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Release of choline by phospholipase D and a related phosphoric diester hydrolase in human erythrocytes

^{1}H spin-echo n.m.r. studies

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A previously detected phosphatidylcholine-specific phospholipase D from lysates of human red blood cells has been further characterized by ^{1}H spin-echo n.m.r. spectroscopy. A second choline-releasing enzymic activity was observed after addition of glycerophosphocholine. Both of these phosphoric diester hydrolase activities were activated to different extents by different concentrations of calcium ions. Differences between the two activities were also observed on inhibition by barium and phosphate ions. These distinct, choline-yielding, reactions which occur in the cytoplasm of red blood cells may be involved in the regulation of the levels of membrane phosphatidylcholine.

INTRODUCTION

One means of transfer of extracellular signals across the plasma membrane of mammalian cells is via a receptor-mediated process which causes the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C and the release of the intracellular messengers inositol 1,4,5-trisphosphate and diacylglycerol. This process is induced by Ca^{2+}-mobilizing agonists, including growth factors, hormones and neurotransmitters, which bind to specific receptors on the surface of target cells [1–3]. Phospholipase C enzymes which act preferentially, but not exclusively, on phosphatidylinositol and its higher phosphorylated derivatives are common [4–6]. A phospholipase C which cleaves phosphatidylcholine (PC) with high activity has been partially purified from dog myocardium [7]. Recently it was confirmed that the hydrolysis of PC, the main lipid component of plasma membranes, is also triggered by a receptor-linked mechanism. This process is initiated by Ca^{2+}-mobilizing agents and catalysed by phospholipases of the C- and D-type [8,9]. Phospholipase D, common in plant cells, has recently been described in a number of mammalian cells [9], but it seldom has been isolated from mammalian systems so far. Hübscher first suggested the existence of this enzyme in rat liver [10,11], and it has since been partially purified from rat brain [12,13] and human eosinophils [14].

The product of phospholipase D-catalysed PC hydrolysis is choline, which plays a central role in acetylcholine metabolism. It is a precursor and cleavage product of acetylcholine, a neurotransmitter in synapses in the central and peripheral nervous systems [15]. Its concentration in the red blood cell (RBC) has been used as an indirect parameter of cholinergic activity in the central nervous system as well as a test for irregularities in acetylcholine metabolism [16]. RBC choline levels are increased in various neuropsychiatric disorders [16–18]. A dramatic elevation in the choline concentration was observed in manic-depressive patients on lithium therapy in comparison with lithium-free patients and healthy volunteers [17–20]. Lithium ions inhibit the choline transport protein in RBC membranes, causing accumulation of choline [21]. The release of choline by a Ca^{2+}-activated phospholipase from PC in haemolysates was first monitored using ^{1}H spin-echo n.m.r. spectroscopy [22].

Spin-echo n.m.r. spectroscopy allows the direct determination of metabolite concentrations in whole cells and haemolysates [23,24]. Using this technique we measured the Ca^{2+}-induced release of choline in human haemolysates from PC and L-α-glycerophosphocholine (GPC). Based on the different responses of these two reactions to activatory Ca^{2+} and inhibitory Ba^{2+} and monophosphate ions, we propose the existence of both a phospholipase D (EC 3.1.4.4) and a GPC phosphodiesterase (PDE) (EC 3.1.4.2) in the cytoplasm of human RBC.

MATERIALS AND METHODS

Preparation of RBC lysates

Freshly drawn venous blood was washed twice (2000 *g*, 10 min, 5 °C) in 2 vol. of iso-osmotic saline (0.154 M-NaCl); the RBC were resuspended in saline and gassed with CO for 3 min. The cells were washed once more in saline containing ^2H_2O (99.75 atom% ^2H; Australian Institute of Nuclear Science and Engineering, Lucas Heights, N.S.W., Australia) and the haematocrit (Hc) was adjusted to 0.85. The cells were lysed by three freeze–thaw cycles.

For control experiments, leucocytes were completely removed as follows. Freshly drawn venous blood was heparinized (10 units/ml of whole blood), diluted 1:3 with iso-osmotic saline and filtered through an α-cellulose/microcrystalline cellulose (1:1, w/w) column, pre-equilibrated with saline [25]. After elution with saline, the cells were collected by centrifugation (2000 *g*, 10 min, 5 °C), gassed with CO and washed once with CO-bubbled saline/^2H_2O. The packed cells (2000 *g*, 10 min, 5 °C, Hc = 0.85) were lysed by freeze–thawing.

N.m.r. measurements

All acquisitions were carried out at 37 °C with a Bruker AMX 400 wb spectrometer in the pulse-Fourier mode. For ^{1}H spin-echo experiments, 450 μl of lysate was placed with 20 mM-nicotinamide, 2 mM-D-[2,3,4,6,6-^{2}H]glucose (Merck, Sharp and Dohme, Point Claire-Dorval, Quebec, Canada) and various

Abbreviations used: GPC, L-α-glycerophosphocholine; Hc, haematocrit; HDL, high-density lipoprotein; HPCHA, hereditary high red cell membrane phosphatidylcholine haemolytic anaemia; PC, phosphatidylcholine; PDE, phosphodiesterase; PDH, phosphoric diester hydrolase; PE, phosphatidylethanolamine; RBC, red blood cell(s); TSP, sodium 3-(trimethylsilyl)[2,2,3,3-^{2}H]propionate.

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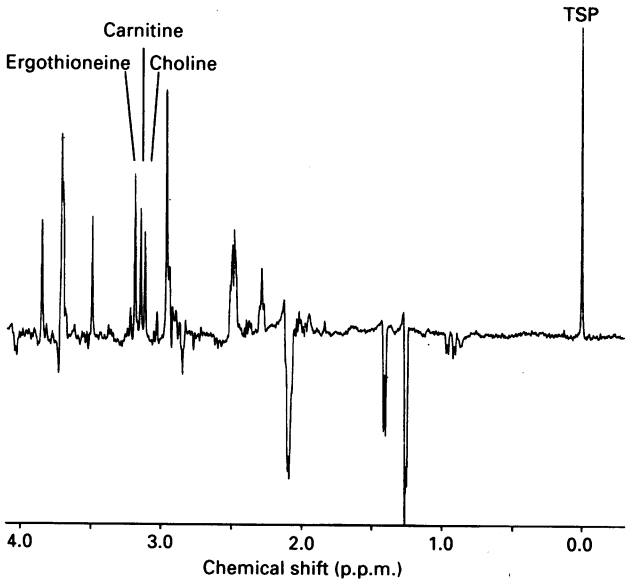

Fig. 1. Aliphatic region (0–4 p.p.m.) of a ^1H spin-echo n.m.r. spectrum of a haemolysate

The spectrum was acquired at 37 °C with a standard spin-echo sequence with $\tau = 60$ ms. N.m.r. parameters: spectral width, 5000 Hz; 16000 data points; 128 transients. Chemical shifts were measured relative to TSP (0.000 p.p.m.) as external standard.

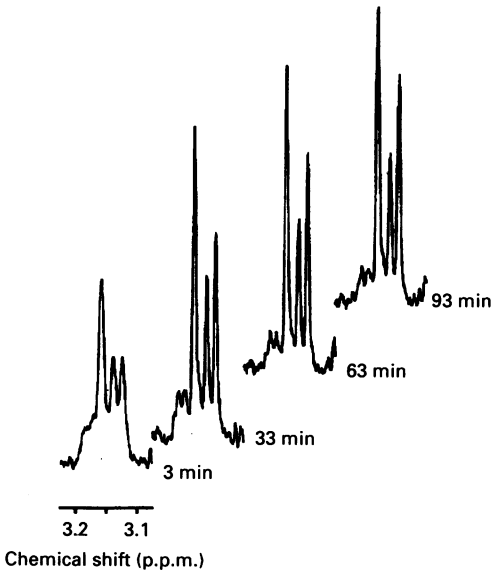

Fig. 2. Time course of ^1H spin-echo spectra of the quaternary amino methyl region of a haemolysate

The reaction was started by addition of 1 mM-$CaCl_2$ to the haemolysate (Hc = 0.85). The amplitude of the choline signal increased with time; the time indicated is that of the mid-point of the spectral accumulation. For signal assignment, see Fig. 1. The spectra were acquired as described in the Materials and methods section.

additives (as indicated in the Results section) in a 5 mm (outer diam.) n.m.r. tube. The enzymic reaction was started by adding different amounts of $CaCl_2$ (see the Results section); the final sample volume was 0.5 ml.

A standard spin-echo pulse sequence was used with $\tau = 60$ ms [23]. A total of 16 spectra were acquired over 97 min; in each, 128 transients were averaged into 16 K memory locations over a spectral width of 5000 Hz. Chemical shifts were measured relative

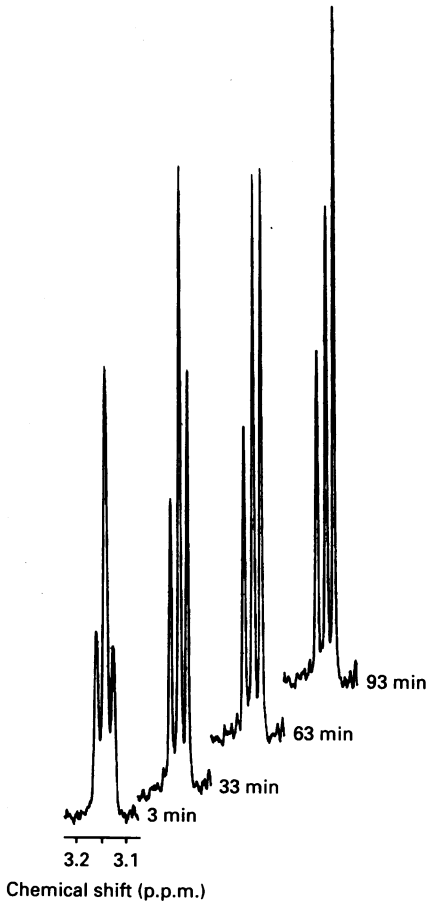

Fig. 3. Time course of ^1H spin-echo spectra of the quaternary amino methyl region of a haemolysate with added GPC

After addition of 0.185 mM-GPC, the reaction was started by addition of 0.1 mM-$CaCl_2$ to the haemolysate (Hc = 0.85). The amplitude of the choline signal increases relative to the ergothioneine signal with time, while the amplitude of the GPC signal decreases. For assignment of the signals, see Fig. 1. The spectra were acquired as described in the Materials and methods section.

to 3-(trimethylsilyl)[2,2,3,3-^2H]propionate sodium salt (TSP; 0.000 p.p.m.; Australian Atomic Energy Commission, Sutherland, N.S.W., Australia) in ^2H_2O in a capillary inserted in the n.m.r. tube.

The assignment of resonances was based on previous work [22,26].

RESULTS

The 'aliphatic' (0–4 p.p.m.) section of a ^1H spin-echo n.m.r. spectrum of human haemolysate is shown in Fig. 1. The resonances due to the $-N^+(CH_3)_3$ groups of choline, carnitine and ergothioneine are labelled.

Release of choline

Fig. 2 shows a time course of ^1H spin-echo n.m.r. spectra, expanded in the chemical shift scale, around the $-N^+(CH_3)_3$ region, acquired after the addition of 1 mM-$CaCl_2$ to the haemolysate. The intensity (peak area) of the choline signal increases relative to the ergothioneine signal. The latter signal remained of constant intensity in the present experiments, in contrast to one report of variations which were only observed in strongly oxidative environments [27]. (Note that the first spectrum of this time course and that in Fig. 3 show broader resonances as a

Table 1. Ca^{2+}-dependent release of choline from GPC and PC

The increase in choline levels in haemolysates relative to the zero-time value is expressed in $\mu M \cdot h^{-1}$. One spectrum was acquired 5 min after starting the reaction with the indicated amount of $CaCl_2$. The acquisition of a second spectrum followed 10 min later. The choline concentrations were calculated, having calibrated the external intensity standard TSP, and the rates were calculated from the time-dependent differences of the estimated concentration values. The data for the release from GPC and from PC were obtained from three samples of freshly drawn blood from the same donor, and the values indicated are means ± S.E.M. n.d., not detected.

	Choline release ($\mu M \cdot h^{-1}$)	
$CaCl_2$ (mM)	Haemolysate	Haemolysate plus 185 μM-GPC
0	n.d.	37 ± 5
0.1	10 ± 4	88 ± 2
0.5	43 ± 10	49 ± 3
1.0	192 ± 6	172 ± 37
5.0	98 ± 13	89 ± 9
10.0	49 ± 6	37 ± 14

Table 2. Inhibition of Ca^{2+}-dependent choline release from GPC and PC

Choline release, in $\mu M \cdot h^{-1}$, was calculated as described in Table 1. The data show the choline release after inhibition as a percentage of that in control experiments without added inhibitors. The results are the means from three freshly drawn blood samples from one donor.

	Choline release (% of control)	
Inhibitor	Haemolysate plus 1.0 mM-$CaCl_2$	Haemolysate plus 185 μM-GPC and 0.1 mM-$CaCl_2$
1 mM-$BaCl_2$	39	83
2 mM-$BaCl_2$	2	69
10 mM-K_2HPO_4	34	44

result of incomplete temperature equilibration; however, the peak areas rather than the peak heights bear a constant relationship to concentration.) An increase in the choline resonance in a haemolysate (3.13 p.p.m. at pH 7.4) was also observed when $CaCl_2$ (0.1 mM) and GPC (0.185 mM) were added (Fig. 3). The methyl resonances of choline phosphate (3.16 p.p.m.), GPC (3.16 p.p.m.) and lyso-PC (3.17) were found, by adding the authentic compounds, to be co-resonant with the carnitine resonance (3.16 p.p.m.).

The rate of choline release from PC and GPC was dependent on the concentration of added Ca (Table 1). GPC breakdown, already observed at physiological concentrations (< 1 μM free cytoplasmic Ca, up to 0.05 mM total cellular Ca [28]), was accelerated by 0.1 mM-$CaCl_2$ and inhibited by 0.5 mM-$CaCl_2$. This was verified in a less extensive set of experiments with Ca^{2+} concentrations of 0.05 mM and 0.3 mM, and the activity at 0.1 mM-Ca^{2+} was found to be the highest. In contrast, no PC-specific phospholipase activity was observed at physiological $CaCl_2$ concentrations, but occurred with Ca^{2+} levels in excess of 0.1 mM. No activity differences were detected as a result of the different techniques used for RBC preparation (see the Materials and methods section).

A large variation in the rate of choline release was observed for blood from different donors, as well as for different donations from one person. Over a period of 6 months haemolysates from one donor showed rates for the release of choline from PC, with 1 mM-Ca^{2+}, of 97 ± 26 $\mu M \cdot h^{-1}$ (mean ± S.E.M., $n = 9$) and from GPC, with 0.1 mM-Ca^{2+}, of 88 ± 14 $\mu M \cdot h^{-1}$ ($n = 8$). The mean rates for haemolysates from different donors were 40 ± 5 $\mu M \cdot h^{-1}$ ($n = 7$) with PC and 85 ± 17 $\mu M \cdot h^{-1}$ ($n = 7$) with GPC as substrate.

Inhibition of choline release

The addition of 1 mM-EGTA to haemolysates strongly decreased the choline release from GPC (results not shown), whereas choline release from PC was not detected without added $CaCl_2$ (Table 1). The results showing inhibition by $BaCl_2$ and inorganic monophosphate [29] are shown in Table 2. In contrast, in the presence of 1 mM-$CaCl_2$ no inhibition was detected after the addition of 3.33 mM-GDP.

Determination of type of phospholipase

After the addition of choline phosphate (0.125 mM and 0.5 mM) and $CaCl_2$ (0.5 mM), no hydrolysis of the phosphorylated choline was observed.

DISCUSSION

The first description of Ca^{2+}-dependent choline release in human haemolysates led to the postulation of an intracellular phospholipase D, cleaving cellular PC [22]. The present study confirmed these findings and indicated a second PDH activity, releasing choline from GPC.

There was no evidence in the ^1H spin-echo n.m.r. spectra of the release of choline phosphate from PC, thus suggesting that human RBC do not contain phospholipase C (EC 3.1.4.3) activity. Furthermore, no choline phosphate phosphatase activity was detected. Therefore the conclusion is drawn that a phospholipase of the D-type catalyses the release of choline from PC

The Ca^{2+}-dependency of the PDH reactions was demonstrated by inhibition with EGTA. The activity of each enzyme was only detectable in distinct but separate ranges of $CaCl_2$ concentration (Table 1). After adding GPC and 0.1 mM-$CaCl_2$, choline was released from GPC rather than from PC, as the data in Table 1 indicate. Choline release from GPC with Ca^{2+} concentrations of 1.0 mM and above is likely to be due to hydrolysis of PC. An additional experiment showed a decrease in the choline release with increased addition of GPC (results not shown). Therefore this indicates that GPC inhibits PC cleavage. Inhibition experiments with $BaCl_2$ revealed quantitative differences between the breakdown rates of the phospholipid and of the hydrophilic GPC. This suggests the possibility that two similar but distinct PDH activities are present in human RBC.

The hydrophilic GPC is soluble in the cytoplasm, which is confirmed by its detection in haemolysates by ^1H spin-echo n.m.r. spectroscopy. This suggests that the corresponding PDH is a cytoplasmic enzyme. The phospholipase D which is described here appears not to be controlled by a G protein, since high concentrations of GDP (3.33 mM), which prevent the activation of G proteins [30] in haemolysates, did not inhibit choline release from PC. G proteins are involved in the regulation of phospholipases probably located in the plasma membrane [8]. Moreover, Ca^{2+}-regulated phospholipases are likely to be located at other subcellular sites [8], i.e. the cytoplasm, in RBC. Thus the phospholipase D appears to be a soluble, cytoplasmic enzyme. This point, as well as the simplicity of the cytological structure of RBC, indicates that the involvement of phospholipase D in a PC-cleaving signal transducing mechanism, as described by Exton [8] for membrane-bound phospholipases, is unlikely; more likely, it takes part in the regulation of the cellular PC content.

RBC do not carry out *de novo* synthesis of phospholipids [31,32]. Although cellular phospholipid content and composition change with cell age, the levels are influenced by lipid uptake from the blood plasma and by ester exchange reactions. The uptake of PC from the plasma via high-density lipoprotein (HDL) is well documented and characterized [21]. Its incorporation in the outer leaflet of the RBC membrane occurs with a $t_{\frac{1}{2}}$ of about 54 h [33], followed by a flip into the inner leaflet with an estimated $t_{\frac{1}{2}}$ of 2 h [34,35]. The Ca^{2+}-dependent control of the described phospholipase D prevents a significant breakdown at low physiological Ca^{2+} concentrations which cannot be compensated for by the slow phospholipid uptake from blood plasma. Thus the enzyme is switched on under conditions of elevated intracellular free Ca^{2+}.

An accumulation of PC in the plasma membrane can be pathogenic: hereditary high red cell membrane phosphatidylcholine haemolytic anaemia (HPCHA), first described by Jaffé & Gottfried [36], is characterized by high levels of PC and cholesterol in RBC membranes, in spite of normal lipid composition in the blood plasma. It has been suggested that the blockage of fatty acid transfer from PC to phosphatidylethanolamine (PE), which is responsible for one-third of the turnover of PC fatty acids [37–40], results in an increase of PC and a decrease of PE in RBC membranes, consequently causing the phenomena of HPCHA [40,41]. Otsuka *et al.* [42] described, apart from a normal uptake and a diminished efflux of PC in RBC, an increased lyso-PC influx from the plasma followed by its increased intracellular conversion to PC. The mechanism of HPCHA has not been elucidated [42], and it would be of interest to measure the phospholipase D activity in erythrocytes from HPCHA patients.

Lyso-PC arises in blood plasma from PC by the esterification of cholesterol in the lecithin-cholesterol acyltransferase reaction, as well as by phospholipase A activity [28]. It enters the RBC via HDL and only exists in the cell in small amounts (2% of the cellular phospholipid fraction [43]). Since intracellular accumulation of lyso-PC causes lysis of erythrocytes [44], biochemical conversion of this haemolysin is necessary for cell survival; so far, the mechanism of conversion is unknown, but reacylation to PC has been observed [31,45] and can result in an enrichment of PC in the membrane [46]. Likewise, deacylation by lysophospholipase (EC 3.1.1.5) has been suggested [28], and GPC as one breakdown product might be recycled, by the GPC PDE activity described here, into glycolysis. The constitutive and highly active PDH would allow a rapid removal of the haemolytic lyso-PC; the reaction would be switched off by an increase in the Ca^{2+} concentration in favour of reacylation. Activated phospholipase D then might control the PC levels to obviate pathogenic overenrichment of the membrane with PC.

We suggest that a decrease in the phospholipase D and GPC PDE activities would explain elevated membrane PC levels in RBC in a situation in which phospholipid levels in the plasma show no significant changes. The increase of intracellular choline which occurs during lithium therapy in RBC could be related to a second intracellular PDH activity apart from phospholipase D. If deacylation of PC and lyso-PC by A-type phospholipases takes place in RBC, the GPC PDE would release choline at physiological Ca^{2+} concentrations. Since the Ca^{2+} content of RBC increases with cell age [28], the phospholipase D activity would increase during this process as well. Therefore the described PDH activities may play a role in the regulation of PC levels in RBC during cell senescence as well as in the manifestation of diseases related to PC turnover in the plasma membrane.

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