

# Involvement of Phospholipids in the Mechanism of Insulin Action in HEPG2 Cells

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## Summary

The mechanism of action by which insulin increases phosphatidic acid (PA) and diacylglycerol (DAG) levels was investigated in cultured hepatoma cells (HEPG2). Insulin stimulated phosphatidylcholine (PC) and phosphatidylinositol (PI) degradation through the activation of specific phospholipases C (PLC). The DAG increase appears to be biphasic. The early DAG production seems to be due to PI breakdown, probably through phosphatidylinositol-3-kinase (PI3K) involvement, whereas the delayed DAG increase is derived directly from the PC-PLC activity. The absence of phospholipase D (PLD) involvement was confirmed by the lack of PC-derived phosphatidylethanol production. Experiments performed in the presence of R59022, an inhibitor of DAG-kinase, indicated that PA release is the result of the DAG-kinase activity on the DAG produced in the early phase of insulin action.

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## Key words

Insulin • Phosphatidic acid • Diacylglycerol • Phospholipases • HEPG2 cells

## Introduction

Membrane phospholipids are the basic structural components of eukaryotic cells which also play an important role in the control of diverse cellular responses (Exton 1994, Nishizuka 1995). One of the best characterized membrane-derived phospholipids is phosphatidylinositol-1,4-bisphosphate (PIP<sub>2</sub>), whose hydrolysis by a phosphoinositide (PI)-specific phospholipase C (PI-PLC) results in the generation of diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP<sub>3</sub>) (Cockcroft and Thomas 1992). Pathways for lipid-derived second messengers other than those produced by PI-PLC have been reported (Gomez-Cambronero and Keire 1998), in particular, those involving agonists able to stimulate phosphatidylcholine (PC) hydrolysis through

specific phospholipases (Pyne and Pyne 2000). Phosphatidylcholine may be a substrate for phospholipases PLC and PLD. Activation of the PC-PLC increases the concentration of phosphocholine and DAG, a known activator of protein kinase C (PKC) (Lacal 1997). Activation of PLD leads to the formation of choline and phosphatidic acid (PA) which in turn may be hydrolyzed by a phosphatidate phosphatase (PAP) to yield DAG (Donchenko *et al.* 1994). On the other hand, phosphatidic acid formation can also be derived indirectly from the sequential activation of PLC and DAG-kinase (DAGK) (Zanetti *et al.* 1997). Propranolol, well known  $\beta$ -adrenergic receptor antagonist, blocks PA conversion to DAG by inhibiting PAP (Zanetti *et al.* 1997). It has been therefore frequently used for assessing the part of DAG derived from PA (Meier *et al.* 1998).

Many hormones cause a biphasic production of DAG (Donchenko *et al.* 1994, Zanetti *et al.* 1997). The first DAG production wave occurs immediately after stimulation, whereas the second wave is observed later (Lacal 1997). The early DAG peak is associated with PI hydrolysis by activation of a specific phospholipase PI-PLC, while the late DAG peak cannot be associated with IP<sub>3</sub> release (Billah and Anthes 1990). The DAG source different from PI hydrolysis was identified with the hydrolysis of PC by Exton (1999). These results suggest the existence of two different DAG sources.

The presence of both phospholipases PC-PLC and PC-PLD as active participants in signal transduction pathways has been described in many tissues (Plo *et al.* 2000) and participation of both these enzymes in signal transduction have also been reported in rat hepatocytes (Donchenko *et al.* 1994).

A unique property of PLD which provides a specific assay for this enzyme activity determination is the transphosphatidyl transfer reaction. In the presence of a primary alcohol PLD can catalyze the exchange of the polar head group of the phospholipid substrate with a given alcohol to form the corresponding phosphatidyl alcohol (Davis *et al.* 1998). This specific transphosphatidyl transfer reaction has been used in many studies as a marker for estimating PLD activation and to discriminate it from PLC activity.

In the present study we have examined the insulin effect on the mechanism leading to PA, DAG and choline metabolites formation in cultured human hepatoblastoma cells (HEPG2) with special consideration for the possible involvement of specific PC-PLD and PC-PLC activation. Our results indicate the absence of PLD involvement in DAG production, whose early concentration increase results from the PI breakdown by activation of a phosphatidylinositol-3-kinase (PI-3-kinase), which is a known PI-PLC activator (Eichhorn *et al.* 2001). This pathway of the DAG formation appears to be different from that observed in rat hepatocytes after insulin treatment (Etindi and Fain 1989, Pittner and Fain 1990, Donchenko *et al.* 1994).

## Methods

### Cell Culture

HEPG2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in 32-mm plastic tissue culture plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum, 200 mM

glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin in a humidified atmosphere of 5 % CO<sub>2</sub>, 95 % O<sub>2</sub> at 37 °C. The cells were subcultured at 3 to 4-day intervals and the experiments were performed at 80-90 % confluence (1x10<sup>6</sup> cells/plate).

### Radioactive labeling

Preliminary experiments on [<sup>3</sup>H] myristate and [<sup>3</sup>H] choline incorporation indicated that optimal labeling for [<sup>3</sup>H] myristate occurs at 3 h (1 µCi/plate) and for [<sup>3</sup>H] choline at 24 h (1 µCi/plate). After labeling, HEPG2 cells were left for 1 h in the medium without fetal bovine serum. The cells were then washed with buffer containing 20 mM Hepes (pH 7.4), 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 1 mg/ml bovine serum albumin and 5.6 mM glucose. When required, cells were preincubated for 15 min with 100 µM propranolol (inhibitor of PAP) and/or 10 µM R59022 (inhibitor of DAGK) at 37 °C and for 30 min with 10 µM 1-O-octadecyl-2-O-methyl-sn-glycero-3-phosphocholine (ET-18-O-CH<sub>3</sub>) (inhibitor of PI-PLC), or with 50 nM wortmannin (inhibitor of phosphatidylinositol-3-kinase). Insulin was added at the final concentration of 10<sup>-9</sup> M. Incubation was stopped with 1 ml of ice-cold methanol.

### Thin layer chromatography of lipids

Cells were scraped off the plates and lipids were extracted by the method of Bligh and Dyer (1959). The dried lipids were redissolved in 20 µl of CH<sub>2</sub>Cl<sub>2</sub>. DAG was separated by chromatography on 10x10 cm Merck silica gel-60 precoated glass plates, activated at 100 °C for 1 h immediately before use. The solvent system consisted of light petroleum (b.p. 40-60 °C)/diethyl ether/acetic acid (80/20/1, v/v). Phospholipids were identified by two dimensional chromatography on 10x10 cm silica gel-60 coated glass plates activated as previously described. The solvent system consisted of CHCl<sub>3</sub> / methanol / acetone / 25 % NH<sub>4</sub>OH/H<sub>2</sub>O (16.4/4/9.8/1/1, v/v) in the first dimension and CHCl<sub>3</sub> / methanol / acetone / acetic acid / H<sub>2</sub>O (15/5/6/3/1.5, v/v) in the second dimension (Donchenko *et al.* 1994). After chromatography, plates were dried under N<sub>2</sub> stream. The spots revealed under iodine vapor were scraped off, eluted with 0.2 ml ethanol and counted for radioactivity after the addition of 7 ml of Optifluor (Packard Instruments, Downers Grove, IL, USA). Lipids were identified by comparing their R<sub>f</sub> values with those of authentic standards obtained from Supelco (Bellefonte, PA, USA).

### Intra- and extracellular [ $^3\text{H}$ ] choline metabolites

Intracellular water-soluble [ $^3\text{H}$ ] choline metabolites were obtained by removing the aqueous phases according to Bligh and Dyer (1959). The aqueous phase was dried and resuspended in 200  $\mu\text{l}$  methanol, containing a standard mixture of choline, phosphocholine and glycerophosphocholine. The choline metabolites were separated by chromatography on 20x20 cm silica gel-60 coated plates, activated at 100  $^{\circ}\text{C}$  for 1 h immediately before use. The solvent system consisted of 0.5 % NaCl/methanol/ethanol/conc. $\text{NH}_4$  (50/20/30/5, v/v) (Donchenko *et al.* 1994). The plates were dried under  $\text{N}_2$  stream and the spots revealed under iodine vapour were scraped off into vials, extracted with 1 ml of 0.4 M NaOH and after 1 h neutralized with 0.15 ml of 1 M acetic acid. The samples were counted for radioactivity after addition of 7 ml of Optifluor. For assay of extracellular [ $^3\text{H}$ ] choline metabolites, the medium was collected and centrifuged for 5 min at 900 rpm to precipitate detached cells and debris. The [ $^3\text{H}$ ] choline metabolites in the medium were extracted with tetraphenylboron (1.5 % w/v) in 3-heptanone as described by Martinson *et al.* (1989).

### Transphosphatidylolation reaction

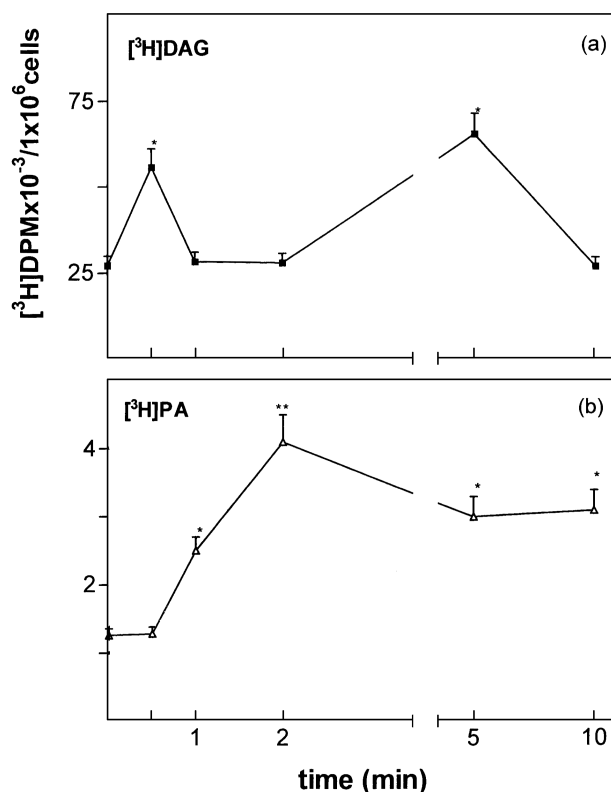
To determine phospholipase D (PLD) activity, 1 % ethanol was added to prelabeled cells 15 min before insulin addition. Phosphatidylethanol (PetOH) and PA were separated by thin layer chromatography on 10x20 cm silica gel-60 plates; the solvent system consisted of ethyl acetate/isooctane/acetic acid/ $\text{H}_2\text{O}$  (130/20/30/100, v/v). PA was identified by comparing its  $R_f$  value with that of an authentic Sigma standard, PetOH was identified by comparing its  $R_f$  value with that of a standard prepared according to Chattopadhyay *et al.* (1991).

### Materials

Radioactive compounds were from Amersham (Amersham, Bucks., UK): 9,10(n)-[ $^3\text{H}$ ] myristic acid (spec. activity 53 Ci/mmol); [methyl- $^3\text{H}$ ] choline chloride (spec. activity 75 Ci/mmol). Porcine insulin, propranolol, R59022, Wortmannin, ET-18-O- $\text{CH}_3$  and other chemicals and reagents were purchased from Sigma (St. Louis, MO, USA). Cell culture media were obtained from Flow Laboratories (Scotland). Plates for thin-layer chromatography were obtained from Merck (Darmstadt, Germany).

## Results

The effect of insulin on DAG and PA production in [ $^3\text{H}$ ] myristate prelabeled HEPG2 cells was studied in the concentrations from range  $10^{-8}$  M to  $10^{-12}$  M. Maximal effect of insulin was achieved at  $10^{-9}$  M concentration, which was used in all the following experiments (data not shown).

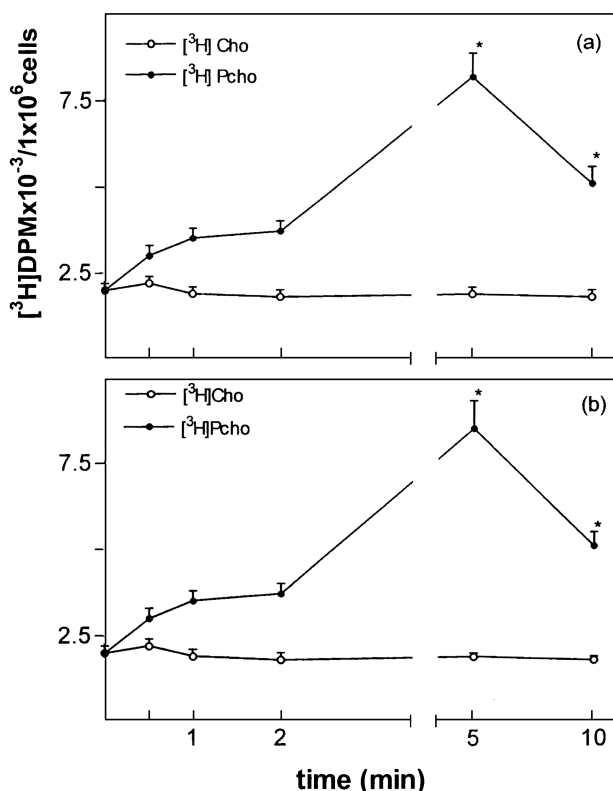


**Fig. 1.** Time-course of insulin effect on [ $^3\text{H}$ ] myristate incorporation into DAG (a) and PA (b) in cultured HEPG2 cells. Cells were prelabeled for 3 h with [ $^3\text{H}$ ] myristate as described in the text. The results are reported as [ $^3\text{H}$ ] DPM  $\times 10^{-3}$  /  $1 \times 10^6$  cells and are means  $\pm$  S.D. of the three different experiments carried out in duplicate. \* $p < 0.05$  evaluated by the unpaired *t*-test with respect to time 0 (controls); \*\* $p < 0.05$  with respect to time 0 and times at 1 and 5 min.

Insulin elicited a biphasic response in DAG formation in HEPG2 cells (Figs. 1a and 1b). The DAG formation showed an early peak 30 s after stimulation with a subsequent (about 1 min) decrease to the basal level. Later, a second DAG increase was observed 5 min after insulin addition. Insulin also caused a significant

increase in the concentration of PA. The maximal effect was observed 2 min after insulin application.

In order to assess the possible involvement of PC-PLC or PC-PLD in DAG and PA production, the effect of insulin on the release of [ $^3\text{H}$ ] choline and [ $^3\text{H}$ ] phosphocholine from prelabeled HEPG2 cells was investigated in further experiments. Cells were prelabeled with [ $^3\text{H}$ ] choline for 2 hours. The distribution of radioactive labeling was found 85 % into PC, 8 % into sphingomyelin and 4 % into lysophosphatidylcholine (data not shown).

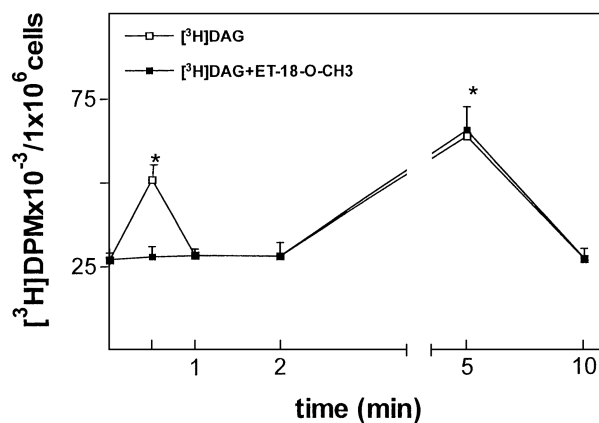


**Fig. 2.** Time-course of insulin effect on water soluble [ $^3\text{H}$ ] choline metabolites in intracellular (a) and extracellular (b) medium in cultured HEPG2 cells. Cells were prelabeled for 24 h with [ $^3\text{H}$ ], as described in text. Results are reported as [ $^3\text{H}$ ] DPM  $\times 10^3/1 \times 10^6$  cells and are means  $\pm$  S.D. of three different experiments carried out in duplicate. \* $p < 0.05$  employing the unpaired  $t$  test with respect to the time 0.

The time-course of the insulin effect on water-soluble choline metabolites released into intracellular (panel a) and extracellular medium (panel b) is shown in Figure 2. Insulin caused a significant intracellular release

of [ $^3\text{H}$ ] phosphocholine (Pcho) with a maximum at 5 min, whereas the intracellular release of [ $^3\text{H}$ ] choline (Cho) proceeded more slowly and no significant changes were observed with respect to its basal level.

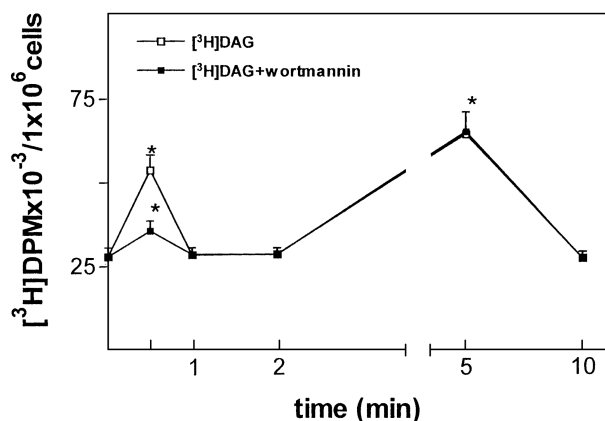
The time-course of intracellular release of [ $^3\text{H}$ ] Pcho was superimposed to the second [ $^3\text{H}$ ] DAG increase observed at 5 min after insulin stimulation (Fig. 1a). The level of [ $^3\text{H}$ ] Pcho in the extracellular medium significantly increased 5 min after addition of insulin following a pattern similar to that observed in the intracellular medium (Figs 2a and 2b). No variation in the aqueous choline metabolites was observed during the first 30 s. These data suggest the involvement of insulin in specific PC-PLC activation.



**Fig. 3.** Time-course of insulin effect on [ $^3\text{H}$ ] myristate incorporation into DAG in the absence and in the presence of ET-18-O-CH<sub>3</sub> in cultured HEPG2 cells. Cells were prelabeled for 3 h with [ $^3\text{H}$ ] myristate and 10  $\mu\text{M}$  ET-18-O-CH<sub>3</sub> was added 30 min before insulin addition. Results are expressed as [ $^3\text{H}$ ] DPM  $\times 10^3/1 \times 10^6$  cells and means  $\pm$  S.D. of three different experiments carried out in duplicate. \* $p < 0.05$  evaluated by the unpaired  $t$  test with respect to time 0.

In order to examine the mechanism of insulin action on DAG production in HEPG2 cells, we have investigated the possible role of different pathways of DAG production, such as PI-PLC activation. Involvement of PI-PLC in early DAG production was studied using 10  $\mu\text{M}$  specific PI-PLC inhibitor ET-18-O-CH<sub>3</sub> (Fig. 3). HEPG2 cells were pretreated for 30 min with ET-18-O-CH<sub>3</sub> and 30 s after insulin addition. We observed a significant decrease of early DAG production, without any influence on delayed second peak of DAG formation.

HEPG2 cells were then pretreated with 50 nM wortmannin, a specific inhibitor of phosphatidylinositol-3-kinase (PI3K) which in turn is known to activate PI-PLC. Wortmannin partially abolished the effect of insulin on early DAG production (Fig. 4). These results support the hypothesis of the involvement of PI-PLC in the insulin action in HEPG2 cells.

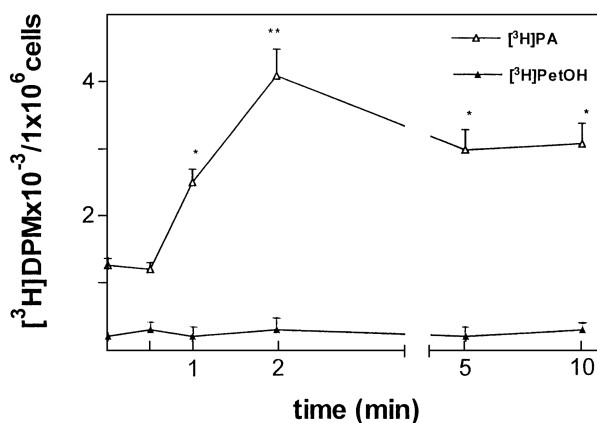


**Fig. 4.** Time course of insulin action on [<sup>3</sup>H] myristate incorporation into DAG in the absence or presence of wortmannin in cultured HEPG2 cells. Cells were prelabeled for 3 h with [<sup>3</sup>H] myristate and 50 nM wortmannin was added 30 min before insulin addition. Results are reported as [<sup>3</sup>H] DPM x 10<sup>-3</sup> / 1 x 10<sup>6</sup> cells and are means ± S.D. of three different experiments carried out in duplicate. \**p* < 0.05 evaluated by the unpaired *t* test with respect to time 0.

In order to assess the origin of PA peak at 2 min after hormone application, we studied the transphosphatidyl reaction which is a specific marker of PLD activity and catalyzes the formation of PetOH from PC in the presence of ethanol. Figure 5 shows the effect of insulin on PetOH formation in cells prelabeled with [<sup>3</sup>H] myristate at 37 °C and incubated for 15 min with 1 % ethanol before insulin addition. In the presence of insulin we did not observe any changes in PetOH formation with respect to its basal level. PA formation showed a pattern which suggests that the involvement of PC-PLD can be ruled out.

To investigate another possible pathway of PA formation in insulin-stimulated HEPG2 cells we focused our experiments on the DAG kinase activity, an enzyme able to phosphorylate DAG to PA. We found the effect of R59022, a specific DAG kinase inhibitor, on PA and DAG production in insulin-stimulated HEPG2 cells. In

cells pretreated with inhibitor significant decrease of PA production was found (Fig. 6a), suggesting that DAG kinase is involved in PA formation at 2 min after the stimulation. Accordingly, a significant DAG increase was found 30 s after insulin treatment in the presence of the DAG kinase inhibitor R59022 (Fig. 6b).



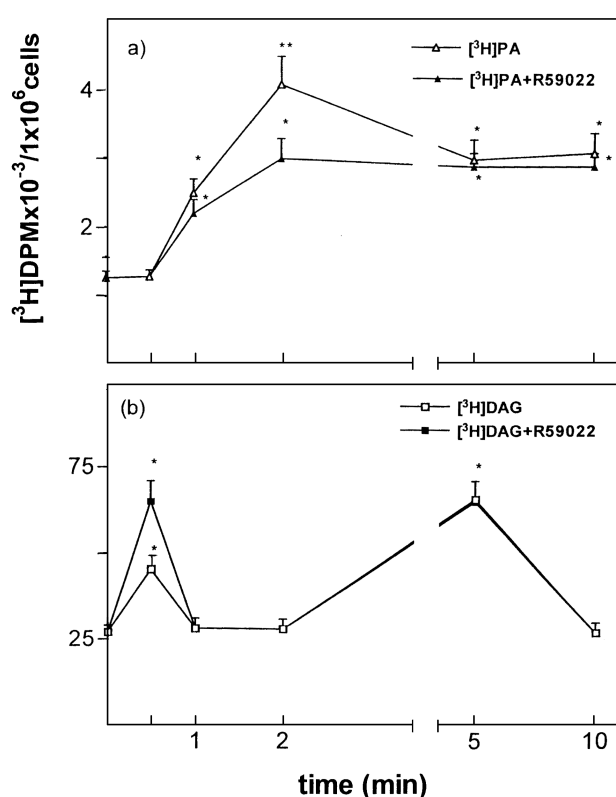
**Fig. 5.** Time course of [<sup>3</sup>H] PA and [<sup>3</sup>H] PetOH formation in the presence of ethanol in cultured HEPG2 cells. 1 % ethanol was added 15 min before insulin addition. Results are reported as [<sup>3</sup>H] DPM x 10<sup>-3</sup> / 1 x 10<sup>6</sup> cells and are means ± S.D. of three different experiments carried out in duplicate. \**p* < 0.05 evaluated by the unpaired *t* test with respect to time 0; \*\**p* < 0.05, with respect to time 0 and time 1 and 5 min.

Finally, experiments were carried out in the presence of phosphatidate phosphatase (PAP) inhibitor propranolol, to rule out that the second peak of DAG might be derived from PA through PAP activity. In this regard we observed that propranolol did not affect the DAG increase (data not shown).

## Discussion

In the present study we obtained data which indicate that after insulin stimulation PA and DAG are formed by different enzymatic pathways at different times in HEPG2 cells. The initial increase of DAG production in HEPG2 cells stimulated by insulin is associated with PI-PLC activation and PI breakdown, while the second DAG increase is derived from PC hydrolysis due to PC-PLC activation. These results were also confirmed by analysis of the release of aqueous choline metabolites, as already reported for many receptor agonists in different tissues (Martin *et al.* 1993).

With regard to this, the metabolic pathways of the DAG and PA formation in insulin-stimulated HEPG2 cells appear to be different from those reported for rat hepatocytes (Donchenko *et al.* 1994). In fact, we have previously reported in rat hepatocytes that i) insulin initially stimulated PC-PLD activity with a significant increase in PA concentration (at 30 s) as well as intracellular and extracellular choline release; ii) the phosphatase phosphohydrolase (PAP) is involved in the transformation of the PA into DAG, suggesting that the early DAG increase is due to the PLD-PAP pathways activation (Donchenko *et al.* 1994).



**Fig. 6.** Time-course of the insulin effect on  $[^3\text{H}]$  PA (a) and  $[^3\text{H}]$  DAG (b) formation in the presence or absence of DAGK inhibitor R59022 in cultured HEPG2 cells. Cells were prelabeled with  $[^3\text{H}]$  myristate for 3 h and 10  $\mu\text{M}$  R59022 was added 15 min before insulin application. The results are given as  $[^3\text{H}]$  DPM  $\times 10^3$  /  $1 \times 10^6$  cells and are means  $\pm$  S.D. of three different experiments carried out in duplicate. \* $p < 0.0$  evaluated by the unpaired *t*-test with respect to the time 0; \*\* $p < 0.05$ , with respect to time 0 and time 1 and 5 min.

On the contrary, we have observed that early DAG production in HEPG2 cells depends on specific PI-PLC activation as confirmed by the decrease of early DAG production after treatment of the cells with ET-18-O-CH<sub>3</sub>, a specific PI-PLC inhibitor. The involvement of PI-PLC in DAG production has also been demonstrated for the other extracellular mediators such as bradykinin (van Blitterswijk *et al.* 1991), histamine (Resink *et al.* 1987) and endothelin (Resink *et al.* 1988). Our results also suggest the involvement of PI3K on PI-PLC activation and early DAG production, as reported in different cellular systems (Eichhorn *et al.* 2001), because wortmannin abolished the early DAG formation in insulin-stimulated HEPG2 cells.

The involvement of PI3K in insulin-mediated phospholipid signal transduction in HEPG2 cells seems to be of special significance. This enzyme has been shown to be a major regulator of cell survival in response to growth factors as EGF (Roberts *et al.* 2000).

The insulin-stimulated release of intracellular phosphocholine together with DAG production at 5 min, confirms the interesting role of PC-PLC in HEPG2 cells. A specific PC-PLC activation was also responsible in delayed DAG production in rat hepatocytes but this DAG was detected 2 min after insulin stimulation (Donchenko *et al.* 1994). On the basis of this, it is conceivable that insulin may activate PC-PLC in both HEPG2 cells and hepatocytes at later times.

Another difference in the second messenger pathways between rat hepatocytes and HEPG2 cells resides in the PA production. In rat hepatocytes Donchenko *et al.* (1994) observed an initial stimulation of PC-PLD with a significant increase of PA 30 s after insulin stimulation, while the PA production in HEPG2 cells was delayed to 2 min and no involvement of the PC-PLD activation was observed. These findings were confirmed by the results of the specific transphosphatidylations reactions and by choline release after insulin treatment.

When HEPG2 cells were pretreated with the DAG inhibitor R59022, the PA formation induced by insulin was diminished, while the level of DAG increased at 30 s. Our data confirmed that such an increase might be due to PI-PLC activation and to the subsequent DAG phosphorylation derived from PI hydrolysis. It is conceivable that PA itself can act as a second messenger (Grange *et al.* 1998, Zhou *et al.* 1999) which is able to modulate short-term cellular responses (Zakharoff-Girard *et al.* 1999) and to activate cellular kinases, as widely reported for DAG (Lassegue *et al.* 1993). It has also been

reported that PA could be an important element of a signal transduction mechanism in hepatocytes (Dajani *et al.* 1999). PA probably plays a role in the regulation of PLC activity (Lithosch 2000). Moreover, it is known among these various biological PA effects that PA stimulates the MAP kinases signal transduction pathway as was also reported for insulin (Kariya *et al.* 1987).

In conclusion, we suggest that insulin acts in cultured HEPG2 cells at variance with respect to hepatocytes, through subsequent activation of specific PI-PLC and PC-PLC and production of two different DAG pools, which are able to mediate early and late hormone responses. Moreover, our data show that PA is not derived from PC-PLD activation, as in rat hepatocytes. Phosphatidic acid is produced by PI-PLC and DAGK pathways. These results show that the second

messenger production pathways are different in insulin stimulated HEPG2 cells with respect to that observed in rat hepatocytes, with particular relevance for the involvement of PI3K as mediator of the downstream effects of insulin.

### Abbreviations

DAG – diacylglycerol, DAGK – diacylglycerol kinase, HEPG2 – hepatoblastoma cells, IP<sub>3</sub> – inositol-1,4,5-tris-phosphate, PA – phosphatidic acid, PAP – phosphatidate phosphohydrolase, PC – phosphatidylcholine, Pcho phosphocholine, PC-PLC – phosphatidylcholine phospholipase C, Pet – OH phosphatidylethanol, PI – phosphatidylinositol, PI3K – phosphatidylinositol-3-kinase, PI-PLC – phosphatidylinositol phospholipase C, PLD – phospholipase D, PKC – protein kinase C.

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