in Figure 7, incubated with (+ W) or without (– W) 0.1 μ M wortmannin and 10 nM insulin as indicated. Values depicted are means $\pm$ S.E.M. of three or four determinations.

from the present studies in the GK rat adipocyte that PC hydrolysis is a (or the) major cause of insulin effects on DAG/PKC signalling in the plasma membrane, as glycosylphosphatidylinositol hydrolysis and *de novo* PA synthesis are markedly deficient or absent in GK rat adipocytes [22].

Hydrolytic decreases in PC were particularly prominent in studies of plasma membranes of GK rat adipocytes, in which insulin effects on *de novo* PA/PC synthesis are compromised. Nevertheless, it seems clear that insulin provokes increases in PC hydrolysis in ND rat adipocytes (present results, and see ref. [3]), as well as in rat hepatocytes [5] and BC3H-1 myocytes [3,4]. Moreover, in each of these cell types, PC-PLD activation appears to play an important role in the initial phase (i.e. during the first 15–60 s) of insulin action, and is not dependent upon antecedent PKC activation or Ca^{2+} mobilization [3–5].

Of particular interest, the PI 3-kinase inhibitor, wortmannin, inhibited insulin-induced PLD-dependent PC hydrolysis and consequent PKC translocation. For the same reasons stated above, this inhibition was more clearly observed and more readily interpreted, with respect to interrelatedness between PI 3-kinase activation and PC hydrolysis, in GK rat adipocytes. Nevertheless, wortmannin-induced inhibition of insulin-stimulated PC hydrolysis and PKC translocation was also apparent in ND rat adipocytes, as shown by inhibition of (a)

[³H]choline release, (b) [³H]PET production, and (c) translocation of PKC- α and PKC- β to the plasma membrane.

Our findings with wortmannin therefore suggested that the effect of insulin on phospholipase D-mediated hydrolysis of PC is largely dependent upon PI 3-kinase activation. In contrast, the effects of insulin on G3PAT activation and *de novo* PA synthesis (presumably secondary to glycosylphosphatidylinositol hydrolysis [3,22,25,30]) did not appear to be dependent upon PI 3-kinase activation. Comparable separation of these phospholipid effects of insulin was observed in studies of BC3H-1 myocytes in which pertussis toxin inhibits insulin effects on glycosylphosphatidylinositol hydrolysis [31] and *de novo* PA synthesis [3,30], but not phospholipase D-mediated PC hydrolysis [3,4]. Also consistent with the present findings, increases in DAG and concomitant PKC activation within the first minute of insulin stimulation are only slightly diminished by pertussis toxin treatment of BC3H-1 myocytes [3,4]. Accordingly, in the present experiments in which glycosylphosphatidylinositol hydrolysis and *de novo* PA synthesis were compromised in GK rat adipocytes through genetic mechanisms [22], insulin-induced PC hydrolysis and increases in plasma membrane DAG and PKC- α and PKC- β translocation were apparently largely intact, particularly during the first minute of insulin treatment.

The present finding that wortmannin inhibited insulin-stimulated PC-PLD activation is similar to a previous report in which wortmannin inhibited formylmethionyl-leucyl-phenylalanine-induced activation of a phospholipase D and the respiratory burst in neutrophils [32]. The involvement of PI 3-kinase as the wortmannin-sensitive step was not recognized at that time, nor was it realized that PKC activation may have been a consequence of PI 3-kinase-dependent phospholipase D activation. It is tempting to suggest that PI 3-kinase activation may serve as a general mechanism for the activation of PC-PLD in the action of a variety of agonists.

There are two other noteworthy points that emerge from the present findings. First, the PI 3-kinase-dependent increases in PKC- α and PKC- β activation observed in the present study, particularly in the GK rat adipocytes, presumably reflect DAG derived from PC hydrolysis. This mode of PKC activation would therefore complement any PKC activation that may be occurring secondary to increases in 3'-phosphorylated derivatives of phosphatidylinositol. Secondly, inhibitory effects of RO 31-8220 on glucose transport are apparently not due to inhibition of PI 3-kinase, or other kinases that precede the activation of PI 3-kinase.

To summarize, our findings suggest that phospholipase D-dependent PC hydrolysis is a downstream signalling event that follows, and is dependent upon, the activation of PI 3-kinase by insulin. The mechanism(s), or factor(s), that couples PI 3-kinase activation to PC hydrolysis, however, is(are) unclear. Conceivably, wortmannin-sensitive protein–protein interactions between PI 3-kinase and other downstream proteins may be operative, or PI 3-kinase may activate a PC-PLD through increases in 3'-phosphorylated derivatives of inositol lipids. Along the latter lines, phosphatidylinositol 3,4,5-trisphosphate apparently activates PC-PLD, but this is not specific for 3'-phosphorylated inositol lipids [33]. Of further interest, small G-proteins, e.g. rho [34] and ARF [35], serve to activate PC-PLD in neutrophils; this raises the possibility that small G-proteins may operate between PI 3-kinase and PC-PLD. On the other hand, rho [36,37] and $\beta\gamma$ subunits of heterotrimeric G-proteins [38] have been suggested to operate upstream of PI 3-kinase in platelets. Perhaps similar mechanisms involving PI 3-kinase and these or other GTP-binding proteins are operative in insulin-induced activation of PC-PLD in rat adipocytes.

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REFERENCES

- Shun-ichi, N. and Nishizuka, Y. (1994) *J. Biochem. (Tokyo)* **115**, 1029–1034
- Bocckino, S. B., Blackmore, P. F., Wilson, P. B. and Exton, J. H. (1987) *J. Biol. Chem.* **262**, 15309–15315
- Hoffman, J., Standaert, M. L., Nair, G. P. and Farese, R. V. (1991) *Biochemistry* **30**, 3315–3322
- Standaert, M. L., Musunuru, K., Yamada, K., Cooper, D. R. and Farese, R. V. (1994) *Cell. Signal.* **6**, 707–716
- Donchenko, V., Zannetti, A. and Baldini, P. M. (1994) *Biochim. Biophys. Acta* **1222**, 492–500
- Farese, R. V., Standaert, M. L., Francois, A. J., Ways, K., Arnold, T. P., Hernandez, H. and Cooper, D. R. (1992) *Biochem. J.* **288**, 319–323
- Arnold, T. P., Standaert, M. L., Hernandez, H., Watson, J., Mischak, H., Kazanietz, M. G., Zhao, L., Cooper, D. R. and Farese, R. V. (1993) *Biochem. J.* **295**, 155–164
- Egan, J. J., Saltis, J., Wek, S. A., Simpson, I. A. and Londos, C. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1052–1056
- Giorgetti, S., Ballotti, R., Kowalski-Chauvel, A., Cormont, M. and Van Obberghen, E. (1992) *Eur. J. Biochem.* **207**, 599–606
- Kelly, K. L. and Ruderman, N. B. (1993) *J. Biol. Chem.* **268**, 4391–4398
- Kanai, F., Ito, K., Todaka, M., Hayashi, H., Kamohara, S., Ishii, K., Okada, T., Hazeki, O., Ui, M. and Ebina, Y. (1993) *Biochem. Biophys. Res. Commun.* **195**, 762–768
- Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O. and Ui, M. (1994) *J. Biol. Chem.* **269**, 3568–3573
- Hara, K., Yonezawa, K., Sakaue, H., Ando, A., Kotai, K., Kitamura, T., Kitamura Y., Ueda, H., Stephens, L., Jackson, T. R., Hawkins, P. T., Dhand, R., Clark, A. E., Holman, G. D., Waterfield, M. D. and Kasuga, M. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 7415–7419
- Kanai, F., Nishioka, Y., Hayashi, H., Kamohara, S., Todaka, M. and Ebina, Y. (1993) *J. Biol. Chem.* **268**, 14523–14526
- Robertson, D. G., DiGirolamo, M., Merrill, Jr., A. H. and Lambeth, J. D. (1989) *J. Biol. Chem.* **264**, 1–7
- Standaert, M. L., Buckley, D. J., Ishizuka, T., Hoffman, J. M., Cooper, D. R., Pollet, R. J. and Farese, R. V. (1990) *Metabolism* **39**, 1170–1179
- Yano, Y., Sumida, Y., Benzing, C. F., Robinson, F. W. and Kono, T. (1993) *Biochim. Biophys. Acta* **1176**, 327–332
- Nishimura, H. and Simpson, I. A. (1994) *Biochem. J.* **202**, 271–277
- Cherqui, G., Caron, M., Wicek, D., Lascols, O., Capeau, J. and Picard, J. (1987) *Endocrinology* **120**, 2192–2194
- Ishizuka, T., Cooper, D. R., Arnold, T., Hernandez, H. and Farese, R. V. (1991) *Diabetes* **40**, 1274–1281
- Farese, R. V., Standaert, M. L., Ishizuka, T., Yu, B., Hernandez, H., Waldron, C., Watson, J., Farese, J. P., Cooper, D. R. and Wickstrom, E. (1991) *Antisense Res. Dev.* **1**, 35–42
- Farese, R. V., Standaert, M. L., Yamada, K., Huang, L. C., Zhang, C., Cooper, D. R., Wang, Z., Yang, Y., Suzuki, S., Toyota, T. and Larner, J. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 11040–11044
- Villar-Palasi, C. and Farese, R. V. (1994) *Diabetologia* **37**, 885–888
- Bisbis, S., Bailbe, D., Tormo, M. A., Picarel-Blanchot, F., Derouet, M., Simon, J. and Portha, B. (1993) *Am. J. Physiol.* **265**, E807–E813
- Vila, M. C. and Farese, R. V. (1991) *Arch. Biochem. Biophys.* **284**, 366–368
- Hoffman, J. M., Ishizuka, T. and Farese, R. V. (1991) *Endocrinology* **128**, 2937–2948
- Roth, B. L., Mehegan, J. P., Jacobowitz, D. M., Robey, F. and Iadarola, M. J. (1989) *J. Neurochem.* **52**, 215–221
- Ishizuka, T., Cooper, D. R. and Farese, R. V. (1989) *FEBS Lett.* **257**, 337–340
- Simpson, J., Johnson, M. S. and Mitchell, R. (1993) *Biochim. Biophys. Acta* **1220**, 69–75
- Vila, M. C., Milligan, G., Standaert, M. L. and Farese, R. V. (1990) *Biochemistry* **29**, 8735–8740
- Luttrell, L. M., Hewlett, E. L., Romero, G. and Rogol, A. D. (1988) *J. Biol. Chem.* **263**, 6134–6141
- Reinhold, S. L., Prescott, S. M., Zimmerman, G. A. and McIntyre, T. M. (1990) *FASEB J.* **4**, 208–214
- Liscovitch, M., Chalifa, V., Pertile, P., Chen, C. S. and Cantly, L. C. (1994) *J. Biol. Chem.* **269**, 21403–21406
- Bowman, E. P., Uhlinger, D. J. and Lambeth, J. D. (1993) *J. Biol. Chem.* **268**, 21509–21512
- Cockcroft, S., Thomas, G. M., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N. F., Truong, O. and Hsuan, J. J. (1994) *Science* **263**, 523–526
- Kumagai, N., Morii, N., Fujisawa, K., Nemoto, Y. and Narumiya, S. (1993) *J. Biol. Chem.* **268**, 24535–24538
- Zhang, J., King, W. G., Dillon, S., Hall, A., Feig, L. and Rittenhouse, S. E. (1993) *J. Biol. Chem.* **268**, 22251–22254
- Thomason, P. A., James, S. R., Casey, P. J. and Downes, C. P. (1994) *J. Biol. Chem.* **269**, 16525–16528