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PHOSPHATE DEPLETION DECREASES MITOGEN-MEDIATED STIMULATION OF PHOSPHOLIPID SYNTHESIS IN HUMAN PERIPHERAL LYMPHOCYTES

C. Rampini, C. Dubois and V. Barbu

Laboratoire de Biochimie Cellulaire
Faculté de Médecine Saint-Antoine
27 rue Chaligny, 75012 Paris, France

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Concanavalin A-mediated stimulation of ^{32}P-phosphate incorporation into phospholipids of human peripheral lymphocytes is comparatively studied in normal and phosphate-depleted media. In the phosphate-depleted medium, 2 hours after the start of cell activation, the stimulation sharply decreases for phosphatidylinositol (6.5-fold) and for phosphatidylcholine (in the latter case, the stimulation is even replaced by a slight inhibition of the incorporation). These results must be related to the rate-limiting effect of inorganic phosphate on ATP formation and thus on phospholipid synthesis, an effect which may be particularly pronounced when there is both phosphate depletion and cell activation.

The lectin-mediated stimulation of ^{32}P-phosphate incorporation into phospholipids of lymphocytes has generally been studied in phosphate-free media (1-7), in order to enhance the specific activity of the marker. In other studies, a normal phosphate-containing medium was used (8-10). Now, differences in the phosphate content of the medium may change the phosphate transport into the cell (11,12), the ATP formation (11,13), and thus it may be thought that the incorporation of the marker into phospholipids and the phospholipid synthesis itself are also influenced. To examine this point, concanavalin A -triggered stimulation of ^{32}P-phosphate incorporation into phospholipids of human peripheral lymphocytes is here comparatively studied in normal and phosphate-depleted media. We observe that phosphate depletion can sharply decrease the stimulation for both phosphatidylinositol (PI) and phosphatidylcholine (PC).

MATERIALS AND METHODS

Preparation of lymphocytes.
Normal human veinous blood is withdrawn on 250 I.U. of heparin per 10 ml of blood. After dilution at 1/2 in McCoy medium supplemented with 25 mM HEPES buffer per liter, 20 ml are layered on 10 ml of Lymphoprep (NYEGAARD, Oslo) - a solution of 9.6 % metrizoate and 5.6 % Ficoll (14) - and centrifuged at 22,000 rev./min. for 35 minutes. The fluffy interface layer is collected by aspiration. The

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cells are then washed twice in the McCoy medium and once in the incubation medium (see below), in which they are finally re-suspended. The average proportion of lymphocytes in this preparation is about 95 %.

Incubation of lymphocytes and pulse experiments.

The incubation medium is either RPMI or phosphate-free RPMI, supplemented with 25 mM HEPES buffer, 3×10^5 I.U. of penicillin and 0.3 g of streptomycin per liter, and with 10 per cent autologous plasma. 4×10^6 cells are incubated in 1.5 ml of medium, at 37°c, in glass tubes (in which further direct extraction of phospholipids will be feasible). For cell activation, the medium contains, in addition, 30 µg of concanavalin A (SIGMA) per tube.

Cells are first incubated for 2 hours with or without concanavalin A. Then, activated and non-activated cells receive 100 µCi (50 µl, i.e. 5 µg) of ^{32}P-phosphate (CEA, Saclay), for a further 1-hour incubation. The incorporation is arrested by addition of 10 ml of methanol-chloroform, 1/1, vol./vol. (15). In other experiments, the marker is directly added at the start of the incubation with or without lectin, and the incorporation is arrested after 2 1/2 hours.

Phospholipids are extracted as previously described (15), except that the extracts are washed three times (10 ml of extract, 1.5 ml of aqueous phase, and then 20 ml of chloroform). After bidimensionnal chromatography (16) on silica gel (SCHLEICHER and SCHÜLL), the separated phospholipids are counted for radioactivity by liquid scintillation.

RESULTS

The stimulation of the incorporation is evaluated by the ratio :

$$\frac{\text{cpm activated cells} - \text{cpm non-activated cells}}{\text{cpm non-activated cells}} \text{ per cent}$$

For both the 1 hour and 2 1/2 hours pulse times (especially in phosphate-containing medium), ^{32}P-phosphate incorporation is perceptible in PI and PC exclusively.

We can see Table I that upon phosphate depletion, 2 hours after the start of the cell activation, the 1-hour pulse reveals a sharp decrease in the stimulation of the incorporation for both PI and PC. For PI, the stimulation is lowered 6.5-fold. For PC, the stimulation is completely annulled, and replaced by a slight inhibition of the incorporation (30 %).

If the incorporation is performed as soon as the lectin is added and continued for a further 2 1/2 hours, Table II shows that in phosphate-containing medium, the values of the stimulation are lower than those of Table I. This result is expected, considering that, in activated cells, the specific activity of the marker increases more slowly after a 2 1/2-hours incorporation than after a shorter pulse time. More importantly, the effect of phosphate depletion is less pronounced if, as in Table II, the incorporation is effected at the start of the incubation with the lectin, than if it is effected after

TABLE I

Stimulation of ^{32}P-phosphate incorporation into PI and PC, evaluated by a 1-hour pulse performed 2 hours after the start of cell activation

	Phosphate-containing medium		Phosphate-depleted medium	
	PI	PC	PI	PC
Incorporation rate (cpm / 10^6 cells)				
non-activated cells	57 (±8)	44 (±23)	232 (±30)	146 (±13)
activated cells	188 (±32)	70 (±21)	312 (±56)	100 (±11)
Stimulation*	230 % (±32)	85 % (±49)	35 % (±19)	-33 % (±5)

PI : phosphatidylinositol. PC : phosphatidylcholine. For experimental procedure, see the text. Means of 6 experiments.

* The stimulation is expressed, for each experiment, by the ratio :

$$\frac{\text{cpm activated cells - cpm non-activated cells}}{\text{cpm non-activated cells}} \text{ per cent}$$

a lag time of 2 hours, as in Table I. This result may explain the fact that the values of the stimulation in phosphate-depleted medium, in the case of short pulses of 1 hour or less, are lower in our study (Table I) than in previous ones (1-7), in which there was no or little incubation with the lectin before the marker incorporation, and thus the effect of phosphate depletion could not be expressed.

In phosphate-depleted medium (which contains a small amount of phosphate from plasma and ^{32}P-phosphate origin), the phosphate concentration is 60-fold lower than in phosphate-containing medium (Table III), and thus the marker is 60-fold less diluted. Nevertheless, Tables I and II indicate that, for non-activated cells (1st line), the incorporation rate, in cpm, into phospholipids is only about 4- or 5-fold higher, whatever the pulse time, and equally so for PI and PC. It can be concluded that, in the phosphate-depleted medium, phosphate transport is greatly decreased, 12- to 15-fold. Similar results have been described in Erlich cells (11) and in 3T3 fibroblasts (12).

DISCUSSION

Phosphate depletion decreases concanavalin A-mediated stimulation of ^{32}P-phosphate incorporation into phospholipids of lymphocytes (Tables I and II). It is probable that phosphate deple-

TABLE II

Stimulation of ^{32}P-phosphate incorporation into PI and PC, evaluated by a 2 1/2-hours pulse performed at the start of cell activation

	Phosphate-containing medium		Phosphate-depleted medium	
	PI	PC	PI	PC
Incorporation rate (cpm / 10^6 cells)				
non-activated cells	166 (±23)	237 (±53)	845 (±38)	1287 (±181)
activated cells	355 (±75)	261 (±38)	1367 (±95)	1218 (±163)
Stimulation*	117 % (±45)	14 % (±10)	62 % (±7)	-5 % (±3)

PI : phosphatidylinositol. PC : phosphatidylcholine. For experimental procedure, see the text. Means of 6 experiments.

* The stimulation is expressed, for each experiment, by the ratio :

$$\frac{\text{cpm activated cells - cpm non-activated cells}}{\text{cpm non-activated cells}}$$

tion does not act by decreasing the stimulation of the marker transport into the cell. Indeed, it was observed, in the case of serum-activated 3T3 fibroblasts, that phosphate transport is stimulated, but the stimulation remains unchanged whatever the phosphate content in the medium (12), and the same phenomenon most likely occurs in lectin-activated lymphocytes. In addition, our results indicate that phosphate depletion affects the stimulation of the incorporation into PI and PC unequally. Thus, we rather conclude that

TABLE III

Final concentration of phosphate in the incubation media*

Phosphate origin	Phosphate-containing medium	Phosphate-depleted medium
RPMI	8.34	—
Plasma (10 per cent)	0.13	0.13
^{32}P-phosphate	0.02	0.02
Total	8.49	0.15

* µM/ml

phosphate depletion decreases the stimulation of the phospholipid synthesis pathway itself.

To explain this fact, it may be considered that, on one hand, phosphate depletion decreases phosphate transport into the cell (see above in § Results), that tends to lower the phosphate content in cells and to consequently restrict ATP formation (11), and on the other, cell activation stimulates ATP formation and phosphorylated metabolites synthesis (11,13,17,18,19). Thus, it is expected that the rate-limiting effect of phosphate depletion on ATP formation is particularly pronounced in activated cells. This result is clearly observed in Erlich cells (11), and we believe it is the same in human lymphocytes (the preparation of lymphocytes from a single donor provides an insufficient amount of cells to perform as well ATP determination). We also hypothesize that the generation of ATP is itself a limiting step in phospholipid synthesis. Finally, it may be thought that phosphate depletion restricts ATP formation, and hence phospholipid synthesis, to a greater extent in activated lymphocytes than in non-activated ones. In addition, although both, PI and PC formation require ATP, the synthesis of PI (through CDP-diglyceride) and the synthesis of PC (through CDP-choline) are very dissimilar, and so, changes in the ATP content of cells may affect these two metabolic pathways to a different degree.

Changes in PI metabolism - "PI response" - are observed by activating the division of various kinds of cells, such as lymphocytes (1,2,3), fibroblasts (20), virus-transformed fibroblasts (21). Upon lectin-activation of lymphocytes, it has been observed that the PI response, which consists in the stimulation of the PI breakdown and re-synthesis cycle, is related to early cellular events (particularly calcium influx) involved in the initial steps of the lectin action (22). It may be thought that phosphate depletion, since it decreases the stimulation of PI synthesis, is able to secondarily restrict the whole PI metabolic cycle, and thus the early blastogenic effects of lectin.

In addition, in activated lymphocytes, PC synthesis is stimulated (1,23), which corresponds to PC accumulation and membrane neo-formation (24). Between G_0 and S in the cell cycle, i.e. before the restriction point described in G_1 (25), this phenomenon is also lectin-dependent and related to the triggering of cell division (26, 27). Table IV indicates that, when ^{32}P-phosphate incorporation is evaluated, in phosphate-containing medium, by a continuous labelling performed from the start of cell activation at various intervals up

TABLE IV

Stimulation of ^{32}P-phosphate incorporation into PC, evaluated by a continuous labelling performed from the start of cell activation, in phosphate-containing medium

Time, in hours, after cell activation	2 1/2*	5	24	48
Incorporation rate (cpm / 10^6 cells)				
non-activated cells	237 (±53)	1198 (±325)	10076 (±2616)	17712 (±4304)
activated cells	261 (±38)	1576 (±377)	16377 (±3565)	27852 (±4832)
Stimulation**	14 % (±10)	34 % (±6)	62 % (±3)	57 % (±3)

PC : phosphatidylcholine. For experimental procedure, see the text. Means of 3 experiments.

* From Table II.

** The stimulation is expressed, for each experiment, by the ratio :

$$\frac{\text{cpm activated cells} - \text{cpm non-activated cells}}{\text{cpm non-activated cells}} \text{ per cent}$$

to 48 hours, the stimulation of PC synthesis is not visible early in G_1 (for instance 2 1/2 hours after cell activation). Thus, the changes we have noted in Table I, i.e. in early G_1 also (2 hours after cell activation) but by means of a short pulse of 1 hour, reveal changes in the specific activity of the phosphorylated metabolites in the PC synthesis pathway, rather than changes in PC accumulation. Nevertheless, Table I indicates that phosphate depletion triggers an early alteration in PC metabolism. Therefore, we can hypothesize that phosphate depletion would be also involved, later in G_1, in the inhibition of PC accumulation and, as in fibroblasts (28), in the inhibition of the cell division process.

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