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β-Adrenergic control of phosphatidylcholine synthesis by transmethylation in hepatocytes from juvenile, adult and adrenalectomized rats

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Changes in isoprenaline-sensitive phospholipid methyltransferase were studied in hepatocytes isolated from juvenile, mature and adrenaletomized rats. Isoprenaline produced greater stimulation of cyclic AMP accumulation in juvenile and mature adrenalectomized rats than in mature animals. Similarly, isoprenaline stimulated phospholipid methyltransferase in juvenile and mature adrenalectomized rats but had no effect in mature animals. Isoprenaline-mediated activation of phospholipid methyltransferase in adrenalectomized rats was time- and dose-dependent. In hepatocytes isolated from adrenalectomized rats incubated with [*Me*-^{3}H]methionine or [^{3}H]-ethanolamine the addition of isoprenaline increased the amount of radioactivity incorporated into phosphatidylcholine. The activation by isoprenaline of phospholipid methyltransferase was abolished by the β-blocker propranolol and by insulin. These results indicate that in rat liver the occupation of functional β-receptors causes a stimulation of phospholipid methylation. It is suggested that, as reported previously, cyclic AMP activates phospholipid methyltransferase.

Phosphatidylcholine can be synthesized by two different pathways: by the CDP-choline pathway (Kennedy & Weiss, 1956) and by the transmethylation pathway (Bremer *et al.*, 1960). In most cells and tissues the contribution of the transmethylation pathway to the total synthesis of phosphatidylcholine is less than 5%. This has led to a controversial hypothesis about the function of the transmethylation pathway in these systems (Hirata & Axelrod, 1980; Vance & Kruijft, 1980; Mato & Alemany, 1983). In the rat liver, however, the transmethylation pathway synthesizes 20–40% of the total phosphatidylcholine (Sundler & Åkesson, 1975). One of the functions of this pathway in this organ is therefore to contribute to the maintenance of the structure of the hepatocyte and the secretion of bile and lipoproteins.

In the liver there is evidence indicating a hormonal control of the synthesis of phosphatidylcholine by both pathways. Thus the addition of angiotensin and vasopressin to isolated rat hepatocytes activates phospholipid methyltransferase (Alemany *et al.*, 1981) through a process that requires Ca^{2+}, ATP and calmodulin (Alemany *et al.*, 1982*a*). These same hormones inhibit the synthesis of phosphatidylcholine by the CDP-choline pathway through a process that also requires Ca^{2+} (Alemany *et al.*, 1982*b*). This co-ordinated mechanism of regulation of phosphatidylcholine synthesis is also observed when 3-deaza-adenosine, an inhibitor of the transmethylation pathway, is administered to rats (Pritchard *et al.*, 1982) or by using a diet poor or rich in choline (Lombardi *et al.*, 1969; Thompson *et al.*, 1969; Skurdal & Cornatzer, 1975; Schneider & Vance, 1978). The results with glucagon are more controversial. Thus the addition of this hormone to rat hepatocyte has been reported variously to stimulate (Geelen *et al.*, 1979; Castaño *et al.*, 1980) or to have no effect (Schanche *et al.*, 1982) on the transmethylation pathway. The addition of cyclic AMP (Castaño *et al.*, 1980) or chlorophenylthio-cyclic AMP (Pritchard *et al.*, 1981) to isolated rat hepatocytes stimulates phospholipid methyltransferase. However, chlorophenylthio-cyclic AMP inhibits the incorporation of radioactivity into phosphatidylcholine in hepatocytes incubated with [*Me*-^{3}H]methionine and [^{3}H]ethanolamine (Pritchard *et al.*, 1981). To clarify the role of cyclic AMP in phospholipid methylation we have studied the effect of isoprenaline, a β-adrenergic ligand, on phospholipid methyltransferase from isolated rat hepatocytes. The effect of isoprenaline on the incorporation of radioactivity into phosphatidylcholine in cells incubated with [*Me*-^{3}H]methionine and [^{3}H]ethanol-

amine has been studied also. In these studies we have used hepatocytes isolated from juvenile and mature rats and mature adrenalectomized rats, since rats under these conditions have different amounts of functional β-receptors (Wolfe *et al.*, 1976; Blair *et al.*, 1979; Chan *et al.*, 1979).

Experimental procedures

Isolation and incubation of hepatocytes

Juvenile (25–30 days; 60–80 g), mature (60–90 days; 300–350 g) and bilaterally adrenalectomized mature male Wistar rats were used. Rats were adrenalectomized 3–6 days before being used. All animals had free access to food and water or iso-osmotic 0.9% NaCl (adrenalectomized). Hepatocytes were isolated as described by Castaño *et al.* (1980).

Measurement of phospholipid methyltransferase

Hepatocytes were incubated as previously described (Castaño *et al.*, 1980). After 30 min of pre-incubation isoprenaline or iso-osmotic 0.9% NaCl was added. At the time indicated the suspension of hepatocytes was poured into precooled centrifuge tubes and immediately centrifuged at 100 ***g*** for 20 s. The supernatant was discarded and the pellet was immediately frozen in a solid CO_2/acetone bath. The pellet was homogenized and phospholipid methyltransferase was assayed as described by Castaño *et al.* (1980).

Incorporation of radioactivity from precursors into phosphatidylcholine

After 30 min pre-incubation hepatocytes were incubated with 10 μCi of [*Me*-^{3}H]methionine/ml (Amersham International; 15 Ci/nmol). Isoprenaline was added simultaneously with the isotope. At the time indicated the hepatocytes were centrifuged at 100 ***g*** for 20 s and the pellet was immediately frozen. For the determination of phospholipid methylation the frozen pellet was extracted with 10 ml of chloroform/methanol/2 M-HCl (6:3:1, by vol.). The chloroform phase was washed three times with 3 ml of 0·5 M-KCl in 50% methanol and finally dried under a stream of N_2 gas. The residue was dissolved in 200 μl of chloroform and 100 μl was applied to a silica-gel plate (Silica Gel 60; Merck) and developed with propionic acid/propan-1-ol/chloroform/water (2:2:1:1, by vol.) for phospholipid separation. The silica gel was then scraped into 5 mm bands and each band was counted as described by Castaño *et al.* (1980).

In those experiments in which [^{3}H]ethanolamine was used as a precursor for phosphatidylcholine synthesis cells were incubated for 60 min with 10 μCi of [1-^{3}H]ethanolamine/ml (Amersham International; 8.8 Ci/mmol). At the end of this period isoprenaline or iso-osmotic 0.9% NaCl were added and at the time indicated the hepatocytes were treated as described above for phospholipid extraction.

For the determination of cyclic AMP frozen hepatocyte pellets were treated with trichloroacetic acid. After centrifugation, the protein-free supernatant was extracted four times with diethyl ether, freeze-dried and the amount of cyclic AMP determined by radioimmunoassay as described by Alonso *et al.* (1982). Proteins were determined as described by Lowry *et al.* (1951).

Results

Effect of age and adrenalectomy on basal and isoprenaline-sensitive phospholipid methyltransferase of rat liver

Whereas the addition of 1 μM-isoprenaline stimulates phospholipid methyltransferase of hepatocytes isolated from juvenile rats, the same treatment has no effect on phospholipid methylation by mature rate hepatocytes (Table 1). A striking difference between hepatocytes from young and adults rats is the magnitude of the rise in cyclic AMP in response to 1 μM-isoprenaline. With hepatocytes from juvenile rats isoprenaline increases cyclic AMP levels by approx. 2.4-fold and only 1.4-fold with hepatocytes from mature rats (Table 1). With hepatocytes from

Table 1. *Effect of age and adrenalectomy on isoprenaline-sensitive phospholipid methyltransferase and cyclic AMP accumulation*

Incubation of hepatocytes with 1 μM-isoprenaline was carried out for 5 min as described in the Experimental procedures section. Assay of enzyme activity and cyclic AMP was carried out as described in the Experimental procedures section. Results are expressed as means ± S.E.M. from at least three independent experiments in triplicate; 100% activity corresponds to 30 ± 2, 90 ± 5 and 90 ± 4 pmol/min per mg of protein in hepatocytes from respectively juvenile, mature and adrenalectomized rats. Control levels of cyclic AMP were 0.24, 0.29 and 0.30 pmol/mg of protein in hepatocytes from respectively juvenile, mature and mature adrenalectomized rats.

	Proportion of control value (%)	
Rat hepatocytes	Phospholipid methyltransferase	Cyclic AMP
Juvenile	166 ± 8	236 ± 5
Mature	106 ± 6	145 ± 17
Mature adrenalectomized	160 ± 4	420 ± 15

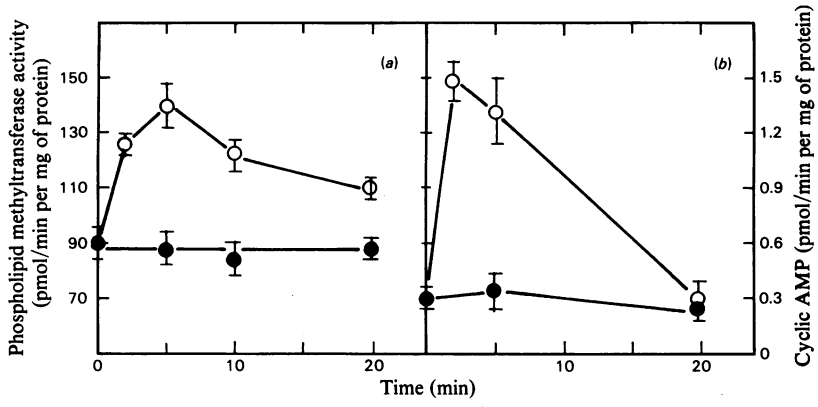

Fig. 1. *Time course of the effect of isoprenaline on phospholipid methyltransferase and cyclic AMP levels of adrenalectomized rat hepatocytes*

●, Control hepatocytes; ○, isoprenaline (1 μM)-treated hepatocytes. (*a*), Phospholipid methyltransferase; (*b*), cyclic AMP levels. Incubation of hepatocytes and assay of enzyme activity and cyclic AMP were carried out as described in the Experimental procedures section. Results are means ± S.E.M. from three independent experiments in triplicate.

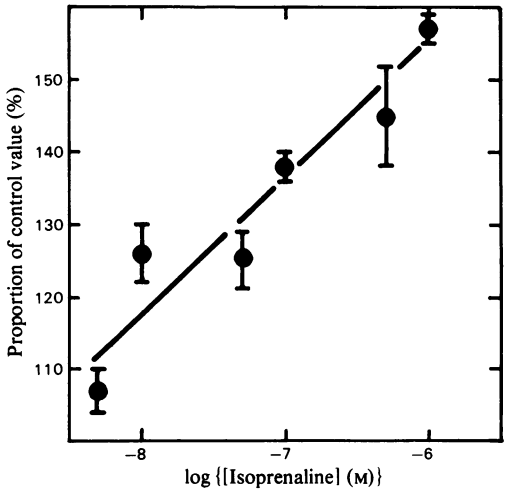

Fig. 2. *Effect of various concentrations of isoprenaline on phospholipid methyltransferase of adrenalectomized rat hepatocytes*

Portions of hepatocytes for enzyme assay were taken 5 min after the addition of isoprenaline. Incubation of hepatocytes and assay of enzyme activity were carried out as described in the Experimental procedures section. Results are means ± S.E.M. of three independent experiments in triplicate.

adrenalectomized mature rats 1 μM-isoprenaline increases cyclic AMP levels by 4.2-fold. This improved cyclic AMP response in adrenalectomized rats in response to isoprenaline is also accompanied by a stimulation of phospholipid methyltransferase (Table 1). Phospholipid methyltransferase is up to 3-fold higher in mature rats than in juvenile rats (Table 1). Double-reciprocal plots of phospholipid methyltransferase indicate that age increases the $V_{max.}$ of the enzyme without significantly affecting the apparent K_m value (about 6 μM) for *S*-adenosylmethionine (results not shown). The kinetics of phospholipid methyltransferase has been extensively studied by other workers (Schneider & Vance, 1979; Hoffman & Cornatzer, 1981). The first methylation reaction is the rate-limiting step and has a K_m for *S*-adenosylmethionine similar to that reported in the present paper. This value is also similar to that previously shown using rat hepatocytes (Castaño *et al.*, 1980) or isolated rat liver microsomes (Alemany *et al.*, 1982*a*). Treatment with 1 μM-isoprenaline of isolated hepatocytes from adrenalectomized rats produces a time-dependent activation of phospholipid methyltransferase (Fig. 1). Maximal activation is attained about 5 min after the addition of the hormone to slowly recover prestimulation activity. The time course of cyclic AMP accumulation in response to 1 μM-isoprenaline follows similar kinetics to those shown for phospholipid methyltransferase activation (Fig. 1). A transient accumulation of cyclic AMP by rat hepatocytes in response to adrenergic agonists has been shown previously (Blair *et al.*, 1979). The dose-dependent activation of phospholipid methyltransferase by isoprenaline is shown in Fig. 2. Concentrations of isoprenaline lower than 5 nM had no effect on the transmethylation reaction. Concentrations higher than 1 μM-isoprenaline were not used.

Effect of isoprenaline on the incorporation of radioactivity from [Me-^{3}H]methionine and [^{3}H]-ethanolamine into phosphatidylcholine

To examine the effect of isoprenaline addition on phospholipid methylation in intact hepatocytes from adrenalectomized rats two different approaches were taken. The first approach consists of the addition to isolated hepatocytes of [*Me*-^{3}H]methionine simultaneously with 1 μM-isoprenaline followed by the isolation and separation of the labelled phospholipids 10 min after stimulation. As shown in Fig. 3 the amount of radioactivity recovered into phospholipids was greater in hepatocytes treated with isoprenaline than in control cells. Fig. 3 is a representative experiment. The average incorporation of radioactivity into phospholipids was almost twice as high (1.8 ± 0.2, $n = 10$, $P < 0.01$) in cells treated with isoprenaline than in control hepatocytes. In the second approach, hepatocytes pre-incubated with [^{3}H]ethanolamine for 60 min were stimulated with 1 μM-isoprenaline or iso-osmotic 0.9% NaCl and 10 min later the labelled phospholipids were isolated and separated by t.l.c. As shown in Fig. 4 the amount of radioactivity associated with phosphatidylcholine and phosphatidyl-*NN*-dimethylethanolamine increased with the addition of isoprenaline. The addition of isoprenaline had no significant effect on the amount of radioactivity incorporated into phosphatidylethanolamine. Pre-incubation for 60 min with [^{3}H]-ethanolamine was enough to maintain the amount of radioactivity incorporated into phosphatidylcholine and phosphatidyl-*NN*-dimethylethanolamine in control cells constant. The amount of radioactivity in phosphatidylcholine and phosphatidyl-*NN*-dimethylethanolamine in control cells treated with [^{3}H]-ethanolamine was approx. 10% of the amount present in phosphatidylethanolamine. Fig. 4 is a representative experiment. The average incorporation of radioactivity into phosphatidylcholine and phosphatidyl-*NN*-dimethylethanolamine was twice as high (2.2 ± 0.4, $n = 7$, $P < 0.01$) in hepatocytes treated with isoprenaline than in control cells.

Effect of propranolol and insulin on isoprenaline-sensitive phospholipid methyltransferase

Whereas the addition of 5 μM-propranolol to hepatocytes isolated from adrenalectomized rats had only a small effect on basal phospholipid methyltransferase, the response to 1 μM-isoprenaline is blocked by propranolol (Table 2). As expected, the rise in cyclic AMP concentration induced by

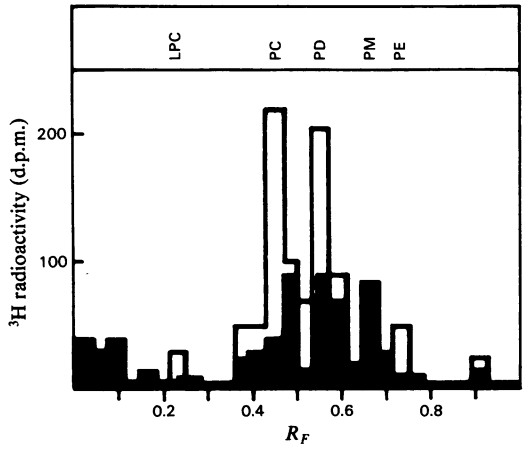

Fig. 3. *Separation of methylated phospholipids of hepatocytes from adrenalectomized rats*

Filled columns, control hepatocytes; open columns, isoprenaline (1 μM)-treated hepatocytes. Portions of hepatocytes were taken 10 min after the addition of [*Me*-^{3}H]methionine in the presence or in the absence of isoprenaline. Incorporation of radioactivity from [*Me*-^{3}H]methionine into phospholipid was carried out as described in the Experimental procedures section. Abbreviations used: LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PD, phosphatidyl-*NN*-dimethylethanolamine; PM, phosphatidyl-*N*-methylethanolamine; PE, phosphatidylethanolmine.

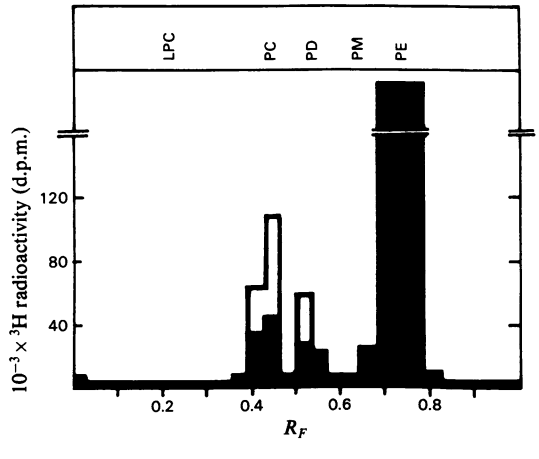

Fig. 4. *Separation of labelled lipids of hepatocytes from adrenalectomized rats incubated with [^{3}H]ethanolamine*

Filled columns, control hepatocytes; open columns, isoprenaline (1 μM)-treated hepatocytes. Hepatocytes were pre-incubated for 60 min with [^{3}H]-ethanolamine. At the end of this period cells were stimulated with 1 μM-isoprenaline or iso-osmatic 0.9% NaCl and 10 min later portions were taken to determine the incorporation of radioactivity into phospholipids. Conditions are described in the Experimental procedures section. Abbreviations used are defined in the legend to Fig. 3. The amount of radioactivity recovered in phosphatidylethanolamine was approx. 1.7×10^6 d.p.m.

Table 2. *Effect of propranolol and insulin on isoprenaline-sensitive phospholipid methyltransferase of adrenalectomized rat hepatocytes*

Incubations of hepatocytes with 1 μM-isoprenaline were carried out for 5 min as described in the Experimental procedures section. Propranolol was added 5 min before the addition of isoprenaline. Insulin was added together with isoprenaline. Results are expressed as means ± S.E.M. of three independent experiments in triplicate.

Addition	Proportion of control phospholipid methyltransferase activity (%)
Isoprenaline (1 μM)	160 ± 4
Propranolol (5 μM)	108 ± 3
Isoprenaline (1 μM) + propranolol (5 μM)	106 ± 3
Insulin (10 nM)	92 ± 14
Insulin (10 pM)	133 ± 6
Isoprenaline (1 μM) + insulin (10 nM)	76 ± 5
Isoprenaline (1 μM) + insulin (10 pM)	127 ± 6

isoprenaline [4.2 (± 1.5)-fold] is also reduced [1.6 (± 0.8)-fold] by treatment with 5 μM-propranolol.

The results of studies using insulin and isoprenaline are presented in Table 2. Whereas 10 nM-insulin had no effect on phospholipid methyltransferase, 10 pM gives a small but significant ($P < 0.05$) stimulation of this reaction. The addition of insulin (10 pM) blocked the stimulation by isoprenaline of phospholipid methyltransferase and higher concentrations of insulin (10 nM) induced a small but significant ($P < 0.05$) decrease in the enzyme activity.

Discussion

The results of the present investigation indicate that in rat liver the occupation of β-receptors results in phospholipid methyltransferase activation. This conclusion is based on the following observations. First, the activation by isoprenaline of phospholipid methyltransferase depends on the existence of functional β-receptors. With hepatocytes from juvenile rats 1 μM-isoprenaline stimulates phospholipid methyltransferase approx. 1.7-fold, whereas with hepatocytes from mature rats this activation was not significant. These results are consistent with the reported loss of functional β-receptors in hepatocytes from adult rats (Blair *et al.*, 1979) and with the magnitude of the rise in cyclic AMP observed in these experiments. A similar situation is observed with adrenalectomized rats. With hepatocytes from adrenalectomized rats 1 μM-isoprenaline activates phospholipid methyltransferase. These results agree with the reported increase in the number of functional β-receptors after adrenalectomy (Wolfe *et al.*, 1976; Chan *et al.*, 1979) and with our data showing an increase in the magnitude of cyclic AMP accumulated in response to isoprenaline. Secondly, isoprenaline-sensitive phospholipid methyltransferase in hepatocytes from adrenalectomized animals is time- and dose-dependent. The time course of phospholipid methyltransferase activation in response to isoprenaline is consistent with the transient increase in cyclic AMP stimulated by this agent (Blair *et al.*, 1979). Thirdly, β-adrenergically mediated stimulation of phospholipid methyltransferase is also observed in the intact cells incubated with [*Me*-^{3}H]methionine and [^{3}H]-ethanolamine. These results indicate that isoprenaline increases the flux from phosphatidylethanolamine to phosphatidylcholine in isolated hepatocytes. Fourthly, propranolol, a β-adrenergic blocker, blocks the response of cyclic AMP levels to isoprenaline and the stimulation of phospholipid methyltransferase. Insulin also blocks isoprenaline-sensitive phospholipid methyltransferase. Both treatments are known to block other metabolic effects triggered by the occupation of functional β-receptors (Claus & Pilkis, 1976; Blair *et al.*, 1979). The significance of the small activation of phospholipid methyltransferase obtained with 10 pM-insulin or of the small decrease in activity obtained by the addition of 1 μM-isoprenaline plus 10 nM-insulin are difficult to evaluate. It is interesting to note that the addition of insulin to chicken embryos induces lens fibre cell differentiation and a transient stimulation of phospholipid methylation (Zelenka *et al.*, 1982).

The present results are compatible with a role for cyclic AMP as an activator of phospholipid methyltransferase in isolated rat hepatocytes and agree with previous results in which glucagon (Geelen *et al.*, 1979; Castaño *et al.*, 1980), cyclic AMP (Castaño *et al.*, 1980) and chlorophenylthio-cyclic AMP Pritchard *et al.*, 1981) added to hepatocytes before being homogenized have been shown to activate phospholipid methyltransferase. Chlorophenylthio-cyclic AMP has also been reported to inhibit the incorporation of labelled methionine and ethanolamine into phosphatidylcholine (Pritchard *et al.*, 1981). The reason for the difference observed with this cyclic AMP derivative is not clear and the various alternatives have been discussed recently (Mato & Alemany, 1983). In conclusion, we believe that there is strong evidence indicating that cyclic AMP activates phospholipid methylation in rat hepatocytes. Evidence in favour of a cyclic AMP-dependent mechanism of activation of phospholipid methyltransferase has also been obtained with rat Leydig cells (Nieto & Catt, 1983). The addition of human choriogonadotropin to isolated rat Leydig cells before being homogenized activates phospholipid methyltransferase. This effect was reproduced by 8-bromo-cyclic AMP and by cholera toxin. In two other systems (Hirata *et al.*, 1979; Munzel &

Koschel, 1982) the occupation of β-adrenergic receptors has been associated with an increase in phospholipid methylation. In reticulocytes, it has been proposed that phospholipid methylation facilitates the coupling β-receptor–adenylate cyclase (Hirata *et al.*, 1979). In rat hepatocytes, however, adenylate cyclase activation by hormones is not dependent on phospholipid methylation (Schanche *et al.*, 1982). To try to understand these two different mechanisms it is important to remember that phospholipid methyltransferase in rat liver is located in the microsomal fraction (Gibson *et al.*, 1961). Therefore cyclic AMP and Ca^{2+} might serve as the messengers for communication between the plasma membrane and the endoplasmic reticulum.

The addition of chlorophenylthio-cyclic AMP and 8-bromo-cyclic AMP to rat hepatocytes has been shown to inhibit about 40% of the synthesis of phosphatidylcholine from [^{3}H]choline (Pelech *et al.*, 1981). The present results indicate that the β-adrenergic agonist stimulates by up to 160% the activity of phospholipid methyltransferase and previous results with glucagon and cyclic AMP have shown an activation of up to 200% (Geelen *et al.*, 1979; Castaño *et al.*, 1980). Thus the inhibitory effect of cyclic AMP on the CDP-choline pathway seems to be compensated by its stimulatory effect on the transmethylation route. The situation is therefore similar to that found with angiotensin and vasopressin. The addition of these hormones to isolated rat hepatocytes stimulates phospholipid methyltransferase (Alemany *et al.*, 1981) and inhibits the CDP-choline pathway (Alemany *et al.*, 1982*b*) This co-ordinated mechanism of regulation of phosphatidylcholine synthesis is also observed in other circumstances. Thus the inhibition by 3-deazaadenosine of the transmethylation pathway results in an activation of the CDP-choline pathway (Pritchard *et al.*, 1982). Rats maintained on a diet deficient in choline have a reduced activity of the CDP-choline pathway and an increased phospholipid methyltransferase activity (Schneider & Vance, 1978). However, it is important to note that the increased phospholipid methyltransferase activity in choline-deficient rats has not been observed by other workers (Skurdal & Cornatzer, 1975). Although the mechanisms that modulate the synthesis of phosphatidylcholine by the two pathways are not always clear, these results indicate that the activity of both pathways in the liver are co-ordinated to maintain a steady-state level of phosphatidylcholine synthesis under different metabolic conditions.

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