Evidence for existence of tissue-specific regulation of the mammalian pyruvate dehydrogenase complex

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Tissue distribution and kinetic parameters for the four isoenzymes of pyruvate dehydrogenase kinase (PDK1, PDK2, PDK3 and PDK4) identified thus far in mammals were analysed. It appeared that expression of these isoenzymes occurs in a tissue-specific manner. The mRNA for isoenzyme PDK1 was found almost exclusively in rat heart. The mRNA for PDK3 was most abundantly expressed in rat testis. The message for PDK2 was present in all tissues tested but the level was low in spleen and lung. The mRNA for PDK4 was predominantly expressed in skeletal muscle and heart. The specific activities of the isoenzymes varied 25-fold, from 50 nmol/min per mg for PDK2 to 1250 nmol/min per mg for PDK3. Apparent K_i values of the isoenzymes for the synthetic analogue of pyruvate, dichloroacetate, varied 40-fold, from 0.2 mM for PDK2 to 8 mM for PDK3. The isoenzymes were also different with respect to their ability to respond to NADH and NADH plus acetyl-CoA. NADH alone stimulated the activities of PDK1 and PDK2 by 20 and 30% respectively. NADH plus acetyl-CoA activated these isoenzymes nearly 200 and 300%. Under comparable conditions, isoenzyme PDK3 was almost completely unresponsive to NADH, and NADH plus acetyl-CoA caused inhibition rather than activation. Isoenzyme PDK4 was activated almost 2-fold by NADH, but NADH plus acetyl-CoA did not activate above the level seen with NADH alone. These results provide the first evidence that the unique tissue distribution and kinetic characteristics of the isoenzymes of PDK are among the major factors responsible for tissue-specific regulation of the pyruvate dehydrogenase complex activity.

INTRODUCTION

The mammalian pyruvate dehydrogenase complex (PDC) catalyses the physiologically irreversible step in oxidative degradation of carbohydrate fuels [1,2]. It consists of three enzymes organized into a high-molecular-mass complex: pyruvate dehydrogenase (E1), dihydrolipoamide transacetylase (E2) and dihydrolipoamide dehydrogenase (E3) [1]. Besides these catalytic components, mammalian PDC also contains a structural component, protein X or E3-binding protein [3], and two regulatory enzymes, pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase [4]. The activities of the latter two enzymes determine the proportion of PDC in its active dephosphorylated state. These two regulatory enzymes control flux through the complex and contribute to the overall control of aerobic oxidation of carbohydrate fuels [5].

Within the last decade, PDK has been the subject of intensive studies because it appears that, under many circumstances, changes in the phosphorylation state of PDC directly correlate with changes in the activity of PDK [5]. Short- [6,7] and long- [8] term mechanisms have been implicated in the regulation of PDK activity. In the short term, PDK is reversibly regulated by the products and substrates of the pyruvate dehydrogenase reaction. The products, NADH and acetyl-CoA, activate PDK [9,10]. This activation is believed to reflect the reduction and acetylation of the lipoyl moiety of the transacetylase component (E2) occurring as a result of accumulation of NADH and acetyl-CoA [11]. It can be reversed therefore by excess NAD^+ and CoA [10]. The inhibition of kinase activity by pyruvate, in contrast, results from its direct interaction with an unidentified site on the kinase molecule [12]. In the long term, various nutritional conditions and pathological disorders have been shown to produce a stable increase in PDK activity [8,13]. Under some circumstances, this long-term regulation may be part of an adaptive response or vice versa may contribute to the pathogenesis of disease. For example, the stable increase in PDK activity during starvation allows conservation of carbohydrate fuels for tissues solely dependent on this source of energy [5]. A comparable increase in kinase activity in diabetes contributes to pathogenesis of diabetes by preventing the catabolism of carbohydrates [5]. The molecular mechanisms responsible for the long-term control of PDK activity have not been entirely elucidated.

Recent evidence from this and other laboratories indicates the existence of multiple isoenzymes of PDK [14,15]. Thus far, four isoenzymes have been identified in humans, and two isoenzymes homologous with human isoenzymes PDK1 and PDK2 have been found in rodents [16]. The role of multiple PDK isoenzymes in the short-term and long-term control of PDC activity is currently unclear because of the lack of data on their catalytic and regulatory properties, as well as on regulation of their expression. In the present paper we report data on the kinetic parameters of four isoenzymes of PDK obtained as recombinant proteins along with data on their tissue distribution according to Northern-blot analysis. These results provide the first evidence that isoenzymes of PDK are responsible for tissue-specific regulation of PDC activity.

EXPERIMENTAL

Molecular cloning of cDNA encoding rat PDK4

A 5′-Stretch λgt10 rat heart cDNA library obtained from Clontech was screened with random-primed ^{32}P-labelled partial

Abbreviations used: PDK, pyruvate dehydrogenase kinase; PDC, pyruvate dehydrogenase complex; E1, pyruvate dehydrogenase component of PDC; E2, dihydrolipoamide transacetylase component of PDC; E3, dihydrolipoamide dehydrogenase component of PDC; His-Tag, stretch of six consecutive histidine residues added to the recombinant protein to facilitate purification; DCA, dichloroacetate.

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The nucleotide sequence for rat PDK4 has been submitted to GenBank and appears under the accession number AF034577.

cDNA of human PDK4. Approx. 10^6 plaque-forming units of cDNA library were screened essentially as described previously [17]. Six positive plaques were purified through four more cycles of plating and screening. Respective cDNAs ranging in size from 1.0 to 2.5 kb were cut out of λgt10 with *Eco*RI and religated in *Eco*RI-digested pUC18 for sequencing. Sequence analysis of the respective cDNAs revealed that one of the clones contained cDNA (1435 bp long) encoding the full-length polypeptide product. Comparison of the deduced protein sequences of human and rat PDK4 revealed up to 92 % identity (results not shown; nucleotide and deduced protein sequences of rat PDK4 are available through GenBank).

Sequencing

Sequencing of double-stranded plasmid DNA was carried out by the Biochemistry Biotechnology Facility (Indiana University) using a *Taq* DyeDeoxy Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase, FS (Perkin–Elmer Corporation) following the manufacturer's instructions. Both strands were sequenced.

Northern-blot analysis

Rat multiple tissue Northern blots were obtained from Clontech. Blots were probed as previously described [16]. The cDNAs of rat PDK1 [18], rat PDK2 [16], human PDK3 [14] and rat PDK4, cloned in the present study, were labelled to the comparable specific radioactivities of 10^9 c.p.m./μg using a random-primed DNA-labelling kit from Boehringer.

Construction of the bacterial expression vectors

The construction of the bacterial expression vectors for rat PDK1, rat PDK2 and human PDK3 (pPDK1, pPDK2 and pPDK3) was reported previously [14,16]. The bacterial expression vector for rat PDK4 was constructed as follows. *Nco*I and *Xho*I restriction sites flanking the coding region of the PDK4 cDNA were constructed by PCR with *Pfu* DNA polymerase (Stratagene). The sense/antisense PCR primers corresponded to bases 135–153 and 1320–1340 of rat PDK4 cDNA (AAA CCA TGG CCA GCT CGC TGG GCA ACG; TTT CTC GAG CAC TGC CAG TTT CTC CTT CGA); sequences for *Nco*I and *Xho*I restriction sites added to sense and antisense primers respectively are underlined. A corresponding PCR fragment of approx. 1.2 kb was purified, digested with *Nco*I and *Xho*I, and ligated between *Nco*I and *Xho*I restriction sites of pET-28a expression vector (Novagen) to produce C-terminal fusion with His-Tag encoded by the vector. Resulting plasmids were transformed in TG-1 cells. Transformants were selected on TY/Agar (DIFCO) containing 45 μg/ml kanamycin. Colonies that expressed kanamycin-resistance were screened for the presence of inserts by *Nco*I–*Xho*I restriction analysis. Construct fidelity was established by nucleotide sequencing (plasmid PDK4).

To establish expressing cell lines, competent BL21(DE3) cells obtained from Novagen were co-transformed with one of the pPDK expression vectors and pGROESL (plasmid GROESL containing the inducible genes for molecular chaperonins GroEL and GroES was a gift from Dr. Anthony Gatenby at Du Pont). Transformants were selected on TY/Agar containing 45 μg/ml kanamycin and 35 μg/ml chloramphenicol. Several colonies displaying resistance to both antibiotics were tested for their ability to express kinases and were used to prepare glycerol stocks.

Expression and purification of PDK isoenzymes

The following protocol was used to express and purify all four isoenzymes of PDK. Glycerol stock (10 μl) was inoculated into 11 M9ZB medium containing 45 μg/ml kanamycin and 35 μg/ml chloramphenicol. The cells were allowed to grow at 37 °C. After A_{600} reached 0.7–0.8, the culture was transferred at room temperature and induced with 0.4 mM isopropyl β-D-thiogalactopyranoside. Incubation was continued for another 20–24 h at room temperature.

After 20–24 h of induction, cells were harvested by centrifugation at 5000 rev./min (JA-10 rotor) for 20 min at 4 °C. Harvested cells were resuspended in 10 vol. of TN buffer (20 mM Tris/HCl, pH 8.0, 0.1 M NaCl) supplemented with 10 mM 2-mercaptoethanol, 0.5 % (w/v) Triton X-100 and a cocktail of protease inhibitors [0.1 mM PMSF, 0.1 mM benzamidine, 20 μg/ml pepstatin A, 20 μg/ml leupeptin and 1 % (v/v) aprotinin]. Resuspended cells were disrupted by sonication (5 × 20 s with 1 min intervals for cooling on ice). Extracts were clarified by centrifugation at 50000 ***g*** for 30 min at 4 °C. Recombinant kinase was purified on a 5 ml bed volume of Talon resin (Clontech) equilibrated with 3 vol. of TN. The column was sequentially washed with 10 vol. of TN and 5 vol. of TN containing 15 mM imidazole. Bound protein kinase was eluted with TN plus 100 mM imidazole. Purified kinase was supplemented with 5 mM dithiothreitol, desalted on a PD-10 column (Pharmacia) equilibrated in TN containing 0.5 mM EDTA, 0.05 % Triton X-100, 5 mM dithiothreitol and the cocktail of protease inhibitors given above. The final preparation was made 50 % (v/v) with glycerol and stored in small aliquots at −80 °C.

Assay of kinase activity

Kinase activity was determined by following [^{32}P]phosphate incorporation from [γ-^{32}P]ATP into kinase-depleted PDC essentially as described by Stepp et al. [19] and by Pratt and Roche [20] with some modifications as follows. Phosphorylation reactions were set up in a final volume of 100 μl containing 20 mM Tris/HCl, pH 7.4, 5 mM $MgCl_2$, 50 mM KCl, 2 mM dithiothreitol, 0.1 mg/ml BSA, 1.0 mg/ml kinase-depleted porcine heart PDC [21], recombinant kinase and [γ-^{32}P]ATP (specific radioactivity 200–500 c.p.m./pmol) at 37 °C. Phosphorylation reactions were initiated by the addition of ATP after equilibration at 37 °C for 30 s. Aliquots (20 μl) of the phosphorylation reaction mixture were withdrawn after 15, 30, 45 and 60 s of incubation and applied to dry discs of Whatman no. 3MM paper presoaked in solution containing 24 % (w/v) trichloroacetic acid, 0.2 M phosphoric acid, 2 mM sodium pyrophosphate and 1 mM ATP. Protein-bound radioactivity was determined as described previously [21]. A control (minus kinase) was included in each experiment. Kinase activities were calculated on the basis of incorporation of [^{32}P]phosphate during the first 30 s of the reaction. For isoenzymes PDK1, PDK2 and PDK4, the rate of phosphorylation was proportional to the amount of added kinase, when kinase was varied from 0.5 to 2.0 μg, suggesting that PDC is not limiting under the conditions used. Owing to the high specific activity of isoenzyme PDK3, this relationship deviated somewhat from linearity when 2 μg of kinase protein was used. Therefore, in the experiments described here, 0.5 μg of kinase protein per assay was used for isoenzymes PDK1, PDK3 and PDK4. PDK2 was used at 2.0 μg of kinase protein because of its lower specific activity. The concentrations of substrate (ATP) and effectors used in particular experiments are given in the legends to the Tables. Raw kinetic data were fitted and analysed by using Grafit software (Eritacus Software).

Other procedures

SDS/PAGE was performed as described by Laemmli [22]. Protein was determined by the method of Lowry et al. [23] with BSA as a standard.

RESULTS

Expression of PDK isoenzymes in rat tissues

Distribution of PDK isoenzymes in rat tissues was analysed by Northern blotting. The multiple tissue Northern blots were probed with ^{32}P-labelled cDNAs for rat PDK1 [18], rat PDK2 [16], human PDK3 [14] and rat PDK4 (cloned in this study). In accord with previous observations [16], the mRNA for isoenzyme PDK1 was found almost exclusively in heart and showed characteristic multiple bands (Figure 1A). In contrast, the mRNA for isoenzyme PDK2 was highly expressed in all tissues tested except for spleen and lung (Figure 1B). The mRNA for isoenzyme PDK3 was detected in most rat tissues. However, the amount of mRNA for PDK3 was extremely low, especially in comparison with the amount of mRNA for isoenzyme PDK2. Rat testis appeared to be the only tissue with a relatively high level of expression of PDK3 (Figure 1C). It is interesting to note that testis also appeared to be the only tissue where the second PDK3 mRNA (size approx. 2.0 kb) was detected. The molecular nature of this heterogeneity is currently unknown. mRNA for isoenzyme PDK4 was found to be most abundantly expressed in skeletal muscle and heart. Lower amounts of message were found in lung, liver and kidney (Figure 1D). Thus Northern-blot analysis clearly show that the isoenzymes of PDK have unique patterns of tissue distribution, and every tissue expresses its own subset of PDK isoenzymes. In the brain, for example, PDK activity corresponds primarily to isoenzyme PDK2. In other tissues it is a mixture of several isoenzymes: PDK2 and PDK4 in skeletal muscle; PDK2 and PDK3 in testis; PDK1, PDK2, and PDK4 in heart. The level of the message is another factor that has to be taken into consideration. Both skeletal muscle and liver, for example, express isoenzymes PDK2 and PDK4. However, the amount of both mRNAs is relatively high in skeletal muscle, whereas PDK2 mRNA is far more abundant than that of PDK4 in liver.

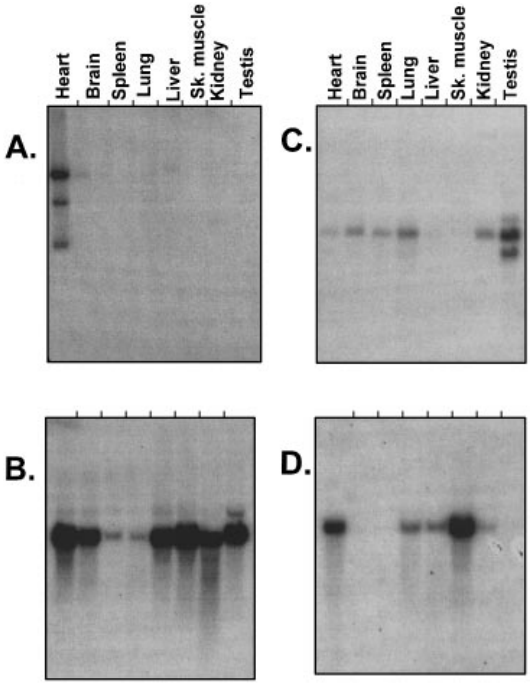

Figure 1 Relative abundancy of mRNA for PDK isoenzymes in rat tissues

(**A**) PDK1; (**B**) PDK2; (**C**) PDK3; (**D**) PDK4. Owing to variations in signal strength, Northern blots were exposed to X-ray film for the following lengths of time: PDK2, 10 h; PDK1 and PDK4, 15–16 h; PDK3, 24 h. mRNA sizes were as follows: PDK1, 5.5 kb (major band), 3.5 and 2.0 kb (minor bands); PDK2, 2.4 kb; PDK3, 2.2 kb (major band) and 2.0 kb (minor band); PDK4, 3.9 kb.

Expression and purification of PDK isoenzymes

The individual isoenzymes of PDK have not previously been characterized enzymically, mainly because of the general difficulties in purification and separation of isoenzymes from one another [19,21]. Therefore we obtained the isoenzymes as recombinant proteins for the present study. The cDNAs for all four isoenzymes were expressed in *Escherichia coli* under the control of the strong bacteriophage T_7 promoter as described in the Experimental section. Several important observations were made during optimization of the expression procedure. First, it was found that strain BL21(DE3) cells, deficient in *lon* protease, produce significantly greater amounts of full-sized soluble kinases than strain HMS174(DE3). Second, the yield of soluble enzymes was also improved when the kinases were co-expressed with molecular chaperonins GroEL and GroES. The presence of chaperonins did not affect the overall level of expression, but rather increased the amount of soluble catalytically active kinase. The effect was especially apparent when induction was allowed to continue for more than 20 h. Third, the highest levels of soluble kinase were obtained when the temperature of expression was lowered to 22–25 °C.

His-tagged kinases were purified by immobilized metal affinity chromatography on Talon resin as described in the Experimental section. The purity of preparations was more than 90% as judged by SDS/PAGE with Coomassie Blue staining (results not shown). Proteolytic degradation of isoenzyme PDK2 and especially PDK4 was encountered in these studies, as evidenced by fragments with a molecular mass of 20–30 kDa. This problem was solved by carrying out all purification in the presence of a battery of protease inhibitors which effectively suppressed the proteolytic degradation. On average, the described procedure yielded approx. 5–10 mg of the recombinant kinase from 1 litre of bacterial culture.

Kinetic parameters of the recombinant isoenzymes of PDK

The kinetic parameters for isoenzymes of PDK are summarized in Table 1. The isoenzymes are most noticeably different with respect to their specific activities. The activity of the most active isoenzyme PDK3 was 25-fold higher than the activity of the least active PDK2 (1250 compared with 50 nmol/min per mg of protein). The specific activities of PDK1 and PDK4 were 650 and 400 nmol/min per mg of protein respectively. These values are still far above the activity of PDK2. The apparent K_m values for ATP were much more uniform, being within the range of 50–65 μM for PDK1, PDK3 and PDK4. That for PDK2 was somewhat lower (10 μM). In accord with previous studies [12,20], ADP was found to be a competitive inhibitor of kinase activity with respect to ATP. For three isoenzymes (PDK2, PDK3 and PDK4), the apparent K_i values for ADP were within the close range of 80–120 μM (Table 1). The apparent K_i value for PDK1 was approximately 3-fold higher (370 μM).

Table 1 Kinetic parameters of PDK isoenzymes

The values for apparent K_m and V_{max} for PDK isoenzymes were determined by measuring the kinase activities at five different concentrations of ATP as described in the Experimental section. ATP concentrations used were within a range of 30–600 μM for isoenzymes PDK1, PDK3 and PDK4, and 5–100 μM for isoenzyme PDK2. Specific activities for PDK isoenzymes were calculated on the basis of the V_{max} values obtained. The apparent K_i values for ADP were determined by measuring the activities of PDK isoenzymes at five different ATP concentrations in the presence of four different concentrations of ADP (25 total data points). Concentrations of ATP were varied as described above. The range of ADP concentrations used was 0–300 μM for isoenzymes PDK2, PDK3 and PDK4. For isoenzyme PDK1 it was between 0 and 800 μM. Phosphorylation reactions were initiated by addition of the appropriate mixtures of ADP and ATP. Data were analysed using GraFit software (Eritacus Software). Error values given are standard deviations for at least three separate preparations. The best fit for all isoenzymes was obtained when ADP was assumed to be a competitive inhibitor with respect to ATP.

Parameter	PDK1	PDK2	PDK3	PDK4
Specific activity (nmol/min per mg)	650 ± 80	50 ± 5	1250 ± 200	400 ± 60
Apparent K_m for ATP (μM)	60 ± 5	10 ± 1	50 ± 5	65 ± 6
Apparent K_i for ADP (μM)	370 ± 20	120 ± 20	80 ± 10	100 ± 15

Table 2 DCA inhibition of the activities of PDK isoenzymes

The assay of PDK activity was conducted as described in the Experimental section. The concentration of ATP was held constant at 0.6, 0.1, 0.5 or 0.65 mM for isoenzymes PDK1, PDK2, PDK3 and PDK4 respectively. The respective concentrations of ADP and DCA were varied as follows: for PDK1, from 0.2 to 1.6 mM and from 0 to 6 mM; for PDK2, from 0.1 to 0.8 mM and from 0 to 2 mM; for PDK3, from 0.1 to 0.8 mM and from 0 to 16 mM; for PDK4, from 0.1 to 0.8 mM and from 0 to 3 mM. ADP was used at four different concentrations. DCA was used at five different concentrations. Kinase reactions were initiated with the appropriate mixture of inhibitors and ATP. Data were analysed by Dixon plot. Error values given are standard deviations for at least three separate preparations. The apparent K_i values for ADP in the presence of DCA were obtained from intercepts for families of primary plots of $1/v$ against [ADP] at different [DCA] on the [ADP] axis [26].

Parameter	PDK1	PDK2	PDK3	PDK4
Apparent K_i for DCA (mM)	1.0 ± 0.2	0.20 ± 0.05	8.0 ± 1.0	0.5 ± 0.2
Apparent K_i for ADP in the presence of DCA (μM)	50 ± 10	35 ± 5	50 ± 10	200 ± 30

Effects of dichloroacetate (DCA) and ADP on the activities of isoenzymes of PDK

DCA is one of the few known highly specific synthetic inhibitors of PDK which is believed to mimic the effect of pyruvate, but, in contrast with pyruvate, does not activate the kinase activity at low concentrations [24]. It is generally believed that DCA binds to the kinase–ADP complex. As a consequence, its effect depends on the presence of ADP [20]. Therefore we studied the inhibition of kinase activity at a large number of ADP and DCA concentrations and analysed the data by Dixon plots [25]. The results for all four isoenzymes of PDK are shown in Table 2. Isoenzyme PDK2 was the most sensitive to inhibition by DCA (apparent K_i of 0.2 mM), a value 40-fold lower than the K_i for the least sensitive isoenzyme PDK3, 8.0 mM. The K_i values for isoenzymes PDK1 and PDK4 were 1.0 and 0.5 mM respectively. For most isoenzymes, DCA increased the effectiveness of ADP as a competitive inhibitor of kinase activity, as would be expected from the kinetic analysis made previously for native PDK [20]. This effect was especially apparent for isoenzymes PDK1 and PDK2, which showed approximately 7- and 4-fold decreases respectively in the apparent K_i for ADP in the presence of DCA (Table 2). In contrast, for isoenzyme PDK4 the apparent K_i value for ADP was somewhat increased (from 100 to 200 μM), suggesting hindered inhibitor binding for this isoenzyme [26].

Table 3 Effects of NADH and NADH plus acetyl-CoA on the activity of PDK isoenzymes

The activity of PDK was determined as described in the Experimental section with the addition of NADH and NAD^+ in a mixture to a final concentration of 0.6 and 0.2 mM respectively, and of acetyl-CoA to 50 μM. Effectors were added 30 s before ATP to allow for equilibration of the reactions catalysed by E2 and E3 components. The final concentrations of ATP were as follows: 0.6 mM for PDK1, 0.1 mM for PDK2, 0.5 mM for PDK3 and 0.65 mM for PDK4. Controls were determined with the addition of a mixture of NADH and NAD^+ (molar ratio 1:200) to the final total concentration of 0.8 mM. Numbers in parentheses are percentage of control. Error values given are standard deviations for at least three separate preparations.

	Kinase activity (nmol/min per mg of protein)			
Effectors	PDK1	PDK2	PDK3	PDK4
NAD^+/NADH (200:1)	460 ± 10 (100)	33 ± 2 (100)	1020 ± 80 (100)	220 ± 15 (100)
NAD^+/NADH (1:3)	540 ± 15 (120)	44 ± 5 (130)	1150 ± 90 (110)	410 ± 20 (190)
NAD^+/NADH (1:3) plus 50 μM acetyl-CoA	820 ± 30 (180)	105 ± 10 (320)	680 ± 50 (60)	340 ± 25 (150)

Effects of NADH and NADH plus acetyl-CoA on the enzymic activities of PDK isoenzymes

The effects of NADH and NADH plus acetyl-CoA on kinase activity are believed to be mediated by the lipoyl-bearing moiety of the transacetylase component [11]. It has been suggested that PDK binds to the E2 core through an association that requires the lipoyl prosthetic group [27]. By the nature of this association, the kinase acquires the ability to sense the reduced and acetylated states of lipoates, which reflect the ratios of NAD^+/NADH and CoA/acetyl-CoA. Therefore, under conditions in which the NAD^+/NADH ratio is high (excess of NAD^+), the lipoates are mainly in the oxidized form and kinase activity is low. When the NAD^+/NADH ratio is low (high NADH), the lipoates are in the reduced form and kinase activity is higher. Finally, in the presence of both acetyl-CoA and NADH, lipoates are in the reduced and acetylated state and this translates into further activation of kinase. Therefore, to evaluate the effects of NADH and NADH plus acetyl-CoA on the activities of PDK isoenzymes, PDC reconstituted with kinase was preincubated in the solutions containing NAD^+/NADH (molar ratio of 1:3) or NAD^+/NADH plus acetyl-CoA (50 μM), essentially as described by Ravindran et al. [27]. After a preincubation period needed for the reactions leading to reduction or reduction and acetylation of the lipoates to equilibrate, phosphorylation reactions were initiated with ATP. The controls were set up in a similar way except the NAD^+/NADH mixture was used at a molar ratio of 200:1 in order to establish the oxidized form of lipoates [28]. Differences in activities among the isoenzymes were quite apparent under these conditions (Table 3). Isoenzymes PDK1 and PDK2 showed approximately 20 and 30% activation respectively in response to NADH. NADH with acetyl-CoA produced further activation of both isoenzymes, reaching approximately 2 and 3 times the control values respectively. In marked contrast with isoenzymes PDK1 and PDK2, PDK3 showed little, if any, activation in response to NADH, whereas the simultaneous presence of NADH and acetyl-CoA resulted in 40–50% inhibition of its activity. It is interesting to note, in this respect, that the latter

anomalous response to NADH plus acetyl-CoA has been reported to occur for the PDK of PDC isolated from plants [29]. Under comparable conditions isoenzyme PDK4 was activated almost 2-fold by NADH. With NADH plus acetyl-CoA, PDK4 did not show activation above that seen with NADH alone.

DISCUSSION

The results of the present study clearly show that isoenzymes of PDK are different with respect to their tissue distribution, kinetic parameters and regulation. Of four isoenzymes studied here, only PDK2 appears to be ubiquitously and abundantly expressed. It is not surprising therefore that its characteristics most closely resemble those of the kinase described in the literature. As reported for the native enzyme, PDK2 has a fairly low specific activity [28,30], a low K_m value for ATP [12], low K_i values for ADP and DCA [20,31], and responds strongly to NADH plus acetyl-CoA [27]. These observations are consistent with the idea that PDK2 is the major isoenzyme responsible for metabolic control over PDC activity.

The physiological significance of the other isoenzymes is currently unknown. Owing to their rather limited tissue distribution, it seems likely that their functions are more specialized than PDK2. Isoenzyme PDK4 is of special interest in this respect. As shown in the following paper [32], the level of expression of PDK4 increases severalfold during starvation. This suggests that PDK4 may contribute to the regulation of the adaptive response or long-term control [5,13] of PDC activity. This role would be consistent with reports of the effect of starvation on the regulatory properties of kinase. It appears that 48 h of starvation increases severalfold the total kinase activity [8,13] and, at the same time, decreases the ability of pyruvate to re-activate PDC by inhibiting the kinase [33]. This outcome is consistent with results reported here. The isoenzyme PDK4 was found to have fairly high specific activity and approx. 3-fold lower sensitivity than PDK2 to inhibition by DCA, the synthetic analogue of pyruvate. Its overexpression during starvation therefore should lead to the changes described by Priestman et al. [33]. It is of interest to see in this respect whether the 48 h starvation affects the response of kinase to NADH plus acetyl-CoA, because isoenzyme PDK4 does not respond to this combination of effectors. Its overexpression in starvation should somewhat diminish the magnitude of total kinase activation by NADH and acetyl-CoA. This, in turn, should affect the regulation of PDC activity by fatty acids.

The isoenzyme PDK3 is unique because it has an extremely high specific activity that is not significantly regulated by most of the effectors. In fact, the specific activity of PDK3 is so high that it reaches the highest specific activity ever reported for pyruvate dehydrogenase phosphatase [34]. It is not surprising therefore that the level of PDK3 expression is extremely low in most tissues. Otherwise, the steady-state level of PDC phosphorylation, which depends on the relative activities in kinase and phosphatase, could not be achieved. This analysis suggests that PDK3 must play a unique role in testes where it is abundantly expressed. It is interesting to note, in this respect, that sperm is the only type of cell that expresses a different form of the E1 component [35]. The gene encoding the E1 α-subunit in mammals is localized on the X chromosome [36,37] and it does not function in sperm cells after meiosis. Evidence indicates that an autosomal gene for E1α becomes transcriptionally active after meiosis [37,38] and its protein product substitutes for the original E1 component. It is attractive to suggest that PDK3 promotes E1 isoenzyme switching by phosphorylating and inactivating old copies of the E1 component. Experiments to check this hypothesis are currently in progress.

The role of isoenzyme PDK1 is the most uncertain. Its tissue distribution is basically limited to heart muscle and it is very similar to isoenzyme PDK2 with respect to its kinetic parameters and regulation. Thus far, apparent differences in only a few parameters have been found between PDK1 and PDK2. Isoenzyme PDK1, in contrast with PDK2, has much higher specific activity and shows stronger synergism in the inhibitory response to DCA and ADP. On the basis of these observations, isoenzyme PDK1 may be responsible for sensing simultaneous changes in intramitochondrial concentrations of pyruvate and ADP. However, it remains to be established whether this regulatory feature is physiologically important or whether there are other unidentified signals characteristic of heart muscle that actually regulate the activity of PDK1.

The results of the present study strongly suggest the existence of tissue-specific regulation of PDC activity. The evidence supporting this idea comes from experiments on tissue distribution and enzymic properties of PDK isoenzymes. Our experiments show that (1) most of the tissues have a unique subset of expressed PDK isoenzymes and (2) the isoenzymes are different with respect to their kinetic parameters and regulation. Therefore in different tissues the activity of PDC, at least in the short term, is probably regulated differently. It also seems reasonable to suggest that some isoenzymes, such as PDK1 and especially PDK2, are specialized for short-term or metabolic control of PDC activity, whereas others, such as PDK4, are more involved in the regulation of adaptive responses of PDC in circumstances such as starvation.

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