Inhibition of phosphatidylcholine synthesis by vasopressin and angiotensin in rat hepatocytes

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The addition of $1\mu\text{M}$ -vasopressin or -angiotensin to isolated rat hepatocytes induced a fast transient inhibition of the rate of incorporation of [$\text{Me-}^3\text{H}$]choline into phosphatidylcholine. The cationophore A23187 induced a similar inhibition of phosphatidylcholine synthesis. The addition of micromolar Ca^{2+} to rat liver microsomes inhibited the activity of CDP-choline:1,2-diacylglycerol cholinephosphotransferase. This inhibition is due a decrease in the V_{max} of the enzyme without affecting the K_m for CDP-choline. It is concluded that Ca^{2+} regulates phosphatidylcholine synthesis in rat liver.

The turnover rate of most phospholipid classes in cell membranes is generally higher than that of proteins (Quinn, 1976). The significance of this 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 stimulation. Phospholipids synthesized in liver are utilized in membrane formation within the organ or are transported into bile or lipoproteins of blood plasma (Coleman, 1973). The most abundant phospholipid in liver is phosphatidylcholine, which accounts for from about 60% of the phospholipid in the endoplasmic reticulum to 33% in that of lysosomal membranes (McMurray & Magee, 1972). The synthesis of phosphatidylcholine in liver can occur by two different pathways: by the CDP-choline pathway (Kennedy & Weiss, 1956) and by the transmethylation pathway (Bremer & Greenberg, 1961). The enzymes required for the synthesis of phosphatidylcholine are, for both pathways, mainly located in the microsomal fraction of the liver (Gibson *et al.*, 1961; Yeselma & Moore, 1978).

Short-term hormonal regulation of liver phosphatidylcholine turnover seems to occur by both cyclic AMP-dependent and cyclic AMP-independent mechanisms. The cyclic AMP-dependent mechanism is characterized for glucagon. The addition of this hormone to rat hepatocytes enhances the incorporation of label from [^{14}C]ethanolamine into phosphatidylcholine, which proceeds via the transmethylation pathway (Geelen *et al.*, 1978) and activates phospholipid methyltransferase (Castano *et al.*, 1980). The addition of a cyclic AMP analogue to isolated rat hepatocytes also stimulates phospho-

lipid methyltransferase (Pritchard *et al.*, 1981). However, this compound reduced the incorporation of labelled precursors into phosphatidylcholine, perhaps by reducing the availability of phosphatidylethanolamine (Pritchard *et al.*, 1981), which makes the interpretation of these data difficult. On the other hand, glucagon and cyclic AMP analogues inhibit the incorporation of precursors via the transcholine pathway into phosphatidylcholine (Gelen *et al.*, 1978; Pelech *et al.*, 1981). The cyclic AMP-independent mechanism is involved in the effects of vasopressin and angiotensin on the liver (Kirtz & DeWulf, 1976; Keppens & DeWulf, 1976; Fain, 1978). The addition of these hormones to rat hepatocytes stimulates, in a Ca^{2+} -dependent manner, phospholipid methyltransferase (Alemany *et al.*, 1981). Furthermore, we have recently shown that Ca^{2+} activates phospholipid methyltransferase in isolated rat liver microsomes (Mato *et al.*, 1982; Alemany *et al.*, 1982). With respect to the transcholine pathway, there is evidence that indicates that Ca^{2+} inhibits the pathway in isolated microsomes from rat liver (Kennedy & Weiss, 1956; Soler Argilaga *et al.*, 1977). In the present study the effect of vasopressin and angiotensin on phosphatidylcholine synthesis by transcholine pathway was investigated in rat hepatocytes. The effect of Ca^{2+} on CDP-choline:1,2-diacylglycerol cholinephosphotransferase activity by isolated rat liver microsomes was also studied.

Materials and methods

Isolation and incubation of hepatocytes

Hepatocytes were isolated from normally fed

Wistar rats (250–300g) as previously described (Castano *et al.*, 1980). Isolated hepatocytes were incubated as follows. Cell suspension (1ml; 30–50mg wet wt./ml of suspension) was shaken (150 strokes/min) in stoppered 20ml vials at 37°C in the presence of 10mM-glucose. The gas phase was O₂/CO₂ (19:1).

*Measurement of [*Me*-³H]choline incorporation into phospholipids*

Hepatocytes were pre-incubated for 30min at 37°C and at the end of this period 0.25μCi of [*Me*-³H]choline was added. After 10min incubation, vasopressin, angiotensin, the cationophore A23187 or saline (0.9% NaCl solution) was added. At the time indicated the suspension of hepatocytes was poured into precooled centrifuge tubes and immediately centrifuged at 100g for 20s. The supernatant was discarded and the pellet was extracted with 2.9ml of methanol/chloroform/water (10:10:9), the aqueous phase treated three times with 2ml of methanol/chloroform (1:1, v/v) and the pooled organic phase dried under a stream of N₂ at 22°C. The measurement of the incorporation of radioactivity into phospholipids and their analysis by t.l.c. was carried out as described by Castano *et al.* (1980) and Garcia Gil *et al.* (1982).

Isolation of microsomes

Normally fed Wistar rats (250–300g) were killed by decapitation, the liver removed and homogenized with a Potter–Elvehjem apparatus in 4vol. of ice-cold 10mM-Tris/HCl (pH 7.4)/0.3M-sucrose. The homogenate was centrifuged at 12500g for 20min at 4°C and the supernatant was centrifuged again at 105000g for 60min at 4°C. The pellet of this last centrifugation, the microsomal fraction, was resuspended in an appropriate volume of 0.25M-sucrose (2.5–5.5mg of protein/ml) and used for the enzyme assay.

Assay of CDP-choline:1,2-diacylglycerol cholinephosphotransferase

CDP-choline:1,2-diacylglycerol cholinephosphotransferase was assayed as previously described by Garcia Gil *et al.* (1982). The basic medium (0.5ml final volume, 30°C) contained 50mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.3, 2mM-dithiothreitol, 10mM-MgCl₂, 0.2mM-EGTA, pH 7.3, 50μM-CDP-choline, 0.1μCi of CDP-[*Me*-¹⁴C]choline (Amersham International, sp. radioactivity 50Ci/mol) and 20μl of homogenate. The reaction was initiated by the addition of the homogenate and terminated by pipetting 100μl of assay mixture into 2ml of chloroform/methanol/2M-HCl (6:3:1, by vol.) for lipid extraction (Castano *et al.*, 1980; Garcia Gil *et al.*, 1982). Samples were taken 5, 10, 15 and 20min after the

addition of the homogenate. The measurement of the incorporation of radioactivity into phospholipids and their analysis by t.l.c. was carried out as described by Castano *et al.* (1980) and Garcia Gil *et al.* (1982). The enzyme activity was linear with time for at least 20min at all concentrations of substrate used. The concentration of protein was determined as described by Lowry *et al.* (1951).

Assay of phospholipase activity

The basic medium was the same as that for the assay of CDP-choline:1,2-diacylglycerol cholinephosphotransferase, except that CDP-choline and CDP-[*Me*-¹⁴C]choline were replaced by the addition of 0.25μCi of 2-[1-¹⁴C]arachidonyl-1-stearoylglycerophosphocholine (Amersham International, sp. radioactivity >50Ci/mol). Immediately after the addition of the microsomes, samples were sonicated for 1.5min at 4°C. After sonication the mixture was incubated at 30°C for 15min. The reaction (500μl) was stopped with 3.75ml of chloroform/methanol (1:2, v/v). After shaking, 1.25ml of chloroform and 1.25ml of 2M-KCl/5mM-EDTA were added to form two phases. The aqueous phase was then removed and the organic phase dried under a stream of N₂. The formation of [¹⁴C]diacylglycerol and [¹⁴C]-arachidonic acid was quantified after separation by t.l.c. in ligroin/diethyl ether/acetic acid (50:50:1, by vol.) as described by Garcia Gil *et al.* (1982).

Results and discussion

The addition of 1μM-vasopressin or -angiotensin, two hormones whose actions in liver are mediated through Ca²⁺ (Kirtz & DeWulf, 1976; Keppens & DeWulf, 1976; Fain, 1978), to a suspension of rat hepatocytes decreased about 2-fold the rate of incorporation of [*Me*-³H]choline into phospholipids (Fig. 1). This effect was transient and the rate of incorporation of radioactivity into phospholipids recovered control values about 4min after the addition of the hormone. The only phospholipid labelled under the present conditions was phosphatidylcholine (results not shown). When the hormones were added, the hepatocytes had been pre-incubated for 10min with [*Me*-³H]choline. Therefore, at the start of the experiment the pool sizes of phosphatidylcholine precursors and the total amount of radioactivity in both the control and the stimulated cells were identical. The reduced incorporation of [*Me*-³H]choline into phosphatidylcholine was therefore not due to a reduced uptake of choline. The addition of the cationophore A23187 also induced a fast, partially reversible, dose-dependent inhibition of the incorporation of [*Me*-³H]choline into phosphatidylcholine (Figs. 2 and 3). These results suggest that the inhibition of the transcholineation reaction is secondary to an in-

Fig. 1. Effect of vasopressin and angiotensin on the incorporation of $[\text{Me-}^3\text{H}]$ choline into phospholipids. At zero time $1\text{ }\mu\text{M}$ hormone (O) or saline (●) was added to a suspension of rat hepatocytes. Conditions were as described in the Materials and methods section. The radioactivity incorporated during the 10 min incubation before the addition of the hormone or saline was subtracted from both control and stimulated cells. Results are means $\pm$ S.E.M. of three independent experiments in triplicate.

Fig. 2. Effect of the addition of the cationophore A23187 on the incorporation of $[\text{Me-}^3\text{H}]$ choline into phospholipids.

At zero time $5\text{ }\mu\text{M}$ -A23187 in dimethyl sulphoxide (O) or dimethyl sulphoxide alone (●) was added to a suspension of rat hepatocytes. Conditions are as described in the Materials and methods section. Results are means $\pm$ S.E.M. of three independent experiments in triplicate.

Fig. 3. Effect of different cationophore A23187 concentrations on $[\text{Me-}^3\text{H}]$ choline incorporation into phospholipids.

The incorporation of radioactivity into phospholipids was measured 4 min after the addition of A23187. Results are means $\pm$ S.E.M. of three independent experiments in triplicate.

crease in the intracellular Ca^{2+} concentration. The studies with isolated microsomes support this view by showing that the addition of $300\text{ }\mu\text{M}$ - Ca^{2+} inhibited, almost completely, the synthesis of phosphatidylcholine by transcholineation. These results confirm a previous report showing that Ca^{2+} inhibits CDP-choline:diacylglycerol cholinephosphotransferase (Kennedy & Weiss, 1956), and extend them by showing that this effect is dose-dependent and occurs at physiological Ca^{2+} concentrations (Fig. 4). Treatment of microsomes with Ca^{2+} changed the kinetic properties of CDP-choline:1,2-diacylglycerol cholinephosphotransferase decreasing the

V_{max} value of the enzyme, without affecting the K_m value for CDP-choline (Fig. 5). The addition of $300\text{ }\mu\text{M}$ - Ca^{2+} to isolated microsomes had only a small stimulatory effect (about 1.3-fold) on phos-

Fig. 4. Effect of different Ca^{2+} concentrations on CDP-choline:1,2-diacylglycerol cholinephosphotransferase from rat liver microsomes

In the absence of added Ca^{2+} the activity was 1030 ± 71 pmol/min per mg of protein. Values are means $\pm$ S.E.M. of three independent experiments in triplicate.

Fig. 5. Double-reciprocal plot of CDP-choline:1,2-diacylglycerol cholinephosphotransferase activity

Symbols: $\circ$, control microsomes; $\bullet$, microsomes treated with $10 \mu\text{M-Ca}^{2+}$. Activities were assayed as described in the Materials and methods section.

pholipase activity. These results provide further support for the conclusion that the inhibition by Ca^{2+} of phosphatidylcholine formation cannot be explained as the result of an increased degradation of this phospholipid but rather by a decreased rate of its synthesis.

Calmodulin is a ubiquitous Ca^{2+} -binding protein

that mediates many of the effects of Ca^{2+} (Weiss & Levine, 1978). To discover if calmodulin mediates the inhibition by Ca^{2+} of CDP-choline:1,2-diacylglycerol cholinephosphotransferase, microsomes were isolated in the presence of 5 mM-EGTA to remove endogenous calmodulin. Under these conditions Ca^{2+} still inhibited phosphatidylcholine synthesis (results not shown). Microsomes were also incubated with an antiserum against calmodulin (Wallace & Cheung, 1979), which has been proven to be effective in inhibiting calmodulin-mediated effects (Garcia Gil *et al.*, 1980; Alemany *et al.*, 1982). The addition of this antiserum had no effect on Ca^{2+} inhibition of phosphatidylcholine formation (results not shown). These results suggest that calmodulin is not mediating the inhibition by Ca^{2+} of phosphatidylcholine synthesis by transcholineation. However, if calmodulin is tightly bound to the enzyme, it is possible that the treatment with EGTA or the antiserum does not remove it. Chlorpromazine, an inhibitor of Ca^{2+} -calmodulin actions, inhibited phosphatidylcholine synthesis by transcholineation in the absence of Ca^{2+} , which makes the interpretation of the results with this drug difficult.

In conclusion, the present studies show that vasopressin and angiotensin inhibit phosphatidylcholine synthesis in rat hepatocytes and that this effect can be explained by an inhibition of CDP-choline:1,2-diacylglycerol cholinephosphotransferase. The addition of Ca^{2+} , in the presence of ATP, to rat liver microsomes also inhibits glycerol 3-phosphate 1-acyltransferase (Soler Argilaga *et al.*, 1977), a rate-limiting step in the synthesis of diacylglycerol, which is a precursor of phosphatidylcholine synthesis by transcholineation. These results indicate that Ca^{2+} inhibits the transcholineation pathway by acting at various levels. The inhibitory effect of Ca^{2+} on phosphatidylcholine synthesis by transcholineation contrasts with its stimulatory effect on the transmethylation pathway (Alemany *et al.*, 1981; Mato *et al.*, 1982). A similar situation occurs with cyclic AMP in rat hepatocytes (Geelen *et al.*, 1978; Castano *et al.*, 1980; Pritchard *et al.*, 1981; Pelech *et al.*, 1981), during the phagocytic response of human neutrophils (Garcia Gil *et al.*, 1981, 1982) and in transformed hamster fibroblasts (Maziere *et al.*, 1981, 1982). The purpose of this differential regulation of phosphatidylcholine synthesis by transmethylation and transcholineation is not yet clear. Phosphatidylcholine synthesized in liver is utilized in membrane formation within the organ or is transferred into bile or blood plasma lipoproteins (Coleman, 1973). In rat liver about 70% of the total phosphatidylcholine is synthesized by the transcholineation pathway and the remaining 30% by the transmethylation pathway (Sundler & Akesson, 1975). In situations where the transcholineation pathway is inhibited (i.e. during inhibition of

lipoprotein synthesis) an increased synthesis of phosphatidylcholine by transmethylation would account for the cellular needs of this phospholipid during a period of shortage.

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