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Phorbol 12,13-dibutyrate and mitogens increase fructose 2,6-bisphosphate in lymphocytes

Comparison of lymphocyte and rat-liver 6-phosphofructo-2-kinase

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The influence of tumour promoters and growth factors on glycolysis and on fructose-2,6-bisphosphate concentration was studied in isolated mouse spleen lymphocytes and in purified B-cells. The intracellular concentration of fructose 2,6-bisphosphate and the rate of lactate release were increased 2–3-fold in spleen lymphocytes exposed to active phorbol esters, mitogenic lectins, interleukin 4 or lipopolysaccharide. The maximal effect was observed after 1 h of exposure. In these cells hexose 6-phosphates increased 2-fold and 6-phosphofructo-2-kinase activity remained unchanged after treatment with phorbol 12,13-dibutyrate or with lectins. Exposure of B-cells to phorbol 12,13-dibutyrate, interleukin 4 or lipopolysaccharide increased the glycolytic flux and the concentration of fructose 2,6-bisphosphate without relation to their mitogenic activity.

Lymphocytes and rat liver 6-phosphofructo-2-kinase were partially purified using the same procedure. The lymphocyte enzyme was not inhibited by *sn*-glycerol 3-phosphate in contrast to the potent inhibition observed in liver. Treatment of both enzymes with the catalytic subunit of the cyclic-AMP-dependent protein kinase failed to inactivate 6-phosphofructo-2-kinase from lymphocytes. These differences suggest that lymphocytes and liver contain different forms of this enzyme.

The interaction of isolated lymphocytes with specific mitogens elicits different biochemical and morphological changes including receptor mobilization [1], changes in ionic fluxes [2], translocation of the phospholipid/Ca^{2+}-dependent protein kinase [3] and expression of c-*myc* and c-*fos* genes [4]. One of the early biochemical events observed after exposure of these cells to mitogens such as concanavalin A was an important increase in the glycolytic flux causing the release of large amounts of lactate to the incubation medium. Glucose and glutamine seem to be the main energetic fuels for resting and activated lymphocytes [5].

Fru(2,6)P_2, the most potent stimulator of PFK-1, is involved in the control of glycolysis and gluconeogenesis in liver and other tissues [6, 7]. In the case of PFK-1 partially purified from human B-lymphocytes and B-chronic lymphocytic leukemia cells, a different sensitivity to Fru(2,6)P_2 has been found [8]. In recent years it has been reported [9, 10] that Fru(2,6)P_2 increases after exposure of quiescent chicken embryo fibroblasts to the tumour promoter phorbol 12-myristate 13-acetate, mitogenic concentration of insulin or transformation by the Rous sarcoma virus. This increase in Fru(2,6)P_2 can be related to the increased glycolytic flux observed under these conditions.

The aim of the present work is to determined whether Fru(2,6)P_2 concentration can be altered after exposure of lymphocytes to tumour promoters or mitogens and to study the influence of changes in the concentration of this metabolite on the glycolytic flux. It was therefore decided to use active phorbol esters that directly activate the C-kinase [3, 11], mitogenic lectins specific for T-cells, and the specific B-cell growth factor, interleukin 4 [12–14] and the murine B-cell-specific mitogen, lipopolysaccharide [15, 16].

The kinetic properties of lymphocyte PFK-2 were compared with those of liver, suggesting the presence in lymphocytes of an isoenzymatic form different from liver.

MATERIALS AND METHODS

Materials

[^{3}H]Thymidine (24 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were from Amersham International (Amersham, UK). DEAE-cellulose (DE52) and glass-fiber filters (GF/C) were from Whatman. Blue Sepharose and Sephadex G-25 (medium) were from Pharmacia (Uppsala, Sweden). Hexose phosphates, *sn*-glycerol 3-phosphate and nucleotides were from Boehringer (Mannheim, FRG). Growth medium RPMI 1640 and other biochemicals were from Sigma (St Louis, MO, USA). Chemicals were purchased from Merck (Darmstadt, FRG). Triton X-100 was from Packard (Downers Grove,

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Abbreviations. Fru6*P*, fructose 6-phosphate; Glc6*P*, glucose 6-phosphate; Fru(2,6)P_2, fructose 2,6-bisphosphate; ConA, concanavalin A; LPS, lipopolysaccharide; IL-4, interleukin 4; PDBu, phorbol 12,13-dibutyrate; OAG, oleoylacetyl glycerol; α-PDD, α-phorbol 12,13-didecanoate.

Enzymes. PFK-1, 6-phosphofructo-1-kinase (EC 2.7.1.11); PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105); C-kinase, phospholipid/Ca^{2+}-dependent protein kinase (EC 2.7.1.37).

USA). Sterile filters were obtained from Millipore (Bedford, USA). Lipopolysaccharide (LPS, *Escherichia coli* strain 055:B5W) was from Difco (Detroit, USA) and IL-4 activity was from supernatant from COS cell line transfected with the IL-4 gene kindly provided by Dr R. Palacios (Basel Institut for Immunology, Switzerland).

Cell preparation

Lymphocytes were prepared from BALB/c mice spleens, aged 3 – 4 months. After centrifugation in a Ficoll-Isopaque discontinuous gradient (600 × *g* for 20 min) [17], the mononuclear leucocyte layer was washed and centrifuged (350 × *g* for 5 min) twice in Hepes-buffered medium lacking calcium (150 mM NaCl, 4 mM Na_2HPO_4, 0.6 mM $MgSO_4$, 0.5 mM $CaCl_2$, 6 mM glucose, 50 µM 2-mercaptoethanol, 15 mM Hepes pH 7.2). Lymphocytes were counted and resuspended at 5×10^6 cells/ml in Hepes-buffered medium for short-term incubations (less than 5 h). For long-term incubations lymphocytes were prepared under sterile conditions and incubated at 37°C at 5×10^6 cells/ml in plastic petri dishes (Costar, 6 mm diameter) in 3 ml RPMI 1640 medium (Sigma) buffered with 20 mM Hepes pH 7.2 and supplemented with 50 µM 2-mercaptoethanol, 60 µg/ml penicilin, 100 µg/ml streptomicin and 5% foetal calf serum. B-cells were obtained after depletion of T lymphocytes by incubating for 40 min the lymphocyte preparation with a monoclonal anti-Thy 1 antibody followed by treatment with rabbit complement and maintained at 5×10^6 cells/ml.

Cell incubation

For short-term incubations 5 – 8 ml lymphocyte or B-cell suspensions were placed on sterile plastic flasks (20 ml capacity) and incubated at 37°C for 15 min in a shaking water-bath (30 cycles/min) previous to the addition of the growth factors or other ligands. At appropriate times samples were collected, and the cells were pelleted (800 × *g* for 30 s) and used for the determination of enzyme activities and metabolite concentrations.

For long-term incubations the additions were performed at the beginning of the incubation period. The ligand solutions were sterilised by passing through a 0.22-µm pore-size filter.

Measurement of metabolites

To measure Fru(2,6)P_2, aliquots of 0.3 ml lymphocyte incubations were centrifuged (800 × *g* for 30 s) and the pellets heated with 0.5 ml hot 50 mM NaOH at 80°C for 10 min. Fru(2,6)P_2 was assayed in these alkaline extracts [18]. For the measurement of hexose 6-phosphate, 3-ml cell suspensions were centrifuged (800 × *g* for 30 s) and the pellets were homogenized in 1 ml ice-cold 0.5 M perchloric acid. After centrifugation (5000 × *g* for 5 min) the supernatants were neutralized with saturated K_2CO_3 and used to measure Glc6*P* and Fru6*P* enzymically [9]. Lactate release was measured in the incubation medium treated with 1 vol. 1 M perchloric acid [9].

Purification of PFK-1

PFK-1 activity was partially purified from isolated lymphocytes. Cells (2×10^8) were homogenized at 4°C in 3 ml of 100 mM KCl, 5 mM $MgCl_2$, 1 mM dithioerythritol, 5 mM potassium phosphate and 20 mM Hepes pH 7.4. After centrifugation at 100000 × *g* for 30 min, the supernatant was fractionated with poly(ethylene glycol) 6000 and the 5 – 15% pellet was resuspended in 3 ml homogenization buffer supplemented with 30% (by vol.) glycerol. After passing through a column of ATP-agarose (0.5 × 3 cm) equilibrated with resuspension buffer, PFK-1 activity was eluted with 0.5 mM MgATP, 0.1 mM Fru6*P*. Fractions containing activity were pooled and stored at –20°C. The enzyme activity was measured spectrophotometrically in a reaction mixture that contained 100 mM KCl, 5 mM $MgCl_2$, 3 mM MgATP, 1 mM potassium phosphate, 0.05 mM AMP, 20 mM Hepes pH 7.2 and the indicated concentrations of Fru(2,6)P_2. After a 5-min incubation at 37°C, the reaction was started by the addition of various concentrations of Fru6*P*.

Purification of PFK-2 from lymphocytes and rat liver

PFK-2 was partially purified from isolated lymphocytes (2×10^8 cells) and from liver of rat fed *ad libitum*. Both purifications were run in parallel essentially as previously described [19, 20]. Cells or liver were homogenized at 4°C in the presence of 4 ml ice-cold 100 mM KCl, 2 mM EDTA, 1 mM dithioerythritol, 20 mM potassium phosphate pH 7.4. After centrifugation at 100000 × *g* for 30 min, the supernatant was fractionated with poly(ethylene glycol) 6000 and the corresponding 3 – 15% pellet was resuspended in 2 ml of a medium containing 20% glycerol (by vol.), 100 mM KCl, 5 mM $MgCl_2$, 1 mM dithioerythritol and 20 mM Hepes pH 7.2 (buffer A). Both fractions were applied to columns of blue Sepharose (0.5 × 4 cm) equilibrated with buffer A. The columns were washed with buffer A and buffer A supplemented with 0.4 M KCl until the protein concentration in the eluates was negligible. PFK-2 was eluted (at 8 – 12 mM potassium phosphate) with a gradient of 0 – 25 mM potassium phosphate (pH 7.2). Fractions containing activity were pooled and stored at –30°C.

Measurement of PFK-2 activity

For the measurement of PFK-2 activity, cell extracts were prepared from lymphocyte pellets (5 – 10 × 10^6 cells) as previously indicated for the purification of the enzyme until the fractionation with poly(ethylene glycol). The 3 – 15% pellet was resuspended in buffer A lacking glycerol. PFK-2 was assayed in this buffer at 30°C in the presence of 2 mM MgATP, 2 mM Fru6*P* and 6 mM Glc6*P* in a final volume of 300 µl. Aliquots were taken at 0, 2, 5 and 10 min and were immediately treated with 1 vol. 100 mM NaOH at 80°C for 10 min. The amount of Fru(2,6)P_2 formed was measured as previously described. The reaction was linear during the time observed.

The sensitivity to inhibition by *sn*-glycerol 3-phosphate [20] was measured in blue Sepharose-purified PFK-2 from lymphocytes and liver. The assay was carried out at 30°C in the presence of buffer A lacking glycerol, and supplemented with 0.5 mM MgATP, 0.1 mM Fru6*P* and various concentrations of *sn*-glycerol 3-phosphate. The amount of Fru(2,6)P_2 formed was measured as previously described.

Incubation of PFK-2 with the catalytic subunit of cAMP-dependent protein kinase and C-kinase

Partially purified PFK-2 from lymphocytes or from liver (50 µU) was incubated at 30°C with catalytic subunit (1.5 µg) in a final volume of 0.2 ml containing buffer A without

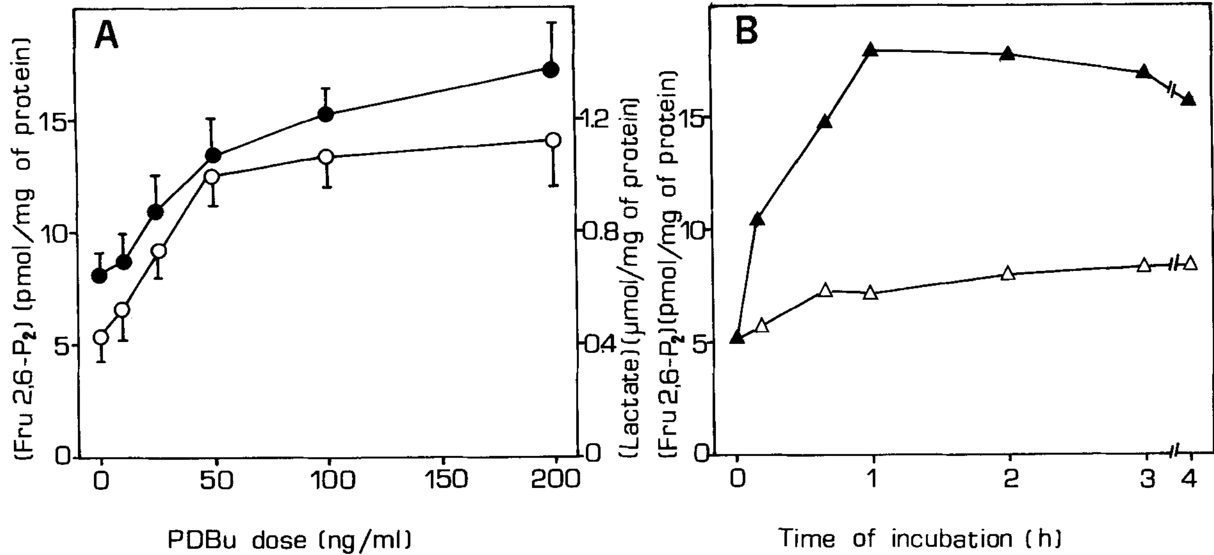

Fig. 1. *Effect of phorbol 12,13-dibutyrate (PDBu) on Fru(2,6)*P_2 *concentration and on lactate release in isolated lymphocytes.* Spleen lymphocytes were isolated and incubated at 5×10^6 cells/ml in Hepes-buffered medium. (A) Fru(2,6)P_2 (●) and lactate release (○) after a 4-h incubation. (B) Time course for the increase in Fru(2,6)P_2 concentration in lymphocytes incubated in the absence (△) or in the presence (▲) of 100 ng/ml phorbol dibutyrate. Results are means ± SEM for three separate experiments (A), or the means of two epxperiments (B)

glycerol. The reaction was started by the addition of 0.5 mM MgATP. After 10 min the reaction was stopped by the addition of 5 mM EDTA. Samples (40 µl) were assayed for PFK-2 activity at pH 6.6 (active form) and at pH 8.5 (total activity) in the presence of 100 mM KCl, 1 mM dithiothreitol, 2 mM MgATP, 1 mM potassium phosphate and 1 mM Fru6*P*. The buffers used were 50 mM Pipes pH 6.6 or 50 mM Tris/HCl pH 8.5 [21].

C-kinase activity was purified from rat brain by DEAE-cellulose (DE52) and protamine-agarose chromatography, and the activity was followed by its histone kinase activity as previously described [22]. The enzyme had a specific activity of 0.73 U/mg protein when assayed with histone H1 as substrate. PFK-2 from lymphocytes was also incubated with 10 µU C-kinase at 30°C and in a medium containing 20 µg/ml leupeptin, 100 mM KCl, 5 mM dithiothreitol, 0.5 mM MgATP and 20 mM Hepes pH 7.2, in the presence or absence of 0.5 M $CaCl_2$, 10 µg/ml phosphatidylserine and 0.5 µg/ml oleoylacetyl glycerol. Aliquots were taken after a 5-min incubation and PFK-2 was assayed for the affinity of Fru6*P* at different pH values.

Thymidine incorporation

Thymidine incorporation was measured in lymphocytes cultured for a period of 25 h. The culture was pulsed by adding 1 µCi/mol of [^{3}H]thymidine to the growth medium. After a 5-h incubation, the medium was aspirated and the cells were collected by centrifugation (800 × *g* for 30 s) and lysed in 9 ml ice-cold 0.1% SDS, 0.5% Triton X-100. After absorbing in GF/C filters, the filters were washed three times, oven-dried and counted in 2 ml scintillation medium.

RESULTS

Effect of phorbol 12,13-dibutyrate and mitogens on Fru(2,6)P_2

Initial experiments were carried out in spleen lymphocytes. As shown in Fig. 1A, when these cells were exposed to phorbol 12,13-dibutyrate in Hepes-buffered medium, a time- and dose-dependent increase was observed both in the intracellular concentration of Fru(2,6)P_2 and in the rate of lactate release. The half-maximal effect of phorbol dibutyrate was obtained at a dose of 30 ng/ml. This dose was in the range of the values to obtain the half-maximal of both translocation of the C-kinase in lymphocytes [3] and the increase in the concentration of Fru(2,6)P_2 in fibroblasts [9]. The time course of the effect of phorbol dibutyrate at 100 ng/ml of Fru(2,6)P_2 concentration is shown in Fig. 1 B. To elicit the half-maximal increase in concentration 30 min was required, and about 1 h for stability, reaching a value of 18 pmol/mg protein. Similar results were obtained with 12-O-tetradecanoyl phorbol 13-acetate.

Phorbol esters act through the activation of the C-kinase as a substitute for the requirements of diacylglycerol, without affecting the intracellular concentration of calcium [16]. Calcium mobilization together with C-kinase translocation stimulated lymphocyte growth [23, 24]. To study the effect of an increase in the cytosolic concentration of calcium on Fru(2,6)P_2, isolated lymphocytes were exposed for 45 min to the calcium ionophore A23187. As shown in Fig. 2, at concentrations lower than 0.5 µM of this ionophore, the Fru(2,6)P_2 concentration and the rate of lactate release were increased. However, higher concentrations of ionophore and longer periods of exposure decreased the cellular viability as determined by the release of lactate dehydrogenase activity to the incubation medium. The exposure of lymphocytes to both phorbol dibutyrate (50 ng/ml) and the calcium ionophore (0.5 µM) did not produce any synergistic effect on the concentration of this metabolite.

The effect of mitogenic lectins such as ConA on lymphocytes was also studied. When total spleen lymphocytes were exposed to ConA, a concentration-dependent increase was observed both in the rate of lactate release and in the concentration of Fru(2,6)P_2 (Fig. 3). Both parameters exhibited the half-maximal effect at the same concentration of ConA (0.3 – 0.4 µg/ml). Moreover, the half-maximal mitogenic activity of ConA determined by the incorporation of [^{3}H]thymidine in

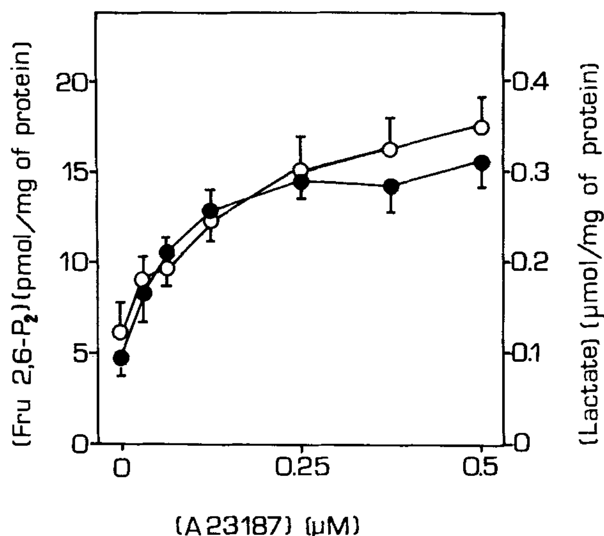

Fig. 2. *Effect of A23187 on Fru(2,6)*P$_2$ *concentration (●) and on lactate release (○) in isolated lymphocytes.* Cells were incubated for 45 min with different concentrations of a freshly prepared solution of ionophore. Results are mean ± SEM for three separate experiments

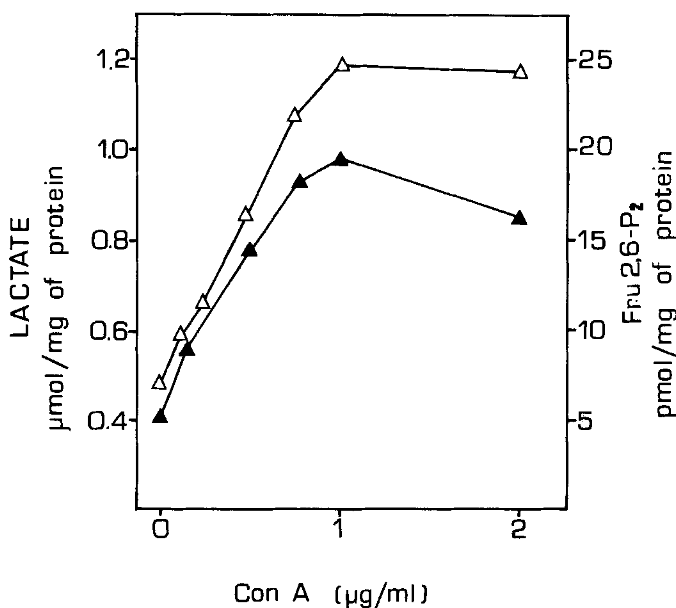

Fig. 3. *Effect of ConA on Fru(2,6)*P$_2$ *concentration and on lactate release in isolated lymphocytes.* Spleen lymphocytes (5×10^6 cells/ml) were incubated for 3 h in Hepes-buffered medium. (▲), Fru(2,6)P_2 concentration; (△) lactate release. Results are means ± SEM of three separate experiments

the macromolecular fraction over a period of incubation of 30 h was 0.30 µg/ml. This is in agreement with the observed effects on glycolysis, suggesting a correlation between activation of glycolysis and growth. Phytohemagglutinin, another mitogenic lectin for lymphocytes, induced the same pattern of changes in Fru(2,6)P_2 and on lactate release. The half-maximal effect was obtained at 1 µg/ml.

In liver and other cells a decrease has been described in Fru(2,6)P_2 concentration concomitant with an increase in the intracellular concentration of cyclic AMP [6, 25]. When lymphocytes were incubated with the permeable chlorophenylthio derivative of cyclic AMP no changes were observed in the concentration of Fru(2,6)P_2 nor in the rate of lactate release. However, a dose-dependent decrease in the concentration of Fru(2,6)P_2 was found when isolated hepatocytes were incubated with this analogue in the range of concentrations used for the incubation of lymphocytes (data not shown).

Fructose 2,6-bisphosphate in B-cells

Since spleen cell populations are a mixture of B and T lymphocytes, the results previously reported may be the consequence of activation involving one of these different types of cells. For this reason B-cells were purified and stimulated either with their specific cell growth factor (IL-4) or with a polyclonal B-cell mitogen in order to obtain cell-type-specific responses. When B-cells were incubated with phorbol dibutyrate, an increase was observed, time- and dose-dependent, in Fru(2,6)P_2 concentration and in lactate release (data not shown). This phenomenon was essentially similar to that observed in spleen lymphocytes. To establish the involvement of C-kinase activation on the acceleration of glycolysis and in the rise in the concentration of Fru(2,6)P_2, B-cells were incubated for three time periods (30 min, 4 h or 30 h) in the presence of either phorbol dibutyrate, B-cell growth factor (IL-4) or LPS. As summarized in Table 1, when B-cells were exposed to these ligands Fru(2,6)P_2 concentration was increased to different extent in all cases although without relation to their mitogenic capacity as estimated by the ability to incorporate [^{3}H]thymidine. Combination of phorbol dibutyrate with the mitogen LPS failed to act synergistically on Fru(2,6)P_2, suggesting a common way to increase its concentration.

The inactive α-phorbol 12,13-didecanoate failed to provoke changes in the rate of lactate release and Fru(2,6)P_2 concentration. These results suggest that activation of C-kinase in an early event that activates glycolysis in B-cells.

PFK-1, PFK-2 and hexose phosphates concentrations

The above-reported results suggest a correlation between lactate production and Fru(2,6)P_2 concentration in lymphocytes and in B-cells. The increase in the concentration of the bisphosphate may be a consequence either of an increase in the activity of PFK-2, or in the concentration of its substrates, or both. As shown in Table 2 no significant changes in PFK-2 activity were found, whether the enzyme was assayed at saturating (5 mM) or subsaturating (100 µM) concentrations of Fru6P. No changes in PFK-1 activity were observed under these conditions (data not shown). However, the concentration of the hexose phosphates increased after exposure of lymphocytes to 1 µg/ml of ConA (2.5-fold increase), 50 µg/ml phorbol dibutyrate (2-fold increase) and to a lesser extent when LPS was used (1.5-fold). The increase in the concentration of hexose phosphates was parallel to the changes in Fru(2,6)P_2 concentration. Moreover, these changes persisted for at least 4 h. This was in agreement with previous reports [26, 27]. The rise in the concentration of Fru(2,6)P_2 can be explained, at least in part, by the changes in Fru6P concentration. To explain the rise in the glycolytic flux, it was decided to investigate the influence on PFK-1 activity of the changes in Fru6P and Fru(2,6)P_2, substrate and effector, respectively, of this enzyme. Fig. 4 shows the relative effects of both hexoses on the activity of this enzyme, purified from lymphocytes by ATP-agarose affinity chromatography.

Table 1. *Fru(2,6)-P₂ concentration, lactate release and [³H]thymidine incorporation in isolated B-cells*

Lymphocytes (5×10^6 cells/ml) were exposed to phorbol 12,13-dibutyrate (PDBu), α-phorbol 12,13-didecanoate (α-PDD), IL-4 (1%, by vol., of supernatant containing IL-4 activity) and LPS. B-cells were incubated in Hepes-buffered medium, and with RPMI 1640 medium for thymidine incorporation. Values are means ± SEM for at least three separate experiments. [^{3}H]Thymidine uptake is given as mean of two separate experiments. n. d., not determined

Addition	Fru(2,6)P_2 after				Lactate after 4 h	[^{3}H]-Thymidine after 30 h
	10 min	20 min	30 min	4 h		
	pmol/mg protein				µmol/mg protein	dpm/mg protein
None	11 ± 1	10 ± 1	10 ± 1	9 ± 3	0.41 ± 0.09	5,100
PDBu, 50 ng/ml	10 ± 1	10 ± 2	25 ± 3	28 ± 4	0.67 ± 0.11	5,600
α-PDD, 100 ng/ml	9 ± 1	14 ± 2	10 ± 2	8 ± 3	0.40 ± 0.10	5,300
IL-4, 1%	17 ± 2	21 ± 3	16 ± 2	17 ± 2	0.54 ± 0.10	8,700
LPS, 50 µg/ml	16 ± 1	23 ± 2	23 ± 2	26 ± 3	0.94 ± 0.14	40,000
LPS + PDBu, 50 µg/ml + 50 ng/ml	n.d.	n.d.	31 ± 4	30 ± 4	0.93 ± 0.17	n.d.

Table 2. *Fru(2,6)P₂ and hexose-phosphate concentrations and PFK-2 activity in lymphocytes*

Spleen lymphocytes were incubated for 4 h in the presence of appropriate additions in Hepes-buffered medium. At the end of the incubation time, cells were centrifuged ($800 \times g$ for 30 s) and the pellets processed as described. Hexose 6-phosphates were measured from perchloric acid extracts. PFK-2 activity was assayed at pH 7.2 in the presence of 2 mM MgATP, 2 mM Fru6*P*, 6 mM Glc6*P*; or 0.1 mM Fru6*P*, 0.3 mM Glc6*P*

Addition	Fru(2,6)P_2	Glc6*P* + Fru6*P*	PFK-2 with Fru6*P* at	
			2 mM	0.1 mM
	pmol/mg protein	nmol/mg protein	µU/mg protein	
None	8 ± 2	3.2 ± 0.3	5.3 ± 0.2	3.0 ± 0.1
PDBu, 50 ng/ml	18 ± 3	6.8 ± 0.4	5.9 ± 0.3	3.2 ± 0.2
ConA, 1 µg/ml	21 ± 2	8.2 ± 0.6	5.7 ± 0.3	3.3 ± 0.2
LPS, 50 µg/ml	16 ± 3	4.8 ± 0.3	5.7 ± 0.4	3.4 ± 0.2

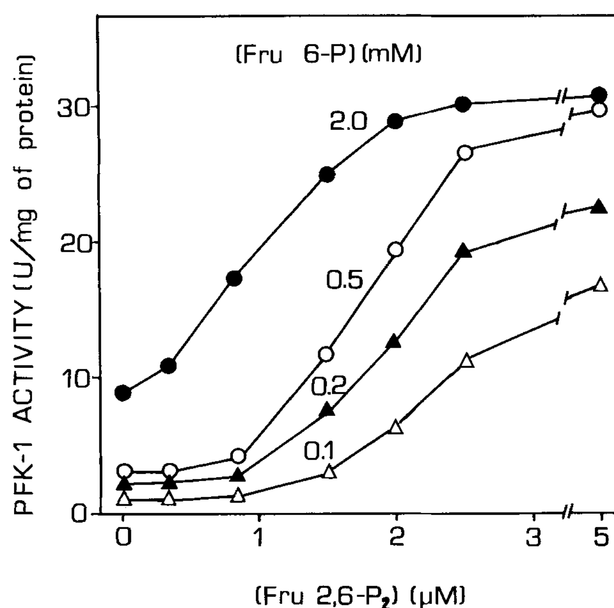

Fig. 4. *Stimulation of PFK-1 activity by Fru(2,6)P₂*. The enzyme was partially purified from lymphocytes by ATP-agarose affinity chromatography as described in Materials and Methods. The assay was performed at 37°C and pH 7.2 with 0.8 µg/ml enzyme in the presence of 3 mM MgATP, 0.2 mM AMP, 1 mM potassium phosphate and various concentrations of Fru(2,6)P_2. The reaction was started by the addition of Fru6*P*

Characterization of lymphocyte PFK-2

To characterize the nature of the PFK-2 present in mouse spleen lymphocytes, the enzyme was partially purified by blue-Sepharose affinity chromatography. Rat liver PFK-2, whose more relevant kinetic properties are known in detail [28, 29], was also purified in parallel with the lymphocyte enzyme in order to compare the physical and kinetic properties of both activities. The behaviour of both enzymes on fractionation by poly(ethylene glycol) and on the retention and elution in the blue-Sepharose column were similar. The comparison of the kinetic properties between both preparations showed important differences in some parameters. Both enzymes exhibited hyperbolic kinetics for the affinity for MgATP (K_m 0.1 M) and for Fru6*P* (K_m 80 and 50 µM for lymphocyte and liver respectively) when assayed in the presence of 1 mM potassium phosphate (Fig. 5A). However, the enzymes were different in the response to *sn*-glycerol 3-phosphate. Whereas lymphocyte PFK-2 activity remained unchanged when assayed in the presence of concentrations up to 1 mM of this metabolite, the liver enzyme exhibited the reported inhibition [30] with an apparent K_i value of about 100 µM (Fig. 5B). The lack of inhibition by *sn*-glycerol 3-phosphate has been previously reported for the purified bovine heart enzyme [20]. One of the most relevant properties of rat liver PFK-2 is to exhibit kinetic changes when the enzyme is phosphorylated by the cyclic-AMP-dependent protein kinase. These changes in the maximal activity of the enzyme are observed at acidic pH (6.6), whereas at pH 8.5 the kinetic differences between the phosphorylated and dephosphorylated enzyme are minimized [21]. When the effect of treatment with the catalytic subunit of the cyclic-AMP-dependent protein kinase was studied, it was observed that the lymphocyte enzyme failed to show kinetic changes, while under the same conditions the purified liver enzyme exhibited the reported inhibition at pH 6.6. These results are shown in Table 3. The absence of response to cyclic-AMP-dependent protein kinase is in agreement with the lack of changes observed in Fru(2,6)P_2 concentration and in lactate release in isolated lymphocytes incubated with a permeable cyclic AMP derivative.

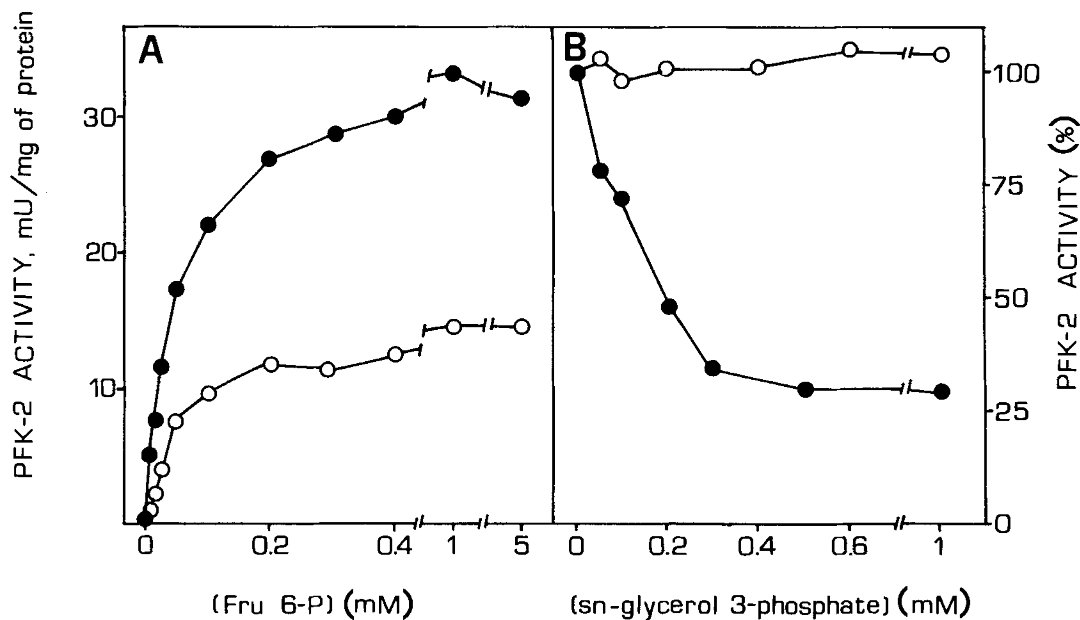

Fig. 5. *Affinity for Fru(2,6)*P_2 *and inhibition by* sn*-glycerol 3-phosphate of PFK-2 from mouse spleen lymphocyte and rat liver.* The enzyme was purified by blue-Sepharose affinity chromatography. (A) Affinity for Fru6*P*. The enzymes were assayed at pH 7.2 in the presence of 2 mM MgATP and 1 mM potassium phosphate. (B) Inhibition by *sn*-glycerol 3-phosphate. The enzymic activity was measured at pH 7.2 in the presence of 0.5 mM MgATP and 0.1 mM Fru6*P*. The activities in the absence of *sn*-glycerol 3-phosphate were 6 and 22 mU/mg protein for the lymphocyte and liver enzyme respectively. (○) Lymphocytes preparation; (●) liver enzyme

Table 3. *Effect of treatment with the catalytic subunit of the cyclic-AMP-dependent protein kinase on purified PFK-2 from mouse spleen lymphocytes and rat liver*

PFK-2 was purified by blue-Sepharose affinity chromatography. After incubation with the catalytic subunit for 10 min in the presence of 0.5 mM MgATP, PFK-2 activity was assayed at pH 6.6 and at pH 8.5 in the presence of 1 mM Fru6*P* and 2 mM MgATP. The values shown are means ± SEM for three separate experiments

PFK-2	Addition		PFK-2 activity at	
	catalytic subunit	1 mM MgATP	pH 6.6	pH 8.5
			mU/mg protein	
Lymphocyte	–	–	8.8 ± 0.9	8.4 ± 0.7
	+	–	9.1 ± 1.0	8.7 ± 1.0
	+	+	9.3 ± 1.1	8.5 ± 0.9
Liver	–	–	24 ± 3	33 ± 4
	+	–	20 ± 3	34 ± 3
	+	+	8 ± 1	32 ± 3

To ascertain whether the affinity of lymphocyte PFK-2 for Fru6*P* may be affected by C-kinase activity, partially purified enzyme (blue-Sepharose chromatography) was incubated with C-kinase purified from rat brain in the presence or absence of Ca^{2+} and phospholipids. PFK-2 activity was then assayed both at saturating (1 and 0.5 mM) and subsaturating (50 and 100 µM) concentrations of Fru6*P* and at pH 6.6, 7.2 and 8.6. Under these conditions no changes were observed in the enzyme activity, in agreement with the results obtained after exposure of lymphocytes to phorbol dibutyrate (Table 2).

DISCUSSION

The data presented in this study show that in total mouse spleen lymphocytes and in B-cells both the rate of lactate release and Fru(2,6)P_2 concentration were increased when cells were exposed either to phorbol esters, to a calcium ionophore or activated by specific lymphocyte mitogens such as lectins or LPS. The mechanism by which these different ligands act increasing the glycolytic flux may be related, at least in part, to an increase in the concentration of Fru6*P* and Fru(2,6)P_2, the substrate and effector, respectively, of PFK-1. In fact, at concentrations of substrates [ATP and Fru6*P*] and effectors [AMP, phosphate and Fru(2,6)P_2] similar to those prevailing in resting lymphocytes, this enzyme is very sensitive to simultaneous changes in Fru6*P* and Fru(2,6)P_2 concentrations. Consistent with these findings is the observation that the half-maximal effects on lactate release and on Fru(2,6)P_2 were obtained with the same doses either of phorbol dibutyrate, calcium ionophore or lectins.

The observed rise in Fru6*P* may be the consequence of the increased glucose uptake after exposure of lymphocytes to different growth factors [31, 32]. This increase, in turn, may be related to the rise in Fru(2,6)P_2 as expected for the relative values of the K_m of PFK-2 for Fru6*P* (80 µM) and the changes in substrate concentration (from 70 to 250 µM).

One of the most rapid changes induced after exposure of lymphocytes to active phorbol esters is the translocation of the C-kinase activity from cytosol to membranes, although activation of C-kinase is sufficient to increase Fru(2,6)P_2 and glycolysis either in spleen lymphocytes or in B-cells. These changes cannot be attributed to stable modifications in the kinetic properties of PFK-2 activity (i.e. phosphorylation by C-kinase). Accordingly, the increase in Fru(2,6)P_2 after exposure of lymphocytes to phorbol esters requires more than 30 min to reach stability. The changes in hexose phosphate

concentration may account for the observed acceleration of glycolysis.

The effect of phorbols on lymphocyte growth is quite different: T-cells are induced to proliferate after exposure to phorbols and to calcium ionophore [24] whereas B-cells do not undergo proliferation under these conditions [33]. ConA and phytohemoglutinin, mitogens for T-cells, increased phosphoinositol lipid turnover resulting in translocation of C-kinase activity and in mobilization of intracellular calcium [34–36]. Consistent with the translocation of C-kinase is the accumulation of Fru(2,6)P_2 and the enhancement of glycolysis observed after exposure of lymphocytes to these lectins. One of the proliferative signals elicited by the polyclonal mitogen of B-cell LPS is the translocation of C-kinase from cytosol to membranes and, as would be expected, the pattern of glycolytic changes resembled those obtained after exposure of lymphocytes to phorbol dibutyrate. When B-cell growth factor (IL-4) was used, glycolysis and Fru(2,6)P_2 were also moderately increased (60%) despite the fact that this growth factor was unable to decrease C-kinase activity from cytosol. In accordance with this, it has been shown that LPS and IL-4 bypass early mitogenic signals, such as phosphoinositol lipid degradation and calcium mobilization through an unknown mechanism [16, 37].

Lymphocyte PFK-2 was characterized and compared with the rat liver enzyme. Both enzymes were completely absorbed on a blue-Sepharose column ad were eluted under analogous conditions, suggesting a similarity between the proteins. In the presence of phosphate as effector, both enzymes, exhibited roughly the same affinity for Fru6*P*. However, the purified lymphocyte enzyme failed to be inhibited by treatment with the catalytic subunit of the cyclic-AMP-dependent protein kinase. Moreover, the enzyme does not respond to the inhibitory action of *sn*-glycerol 3-phosphate. Taken together, these results suggest important differences in the activity of both enzymes. The properties of lymphocyte PFK-2 are very similar to those described for the beef heart and foetal hepatocyte isoenzymes [20, 38].

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