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REVIEW ARTICLE

What is the function of phospholipid *N*-methylation?*

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Introduction

Most if not all membrane functions are made possible by the unique composition of proteins and lipids in biological membranes. Although most biological membranes carry out similar functions, their composition varies largely (reviewed by Quinn, 1977). Thus, the protein/lipid ratio of rat plasma membranes can vary from 0.25 in myelin to 1.5 in the liver. The ratio cholesterol/phospholipid can also vary from 1.0 in rat erythrocytes to 0.5 in rat liver plasma membranes. The phospholipid composition is also very variable; rat erythrocytes contain about 30% phosphatidylcholine but sheep erythrocytes contain only 5%. Variations in the phospholipid content are also found within a cell. Thus, the content of phosphatidylcholine of liver cell membranes varies from 61% in rough endoplasmic reticulum to 35% in plasma membranes, while the content of phosphatidylethanolamine is about 20% in all liver membrane fractions (McMurray & McGee, 1972). It is important to remember these data when making generalizations. Another important feature of membranes is the rapid turnover of their phospholipids. The half-life of phosphatidylcholine in rat liver varies from 1h to 10h depending on the type of precursor used (Tolbert & Okey, 1952; Arvidson, 1968). Different cell fractions of rat liver have also different phospholipid turnover. Thus, the half-life of phosphatidylcholine in rat liver mitochondria and microsomes is 16h and 8h respectively (Bygrave, 1969). The significance of this rapid turnover is not well understood, but may result from membrane repair mechanisms as well as from structural changes which may render cell membranes more sensitive to activation. In this Review we will deal with the control mechanisms of phosphatidylcholine synthesis by the CDP-choline and transmethylation pathways. The catabolism of phosphatidylcholine has been recently reviewed (Van den Bosch, 1980).

Abbreviations used: AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; deaza-SIBA, 5'-deoxy-5'-isobutylthio-3-deaza-adenosine; CPT-cAMP, chlorophenylthioadenosine 3',5'-monophosphate; RBL cells, rat basophilic leukaemia cells.

* This Review is dedicated to Dr. Francisco Vivanco on his 72nd birthday.

Enzymes involved in phosphatidylcholine synthesis

The synthesis of phosphatidylcholine can occur by two different pathways: by the CDP-choline pathway (Kennedy & Weiss, 1956; Kennedy, 1962) and by the transmethylation pathway (Bremer *et al.*, 1960; Bremer & Greenberg, 1961). The CDP-choline pathway is the major pathway for the synthesis of this phospholipid and involves the transfer of phosphocholine from CDP-choline to diacylglycerol. The enzyme which catalyses this reaction is called phosphocholine transferase and in the rat liver is located in the microsomal fraction (Gibson *et al.*, 1961; Wilgram & Kennedy, 1963). The rate-limiting step in the synthesis of phosphatidylcholine by this pathway is the cellular content of CDP-choline (Infante & Kinsella, 1978; Schneider & Vance, 1978; Vance & Choy, 1979; Pritchard & Vance, 1981). The synthesis of CDP-choline involves the phosphorylation of choline and its conversion to CDP-choline with CTP.

The transmethylation pathway consists of the addition of three methyl groups to the amino moiety of a phosphatidylethanolamine molecule via AdoMet, the methyl donor. The enzyme which catalyses this reaction is called phospholipid *N*-methyltransferase and in the rat liver is mainly located in the microsomal fraction (Bjornstad & Bremer, 1966; Skurdal & Cornatzer, 1975; Yeselma & Moore, 1978). Phosphatidyl-*N*-methylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine are the two intermediate products of this reaction.

The question has arisen as to whether the conversion of phosphatidylethanolamine to phosphatidylcholine is catalysed by one or two enzymes. In three studies using solubilized methyltransferase from rat liver microsomes the results indicate that a single enzyme catalyses the stepwise methylation of phosphatidylethanolamine to phosphatidylcholine (Rehbinder & Greenberg, 1965; Tanaka *et al.*, 1979; Schneider & Vance, 1979). Sastry *et al.* (1981) have concluded that in rat liver microsomes the conversion of phosphatidylethanolamine to phosphatidylcholine is catalysed by two different methyltransferases. The first enzyme incorporates the first methyl group and the second methyltransferase the remaining two methyl groups. This conclusion is mainly based on kinetic data showing

non-linear kinetics when microsomes are incubated with different concentrations of labelled AdoMet. At low concentrations of AdoMet, phosphatidyl-*N*-methylethanolamine is the main product of the transmethylation reaction, whereas at high concentrations of AdoMet phosphatidyl*N*,*N*-dimethylethanolamine and phosphatidylcholine are synthesized. Phospholipid *N*-methyltransferase showing non-linear kinetics has been described in a number of cells and tissues, including bovine adrenal medulla (Hirata & Axelrod, 1978*a*), rat brain synaptosomes (Crews *et al.*, 1980*a*) and *Dictyostelium discoideum* (García Gil *et al.*, 1980) and in some cases this behaviour has been regarded as indicative of the existence of two methyltransferases. As mentioned by Vance *et al.* (1982), these kinetic studies did not consider the converson of phosphatidyl-*N*-methylethanolamine to phosphatidyl-*N*,*N*-dimethylethanolamine during the enzyme assay. Since this reaction is relatively fast and the initial methylation of phosphatidylethanolamine to phosphatidyl-*N*-methylethanolamine is relatively slow (Schneider & Vance, 1979), this introduces an error in the determination of the kinetic parameters for the initial methylation reaction. The claims of two methyltransferases based only on kinetic data are therefore not justified. Another source of error in the determination of the kinetics of phospholipid *N*-methyltransferase is the possible methylation of lipids other than phospholipids. Several neutral lipids are formed by incubation with AdoMet (Zatz *et al.*, 1981; Alemany *et al.*, 1982*a*) or by incubation of intact cells with methionine (Rabe & McGee, 1982). Some of these lipids have been identified as methyl esters of fatty acids (Zatz *et al.*, 1981) and as *S*-methyl-*N*-oleoyl-mercaptoethylamide (Zatz *et al.*, 1982).

Evidence indicating the presence of at least two phospholipid *N*-methyltransferases has been obtained with rat pituitary extracts (Prasad & Edwards, 1981*a*). The supernatant fraction after 20 min centrifugation at 20000 *g* of pituitary extracts catalyses the transfer of the first methyl group into phosphatidylethanolamine, and the particulate fraction incorporates methyl groups into phosphatidyl-*N*-methylethanolamine, phosphatidyl-*N*,*N*-dimethylethanolamine and phosphatidylcholine. Although these results suggest the existence of two methyltransferases in rat pituitary extracts, it is possible that there is only one enzyme and that the substrate specificity changes depending on its particulate state. Evidence in favour of two phospholipid *N*-methyltransferases has also been obtained with RBL cells (McGivney *et al.*, 1981). At low concentrations of AdoMet, RBL cell extracts incorporate methyl groups preferentially into phosphatidyl-*N*-methylethanolamine and at high concentrations of substrate into phosphatidylcholine. RBL cell variants have been isolated which methylate phosphatidylethanolamine to phosphatidyl-*N*-monomethylethanolamine but have an impaired transfer to phosphatidyl-*N*,*N*-dimethylethanolamine and phosphatidylcholine (20% of that of the parental line). Similarly, variants have been obtained which could methylate phosphatidyl-*N*-methylethanolamine or phosphatidyl-*N*,*N*-dimethylethanolamine to phosphatidylcholine but have an impaired transfer (18% of that of the parental line) to phosphatidyl-*N*-methylethanolamine. Reconstitution by fusion of the two RBL cell variants results in normal phospholipid *N*-methylation. These results are very suggestive of the presence of more than one phospholipid *N*-methyltransferase in RBL cells. However, none of the variants has exclusively one of the methyltransferase activities and the possibility that the differences in phospholipid *N*-methylation are due to other unknown defects cannot be excluded.

In conclusion, whether there is more than one enzyme that catalyses the three-step methylation of phosphatidylethanolamine to phosphatidylcholine is undecided and will have to await till the enzyme(s) is purified and its substrate specificity assayed.

Regulation of phospholipid *N*-methylation

During the last few years evidence has accumulated which indicates that several signals acting on cell-surface receptors regulate the methylation of phosphatidylethanolamine (Tables 1 and 2). With hepatocytes isolated from mature rats the addition of glucagon before homogenization produces a time- and dose-dependent activation of the methylation reaction (Castaño *et al.*, 1980). The activation is about two-fold, occurs at physiological concentrations of glucagon and is due to an increase in the $V_{max.}$ of the enzyme without affecting the apparent K_m value for AdoMet. Cyclic AMP added exogenously to rat hepatocytes reproduces the effect of glucagon on phospholipid *N*-methylation (Castaño *et al.*, 1980). The addition of isoprenaline to hepatocytes isolated from mature adrenalectomized rats, which contain a larger number of functional β-receptors, also stimulates the activity of phospholipid *N*-methyltransferase (D. Marin Cao, V. Alvarez Chiva & J. M. Mato, unpublished work). This effect is dose- and time-dependent and is inhibited by the β-blocker propranolol and by insulin. CPT-cAMP added exogenously to rat hepatocytes also stimulates phospholipid *N*-methyltransferase by about two-fold (Pritchard *et al.*, 1981). Finally, the addition of cyclic AMP in the presence of micromolar concentrations of ATP to rat liver microsomes stimulates phospholipid *N*-methyltransferase (Mato *et al.*, 1982) which suggests the involvement of a cyclic AMP-dependent

Table 1. *Effect of different additives on phospholipid N-methylation by hepatocytes*
Phospholipid *N*-methyltransferase is measured in cells stimulated before homogenization. Phospholipid *N*-methylation is measured as the incorporation of labelled precursors into phospholipids. Abbreviation: n.d., not determined.

	Effect on phospholipid:		
	N-Methyltransferase	Methylation	Reference
Glucagon	Activation	n.d.	Castaño *et al.* (1980)
Glucagon	n.d.	Activation	Geelen *et al.* (1979)
Glucagon	n.d.	No effect	Schanche *et al.* (1982)
Isoprenaline	Activation	Activation	D. Marin Cao, V. Alvarez Chiva & J. M. Mato, unpublished work
CPT-cAMP	Activation	Inhibition	Pritchard *et al.* (1981)
Cyclic AMP	Activation	n.d.	Castaño *et al.* (1980)
Angiotensin	Activation	n.d.	Alemany *et al.* (1981)
Vasopressin	Activation	n.d.	Alemany *et al.* (1981)
A23187	Activation	n.d.	Alemany *et al.* (1981)

Table 2. *Effect of different additives on phospholipid N-methylation*
Phospholipid *N*-methyltransferase is measured in cells stimulated before homogenization. Phospholipid *N*-methylation is measured as the incorporation of labelled precursors into phospholipids. Abbreviation: n.d., not determined.

		Effect on phospholipid:		
System	Stimulus	*N*-Methyltransferase	Methylation	Reference
Leydig cells	Gonadotropin	Activation	n.d.	Nieto & Catt (1983)
Pancreatic islets	Glucose	Activation	Activation	M. Saceda, P. Garcia Morales, J. M. Mato, I. Valverde & W. J. Malaisse, unpublished work
Reticulocytes	Isoprenaline	Activation	n.d.	Hirata *et al.* (1979*a*)
Glioma cells	Isoprenaline	n.d.	Activation	Munzel & Kosehel (1982)
RBL cells	Immunoglobulin E	n.d.	Activation	Crews *et al.* (1980*b*)
Mast cells	Immunoglobulin E	Activation	Activation	Ishizaka *et al.* (1980, 1981)
Dictyostelium	Cyclic AMP	n.d.	Activation	Alemany *et al.* (1980)
Neuritis	Nerve growth factor	n.d.	Activation	Pfenninger & Johnson (1981)
Pituitary	Corticotropin releasing factor	n.d.	Activation	Hook *et al.* (1982)
Chicken embryos	Differentiation	n.d.	Activation	Zelenka *et al.* (1982)
Pituitary	Vasopressin	Activation	n.d.	Prasad & Edwards (1981)
Neutrophils	Attractant	n.d.	Inhibition	Hirata *et al.* (1979*b*)
Neutrophils	Phagocytosis	Inhibition	Inhibition	García Gil *et al.* (1981)
Macrophages	Attractant	n.d.	Inhibition	Pike *et al.* (1979)
Platelets	Aggregation	n.d.	Inhibition	Shattil *et al.* (1981)

protein kinase. In conclusion, these results indicate that in rat liver a stimulation of the intracellular content of cyclic AMP results in an activation of the enzyme phospholipid *N*-methyltransferase.

The results with hepatocytes incubated with [*methyl*-^{3}H]methionine or [^{3}H]ethanolamine are more controversial. In these experiments the hepatocytes are incubated with the labelled precursor and after stimulation the incorporation of radioactivity into the various phospholipids is analysed. Under these conditions, the addition of glucagon to rat hepatocytes has been reported to stimulate (Geelen *et al.*, 1979) or to have no effect on (Schanche *et al.*, 1982) phospholipid *N*-methylation, and addition of CPT-cAMP to rat hepatocytes has been shown to inhibit phospholipid *N*-methylation (Pritchard *et al.*, 1981). In hepatocytes from adrenalectomized rats incubated with [*methyl*-^{3}H]methionine or [^{3}H]ethanolamine the addition of isoprenaline increases phospholipid *N*-methylation (D. Marin Cao, V. Alvarez Chiva & J. M. Mato, unpublished work).

The reasons for these differences are not obvious. It is interesting to note that CPT-cAMP added exogenously to rat hepatocytes decreases the formation of phosphatidylcholine from labelled precursors and stimulates phospholipid *N*-methyltransferase by two-fold (Pritchard *et al.*, 1981). To explain this contradiction the authors suggest that the increased phospholipid *N*-methyltransferase observed with CPT-cAMP may result from an increased avail-

ability of phosphatidylethanolamine as it accumulates in the hepatocyte after the inhibition of the *N*-methylation reaction. No evidence supporting this hypothesis was however given. Furthermore, the addition of exogenous phosphatidylethanolamine (2 mg/ml) does not change the enzyme activity of homogenates from either control or glucagon-treated hepatocytes (Castaño *et al.*, 1980); similarly, phosphatidylethanolamine added to rat liver microsomes does not stimulate methylation (Schneider & Vance, 1979); and the *N*-methylation of phosphatidylethanolamine to phosphatidylcholine in rat hepatocytes remains constant when the rate of phosphatidylethanolamine synthesis is varied up to 8-fold (Sundler & Akesson, 1975). These results indicate that, as reported by Bremer (1969), in hepatocytes isolated from normally fed rats the concentration of phosphatidylethanolamine is enough to saturate the methylation reaction. Under these conditions, a stimulation of the rate of phospholipid *N*-methylation could not be explained by an increase in the amount of phosphatidylethanolamine. In hepatocytes maintained in the absence of ethanolamine for 24 h the amount of phosphatidylethanolamine decreases by about 30% (Akesson, 1978). Under these conditions an increase in the concentration of phosphatidylethanolamine results in an increased rate of methylation until a plateau is reached (Akesson, 1978). There is no evidence however that might indicate that a similar situation applies to the results of Pritchard *et al.* (1981). Another possibility to explain the results with CPT-cAMP is that in addition to its effect on phospholipid *N*-methyltransferase this compound may alter the metabolism of AdoMet. Although CPT-cAMP is more resistant than cyclic AMP to hydrolysis (Miller *et al.*, 1975), the incubation of hepatocytes for 1 h with 0.5 mM-CPT-cAMP may result in intracellular accumulation of different adenosine derivatives. Adenosine and its derivatives are known to inhibit phospholipid *N*-methylation by affecting the ratio AdoMet/nucleosidylhomocysteine (Cantoni & Chiang, 1980; Hoffman *et al.*, 1980; García Castro *et al.*, 1983). In conclusion, we believe that there is strong evidence indicating that the addition to rat hepatocytes of hormones that cause the accumulation of cyclic AMP stimulates phospholipid *N*-methylation.

Evidence in favour of a cyclic AMP-dependent mechanism of activation of phospholipid *N*-methyltransferase has also been obtained with rat Leydig cells (Nieto & Catt, 1983). The addition of human chorionic gonadotropin to isolated rat Leydig cells before homogenization induces a time- and dose-dependent activation of phospholipid *N*-methyltransferase. This effect is reproduced by the addition of 8-bromo cyclic AMP or cholera toxin. In pancreatic islets phospholipid *N*-methyltransferase and the incorporation of radioactivity into phospholipids in cells incubated with [*methyl*-^{3}H]-methionine is stimulated by glucose (M. Saceda, P. García-Morales, J. M. Mato, I. Valverde & W. J. Malaisse, unpublished work). Phospholipid *N*-methyltransferase also increases in islets exposed to glucagon before homogenization (M. Saceda, P. García-Morales, J. M. Mato, I. Valverde & W. J. Malaisse, unpublished work), which suggests that cyclic AMP may play a role in the activation of this reaction.

In rat reticulocyte ghosts isoprenaline induces a dose-dependent stimulation of phospholipid *N*-methyltransferase (Hirata *et al.*, 1979*a*). On the other hand, this effect is not reproduced by 8-bromo cyclic AMP, NaF or cholera toxin. These results indicate that the stimulation of phospholipid *N*-methylation is mediated by the binding of isoprenaline to its receptor but not by cyclic AMP. In rat glioma cells isoprenaline stimulates the incorporation of radioactivity from [*methyl*-^{3}H]methionine into phospholipids (Munzel & Koschel, 1982); and in pineal glands of spontaneously hypertensive rats increased levels of adrenaline correlate with a stimulation of phospholipid *N*-methyltransferase (Saavedra, 1980). Whether these β-adrenergic effects are mediated by the interaction of hormone and receptor directly or by the cyclic AMP generated by the hormone is not known. Evidence indicating that the stimulation of phospholipid *N*-methyltransferase is mediated by the binding of a ligand to its receptor and not by the cyclic AMP formed has also been obtained in rat mast cells. Bridging of immunoglobulin E receptors to isolated plasma membranes from rat mast cells stimulates phospholipid *N*-methyltransferase in the absence of cyclic AMP (Ishizaka *et al.*, 1981). In rat hepatocytes, as mentioned earlier, phospholipid *N*-methyltransferase is mainly located in the microsomal fraction. Therefore, to activate phospholipid *N*-methylation a messenger that moves from the plasma membrane, where the interaction of ligand and receptor takes place, to the endoplasmic reticulum, is necessary. As mentioned above, there is evidence indicating that one of these messengers is cyclic AMP. In addition, there is also evidence that indicates that Ca^{2+} regulates phospholipid *N*-methyltransferase in rat hepatocytes.

In rat hepatocytes the addition of vasopressin or angiotensin, two hormones whose actions in this organ are mediated through Ca^{2+} (Fain, 1978; Kirk & Hems, 1974; Keppens & De Wulf, 1976), induce a dose- and time-dependent activation of phospholipid *N*-methyltransferase (Alemany *et al.*, 1981). This effect is reproduced by the cationophore A23187. In the absence of external Ca^{2+} none of the above hormones stimulate phospholipid *N*-methyltransferase. The addition of micromolar

Ca^{2+} in the presence of ATP to rat liver microsomes also stimulates phospholipid *N*-methyltransferase (Alemany *et al.*, 1982*a*). Different lines of evidence indicate that calmodulin, a ubiquitous Ca^{2+}-binding protein (reviewed by Cheung, 1982), mediates the effect of Ca^{2+} on phospholipid *N*-methylation by isolated rat liver microsomes (Alemany *et al.*, 1982*a*). A similar mechanism seems to operate in extracts of *Dictyostelium discoideum* (Garcia Gil *et al.*, 1980). Vasopressin added to rat pituitary extracts also activates phospholipid *N*-methyltransferase (Prasad & Edwards, 1981*b*). The mechanism of this activation remains unknown. Results showing the effect of angiotensin or vasopressin on the incorporation of labelled precursors into phospholipids in intact cells are not yet available. Although it can be argued that the results obtained from assaying phospholipid *N*-methyltransferase do not necessarily indicate the situation with intact cells, the present results strongly indicate that the addition to rat hepatocytes of hormones that increase the intracellular concentration of Ca^{2+} stimulates phospholipid *N*-methylation.

Different ligands (Table 2) enhance the incorporation of radioactivity from [*methyl*-^{3}H]methionine into phospholipids in a variety of cells, including attractants in *Dictyostelium discoideum* (Alemany *et al.*, 1980); immunoglobulin E in RBL cells (Crews *et al.*, 1980*b*) and rat mast cells (Ishizaka *et al.*, 1980); nerve growth factor in rat neuritis (Pfenninger & Johnson, 1981); corticotropin-releasing factor in mouse pituitary tumour cells (Hook *et al.*, 1982); and agents that induce differentiation in chicken embryos (Zelenka *et al.*, 1982). The mechanisms by which these signals activate phospholipid *N*-methylation are not known. Hirata *et al.* (1979*b*, 1980) have reported that the addition of concanavalin A to rat mast cells and lymphocytes induces a transient stimulation of phospholipid *N*-methylation. However, Moore *et al.* (1982) have recently shown that concanavalin A, at optimal concentrations for mitogenic stimulation, had no effect on phospholipid *N*-methylation in three types of lymphocytes. Under the same conditions concanavalin A stimulated phosphatidylinositol turnover (Moore *et al.*, 1982). This and the numerous control experiments carried out by Moore *et al.* (1982) strongly indicate that there are no changes in phospholipid *N*-methylation during mitogenic stimulation of lymphocytes.

Inhibition of phospholipid *N*-methylation in response to a stimulus has also been observed (Table 2). Decreased incorporation of radioactivity from [*methyl*-^{3}H]methionine into phospholipids has been observed in rabbit leucocytes (Hirata *et al.*, 1979*b*) and macrophages (Pike *et al.*, 1979) in response to attractants. Whereas the data with rabbit leucocytes were interpreted as the result of an accelerated degradation, an inhibition of the rate of synthesis of methylated phospholipids was postulated to occur in macrophages. In none of these cases was the activity of phospholipid *N*-methyltransferase assayed following stimulation. In human platelets the addition of thrombin causes a fast decrease in phospholipid *N*-methylation (Shattil *et al.*, 1981). This effect seems to be due to a decreased synthesis of methylated lipids rather than to an increase in their degradation. Finally, the addition to human neutrophils of zymosan particles coated with complement also inhibits phospholipid *N*-methyltransferase and the incorporation of radioactivity from [*methyl*-^{3}H]methionine into phospholipids (Garcia Gil *et al.*, 1981).

In conclusion, a variety of signals acting through cell surface receptors modulate phospholipid *N*-methylation in several cells and tissues and the effect can be either an activation or an inhibition of the rate of transmethylation.

What is the function of phospholipid *N*-methylation?

Phosphatidylcholine synthesized in the liver is utilized in membrane formation within the organ or is transferred into bile or blood plasma lipoproteins (reviewed by Coleman, 1973). Before considering the possible role of phospholipid *N*-methylation during these functions it is important to examine how the synthesis of phosphatidylcholine by the CDP-choline pathway is regulated in the liver.

As mentioned earlier in this Review the CDP-choline pathway is the major route for the synthesis of phosphatidylcholine. In isolated hepatocytes, treatment with CPT-cAMP or 8-bromo cyclic AMP decreases by about 40% the synthesis of phosphatidylcholine from [^{3}H]choline (Pelech *et al.*, 1981). This effect seems to be due to an inhibition of phosphocholine cytidyltransferase (Pelech *et al.*, 1981; Pelech & Vance, 1982), the enzyme that catalyses the synthesis of CDP-choline and the rate-limiting step in the synthesis of phosphatidylcholine by the CDP-choline pathway (Infante & Kinsella, 1978; Schneider & Vance, 1978; Vance & Choy, 1979; Pritchard & Vance, 1981). Aminophylline, a cyclic AMP phosphodiesterase inhibitor, also inhibits [^{3}H]choline incorporation into phosphatidylcholine in rat liver (Pelech *et al.*, 1981). Finally, recent results with rat liver cytosol indicate that a cyclic AMP-dependent protein kinase is involved in the regulation of phosphocholine cytidyltransferase (Pelech & Vance, 1982). In all these experiments the hepatocytes were exposed to the cyclic AMP derivatives for up to 5h. Prolonged treatment, up to 15h, with cyclic AMP derivatives increases the rate of phosphatidylcholine synthesis by rat hepatocytes

(Pelech *et al.*, 1982). The mechanisms by which a prolonged treatment of rat hepatocytes with cyclic AMP derivatives stimulates phosphatidylcholine synthesis, thereby reversing the initial effect of these compounds, are not known. As in the liver, in the lung the synthesis of phosphatidylcholine by the CDP-choline pathway is stimulated after 12h treatment with aminophylline, 8-bromo cyclic AMP and dibutyryl cyclic AMP (Gross & Rooney, 1977; Niles & Makarski, 1979). Results showing the initial effect of cyclic AMP on phosphatidylcholine synthesis by lung are not available.

The inhibitory effect in hepatocytes of an initial exposure to cyclic AMP on the biosynthesis of phosphatidylcholine by the CDP-choline pathway contrasts with the previously discussed stimulation of the transmethylation pathway by hormones that increase intracellular cyclic AMP levels. A similar behaviour seems to occur in response to hormones that increase intracellular Ca^{2+} levels. We have mentioned earlier that in rat hepatocytes the addition of vasopressin and angiotensin before homogenization stimulates phospholipid *N*-methyltransferase. These two hormones and the cationophore A23187 also induce a rapid inhibition of the rate of incorporation of [^{3}H]choline into phosphatidylcholine in rat hepatocytes (Alemany *et al.*, 1982*b*). Results with liver microsomes indicate that this effect is due, at least in part, to the inhibition of phosphocholine transferase by Ca^{2+} (Kennedy & Weiss, 1956; Alemany *et al.*, 1982*b*). Results from different laboratories indicate that cyclic AMP and Ca^{2+} also inhibit the formation of diacylglycerol (Soler-Argilaga *et al.*, 1977; Sugden *et al.*, 1980; Declerq *et al.*, 1982), one of the two substrates necessary for the synthesis of phosphatidylcholine by the CDP-choline pathway. These results indicate that cyclic AMP and Ca^{2+} inhibit the CDP-choline pathway by acting at various levels.

A plausible model showing the effects of Ca^{2+} and cyclic AMP on phosphatidylcholine synthesis by the CDP-choline and transmethylation pathways in the liver is shown in Fig. 1. This model indicates that both pathways are regulated in a co-ordinated way and that an inhibition of the CDP-choline pathway is accompanied by a stimulation of the transmethylation route. In support of this co-ordinated mechanism of regulation of phosphatidylcholine synthesis are the following observations: the intraperitoneal injection of choline increases phosphocholine transferase and decreases phospholipid *N*-methyltransferase in rat liver microsomes (Skurdal & Cornatzer, 1975); choline deficiency increases phospholipid *N*-methyltransferase and inhibits the CDP-choline pathway (Lombardi *et al.*, 1969; Thompson *et al.*, 1969; Schneider & Vance, 1978), and the inhibition of the transmethylation pathway by intraperitoneal injection of 3-deaza-adenosine in rats is accompanied by a stimulation of the CDP-choline pathway (Pritchard *et al.*, 1982). Since phospholipid *N*-methylation accounts for 20–40% of the total synthesis of phosphatidylcholine in rat liver (Sundler & Akesson, 1975) and since the turnover of this phospholipid in this organ is very rapid, with a half-life of 1–10h (Tolbert & Okey, 1952; Arvidson, 1968), we believe that the purpose of this co-ordinated mechanism is to maintain a steady state level of phosphatidylcholine synthesis under different metabolic conditions.

In other cells and tissues there is also evidence indicating a co-ordinated mechanism of regulation of phosphatidylcholine synthesis by the CDP-choline and transmethylation pathways. During viral transformation of hamster fibroblasts the transmethylation pathway increases (Maziere *et al.*, 1981) and the CDP-choline pathway decreases (Maziere *et al.*, 1982). Differentiation of myeloid leukaemia cells is accompanied by a decrease in methylation of phospholipids and an increase in the CDP-choline pathway (Honma *et al.*, 1981). Antitubulins decrease phospholipid *N*-methylation and stimulate the CDP-choline pathway in phagocytes (Pike *et al.*, 1980). The addition to human neutrophils of zymosan particles coated with complement decreases phospholipid *N*-methyltransferase and stimulates the CDP-choline pathway (Garcia Gil *et al.*, 1981, 1982). Although more examples may appear showing that these two pathways are regulated in a co-ordinated way, there are also exceptions to this control mechanism. Thus, the addition of thrombin to platelets inhibits both the transmethylation and the CDP-choline pathway (Shattil *et al.*, 1981); chemoattractants inhibit the incorporation of radioactivity from [^{3}H]methionine into phospholipids in rabbit neutrophils (Hirata *et al.*, 1979; Pike *et al.*, 1979), whereas the CDP-choline pathway remains constant (Hirata *et al.*, 1979*b*); and treatment of rabbit neutrophils with inhibitors of receptor-mediated endocytosis inhibits both the transmethylation and CDP-choline pathways (Mato *et al.*, 1983).

The purpose of a co-ordinated mechanism of regulation of phosphatidylcholine synthesis in cells and organs other than the liver is not to maintain a steady-state level of this lipid. This becomes clear when we consider that the activity of phospholipid *N*-methyltransferase in the liver is 10–1000-fold higher than in most, if not all, other cells and tissues and that in these systems the relative contribution of the transmethylation pathway to the total synthesis of phosphatidylcholine is negligible (see Vance & Kruijff, 1980). This raises the question of what is the function of phospholipid *N*-methylation in systems other than liver.

Based on the numerous examples showing changes in phospholipid *N*-methylation during cellu-

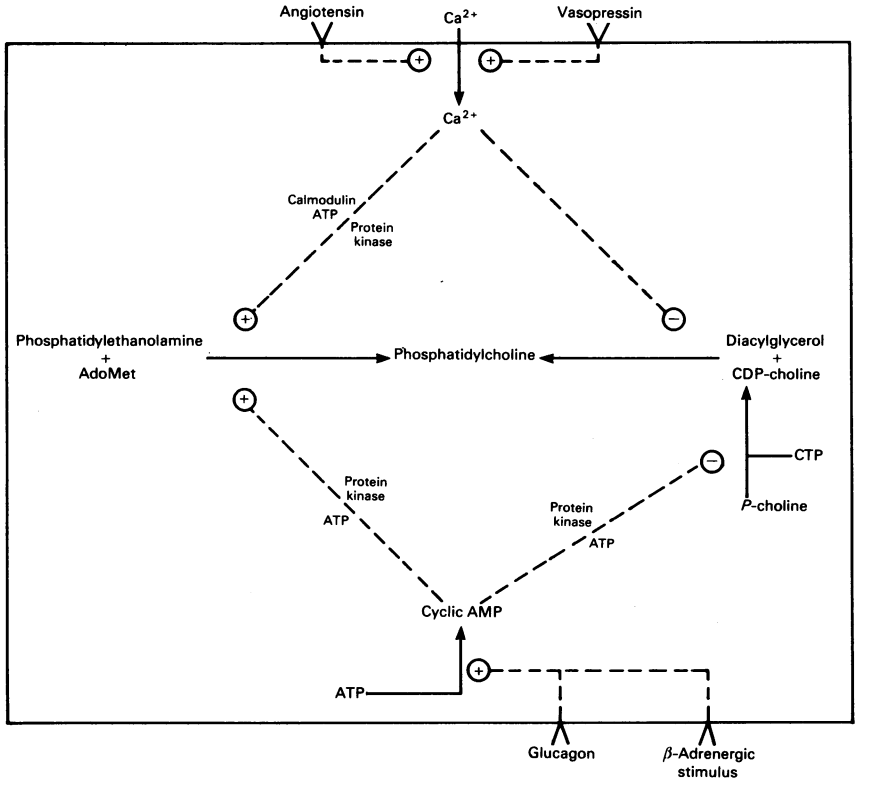

Fig. 1. *A plausible model showing the effects of Ca^{2+} and cyclic AMP on phosphatidylcholine synthesis by the CDP-choline and transmethylation pathways in rat hepatocytes*

The occupation of glucagon or β-adrenergic receptors elevates cyclic AMP, which in its turn inhibits the synthesis of CDP-choline and stimulates the *N*-methylation of phosphatidylethanolamine. In both cases ATP and a protein kinase seem to be required. The occupation of angiotensin or vasopressin receptors increases intracellular Ca^{2+}. This elevation in the levels of Ca^{2+} causes an inhibition of the synthesis of phosphatidylcholine by the CDP-choline pathway. Furthermore, Ca^{2+} stimulates the transmethylation of phosphatidylethanolamine in a process that requires calmodulin, ATP and probably a protein kinase.

lar activation (Table 2), it has been proposed that the transmethylation pathway is involved in signal transduction. Thus, in RBL and mast cells bridging of immunoglobulin E receptors induces a fast, transient activation of phospholipid *N*-methylation (Crews *et al.*, 1980*b*; Ishizaka *et al.*, 1980). RBL cell variants have been isolated which are deficient in phospholipid *N*-methylation and immunoglobulin E-mediated, but not ionophore-mediated, histamine release (McGivney *et al.*, 1981). Reconstitution by hybridization of these variants results in normal phospholipid *N*-methylation and immunoglobulin E-mediated histamine release. These results indicate that phospholipid *N*-methylation is required for the initial activation in these cells.

To explore the functions of phospholipid *N*-methylation one of the experimental approaches has been to study the effect of inhibitors of transmethylation on the behaviour of cells in response to stimulation. 3-Deaza-adenosine and 5′-deoxy-5′-isobutylthio-3-deaza-adenosine (deaza-SIBA) are the two compounds most frequently used. 3-Deaza-adenosine raises the cellular content of AdoHcy and 3-deaza-AdoHcy by inhibiting AdoHcy hydrolase; both these compounds are inhibitors of most if not all transmethylation reactions (Kerr, 1977; Chiang *et al.*, 1977; Zimmerman *et al.*, 1978; Chiang & Cantoni, 1979; Cantoni & Chiang, 1980). Deaza-SIBA, an analogue of AdoHcy (Chiang *et al.*, 1978) inhibits thymidine incorporation in response to concanavalin A in mouse lymphocytes (Hirata *et al.*, 1980) and the release of histamine in response to immunoglobulin E in RBL cells (Crews *et al.*, 1980*b*) and rat mast cells (Ishizaka *et al.*, 1980). However, in addition to inhibiting phospholipid *N*-methylation, deaza-SIBA is a potent inhibitor of the CDP-choline pathway for the synthesis of phosphatidylcholine in RBL cells (Morita *et al.*,

1982) and NG108-15 neuroblastoma cells (De Blas *et al.*, 1981), which invalidates the use of this compound to study the cellular functions regulated by phospholipid *N*-methylation. 3-Deazaadenosine inhibits phospholipid *N*-methylation and a number of cell functions, including immunoglobulin E-mediated histamine release (Crews *et al.*, 1980*b*, 1981) and rabbit neutrophil chemotaxis (Zimmerman *et al.*, 1979; Bareis *et al.*, 1982; Garcia Castro *et al.*, 1983). However, this compound increases cyclic AMP levels (Zimmerman *et al.*, 1979, 1980) which may account for some of its effects. This compound inhibits phospholipid *N*-methylation and chemotaxis in rabbit neutrophils (Bareis *et al.*, 1982; Garcia Castro *et al.*, 1983). However, adenosine, which also inhibits phospholipid *N*-methylation in rabbit neutrophils by drastically changing the ratio AdoMet/AdoHcy, has no effect on chemotaxis (Garcia Castro *et al.*, 1983). Furthermore, preincubation with adenosine protects rabbit neutrophils against the inhibitory effect of 3-deaza-adenosine on chemotaxis but not on phospholipid *N*-methylation. None of these treatments has any effect on the CDP-choline pathway. These results indicate that phospholipid *N*-methylation is not primarily involved in neutrophil chemotaxis. Similarly, while 3-deaza-adenosine inhibits phospholipid *N*-methylation and potentiates platelet aggregation, 3-deazaaristeromycin, which is equipotent in inhibiting phospholipid *N*-methylation, has no effect on aggregation (Shattil *et al.*, 1982). This and other studies (Random *et al.*, 1981; Hotchkiss *et al.*, 1981; Lecompte *et al.*, 1982) indicate that phospholipid *N*-methylation is not required for the initial activation of platelets. Finally, it should be remembered that 3-deaza-adenosine is also an inhibitor of methylation reactions other than those of phospholipids, which might account for some of its effects. To conclude, although 3-deaza-adenosine is a good tool to explore the functions of phospholipid *N*-methylation, caution should be exercised in interpreting the results obtained with this compound.

Phospholipid *N*-methylation has been reported to increase membrane fluidity in rat erythrocyte membranes (Hirata & Axelrod, 1978*b*) and rat reticulocyte ghosts (Hirata *et al.*, 1979*b*). These results have been criticized on the basis that methylation of very small amounts of phosphatidylethanolamine could not produce large changes in membrane fluidity (Vance & Kruijff, 1980). Furthermore, neither phospholipid *N*-methylation nor the addition of phosphatidyl *N*-methylethanolamine produce large changes in membrane fluidity (Chauhan *et al.*, 1982). With LM cells alteration by more than 50% of the polar head group of the phospholipids had no effect on membrane fluidity (Schroeder *et al.*, 1976). We therefore conclude that there is no clear evidence linking phospholipid *N*-methylation and membrane fluidity.

Phospholipid *N*-methylation has also been related to adenylate cyclase activation by isoprenaline and immunoglobulin E in reticulocyte ghosts (Hirata *et al.*, 1979*a*) and rat mast cells (Ishizaka *et al.*, 1981) respectively. However, phospholipid *N*-methylation has no effect on adenylate cyclase from rat liver plasma membranes (Colard & Breton, 1981) or cyclic AMP production by rat hepatocytes (Schanche *et al.*, 1982). These results indicate that at present it is difficult to make generalizations about the functions of phospholipid *N*-methylation. Another function attributed to phospholipid *N*-methylation is the control of the number of cell surface receptors. Incubation with AdoMet increases the number of β-adrenergic receptors in reticulocytes (Strittmatter *et al.*, 1979) and of lactogenic receptors in mammary gland membranes (Bhattacharya & Vonderhaar, 1979). The mechanism by which these changes may take place is not known.

Evidence indicating that phospholipid *N*-methylation provides phosphatidylcholine molecules rich in arachidonic acid has been obtained in platelets (Kannagi *et al.*, 1980) and RBL cells (Crews *et al.*, 1981). This led to the hypothesis that arachidonic acid is liberated during cellular activation from a pool of phosphatidylcholine that is synthesized by methylation of phosphatidylethanolamine (reviewed by Hirata & Axelrod, 1980). This hypothesis has been recently criticized (Irvine, 1982). Here we should only mention that liberation of arachidonic acid during cellular activation has been observed both during stimulation (Crews *et al.*, 1980*b*; Ishizaka *et al.*, 1980) and inhibition (Shattil *et al.*, 1981; Rittenhouse-Simmons, 1979; Garcia Gil *et al.*, 1981, 1982; Pike & Snyderman, 1981) of phospholipid *N*-methylation. These results make it difficult to visualize a general mechanism linking phospholipid *N*-methylation to arachidonate liberation.

To summarize, although the data shown in Table 2 strongly suggest that in a variety of cells phospholipid *N*-methylation is involved in signal transduction, the biochemical or biophysical signals which might be triggered by changes in the rate of phospholipid *N*-methylation remain to be determined. In the liver, however, the present evidence indicates that this pathway is important in meeting the cellular requirements for phosphatidylcholine.

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