Phosphoinositide reorganization in human erythrocyte membrane upon cholesterol depletion

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The effect of cholesterol depletion on the activity of phosphatidylinositol/phosphatidylinositol 4-phosphate and diacylglycerol kinases and polyphosphoinositide phosphodiesterase has been studied in isolated membranes of human normal and cholesterol-depleted erythrocytes. Polyphosphoinositide synthesis (phosphatidylinositol/phosphatidylinositol 4-phosphate kinase activities) were found to depend on the permeability and sidedness characteristics of the membrane vesicles, which could limit the accessibility of ATP for the enzymes. When measured under proper conditions, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate synthesis were decreased in cholesterol-depleted membranes as compared with control membranes. The same level of synthesis could be obtained in both membranes by the addition of phosphatidylinositol (and Triton X-100) or of phosphatidylinositol 4-phosphate. Phosphatidic acid synthesis (diacylglycerol kinase activity) was also decreased in cholesterol-depleted membranes as compared with control membranes when measured in the presence of Ca^{2+} . Addition of diolein (and Triton X-100) caused a large increase in phosphatidic acid synthesis which reached approximately the same level in both membranes. This showed that the apparent inhibition of polyphosphoinositide and phosphatidic acid synthesis was not due to a loss or to an inactivation of the kinases. Ca^{2+} -activated polyphosphoinositide phosphodiesterase promoted the hydrolysis of 65–70% of the polyphosphoinositides in control and of only 45–55% in cholesterol-depleted membranes without changing the Ca^{2+} concentration for half-maximum hydrolysis (1 μM). Upon addition of sodium oleate, the extent of polyphosphoinositide hydrolysis became identical in both membranes, indicating again that there was no loss nor inactivation of the polyphosphoinositide phosphodiesterase in the cholesterol-depleted membranes. Since the concentration of the polyphosphoinositides was not changed by cholesterol depletion [Giraud, M'Zali, Chailley & Mazet (1984) *Biochim. Biophys. Acta* 778, 191–200], the reduction in both their synthesis and degradation observed here could be attributed to a reorganization of the phosphoinositides in membrane domains where they were not accessible to the kinases and phosphodiesterase. The reduction in phosphatidic acid synthesis was likely caused by a reduction in the total amount of the substrate diacylglycerol in cholesterol-depleted membranes as already shown [Giraud, M'Zali, Chailley & Mazet (1984) *Biochim. Biophys. Acta* 778, 191–200].

INTRODUCTION

The PPI, PIP_2 and PIP, and PA are metabolically active but quantitatively minor components of the erythrocyte membrane. They undergo a rapid turnover of their monoesterified phosphate group by phosphorylation and dephosphorylation reactions catalysed by specific kinases and phosphatases. Except for PIP_2 phosphatase, all these enzymes are membrane-bound (for a review see Irvine, 1982). There is also a membrane-bound Ca^{2+} -activated PPI phosphodiesterase (phospholipase C) but this latter is thought to be inactive in normal erythrocytes because of their low cytosolic Ca^{2+} concentration (Downes & Michell, 1982).

Cholesterol, a major component in the plasma membrane of animal cells, modulates bilayer fluidity (Oldfield & Chapman, 1972) and is probably unevenly distributed within the membrane because of its preferential interaction with specific phospholipids (Demel *et al.*, 1977; Van Dijk, 1979). We have recently shown that partial cholesterol depletion in intact erythrocytes

induced a decrease in the turnover rate of PPI and PA without changing their concentration (Giraud *et al.*, 1984). Although the effect of cholesterol on a number of enzymic activities is well documented (for a review see Sabine, 1983), its possible role on the enzymes involved in the turnover of PPI and PA has only been reported briefly (Vickers & Rathbone, 1979). In the present study, inhibition of the activity of PI, PIP and diacylglycerol kinases was observed in cholesterol-depleted erythrocyte membranes. The activity of the Ca^{2+} -activated PPI phosphodiesterase was also inhibited in these membranes. Experiments were designed to show that these effects were not due to a loss or to an inactivation of the enzymes and that they resulted from partial inaccessibility of the PPI to these enzymes.

MATERIALS AND METHODS

Reagents

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was obtained from Amersham International and $^{32}\text{P}_i$ (sodium salt) from CEA (France). Egg

Abbreviations used: PPI, polyphosphoinositides; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2 , phosphatidylinositol 4,5-bisphosphate; PA, phosphatidic acid; C/P, cholesterol/phospholipid molar ratio.

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phosphatidylcholine, 1,2-diolein, Triton X-100 and phosphoinositides were purchased from Sigma. Silica gel t.l.c. plates were obtained from Schleicher and Schull (Dassel, Germany). X-ray films for autoradiography (X-Omat AR) were from Kodak. All the other chemicals were of reagent grade.

Lipid vesicle preparation and cholesterol depletion of erythrocytes

These were as previously reported (Giraud *et al.*, 1984). After incubation (15 h, 37 °C) with or without vesicles, human erythrocytes were washed three times in the appropriate buffer [140 mM-NaCl, 10 mM-KCl, 1 mM-MgCl₂, 2.5 mM-Na₂HPO₄/NaH₂PO₄ (pH 7.4), 10 mM-glucose and 35 mM-sucrose for control and cholesterol-maintained erythrocytes and 20 mM-NaCl, 130 mM-KCl and the same concentrations of the other constituents for cholesterol-depleted erythrocytes].

Membrane preparation

Membranes were prepared from control, cholesterol-maintained and cholesterol-depleted erythrocytes by haemolysis at 4 °C either in 20 mM-Tris/HCl/0.1 mM-EDTA (pH 7.4) or 2.5 mM-Hepes/NaOH/1 mM-EGTA (pH 7) (1 vol. of packed cells in 40 vol. of lysing buffer). Membranes were sedimented at 29000 *g* for 10 min. The red button was discarded and the remaining creamy membranes were washed with the lysing buffer until they were white (usually four or five washes) and used immediately for the enzymic assays. With Hepes/EGTA buffer the membranes remained pink, indicating haemoglobin retention. For this reason, enzymic activities were expressed relative to phospholipid content and not to protein content. All enzymic assays were carried out in duplicate which did not vary by more than 10%.

PI and PIP kinase assay

Membranes were incubated with [γ -³²P]ATP and the synthesis of PIP and PIP₂ was calculated from their radioactivity and the specific radioactivity of ATP (Garrett & Redman, 1975). Membranes prepared as above (150 μ l, 100–300 nmol of phospholipid) were preincubated in their respective 'incubation buffer' [either 20 mM-Tris/HCl/0.1 mM-EDTA (pH 7.4) or 30 mM-Hepes/NaOH/1 mM-EGTA (pH 7) for Tris and Hepes membranes respectively] for 10 min at 37 °C. When required MgCl₂ (2 mM), PI and Triton X-100 (0.6%) or PIP were added to the preincubation medium. The reactions were started by the addition of [γ -³²P]ATP (100 μ M, 2–3 μ Ci) and MgCl₂ (when not present) (final volume 1 ml). They were stopped at the indicated times by adding 3.75 ml of chloroform/methanol/conc. HCl (20:40:1, by vol.) and 1.25 ml each of chloroform and water. Lipids were extracted and phospholipids separated by t.l.c. on silica gel plates treated with EDTA as previously described (Giraud *et al.*, 1984). The spots corresponding to PIP and PIP₂ were located by autoradiography, scraped and mixed with water for Cerenkov counting.

Diacylglycerol kinase assay

Membranes were incubated with [γ -³²P]ATP and PA synthesis was calculated from its radioactivity and the specific radioactivity of ATP. The protocol used was the same as that described for the PI/PIP kinase assay. When

required diolein (1 mM) and diolein + Triton X-100 (1 mM and 0.0015% respectively) were preincubated with the membranes for 10 min at 37 °C before ATP addition. Diolein alone or the mixture diolein + Triton X-100 in Hepes/EGTA buffer was sonicated in a probe sonicator (5 min, 4 °C) on the day of the experiment (Sagawa *et al.*, 1982). The effect of Ca²⁺ (50 or 450 μ M free concentrations) added together with ATP on PA synthesis was also tested both in the absence and in the presence of diolein and Triton X-100.

PPI phosphodiesterase assay

Membranes prepared from PPI-prelabelled erythrocytes were incubated with CaCl₂ and the percentage of PIP₂ and PIP hydrolysed was calculated from the Ca²⁺-induced decrease in their radioactivity (Allan & Michell, 1978). Washed erythrocytes were incubated with ³²P_i (50–100 μ Ci/ml of packed cells) in their respective buffer (90 min, 37 °C, haematocrit 35%). Membranes were prepared as described above. The 'membrane incubation buffers' containing Ca²⁺ (up to 150 μ M), chlorpromazine (100 μ M), sodium deoxycholate (1.2 mM) or sodium oleate (400 μ M) were preincubated for 10 min at 37 °C. The reactions were started by addition of the membranes (100–300 nmol of phospholipids in a final volume of 1 ml). At the indicated times the reactions were stopped by addition of solvent as reported above for the kinase assays.

Other assays

Membrane protein, phospholipids and cholesterol were assayed as previously described (Giraud *et al.*, 1984). Contamination of membranes by phospholipid vesicles used for cholesterol depletion was estimated from the ³H radioactivity found in membranes prepared from erythrocytes incubated with phosphatidylcholine vesicles containing [³H]glycerol trioleate (a non-exchangeable marker of the vesicles). Free Ca²⁺ concentrations in EGTA buffers were calculated by using the stability constant $K = 4 \times 10^6 \text{ M}^{-1}$ for the Ca-EGTA complex (Bartfai, 1979).

RESULTS

Cholesterol depletion

Native erythrocyte membranes have a cholesterol to phospholipid molar ratio (C/P) of 0.89 ± 0.02 . Incubation of erythrocytes without (control) or with lipid vesicles having a C/P of 0.90 failed to exert any effect on erythrocyte C/P (cholesterol-maintained) (Chailley *et al.*, 1981). Incubation, under the same conditions, of erythrocytes with phosphatidylcholine vesicles caused a reduction in the cholesterol content of their membranes without affecting the overall composition in phospholipids (cholesterol-depleted), resulting in the present experiments in a C/P of 0.71 ± 0.01 ($n = 20$). When vesicles containing [³H]glycerol trioleate (as an index of phospholipid contamination) were used for the incubation with erythrocytes, the resulting membranes were found to contain less than 0.02% of exogenous phospholipid relative to the endogenous pool (not shown).

PI and PIP kinases

In initial experiments, PPI synthesis from [γ -³²P]ATP was measured on membranes prepared in different

Table 1. Effects of membrane preparation and preincubation conditions on PPI synthesis

Membranes prepared in Tris/EDTA or Hepes/EGTA from control, cholesterol-maintained or cholesterol-depleted erythrocytes were preincubated with or without Mg^{2+} for 10 min at 37 °C and incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (and Mg^{2+} when not present) for a further 10 min, at 37 °C (see the Materials and methods section). Values are means $\pm$ S.E.M. for n experiments. ^aSignificantly different from control membranes; ^bsignificantly different from corresponding Tris membranes; ^csignificantly different from corresponding Hepes membranes preincubated with Mg^{2+} ($P < 0.05$); n.d., not determined.

Haemolysis		PPI synthesis (nmol/ μ mol of phospholipid)			n
		Control	Cholesterol-maintained	Cholesterol-depleted	
Tris/EDTA + Mg^{2+}	PIP ₂	0.056 $\pm$ 0.007	0.048 $\pm$ 0.011	0.093 $\pm$ 0.035	5
	PIP	0.036 $\pm$ 0.007	0.045 $\pm$ 0.011	0.185 $\pm$ 0.066 ^a	5
Hepes/EGTA + Mg^{2+}	PIP ₂	0.27 $\pm$ 0.07 ^b	0.27 $\pm$ 0.01 ^b	0.11 $\pm$ 0.02 ^a	5
	PIP	0.32 $\pm$ 0.01 ^b	0.37 $\pm$ 0.03 ^b	0.19 $\pm$ 0.04 ^a	5
Hepes/EGTA	PIP ₂	0.58 $\pm$ 0.07 ^c	n.d.	0.27 $\pm$ 0.03 ^{a,c}	6
	PIP	1.82 $\pm$ 0.26 ^c	n.d.	1.12 $\pm$ 0.08 ^{a,c}	6

Fig. 1. Effect of PI addition on the time course of PPI synthesis

Control (●, ▲) and cholesterol-depleted (○, △) membranes prepared in Hepes/EGTA were preincubated for 10 min in the absence (a) or presence (b) of PI (0.16 mM) and Triton X-100 (0.6%). At zero time $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and Mg^{2+} were added. The quantity of PIP₂ (●, ○) and PIP (▲, △) formed were calculated (see the Materials and methods section). Each tube contained 144 nmol of membrane phospholipids in control, and 82 nmol in cholesterol-depleted, samples. Results presented are means of duplicate determinations in a single experiment. One further experiment gave similar results.

haemolysing buffers and preincubated with or without Mg^{2+} for 10 min (Table 1). It was found that PPI synthesis was larger in control membranes when they were prepared in Hepes/EGTA than when they were prepared in Tris/EDTA, a difference which was not observed in cholesterol-depleted membranes. When Mg^{2+} was omitted from the preincubation medium, PPI synthesis was increased to a similar extent in both control and cholesterol-depleted membranes. Cholesterol depletion always induced a decrease in PPI synthesis in Hepes membranes. In cholesterol-maintained membranes, PPI synthesis was the same as that in control membranes under all conditions (Table 1). All the other experiments

were carried out on Hepes membranes preincubated without Mg^{2+} .

The time-course of PPI synthesis is illustrated in Fig. 1(a). PIP synthesis reached its maximum at 30 min and then started to decline. PIP₂ synthesis proceeded more slowly. Initial rates and maximal amounts of PIP₂ and PIP synthesized were reduced in cholesterol-depleted membranes. Table 1 shows that on average, after 10 min, the synthesis of PIP₂ was reduced by 54%, and that of PIP by 38%. As the concentrations of phosphoinositides were not changed in the cholesterol-depleted membranes (Giraud *et al.*, 1984), this diminution in PPI synthesis might be due to a loss of PI and PIP kinases. To explore

Fig. 2. Effect of PI concentration on PIP synthesis

Control (▲) and cholesterol-depleted (△) membranes prepared in Hepes/EGTA were preincubated for 10 min with Triton X-100 (0.6%) and PI, and incubated for 10 min with [γ - 32 P]ATP and Mg^{2+} . The quantity of PIP (▲, △) formed was calculated (see the Materials and methods section). Each tube contained 260 nmol of membrane phospholipids in control, and 282 nmol in cholesterol-depleted, samples. Results presented are means of duplicate determinations in a single experiment.

this possibility, PPI synthesis was measured in the presence of exogenous substrates and detergents. Triton X-100 alone caused a large inhibition of both PIP and PIP_2 synthesis (results not shown). Addition of PI and Triton X-100 caused a very large increase in PIP synthesis and an inhibition of PIP_2 synthesis (Fig. 1b) as reported before (Buckley, 1977; Marche *et al.*, 1982). Interestingly, under these conditions both the time course (Fig. 1b) and the dose-dependence (Fig. 2) of PIP synthesis were similar in control and in cholesterol-depleted membranes. To compare the effect of adding PI on the same amount of each type of membrane (i.e. same amount of enzyme) the values of PIP synthesis obtained in four experiments were plotted as a function of the amount of membrane phospholipids per assay (results not shown). A linear relationship was obtained ($y = 0.033x + 0.04$, $r = 0.84$) regardless of the cholesterol content of the membranes, indicating that PI kinase was not lost from cholesterol-depleted membranes.

To investigate whether the same conclusion also holds for PIP kinase activity, the effect of PIP addition (without detergent) on PPI synthesis was tested. It did not affect PIP synthesis but increased PIP_2 synthesis in cholesterol-depleted membranes and slightly decreased it in control membranes, so that PIP_2 synthesis became identical in

both types of membranes (Fig. 3). Again these results indicate that PIP kinase activity was retained in cholesterol-depleted membranes.

Diacylglycerol kinase

No difference between control and cholesterol-depleted membranes was observed for the time-course of PA synthesis in the absence of Ca^{2+} (results not shown). Maximal synthesis occurred after about 40 min. In the presence of Ca^{2+} (50 or 450 μM) PA synthesis was largely increased in control and in cholesterol-maintained membranes, as expected from the Ca^{2+} -induced production of diacylglycerol by the PPI phosphodiesterase (Allan *et al.*, 1976) but this increase was not observed in cholesterol-depleted membranes (Table 2). This lack of activation of PA synthesis might be due to a smaller production of diacylglycerol by the PPI phosphodiesterase and/or to a loss of diacylglycerol kinase from cholesterol-depleted membranes. Using the same strategy as above, PA synthesis was measured in the presence of an exogenous substrate, dioleoin. In the absence of detergent, dioleoin had no effect on PA synthesis (results not shown). Addition of dioleoin plus Triton X-100 caused a large increase in this synthesis both in control and in cholesterol-depleted membranes (Table 2). The results show a large scatter, probably due to different levels of dioleoin incorporation in the different experiments. However, under these conditions Ca^{2+} addition had no significant effect on PA synthesis either in control or in cholesterol-depleted membranes. This shows that diacylglycerol kinase was not activated by Ca^{2+} , as already reported (Allan *et al.*, 1976). In cholesterol-depleted membranes, PA synthesis reached higher levels than in control membranes, but the effect was not statistically significant. Although no firm conclusion could be drawn on this point, at least these results indicate that diacylglycerol kinase was not lost from cholesterol-depleted membranes.

PPI phosphodiesterase

Ca^{2+} -activated PPI hydrolysis was measured on membranes prepared in different haemolysing buffers from erythrocytes prelabelled with $^{32}P_i$ (see the Materials and methods section). The percentage of PPI hydrolysed upon Ca^{2+} addition was the same in Tris/EDTA and in Hepes/EGTA membranes (results not shown). In cholesterol-maintained membranes this percentage was not significantly different from that in control membranes (Table 3).

The time-course of PPI hydrolysis is shown in Fig. 4. Maximum hydrolysis was observed after 40–50 min in both types of membranes. However, 65–70% of the PPI was hydrolysed in control and only 45–55% in cholesterol-depleted membranes. At the latest times, in all membranes, significantly more PIP than PIP_2 was hydrolysed (see also Fig. 6a). This was likely due to the activity of the membrane-bound PIP phosphatase (Mack & Palmer, 1984). By 10 min, the time chosen for most of the other experiments, this effect was small and nearly the same percentage of PIP and PIP_2 were degraded. On average this value was 50.3% and 35.5% respectively in control and cholesterol-depleted membranes in 10 min (Table 3).

The requirement for Ca^{2+} is illustrated in Fig. 5. Maximum hydrolysis was seen at 10 μM - Ca^{2+} in control and at 5 μM in cholesterol-depleted membranes. The

Fig. 3. Effect of PIP addition on the time course of PPI synthesis

Control (●, ▲) and cholesterol-depleted (○, △) membranes prepared in Hepes/EGTA were preincubated for 10 min in the absence (a) or presence (b) of PIP (0.25 mM). At zero time [γ -³²P]ATP and Mg²⁺ were added. The quantity of PIP₂ (●, ○) and PIP (▲, △) formed were calculated (see the Materials and methods section). Each tube contained 274 nmol of membrane phospholipids in control, and 200 nmol in cholesterol-depleted, samples. Results presented are means of duplicate determinations in a single experiment. One further experiment gave similar results.

Table 2. Effects of Ca²⁺ and diolein addition on PA synthesis

Membranes prepared in Hepes/EGTA from control, cholesterol-maintained or cholesterol-depleted erythrocytes were incubated with [γ -³²P]ATP and Mg²⁺, in the presence or absence of Ca²⁺ and diolein and Triton X-100, for 60 min at 37 °C (see the Materials and methods section). Values are means $\pm$ S.E.M. for *n* experiments. ^aSignificantly different from control membranes; ^bsignificantly different from corresponding membranes without Ca²⁺ (*P* < 0.05); n.d., not determined.

Ca ²⁺ (μM)	Diolein + Triton X-100	PA synthesis (nmol/μmol of phospholipid)			<i>n</i>
		Control	Cholesterol-maintained	Cholesterol-depleted	
0	—	0.054 $\pm$ 0.011	0.075 $\pm$ 0.024	0.049 $\pm$ 0.006	4
50	—	0.130 $\pm$ 0.020 ^b	0.118 $\pm$ 0.023	0.037 $\pm$ 0.005 ^a	4
450	—	0.174 $\pm$ 0.022 ^b	0.202 $\pm$ 0.045 ^b	0.075 $\pm$ 0.024 ^a	4
0	+	0.93 $\pm$ 0.43	n.d.	1.94 $\pm$ 0.53	3
50	+	1.39 $\pm$ 0.27	n.d.	2.42 $\pm$ 0.65	3
450	+	1.15 $\pm$ 0.07	n.d.	1.74 $\pm$ 0.37	3

Ca²⁺ concentration for half-maximum activation was not changed by cholesterol depletion (about 1 μM). If diacylglycerol produced by PPI hydrolysis was the only substrate for PA synthesis, the Ca²⁺ requirements for both processes should be the same. However PA synthesis was increased when the Ca²⁺ concentration was increased from 50 to 450 μM (see Table 2). This apparent

contradiction is likely to be due to the addition of Mg²⁺ in the incubation buffer when PA synthesis was measured. Indeed this has been reported to shift the Ca²⁺ activation curve for PPI hydrolysis to higher concentrations (Downes & Michell, 1982). To investigate whether this inhibition of PPI hydrolysis was caused by a loss of PPI phosphodiesterase from

Table 3. Effects of Ca^{2+} and oleate addition on PPI hydrolysis

Membranes prepared in Tris/EDTA from ^{32}P -labelled control, cholesterol-maintained and cholesterol-depleted erythrocytes were incubated for 10 min at 37 °C with Ca^{2+} (150 μM) or with Ca^{2+} (150 μM) and sodium oleate (400 μM) (see the Materials and methods section). The values are the percentages of PPI hydrolysed (means $\pm$ S.E.M. for n experiments). ^aSignificantly different from control membranes; ^bsignificantly different from corresponding membranes without oleate ($P < 0.05$); n.d., not determined.

Addition		PPI hydrolysis (%)			n
		Control	Cholesterol-maintained	Cholesterol-depleted	
Ca^{2+}	PIP_2	48.9 $\pm$ 3.1	45.8 $\pm$ 2.5	34.5 $\pm$ 3.8 ^a	3
	PIP	51.8 $\pm$ 2.2	53.5 $\pm$ 2.5	36.5 $\pm$ 3.3 ^a	3
Ca^{2+} + oleate	PIP_2	51.5 $\pm$ 3.0	n.d.	46.9 $\pm$ 3.6 ^b	3
	PIP	55.6 $\pm$ 5.4	n.d.	51.8 $\pm$ 2.1 ^b	3

Fig. 4. Time course of Ca^{2+} -activated PPI hydrolysis

Control (●, ▲) and cholesterol-depleted (○, △) membranes prepared in Tris/EDTA, from ^{32}P -labelled erythrocytes, were incubated with Ca^{2+} (150 μM). The percentage of PIP_2 (●, ○) and PIP (▲, △) hydrolysed was calculated (see the Materials and methods section). Results presented are means of duplicate determinations in a single experiment. Two other experiments gave similar results.

cholesterol-depleted membranes, we have used various agents which were reported to activate PI hydrolysis by phospholipase C in other membranes: chlorpromazine (Allan & Michell, 1974), sodium deoxycholate (Irvine & Dawson, 1978), and sodium oleate (Irvine *et al.*, 1979). Chlorpromazine (100 μM) and sodium deoxycholate (1.2 mM) inhibited Ca^{2+} -activated PPI breakdown (results not shown). In the presence of oleate (400 μM), the initial rate of PPI hydrolysis was increased in both types of membranes whereas the maximum percentage of hydrolysis was not changed (or slightly decreased for PIP_2) in control membranes but clearly increased in cholesterol-depleted membranes (Fig. 6). Consequently, the time course of hydrolysis became very similar in control and

Fig. 5. Effect of Ca^{2+} on PPI hydrolysis

Control (●) and cholesterol-depleted (○) membranes prepared in HEPES/EGTA, from ^{32}P -labelled erythrocytes, were incubated for 10 min with different Ca^{2+} concentrations. The mean percentage of PIP_2 and PIP hydrolysed was calculated (see the Materials and methods section). Results presented are means of duplicate determinations in a single experiment. Three other experiments gave similar results.

in cholesterol-depleted membranes. By 10 min the percentages of both PPI hydrolysed were not significantly different in oleate-treated control or cholesterol-depleted membranes (Table 3). Thus PPI phosphodiesterase was not lost from nor inactivated in cholesterol-depleted membranes.

DISCUSSION

The membranes used in this study were prepared from erythrocytes that had been incubated without vesicles (control), with cholesterol/phospholipid vesicles (cholesterol-maintained) or with phospholipid vesicles (cholesterol-depleted) (Giraud *et al.*, 1984). This method allows the cholesterol content to be manipulated without incorporation of exogenous phospholipid or change in the overall composition of phospholipids. The enzymic activities measured on cholesterol-maintained membranes

Fig. 6. Effect of oleate addition on the time course of Ca^{2+} -activated PPI hydrolysis

Control (●, ▲) and cholesterol-depleted (○, △) membranes prepared in Tris/EDTA from ^{32}P -labelled erythrocytes were incubated with Ca^{2+} ($150\ \mu\text{M}$) and in the absence (a) or presence (b) of sodium oleate ($400\ \mu\text{M}$). The percentage of PIP_2 (●, ○) and PIP (▲, △) hydrolysed was calculated (see the Materials and methods section). Results presented are means of duplicate determinations in a single experiment. One further experiment gave similar results.

were always the same as those measured on control membranes, indicating that the effects observed on the cholesterol-depleted membranes could be attributed specifically to the removal of cholesterol.

The conditions attending membrane preparation, which influence the permeability and the sidedness characteristics of the membrane vesicles (Bramley *et al.*, 1971), were found to affect the level of PPI synthesis from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. PPI synthesis was increased in Hepes/EGTA membranes (more permeable to ATP; Downes *et al.*, 1981) in comparison to Tris/EDTA membranes. PPI synthesis was decreased upon short preincubation with Mg^{2+} , which promotes the formation of right-side-out vesicles (Kant & Steck, 1972). Garrett & Redman (1975) obtained similar results and concluded that PI and PIP kinases and possibly PPI were located on the inner surface of the membrane in normal erythrocytes. This latter conclusion also applied to the cholesterol-depleted membranes, the only difference being that they exhibited the same permeability to ATP either prepared in Tris or in Hepes buffer.

Synthesis of both PIP and PIP_2 was markedly inhibited in cholesterol-depleted membranes. However, PPI synthesis could be increased to the same level as that in control membranes by addition of exogenous substrates, PI or PIP, indicating that the effect was not due to a loss or to an inactivation of PI or PIP kinase. Since the concentration of endogenous phospholipids was not changed by cholesterol depletion (Giraud *et al.*, 1984) it seemed that the apparent inhibition of the enzyme activity could result from the inaccessibility of a fraction of PI and PIP to their kinases.

To test this possibility, we have measured, on the same membranes, the Ca^{2+} -activated PPI hydrolysis. This

reaction is catalysed by the PPI phosphodiesterase, an enzyme which presumably is not active under physiological conditions (Downes & Michell, 1982). However, the activity of this enzyme was investigated to provide further evidence for the reduced accessibility of the PPI in cholesterol-depleted membranes. The maximum percentage of PPI hydrolysed and the Ca^{2+} concentration for maximum hydrolysis were both decreased as compared to control membranes. However, the Ca^{2+} concentration for half-maximum hydrolysis was not changed. Furthermore in the presence of sodium oleate, PPI hydrolysis occurred to a similar extent in both types of membranes. The stimulatory action of oleate on PPI hydrolysis can be due in principle to a direct action on the PPI phosphodiesterase, to an increased availability of the substrates or to a combination of both mechanisms. The similarities of the action of oleate on the initial rate of PPI hydrolysis in both control and cholesterol-depleted membranes could indicate a direct action on the enzyme. However, the differential effect of oleate on the maximum percentage of hydrolysis can be better interpreted as resulting from an increased availability of previously disorganized substrates in the cholesterol-depleted membranes. In any case, oleate treatment allowed the enzyme to express the same activity as in control membranes and showed that the reduction of PPI hydrolysis was not due to a loss or to an inactivation of the enzyme.

These latter results are indicative of a reorganization of PIP and PIP_2 in cholesterol-depleted membranes, whereas the kinase data suggested the reorganization of PI and PIP. From the results presented in Tables 1 and 3, it can be calculated that only about 60% of PI and 46% of PIP were accessible to the kinases and about 70% of the PPI to the phosphodiesterase in these membranes.

Phosphoinositides are believed to be located on the inner surface of the membrane (Garrett & Redman, 1975). Our results do not provide any indication about their new location in cholesterol-depleted membranes. They could stay in the inner surface or be transferred to the outer surface. The inaccessibility of a fraction of the PPI to their kinases can explain also why, in cholesterol-depleted relative to control erythrocytes, ^{32}P incorporation into PPI was reduced without changes in their concentration or the specific activity of ATP (Giraud *et al.*, 1984). The possibility that the membrane cholesterol content can influence the phospholipid organization has been suggested previously in different situations: shape change in erythrocytes (Lange *et al.*, 1982), thrombin-induced changes in platelet phospholipid metabolism (Kramer *et al.*, 1982), adenylate cyclase activity modulation in hepatocytes (Whetton *et al.*, 1983), phosphatidylcholine and phosphatidylethanolamine hydrolysis rate by phospholipase A_2 in fibroblasts (Fisher *et al.*, 1983).

Synthesis of PA was also inhibited in cholesterol-depleted membranes when measured in the presence of Ca^{2+} . However, this effect was suppressed by the addition of exogenous substrate diolein, indicating that the diacylglycerol kinase was not lost nor inactivated upon cholesterol depletion. Thus the reduction in PA synthesis observed in cholesterol-depleted erythrocytes is probably due to a diminution of the diacylglycerol pool, which has indeed been observed in these erythrocytes (Giraud *et al.*, 1984).

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