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Inhibition of foetal pulmonary choline-phosphate cytidylyltransferase under conditions favouring protein phosphorylation

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Choline-phosphate cytidylyltransferase (EC 2.7.7.15) activity from 25- and 29-day-foetal rabbit lungs was inhibited in both the cytosolic and the microsomal fractions by preincubation with MgATP. The inhibition of the cytosolic enzyme was greater when measured with added phosphatidylglycerol (PG) than without (78–89% versus 50–55%), whereas the inhibition of the microsomal enzyme did not exhibit this distinction (66–72% versus 60–70%). When preincubated with the buffer alone, the cytosolic enzyme was activated to a greater extent by added PG than was the microsomal enzyme (13–14-fold versus 2–3-fold). However, after preincubation with MgATP, the cytosolic enzyme was activated to a smaller extent by added PG (3–6-fold). The inhibition of the enzyme by MgATP required a preincubation and was absent when ADP or AMP was substituted for ATP. Moreover, ATP analogues such as adenosine 5′-[β,γ-methylene]triphosphate and adenosine 5′-[γ-thio]triphosphate also failed to inhibit the enzyme when substituted for ATP in the preincubation. The inhibition by MgATP was not affected by including cyclic AMP in the preincubation, but Ca^{2+} ions alone or plus diacylglycerol in the preincubation increased the inhibition slightly. The inhibition was abolished by including an inhibitor of cyclic-AMP-dependent protein kinase in the preincubation. These observations, taken collectively, point to the inhibition of foetal pulmonary cytidylyltransferase through the phosphorylation of a protein and suggest that this key enzyme in lung surfactant production may be regulated through this mechanism.

INTRODUCTION

Choline-phosphate cytidylyltransferase (CTP:choline-phosphate cytidylyltransferase, EC 2.7.7.15) catalyses the transfer of a cytidylyl group from CTP to choline phosphate, forming CDP-choline and pyrophosphate. This is an important regulatory enzyme in the biosynthesis of phosphatidylcholine (PC) in several organs (Vance *et al.*, 1980; Zelinski *et al.*, 1980; Pritchard & Vance, 1981), including the lung (Post *et al.*, 1982). In the latter, PC is the major constituent of the lung surfactant, which stabilizes the alveoli during breathing (Goerke, 1974). During foetal development, the amount of PC in the alveolar lavage as well as in the whole lung increases markedly near term (Clements & Tooley, 1977; Ohno *et al.*, 1978). Studies of pool sizes indicate that there is a concomitant increase in the rate of the step catalysed by the cytidylyltransferase during the perinatal period (Tokmajian & Possmayer, 1980). Studies with prematurely delivered rat foetuses suggest that the total amount of cytidylyltransferase itself does not change during this period, but that a cytosolic form of low activity is converted into a microsomal form of high activity (Weinhold *et al.*, 1981). The mechanism whereby such a process may occur is of obvious interest, considering that lung maturation is of paramount importance for the survival of the newborn and that the regulation of cytidylyltransferase is an important part of the lung maturation.

One of the mechanisms suggested for the regulation of cytidylyltransferase is that of reversible protein phosphorylation. Previous studies in liver indicate that this enzyme may be interconverted between two forms: a phosphorylated form, which does not associate with the endoplasmic reticulum and is of low activity, and a dephosphorylated form, which associates with the membrane and is of high activity (Vance & Pelech, 1984).

There is increasing evidence that biosyntheses of fatty acids and other lipids can be controlled by protein phosphorylation (Hardie, 1981). In general, biosynthetic pathways which are regulated through protein phosphorylation are inhibited when anabolic enzymes are phosphorylated. However, although incubation of hepatocytes with cyclic AMP analogues for short periods resulted in an inhibition of PC biosynthesis, further incubation resulted in a stimulation (Pelech *et al.*, 1982). In the lung studies, treatment of pregnant rabbits with hormones that promote protein phosphorylation and treatments of foetal-rat lung explants as well as transformed adult human lung cell lines with cyclic AMP analogues were conducted for prolonged periods, and an increase in PC biosynthesis was seen (Barrett *et al.*, 1976; Gross & Rooney, 1977; Niles & Makarski, 1979).

These observations suggest that the biosynthesis of PC

Abbreviations used: PC, phosphatidylcholine; PG, 3-*sn*-phosphatidylglycerol; ATP[S], adenosine 5′-[γ-thio]triphosphate; p[CH_2]ppA, adenosine 5′-[β,γ-methylene]triphosphate.

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in the foetal lung (and in the liver) may be regulated through protein phosphorylation, though other complex phenomena may be occurring as well. Such a possibility led us to study the effect of conditions which favour protein phosphorylation on foetal pulmonary cytidylyltransferase, an important enzyme in foetal pulmonary maturation (Tokmajian & Possmayer, 1980).

EXPERIMENTAL

Materials

Pregnant New Zealand White rabbits were obtained from Don Reimen's Fur Ranch, St. Petersburg, Ontario, Canada. [*methyl*-^{14}C]Choline phosphate (55 Ci/mol) was purchased from New England Nuclear Canada, Lachine, Quebec, Canada. Hepes, ATP[S] and p[CH_2]ppA were purchased from Boehringer Mannheim Canada, Dorval, Quebec. KCl and $MgCl_2$ were from Fisher Scientific Co., Fairlawn, NJ, U.S.A., and $CaCl_2$ was from J. T. Baker Co., Phillipsburg, NJ, U.S.A. All other biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Preparation of lung homogenate

Pregnant does were killed on day 25 or 29 of gestation by an injection of sodium pentobarbital into the lateral ear vein. The foetuses were removed by hysterectomy and decapitated. The lungs were excised, washed in ice-cold homogenization buffer (1 mM-Hepes, 1 mM-EDTA/0.15 M-KCl/5 mM-dithiothreitol, adjusted to pH 7.0 with KOH), and the surrounding mediastinal structures were dissected away. The lungs were weighed and chopped on a McIlwain Mechanical Tissue Chopper into 0.5 mm cubes. The chopped lungs were homogenized in the homogenization buffer with a Teflon pestle. The lung homogenate was diluted 10-fold in the same buffer and then subjected to subcellular fractionation.

Preparation of subcellular fractions

The homogenate was centrifuged at 1500 *g* for 5 min and the resulting supernatant was centrifuged at 12000 *g* for 10 min. The (post-mitochondrial) supernatant from the latter procedure was centrifuged at 100000 *g* for 60 min. The (microsomal) pellet was resuspended in the homogenization buffer at a protein concentration of approx. 3 mg/ml. Protein concentrations were measured by the Bradford assay (Bradford, 1976; Spector, 1978), with essentially fatty-acid- and globulin-free bovine serum albumin (Sigma) as the standard. The cytosolic and the microsomal fractions were assayed while fresh for the phosphorylation and the cytidylyltransferase activities (Table 1 and Fig. 4), and frozen in small portions in a solid-CO_2/acetone bath and stored at −70 °C for further studies of phosphorylation (Figs. 1, 2, 3 and 5).

Protocol for phosphorylation

A portion (50 μl) of either the cytosolic or the microsomal fraction was preincubated with 2 mM-ATP, 10 mM-$MgCl_2$ and 50 mM-Hepes at pH 7.0 in a total volume of 100 μl at 37 °C for 10 min in a Dubnoff metabolic shaking incubator. In the control assays, the same volume of protein (50 μl) was preincubated with the buffer alone in a similar fashion. In assays where effects of other compounds were to be tested on the phosphorylation, they were included in the standard phosphorylation preincubation, but the total volume was maintained at 100 μl. At the end of the preincubation period, the samples were placed for 10 min in an ice/water bath before being assayed for cytidylyltransferase activity.

Assay of cytidylyltransferase activity

Assay mixtures contained 3 mM-[*methyl*-^{14}C]choline phosphate (1.0 Ci/mol), 3.75 mM-CTP, 10 mM-$MgCl_2$ and 50 mM-Hepes at pH 7.0 in a reaction volume of 50 μl. Assays with added PG contained 0.5 mg of PG (from egg lecithin; Sigma)/ml, a stock preparation of which was made by sonicating 5 mg of PG in 1 ml of 50 mM-Hepes buffer, pH 7.0.

Each assay was started by the addition of 20 μl of enzyme and incubated for 20 min at 37 °C with shaking. The samples were placed in a boiling-water bath for 3 min to stop the reaction. They were then centrifuged in an Eppendorf 5412 centrifuge for 5 min, and the supernatant

Table 1. Inhibition of choline-phosphate cytidylyltransferase from foetal rabbit lung under phosphorylating conditions

Fresh subcellular fractions from foetal rabbit lungs were incubated for 10 min at 37 °C with 50 mM-Hepes buffer, pH 7.0 ('Control'), or 10 mM-$MgCl_2$ and 2 mM-ATP in the same buffer ('Phos.'). The samples were then assayed for cytidylyltransferase activity without and with added PG (0.5 mg/ml). The values presented are means ± S.D. for duplicate assays with samples from four separate litters of foetuses. *P* values were calculated by Fisher's paired *t* test.

Gestation (days)	Subcellular fraction	Type of activity	Activity (nmol/min per mg)		Inhibition (%)	P (n = 4)
			Control	Phos.		
25	Cytosolic	−PG	0.27 ± 0.11	0.15 ± 0.13	50 ± 23	< 0.05
		+PG	3.68 ± 2.03	0.43 ± 0.38	89 ± 6	< 0.05
	Microsomal	−PG	1.18 ± 0.64	0.29 ± 0.22	66 ± 25	< 0.10
		+PG	1.83 ± 0.79	0.48 ± 0.45	70 ± 33	< 0.01
29	Cytosolic	−PG	0.23 ± 0.06	0.11 ± 0.06	55 ± 21	< 0.01
		+PG	3.04 ± 0.95	0.64 ± 0.25	78 ± 8	< 0.01
	Microsomal	−PG	2.09 ± 1.08	0.64 ± 0.52	72 ± 19	< 0.02
		+PG	3.81 ± 1.69	1.72 ± 1.45	60 ± 26	< 0.02

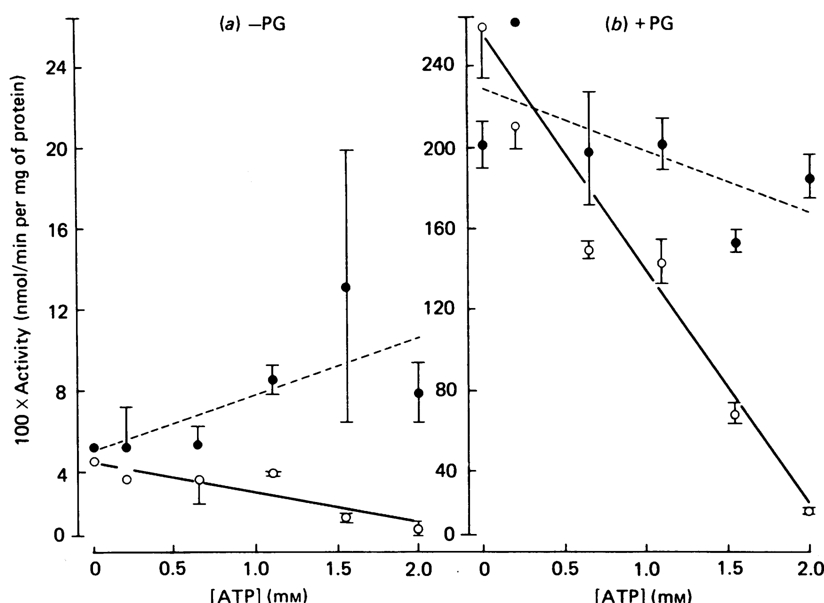

Fig. 1. Inhibition of cytidylyltransferase by preincubations at different ATP concentrations

Cytosol from 29-day-foetal rabbit lung was mixed with 10 mM-$MgCl_2$ and various concentrations of ATP. The samples were assayed for cytidylyltransferase activity before (●) and after (○) preincubation at 37 °C for 10 min. The data in panel (*a*) are from assays containing no added PG and those in panel (*b*) from assays containing 0.5 mg of PG/ml. The lines are derived by a linear correlation of activities with ATP concentrations. The values presented are means ± S.D. for data obtained with frozen cytosol from one litter of foetuses.

was subjected to t.l.c. on Linear-K5D silica-gel plates (Whatman) along with the unlabelled CDP-choline carrier. The plates were developed in the solvent system 0.45% (w/v) NaCl/methanol/conc. NH_3 (50:50:1, by vol.). The CDP-choline was detected under u.v. light by a spray of 0.01% rhodamine. The silica gel from the spots thus located was scraped into vials, 0.2 ml of water and 5 ml of Scinti-Verse E (Fisher) scintillant were added, and the radioactivity was determined in a Nuclear Chicago PDS/3-Isocap scintillation counter. All enzymic activities are expressed in units of nmol of CDP-choline produced/min.

RESULTS

When either the cytosolic or the microsomal fraction from 25- or 29-day-foetal rabbit lung was preincubated with 10 mM-$MgCl_2$ and 2 mM-ATP, the cytidylyltransferase activity was inhibited (Table 1). The enzymic activity in the cytosol was lower than that in the microsomal fraction after preincubation with buffer alone ('Control' values), but the former was activated more than the latter by added PG. On the other hand, the cytosolic activity was inhibited more by the preincubation with MgATP when it was measured with added PG than without. Such a distinction was not displayed by the microsomal activity. Inhibition of the foetal cytidylyltransferase required the simultaneous presence of Mg^{2+} ions and ATP (results not shown). Preliminary work showed that the inhibited enzyme remained inhibited, even after being subjected to gel filtration and freed of small molecules (P. McGarry & F. Possmayer, unpublished work). We found that the inhibition of the cytidylyltransferase by preincubation with MgATP as well as activation of the enzyme by PG were more reproducible when 5 mM-dithiothreitol was included in the homogenization buffer.

The foetal cytosolic cytidylyltransferase was inhibited by MgATP only after preincubation with MgATP (Fig. 1). This is seen by comparing the steep negative slopes of the continuous lines (preincubations) with the shallow negative or positive slopes of the broken lines (no preincubation). Both sets of lines were obtained by a linear correlation of activities with ATP concentrations. Only the continuous lines show negative linear correlation coefficients which are significant at a 95% confidence level. In Figs. 1(*a*) and 1(*b*), the linear correlation coefficients for the continuous lines are −0.8014 and −0.9780, and for the broken lines +0.4821 and −0.6024, respectively. Therefore the inhibition of cytidylyltransferase by MgATP was due to a time-dependent phenomenon, was in direct proportion to the ATP concentration and was not due to a simple inhibition by MgATP. Once again, the cytosolic activity measured with added PG was more sensitive to inhibition by preincubation with MgATP than was the activity measured without added PG.

The inhibition of cytidylyltransferase by a preincubation of the cytosol with Mg^{2+} and an adenine nucleotide required ATP specifically (Fig. 2). Neither 2 mM-ADP nor 2 mM-AMP inhibited the enzyme when the same protocol was used. Moreover, the requirement of the inhibition for ATP appears to be due to a requirement for a transferable γ-phosphate group on the adenine nucleotide (Fig. 3). The ATP analogue p[CH_2]ppA contains a non-transferable γ-phosphate group, and ATP[S] contains a transferable γ-thiophosphate group. Of the three compounds tested, only ATP

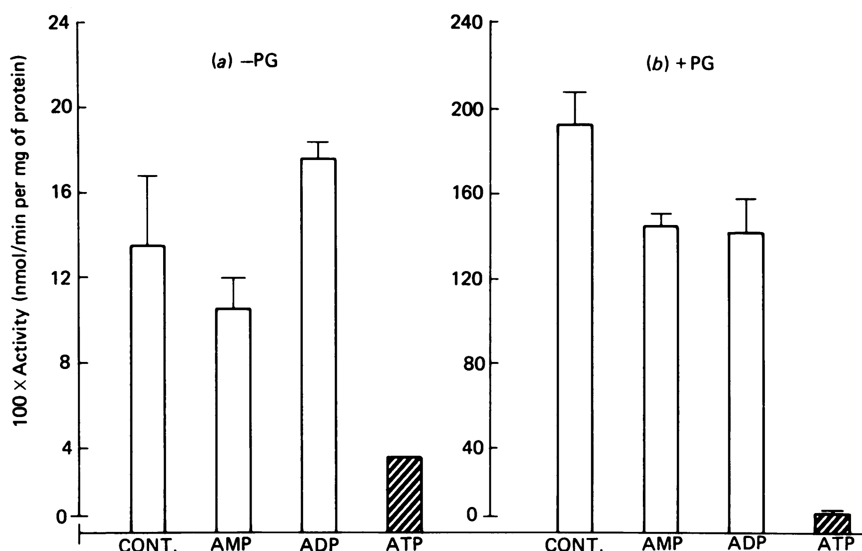

Fig. 2. Adenine nucleotides and the inhibition of cytidylyltransferase

Cytosol from 29-day-foetal rabbit lung was incubated at 37 °C for 10 min with 10 mM-$MgCl_2$ and 2 mM-AMP, 2 mM-ADP or 2 mM-ATP, and then assayed for cytidylyltransferase activity. The control (CONT.) values were obtained by assays of a sample of cytosol which was incubated with 50 mM-Hepes, pH 7.0, in a manner identical with that for the other samples. The data in panels (*a*) and (*b*) were obtained as described for Fig. 1. The values presented are means ± S.D. for data obtained with frozen cytosol from one litter of foetuses.

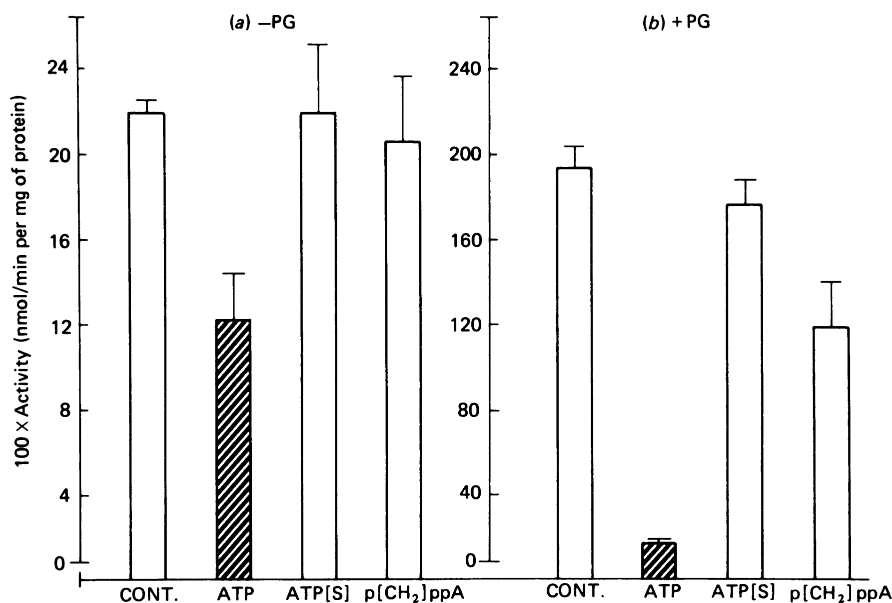

Fig. 3. Inhibition of cytidylyltransferase: effect of the transferable γ-phosphate group on the adenine nucleotide

Cytosol from 29-day-foetal rabbit lung was preincubated at 37 °C for 10 min with 10 mM-$MgCl_2$ and 2 mM-ATP (with a transferable γ-phosphate group) or ATP[S] (with a transferable γ-thiophosphate group) or p[CH_2]ppA (with a non-transferable γ-phosphate group), and then assayed for cytidylyltransferase activity. The control (CONT.) values were obtained as described for Fig. 2. The data in panels (*a*) and (*b*) were obtained as described for Fig. 1. The values presented are means ± S.D. for data obtained with frozen cytosol from one litter of foetuses.

could inhibit the cytidylyltransferase by a preincubation with Mg^{2+} and foetal lung cytosol, and ATP alone contains a transferable γ-phosphate group. Though p[CH_2]ppA inhibited the activity measured in the presence of PG to a small extent (37%), this inhibition was much less than that by ATP (95%). Similar results were obtained in three trials of this experiment. Taking these results together, it appears that the transfer of a γ-phosphate group from an adenine nucleotide, such as that catalysed by protein kinases, is required for the inhibition of foetal pulmonary cytidylyltransferase.

We attempted to identify the protein kinase(s)

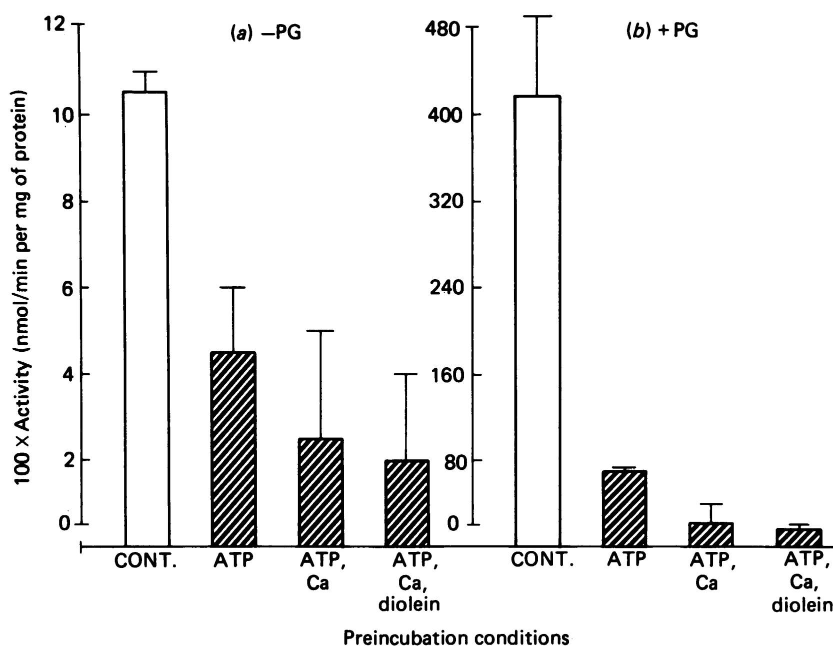

Fig. 4. Identity of the protein kinase implicated in the regulation of cytidylyltransferase

Cytosols from one set of 25-day and another set of 29-day foetuses were preincubated at 37 °C for 10 min with 10 mM-$MgCl_2$ and 2 mM-ATP with no further additions, or with 2 mM-$CaCl_2$ or with 2 mM-$CaCl_2$ plus 4 μg of 1,2-dioleoyl-*sn*-glycerol (diolein)/ml, and then assayed for cytidylyltransferase activity. The control (CONT.) values were obtained as described for Fig. 2. The data in panels (*a*) and (*b*) were obtained as described for Fig. 1. The values presented are means ± S.D. for data obtained with fresh cytosol from two litters of foetuses (one of 25 days and one of 29 days gestation).

implicated in the inhibition of cytidylyltransferase. Mg^{2+} plus ATP were necessary and sufficient in the preincubation to cause the observed inhibition of the enzyme. Adding cyclic AMP (0.01 and 0.1 mM) to the incubation did not alter the observed inhibition significantly in four trials (results not shown). However, adding Ca^{2+} (0.5 and 2.0 mM) to the preincubation increased the inhibition of the cytosolic enzyme, and adding both Ca^{2+} (0.5 and 2.0 mM) and 1,2-dioleoyl-*sn*-glycerol (4 μg/ml) increased the inhibition even further, but only to a small degree (Fig. 4). [The results presented in Fig. 4 were obtained with the pulmonary cytosol from two different sets of foetal rabbits, one set of 25 days gestation and the other of 29 days. Similar results were obtained with the microsomal enzymes also (results not shown).] Therefore it appears that the inhibition of cytidylyltransferase under conditions that favour protein phosphorylation may be due to a Ca^{2+}-mediated, but not cyclic-AMP-requiring, protein kinase.

The inhibition of cytosolic cytidylyltransferase by MgATP is prevented by including an inhibitor of cyclic-AMP-dependent protein kinase (Sigma) in the preincubation (Fig. 5). Moreover, preincubation of the cytosol with the inhibitor alone, in the absence of MgATP, results in a mild stimulation of the cytidylyltransferase (20%), whether the activity is measured with or without added PG.

DISCUSSION

The studies described herein were undertaken to investigate whether the regulation of choline-phosphate cytidylyltransferase by phosphorylation was a general phenomenon in organs other than liver, and to examine the possibility that control of cytidylyltransferase through phosphorylation could be involved in the regulation of surfactant production during foetal maturation. We were also interested to find out whether the anomalous increase in pulmonary PC biosynthesis in response to cyclic AMP analogues and hormones which operate through cyclic-AMP-mediated phosphorylation (Barrett *et al.*, 1976; Gross & Rooney, 1977; Niles & Makarski, 1979) was due to an activation of the critical cytidylyltransferase by protein phosphorylation, or whether phosphorylating conditions inhibited the pulmonary cytidylyltransferase, commensurate with the known effect of phosphorylation on other anabolic enzymes.

The present results demonstrate that foetal pulmonary cytidylyltransferase is inhibited by preincubation under phosphorylating conditions, both in the cytosolic and in the microsomal fractions. In the foetal rabbit (term is 31 days), choline incorporation into PC in lung slices increases 4-fold between days 25 and 29 of gestation (Rooney *et al.*, 1979). The amount of surfactant stored as lamellar bodies in type II cells increases sharply around 28 days of gestation (Ballard *et al.*, 1977). Consequently, we chose the gestational ages of 25 and 29 days to examine the response of cytidylyltransferase to phosphorylating conditions. The results demonstrate that the enzyme is equally susceptible to inhibition whether it is from foetuses before or after this critical transition.

No other exogenous chemical besides MgATP is necessary in the preincubation to inhibit the pulmonary cytidylyltransferase. In this feature, the protein kinase implicated in the inhibition of pulmonary cytidylyl-

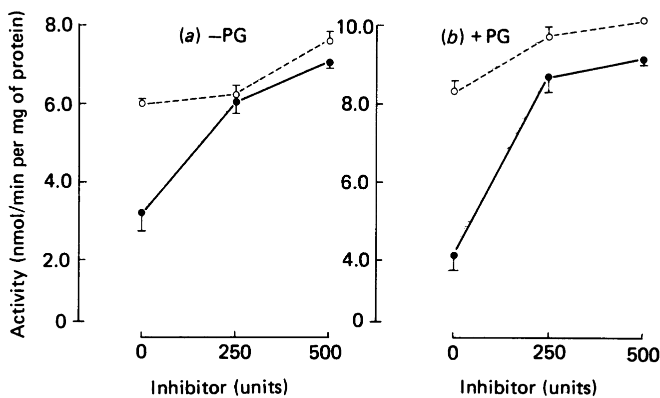

Fig. 5. Effect of inhibitor of protein kinase on inhibition of cytidylyltransferase

Cytosol from 29-day-foetal rabbit lung was incubated at 37 °C for 10 min with various amounts of an inhibitor of cyclic-AMP-dependent protein kinase (from rabbit muscle), and then assayed for cytidylyltransferase activity. ○, Activities from preincubations containing MgATP; ●, activities from preincubations containing 10 mM-$MgCl_2$ and 2 mM-ATP. One unit of inhibitor activity is defined as the amount which will block the transfer of 1.0 pmol of phosphate from [γ-^{32}P]ATP to hydrolysed and partially dephosphorylated casein at 37 °C by cyclic-AMP-dependent protein kinase/min. The values presented are means ± S.D. for data obtained with frozen cytosol from one litter of foetuses.

transferase seems similar to those implicated in the regulations of diacylglycerol acyltransferase (Haagsman *et al.*, 1982) and hepatic cytidylyltransferase (Pelech & Vance, 1982). However, in the case of acyltransferase, which is a microsomal enzyme, the protein kinase implicated appears to reside in the cytosol exclusively. With the foetal pulmonary cytidylyltransferase, both the cytosolic and the microsomal fractions contain the relevant protein kinase. Over 75% of the cytidylyltransferase in normal rat liver is in the cytosolic fraction, and, when incubated under phosphorylating conditions, this enzyme is not inhibited (Pelech & Vance, 1982). However, the liver cytosolic enzyme is known to undergo an activation when allowed to age without added phospholipid (Fiscus & Schneider, 1966; Choy *et al.*, 1977), and this activation is prevented or decreased by the preincubation with MgATP. The foetal pulmonary cytosolic cytidylyltransferase is not activated through aging (Fig. 1*a*,–PG) and is inhibited by the preincubation under phosphorylating conditions. The microsomal enzyme in both the foetal lung and adult liver are inhibited by the same preincubation.

The inhibition of pulmonary cytidylyltransferase by MgATP requires a preincubation. Therefore it is not a simple inhibition of cytidylyltransferase by a non-substrate nucleotide. The inhibition is caused only by MgATP, and not by either MgADP or MgAMP. Both p[CH_2]ppA and ATP[S] do not inhibit the cytidylyltransferase when they are preincubated with foetal pulmonary cytosol and Mg^{2+} ions. p[CH_2]ppA is known to be a substrate for phosphodiesterase, which requires a hydrolysable α–β bond, but not for hexokinase and myokinase, which require a hydrolysable β–γ bond (Yount *et al.*, 1971*a*, *b*). ATP[S], on the other hand, is known to be a substrate for thiophosphorylation by cyclic-AMP-dependent and calmodulin-mediated protein kinases (Gratecos & Fischer, 1974; Kerrick *et al.*, 1981). These results do not preclude the possibility that a thiophosphorylation of a polypeptide has taken place, but they do show that, even if that process had occurred, it did not lead to the inhibition of the cytidylyltransferase in the foetal pulmonary cytosol. Among the adenine nucleotides and analogues tested, only ATP has a transferable γ-phosphate group, and it is the only nucleotide to bring about a significant inhibition of foetal pulmonary cytidylyltransferase, and this inhibition requires a preincubation along with Mg^{2+} ions. Collectively, these results indicate that the inhibition of pulmonary cytidylyltransferase under phosphorylating conditions is mediated through protein phosphorylation.

Phosphorylating conditions also affect the foetal pulmonary enzyme's interaction with phospholipid activators. Cytosolic enzymes from both lung and liver are activated by anionic phospholipids, especially phosphatidylglycerol and lysophosphatidylethanolamine (Fiscus & Schneider, 1966; Choy & Vance, 1978; Feldman *et al.*, 1980). The presence of anionic phospholipid domains within intracellular membranes presumably provides an environment best suited for the activity of cytidylyltransferase (Feldman *et al.*, 1980; Weinhold *et al.*, 1983). Preincubation of the pulmonary cytosolic enzyme with MgATP inhibits the activity measured with added PG to a greater extent (80–88%) than that measured without PG (44–52%). The activation caused by PG itself is decreased from 13–14-fold to 3–6-fold by the preincubation with MgATP. In contrast, the microsomal activity is inhibited to the same extent by the preincubation with MgATP, whether it is measured with or without added PG. The microsomal enzyme is activated less (2–3-fold) than the cytosolic enzyme (13–14-fold) by PG, and it is the latter activity that is more sensitive to inhibition when it is measured with added PG than without. Even though we have added a fairly high concentration of phospholipid (0.5 mg/ml) in our assay of the foetal cytosolic enzyme [concentration of phospholipid in adult lung cytosol, if prepared by our protocol, is 0.2 mg/ml for the rat, based on values reported by Feldman *et al.* (1980)], there is no reversal of inhibition from phosphorylating conditions. Therefore, the phosphorylating conditions inhibit not only the pulmonary cytidylyltransferase itself, but also prevent the activation of the foetal cytosolic form through a mechanism which has been demonstrated to operate *in vitro*, but may operate *in vivo* as well (Chu & Rooney, 1985). The conversion of the enzyme into a more active form, rather than an increased production of the enzyme, is considered critical in foetal pulmonary maturation, resulting in an increased production of phosphatidylcholine (Khosla *et al.*, 1980; Possmayer *et al.*, 1981). Hence, the regulation of this important regulatory enzyme through protein phosphorylation can provide a means for a fine control of foetal pulmonary maturation.

These results differ from those obtained with the hepatic cytidylyltransferase (Pelech & Vance, 1982). The rapid activation of the liver cytosolic enzyme by the phospholipid vesicles in the hepatic cytosol at 37 °C is inhibited by including MgATP in the preincubation. Addition of 1 mg of phospholipid/ml to the assay, however, restores the activity to control values. Further addition of phospholipid increases the activity considerably.

The inhibition of foetal pulmonary cytidylyltransferase by the preincubation with MgATP is not affected by including 0.01 mM- and 0.1 mM-cyclic AMP in the preincubation. But adding Ca^{2+} ions or Ca^{2+} ions plus dioleoylglycerol to the preincubation inhibited the enzyme further, though with some sample-to-sample variation. Therefore the putative protein kinase which brings about the inhibition of pulmonary cytidylyltransferase does not seem to be cyclic-AMP-dependent, but may be Ca^{2+}-mediated. The augmented PC biosynthesis observed after prolonged treatment of foetal lung cells with cyclic AMP analogues or hormones causing an increase in intracellular cyclic AMP (Barrett *et al.*, 1976; Gross & Rooney, 1977; Niles & Makarski, 1979) can be explained most easily through the acceleration of pulmonary maturation, leading to an increase of type II cells (Rooney, 1979).

The inhibition of foetal pulmonary cytidylyltransferase under phosphorylating conditions is reversed by an inhibitor of cyclic-AMP-dependent protein kinase, even though the inhibition itself does not require cyclic AMP. The same effect has been observed with the hepatic cytidylyltransferase also (Pelech & Vance, 1982). An explanation offered in the latter case (Pelech & Vance, 1984) is that the inhibitor may exert its effect on cytidylyltransferase by inhibiting the phosphorylation of another well-known protein, Inhibitor I. When Inhibitor I is phosphorylated, it becomes inhibitory to phosphoprotein phosphatase 1 (Cohen, 1982). Therefore, when the phosphorylation of Inhibitor I is blocked, phosphoprotein phosphatase 1 becomes active. This phosphatase, which is known to act on glycogen synthase, glycogen phosphorylase and phosphorylase kinase (Cohen, 1982), may also be involved in the dephosphorylation of cytidylyltransferase. This would explain the results observed with the inhibitor of cyclic-AMP-dependent protein kinase. The availability of methods of preparation for phosphoprotein phosphatase 1 and its inhibitors (Resink *et al.*, 1983) should provide the means for testing this concept. This is an attractive idea, because it brings together the regulation of glycogen and phospholipid metabolism and because it has been suggested that glycogen metabolism is important to phosphatidylcholine biosynthesis in the foetal lung during the late part of gestation (Maniscalco *et al.*, 1978).

The foregoing results show that foetal pulmonary cytidylyltransferase is inhibited under conditions which favour protein phosphorylation, and that this inhibition also affects its interaction with phospholipid activators. Likely identities for the protein kinase and the phosphatase which may be involved in the regulation of this enzyme are suggested by our results, and further experiments are needed for confirmation. However, as with the hepatic enzyme, the results of studies on foetal pulmonary cytidylyltransferase have not demonstrated the direct phosphorylation of the enzyme. The results obtained so far can be explained by either a direct phosphorylation of the enzyme itself or that of a modulator protein, but in either case the results are consistent with a regulation through phosphorylation of a polypeptide. The isolation and identification of the phosphopeptide is necessary to prove and resolve this issue.

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