Mechanism of activation of pyruvate dehydrogenase by mitogens in pig lymphocytes

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1. The activity of pyruvate dehydrogenase in extracts of pig mesenteric lymphocytes was measured under different preincubation conditions. 2. The mitogens concanavalin A and ionophore A23187 both increased pyruvate dehydrogenase activity. 3. In both cases activation required extracellular Ca^{2+} . 4. Digitonin-permeabilized cells required $0.5\ \mu\text{M}$ free Ca^{2+} for half-maximal activation of pyruvate dehydrogenase. 5. The stimulation by concanavalin A in intact cells was probably not due to changes in effectors of pyruvate dehydrogenase kinase. 6. This evidence suggests that activation of pyruvate dehydrogenase is by Ca^{2+} activation of pyruvate dehydrogenase phosphatase and supports the view that the cytoplasmic free $[\text{Ca}^{2+}]$ rises to something less than $1\ \mu\text{M}$ on stimulation with mitogens.

Increased utilization of pyruvate is a well-documented response of lymphocytes to mitogenic stimulation. The effect has been attributed to a mitogen-dependent stimulation of mitochondrial PDH, though the mechanism of stimulation remains unknown (see Hume & Weidemann, 1980).

In many tissues the activity of the intramitochondrial PDH enzyme complex is regulated by a phosphorylation–dephosphorylation cycle, catalysed by a Mg^{2+} –ATP-requiring kinase (PDH kinase, EC 2.7.1.99) and a phosphatase (PDH phosphatase, EC 3.1.3.43), which inactivate and activate the enzyme complex respectively (Denton & Halestrap, 1979). Although these enzymes are confined to the mitochondria, it has been shown that a good correlation exists between the extramitochondrial free Ca^{2+} concentration and the activity of PDH in well-coupled rat heart mitochondria (Denton *et al.*, 1980). The phosphatase is stimulated by Ca^{2+} , and the kinase is inhibited by Ca^{2+} . The kinase is also inhibited by increases in intramitochondrial [pyruvate] and increases in $[\text{NAD}^+]/[\text{NADH}]$ and $[\text{CoA}]/[\text{acetyl-CoA}]$ ratios or decreases in the

$[\text{ATP}]/[\text{ADP}]$ ratio (Cooper *et al.*, 1974; Denton & Halestrap, 1979).

Activation of PDH by mitogens in lymphocytes has not been unambiguously demonstrated. We have investigated this activation to help to clarify how the mitogenic message generated on the plasma membrane is translated into a signal capable of activating an enzyme located within the mitochondrial matrix, in particular to gain further information about the role of Ca^{2+} in this process.

Many authors (see Hesketh *et al.*, 1977; Metcalfe *et al.*, 1980) have proposed that an increase in the cytoplasmic free Ca^{2+} concentration is one of the primary events following the mitogenic stimulus of lymphocytes. Evidence for this hypothesis is based mainly on the fact that the Ca^{2+} ionophores A23187 and ionomycin can act as mitogens for these cells (Maino *et al.*, 1974; Lyall *et al.*, 1980; Metcalfe *et al.*, 1980) and by analogy with the stimulation of other cell types where extracellular Ca^{2+} is required for the stimulus at cell surface receptors (Freedman, 1979; Metcalfe *et al.*, 1980). Recently, by using a novel technique of introducing a Ca^{2+} -sensitive dye into isolated lymphocytes, changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ have been monitored continuously during the first $\frac{1}{2}$ h after mitogenic stimulus (Tsien *et al.*, 1982a,b). These authors demonstrated that at least an average doubling in $[\text{Ca}^{2+}]_{\text{cyt}}$, from 0.125 to $0.250\ \mu\text{M}$, occurred within 2 min of addition of concanavalin A to mouse thymocytes.

Lymphocyte mitochondria have Ca^{2+} -transporting systems very similar to those of liver

Abbreviations used: PDH, pyruvate dehydrogenase (EC 1.2.4.1); $[\text{Ca}^{2+}]_{\text{cyt}}$, cytoplasmic free calcium concentration; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid.

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mitochondria, and are capable of buffering the extramitochondrial free Ca^{2+} concentration at around $0.6 \mu\text{M}$ (Dippenaar & Brand, 1982). Any rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ should therefore cause a rise in intramitochondrial $[\text{Ca}^{2+}]$, which might be sensed by PDH.

Measurement of changes in the activity of Ca^{2+} -sensitive enzymes after mitogenic stimulation provides an indirect method of monitoring $[\text{Ca}^{2+}]_{\text{cyt}}$, allowing confirmation and estimation of the magnitude of the proposed rise in $[\text{Ca}^{2+}]_{\text{cyt}}$. Glycogen phosphorylase has been investigated in lymphocytes by Ruf & Gella (1982), although they did not attempt to show whether the activation they measured was due to increased $[\text{Ca}^{2+}]_{\text{cyt}}$ or to other factors such as cyclic AMP. The approach has not been used with PDH in lymphocytes.

In the present paper we show directly that an activation of PDH is an early event following mitogenic stimulation of pig lymphocytes. Our measurements of the concentrations of PDH kinase effectors tend to exclude the possibility that inhibition of PDH kinase causes PDH activation, and suggest that this effect is mediated by an activation of PDH phosphatase by Ca^{2+} . A Ca^{2+} titration of PDH activity in permeabilized cells allows an indirect calculation of the values of $[\text{Ca}^{2+}]_{\text{cyt}}$ in intact cells under resting and mitogen-stimulated conditions; the values obtained are in good agreement with the direct measurement of $[\text{Ca}^{2+}]_{\text{cyt}}$ reported by Tsien *et al.* (1982a,b).

Experimental

Materials

ATP, NAD^+ , NADH, sodium pyruvate, thiamin pyrophosphate and purified enzymes were purchased from Boehringer Corp. (London) Ltd., Lewes, East Sussex, U.K. Acetyl-CoA, EGTA, dithiothreitol, gentamycin and pigeon liver acetone-dried powder for extraction of arylamine acetyltransferase (EC 2.3.1.5) were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.; EDTA, silicone fluid MS 550 and dinonyl phthalate were from Hopkin and Williams, Chadwell Heath, Essex, U.K. RPMI medium and glutamine were from Flow Laboratories, Irvine, Scotland, U.K., and Ficoll was from Pharmacia Fine Chemicals, Uppsala, Sweden. Concanavalin A was obtained from Miles Laboratories, Slough, Bucks., U.K., and ionophore A23187 from Calbiochem, Bishops Cleeve, Herts., U.K. $[6\text{-}^3\text{H}]\text{Thymidine}$ was supplied by Amersham International, Amersham, Bucks., U.K. $p\text{-(p-Aminophenylazo)benzenesulphonic acid}$ was generously given by Dr. R. M. Denton, University of Bristol. All other reagents were of analytical grade. The modified Eppendorf Minifuge rotor described by Scott *et al.* (1980) was

purchased from the workshop of Ninewells Medical School, University of Dundee.

Preparation of pig mesenteric lymphocytes

These were prepared in standard medium (RPMI 1640) containing 20 mM-Hepes, 0.2% NaHCO_3 , 2 mM-glutamine and $10 \mu\text{g}$ of gentamycin/ml, pH 7.3 (filtered through a $0.45 \mu\text{M}$ Millipore filter), as described by Felber & Brand (1982). Cell viability was assessed by the exclusion of sodium eosin and was always higher than 93%.

Incubation of cells with mitogens and transformation assays

Samples of filtered concanavalin A stocks (made up in RPMI medium) or ionophore A23187 incorporated in vesicles of dioleoyl phosphatidylcholine (Pozzan *et al.*, 1980) were added to 10 ml of cell suspension in RPMI medium (6×10^6 cells/ml) in 15 ml sterilized glass culture tubes, pre-equilibrated at 37°C . The cell suspension was further incubated at 37°C for the times indicated, either in a humidified atmosphere of CO_2/air (1:19) or in air (in this case NaHCO_3 was replaced by 20 mM-Hepes). When parallel $[^3\text{H}]\text{thymidine}$ -incorporation assays were performed, 200 μl samples of the cell suspension were transferred (a few minutes before the end of the incubation) to wells of microplates, and mitogenic stimulation was assayed by the incorporation of $[6\text{-}^3\text{H}]\text{thymidine}$ into DNA 48 h later as described by Hesketh *et al.* (1977). The remaining cells were then pelleted and PDH was extracted as described below. Incubations of cells with mitogens were always performed under conditions optimal for subsequent thymidine incorporation.

Extraction and assay of pig lymphocyte PDH

Because of the low activity of PDH in lymphocytes [approx. 3 munits/mg of total protein (i.e. 3 nmol/min per mg), compared with around 20 munits/mg for rat heart or fat-pad], a large number of cells (around 5×10^7) was needed for each assay. Pig lymphocytes, which can be obtained easily and in quantity, were therefore used in our experiments.

Cells were pelleted by centrifugation for 2 min at 5000 g in an MSE bench centrifuge at room temperature, and the supernatant was quickly aspirated by vacuum suction. The bottom of the tube containing the cell pellet was quickly immersed in liquid N_2 . Then 100–150 μl of ice-cold extraction buffer (20 mM-Hepes, pH 7.0, 3 mM- MgCl_2 , 3 mM-EGTA, 0.5 mM-EDTA, 0.25% Triton X-100 and 3 mM-dithiothreitol) was added to the frozen cell pellet, which was then vortex-mixed vigorously until thawed, and refrozen and thawed twice to ensure complete disruption of mitochondria and extraction

of membrane-bound PDH. Mg^{2+} alone did not activate the PDH complex at any temperature tested. Addition of rat serum (10%), defatted bovine serum albumin (1%) or phenylmethanesulphonyl fluoride (50 μM) to the extraction medium did not alter the basal activity measured. The extract was kept frozen until use.

To measure total activity, the same procedure was used but the extraction buffer contained 20 mM-Hepes, pH 7.0, 20 mM- $MgCl_2$, 1 mM- $CaCl_2$, 0.5 mM-EDTA and 3 mM-dithiothreitol. The extract was incubated at 37°C for 20–30 min to convert all the enzyme into its active form. Triton (0.25%) was then added and the enzyme was extracted as above. Before assay of the samples, the frozen cell extract was thawed while being vigorously mixed and then immediately centrifuged at 14 000 g for 3 s.

PDH was assayed spectrophotometrically by coupling with arylamine acetyltransferase, essentially as described by Coore *et al.* (1971). Cell-extract supernatant (75 μl) was added to 1 ml of assay 'cocktail' [50 mM-Tris/HCl, pH 7.8, 3 mM- $MgCl_2$, 3 mM-EGTA, 0.5 mM-EDTA, 3 mM-dithiothreitol, 0.1 mM-CoA, 5 mM-NAD⁺, 0.1 mM-thiamin pyrophosphate, 1.0 mM-pyruvate, 150 μg of *p*-(*p*-aminophenylazo)benzenesulphonic acid/ml, about 100 munits of arylamine acetyltransferase, preincubated at 37°C]. The reaction rate was proportional to the volume of supernatant added. Addition to the assay of either ATP-depleting systems or NaF (to inhibit the action of PDH kinase or non-specific phosphatases respectively) did not alter the measured activity.

Ca²⁺-EGTA buffers and digitonin permeabilization of lymphocytes

Concentrations of free Ca^{2+} were controlled by the use of Ca^{2+} -EGTA buffers and calculated by using stability constants from Martell & Smith (1977).

The composition of the medium was designed to mimic roughly the cytoplasmic ionic composition, and contained 130 mM-KCl, 4 mM-NaCl, 1 mM- $MgCl_2$, 1 mM- KH_2PO_4 and 3 mM- Mg^{2+} -ATP; 3 mM-succinate was also added to ensure that the mitochondria had sufficient respiratory substrate. Double-distilled water treated by passage through a Milli-Q ion-exchange apparatus was used to decrease contaminant Ca^{2+} . All glassware was washed with 5 mM-EGTA and thoroughly rinsed with Milli-Q-treated water.

Lymphocytes (6×10^7 cells/ml) preincubated at 37°C in normal RPMI medium were centrifuged on an Eppendorf bench centrifuge for 3 s and washed once in the appropriate Ca^{2+} buffer at 37°C. The cell pellet was carefully resuspended in the same Ca^{2+} buffer pre-equilibrated at 37°C, and digitonin was added from a stock in dimethyl sulphoxide to

give a final concentration of 60 μg of digitonin/ml (around 10 μg of digitonin/ 10^7 cells). This concentration of detergent produced 100% release of lactate dehydrogenase and negligible release of glutamate dehydrogenase and PDH (results not shown). The cells were incubated in the various Ca buffers for 5–10 min at 37°C and then pelleted in an Eppendorf bench centrifuge for 3–5 s. PDH was extracted and assayed as described above.

Rapid separation of lymphocyte mitochondria

The technique used by Scott *et al.* (1980) to separate mitochondria rapidly (about 15 s) from other cellular components of synaptosomes has been used for lymphocytes. Briefly, 1 ml of a lymphocyte suspension (1×10^7 – 2×10^7 cells/ml), incubated at 37°C with or without mitogen in Hepes-buffered RPMI medium for the times indicated, was rapidly mixed with digitonin dissolved in dimethyl sulphoxide (1–7 μl) in a plastic 2 ml syringe. This suspension was immediately injected through a 23-gauge needle into Eppendorf tubes in a specially adapted rotor of an Eppendorf bench Minifuge (model 5412), which was already spinning at maximum speed at 4°C. The joint action of the detergent and the shear forces resulting from the passage of the cells through the needle disrupts the plasma membrane of the cells, liberating intact mitochondria and other intracellular organelles.

Fig. 1. *Rapid separation of lymphocyte mitochondria*

Lymphocytes (2.5×10^7 cells/ml) were fractionated as described in the Experimental section with increasing digitonin concentrations. Volume of solvent (dimethyl sulphoxide) was never higher than 0.8%. Activities of lactate dehydrogenase (●), adenylate kinase (Δ) and citrate synthase (■) measured in the supernatant from the fractionation are expressed as percentages of total activity.

These heavy intact structures are spun for 1 min through 250 μ l of an oil layer of density 1.03 g/ml [a 3:2 (v/v) mixture of silicone oil and dinonyl phthalate] into 40 μ l of 0.25 M-sucrose/4% (w/v) HClO_4 /1 mM- CaCl_2 , while the soluble cytoplasmic contents remain in the supernatant. Activities of marker enzymes from the cytoplasm (lactate dehydrogenase), mitochondrial matrix (citrate synthase) and mitochondrial intermembrane space (myokinase) measured in the supernatant (plus 0.1% Triton) were taken as a measure of the disruption of the plasma membrane and damage caused to the mitochondria by increasing digitonin concentrations (Fig. 1). A concentration of 20 μ g of digitonin/ 2.5×10^7 cells per ml was routinely used to separate the mitochondria from the cells, with about 80% of the mitochondria intact. When measuring the metabolite concentrations associated with the infranatant, the amount of metabolite released into the supernatant owing to damage to a fraction of the mitochondria (as judged by citrate synthase and myokinase released into the supernatant) was variable. A correction was made for each experiment.

Extraction and assay of metabolites

Immediately after centrifugation the bottom of the Eppendorf tube was immersed in liquid N_2 . The supernatant and oil layer were removed by vacuum suction. After careful removal of any remaining traces of oil with paper tissue, the infranatants were slowly thawed by constant vortex-mixing at room temperature. The precipitated protein was removed by centrifugation for 2 min in the bench centrifuge, and the supernatant was adjusted to pH 7.0 (for pyruvate analysis) or pH 6.0 (for CoA and acetyl-CoA analysis), with 0.6 M-KOH and either 1.0 M- K_2HPO_4 or 0.1 M-Mops. Metabolites were analysed in this extract after removal of precipitated KClO_4 .

Pyruvate was analysed fluorimetrically (Passonneau & Lowry, 1974), as quickly as possible after neutralization of the infranatant, which was kept frozen in liquid N_2 until the assay was performed (never longer than 2 h). Neutralized infranatants for analysis of CoA and acetyl-CoA were also analysed by a fluorimetric method, by coupling with phosphate acetyltransferase (EC 2.3.1.8) and citrate synthase, by using the reaction of malate dehydrogenase as the indicator (Michal & Bergmeyer, 1974; Garland, 1974). Neutralized infranatants were kept frozen for a maximum of 24 h in liquid N_2 before the assay was performed.

The volume of lymphocytes remains unchanged during the first 1 h of mitogenic stimulation, and mitochondria make up 5% of the volume of pig lymphocytes (Brand & Felber, 1983). Cytoplasmic and mitochondrial volumes of 1.8 μ l/ 10^7 cells (Felber & Brand, 1982) and 0.09 μ l/ 10^7 cells were used

throughout to calculate the cellular concentrations of metabolites.

Other enzymic analyses

Lactate dehydrogenase (Bergmeyer & Bernt, 1974), citrate synthase (Bergmeyer *et al.*, 1974), glutamate dehydrogenase (Schmidt, 1974) and adenylate kinase (Bergmeyer *et al.*, 1974) were assayed spectrophotometrically by classical methods as indicated. Enzyme activities are expressed as percentages of the total amount present in a non-disrupted cell suspension treated with 0.1% Triton for 10 min on ice.

Results

Effect of concanavalin A on pig lymphocyte PDH

Concanavalin A activated PDH in intact pig mesenteric lymphocytes at both mitogenic and supramitogenic concentrations (Fig. 2). A consistent, albeit small, maximal increase in PDH activity of around 40% over controls was observed. Maximal activation was approximately similar for both

Fig. 2. Time-dependence of lymphocyte PDH activation induced by concanavalin A

Lymphocytes (6.5×10^6 cells/ml) were incubated in glass culture tubes with 2.5 (●) or 25.0 (○) μ g of concanavalin A/ml. At the times indicated, cells were pelleted and extracted as described in the Experimental section. Values are means for two different incubations assayed in duplicate. Maximal activity was 122.3 ± 4.2 nmol/h per 10^7 cells (mean $\pm$ S.E.M. for three assays).

Fig. 3. Effect of [concanavalin A] on lymphocyte PDH activity

Cells were incubated with increasing concanavalin A concentrations for 1 h, as described in Fig. 2. A few minutes before the end of the incubation, 0.2 ml samples were transferred into micro-plates and [³H]thymidine incorporation was measured after 48 h culture (●). Remaining cells were extracted and PDH was assayed as described in the Experimental section (○). Maximal PDH activity was 123.0 ± 4.8 nmol/h per 10^7 cells. Results are means of two incubations of a representative experiment assayed in duplicate.

concanavalin A concentrations tested, although activation was faster with supramitogenic concentrations. The activation remained for over 5 h.

Fig. 3 shows the effect of different concentrations of concanavalin A on PDH activity after incubation for 1 h. Superimposed is the dose-dependence of [³H]thymidine incorporation measured 48 h later in the same cells. PDH activity increased linearly with concanavalin A concentration, whereas [³H]thymidine incorporation was maximal at 2–3 μg/ml and declined to background values at 20 μg/ml. The decrease in [³H]thymidine incorporation at high concanavalin A concentration is a well-established phenomenon and probably reflects a late inhibition of DNA synthesis, since most early events in mitogenic stimulation, as well as the PDH activation seen here, are triggered by high concanavalin A concentrations. The maximal magnitude of the stimulation of PDH varied from 20 to 50% activation with respect to controls, depending on the cell preparation [average increase was $32 \pm 3\%$ over controls (S.E.M.; $n = 10$)], although basal values were usually similar (20–30% of total activity).

Table 1 shows that PDH activation induced by either optimal or supraoptimal concanavalin A

Table 1. Ca^{2+} -dependence of concanavalin A-induced activation of PDH in lymphocytes

Cells were incubated for 1 h in medium with additions as shown, then PDH was extracted and assayed. Total activity was 99.05 ± 2.15 nmol/h per 10^7 cells. Results are means of duplicate measurements of one representative incubation. At 2.5 μg/ml, concanavalin A is mitogenic; 25 μg/ml is supramitogenic.

Addition	PDH activity (nmol/h per 10^7 cells)
None (control)	29.80
10 mM-EGTA	30.95
Concanavalin A (2.5 μg/ml)	43.85
Concanavalin A + 10 mM-EGTA (2.5 μg/ml)	31.70
Concanavalin A (25.0 μg/ml)	46.15
Concanavalin A + 10 mM-EGTA (25.0 μg/ml)	30.95

concentrations in pig lymphocytes was strictly dependent on the presence of Ca^{2+} in the extracellular medium. Addition of high concentrations of EGTA to the incubation medium had no appreciable effect on the basal activity.

Thus mitogens activate PDH. Was the activation via inhibition of PDH kinase or stimulation of PDH phosphatase?

Effect of mitogens on PDH kinase

Dichloroacetate is an inhibitor of PDH kinase. It increases the proportion of the active dephosphorylated form of PDH from different sources, binding probably to the same site as pyruvate (Whitehouse *et al.*, 1974). Fig. 4 shows that PDH activity was doubled in intact cells in 15 min by dichloroacetate; 10 mM-pyruvate also doubled PDH activity (results not shown). Maximal activity could not be induced by either of the two agents tested, which indicates that the kinase remained partially active. Thus pig lymphocyte PDH kinase was responsive to at least two effectors described for the enzyme from other sources.

Activation of glycolysis and an increased lactate output are well-known responses of lymphocytes after mitogenic stimulation (Hume & Weidemann, 1980); this would be expected to be correlated with an increased cellular concentration of pyruvate. Since pyruvate is an inhibitor of PDH kinase activity, an increase of mitochondrial pyruvate concentration could be the mechanism of mitogen-induced activation of PDH. To determine whether concanavalin A-dependent PDH activation was due to an effect on PDH kinase activity, the concentrations of pyruvate and of some of the other known PDH kinase effectors in the cytoplasm and in the mitochondria of lymphocytes treated with concanavalin A were measured.

The amount of pyruvate associated with these lymphocytes did not change measurably even after 2 h of mitogenic stimulation. A constant 3.8-fold gradient of pyruvate across the mitochondrial membrane was calculated from the pyruvate concentrations in the cytoplasm (0.88 mM) and mitochondrial matrix (3.34 mM), values which are slightly higher than those found for hepatocytes (0.64 and 1.08 mM respectively; Siess *et al.*, 1977). When this experiment was repeated with mouse thymocytes, a doubling in the total pyruvate content of cells was detected (results not shown).

An increase in the mitochondrial [CoA]/[acetyl-CoA] ratio in the mitochondria might also activate PDH. However, Table 2 shows that, although a change in this ratio in intact cells could not be detected, a redistribution of these metabolites occurred inside the cell after mitogenic stimulation, and

was reflected by a 20% fall in the [CoA]/[acetyl-CoA] ratio within the mitochondria. Although the cytoplasmic [CoA]/[acetyl-CoA] ratio is similar to the value of 1.2 published for hepatocytes (Siess *et al.*, 1977), the mitochondrial ratio measured in the present work was lower (1.5, compared with 5). Thus PDH activation could not be explained by an altered [CoA]/[acetyl-CoA] ratio.

Two other parameters are known that could affect the activity of PDH kinase. Decreases in the [ATP]/[ADP] ratio and increases in the [NAD⁺]/[NADH] ratio of mitochondria can inactivate the kinase, activating the complex. The rate of oxygen consumption by pig mesenteric lymphocytes does not change over the first 1 h of concanavalin A activation (Corps, 1982), which implies that the mitochondrial [ATP]/[ADP] ratio is kept constant (see, e.g., Jacobus *et al.*, 1982). Also, no change in the total [ATP]/[ADP] ratio has been observed under the same conditions (Corps, 1982).

The intramitochondrial [NAD⁺]/[NADH] ratio in lymphocytes is likely to be considerably less than 4, since in human peripheral lymphocytes the total [NAD⁺]/[NADH] ratio is constant at about 4 for the first few hours of stimulation (Blomquist *et al.*, 1976), and in those cells studied the ratio is lower in the mitochondrial matrix than in the cytoplasm (Williamson *et al.*, 1967; Siess *et al.*, 1977). Extrapolating studies on the rat heart enzyme (Williamson *et al.*, 1967; Hansford, 1976; Siess *et al.*, 1977) to lymphocytes, we calculate that there would be no effect of the intramitochondrial [NAD⁺]/[NADH] ratio on PDH activity below [NAD⁺]/[NADH] ratios of about 7. If these assumptions hold, then the concanavalin A stimulation of PDH is not caused by changes in the intramitochondrial [NAD⁺]/[NADH] ratio.

Fig. 4. PDH activation by dichloroacetate. Cells were incubated with 10 mM-dichloroacetate, at a cell density of 6×10^6 cells/ml, for the times indicated. Maximal PDH activity was 106.4 ± 8.0 nmol/h per 10^7 cells. The insert demonstrates the effect of [dichloroacetate] on pig lymphocyte PDH after the cells were incubated for 30 min with this agent.

Effect of mitogens on PDH phosphatase

The lack of evidence for activation of PDH via inhibition of the kinase suggests that mitogens may act by activation of the phosphatase. The most obvious candidate for PDH phosphatase activation is an increase in intramitochondrial free $[Ca^{2+}]$.

To check that PDH phosphatase in lymphocytes

Table 2. Concanavalin A-induced change of mitochondrial [CoA]/[acetyl-CoA] ratio in lymphocytes. Lymphocytes (2×10^7 cells/ml) were incubated with mitogenic concentrations of concanavalin A ($3 \mu\text{g/ml}$) at 37°C . After 1 h the content of metabolites associated with the total sample or with the mitochondria rapidly separated from the soluble cytoplasmic fraction as described in the Experimental section was assayed. The results are means ($\pm$ S.E.M.) for three incubations assayed in duplicate.

Sample	Total			Mitochondrial		
	CoA (mM)	Acetyl-CoA (mM)	Ratio	CoA (mM)	Acetyl-CoA (mM)	Ratio
Control	0.60 ± 0.03	0.33 ± 0.01	1.8	5.1 ± 0.2	3.33 ± 0.1	1.53
Concanavalin A	0.60 ± 0.05	0.33 ± 0.02	1.8	6.7 ± 0.1	5.55 ± 0.3	1.20

Fig. 5. Effect of free $[Ca^{2+}]$ on PDH activity of lymphocytes treated with digitonin

Cells (6×10^7 cells/ml) were incubated in medium containing Ca^{2+} -EGTA buffers, supplemented with 2 mM-succinate, 3 mM- Mg^{2+} -ATP and 60 μg of digitonin/ml, for 10 min at 37°C. PDH was then extracted and assayed as described in the Experimental section. Maximal activity was 119.3 ± 2.1 nmol/h per 10^7 cells. Resting-state activity was 30% of maximal activity. Results are means of duplicate assays from two different incubations.

Fig. 6. Effect of $[A23187]$ on lymphocyte PDH

Cells were incubated as described in Fig. 3 but with A23187. PDH (O) and $[6\text{-}^3H]$ thymidine incorporation (●) were measured as described in the Experimental section. Foetal-calf serum (10%) was added at 30 h of culture to improve cell viability. Maximal activity of PDH was 116.7 ± 2.3 nmol/h per 10^7 cells. Results are means of two incubations assayed in duplicate.

Fig. 7. Time-dependence of lymphocyte PDH activation induced by A23187

Lymphocytes were incubated with (∇) 43 nM-, ($\square$) 86 nM- or (O) 830 nM-A23187 for the times indicated, under the same conditions as described in Fig. 6. Maximal activity was 116.7 ± 2.3 nmol/h per 10^7 cells (mean $\pm$ S.E.M. for three different assays). Two incubations assayed in duplicate were performed for each point.

was Ca^{2+} -sensitive, and to ascertain its sensitivity to extramitochondrial free Ca^{2+} concentrations in the physiological range, we performed a Ca^{2+} titration on lymphocytes whose plasma membrane was rendered permeable by treatment with digitonin, as described for hepatocytes (Murphy *et al.*, 1980). Lymphocytes were incubated in a medium designed to mimic the cytoplasmic ionic composition containing different free $[Ca^{2+}]$ (EGTA-buffered). Succinate and ATP were added to ensure that the mitochondria were respiring and that PDH kinase was functional. Oxygen-electrode measurements indicated that the mitochondria were as well coupled as in untreated cells (respiratory control index of 3) (results not shown). Fig. 5 shows that there was a clear response of PDH to increasing extra-mitochondrial free $[Ca^{2+}]$, with a half-maximal activation at around $0.5 \mu M$.

Thus the sensitivity of PDH *in situ* to $[Ca^{2+}]_{cyt}$ is compatible with the hypothesis that the mitogen-stimulated rise in $[Ca^{2+}]_{cyt}$ from about 100 nM to about 250 nM (Tsien *et al.*, 1982a,b) causes the increase in PDH activity that we observe.

To investigate this further, we examined the effect of Ca^{2+} ionophores on PDH activity. Fig. 6 shows

Table 3. Ca^{2+} -dependence of A23187-induced PDH activation in lymphocytes

Cells were incubated with mitogenic concentrations of ionophore A23187 for the times indicated at 6.5×10^6 cells/ml in medium with or without 10 mM-EGTA, after which PDH was extracted and assayed. Maximal activity was 122.4 ± 1.3 nmol/h per 10^7 cells. Results are expressed as percentage of maximal activity and are means of duplicate assays of two different incubations.

A23187 (80 nM)	EGTA	Time (min)	Activity (% of maximal)
—	—	30	29.4
—	+	30	28.8
+	—	30	57.0
+	+	30	22.2
—	—	60	28.8
—	+	60	29.0
+	—	60	67.0
+	+	60	20.0

that ionophore A23187 at mitogenic concentrations caused activation of the enzyme. Fig. 7 shows that mitogenic doses of A23187 caused doubling of enzyme activity in 1 h; supramitogenic doses gave greater stimulation more quickly. The viability of the cells (measured by eosin exclusion) was unaltered during the incubation period.

The activation was again strictly dependent on the presence of Ca^{2+} in the extracellular medium (Table 3). PDH activation could also be induced by another mitogenic Ca^{2+} ionophore, ionomycin (Lyll et al., 1980) (results not shown).

Discussion

In the experiments described in the present paper, we show that pig mesenteric lymphocyte PDH is similar to the enzyme from other sources, since it is activated by increased Ca^{2+} (phosphatase activation) and by dichloroacetate or pyruvate (kinase inhibition).

We then show that concanavalin A or ionophore A23187, which are mitogens for these cells, activate PDH. A similar activation of pyruvate oxidative metabolism by concanavalin A has been reported in rat thymocytes (Hume et al., 1978a,b) and in rat mesenteric lymphocytes (Beachy et al., 1981), although these studies did not unambiguously show activation of PDH. One question not answered by these earlier studies is whether the activation reflects a rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ or whether it is due to changes in the concentrations of other effectors of the complex.

Our experiments show that PDH stimulation is not caused by changes in [pyruvate] or the $[\text{CoA}]/[\text{acetyl-CoA}]$ ratio; neither does activation by alteration of $[\text{ATP}]/[\text{ADP}]$ or $[\text{NAD}^+]/[\text{NADH}]$ ratios seem likely. Therefore an inhibition of PDH

kinase is probably not involved in the mechanism of concanavalin A-induced stimulation of PDH activity [except perhaps via Ca^{2+} inhibition of the enzyme (Cooper et al., 1974)].

We cannot exclude an activation by other less well-characterized factors (see, e.g., Seals & Czech, 1980), but the simplest interpretation remains that concanavalin A-induced PDH activation is due to an effect on PDH phosphatase through an increase in mitochondrial $[\text{Ca}^{2+}]$. Although for technical reasons we have not been able to provide direct definitive evidence for a rise in mitochondrial free $[\text{Ca}^{2+}]$, our interpretation is supported by several pieces of indirect evidence.

(a) A rapid rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ has been shown, by using an intracellularly trapped Ca^{2+} indicator, to be an early event in mitogenic stimulation in pig lymphocytes (Tsien et al., 1982a,b).

(b) Mitochondria from pig lymphocytes take up Ca^{2+} in the physiological range (Dippenaar & Brand, 1982).

(c) Two chemically unrelated Ca^{2+} ionophores, A23187 and ionomycin, can both activate PDH in the short term at mitogenic concentrations.

(d) The effects of concanavalin A and of the Ca^{2+} ionophores are strictly dependent on extracellular Ca^{2+} .

(e) PDH activity in digitonin-treated cells is extremely sensitive to extramitochondrial free $[\text{Ca}^{2+}]$ in the range 0.1–1 μM .

From the data in Fig. 5 we can, in principle, calculate the rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ caused by mitogenic concentrations of concanavalin A or ionophore A23187. If we assume that all the cells in the preparation respond in the same way, the rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ would be from 100 nM to 250 nM for concanavalin A, or from 100 nM to 1 μM with A23187. However, the calculation is complicated by the heterogeneity of our cell preparation, in which we estimate that only about 50% of the cells are T-cells responsive to concanavalin A. If we assume that only these cells change PDH activity with concanavalin A, then the rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ in this case would be from 100 nM to 400 nM. These values agree remarkably well with those obtained by direct measurement in mouse thymocytes (Tsien et al., 1982a,b) and in pig lymphocytes (T. Pozzan, R. Y. Tsien & T. J. Rink, unpublished work).

We can also estimate intramitochondrial free $[\text{Ca}^{2+}]$. If pig lymphocyte PDH is half-maximally activated at about 1 μM intramitochondrial $[\text{Ca}^{2+}]$, as it is in other systems (Denton & Halestrap, 1979), then the Ca^{2+} gradient across lymphocyte mitochondria is small, about 2-fold, as it is in rat heart mitochondria (Denton et al., 1980).

In conclusion, we have shown directly for the first time that the polyclonal T-cell mitogen concanavalin A activates the key enzyme of oxidative

metabolism. PDH. We have shown that this mitogen-dependent PDH activation is probably not caused by inhibition of PDH kinase activity, and we have therefore concluded that it is due to an increase in PDH phosphatase. Finally we have shown that PDH activity in permeabilized cells responds to variations of extramitochondrial Ca^{2+} in the range 0.1–1 μM , i.e. the range of cytosolic Ca^{2+} changes that have been reported to occur in response to polyclonal mitogens.

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