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## ARE POLYPHOSPHORYLATED PHOSPHOLIPIDS INVOLVED IN THE HORMONAL CONTROL OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY IN TUMOUR LEYDIG CELLS?

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**Summary**—The possible role of LH or dbcAMP induced changes in polyphosphorylated phospholipid metabolism in the regulation of cholesterol side-chain cleavage activity has been studied in tumour Leydig cells. Mitochondria isolated from LH-stimulated Leydig cells were 400% more active in pregnenolone production than mitochondria from control cells. Steroid production in isolated mitochondria from control cells could be stimulated only 25% by cytosol fractions from stimulated cells and 100  $\mu$ M phosphatidyl inositol-4'-phosphate (PtdIns4P). Other polyphosphorylated phospholipids were either inactive or showed aspecific effects. During a preincubation period tumour cells were labelled with [<sup>32</sup>P]phosphate and steady-state labelling was obtained for the polyphosphorylated phospholipids after 40–60 min. [<sup>32</sup>P]Phosphate incorporation in Ptd Ins4P, phosphatidyl inositol (PtdIns), phosphatidyl choline (PtdChl), phosphatidyl ethanolamine (PtdEtn) and cardiolipin (CL) was not affected by treatment of the Leydig cells with LH which stimulated (6-fold), or with cycloheximide which suppressed (4-fold) steroid production. A 25% increase of phosphate incorporation by LH was observed only in phosphatidyl inositol-4',5'-biphosphate (PtdIns4,5P<sub>2</sub>). <sup>32</sup>P Incorporation in PtdIns4,5P<sub>2</sub>, PtdIns,PtdEtn and CL was stimulated by quinacrine 50  $\mu$ M. Under these conditions the LH-stimulated pregnenolone production but not the 25-hydroxycholesterol dependent pregnenolone production, was completely inhibited. The results obtained with isolated mitochondria and intact cells indicate that increased levels of polyphosphorylated phospholipids are not consistently correlated with increased mitochondrial pregnenolone production. This argues against an important role of polyphosphorylated phospholipids in the hormonal regulation of cholesterol side-chain cleavage activity in tumour Leydig cells.

### INTRODUCTION

Stimulation of steroid production in Leydig cells by lutropin (LH) is correlated with a sharp rise in the cAMP level [1–4] and phosphorylation of specific proteins by cAMP-dependent kinases [5–7]. However it is not yet which phosphorylated proteins are involved in regulation of steroid production and how the activation signal is propagated in the cell.

The rate-limiting step in the control of steroidogenesis is the cholesterol side-chain activity which requires cytochrome P450 (P450<sub>sc</sub>), located in the inner mitochondrial membrane. Trophic hormones

increase the binding of cholesterol to P450<sub>sc</sub>, which is followed by an increased synthesis of pregnenolone [8]. As in the adrenal cortex [9, 10] inhibitors of protein synthesis, like cycloheximide and puromycin rapidly terminate the stimulated steroid production in the testis [11, 12]. This indicates that a rapidly turning-over protein may be involved in the regulation of cytochrome P450<sub>sc</sub>. Recently, an ACTH-dependent peptide that can stimulate cytochrome P450<sub>sc</sub> in isolated mitochondria has been isolated from adrenal tissue [13]. Changes in phospholipid metabolism may also play an important role in the transduction of signals through cellular membranes. It has been shown in the last years that rapid changes in phosphoinositol metabolism in the plasma membrane can occur after binding of particular hormones to receptors. The key event seems to be the receptor mediated hydrolysis of PtdIns4,5P<sub>2</sub>, which may take place within a minute after binding of the hormone. This is followed by an increase in the intracellular free calcium concentration by inositol triphosphate and activation of protein kinase C by diacylglycerol [14].

Farese *et al.* [15–17] have suggested, that phosphoinositides may also play a role at the level of the mitochondria in the regulation of the cholesterol side-chain cleavage activity. They have observed that

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**Abbreviations:** LH: lutropin. ACTH: adrenocorticotrophic hormone. dbcAMP: N<sup>6</sup>-2'-O-dibutyryl adenosine cyclic-3', 5'-monophosphate. PtdIns: phosphatidyl inositol. PtdIns4P: phosphatidyl inositol-4'-phosphate. PtdIns4,5P<sub>2</sub>: phosphatidyl inositol-4',5'-biphosphate. PtdChl: phosphatidyl choline. PtdEtn: phosphatidyl ethanolamine. PA: phosphatidic acid. CL: cardiolipin. P450<sub>sc</sub>: cholesterol side-chain cleavage cytochrome P450<sub>sc</sub>.

the amount of polyphosphorylated phospholipids, such as PtdIns4,5P<sub>2</sub> and PrdIns4P, increased concomitantly with the hormonal stimulation of steroid production in adrenal cells and Leydig cells [15–18]. These sustained effects of hormones on increased levels of cellular polyphosphorylated phospholipids are different from the rapid and transient effects of particular hormones on hydrolysis of polyphosphorylated phospholipids in the plasma membranes. It was also found that isolated mitochondria from adrenals could be stimulated directly by these phospholipids [19]. Moreover purified cytochrome P450<sub>sc</sub> in liposomes could be activated by phospholipids, especially cardiolipin [20]. These observations indicate that polyphosphorylated phospholipids may be involved in regulation of mitochondrial pregnenolone production in steroidogenic tissues in general. However, data obtained from experiments with ovarian tissue [12, 22], did not support this model. It seemed of interest therefore to investigate the role of polyphosphorylated phospholipids in the hormonal regulation of cholesterol side-chain cleavage in tumour Leydig cells, which can be obtained pure and in large quantities and which have been used previously for investigating the mechanism of action of LH on steroid production [7, 12].

## EXPERIMENTAL

### Materials

Sheep lutropin (NIH-LH-S18; 1.03 i.u./mg) was a gift from the NIAMDD, Bethesda, MD, U.S.A. ACTH<sub>1-24</sub> was a gift from Organon, Oss, The Netherlands [<sup>32</sup>P]Orthophosphate (carrier-free) was purchased from Amersham, U.K. Cyanoketone (2 $\alpha$ -cyano-17 $\beta$ -hydroxy-4,4',17-trimethylandroster-5-en-3-one), an inhibitor of 3 $\beta$ -hydroxysteroid dehydrogenase activity and SU-10603 (7-chloro-3,4-dihydro-2-(3-pyridyl)-naphthalen-1(2H)-one), an inhibitor of 17 $\beta$ -hydroxylase activity, were kindly donated by Dr R. Neher from the Friedrich Miescher Institute, Basle, Switzerland. Crude collagenase was obtained from Worthington, U.S.A. quinacrine (mepacrine, N<sup>4</sup>-(6-chloro-2-methoxy-9-acridinyl)-N<sup>1</sup>,N<sup>1</sup>-diethyl-1,4-pentanediamine) and phospholipids were obtained from Sigma, U.S.A. Dibutyl-*c*-AMP was obtained from Boehringer, F.R.G.

### Isolated Leydig cells

Isolation of the tumour cells and the properties of these cells have been described previously [12]. Cells were preincubated and incubated under an O<sub>2</sub>/CO<sub>2</sub> (19:1) atmosphere in a shaking waterbath (80 cpm) at 32°C in Krebs–Ringer buffer without phosphate (118 mM NaCl; 4.75 mM KCl/25 mM NaHCO<sub>3</sub>/1.2 mM MgSO<sub>4</sub>/2.5 mM CaCl<sub>2</sub>), pH 7.3, containing 0.2% glucose. Unless indicated otherwise, the preincubation time was 1 h. In some experiments 1 mM *p*-aminogluthetamide was added during this preincubation and washed out thereafter. Incu-

bations were carried out in 200  $\mu$ l Krebs–Ringer buffer, containing 1–2  $\times$  10<sup>6</sup> cells. The time of addition of lutropin (1000 ng/ml final concentration) or dbcAMP (130  $\mu$ M, final concentration) was taken as time zero. When pregnenolone production was measured, cyanoketone and SU-10603 were added also at time zero at a final concentration of 5  $\mu$ M and 19  $\mu$ M respectively. After 1 h the incubation was terminated through addition of ethyl acetate (3 ml) and pregnenolone was measured by radioimmunoassay [23].

### Subcellular fractions

To study the effect of Leydig tumour supernatant fractions on pregnenolone production by mitochondria, the following procedure was used. Tumour Leydig cells were divided into two equal portions. At time zero lutropin was added to one portion, medium to the other. After 30 min the cells were spun down (10 min at 100 *g*) and resuspended in 250 mM sucrose/10 mM Tris–HCl/0.05 mM EDTA, pH 7.4 (STE buffer), at a concentration of 30  $\times$  10<sup>3</sup> cells/ml. The cell suspension (15 ml) was homogenized at 0°C with a Dounce glass homogenizer (clearance 0.03 mm).

After 8–10 strokes 65% of the cells were broken. Intact cells were removed by centrifugation for 5 min at 100 *g*. The nuclei were sedimented after centrifugation for 10 min at 1000 *g*. The mitochondria and the postmitochondrial supernatant (PMS) were isolated after 10 min at 15000 *g* (for characterization see Bakker *et al.*, 6). The mitochondrial pellet was carefully resuspended (with a glass–Teflon homogenizer) in 12 ml of incubation medium, containing 100 mM sucrose–50 mM Tris–HCl 60 mM KCl 5 mM MgCl<sub>2</sub>/5 mM isocitrate pH 7.4 [24]. One tenth volume of a solution containing 55 mM MgCl<sub>2</sub> and 55 mM isocitrate was added to the PMS fraction or a portion of STE buffer. Mitochondrial suspensions (0.25 ml) from stimulated or unstimulated cells were combined with an equal volume of PMS from stimulated or unstimulated cells or with STE buffer supplemented as described above. When production of pregnenolone was measured in subcellular fractions cyanoketone and SU-10603 were added as described above. The incubation was terminated after 15 min with the addition of ethylacetate. Addition of NADPH to the incubation mixture had no effect on pregnenolone production. This indicates that the inner mitochondrial membranes were intact. The pregnenolone production of PMS alone was less than 1% of the mitochondrial pregnenolone production.

To study the effect of added phospholipids, mitochondria were isolated directly from unstimulated tumour tissue by homogenization in STE buffer, using a glass–Teflon potter tube (800 rpm, 5 strokes, 0°C), followed by differential centrifugation. The mitochondrial pellet was resuspended in incubation medium. Incubation (15 min at 32°C) was started with the addition of a concentrated solution, containing phospholipid, cyanoketone and SU-10603. The

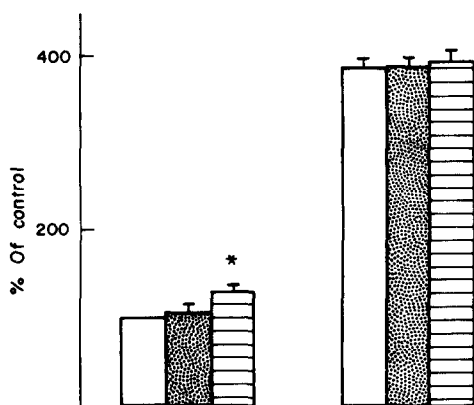


Fig. 1. Effect of supernatant fractions from tumour Leydig cells on pregnenolone production by isolated mitochondria. The three bars at the left represent mitochondria from control cells; the three bars at the right mitochondria from lutropin-stimulated cells. Open bars: addition of supernatant medium only; stippled bars: addition of supernatant from control cells; hatched bars: addition of supernatant from lutropin-stimulated cells. Values are means  $\pm$  SD of 4 different mitochondrial preparations. \*Significantly different ( $P < 0.01$ ) when compared to mitochondria with supernatant from control cells. One-hundred represents 140 ng pregnenolone/mg mitochondrial protein/15 min; protein concentration during incubation: mitochondrial protein 250  $\mu$ g/ml; supernatant protein 600  $\mu$ g/ml.

phospholipids were dissolved in chloroform-methanol- $H_2O$  or ethanol. Before addition they were concentrated in 35  $\mu$ l propyleneglycol by evaporation of the solvents with  $N_2$ . Then 315  $\mu$ l incubation medium was added and after extensive vortexing the appropriate amount was added to the incubation media.

#### Phospholipids

Cellular phospholipids were labelled as follows: after a 30 min preincubation of the cells 50  $\mu$ Ci [ $^{32}$ P]orthophosphate was added to the cells and the preincubation was continued for 60 min. At time zero stimulators or inhibitors were added. After 60 min the incubations were terminated by addition of ice-cold extraction mixture. Parallel incubations without [ $^{32}$ P]phosphate but with cyanoketone and SU-10603 added at time zero were used to measure the pregnenolone production of the cells.

Phospholipids were extracted using the acid chloroform/methanol procedure described by Jolles *et al.* [25]. In our hands this method worked better than a neutral extraction procedure [15, 26], because twice the amount of phospholipids was extracted from the same batch of labelled cells. Re-extraction of a freshly labelled phospholipid extract added back to unlabelled cells gave a recovery of 85% for the acid extraction procedure without specific losses for individual phospholipids, as compared to 70% for the neutral extraction procedure with specific losses for polyphosphorylated phospholipids.

Analysis of phospholipids was carried out immediately after extraction on Merck HPTLC plates (Kieselgel 60, without fluorescence indicator) impregnated with potassium oxalate and developed in chloroform-acetone-methanol-acetic acid- $H_2O$  (40:15:13:12:8, by vol) for 45 min [27, 28]. Phospholipids were visualized using  $I_2$  vapour and autoradiography. Individual spots were scraped off the plate and their radioactivity was determined in Picofluor 15 scintillation fluid (Packard).  $R_f$  values for individual phospholipids in this system are: phosphatidyl-inositol-4'-5'-bisphosphate: 0.21; phosphatidyl-inositol-4'-phosphate: 0.27; phosphatidyl-inositol: 0.37; phosphatidyl-choline: 0.41-0.48; phosphatidyl-serine: 0.61; phosphatidyl-ethanolamine: 0.70; phosphatidic acid and cardiolipin: 0.78. After chromatography no radioactivity was detected at the application point. Recovery of radioactivity from the TLC plates was greater than 90%. The  $^{32}$ P-label incorporated in the individual phospholipids by  $10^6$  cells incubated under different conditions was expressed relative to the activities obtained from cells incubated with cycloheximide; the latter was taken as 100%. The different incubation conditions of the cells did not influence the amount of  $^{32}$ P-label incorporated in the total phospholipid fraction extracted from the cells. Incorporation of  $^{32}$ P in this total phospholipid fraction varied not more than 10% between duplicate incubations. Protein was determined by the Lowry method, with BSA as standard. Statistical significance of results was calculated using the Student's *t*-test.

#### RESULTS

The pregnenolone production in the cells from which the mitochondria were isolated was stimulated 6-fold by LH when compared with controls. Mitochondria isolated from LH-stimulated tumour Leydig cells in the presence of isocitrate retain their increased capacity to produce pregnenolone (Fig. 1). There was a 4-fold difference in specific activity of steroid production between mitochondria of stimulated and unstimulated cells.

Addition of supernatant fractions from LH-treated cells to mitochondria from untreated cells gave a small (25%) but significant rise in pregnenolone production (Fig. 1).

The effect of added phospholipids on the pregnenolone production of isolated mitochondria from unstimulated tumour cells is shown in Fig. 2. Phosphatidyl choline had no significant effect. Cardiolipin stimulated pregnenolone production approx 20% at 0.1 mM, but this effect disappeared at 0.4 mM. PtdIns4,5P<sub>2</sub> had no effect, but PtdIns4P caused a 50% stimulation. Pregnenolone production could not be increased by addition of cholesterol (50  $\mu$ M) in the incubation medium or by pretreatment of the cells with *p*-aminogluthetimide (data not shown).

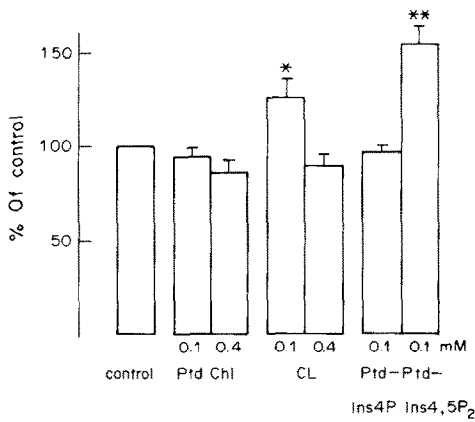


Fig. 2. Effect of phospholipids on pregnenolone production by mitochondria isolated from tumour Leydig cells. Mitochondria from tumour tissue were incubated with various phospholipids at the indicated concentrations. 100% = 150 ng pregnenolone/mg mitochondrial protein/15 min; protein concentration during incubation: 500  $\mu$ g/ml. Values are means  $\pm$  SD of 4 different mitochondrial incubations. \*\* $P$  < 0.01 vs control; \* $P$  < 0.05 vs control.

Addition of phospholipids to intact isolated tumour Leydig cells caused a significant stimulation of pregnenolone production, but this stimulation was non-specific with respect to the nature of the phospholipid added and was much smaller than the stimulation that could be reached by addition of dibutyryl cAMP (Fig. 3).

In the next series of experiments we have studied the effect of compounds that inhibit or stimulate steroid production on phospholipid metabolism and steroid production in intact cells. Quinacrine is an inhibitor of phospholipase  $A_2$  [29, 39] and was added because inhibition of the metabolism of phospholipids might increase the steady state concentrations of polyphosphorylated phospholipids. Quinacrine, however, inhibited steroid production in control and dbcAMP-stimulated cells progressively (Fig. 4). Similar results were obtained when 1  $\mu$ g LH/ml was used instead of dcAMP (data not shown). At a quinacrine concentration of 6  $\mu$ M LH- or dcAMP-dependent steroid production was inhibited by 50%. However, in the presence of 32  $\mu$ M 25-OH-cholesterol none of the quinacrine concentrations used inhibited pregnenolone production more than 15%, indicating that quinacrine does not inhibit cytochrome P450<sub>sec</sub> activity itself. Effects of LH, cycloheximide and quinacrine on the metabolism of phospholipids in cells were investigated after cells had been labelled with [ $^{32}$ P]phosphate until a steady state incorporation of [ $^{32}$ P] into PtdIns4P, PtdIns4,5P<sub>2</sub>, PA and CL was reached (Fig. 5). Due to the short labelling period the PtdEtn, PtdChl and to a lesser extent PtdIns were not labelled to constant specific activity. Cycloheximide inhibited the steroid production in tumour Leydig cells incubated without LH [31]. We have therefore taken the cycloheximide incubated cells as a reference point [100%] (Fig. 6:

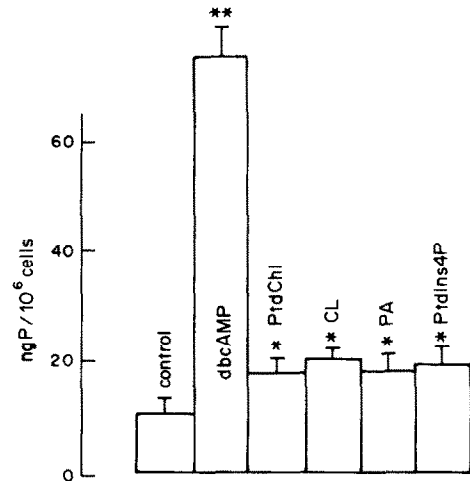


Fig. 3. Effect of dbcAMP and phospholipids on pregnenolone production by tumour Leydig cells. Phospholipids were added to the cells to a final concentration of 200  $\mu$ M; dbcAMP to a concentration of 130  $\mu$ M. The incubation time was 1 h; thereafter pregnenolone was determined. 10<sup>6</sup> Leydig cells contain approx 170  $\mu$ g protein. Values are means  $\pm$  SD from 3 cells preparations. \*\* $P$  < 0.005 vs control; \* $P$  < 0.05 vs control.

right panel). The change in ( $^{32}$ P)-labelling of individual phospholipids under various conditions was expressed in the same way: cycloheximide treated cells were taken as 100% (Fig. 6: left panel). No significant differences in phospholipid labelling could be detected when the untreated and cycloheximide-treated cells were compared. Cells stimulated with LH showed a 80-fold increase in pregnenolone production with respect to cycloheximide-treated metabo-

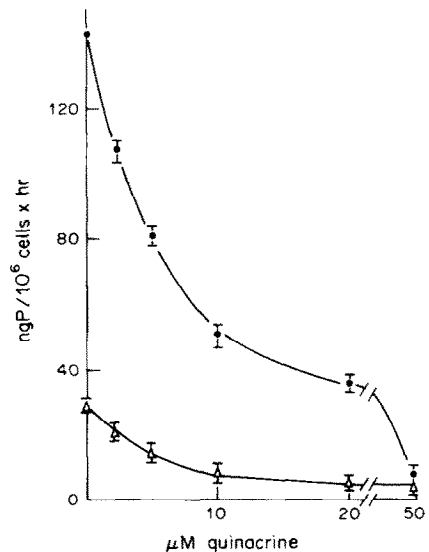


Fig. 4. Effect of quinacrine on control and dbcAMP-stimulated pregnenolone production by tumour Leydig cells. At time zero quinacrine alone ( $\Delta$ — $\Delta$ ) or quinacrine with dbcAMP (130  $\mu$ M) ( $\bullet$ — $\bullet$ ) was added and the incubation was carried out for 1 h. Values are means  $\pm$  SD from three different cell preparations.

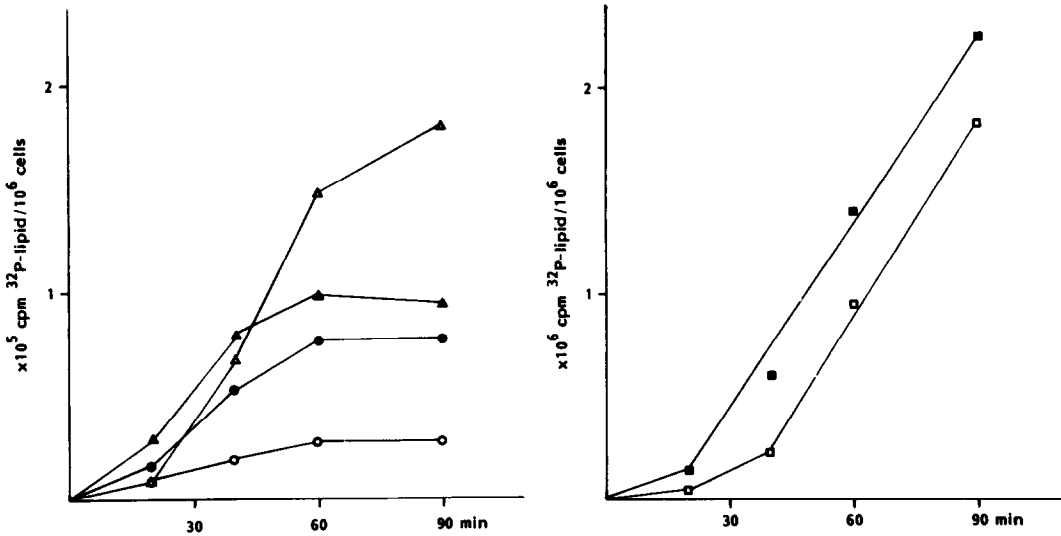


Fig. 5. Kinetics of  $^{32}\text{P}$  phosphate incorporation into individual phospholipids from tumour Leydig cells. Tumour Leydig cells were incubated with  $^{32}\text{P}$  phosphate for various time periods. Phospholipids were extracted and analyzed as described in the Experimental Section. No hormones or inhibitors were added. Open circles: phosphatidic acid and cardiolipin; closed circles: phosphatidyl-inositol 4 phosphate; open triangles: phosphatidyl-inositol; closed triangles: phosphatidyl-inositol 4,5 bis phosphate; open squares: phosphatidyl-ethanolamine; closed squares: phosphatidyl-choline.

lism were not significant, except for a small increase (25%) for PtdIns4,5P<sub>2</sub>. Similar data were obtained when effects of LH were measured 10 or 60 min after addition of the hormone, or when dibutyl cAMP was used instead of LH. Due to the low concentration of polyphosphorylated phospholipids in extracts of  $2 \times 10^7$  tumour Leydig cells, it was not possible to

determine the effects of LH on the mass of individual phospholipids directly, but due to the fact that the polyphosphorylated phospholipids were labelled to constant specific activity it seems reasonable to conclude that changes in incorporation of label reflect changes in the mass of these phospholipids. This does not hold for PtdIns, PrdEtn and PtdChl. The discrep-

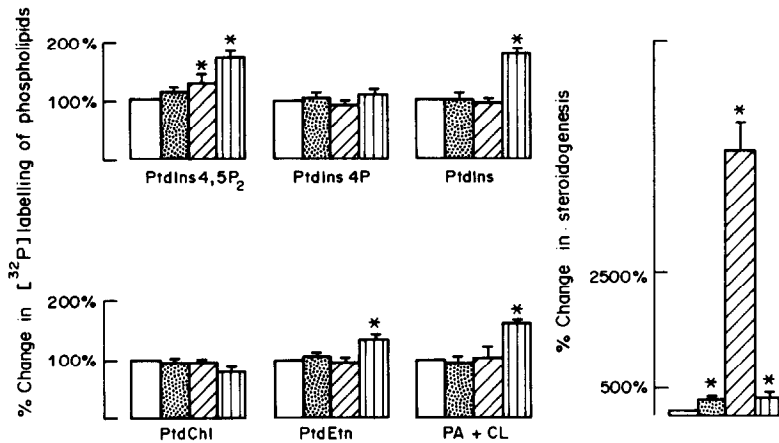


Fig. 6. Effect of cycloheximide, lutropin and quinacrine on steroidogenesis and [ $^{32}\text{P}$ ] incorporation in phospholipids in tumour Leydig cells. Sixty min after addition of [ $^{32}\text{P}$ ]phosphate the following additions were made to the cells: *open bars*: cycloheximide (80  $\mu\text{M}$ ); *stippled bars*: medium; *diagonally hatched bars*: lutropin (1  $\mu\text{g}/\text{ml}$ ); *vertically hatched bars*: quinacrine (50  $\mu\text{M}$ ) + LH (1  $\mu\text{g}/\text{ml}$ ) and the incubations were continued for 1 h. The phospholipids were extracted and analyzed using thin-layer chromatography. The amount of label in the individual phospholipid spots from the cycloheximide incubation was set as 100% (see Results sections). Values are means  $\pm$  SD from 4 different cell preparations. The distribution of  $^{32}\text{P}$ -label in the individual phospholipids was on the average (expressed as % from the total  $^{32}\text{P}$ -label extracted): PtdIns4,5P<sub>2</sub>: 4%; PtdIns4P: 3%; PtdIns: 15%; PtdChl: 67%; PtdEtn: 8%; PA and CL: 3%. \*(Left panel): significantly different ( $P < 0.05$ ) when compared to cycloheximide incubation. The 100% in the right panel represents: 1.5 ng pregnenolone/ $10^6$  cells/h. \*Right panel): significantly different from the cycloheximide incubation:  $P < 0.001$  (lutropin) and  $P < 0.01$  (control and quinacrine).

ancies between phospholipid metabolism and steroid production were most marked when the effect of quinacrine was investigated in the presence of LH. A significant increase in the labelling of PtdIns4,5P<sub>2</sub>, ptdIns, PtdEtn and PA plus Cl was observed, whereas LH stimulated pregnenolone production was strongly inhibited by quinacrine (Fig. 6).

#### DISCUSSION

It has been shown that protein synthesis is required for LH action on pregnenolone production [7, 11] and that the 22000, 24000 and 33000 Da phosphoproteins in the microsomal fraction may be involved in the regulation of protein synthesis and steroidogenesis by LH [7]. Farese *et al.* have proposed that polyphosphorylated phospholipids may also be involved in the control of cytochrome P450<sub>sc</sub> in Leydig cells and adrenals [14–18]. It has been suggested that LH-dependent rapidly turning over proteins control the changes in polyphosphorylated phospholipid metabolism [32].

We have investigated whether phospholipids can regulate the mitochondrial cholesterol side-chain cleavage in tumour Leydig cells. The present results show that mitochondria isolated from LH-stimulated cells were 4-fold more active than mitochondria from control cells. Thus, Leydig cell mitochondria can retain their stimulated activity after the isolation procedure, which makes it possible to compare properties of isolated mitochondria with those of mitochondria *in situ*. Only a small stimulation (25%) by cytosols from activated cells on mitochondria from control cells could be detected. These data confirm results from previous studies with whole testis mitochondria [33]. That only a small stimulation is measured may be explained by turnover of active compounds or by the presence of inhibitors of cholesterol side-chain cleavage in the cytosols [34, 35]. The cytochrome P450<sub>sc</sub> in isolated Leydig cell mitochondria was stimulated with PtdIns4P and to some degree also with CL, but other phospholipids were not active. The stimulating effects could be obtained only at unphysiologically high concentrations (100 μM) of phospholipids. In addition, phospholipids at these high concentrations may have a nonspecific effect, as reflected in the small stimulatory effects of all phospholipids on intact Leydig cells. Such results do not support the general conclusion of Farese [32], that the specific polyphosphorylated state of the phospholipid is the essential factor in the regulation of cholesterol side-chain cleavage in isolated mitochondria. A similar discrepancy between adrenal and gonadal steroid production was observed by Tanaka *et al.* [21], who could stimulate corpus luteum mitochondria only with 100 μM concentrations of cardiolipin but not with PtdIns4P. The high concentrations of phospholipids required for stimulation of isolated mitochondria and the conflicting results on the specificity render the im-

portance of polyphosphorylated phospholipids for the regulation of cholesterol side-chain cleavage activity in intact cells questionable.

If phospholipids are mediators of the action of trophic hormones on mitochondrial steroid production, a correlation between hormonal effects on phospholipid metabolism and steroid production would be expected. Changes in phospholipid metabolism and steroid production were therefore investigated in intact cells incubated in the presence of compounds which stimulate or inhibit steroidogenesis. A small stimulation of only PtdIns4,5P<sub>2</sub> synthesis could be measured after addition of LH. However, this stimulation could only be shown when compared with cycloheximide inhibited cells and not when compared with untreated cells. The production of other phospholipids was not affected by addition of LH, whereas the activity of steroid production could be increased more than 80-fold. Therefore, the functional importance of this stimulatory effect of LH on PtdIns4,5P<sub>2</sub> synthesis in the regulation control of cholesterol side-chain cleavage activity is not clear. These results are different from those published by Lowitt *et al.* [18] who extracted phospholipids from a few million testicular Leydig cells and reported a significant increase in cellular levels of phospholipids after stimulation of the cells with LH or 1 mM dcAMP, but not with 0.1 mM dcAMP. We have not been able to measure significant effects of LH on the mass of polyphosphorylated phospholipids after extraction of twenty million tumour Leydig cells (unpublished results).

Cholesterol side-chain cleavage activity in the presence of 25-hydroxycholesterol, which reflects the maximal mitochondrial steroid production [33, 36] was not inhibited by quinacrine. This suggests that quinacrine does not inhibit mitochondrial activities which are important for steroidogenesis. Addition of quinacrine resulted in increased labelling of PtdIns4,5P<sub>2</sub>, PA and CL in tumour Leydig cells and therefore probably increased the levels of these phospholipids. This may be expected since quinacrine is an inhibitor of phospholipase A<sub>2</sub> [29, 30] and thus prevents breakdown of precursors of polyphosphorylated phospholipids. The increase in the levels of polyphosphorylated phospholipids should cause a stimulation of cholesterol side-chain cleavage activity if these phospholipids were the final mediators of the hormone-dependent activation of the mitochondrial steroid production. However, pregnenolone production in the presence of quinacrine was not stimulated but inhibited.

These results obtained with tumour Leydig cells show that there is no clear-cut correlation between the level of polyphosphorylated phospholipids and the activity of cytochrome P450<sub>sc</sub> under different experimental conditions. We conclude therefore that polyphosphorylated phospholipids apparently do not play an important role in the regulation of cholesterol side-chain cleavage activity after stimulation of

tumour Leydig cells with LH. The exact role of polyphosphorylated phospholipids in the regulation of steroidogenesis in corpus luteum cells is also disputed [21, 22], although effects of LH on phosphate incorporation in phospholipids have been observed [22]. A number of papers have been published on the role of phosphoinositides in the hormonal control of adrenal steroidogenesis [15–19, 24, 32], and the authors suggest that polyphosphorylated phospholipids play an important role in the regulation of  $\text{cytP450}_{\text{sc}}$  in the adrenal gland.

The discrepancies between the results obtained by Tanaka and Strauss [21], Davis *et al.* [22] and in the present paper on one hand and those obtained by Farese *et al.* [15–19, 24, 32] on the other hand cannot be explained at the moment. More experiments should be carried out in order to test the hypothesis whether trophic hormones increase  $\text{cytP450}_{\text{sc}}$  activity via sustained increased levels of polyphosphorylated phospholipids, especially since stimulating effects of certain hormones can be expressed via rapid hydrolysis of polyphosphorylated phospholipids in cell membranes [14].

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