Regulation of pyruvate dehydrogenase kinase activity from pig kidney cortex

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The activity of pyruvate dehydrogenase (PDH) kinase in the purified PDH complex from pig kidney is sensitive to changes in ionic strength. The enzyme has optimum activity within a small range of ionic strength (0.03–0.05 M). An increase in ionic strength from 0.04 M to 0.2 M lowers the activity of PDH kinase by 32 % and decreases the K_m for ATP from 25 μ M to 10 μ M. At constant ionic strength (0.15 M) the enzyme has optimum activity over a broad pH range (7.2–8.0). The PDH kinase is stimulated 2.2-fold by 20 mM- K^+ , whereas Na^+ even at high concentration (80 mM) has no effect on the enzyme activity. The stimulation of PDH kinase by K^+ is not dependent on pH and ionic strength. PDH kinase is inhibited by HPO_4^{2-} in the presence of K^+ , whereas HPO_4^{2-} has no effect on the activity of this enzyme in the absence of K^+ . HPO_4^{2-} at concentrations of 2 and 10 mM inhibits PDH kinase by 28 % and 55 % respectively. The magnitude of this inhibition is not dependent on the ATP/ADP ratio. Inhibition by HPO_4^{2-} in the concentration range 0–10 mM is non-competitive with respect to ATP, and becomes mixed-type at concentrations over 10 mM. The K_i for HPO_4^{2-} is 10 mM. When HPO_4^{2-} is replaced by SO_4^{2-} , the same effects on the activity of PDH kinase are observed. PDH kinase is also inhibited by Cl^- . In the presence of 80 mM- Cl^- the PDH kinase is inhibited by 40 %. The inhibition by Cl^- is not dependent on K^+ . In conclusion, we postulate that changes in phosphate concentrations may play a significant role in the regulation of PDH kinase activity *in vivo*.

INTRODUCTION

Regulation of the pyruvate dehydrogenase (PDH) complex by its kinase/phosphatase system has been investigated extensively in many laboratories (for reviews, see [1,2]). It has been shown that cofactors, uni- and bi-valent cations, and reaction products regulate both the kinase and the phosphatase activity. PDH kinase requires Mg^{2+} or Mn^{2+} [3] and is stimulated by NADH and acetyl-CoA [4–7]. Previous reports have shown that NAD^+ , CoA, thiamin pyrophosphate, pyruvate and ADP inhibit PDH kinase activity [4–9]. In addition, inhibition by ADP and pyruvate requires the presence of K^+ or NH_4^+ [3,6,10–13]. Various studies have shown that univalent ions affect the activity of the PDH complex and PDH kinase [9,14,15]. Both inhibitory and stimulatory effects of K^+ on the activity of PDH kinase have been reported [5,9,13,14]. It has been shown that bovine heart PDH kinase is stimulated 3-fold by 20 mM-KCl, but this stimulation declined as the concentration of KCl increased [9,14]. Studies of the bovine kidney PDH kinase demonstrated that 100 mM- K^+ inhibits this enzyme by as much as 70 % [5]. Na^+ ions at 10–100 mM concentration have little effect on the bovine heart PDH kinase activity [9,14], although it has been shown that concentrations of NaCl higher than 50 mM inhibit the kidney PDH kinase [13].

Our previous reports have shown that the activity of pig kidney PDH complex is regulated by ionic strength and various bivalent and univalent ions [15,16]. In this context, it has been suggested that the previously reported contradictory effects of K^+ and Na^+ on the activity of PDH kinase could be a result of changes in ionic strength followed by increasing concentrations of KCl and NaCl. In the present study we have investigated the effects of changes of ionic strength on PDH kinase activity in purified PDH complex from pig kidney. Also we have

characterized the stimulation of PDH kinase by K^+ at different ionic strengths and pH.

Studies of the purified PDH complex and experiments performed with isolated mitochondria have shown that changes in ATP/ADP ratio can regulate PDH kinase activity [13,17,18]. On the other hand, under physiological conditions the changes observed in the ATP/ADP ratio are not sufficiently large to affect significantly the phosphorylation state of the PDH complex [19–23]. The present study demonstrates that PDH kinase is inhibited by HPO_4^{2-} within a physiological range of concentrations. The possible role of HPO_4^{2-} in the regulation of PDH kinase *in vivo* is discussed.

EXPERIMENTAL

Materials

Tris, Hepes, Mops, Mes, ATP and ADP were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Triethanolamine was purchased from Fisher Scientific. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was purchased from ICN-Biomedical Inc., Irvine, CA, U.S.A. All other chemicals were of the purest grade commercially available. Highly purified preparations of PDH complex from pig kidney cortex (11–14 μ mol/min per mg of PDH-complex protein) were prepared as described previously [15].

Methods

PDH kinase activity was measured as the initial rate of transfer of $[\text{P}^{32}]\text{P}_i$ into PDH complex from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Separation of protein-bound radioactivity was achieved by trichloroacetic acid precipitation on Whatman 3MM paper by the method of Wastila *et al.* [24]. Incubations were made in Tris/Hepes buffer with additions of 0.5 mM-ATP (60000–80000 c.p.m./nmol). The concentrations of various additions,

Abbreviations used: PDH, pyruvate dehydrogenase.

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pH and ionic strength are noted in the Figure legends. The assay of the kinase reaction was initiated by addition of ATP after a preincubation period of 1 min at 30 °C. At 20 s, 50 μ l of reaction mixture was spotted on 1.5 cm squares of Whatman 3MM paper and the papers were quenched in cold 10 % (v/v) trichloroacetic acid containing 20 mM- $\text{Na}_4\text{P}_2\text{O}_7$. All assays were conducted in duplicate. A stock solution containing PDH complex (5.0 mg/ml) in Tris/Hepes buffer (pH 7.8)/1.0 mM- MgCl_2 /1.0 mM-dithiothreitol was incubated for 30 min at 0 °C before use. The ionic strength and pH were adjusted as required by varying the concentration of buffer. Protein was determined by the biuret procedure [25] with BSA as a standard.

Calculations

Estimations of the concentrations of conjugate base and conjugate acid for all buffers were calculated from the Henderson-Hasselbalch equation. The ionic strengths of assay mixtures were calculated on the basis of the concentration of the ionic form of all additions.

RESULTS

Effect of ionic strength and pH on the PDH kinase activity

In a previous publication we reported that the activity of PDH complex from pig kidney cortex is sensitive to changes in ionic strength [16]. Since PDH kinase is tightly bound to the enzyme complex, we assumed that the activity of this regulatory enzyme may be dependent on ionic strength. Measurements of the activity of PDH kinase at different concentrations of various buffers showed that the kinase activity is dependent on the molar concentration of the buffer. Experiments in which mannitol and sucrose were used to generate various molarities in the reaction mixture showed that activity of PDH kinase is not sensitive to changes in osmolality of the assay medium (results not shown). These results suggested that the activity of PDH kinase was sensitive to changes of ionic strength. Consistent with this hypothesis, Fig. 1 shows that the PDH kinase has optimum activity in a small range of ionic strength, 0.03–0.02 M. Thus an increase in the ionic strength of the reaction medium from 0.04 to 0.2 M caused a 32 % decrease in the activity of PDH kinase (Fig. 1). Changes in the ionic strength affect the K_m for ATP.

Fig. 1. Effect of ionic strength on pig kidney PDH kinase activity

Assays of PDH kinase activity were performed as described in the Experimental section. The activity of PDH kinase was determined at pH 7.4 in the presence of triethanolamine/Mops buffer (○) or Tris/Hepes buffer (●); 100 % represents an activity of 6.8 nmol of ^{32}P incorporated/min per mg of PDH complex. The results are means $\pm$ S.D. of five determinations for three different enzyme preparations.

Fig. 2. Effect of pH on PDH kinase activity at constant ionic strength

Assay conditions for PDH kinase activity were as described in the Experimental section. The activity of PDH kinase was determined at $I = 0.15$ M in the presence of Mes/NaOH (○), Mops/NaOH (●), Hepes/NaOH (□), triethanolamine/Mes (■), triethanolamine/Mops (△) or Tris/Hepes (▲) buffer. The results are means of three separate determinations.

Increasing the ionic strength from 0.04 to 0.2 M decreased the K_m for ATP from 25 μM to 10 μM (results not shown).

Previous reports have demonstrated that the optimum activity of kidney and heart PDH kinase is in the pH range 6.9–7.2 [3,12,14]. In the present study various buffers were used to investigate the pH-dependence of PDH kinase activity. From the data presented in Fig. 2 it appeared that at constant ionic strength the optimum activity of PDH kinase was in a broad range of pH. At an ionic strength of 0.15 M the PDH kinase exhibited 95–100 % of maximal activity in the pH range 7.0–8.0 (Fig. 2).

Effect of K^+ and Na^+ on the activity of PDH kinase

Several reports have shown that Na^+ and K^+ affect the activity of PDH kinase [5,9,13,14]. It has been shown that bovine kidney PDH kinase was inhibited by 28 % and as much as 70 % by 60 mM- [13] and 100 mM- [5] KCl respectively. In the present study, however, it was discovered that K^+ stimulates the activity of kidney PDH kinase (Fig. 3). Maximal stimulatory effects of K^+ on the activity of pig kidney PDH kinase were observed at 20 mM concentration. At constant ionic strength (0.15 M) and in the presence of 20 mM- K^+ the activity of PDH kinase was stimulated 2.1-fold (Fig. 3). In contrast with previous reports [9,14], the maximal effect of K^+ was at 20 mM, and above this concentration K^+ ions had no additional stimulatory or inhibitory effect on kinase activity (Fig. 3). As previously reported for bovine heart PDH kinase [14], the effect of K^+ on the activity of pig kidney PDH kinase is not dependent on pH (Fig. 3). The stimulatory effect of K^+ on the PDH kinase activity remained constant over a broad range of ionic strength, 0.03–0.2 M. The inhibition of pig kidney PDH kinase by ADP was abolished when K^+ was replaced by Na^+ (results not shown).

A previous report indicated that NaCl at concentrations above 50 mM markedly inhibits the activity of bovine kidney PDH kinase [5]. In contrast, in the present study Na^+ were shown to have no effect on the activity of pig kidney PDH kinase. At

Fig. 3. Stimulation of PDH kinase activity by K^+

Assay conditions for PDH kinase activity were as described in the Experimental section. The activity of PDH kinase was determined at $I = 0.15$ M and pH 7.8 ($\circ$) or 7.4 ($\bullet$). The reaction mixture contained potassium as its Hepes salt. The results are means of four separate determinations.

Fig. 4. Effect of HPO_4^{2-} on PDH kinase activity

Assay conditions for PDH kinase activity were as described in the Experimental section. The activity of PDH kinase was determined at pH 7.8 and $I = 0.15$ M. The reaction mixture contained: 0.5 mM-ATP ($\blacktriangle$); 0.5 mM-ATP, 0.5 mM-ADP ($\triangle$); 80 mM- K^+ , 0.5 mM-ATP ($\circ$); 80 mM- K^+ , 0.5 mM-ATP, 0.5 mM-ADP ($\bullet$). K^+ was used as its Hepes salt, and HPO_4^{2-} as its Tris salt. The results are means $\pm$ S.D. of five separate determinations.

pH 7.4 Na^+ in concentrations of 20–80 mM neither stimulate nor inhibit PDH kinase over a broad range of ionic strength (0.03–0.2 M). Likewise, at constant ionic strength (0.15 M) Na^+ had no effect on PDH kinase activity in the pH range 7.0–8.0 (results not shown). The results presented demonstrate that K^+ have specific effects, compared with Na^+ , on the activity of the intrinsic PDH kinase of the PDH complex from pig kidney.

Effect of HPO_4^{2-} on PDH kinase activity

At constant ionic strength (0.15 M) and pH 7.8, HPO_4^{2-} anions inhibit the activity of PDH kinase. Fig. 4 shows that K^+ were required for inhibition of the kinase activity by HPO_4^{2-} . In the

Fig. 5. Effect of HPO_4^{2-} and SO_4^{2-} on PDH kinase activity as a function of Mg^{2+} concentration

Assays of PDH kinase activity were performed as described in the Experimental section. The activity of PDH kinase was determined at pH 7.8 and $I = 0.15$ M in the presence of 80 mM- K^+ and HPO_4^{2-} ($\circ$, $\bullet$) or SO_4^{2-} ($\square$, $\blacksquare$). The symbols $\circ$, $\square$ and $\bullet$, $\blacksquare$ represent kinase activity in the presence of Mg^{2+} at 1.5 mM and 20 mM respectively; 100% represents an activity of 9.8 nmol of ^{32}P incorporated/min per mg of PDH complex. The results are means of four determinations for three different enzyme preparations; reproducibility averaged within $\pm 5\%$ with any particular enzyme preparation.

presence of 80 mM- K^+ , the activity of PDH kinase was inhibited by 65% by 20 mM- HPO_4^{2-} , whereas in the absence of K^+ inhibition of kinase activity by HPO_4^{2-} was not observed (Fig. 4). The same percentage inhibition of kinase by HPO_4^{2-} was observed in the presence and absence of 0.5 mM-ADP. It can be calculated from the data shown in Fig. 4 that in the presence of 5 mM- HPO_4^{2-} the kinase activity was inhibited by $32 \pm 3\%$ and $36 \pm 4\%$ with 0.5 mM-ATP and 0.5 mM-ATP plus 0.5 mM-ADP respectively. These data indicate that the inhibitory effect of HPO_4^{2-} anion was not dependent on the ATP/ADP ratio. Inhibition of PDH kinase by HPO_4^{2-} is not specific for the phosphate anion, which can be replaced by SO_4^{2-} anion (Fig. 5). The same percentage (54%) of kinase inhibition was observed at 10 mM- HPO_4^{2-} and 5 mM- HPO_4^{2-} plus 5 mM- SO_4^{2-} (results not shown). On the basis of the stability constants of the $MgHPO_4$ and $MgATP$ complexes, it can be assumed that kinase inhibition by HPO_4^{2-} is not due to chelating Mg^{2+} in the reaction medium. Experiments in which high (20 mM) and low (1.5 mM) concentrations of Mg^{2+} were used indicate that the inhibitory effect of HPO_4^{2-} on PDH kinase activity was not an effect of Mg^{2+} chelation by phosphate anions. As shown in Fig. 5, the same percentage inhibition of kinase by HPO_4^{2-} or SO_4^{2-} was observed in the presence of Mg^{2+} at 1.5 and 20 mM. Analysis of the data by a Dixon plot showed that HPO_4^{2-} in the concentration range 0–10 mM was a non-competitive inhibitor with respect to ATP. At concentrations higher than 10 mM, phosphate ion became a mixed-type inhibitor with respect to ATP. The inhibitory constant, K_i , for HPO_4^{2-} was determined to be 10 mM (results not shown).

Effect of Cl^- on the PDH kinase activity

Fig. 6 shows the inhibitory effect of Cl^- anions on the PDH kinase activity. At constant ionic strength (0.15 M) and pH 7.8, the PDH kinase activity was inhibited by 40% by 80 mM- Cl^- . In the physiological range of Cl^- concentrations (15–20 mM) [26–28]

Fig. 6. Inhibition of PDH kinase by Cl^-

Assay of PDH kinase activity were performed as described in the Experimental section. The activity of PDH kinase was determined at pH 7.8 and $I = 0.15$ M in the absence (●) or the presence (○) of K^+ (80 mM). In the absence of K^+ , the reaction mixture containing Cl^- as its Tris salt. K^+ was used as its Hepes salt. 0% inhibition in the absence or presence of K^+ represents an activity of 4.7 or 9.5 nmol of ^{32}P incorporated/min per mg of PDH complex respectively. The results are means $\pm$ S.D. of three determinations for three different enzyme preparations.

the activity of PDH kinase was inhibited by only 15–18% (Fig. 6). The inhibitory effect of Cl^- was not dependent on K^+ (Fig. 6). In the presence of 10 mM- HPO_4^{2-} the kinase activity was inhibited by 54%, and addition of 20 mM- Cl^- , although maintaining a constant ionic strength, increased the kinase inhibition up to 71% (results not shown). Results obtained indicate that Cl^- anion inhibited the activity of PDH kinase with different characteristics relative to HPO_4^{2-} .

DISCUSSION

The results presented in this study demonstrate new regulatory properties of kidney PDH kinase activity. The activity of this enzyme is dependent on ionic strength; a decrease in PDH kinase activity was observed as the ionic strength increased. It was shown that this effect was mediated by changes in $V_{\max}$ and K_m for ATP. The observed decrease in $V_{\max}$ of PDH kinase as a consequence of increased ionic strength could be the result of a decrease in the number of catalytic sites or binding sites for ATP as well as a lower availability of the other substrate, i.e. the PDH subunit. Our previous studies have shown that changes in ionic strength affect the activity and structure of the PDH complex. Exposing the PDH complex to increased ionic strength causes a decrease in the radius of the enzyme complex [16]. Thus the observed decrease in the $V_{\max}$ of PDH kinase concomitant with increased ionic strength suggests that changes in the conformation of the complex may occur such that lower availability of PDH subunits for PDH kinase is achieved. Previous reports have shown that PDH kinase exhibits optimal activity within a relative small range of pH (6.9–7.2) [3,12,14]. In contrast, the results presented here show that at constant ionic strength the enzyme preserves maximal activity in a broad range of pH from 7.0 to 8.0. We might suggest that different pH optima for PDH kinase reported previously were a result of changes in the ionic strength of the assay medium during adjustment of pH.

Several reports have shown both an inhibitory [5,13] and a stimulatory [9,14] effect of K^+ on PDH kinase activity. Our present data demonstrate that K^+ at constant ionic strength stimulates the kidney PDH kinase activity (Fig. 3). In contrast

with a previous report [5], we found that Na^+ have no effect on kidney PDH kinase activity. We might suggest that the reported inhibition of PDH kinase activity by increasing concentrations of KCl and NaCl was an effect of increased ionic strength and inhibition caused by Cl^- .

It has been shown that some of the regulatory properties of PDH kinase depend on the presence of K^+ , including the inhibitory effect of ADP [13], the stimulatory effect of acetyl-CoA and NADH [4] and both the inhibitory and stimulatory effects of pyruvate [5]. Our present results demonstrate that K^+ are also required for inhibition of PDH kinase activity by HPO_4^{2-} . This inhibition is not dependent on the ATP/ADP ratio (Fig. 4). Study of the regulatory properties of PDH-complex enzyme components showed that the only components affected by Na^+ , K^+ and HPO_4^{2-} are dihydrolipoamide acetyltransferase and PDH kinase. The activity of PDH is not influenced by Na^+ , K^+ or HPO_4^{2-} (T. Pawelczyk & M. S. Olson, unpublished work). As stressed above, analysis of the kinetic data of PDH kinase showed that HPO_4^{2-} is a non-competitive inhibitor with respect to ATP. It means that HPO_4^{2-} and ATP bind to kinase reversibly, randomly and independently at different sites, yielding catalytically inactive complex. From these observations, we assumed that changes in concentration of phosphate may be a regulatory factor for PDH kinase activity. The intracellular concentration of phosphate has been reported in a number of studies to vary in the range 2–8 mM, depending on the tissue and the method of determination [29–32]. Recent evidence from ^{31}P -n.m.r. studies has indicated that the intracellular P_i concentrations in the kidney, liver and heart are in the range 0.95–1.3 mM [33–36]. It can be assumed that these values represent cytosolic concentration, since only cytosolic (but not mitochondrial) P_i is detected by n.m.r. [36,37]. It is difficult to estimate the free P_i concentration in mitochondria. Some workers have estimated mitochondrial P_i concentrations of 15–20 mM [34,38,39] and others have reported much lower values, 7.9–9.0 mM [36,40]. Nevertheless, mitochondria contain considerable amounts of P_i , which can buffer the cytosolic P_i concentration. The cytosolic P_i level is maintained nearly constant under various physiological conditions (for review see [41]). It is possible that an efflux of P_i from mitochondria plays a role in stabilizing the cytosolic concentration under P_i -depletion conditions. The consequent fall in mitochondrial P_i concentration may affect PDH kinase activity. It was reported that phosphate depletion in cells of the renal proximal tubule causes stimulation of gluconeogenesis from pyruvate, malate and succinate [42]. Under the conditions of our experiments the activity of PDH kinase is inhibited by 54% by HPO_4^{2-} at 10 mM (Fig. 4). If it is assumed that the mitochondrial concentration of P_i is near 10 mM, then it is possible that any change in phosphate concentration can markedly affect the PDH kinase activity *in vivo*. This idea supports data from experiments with isolated cardiac mitochondria in which added phosphate blocked inactivation of the PDH complex during incubation with ATP [43]. Several hormones are known to cause increases in the amount of the active (dephospho) form of the PDH complex. It has been shown that administration of vasopressin, adrenaline or glucagon alone *in vivo* resulted in activation of PDH complex (for review see [1]). These hormones are known to cause increases in the cytoplasmic concentration of Ca^{2+} [44–46]. There is evidence that the tissue ratio of known effectors of PDH kinase activity, i.e. ATP/ADP or CoA/acetyl-CoA, remain essentially unchanged after administration of these hormones [47,48]. Thus it might be postulated that these conditions increase the amount of the active form of PDC as a result of phosphatase stimulation by increasing mitochondrial Ca^{2+} concentration [49,50]. On the other hand, it has been reported that incubation of isolated

hepatocytes with glucagon, and to a lesser extent vasopressin and angiotensin II, results in increased intracellular P_i content [51]. Assuming that under these conditions the mitochondrial concentration of P_i increases, it would seem likely that PDH-complex regulation involves not only phosphatase stimulation but also PDH kinase inhibition. An increase in the amount of the active form of PDH complex can also occur as a result of exposure to insulin in tissues which are important sites of fatty acid synthesis, namely liver, epididymal adipose tissue and lactating mammary gland [52–55]. Under these conditions, activation of PDC is not accompanied by changes in the cellular concentration of any known effectors of PDH kinase activity, namely ATP, ADP, NADH, NAD⁺, acetyl-CoA, CoA, K⁺ and phosphatase effectors such as Ca²⁺ and Mg²⁺ [49,56,57]. On the other hand, incubation of isolated rat hepatocytes with insulin leads to an increase in Na⁺-dependent P_i uptake by 50–100 % over control values [58]. It is hard to predict whether the mitochondrial content of P_i increases under stimulation of cellular uptake of P_i. If one considers such a possibility, it seems likely that the inhibition of PDH kinase activity may occur after insulin administration.

In the end, it is somewhat difficult to estimate the magnitude of the changes in the mitochondrial concentration of phosphate under different physiological conditions. However, it is tempting to speculate that changes in concentration of HPO₄²⁻ may play a significant role in the regulation of PDH kinase activity *in vivo*.

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