FUNCTION OF CALCIUM IONS IN PYRUVATE DEHYDROGENASE PHOSPHATASE ACTIVITY*

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Summary

Phosphorylation and dephosphorylation of the pyruvate dehydrogenase (PDH) component of the mammalian pyruvate dehydrogenase complex are catalyzed, respectively, by a $MgATP^{2-}$-requiring kinase and a Mg^{2+}-requiring phosphatase. The kinase and the PDH, but not the phosphatase, are tightly bound to the dihydrolipoyl transacetylase core of the complex. Evidence is presented that the activity of the PDH phosphatase from bovine kidney and heart is increased about tenfold when it is attached to the transacetylase. Ca^{2+} is required to bind the phosphatase, but not the kinase or the PDH, to the transacetylase, thereby facilitating the Mg^{2+}-dependent dephosphorylation of the phosphorylated PDH. Ca^{2+} lowers the apparent K_m of the phosphatase for phosphorylated PDH about twentyfold. The Ca^{2+}-controlled association of the phosphatase and the transacetylase could provide an important mechanism for regulation of the phosphorylation-dephosphorylation cycle.

Linn et al. (1,2) found that the activity of the pyruvate dehydrogenase complex from bovine kidney and heart and porcine liver mitochondria is regulated by a phosphorylation-dephosphorylation cycle. Phosphorylation and concomitant inactivation of the complex is catalyzed by a $MgATP^{2-}$-requiring kinase, and dephosphorylation and concomitant reactivation is catalyzed by a Mg^{2+}-requiring phosphatase. Similar observations have been made with preparations of the pyruvate dehydrogenase complex from porcine heart muscle (3), porcine and bovine brain (4,5,6), and rat epididymal fat cells (7,8).

The mammalian pyruvate dehydrogenase complex contains a core, consisting of dihydrolipoyl transacetylase, to which pyruvate dehydrogenase (PDH), the kinase, and dihydrolipoyl dehydrogenase are tightly bound (9,10,11). In contrast to the kinase, the phosphatase appeared to be loosely associated with the complex (1,2,3,11,12). Phosphorylation and dephosphorylation occur on a seryl residue in one of the two nonidentical chains of the pyruvate dehydrogenase (1,13,14).

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Siess and Wieland (12) reported a marked inhibition of porcine heart PDH phosphatase by ethylene glycol bis-(2-aminoethyl ether)-N,N'-tetraacetate (EGTA) in the presence of Mg^{2+} and protection by Ca^{2+} against this inhibition. Hucho et al. (15) made similar observations with the bovine kidney and heart PDH phosphatases. Denton et al. (16) observed that use of EGTA in the isolation of fat-cell mitochondria resulted in decreased PDH phosphatase activity in mitochondrial extracts. They showed, with CaEGTA buffers, that the Mg^{2+}-requiring phosphatase from pig heart, pig kidney cortex and rat epididymal fat-cell mitochondria is markedly stimulated by Ca^{2+} (0.1-10 μM). It appeared from their data that Ca^{2+} activates the PDH phosphatase only when Mg^{2+} is present and that the effects of these two cations are not interchangeable. In this paper we present evidence that Ca^{2+} is required to bind PDH phosphatase to the transacetylase, thereby facilitating the Mg^{2+}-dependent dephosphorylation of the phosphorylated PDH.

MATERIALS AND METHODS

Highly purified preparations of the bovine kidney and heart pyruvate dehydrogenase complexes and their component enzymes were obtained essentially as described previously (11).

Phosphorylation of PDH: The reaction mixture contained 12 mg of crystalline PDH and 0.25 mg of purified PDH kinase from bovine kidney (11), 0.02 M potassium phosphate buffer (pH 7.5), 0.013 M dithiothreitol, 1.4 mM $MgCl_2$, and 0.29 mM [γ-^{32}P]ATP (2.4 mCi/mmole) in a final volume of 0.7 ml. The mixture was incubated for 30 min at 30° and then filtered through a column of Sephadex G-25 (1 x 13 cm) which had been equilibrated at 4° with 0.02 M imidazole buffer (pH 7.0). The radioactive protein fraction was collected and dialyzed overnight against the imidazole buffer. Protein was determined by the biuret method (17), and protein-bound radioactivity was measured as described below. The phosphorylated PDH contained 0.60-0.72 nmoles of ^{32}P-labeled phosphoryl groups/nmole of enzyme (mol wt 154,000) (13).

Assay of PDH phosphatase: The assay is based on the initial rate of

dephosphorylation of phosphorylated PDH in the presence of the phosphatase and 10 mM Mg^{2+}. The reaction mixture contained 0.16 mg of ^{32}P-labeled PDH, an appropriate amount of PDH phosphatase, and 10 mM $MgCl_2$ in a final volume of 0.2 ml of 0.02 M potassium phosphate buffer (pH 7.0). Phosphatase was omitted from the control. The mixture was incubated for 5 min at 30° prior to addition of $MgCl_2$, which was added last. Aliquots (0.05 ml) were withdrawn at 1-min intervals after addition of $MgCl_2$ and were applied to 2.2-cm disks of Whatman No. 3MM filter paper. The papers were washed four times with cold 10% trichloroacetic acid, twice with ethanol, and twice with ether (11). The papers were air-dried and then counted in a liquid scintillation spectrometer. The extent of dephosphorylation of the phosphorylated PDH was limited to less than 50% to ensure linearity of the response. Modifications of the phosphatase assay components and conditions are noted in the text.

RESULTS AND DISCUSSION

Linn et al. (1) noted that preparations of phosphorylated PDH (bovine kidney) underwent dephosphorylation at a slow rate in the presence of PDH phosphatase and 10 mM Mg^{2+} and that the rate was markedly accelerated when dihydrolipoyl transacetylase was added to the incubation mixture. Further investigation of this finding has revealed that the stimulatory effect of the transacetylase is inhibited by EGTA and that this inhibition is reversed by Ca^{2+} (Table I). However, EGTA did not inhibit the reaction between the phosphatase and phosphorylated PDH in the absence of the transacetylase. In the presence of Ca^{2+} and absence of Mg^{2+}, dephosphorylation did not occur. Similar results were obtained with the PDH phosphatase and the transacetylase from bovine heart mitochondria (Table I). It appears that Ca^{2+} does not participate directly in the dephosphorylation reaction. Presumably, a PDH phosphate-Mg complex is the substrate for the phosphatase. Ca^{2+} does not appear to be involved in the binding of PDH to the transacetylase, since EGTA did not inhibit the reconstitution of the pyruvate dehydrogenase complex (PDC) from pyruvate dehydrogenase, the transacetylase, and dihydrolipoyl dehydrogenase (Table II).

Table I

Effects of Transacetylase, Ca^{2+}, and Mg^{2+} on PDH Phosphatase Activity

Incubation mixtures[a]	Phosphatase activity nmoles $^{32}P_i$ released/ min/mg phosphatase	
	Kidney	Heart
Phosphatase	2.6	2.1
Phosphatase + Ca^{2+}	2.7	3.2
Phosphatase + EGTA	2.8	3.3
Phosphatase + EGTA + Ca^{2+}	3.3	4.3
LTA	0.0	0.0
Phosphatase + LTA	22.8	20.5
Phosphatase + LTA + Ca^{2+}	34.2	17.5
Phosphatase + LTA + Ca^{2+} - Mg^{2+}	0.0	0.0
Phosphatase + LTA + EGTA	5.8	5.6
Phosphatase + LTA + EGTA + Ca^{2+}	32.1	27.7

[a]The incubation mixtures contained 0.16 mg of ^{32}P-labeled PDH (kidney), 0.02 M potassium phosphate buffer (pH 7.0), 10 mM $MgCl_2$ and, where indicated, 20 µg of kidney or heart PDH phosphatase or 10 µg of PDH phosphatase and 46 µg of kidney or 51 µg of heart dihydrolipoyl transacetylase (LTA), 1 mM EGTA, and 1.25 mM $CaCl_2$ in a total volume of 0.2 ml. PDH phosphatase activity was determined as described in Methods. A larger amount of phosphatase (20 µg) was used in the absence of the transacetylase in order to obtain measurable rates.

These observations suggested the possibility that Ca^{2+} is involved in the binding of the phosphatase to the transacetylase. To test this possibility the transacetylase was incubated with the phosphatase in the presence of various combinations of Ca^{2+}, Mg^{2+}, EGTA, and PDH. The incubation mixtures were layered over 10% sucrose that contained the non-protein components of the individual incubation mixtures, and the mixtures were subjected to ultracentrifugation to separate free phosphatase from phosphatase bound to the transacetylase. The large difference in the molecular weights of the phosphatase (about

Table II

Lack of Ca^{2+} Effect on Reconstitution of PDC Activity

Incubation mixtures[a]	DPN-reduction activity ΔA_{340}/min
PDH + LTA + FP	0.256
PDH + LTA + FP + Ca^{2+}	0.255
PDH + LTA + FP + EGTA	0.293
PDH + LTA + FP + EGTA + Ca^{2+}	0.259

[a]The incubation mixtures contained 60 µg of PDH, 60 µg of transacetylase (LTA), 24 µg of flavoprotein (FP), 1 mM $MgCl_2$, 2 mM dithiothreitol, 0.05 M phosphate buffer (pH 7.5) and, where indicated, 1 mM EGTA and 1.25 mM $CaCl_2$ in a total volume of 0.2 ml. The mixtures were incubated for 15 min at 30°, and then 0.01-ml aliquots were assayed as described previously (15) for DPN-reduction activity with pyruvate as substrate. Combinations of any two of the three enzymes exhibited 10% or less of the DPN-reduction activity observed with the mixtures of all three enzymes. The presence of 1 mM EGTA in the assay mixture (15) had no effect on the DPN-reduction activities.

100,000) and the transacetylase (about 3.12 million) (13) facilitated the separation. The pellets were assayed for phosphatase activity. The representative data in Table III show that Ca^{2+} is required to bind the phosphatase to the transacetylase. Thus, binding of the phosphatase to the transacetylase was increased by Ca^{2+} and was inhibited by EGTA. Inhibition by EGTA was reversed by Ca^{2+}. Mg^{2+} or PDH had little effect, if any, on the binding of the phosphatase to the transacetylase.

Further studies revealed that the stimulation of PDH phosphatase activity in the presence of the transacetylase and Ca^{2+} is due to a twentyfold decrease in the apparent K_m of the phosphatase for phosphorylated PDH (Fig. 1). In the presence of EGTA and the transacetylase, the apparent K_m of the kidney PDH phosphatase for phosphorylated PDH was about 58 µM. In the presence of Ca^{2+} and the transacetylase, this value was decreased to about 2.9 µM. V_{max} appeared to be unaffected by Ca^{2+}. Under similar conditions Ca^{2+} did not affect the apparent K_m of the phosphatase for Mg^{2+} (about 7 mM). It appears that the

Table III

Ca^{2+} Requirement for Binding of PDH Phosphatase to the Transacetylase

Incubation mixtures[a]	Activity of bound phosphatase nmoles $^{32}P_i$ released/min
LTA	0.0
Phosphatase + LTA	0.059
Phosphatase + LTA + Ca^{2+}	0.171
Phosphatase + LTA + EGTA	0.018
Phosphatase + LTA + EGTA + Ca^{2+}	0.165
Phosphatase + LTA + EGTA + Mg^{2+}	0.016
Phosphatase + LTA + EGTA + Mg^{2+} + Ca^{2+}	0.174
Phosphatase + LTA + EGTA + Mg^{2+} + Ca^{2+} + PDH	0.171

[a]The incubation mixtures contained, where indicated, 52 μg of kidney PDH phosphatase, 546 μg of kidney dihydrolipoyl transacetylase (LTA), 66 μg of kidney PDH, 1 mM EGTA, 1.25 mM $CaCl_2$, and 1.25 mM $MgCl_2$ in a total volume of 0.2 ml of 0.02 M phosphate buffer (pH 7.0). After incubation for 15 min at 23°, the mixtures were layered over 1 ml of 10% sucrose that contained the non-protein components of the individual incubation mixtures. The mixtures were centrifuged in polycarbonate tubes for 180 min at 40,000 rpm in the type 40 rotor of a Beckman model L ultracentrifuge. The supernatant fluids were discarded, and the pellets were dissolved in 0.2 ml of 0.02 M phosphate buffer (pH 7.0). Aliquots (0.05 ml) of the solutions were assayed for PDH phosphatase activity as described in Methods, except that $CaCl_2$ was added to the assay mixtures to give a final concentration of 1 mM.

Ca^{2+}-facilitated binding of the phosphatase to the transacetylase increases the affinity of the phosphatase for phosphorylated PDH.

Previous studies in this laboratory (11,15) have shown that the transacetylase also markedly stimulated the rate of phosphorylation of PDH by the PDH kinase and $MgATP^{2-}$. The transacetylase lowered the apparent K_m of the kinase for PDH from about 20 μM to about 0.6 μM and increased V_{max} about two-fold (15). The stimulatory effect of the transacetylase on PDH kinase activity was not inhibited by 1 mM EGTA (Table IV) and apparently does not involve Ca^{2+}.

It appears that specific binding of the kinase and the phosphatase to the transacetylase is necessary to elevate the efficiency of the phosphorylation-

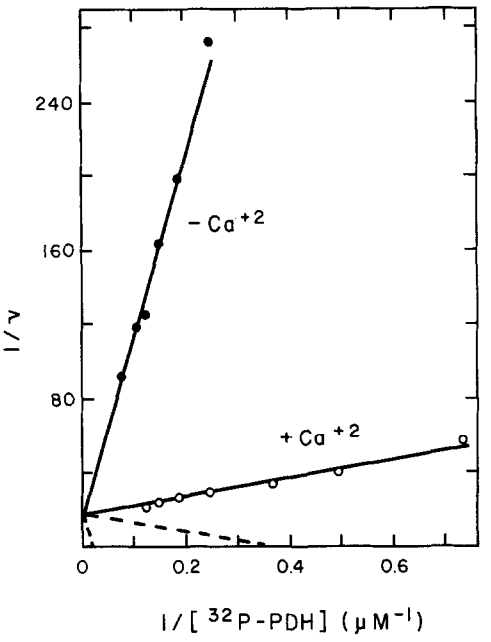

Fig. 1. Effect of Ca^{2+} on apparent K_m of PDH phosphatase for phosphorylated PDH. The incubation mixtures contained 0.02 M phosphate buffer (pH 7.0), 46 μg of kidney transacetylase, 5.8 μg or 17.4 μg of kidney PDH phosphatase, 1 mM EGTA, 0 or 1.25 mM $CaCl_2$, 10 mM $MgCl_2$, and increasing amounts of phosphorylated PDH (0.72 nmole of ^{32}P-labeled phosphoryl groups/nmole of enzyme) in a total volume of 0.2 ml. Aliquots (0.05 ml) of the solutions were assayed for PDH phosphatase activity as described in Methods. Rates are expressed as nmoles of $^{32}P_i$ released/min. A larger amount of phosphatase (17.4 μg) was used in the absence of $CaCl_2$ in order to obtain measurable rates. These rates were divided by three to correspond to the rates obtained in the presence of $CaCl_2$.

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dephosphorylation cycle to a physiologically useful level. Since the activity of the PDH phosphatase varies about tenfold, depending on whether or not it is attached to the transacetylase, it appears that the Ca^{2+}-controlled association of the phosphatase and the transacetylase could provide an important mechanism for the control of the phosphorylation-dephosphorylation cycle.

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Table IV

Lack of Ca^{2+} Effect on PDH Kinase Activity

Incubation mixtures[a]	Kinase activity nmoles ^{32}P incorporated/ min/mg kinase
Kinase	10.0
Kinase + Ca^{2+}	10.2
Kinase + EGTA	9.4
Kinase + EGTA + Ca^{2+}	10.9
LTA	1.4
Kinase + LTA	36.4
Kinase + LTA + Ca^{2+}	26.4
Kinase + LTA + EGTA	37.7
Kinase + LTA + EGTA + Ca^{2+}	38.1

[a]The incubation mixtures contained 0.3 mg of crystalline PDH (kidney), 0.02 M phosphate buffer (pH 7.5), 2 mM dithiothreitol, 0.1 mM EDTA, 1 mM $MgCl_2$, 0.125 mM [γ-^{32}P]ATP (1.32×10^6 cpm/µmole) and, where indicated, 4.7 µg of kidney PDH kinase, 25 µg of kidney dihydrolipoyl transacetylase (LTA), 1 mM EGTA, and 1.25 mM $CaCl_2$ in a total volume of 0.2 ml. The mixtures were incubated for 2 min at 30° prior to addition of ATP, which was added last. A 0.05-ml aliquot was withdrawn 1 min after addition of ATP and was assayed for protein-bound radioactivity.

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