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Diverse mechanisms of inhibition of pyruvate dehydrogenase kinase by structurally distinct inhibitors

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Abstract

The mechanism of action of structurally distinct pyruvate dehydrogenase kinase (PDK) inhibitors was examined in assays with experimental contexts ranging from an intact pyruvate dehydrogenase complex (PDC) with and without supplemental ATP or ADP to a synthetic peptide substrate to PDK autophosphorylation. Some compounds directly inhibited the catalytic activity of PDKs. Some of the inhibitor classes tested inhibited autophosphorylation of recombinant PDK1 and PDK2. During these studies, PDC was shown to be directly inhibited by a novel mechanism; the addition of supplemental recombinant PDKs, an effect that is ADP-dependent and partly alleviated by members of each of the compound classes tested. Overall, these data demonstrate that small molecules acting at diverse sites can inhibit PDK activity.

Keywords: Pyruvate dehydrogenase kinase; Inhibition mechanism; Autophosphorylation

1. Introduction

The pyruvate dehydrogenase multienzyme complex (PDC, 5–10 million Da) catalyzes a key regulatory step in oxidative glycolysis, the irreversible decarboxylation of pyruvate. Its activity is reduced under conditions of increased fatty acid oxidation, i.e. when tissue [acetyl-CoA]/[CoA] and [NADH]/[NAD^+] ratios are elevated. Thus, PDC activity is down-regulated during fasting and in pathological conditions associated with insulin resistance, such as diabetes and obesity [1–4].

The mammalian PDC is composed of multiple copies of three enzymes: pyruvate decarboxylase (E1 subunit), dihydrolipoyl acetyltransferase (E2 subunit, a modular protein which contains a transacetylase and so-called outer and inner lipoyl domains, $E2_c$, $E2_{L1}$ and $E2_{L2}$, respectively), and dihydrolipoyl dehydrogenase (E3 subunit). It is regulated by reversible phosphorylation [5]. Four pyruvate de-

Abbreviations: DCA, dichloroacetate; E1, pyruvate decarboxylase; E2, dihydrolipoyl acetyltransferase; E3, dihydrolipoyl dehydrogenase; E2L, lipoylated $rE2_{L2}$; E2N, non-lipoylated $rE2_{L2}$; PDC, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; PDP, pyruvate dehydrogenase phosphatase; p[NH]ppA, adenosine 5′-[β,γ-imido]triphosphate; $rE2_{L2}$, heterologously expressed E2 inner lipoyl domain; rPDK, recombinant PDK

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hydrogenase kinase (PDK) and two pyruvate dehydrogenase phosphatase (PDP) isozymes have been identified to date [6,7]. PDKs inactivate PDC by catalyzing the ATP-dependent phosphorylation of three serine residues on the PDC E1 subunits; the phosphorylation of one of these serine residues accounts for most of the inhibition of E1 catalytic activity [8,9]. PDPs catalyze the reactivation of phosphorylated E1 subunits [10]. PDK activity is enhanced under conditions of high [acetyl-CoA]/[CoA] and [NADH]/[NAD^+] ratios, conditions which are believed to modulate the binding of PDKs to the PDC. Binding to the inner lipoyl domain E2 subunit ($E2_{L2}$ of E2) is important for the efficient catalytic function of both PDK(s) and PDP(s) [11–13]. PDKs also appear to be subject to autophosphorylation, but the functional consequence of this is unknown [13].

The blood glucose lowering effect of dichloroacetate (DCA) in diabetic rodents [14] and Type 2 diabetic patients [15] has been ascribed to its ability to inhibit PDKs and thereby activate PDC in vivo. The primary structures of PDK isozymes 1–4 share little sequence homology with other eukaryotic protein kinases and are more homologous to prokaryotic histidine kinases [16]. These data led us to embark on an effort to identify novel inhibitors of PDK activity as possible therapeutic agents for diabetes and other pathological conditions in which PDC activity is reduced (e.g. lactic acidosis, ischemia) [17, 18].

Until recently, the only known small molecule inhibitors of PDK activity were pyruvate, DCA, halogenated acetophenones (e.g. **1**) [19] and analogues of ATP (e.g. adenosine 5′-[β,γ-imido]triphosphate (p[NH]ppA), **2**) (Fig. 1). We have recently discovered that appropriately substituted triterpenes (e.g. **3**), lactones (e.g. **4**) and (*R*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionamides (e.g. **5**) are modest to very potent inhibitors of PDK [20–22] (Fig. 1). Previously, Pratt and Roche demonstrated that pyruvate and DCA are inhibitors of PDKs, acting synergistically with ADP [23]. A similar mechanism of action has been ascribed to halogenated acetophenones, although without experimental support [19]. ADP and **2** inhibit PDK competitively with respect to ATP [13,23]. Heterologously expressed lipoylated E2 inner lipoyl domain ($rE2_{L2}$) is also capable of PDK inhibition and may act by affecting the equilibrium between free PDK(s) and PDK(s) bound to the E2 structural core of PDC [13]. Therefore, at least one other binding site(s) other than the pyruvate/DCA and ATP/nucleotide sites is likely to play an important role in PDK regulation.

This report describes the results of studies aimed at determining the mechanism of action of representatives of three recently described series of novel PDK inhibitors, DCA and a halogenated acetophenone. The results point to diverse mechanisms of inhibition. Moreover, PDC is shown to be directly inhibited by the addition of supplemental recombinant PDKs (rPDKs), an effect that is ADP-dependent and partly alleviated by members of each of the compound classes tested.

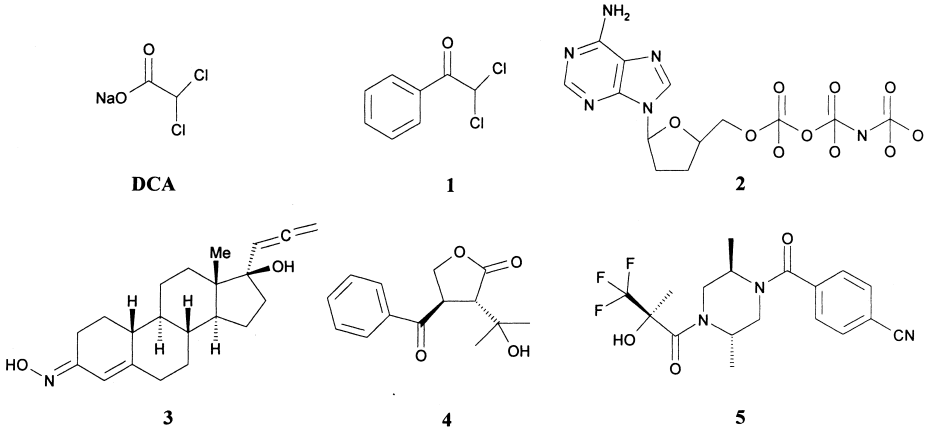

Fig. 1. Structures of PDK inhibitors.

2. Materials and methods

2.1. Chemicals and enzymes

The synthesis of compounds **3**, **4** and **5** is described elsewhere [20–22,24]. All other materials were from the sources previously described [13].

The heterologous expression and purification of proteins was achieved as follows: in brief, $E2_{L2}$ domain cDNA constructs encoding human E2 amino acids 181–276 (in pET17b) were expressed in *Escherichia coli* (strain BL21 (DE3)/pLys S) grown in the presence of 10 μg/ml lipoic acid. Clarified cell lysates were loaded onto a column of Q-Sepharose HP. $rE2_{L2}$ was eluted using a linear gradient of 0–1 M NaCl. The $rE2_{L2}$-containing fraction was adjusted to 40% w/v $(NH_4)_2SO_4$, centrifuged and applied to a column of Phenyl Sepharose HP. Lipoylated and non-lipoylated $rE2_{L2}$ were eluted with a linear gradient of 100–0% w/v $(NH_4)_2SO_4$ in phosphate-buffered saline (PBS), 5 mM DTT. Lipoylated $rE2_{L2}$ eluted 5–10% later in the gradient than non-lipoylated material, which was a baseline separation [13].

N-Terminal hexa-His-tagged rat PDK1 and PDK2 cDNA constructs (in pET28A) were expressed in *E. coli* (strain BL21 (DE3)/ pLys S) with the chaperones GroES/L. Clarified cell lysates were loaded onto a column packed with Pharmacia Chelating Sepharose Fast Flow equilibrated with PBS containing 0.5 M NaCl (buffer I). The column was washed with buffer I until A_{280} of the eluate had stabilized. PDK protein was eluted using a 0–100% gradient of buffer II (buffer I plus 250 mM imidazole, pH 7.5) [13].

2.2. Functional PDK assay

Compounds were assessed for their ability to inhibit the ATP-dependent inactivation of porcine heart PDC as previously described [13]. The assay essentially consisted of three steps. First, a bovine serum albumin (BSA)-stabilized PDC preparation was acetylated to enhance its intrinsic PDK activity. Second, the PDK reaction was initiated by the addition of ATP. Third, after 7 min the PDK reaction was terminated and the residual PDC activity was assessed by monitoring the formation of NADH at 340 nm. In some experiments, the PDC was either supplemented with rPDK and/or ATP was omitted during the second step.

2.3. E1 subunit phosphorylation assay

The phosphorylation of the PDC E1 subunit by endogenous PDK was measured as previously described [13]. In brief, the reaction was initiated by the addition of [γ-^{33}P]ATP (80 mCi/mmol) to intact porcine PDC. The reaction was terminated after 45 s by the addition of trichloroacetic acid. Precipitated protein was recovered by centrifugation, dissolved in 1 M NaOH and the radioactivity of the entire sample was determined by liquid scintillation counting.

2.4. PDK peptide phosphorylation assay

rPDK activity was determined by measuring the rPDK-catalyzed phosphorylation of an acetylated tetradecapeptide substrate, Ac-YHGHSMSDPGV-SYR, as previously described [13], except that the reaction volume was 25 μl. Effects of test compounds on rPDK activity were usually determined in the presence of 0.2 mM [γ-^{33}P]ATP (0.05 μCi/nmol ATP) and 0.5 mM peptide substrate. In experiments where the [γ-^{33}P]ATP (0.05–0.85 μCi/nmol ATP) concentration was varied, the final peptide concentration was 0.5 mM; when the peptide concentration was varied, the final [γ-^{33}P]ATP concentration was 0.2 mM. Results are expressed as specific activity (nmol of phosphate transferred per mg of PDK protein per 30 min).

2.5. Kinase autophosphorylation assay

rPDK1 or rPDK2 (50 μg/ml) was incubated in a 25 μl reaction mixture containing MOPS (40 mM, pH 7.2), KH_2PO_4 (20 mM), EDTA (0.5 mM), $MgCl_2$ (1.8 mM), KCl (30 mM), DTT (2 mM), NaF (10 mM), [γ-^{32}P]ATP (0.2 mM; 0.01 mCi/mmol) and test compound as indicated, at 37°C for up to 5 min. The reaction was terminated by the addition of 0.1 volume of a mixture of 425 mM H_3PO_4 and 5 mM p[NH]ppA. The sample was then adjusted to pH 7.4 by the addition of 125 μl of a mixture of 60 mM Tris (pH 8.7) and NaCl (500 mM). The entire sample was then applied to a nitrocellulose membrane and rinsed with TBS (20 mM Tris, 500 mM

NaCl, pH 7.5) using a Bio-Dot slot blot apparatus according to the manufacturer's instructions. Phosphorylated proteins were visualized using a phosphor screen and a Storm 840 Imager.

2.6. Phosphorylation of E1 protein by rPDKs

rPDK1 or rPDK2 (7.5 μg) was incubated in the presence or absence of 95 μg of acetylated PDC without BSA [13] in a 75 μl reaction mixture containing MOPS (40 mM, pH 7.2), KH_2PO_4 (20 mM), EDTA (0.5 mM), $MgCl_2$ (1.8 mM), KCl (30 mM), DTT (2 mM), NaF (10 mM) and [γ-^{32}P]ATP (0.2 mM; 0.01 mCi/mmol). Following a 45 min incubation at 37°C, the reaction was terminated by the addition of an equal amount of Laemmli buffer; 40 μl portions were subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE).

2.7. Assay for direct inhibition by supplemental rPDKs

rPDK2 was added to the functional PDK assay described above, which was performed in the absence and presence of ATP. Alternatively, a PDC preparation which was neither stabilized with BSA nor preacetylated was incubated (14 μg/ml) in 180 μl of a buffer (pH 7.4) containing 40 mM MOPS, 0.36 mM EDTA, 30 mM KCl, 1.5 mM $MgCl_2$, 120 mM ADP, 2 mM DTT, 10 mM NaF and the additions indicated, at 37°C for up to 10 min. During this step, ADP was omitted in some experiments.

The PDC reaction was initiated by the addition of 20 μl of a buffer (pH 7.4) containing 40 mM MOPS, 0.36 mM EDTA, 1.5 mM $MgCl_2$, 1 mM DTT, 1.1 mM CoASH, 2.2 mM NAD^+, 2.2 mM pyruvate and 2.2 mM thiamine pyrophosphate. NADH formation over 5 min was monitored at 340 nm.

2.8. Data analysis

IC_{50} values were calculated using the growth/sigmoidal, logistic function in the Origin software package, fixing the upper and lower asymptotes at 100 and 0%, respectively. Where compounds exhibited biphasic responses, the upper asymptote was fixed at the concentration at which the highest value of inhibition was attained.

3. Results and discussion

3.1. Compound effects on PDK catalytic activity and autophosphorylation

The functional PDK assay employed in these studies has a spectrophotometric read-out and measures the effect of PDK activity on PDC function; however the PDK isozyme composition and the stoichiometry of PDK relative to PDC are unknown. Obviously this assay was more difficult to optimize and validate than a traditional kinase assay based upon a model fluorescent peptide substrate (e.g. the cAMP kinase assay kit sold by Pierce; catalog number 26500). Nevertheless, the work required to optimize the assay for high throughput screening was undertaken to identify (via high-throughput screening) and to optimize (via structure–activity relationship studies) lead compounds possessing the ability to inhibit PDK. This work was done because in principle, the functional PDK assay should identify not only compounds acting at known or presumed sites on the kinase (ATP, lipoamide, or pyruvate binding sites) but also those interfering with interactions between PDK and other PDC protein components (e.g. E1 subunit or the inner lipoyl domain (E2L2) of the E2 subunit of PDC). Although inhibitors of protein–protein interactions are rare, we felt our initial screening assay must allow for this possibility. The optimization studies of our initial screening hits and designed hits led to potent orally active inhibitors of

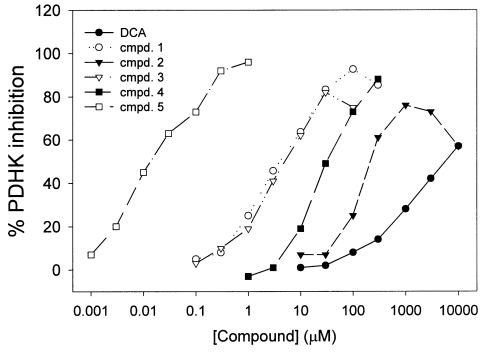

Fig. 2. Effect of compounds in the functional PDK assay. Data shown are mean values from 1–3 independent experiments (triplicate determinations per experiment). Error bars are not shown for the sake of clarity. However, the average coefficient of variation (CV) for individual data points was 3.8% (range 0.18–17%).

PDK [20,22]. Dose-response relationships for the compounds in the functional PDK assay are shown in Fig. 2. The potency of the compounds, from lowest to highest was DCA, p[NH]ppA **2**, the lactone **4**, the triterpene **3**, the dichloroacetophenone **1**, and the (*R*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionamide **5**. The dichloroacetophenone **1**, p[NH]ppA **2** and the triterpene **3** exhibited biphasic responses, with some relief of inhibition being observed at the highest concentrations tested. Approximate IC_{50} values are shown in Table 1. At least compound **5** has physiological relevance, as it has been demonstrated to activate PDC activity in cultured cells and to increase PDH activity ex vivo and to lower blood lactate levels in normal fasted rats following oral administration. Consequently, we attempted to learn more about the mechanism of inhibition of these compounds via additional in vitro assays.

In contrast to the functional PDK assay employed in the optimization, the E1 phosphorylation assay enables PDK activity to be measured directly by testing the compounds for a more specific effect: the ability to inhibit phosphorylation of the E1 subunit by PDK activity intrinsic to the PDC preparation. This assay was used to confirm PDK inhibition in the functional assay. At least one of the concentrations selected for each compound approximated its IC_{50} value determined in the functional assay. The recombinant inner lipoyl domain of the PDC dihydrolipoyl acetyltransferase ($rE2_{L2}$) is a known inhibitor of PDK activity in this assay and was included as a control [13]. Each of the compounds inhibited E1 subunit phosphorylation (Fig. 3). As expected, there was a general concordance between the potency of the inhibition of E1 phosphorylation and the potencies of the compounds in the functional PDK assay. However, the dichloroacetophenone **1**, unlike DCA and the other compounds tested, was much less potent as an inhibitor of PDK-catalyzed phosphorylation of E1 than of the PDK-mediated ATP-dependent inactivation of PDC. At least two possible explanations for this discordance exist: the compound may have exhibited separate PDK inhibition and PDC activation activities. However, at the concentrations tested the dichloroacetophenone **1** and the other compounds do not activate PDC activity in the absence of ATP. The second possibility is that the dichloroacetophenone **1** has greater selectivity for inhibition of PDK(s) phosphorylation of the catalytically relevant serine residue on E1 than the other compounds.

The E1 phosphorylation assay confirmed that apparent PDK inhibition in the functional assay was

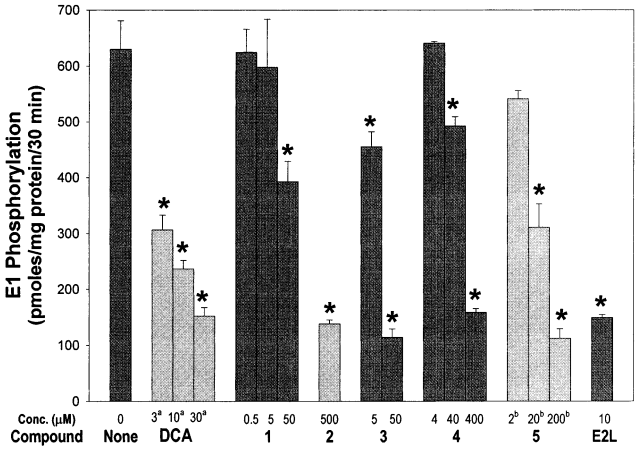

Fig. 3. Effect of compounds and lipoylated human $rE2_{L2}$ on the ability of PDK(s) to phosphorylate porcine PDC E1 protein. [γ-^{32}P]ATP was added to a mixture containing intact porcine PDC containing intrinsic kinase activity. The kinase reaction was terminated after 45 s by the addition of trichloroacetic acid. Precipitated protein was recovered by centrifugation, dissolved in 1 M NaOH and the radioactivity of the entire sample was determined by liquid scintillation counting. Data shown are mean values ± S.D. from a single experiment (triplicate determinations) except for control and p[NH]ppA **2**, where values are mean ± range from two experiments. Concentrations are in μM, except for [a] (mM) and [b] (nM). * Denotes statistically different ($P < 0.05$) from control group in each experiment.

Table 1
Approximate IC_{50} values of compounds in the functional PDK assay

Compound	IC_{50} (mM)
DCA	> 3000
1	5.5 ± 2.6 (S.E.M.)
2	260 ± 62 (range)
3	5.1 ± 0.25 (S.E.)
4	36 ± 4.1 (S.E.)
5	0.017 ± 0.0021 (S.E.)

One to three dose-response experiments were performed for the compounds. IC_{50} values were determined for each dose-response. Where more than one dose-response was performed, the IC_{50} values shown represent the mean value from two or three experiments (errors are indicated as the range or S.E.M.). Where only one dose-response was performed, the standard error of the growth/sigmoidal logistic fit is shown (S.E.).

due to inhibition of E1 phosphorylation. However, this assay suffers the drawback that the PDK isozyme composition and stoichiometry are undefined. Therefore, a much simpler assay with defined stoichiometry of a synthesized tetradecapeptide (a fragment of the E1 subunit which contains all three of the serines which are phosphorylated by PDK) was designed. This assay was modified from work of Reed et al., in which this tetradecapeptide was demonstrated to be a substrate of PDK and was used in assays to demonstrate the selectivity of PDK and the PDH phosphatase [25,26]. Therefore, this assay is in principle the most amenable to kinetic characterization of inhibitors acting at the active site of PDKs.

3.2. Diverse mechanisms of inhibition

The peptide assay was designed to measure the phosphorylation of the tetradecapeptide substrate by heterologously expressed rat rPDK1 or rPDK2 and has previously been validated with DCA and p[NH]ppA **2** (p[NH]ppA was used as a positive control and was the only compound tested which exhibited competitive inhibition with respect to ATP) [13]. At least one of the concentrations chosen for each compound approximated its IC_{50} value determined in the functional PDK. Only DCA and p[NH]ppA **2** inhibited PDK activity in this assay to any appreciable extent (Figs. 4 and 5). Consistent with previous findings [13], DCA was only weakly inhibitory against rPDK2. Interestingly DCA has been reported to exhibit five times greater potency for rPDK2 inhibition than for rPDK1 inhibition in the presence of kinase-depleted PDC as substrate [27]. Also tested against rPDK2 in this assay was heterologously expressed lipoylated or non-lipoylated $rE2_{L2}$ at a concentration known to inhibit PDK activity in both the functional and E1 phosphorylation assays (Fig. 5) [13]. Neither form of the protein inhibited phosphorylation of the peptide substrate by rPDK2. Unlike DCA, the dichloroacetophenone **1** had no effect on phosphorylation of the model peptide substrate with either kinase isoform. Thus, in contrast to previous assumptions [19], it and DCA possess different kinetic mechanisms of action.

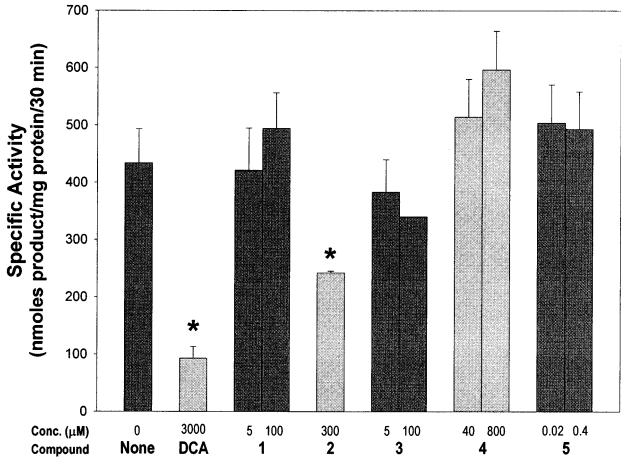

Fig. 4. Effect of compounds on rPDK1 in the peptide phosphorylation assay. Data are mean values ± range from two independent experiments (triplicate determinations per experiment). * Denotes statistically different ($P < 0.05$) from control group in each experiment.

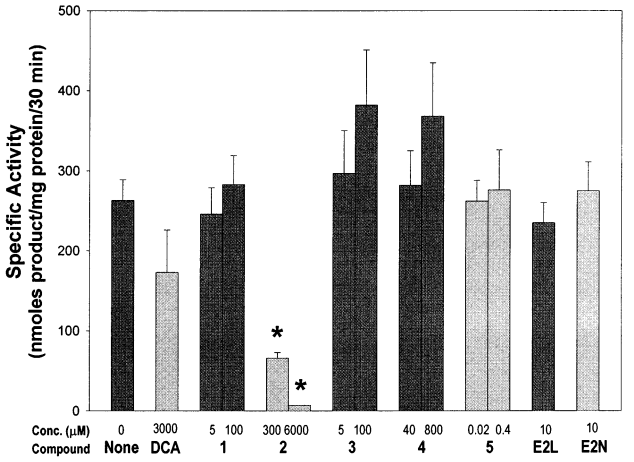

Fig. 5. Effect of compounds and lipoylated and non-lipoylated human $rE2_{L2}$ on rPDK2 in the peptide phosphorylation assay. Data are mean values ± S.E.M. from three independent experiments (triplicate determinations per experiment). E2L and E2N represent lipoylated and non-lipoylated $rE2_{L2}$, respectively. * Denotes statistically different ($P < 0.05$) from control group in each experiment.

Since rPDK2 has previously been demonstrated to be subject to autophosphorylation [13], we investigated whether rPDK1 was also subject to autophosphorylation. A representative result, shown in Fig. 6, demonstrates that both kinases are subject to autophosphorylation (see main bands in lanes marked 'rPDK1' and 'rPDK2'). A comparison of lanes marked 'PDC' (main band represents PDC E1 subunit phosphorylation by endogenous kinase) with lanes marked 'PDC+rPDK1' and 'PDC+rPDK2' (main bands represent phosphorylated PDC E1 sub-

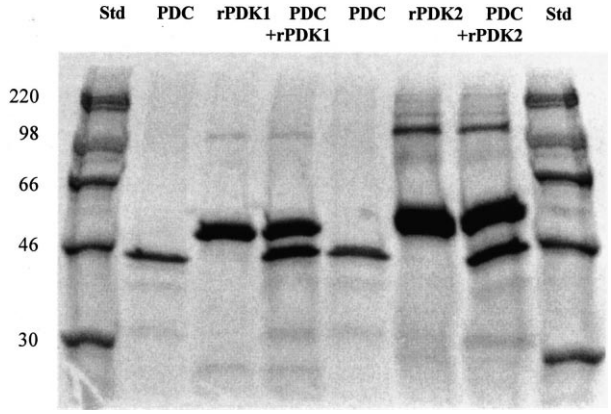

Fig. 6. Autophosphorylation of rPDK1 and rPDK2 and phosphorylation of PDC E1 subunit protein by rPDK1 or rPDK2. rPDK1 or rPDK2 (7.5 μg) was incubated in the presence or absence of 95 μg of acetylated PDC without BSA in a 75 μl reaction mixture containing [γ-^{32}P]ATP. Following a 45 min incubation at 37°C, the reaction was terminated by the addition of an equal amount of Laemmli buffer; 40 μl portions were subjected to SDS/PAGE electrophoresis. Results are from a representative experiment. The outside lanes contain protein standards (labelled Std); molecular masses (in kDa) are shown on the left.

unit and phosphorylated recombinant kinase) reveals that both recombinant kinases are also capable of phosphorylating the PDC E1 subunit. However, the stoichiometry and physiological relevance of PDK autophosphorylation requires exploration. Preliminary data suggest that the doubling time for autophosphorylation was about 2.5 and 1 min for rPDK1 and rPDK2, respectively (data not shown). Since rPDKs displayed apparent Michaelis–Menten kinetics in the peptide phosphorylation assay, autophosphorylation is unlikely to exert a direct effect on PDK catalytic activity as it does on other kinases (e.g. phosphorylase kinase [28]). Furthermore, when phosphorylated rPDKS were added to an intact PDC, they were capable of phosphorylating the PDC E1 subunit. When the compounds were tested for their effects on rPDK2 autophosphorylation, only DCA and p[NH]ppA **2** inhibited rPDK2 autophosphorylation and they did so with potencies concordant with their IC_{50} values in the functional PDK assay (Fig. 7). These findings may provide a basis for exploring the functional relevance of PDK autophosphorylation. For instance, autophosphorylation may be a necessary component of PDK function in the context of an intact PDC and may partly explain the apparent reversal of relative DCA potency against rPDKs in the presence of different substrates (i.e. model peptide or kinase-depleted PDC [27]).

Unlike DCA and p[NH]ppA **2**, the dichloroacetophenone **1**, the triterpene **3**, the lactone **4**, the (*R*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionamide **5**, and heterologously expressed lipoylated or non-lipoylated $rE2_{L2}$ were inactive in the peptide phosphorylation and autophosphorylation assays. Since the peptide phosphorylation assay is a simple model of PDH complex phosphorylation by PDKs, compounds interfering with the known interaction of PDKs with PDH E2 subunits (i.e. lipoylated $rE2_{L2}$) would not be expected to be active in this assay. We therefore directly investigated the effects of the compounds on the interaction(s) between PDKs and the PDC. Despite evidence that PDKs resolved from bovine kidney bind lipoylated human E2 and $rE2_{L2}$ domains [11,12], reports of binding of recombinant rat PDKs to lipoylated human $rE2_{L2}$ are lacking. Indeed, we have previously concluded that binding of PDKs to E2 may occur via a complex binding stoichiometry [13]. We have since extended our attempts to demonstrate an interaction between these heterologously expressed proteins using the technique of surface plasmon resonance [29,30]. These studies, in which either protein was immobilized to the carboxymethylcellulose surface via a variety of techniques, were unsuccessful in demonstrating a

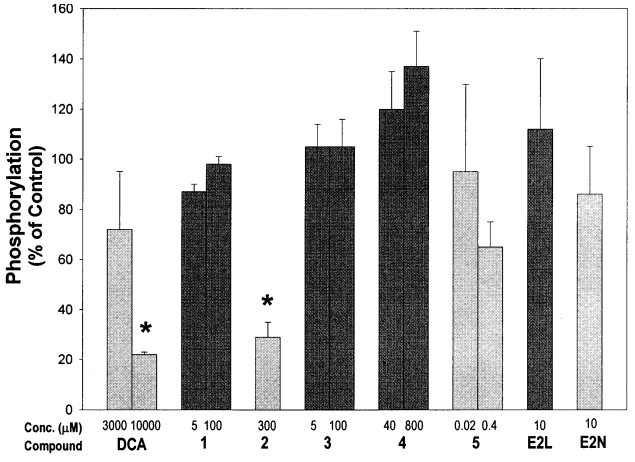

Fig. 7. Inhibition of rPDK2 autophosphorylation. Results are mean values ± S.E.M. from three experiments. Each experimental treatment consisted of triplicate determinations. * Denotes statistically different ($P < 0.05$) from control group in each experiment.

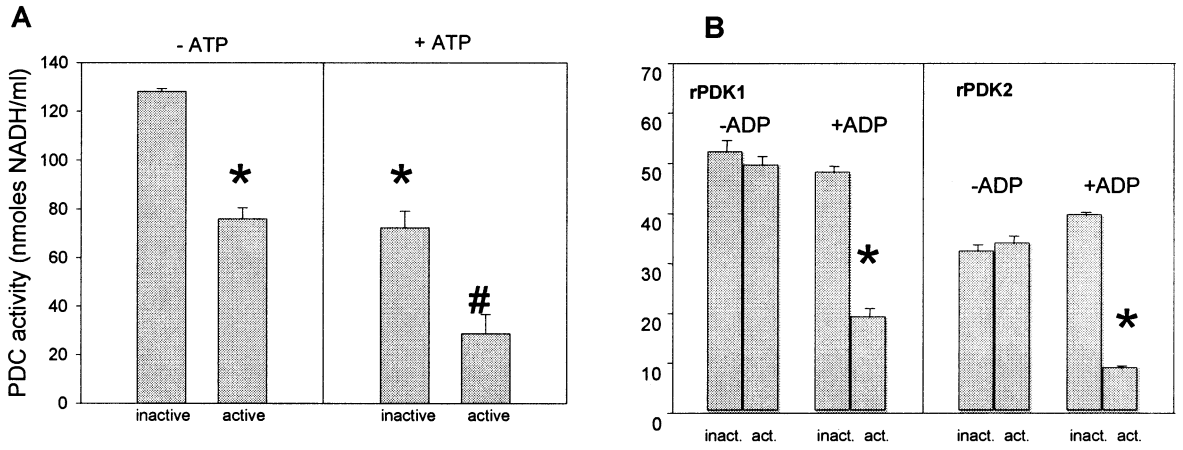

Fig. 8. Inhibition of PDC by rPDKs. (A) Effect of 1 μg/ml rPDK2 on acetylated, BSA-stabilized porcine PDC (21 μg/ml PDC protein) in the presence or absence of ATP. Data are mean ± S.E.M. from three independent experiments (triplicate determinations per experiment), * and # denote statistically different ($P < 0.05$) from heat-inactivated (inactive) minus ATP sample and inactive plus ATP sample groups, respectively. (B) Effect of 10 μg/ml rPDKs on non-preacetylated, BSA-free porcine PDC (14 μg/ml) in the presence or absence of ADP. Data are mean values ± S.D. from a single experiment (triplicate determinations per experiment); * denotes statistically different ($P < 0.05$) from inactivated (inact.) plus ADP sample group.

binding interaction and appeared to confirm our previous conclusion. Therefore, we took the approach to supplement PDC with rPDKs.

3.3. *Identification of a direct effect of rPDKs on PDC, and the effect of compounds*

As expected, ATP-dependent phosphorylation of the PDC occurred at a more rapid rate in the presence of added rPDKs. In early experiments in which the functional PDK assay (PDC with intrinsic PDK activity) was supplemented with rPDK2 in the presence and absence of ATP (ADP present in all incubations), we discovered that addition of 1 μg/ml rPDK2 inhibited acetylated PDC in the absence of ATP (Fig. 8A). This direct effect was confirmed by comparing the effect of active and heat-inactivated rPDKs using PDC that was neither BSA-stabilized nor pre-acetylated and was found to be ADP-dependent (Fig. 8B). The observation that PDC was directly inhibited by rPDKs in an ADP-dependent manner was unexpected. This inhibition is likely due to the direct binding of rPDK2 to the inner and/or outer E2 lipoyl domains. Thus, a requirement for PDK binding to the lipoyl domain appears to be occupancy of a nucleotide site on the kinase.

We therefore next investigated the effect of the compounds on the inhibition caused by the addition of supplemental rPDK2 on acetylated PDC. We found that each of the compounds tested alleviated the nucleotide-dependent inhibitory effect of rPDK2 at concentrations relevant to their potencies in the functional PDK assay (Fig. 9). In addition, DCA and p[NH]ppA **2** also diminished the ability of supplemental rPDK1 to inhibit PDC. Thus, these studies suggest that all of these compounds interfere with the interaction between PDK and PDC. It is tempting to

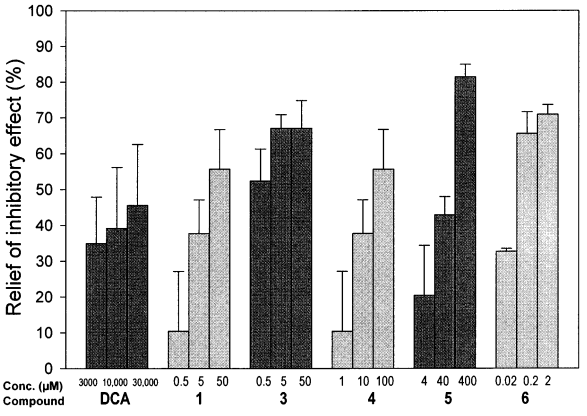

Fig. 9. Effect of compounds on the inhibitory effect of rPDK2 (1 μg/ml) on acetylated, BSA-stabilized porcine PDC (21 μg/ml PDC protein). PDC activity in the absence and presence of 1 μg/ml rPDK2 (representing 100 and 0% relief, respectively) was 130 ± 1.2 and 76 ± 4.4 nmol NADH formation/5 min/ml, respectively. Data shown are mean values ± S.E.M. of three independent experiments (triplicate determinations per experiment), except for compound **4**, where data represent the mean values ± range of two experiments.

speculate that binding at diverse sites (i.e. the nucleotide site by p[NH]ppA, the pyruvate site by DCA, the lipoyl domain site by lipoylated $rE2_{L2}$ and/or **1**, **3**, **4**, **5** or possibly other undefined sites of binding) on PDK can induce conformational changes that impact both on its catalytic activity (DCA and p[NH]ppA) and/or on its interactions with the PDC (all compounds). These findings also provide a possible mechanistic basis for the observation that inhibition of PDK-catalyzed phosphorylation of PDC by DCA and pyruvate is synergistic with ADP [23]. Further studies are required to determine whether the relief of this inhibitory effect is through binding of the compounds to rPDKs or to the PDC, or to both.

The compounds described herein modulated PDK catalytic activity (against the E1 subunit and/or a model peptide substrate), the nucleotide-dependent binding of PDK to the complex and PDK autophosphorylation to varying extents. Effective inhibition of PDK activity was achieved with small synthetic molecules acting at diverse sites and via at least three or more kinetic mechanisms. It is noteworthy that several structural classes of effective PDK inhibitors would not have been identified as active had the peptide phosphorylation assay been used for high throughput screening. For example, compounds such as the (*R*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionamide **5** are potent and effective PDK inhibitors with respect to PDC function when dosed in vivo at concentrations as low as 3 μmol/kg but are inactive against PDK in the stoichiometrically defined PDK peptide phosphorylation assay. The possibility that binding of PDKs to PDC may be augmented by the presence of nucleotides provides a new target for rational drug design.

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