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CHANGES IN MORPHOLOGY AND IN POLYPHOSPHOINOSITIDE TURNOVER OF HUMAN ERYTHROCYTES AFTER CHOLESTEROL DEPLETION

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Human erythrocytes were cholesterol-depleted (5–25%) by incubation with phosphatidylcholine vesicles in media containing Ca^{2+} at different concentrations (0, 28 nM, 5 μM or 1 mM). After removal of the vesicles, the cells were reincubated with [^{32}P]phosphate in the same media. Control (incubated in buffer alone) and cholesterol-maintained erythrocytes (incubated with cholesterol / phosphatidylcholine vesicles) were treated similarly. Cholesterol depletion induced the conversion of the cells into stomatocytes III and spherostomatocytes and decreased the turnover rate of phosphatidylinositol phosphate and of phosphatidylinositol bisphosphate. None of these effects were observed in cholesterol-maintained cells. In cholesterol-depleted cells, they occurred without changes in the ATP specific activity or in the polyphosphoinositide concentrations. Moreover, these modifications of shape and of lipid metabolism were proportional to the extent of the cholesterol depletion and were independent of the external Ca^{2+} concentration. In contrast, other effects of cholesterol depletion, a decrease in the turnover rate of phosphatidic acid, a decrease in diacylglycerol and in phosphatidic acid concentrations were dependent on the external Ca^{2+} concentration. Thus it appears that the shape change was not correlated with a change in the concentrations of these phospholipids or of diacylglycerol and therefore cannot be explained by a bilayer couple mechanism involving these phospholipids. However, the spherostomatocytic transformation was correlated with the decrease in the turnover rate of the polyphosphoinositides, but not with the turnover rate of phosphatidic acid, suggesting a role for the turnover of the polyphosphoinositides in the maintenance of the erythrocyte shape.

Introduction

The biconcave shape of erythrocytes is maintained by ATP-requiring reactions involving membrane components [1,2]. The phosphorylation of spectrin was originally proposed to be responsible for the rearrangements of the membrane skeleton [3,4] but different reports now exclude this possibility [5–7]. Polyphosphoinositides and phosphatidic acid which together represent less than 5% of the total phospholipids of the erythrocyte membrane, are the only phosphorylated lipid species [8]. Polyphosphoinositides undergo a rapid turnover of their phosphomonoester groups, but the role of the turnover of all these minor phospholipids has not been established definitively [8]. In two studies using isolated erythrocyte membranes, it was suggested that the phosphorylation of phosphatidylinositol 4-phosphate [9] or of diacylglycerol [6] – which is phosphorylated into phosphatidic acid in the presence of ATP – could participate in the control of cell membrane curvature.

In order to investigate further the possible in-

volvement of polyphosphoinositides and phosphatidic acid in the erythrocyte shape changes, we have measured the incorporation of ^{32}P into these phospholipids and their concentration and that of diacylglycerol in cholesterol-depleted erythrocytes. In earlier works [10,11], it was shown that this treatment caused the conversion of the cells into stomatocytes. One of the steps involved in the turnover of polyphosphoinositides is Ca^{2+}-actived [12]. Thus experiments with control and cholesterol-depleted cells (preincubation and phosphorylation) were conducted in media of different Ca^{2+} concentrations.

Material and Methods

Reagents. $^{32}PO_4$ (sodium salt) was obtained from CEA (France). Egg phosphatidylcholine, phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 4-phosphate, phosphatidic acid, 1,2- and 1,3-diolein and other lipid standards were purchased from Sigma, Chem. Co. (St Louis, MO, U.S.A.). PEI-cellulose TLC plates were obtained from Merck (Darmstadt, F.R.G.) and silicagel TLC plates with luminescer from Schleicher and Schüll (Dassel, F.R.G.). X-Ray films for autoradiography (no screen, NS-2T or X-Omat AR) were from Kodak (France). All the other chemicals were of reagent grade.

Lipid vesicles. These were prepared as previously reported [13] except that egg phosphatidylcholine vesicles were sonicated in medium II and cholesterol/phosphatidylcholine vesicles (final cholesterol/phospholipid molar ratio, 0.85) in medium I. The composition of medium I was (mM): NaCl, 140/ KCl, 10/ $MgCl_2$, 1/ NaH_2PO_4-Na_2HPO_4 (20 : 80) (pH 7.4), 2.5/ glucose, 10/ sucrose, 35/ penicillin, 5000 units per ml. The composition of medium II was the same except for NaCl (20 mM) and KCl (130 mM).

Cholesterol depletion and ^{32}P labelling of erythrocytes. Human erythrocytes from freshly drawn heparinised blood were isolated by centrifugation and the buffy coat removed by aspiration. The cells were washed three times with isotonic choline chloride solution and resuspended in medium I or II. Erythrocytes were incubated, as previously reported [10], for 15 h at 37°C and 15% haematocrit either with phosphatidylcholine vesicles (1–3 mg phosphatidylcholine/ml packed cells) (cholesterol-depleted) or with cholesterol/ phosphatidylcholine vesicles (1 mg cholesterol, 2 mg phosphatidylcholine/ ml packed cells) (cholesterol-maintained) or without vesicles (controls). After 15 h, adenine and inosine (final concentration 2 and 10 mM, respectively) were added to each suspension for a further hour of incubation. In the previous study [10] the morphology was assessed at that time. Here, the experimental design was slightly modified in a number of ways. After the first incubation with or without vesicles, erythrocytes were washed and reincubated with [^{32}P]phosphate (10–20 μCi/ml) for 3 h at 37°C and 35% haematocrit in order to study their phospholipid metabolism. Their morphology was examined after this second incubation in the absence of vesicles. Cholesterol-depleted erythrocytes are more permeable to monovalent cations than control or cholesterol-maintained cells [13]. Therefore normal intracellular Na^+ and K^+ concentrations in the cholesterol-depleted erythrocytes were maintained by performing all the incubations in medium II rather than in medium I which was only used for the control and the cholesterol-maintained cells. Furthermore to investigate the possible effect of intracellular Ca^{2+} on both the shape change and phospholipid metabolism, the basal composition of media I or II was modified so that they differed in their Ca^{2+} concentration, by adding EGTA (2, 0.1, 0.05 or 0 mM, final concentrations) or $CaCl_2$ (1 mM final concentration). This resulted in final free Ca^{2+} concentrations of, respectively, 0, 13 nM, 28 nM when calculated using the stability constant $K = 4 \cdot 10^{-6}\ M^{-1}$ for the Ca-EGTA complex [14], and of 5 μM (in the absence of EGTA) as measured by atomic absorption spectroscopy. After 1.5 h or 3 h of incubation with ^{32}P, aliquots of the suspensions were centrifuged and the cells were washed three times in their respective media. Cell aliquots were taken for scanning electron microscopy, measurement of Na^+ and K^+ concentrations and ATP content and specific radioactivity.

Under these conditions, the internal Na^+ and K^+ concentrations of cholesterol-depleted erythrocytes were in the same range as that of control or cholesterol-maintained erythrocytes i.e., 7–10 and 90–104 mmol/l cell H_2O for Na^+ and

K^+, respectively. The Ca^{2+} concentration of the external medium did not affect these values except that in cholesterol-depleted erythrocytes, Ca^{2+}-free medium induced a small but significant decrease in the Na^+ concentration relative to that found in the high Ca^{2+} media or to that in the control erythrocytes in the same medium. The ATP content in cholesterol-depleted cells (0.73 ± 0.10 mmol/l cell) was not significantly different from that in control cells (0.88 ± 0.15 mmol/l cell). The Ca^{2+} concentration of the external medium did not affect the ATP content either of control or cholesterol-depleted erythrocytes.

Membrane preparation, lipid extractions and TLC of lipids. After incubation with ^{32}P, washed cell suspensions were lysed at 4°C in 20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA. Membranes were washed three times with the lysing medium.

Neutral extraction (isopropanol/chloroform) [15] was used when both cholesterol and phospholipids were assayed for the estimation of the cholesterol/phospholipid ratio. Acidic extraction was used when phospholipid and diacylglycerol concentrations and phospholipid radioactivity were measured. One vol. of membrane suspension was mixed with 3.75 vol. of chloroform/methanol/conc. HCl (20 : 40 : 1, v/v). Chloroform (1.25 vol.) and H_2O (1.25 vol.) were added [12]. The resulting suspension was kept overnight in the cold. The lower phase was washed three times with 1 M HCl, concentrated under vacuum and aliquots were taken for total phosphorus, radioactivity measurements and for separation of phospholipids by TLC. The latter was performed on silicagel plates soaked in EDTA (2%, pH 7.4) (system A) or in H_2O (system B), air-dried overnight and activated at 120°C for 1 h. Two different solvent systems were used for phospholipid separation: system A (chloroform/methanol/4.3 M NH_4OH, 9 : 7 : 2) [16]; system B (chloroform/pyridine/formic acid, 100 : 60 : 14) [17]. When the radioactivity of the phospholipids was measured, only system A was used. This allowed a good separation of the two polyphosphoinositides and phosphatidic acid which were the only phospholipids labelled under our experimental conditions as revealed by autoradiographs (not shown) and in agreement with previous reports (for a review, see Ref. 8). The areas co-migrating with authentic standards were scraped and counted. However, in this system phosphatidic acid was contaminated with other phospholipids. Thus, when the concentration of phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 4-phosphate and phosphatidic acid was to be measured system A was used for the estimation of the first two and system B for the third one which, in this case, was clearly separated from all the other phospholipids. Lipid spots, visualized with iodine vapor and ultraviolet light, were identified by comparison with authentic standards and scraped for phosphorus measurement. In systems A and B, neutral lipids migrated at the solvent front. They were scraped for further extraction with diethyl ether and TLC with system C (diethyl ether/benzene/ethanol/acetic acid, 40 : 50 : 2 : 0.2) to separate diacylglycerol from other lipids [18]. In this system, 1,2-diacylglycerols were separated from 1,3-diacylglycerols. The erythrocyte membrane contains principally the former species with traces of the latter. Spots corresponding to each species were collected together for measurement of the diacylglycerol content.

Scanning electron microscopy. Cells were fixed for 1 h in glutaraldehyde (1%), post-fixed in osmium tetroxide (1%) [19] and observed in a Cameca MEB 07 scanning electron microscope at 20 kV. The extent of shape change was scored according to the nomenclature of Bessis [20]. At least 200 cells were counted for each determination. Counting variations were found to be very small, within 3–5%.

ATP content and specific radioactivity. Cells were extracted with perchloric acid (0.6 M) at 4°C. Aliquots of the extract were taken for the measurement of the ATP content by an enzymatic method (Test-combination ATP, Beohringer Mannheim GmbH) and for the separation of ATP from other nucleotides by TLC on PEI-cellulose [21]. The spot corresponding to ATP was identified by comparison with a standard and scraped for measuring its ATP content by spectrophotometry and its ^{32}P radioactivity.

Other assays. Na^+ and K^+ were measured by flame photometry after precipitation of the cells with trichloroacetic acid (20%). Membrane protein was assayed according to Lowry et al. [22] using bovine serum albumin as standard. Cholesterol was estimated by the *o*-phthalaldehyde method

[23]. Phospholipids from the silicagel spots or in the lipid extracts were measured according to Rouser et al. [24] after digestion with conc. perchloric acid. Diacylglycerol was measured after saponification in 0.5 M alcoholic KOH of the silicagel spots, by an enzymatic method (Test-combination Triglycerides, Boehringer Mannheim GmbH). Radioactivity of membranes, lipid extracts and silicagel spots was measured by Cerenkov counting in H_2O.

Results

Cholesterol depletion

As previously reported [13], incubation of erythrocytes with phosphatidylcholine vesicles resulted in the reduction of the cholesterol content of the cells without changing their total phospholipid content or the distribution of the major phospholipids. The extent of cholesterol depletion can be monitored by varying the vesicles/cells ratio. Incubation with cholesterol/phosphatidylcholine vesicles (molar ratio = 0.85) did not change any of these compounds and resulted in the so called 'cholesterol-maintained erythrocytes'. Incubation in buffer alone had no effect either on cholesterol or phospholipid content. In the present study, it was found unexpectedly that in the absence of Ca^{2+}, cholesterol depletion was about 2-fold less than that observed in its presence (5 μM or 1 mM). To achieve the same level of cholesterol depletion as that obtained in the presence of high Ca^{2+} solutions, the medium had to contain traces of Ca^{2+} (28 nM) and the vesicles concentration had to be increased 2-fold (results not shown). This observation may indicate that cholesterol exchanges between erythrocytes and lipid vesicles require Ca^{2+}, at least traces, to occur.

Morphology

After the long incubations used in these experiments (16 h + 3 h) control erythrocytes were not all discocytes but a mixture of discocytes (10–20%) and stomatocytes I and II (80–90%) (Fig. 1A). Ca^{2+} concentration of the external medium did not affect their morphology (not shown). Cholesterol depletion induced a dramatic shape change into stomatocytes III and spherostomatocytes (Figs. 1B, C, D). At the same level of cholesterol depletion (about 24% in Fig. 1D), the extent of the shape change, as judged by the percent of spherostomatocytes in the population (70–90%), was the same regardless of the Ca^{2+} concentration of the medium (see Fig. 1E). This effect was specific to cholesterol depletion and was not caused by the presence of the vesicles during

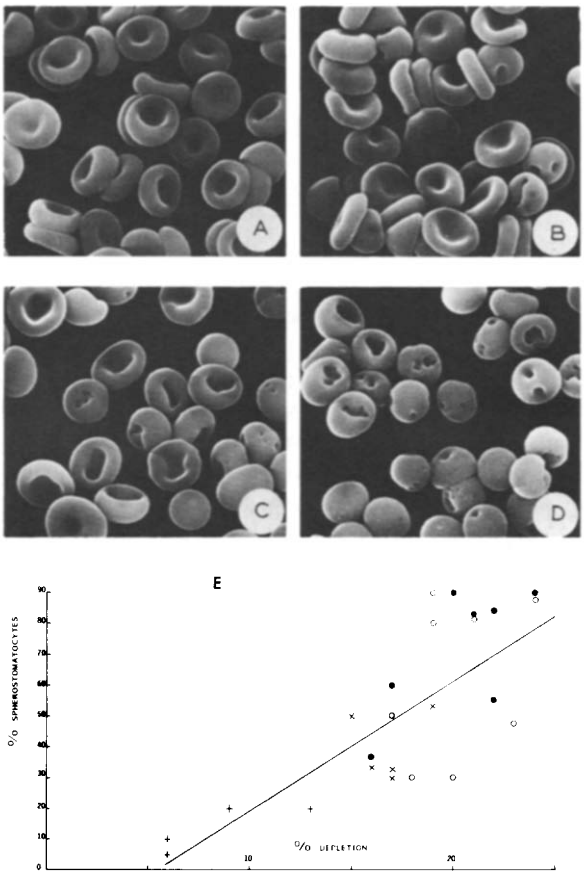

Fig. 1. (A–D) Scanning electron micrographs of control and cholesterol-depleted erythrocytes. Erythrocytes were incubated without (control, A) or with phosphatidylcholine vesicles (cholesterol-depleted, B, C and D) in different Ca^{2+}-containing media and, after removal of the vesicles, reincubated for 3 h in the same media (0–1 mM Ca^{2+}). Cholesterol/phospholipid molar ratio was 0.85 in control (A), 0.80, 0.71 and 0.65, respectively, in cholesterol-depleted erythrocytes (B, C and D). A, C and D were incubated in 1 mM Ca^{2+} and B in Ca^{2+} free solution. Magnification: ×1500. (E) Effect of the extent of cholesterol depletion on the percent of conversion into spherostomatocytes. Erythrocytes were incubated as in Figs. 1A–D and reincubated for 3 h in the presence of 0 (+), 28 nM (×), 5 μM (●) or 1 mM (○) Ca^{2+}.

TABLE I

^{32}P INCORPORATION INTO MEMBRANE (LIPID + PROTEIN) AND LIPID EXTRACTS: LACK OF EFFECT OF THE Ca^{2+} CONCENTRATION OF THE EXTERNAL MEDIUM

Erythrocytes were incubated without (control) or with phosphatidylcholine vesicles (cholesterol-depleted) in different Ca^{2+} containing media and reincubated for 3 h with [^{32}P]phosphate (20 μCi/ml) in the same media. Membranes were prepared by hypotonic haemolysis and either directly used for measuring their radioactivity or extracted with solvent for measuring the radioactivity of the lipids (see Methods). Each value is the mean ± S.E. of five experiments. * $P < 0.05$ relative to corresponding control cells.

Free [Ca^{2+}] in the medium	Cholesterol/ phospholipid (mol/mol)	^{32}P incorporation (cpm/μg membrane protein)	
		Lipid + protein	Lipid
28 nM	0.80 ± 0.05	50.5 ± 2.2	28.4 ± 2.3
5 μM	0.79 ± 0.05	56.7 ± 8.8	32.8 ± 7.0
1 mM	0.79 ± 0.05	57.1 ± 7.4	37.2 ± 6.1
28 nM	0.63 ± 0.05 *	31.5 ± 4.7 *	15.9 ± 1.5 *
5 μM	0.62 ± 0.04 *	32.6 ± 3.0 *	17.2 ± 2.3 *
1 mM	0.62 ± 0.02 *	33.1 ± 2.2 *	16.3 ± 0.9 *

the preincubation since it was not observed in cholesterol-maintained erythrocytes (not shown). Since external Ca^{2+} did not affect the morphology, the effect of the extent of cholesterol depletion on the extent of the shape change can be compared at any Ca^{2+} concentration of the medium. This was done by plotting the percentage of spherostomatocytes versus the percentage of cholesterol depletion (Fig. 1E). Within the observed range of cholesterol depletion (5–25%) a linear positive correlation was obtained ($y = 4.3x - 24.2$; $r = 0.77$). This indicates that the extent of the shape change was proportional to the percentage of the cholesterol depletion and was independent of the Ca^{2+} concentration of the medium. An illustration of this is given in Figs. 1B, 1C and 1D in which cholesterol depletion was, respectively 6, 16 and 24%, either in medium containing 0 (B) or 1 mM Ca^{2+} (C and D).

Incorporation of [^{32}P]phosphate into membranes and lipid extracts

After incubation of erythrocytes with [^{32}P]phosphate, radioactivity was incorporated into ATP (see Table V), membrane proteins and lipids. The Ca^{2+} concentration of the external medium did not affect significantly the level of ^{32}P incorporation into membranes (lipid + protein) and lipid extracts either of control or of cholesterol-depleted cells (Table I). Cholesterol depletion induced a significant diminution of ^{32}P incorporation both into membranes and lipid extracts. At the same level of cholesterol depletion (about 20% for the experiments of Table I) the effect on incorporation occurred to about the same extent regardless of the Ca^{2+} concentration of the medium. This effect was specific of cholesterol depletion and not caused by the presence of the vesicles during the preincubation since it was not observed in cholesterol-maintained erythrocytes (Table II, compare B to

TABLE II

^{32}P INCORPORATION INTO MEMBRANES (LIPID + PROTEIN) AND LIPID EXTRACTS: SPECIFIC EFFECT OF CHOLESTEROL DEPLETION

Erythrocytes were incubated without (control (A)), with cholesterol/phosphatidylcholine vesicles (cholesterol-maintained (B)) or with phosphatidylcholine vesicles (cholesterol-depleted (C)) in media containing 5 μM Ca^{2+} and reincubated for 1.5 h with [^{32}P]phosphate (7 μCi/ml) in the same medium. Membranes were prepared and radioactivity measured as in the legend of Table I. Each value is the mean ± S.E. of four experiments. * $P < 0.05$ relative to corresponding control cells.

Cholesterol/ phospholipid (mol/mol)	^{32}P incorporation (cpm/μg membrane protein)	
	Lipid + protein	Lipid
(A) 0.85 ± 0.03	7.7 ± 0.7	4.2 ± 0.3
(B) 0.83 ± 0.04	8.3 ± 1.5	4.7 ± 0.9
(C) 0.70 ± 0.02 *	3.9 ± 0.3 *	2.1 ± 0.2 *

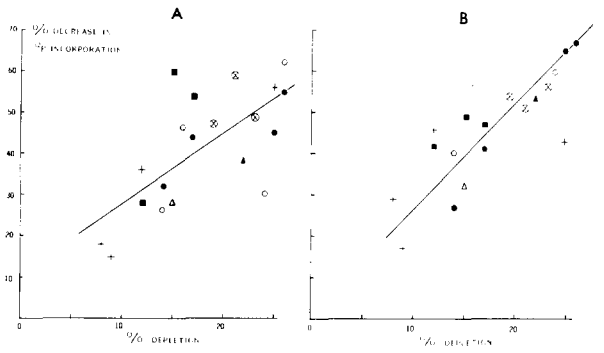

Fig. 2. Effect of the extent of cholesterol depletion on the diminution of ^{32}P incorporation into membranes (A) and lipid extracts (B). Erythrocytes were incubated as in Fig. 1 and reincubated with [^{32}P]phosphate for 1.5 or 3 h in different Ca^{2+} containing media: 0, 1.5 h (×); 13 nM, 1.5 h (△); 28 nM, 1.5 h (▲); 5 μM, 1.5 h (■); 1 mM, 1.5 h (⊗); 0, 3 h (+); 5 μM, 3 h (●); 1 mM, 3 h (○).

A), whereas in the same group of experiments cholesterol depletion resulted in a decrease of the ^{32}P incorporation (Table II, compare C to A). The distribution of radioactivity between lipids and proteins was about 1/1. It was not modified by cholesterol depletion. As above the percentage reduction of ^{32}P incorporation was plotted versus the percentage of cholesterol depletion at any Ca^{2+} concentration of the medium (Figs. 2A and 2B). Linear positive correlations were obtained ($y = 1.7x + 10.7$, $r = 0.68$ and $y = 2.55x + 0.81$, $r = 0.83$, respectively, for membrane and lipid extract) showing that the reduction of the ^{32}P incorporation was proportional to the reduction of the cholesterol content and independent of the concentration of Ca^{2+} in the external medium.

Incorporation of [^{32}P]phosphate into polyphosphoinositides and phosphatidic acid

The Ca^{2+} concentration of the external medium did not affect significantly the level of the incorporation of ^{32}P into the polyphosphoinositides either in control or in cholesterol-depleted erythrocytes (Table III). In contrast, the reduction of this concentration from 1 mM to 28 nM (free Ca^{2+}) significantly modified the labelling of phosphatidic acid in control cells but not in cholesterol-depleted cells. Cholesterol depletion induced a reduction of incorporation into the three phospholipids. At the same level of cholesterol depletion (about 20% in Table III) the extent of this reduction was about 50% in each polyphosphoinositide and varied from 54 to 77% in phosphatidic acid depending on the concentration of the external Ca^{2+}. When, as above, the percentage reduction of ^{32}P incorporation into each polyphosphoinositide was plotted versus the percentage of cholesterol depletion at any Ca^{2+} concentration of medium, linear positive correlations were obtained ($y = 2.7x + 2.8$, $r = 0.70$ and $y = 2.6x - 2.3$, $r = 0.84$, for phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate, respec-

TABLE III

^{32}P INCORPORATION INTO PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE, PHOSPHATIDYLINOSITOL 4-PHOSPHATE AND PHOSPHATIDIC ACID

For experimental conditions see legend of Table I. Phospholipids were isolated from the lipid extracts by TLC (see Methods). Each value is the mean ± S.E. of five experiments. * $P < 0.05$ relative to corresponding control cells; ** $P < 0.05$ relative to control cells preincubated with 28 nM free [Ca^{2+}].

Free [Ca^{2+}] in the medium	Cholesterol/ phospholipid (mol/mol)	^{32}P incorporation (cpm/nmol total phospholipids)		
		Phosphatidylinositol 4,5-bisphosphate	Phosphatidylinositol 4-phosphate	Phosphatidic acid
28 nM	0.78 ± 0.05	40.6 ± 4.0	15.7 ± 2.5	2.7 ± 0.4
5 μM	0.79 ± 0.05	42.9 ± 3.6	14.2 ± 1.9	4.9 ± 1.5
1 mM	0.79 ± 0.05	44.2 ± 3.9	15.8 ± 1.6	7.4 ± 1.2 **
29 nM	0.64 ± 0.02 *	25.4 ± 3.8 *	8.3 ± 2.4 *	1.3 ± 0.2 *
5 μM	0.62 ± 0.04 *	22.1 ± 3.0 *	7.3 ± 1.7 *	1.2 ± 0.2 *
1 mM	0.62 ± 0.04 *	21.8 ± 3.8 *	7.1 ± 1.9 *	1.7 ± 0.4 *

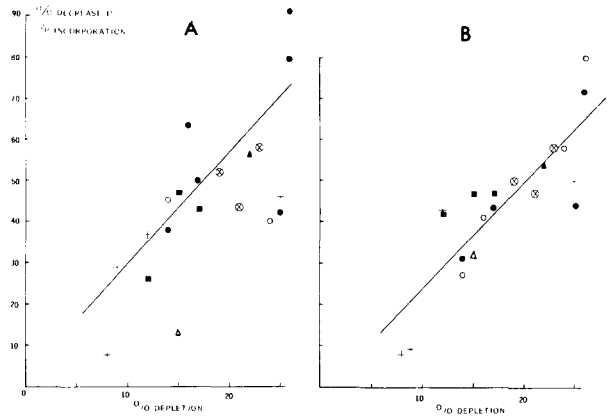

Fig. 3. Effect of the extent of cholesterol depletion on the diminution of ^{32}P incorporation into phosphatidylinositol 4-phosphate (A) and phosphatidylinositol 4,5-bisphosphate (B). Erythrocytes were incubated as in Fig. 1 and reincubated with [^{32}P]phosphate for 1.5 or 3 h. The symbols used are the same as those of Fig. 2.

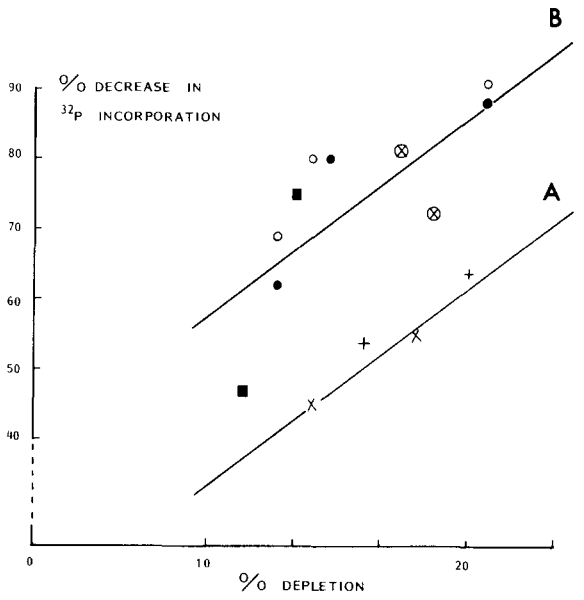

Fig. 4. Effect of the extent of cholesterol depletion on the diminution of ^{32}P incorporation into phosphatidic acid. Erythrocytes were incubated as in Fig. 1 and reincubated with [^{32}P]phospahte for 1.5 h (×, ■, ⊗) or 3 h (+, ●, ○) in different Ca^{2+} containing media. (A) 28 nM (×, +); (B) 5 μM (■, ●) or 1 mM (⊗, ○).

tively) (Figs. 3A and 3B). Thus the effect of cholesterol depletion in reducing the ^{32}P incorporation into polyphosphoinositides was proportional to the extent of cholesterol depletion and independent of the Ca^{2+} concentration of the medium. In contrast, the plot of the percentage reduction of ^{32}P incorporation into phosphatidic acid versus the percentage of cholesterol depletion gave a very poor correlation ($y = 1.45x + 41.2$, $r = 0.47$, not shown). In fact, as mentioned above, the percentage reduction of incorporation into phosphatidic acid, induced by cholesterol depletion was dependent on the external Ca^{2+} concentration. Linear positive correlations were obtained when this percentage was plotted versus the percentage of cholesterol depletion for the erythrocytes incubated in a medium containing 28 nM Ca^{2+} (Fig. 4, curve A, $y = 1.9x + 14.8$, $r = 0.96$) and for those incubated in media containing either 5 μM or 1 mM Ca^{2+} (Fig. 4, curve B, $y = 1.9x + 38.9$, $r = 0.78$). The effect of cholesterol depletion on the reduction of the incorporation of ^{32}P into polyphosphoinositides was thus different from that on the reduction of the incorporation into phosphatidic acid: the former was proportional to the extent of cholesterol depletion and independent of external Ca^{2+}, whereas the latter was also dependent on the external Ca^{2+} concentration.

Concentrations of polyphosphoinositides, phosphatidic acid and diacylglycerol

A decrease in the incorporation of ^{32}P into membrane phospholipids of cholesterol-depleted cells could have arisen from a decrease in the specific radioactivity of the labelled precursor pool, i.e. [γ-^{32}P]ATP. As shown in Table V, this was not the case. A second possibility to explain this decrease would be that the concentration of these phospholipids in the membrane has been reduced by cholesterol depletion. These concentrations were not affected by modifications of the concentration of the external Ca^{2+} either in control or in cholesterol-depleted erythrocytes (Table IV). Cholesterol depletion did not significantly change the concentration of these phospholipids except for a small effect on that of phosphatidylinositol 4-phosphate in the low Ca^{2+} medium and of phosphatidic acid in the high Ca^{2+} medium. Diacylglycerol, the product of the hydrolysis of polyphosphoinositides by the polyphosphoinositide phosphodiesterase [12] and the substrate of the diacylglycerol kinase for its conversion into

TABLE IV

CONCENTRATIONS OF PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE, PHOSPHATIDYLINOSITOL 4-PHOSPHATE, PHOSPHATIDIC ACID AND DIACYLGLYCEROL

For experimental conditions see legend of Table III (with the omission of [^{32}P] in the reincubation). Each value is the mean ± S.E. of seven experiments for the phosphoinositides and of five experiments for phosphatidic acid and diacylglycerol. * $P < 0.05$ relative to corresponding control cells.

Free [Ca^{2+}] in the medium	Cholesterol/ phospholipid (mol/mol)	Concentration (mol/100 mol total phospholipids)			
		Phosphatidylinositol 4,5-bisphosphate	Phosphatidylinositol 4-phosphate	Phosphatidic acid	Diacylglycerol
28 nM	0.77 ± 0.03	0.90 ± 0.06	0.64 ± 0.05	1.72 ± 0.11	0.20 ± 0.06
5 μM	0.77 ± 0.02	1.00 ± 0.05	0.62 ± 0.07	1.71 ± 0.07	0.19 ± 0.02
1 mM	0.78 ± 0.02	0.81 ± 0.07	0.70 ± 0.11	1.85 ± 0.08	0.16 ± 0.02
28 nM	0.65 ± 0.04	0.78 ± 0.04	0.46 ± 0.05 *	1.49 ± 0.06	0.14 ± 0.05
5 μM	0.62 ± 0.02	0.99 ± 0.05	0.54 ± 0.07	1.61 ± 0.07	0.09 ± 0.02 *
1 mM	0.63 ± 0.03	1.03 ± 0.10	0.50 ± 0.04	1.53 ± 0.07 *	0.10 ± 0.01 *

phosphatidic acid [25], was found to be significantly decreased by cholesterol depletion at least in the two high Ca^{2+} media (Table IV).

Specific radioactivities of ATP, polyphosphoinositides and phosphatidic acid

ATP specific radioactivity was close to isotopic equilibrium at 3 h since its value did not change significantly between 1.5 and 3 h (Table V). The Ca^{2+} concentration of the external medium did not significantly modify its value either in control or in cholesterol-depleted cells. Cholesterol depletion did not cause any significant change in the ATP specific radioactivity. In Table V, the specific radioactivity of each phospholipid was calculated from the value of its radioactivity (Table III) and of its concentration (Table IV). The specific radioactivities of phosphatidylinositol 4-phosphate and of phosphatidylinositol 4,5-bisphosphate were always very similar in control cells. This was also the case in the cholesterol-depleted cells, although the specific activities of both polyphosphoinositides were lower. In both types of cells these specific activities were not affected by the Ca^{2+} concentra-

TABLE V

SPECIFIC RADIOACTIVITIES OF [γ-^{32}P]ATP AND OF THE INDIVIDUAL PHOSPHOLIPIDS

Erythrocytes were incubated without (control) or with phosphatidylcholine vesicles (cholesterol-depleted) in different Ca^{2+} containing media and reincubated for 1.5 or 3 h with [^{32}P]phosphate (20 μCi/ml) in the same media. The specific radioactivities of [γ-^{32}P]ATP were measured in four experiments (means ± S.E.) (see Methods). Those of the phospholipids were calculated from the mean radioactivities of Table III (five experiments) and from the mean amounts of each phospholipid of Table IV (5–7 experiments) (because these values were not measured in each separate experiments, no S.E. on the means could be calculated). The values of the C/P are the means ± S.E. of all these experiments.

Free [Ca^{2+}] in the medium	Cholesterol/ phospholipid (mol/mol)	[γ-^{32}P]ATP (cpm/pmol)		cpm/pmol (3 h reincubation)		
		1.5 h	3 h	Phosphatidylinositol 4-phosphate	Phosphatidylinositol 4,5-bisphosphate	Phosphatidic acid
28 nM	0.77 ± 0.03	4.7 ± 0.5	5.6 ± 0.5	2.45	2.25	0.16
5 μM	0.77 ± 0.02	3.9 ± 0.3	5.8 ± 0.8	2.29	2.14	0.29
1 mM	0.78 ± 0.03	5.1 ± 0.5	5.2 ± 0.7	2.25	2.73	0.40
28 nM	0.64 ± 0.04	4.0 ± 0.4	4.2 ± 0.7	1.80	1.62	0.09
5 μM	0.62 ± 0.02	3.9 ± 0.4	4.2 ± 1.3	1.35	1.11	0.07
1 mM	0.63 ± 0.03	3.2 ± 0.1	4.6 ± 0.5	1.42	1.05	0.11

tion of the medium. In contrast, the specific radioactivity of phosphatidic acid was clearly dependent on external Ca^{2+} in control erythrocytes, whereas it was not in cholesterol-depleted erythrocytes. As compared to paired control cells the specific radioactivities of the three phospholipids were decreased in cholesterol-depleted cells.

Discussion

The morphological transformation of erythrocytes induced by cholesterol depletion was more severe than that observed in the earlier work [10]: in addition to the stomatocytes a large number of cells became spherostomatocytes, probably because of the additional 3 h incubation. Furthermore the extent of the shape change was found to be proportional to the extent of the cholesterol depletion and occurred regardless of the Ca^{2+} concentration of the medium (0, 28 nM, 5 μM or 1 mM). Variations in this Ca^{2+} concentration did not affected the shape of control erythrocytes.

We have reported previously that spectrin phosphorylation was decreased by cholesterol depletion whereas lipid phosphorylation was relatively increased [10]. In the present study both lipid and protein phosphorylation were found to be decreased by cholesterol depletion. The reason for this discrepancy may be linked to the absence of a washing procedure for both the lipid extract and the protein layer in our previous experiments. Although the reduction of the phosphorylation was not specific to the phospholipids, it did not result from a comparable reduction in the specific activity of the ATP pool (Table V).

Cholesterol depletion caused a decrease in ^{32}P incorporation into polyphosphoinositides and phosphatidic acid without changing their concentration, indicating that their rates of synthesis and degradation were slowed down to about the same extent, i.e., their turnover rate had been decreased. Evidence for the reduction of the activity of the degradative enzyme, polyphosphoinositide phosphodiesterase (phospholipase C) was provided by the decrease in the level of diacylglycerol in cholesterol-depleted cells in the two highest Ca^{2+} containing media. Diacylglycerol is a product of the breakdown of polyphosphoinositides, catalyzed by this enzyme. A reduction in the turnover rate of phosphatidic acid could be explained also by the inhibition of the polyphosphoinositide phosphodiesterase activity since this led to a decrease in the level of diacylglycerol and consequently by limiting the substrate availability, to a decrease in phosphatidic acid synthesis by the diacylglycerol kinase.

Ca^{2+} has been shown to be an activator of the polyphosphoinositide phosphodiesterase of the erythrocyte membrane [12]. However, Downes and Michell [26] have reported that when the enzyme was assayed under isotonic conditions, it was only activated at Ca^{2+} concentrations greater than 100 μM, suggesting that in normal erythrocytes it did not express any activity. On the other hand, Allan et al. [27] have shown that the rate of labelling of phosphatidic acid by the diacylglycerol kinase directly reflects the rate of production of diacylglycerol by the polyphosphoinositide phosphodiesterase, i.e., the activity of this latter enzyme. In the present study, phosphatidic acid labelling was decreased by 60% in control erythrocytes when the Ca^{2+} concentration in the preincubation medium was reduced from 1 mM to 28 nM (Table III). This indicates that the activity of the phosphodiesterase is detectable at normal cytosolic Ca^{2+} concentrations, i.e., 20–30 nM [28] and is decreased at lower concentrations since reducing external Ca^{2+} will reduce the Ca^{2+} influx and the cytosolic Ca^{2+} concentration. In contrast in cholesterol-depleted cells, the incorporation of ^{32}P into phosphatidic acid was independent of the external Ca^{2+} concentration suggesting that in these cells the cytosolic Ca^{2+} concentration has been decreased to the same low level regardless of the external Ca^{2+} concentration.

It remains to be seen whether the shape change observed after cholesterol depletion is linked with the decrease in the turnover rate of polyphosphoinositides and phosphatidic acid. Recent reports suggest that the ATP requiring reaction involved in the maintenance of erythrocyte shape could be a lipid phosphorylation [6,7,29]. The role of spectrin would be to assume passively the contour of the cell [30] and the transmembrane distribution of the lipids (which could be modified by a change in their rate of phosphorylation) would be at the origin of the shape transformation [6,7,31] according to the bilayer couple theory [32]. Lange

et al. [11] also have proposed that the stomatocytic transformation induced by cholesterol depletion was caused by the modification of the original asymmetrical distribution of cholesterol in the membrane. In our experiments, cholesterol depletion did not change the concentration of polyphosphoinositides except that of phosphatidylinositol 4-phosphate in the low Ca^{2+} containing medium. The concentrations of phosphatidic acid and diacylglycerol were only slightly decreased in the two high Ca^{2+} containing media. Thus the shape change occurring in cholesterol-depleted erythrocytes cannot be adequately explained with the bilayer couple theory involving changes in the phospholipid or diacylglycerol concentrations. However, this shape change can be correlated with the decrease of the turnover rate of polyphosphoinositides but not with that of phosphatidic acid. Indeed both the extent of the spherostomatocytic conversion and the decrease in polyphosphoinositides turnover rate were proportional to the extent of the cholesterol depletion and independent of the concentration of the external Ca^{2+}. In contrast, the decrease in phosphatidic acid turnover rate, although proportional to the cholesterol depletion, was dependent on the concentration of the external Ca^{2+}. That the level of phosphatidic acid synthesis was not involved in the shape change process was also demonstrated from the results obtained in control cells since their morphology was not affected when this synthesis was decreased by 60% (in the medium containing 28 nM Ca^{2+} relative to that containing 1 mM). Interestingly, the final stage of echinocytosis, i.e., the release of microvesicles in the medium induced by Ca^{2+} loading [33] or ATP depletion [34] was correlated with a breakdown of polyphosphoinositides, but neither diacylglycerol nor phosphatidic acid accumulation arising from this breakdown were found to be essential for the shedding of microvesicles. Thus, in agreement with Allan and Thomas [33] we suggest that polyphosphoinositides breakdown may be the important factor for the control of membrane curvature and erythrocyte shape change.

References

1 Nakao, M., Nakao, T., Tatibana, M. and Yoshikawa, H. (1960) J. Biochem. (Tokyo) 47, 694–695
2 Weed, R.I., Lacelle, P.L. and Merril, E.W. (1969) J. Clin. Invest. 48, 795–809
3 Sheetz, M.P. and Singer, S.J. (1977) J. Cell Biol. 73, 638–646
4 Birchmeier, W. and Singer, S.J. (1977) J. Cell Biol. 73, 647–659
5 Anderson, J.M. and Tyler, J.M. (1980) J. Biol. Chem. 255, 1259–1265
6 Fairbanks, G., Patel, V.P. and Dino, J.E. (1981) Scand. J. Clin. Lab. Invest. 41, Suppl. 156, 139–144
7 Patel, V.P. and Fairbanks, G. (1981) J. Cell Biol. 88, 430–440
8 Allan, D. (1982) Cell Calcium 3, 451–465
9 Quist, E. and Reece, K.L. (1980) Biochem. Biophys. Res. Commun. 95, 1023–1030
10 Chailley, B., Giraud, F. and Claret, M. (1981) Biochim. Biophys. Acta 643, 636–641
11 Lange, Y. and Slayton, J.M. (1982) J. Lipid Res. 23, 1121–1127
12 Allan, D. and Michell, R.H. (1978) Biochim. Biophys. Acta 508, 277–286
13 Claret, M., Garay, R. and Giraud, F. (1978) J. Physiol. (Lond.) 274, 247–263
14 Bartfaï, T. (1979) Adv. Cyclic Nuc. Res. 10, 219–242
15 Rose, H.G. and Ocklander, M. (1965) J. Lipid Res. 6, 428–431
16 Steiner, S. and Lester, R.L. (1972) J. Bacteriol. 109, 81–88
17 Farese, R.V., Sabir, M.A. and Larson, R.E. (1980) J. Biol. Chem. 255, 7232–7237
18 Freeman, C.P. and West, D. (1966) J. Lipid Res. 7, 324–327
19 Chailley, B., Weed, R.I., Leblond, P.F. and Maigne, J. (1973) Nouv. Rev. Fr. Hematol. 13, 71–88
20 Bessis, M. (1973) in Red Cell Shape (Bessis, M., Weed, R.I. and Leblond, P., eds.), pp. 1–24, Springer-Verlag, New York
21 Wolfe, L.C. and Lux, S.E. (1978) J. Biol. Chem. 253, 3336–3342
22 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
23 Rudel, L.L. and Morris, M.D. (1973) J. Lipid Res. 14, 364–366
24 Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Lipids 5, 494–496
25 Allan, D., Low, M.G., Finean, J.B. and Michell, R.H. (1975) Biochim. Biophys. Acta 413, 309–316
26 Downes, C.P. and Michell, R.H. (1982) Biochem. J. 202, 53–58
27 Allan, D., Watts, R. and Michell, R.H. (1976) Biochem. J. 156, 225–232
28 Lew, V.L., Tsien, R.T., Miner, C. and Bookchin, R.M. (1982) Nature 298, 478–481
29 Alhanaty, E. and Sheetz, M.P. (1981) J. Cell Biol. 91, 884–888
30 Lange, Y., Hadesman, R.A. and Steck, T.L. (1982) J. Cell Biol. 92, 714–721
31 Lange, Y., Gough, A. and Steck, T.L. (1982) J. Membrane Biol. 69, 113–123
32 Sheetz, M.P. and Singer, S.J. (1974) Proc. Natl. Acad. Sci. USA 71, 4457–4461
33 Allan, D. and Thomas, P. (1981) Biochem. J. 198, 433–440
34 Müller, H., Schmidt, U. and Lutz, H.U. (1981) Biochim. Biophys. Acta 649, 462–470