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ACTIVATION OF PYRUVATE DEHYDROGENASE COMPLEX (PDC) OF RAT HEART MITOCHONDRIA BY GLYBURIDE

Cynthia K. Buffington and Abbas E. Kitabchi

Departments of Medicine and Biochemistry
and Clinical Research Center
The University of Tennessee Center for the Health Sciences
Memphis, Tennessee 38163

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Summary: The effects of the second generation sulfonylurea, glyburide, on the pyruvate dehydrogenase multienzyme complex (PDC) of rat myocardial tissue were examined using rat ventricular slices and isolated mitochondria. Therapeutic concentrations (10^{-7} to 10^{-6}M) of glyburide produced a 30% increase in the decarboxylation of [1^{-14}C] pyruvate by the PDC of ventricular tissue. Addition of glyburide to intact rat heart mitochondria stimulated activity of the PDC in a time- and concentration-dependent manner. Half-maximal stimulation of the enzyme occurred with 6 x 10^{-5}M glyburide and maximal activation of the enzyme was achieved with 1 x 10^{-4}M glyburide. At the height of stimulation, PDC activities were 6-fold greater than those observed under control conditions with succinate alone. When mitochondria were disrupted by sonication or freeze-thawing, glyburide produced no stimulation of pyruvate decarboxylation. We conclude that glyburide directly stimulates the decarboxylation of pyruvate by the PDC of the myocardium. Furthermore, the presence of intact mitochondria is necessary for the stimulatory action of glyburide on the PDC.

INTRODUCTION: Sulfonylureas have been widely used in the treatment of type II diabetes. Acute administration of these agents lowers blood glucose levels by increasing insulin secretion (1-2). The long-term hypoglycemic actions of the sulfonylureas, however, are believed to be of extrapancreatic origin as improvements in glucose tolerance are noted in the presence of normal or decreased insulin secretion (3-5). The mechanisms underlying the extrapancreatic actions of the sulfonylureas are unknown but may involve stimulation of carbohydrate metabolism. The sulfonylureas have been shown to both potentiate and mimic the actions of insulin on glucose uptake (6-9), glycogenesis (9,10), glycolysis (11,12) and glucose oxidation (11,13). Key enzyme systems of carbohydrate metabolism affected by the sulfonylureas include glycogen synthase (10), phosphofructokinase (11,12) and phosphorylase (11).

The pyruvate dehydrogenase multienzyme complex (PDC) is one of the most important regulatory enzymes of carbohydrate metabolism. The PDC is located

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within the mitochondrial membrane and controls the flow of carbon from carbohydrate to the tricarboxylic acid cycle (as reviewed by 14,15). To our knowledge, little information is available as to the effects of the sulfonylureas on the PDC.

The purpose of the present investigation was to determine the effects of the second generation sulfonylurea, glyburide, on the metabolic flux of pyruvate through the PDC of rat ventricular slices and the activity of the PDC of rat heart mitochondria. We report a hitherto unknown direct stimulatory effect of glyburide on the decarboxylation of [1-^{14}C] pyruvate by the PDC of heart ventricular slices and, more specifically, an effect of glyburide on the activation state of the enzyme in a cell-free rat heart mitochondrial system.

MATERIALS AND METHODS

Radiolabelled [1-^{14}C] pyruvic acid (sp. act. = 25.9 mCi/mmol) was purchased from Amersham (Arlington, IL), phenylethylamine and Liquiscint from National Diagnostics (Sommerville, NJ), center wells and serum stoppers from Kontes (Vineland, NJ). Potassium glyburide was a gift from the Upjohn Company. All other materials for the PDC assays were obtained from commercial sources as described previously (16).

Male Sprague-Dawley rats (Sasko) weighing 150-200 g were housed in groups of three and fed ad libitum. Hearts were rapidly excised following decapitation and washed in cold sucrose-mannitol buffer, pH 7.4. Ventricles were trimmed free of fat and connective tissue, sliced into 1 mm portions, and added to 25 ml Erlenmeyer flasks sealed with serum stoppers equipped with center wells. Each flask contained approximately 0.3 g of tissue in 3 ml of oxygenated buffer (115 mM NaCl, 25 mM $NaHCO_3$, 6.0 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM Na_2SO_4, 1.2 mM $MgCl_2$, 1.0 mM pyruvate and 7.5 x 10^5 CPM [1-^{14}C] pyruvate. Glyburide, at concentrations ranging from 2 x 10^{-7}M to 2 x 10^{-5}M, was added to the mixture, and the flasks were incubated at 37°C for 30 minutes. The decarboxylation reaction was stopped with 1 ml of 1 N HCL and phenylethylamine (0.3 ml) was added to the center well to trap $^{14}CO_2$. The flasks were then agitated for 1 hour at room temperature and the center wells were transferred to 10 ml of Liquiscint for counting. Counts were corrected for quinch and for 0 time of the incubation. Values were expressed as µmol $^{14}CO_2$ produced/g of tissue/30 minutes.

Mitochondria were isolated from rat ventricular tissue by a modification of the homogenization methods of Palmer et al (17). Protein was determined by the methods of Bradford (18).

Intact mitochondria were incubated in a shaking water bath at 30°C and activity of the PDC was determined by a modification of the procedures described by Patel et al (19). The incubation media contained at final concentration: 100 mM KCl, 75 mM mannitol, 25 mM sucrose, 10 mM phosphate-tris, pH 7.4, 0.05 mM, K^+-EDTA, pH 7.4, 10 mM tris-HCl, pH 7.4. 10 mM succinate was added to each of the incubation flasks in order to increase the intramitochondrial ATP/ADP ratio and inhibit PDC activity. Glyburide was included at the concentrations described in the figure legends. PDC activity was determined following solubilization of the mitochondrial membranes with Triton X-100 (see below). The PDC assay was initiated by the addition of 0.2 ml aliquots (0.2 to 0.4 mg) of incubated mitochondria to assay media containing the following: 100 mM tris-HCl, pH

7.8; 0.01% v/v Triton X-100, 10 mM NAD+, pH 6.8; 1 mM thiamin pyrophosphate, pH 6.8; 1 mM dithiothreitol, 1 mM CoASH, 5 mM NaF; 1 mM oxalacetate, 10 mM $MgCl_2$, 5 mM dichloroacetate (DCA), pH 6.8; 5 mM pyruvate, pH 6.8; 5 x 10^5 CPM [1-^{14}C] pyruvate; final pH 7.5. The assay medium (1.0 ml) was placed in Erlermeyer flasks sealed with serum stoppers equipped with center wells. The flasks were agitated for 5 minutes at 30°C and the reaction was stopped with 0.5 ml of 1 N HCl. $^{14}CO_2$ was trapped and counted as previously described and values were expressed as nmol $^{14}CO_2$ produced/min/mg of mitochondrial protein.

In order to establish if membrane integrity was necessary for the action of glyburide on the activity of the PDC, a disrupted mitochondrial system was utilized. The mitochondria were prepared as previously described and then either sonicated or quick-frozen in a dry-ice acetone bath. The disrupted mitochondria were incubated at 30°C for 10 minutes in incubation buffer containing 100 mM phosphate buffer, pH 7.4, 1.25 mM ATP, oligomycin (10µg/ml), 50 µM $CaCl_2$, 50 µM $MgCl_2$, and in the presence of either 10 mM DCA or 2 x 10^{-4}M glyburide. PDC activity was determined as described above for the intact solubilized mitochondria.

RESULTS

Table 1 depicts the effects of different concentrations of glyburide on the decarboxylation of [1-^{14}C] pyruvate by the PDC of rat ventricular tissue. Glyburide, at each of the concentrations tested, stimulated the decarboxylation of [1-^{14}C] pyruvate. Therapeutic levels (20) of glyburide (10^{-6}M) produced up to a 30% increase in the production of $^{14}CO_2$. Stimulation of decarboxylation of [1-^{14}C] pyruvate comparable to that observed in the presence of the potent kinase inhibitor, DCA, occurred with 2 x 10^{-5}M glyburide. The results show that the drug, at relatively low concentration, is capable of altering the metabolic flux of pyruvate in rat ventricular tissue.

TABLE 1 Glyburide Effects on the Decarboxylation of Pyruvate by the PDC of Rat Ventricular Slices

Addition	Pyruvate Decarboxylation (µmole $^{14}CO_2$ produced/g/30 min)		% Stimulation Over Control
β-hydroxybutyrate (20 mM)		2.34 ± 0.10	---
β-hydroxybutyrate (20 mM) + DCA (10 mM)		3.46 ± 0.11	48%
β-hydroxybutyrate (20 mM) + Glyburide	2 x 10^{-7}M	2.40 ± 0.02	3%
	1 x 10^{-6}M	2.82 ± 0.06	21%
	2 x 10^{-6}M	3.04 ± 0.10	30%
	1 x 10^{-5}M	3.30 ± 0.11	41%
	2 x 10^{-5}M	3.43 ± 0.01	47%

Rat ventricular slices were prepared and incubated as described in Methods. Control values are those observed for ventricles incubated in the presence of β-hydroxybutyrate. Values are expressed as the mean ± S.E. of triplicate samples and are representative of 3 separate experiments.

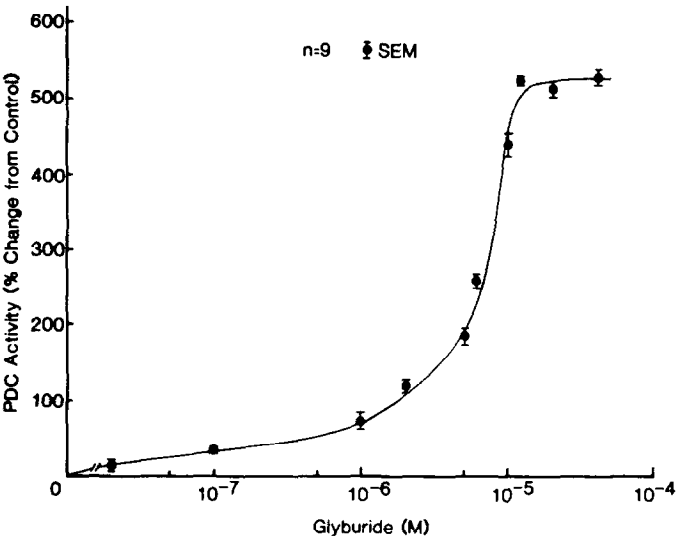

Figure 1: The effect of glyburide concentration on the activity of the PDC. Intact rat heart mitochondria were incubated for 10 minutes in the presence of varying concentrations of glyburide. The mitochondrial membranes were then solubilized by detergent action and PDC activity was assayed as described in Methods. The values are the mean ± S.E. of 9 samples from 3 separate experiments (in triplicate).

In order to determine if a direct effect of the sulfonylurea on the PDC could be established in an isolated, intact mitochondrial system, the experiment shown in Figure 1 was conducted. As can be seen, glyburide stimulated the activity of the PDC of rat heart mitochondria in a dose-dependent manner. Therapeutic levels of the drug stimulated PDC activity up to 35% above those values found for mitochondria treated with 10 mM succinate alone. Half maximal stimulation of enzyme activity occurred with 6 x 10^{-5}M glyburide and full activation of the enzyme was achieved with 1.2 x 10^{-4}M glyburide. PDC activity observed in the presence of glyburide was 6-fold higher than activity found under control conditions (10 mM succinate).

Figure 2 demonstrates the relationship between changes in PDC activity and time of mitochondrial incubation in the presence of 2 x 10^{-4}M glyburide. As is shown, glyburide-induced activation of the PDC was complete within 5 minutes of the incubation period. Interestingly, glyburide produced a 30% greater change in PDC activity than that which occurred with mitochondria incubated in the presence of DCA. This unexpected effect of glyburide was noticed in experiments

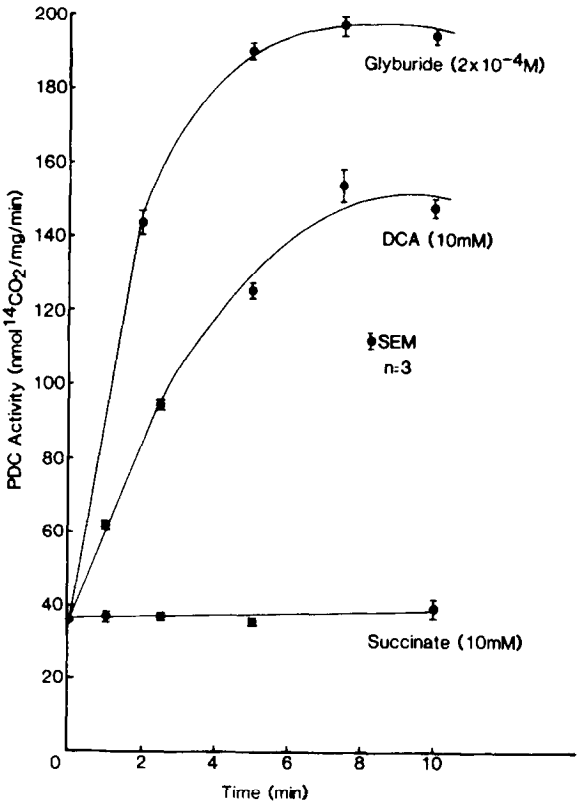

Figure 2: Time course of activation of the PDC by glyburide. Intact rat heart mitochondria were incubated with 10 mM succinate and 10 mM DCA or 2 x 10^{-4}M glyburide for the time periods designated. The mitochondria were disrupted and assayed for PDC activity. Each point is the mean ± S.E. of 3 determinations.

in which mitochondria were incubated with either 10 mM or 40 mM DCA and could not be reversed by increasing the concentration of succinate to 40 mM (data not shown).

In an effort to determine the effects of glyburide on activity of the PDC of a membrane-disrupted mitochondrial system, the mitochondria were disrupted by freeze-thawing or sonication. Figure 3 shows that glyburide is ineffective in altering activity of the PDC in the absence of an intact membrane. Under these same conditions, DCA stimulated decarboxylation of [1-^{14}C] pyruvate 2- to 3-fold over that observed with ATP alone. These observations suggest that an intact mitochondrial membrane is necessary for glyburide-induced stimulation of the PDC.

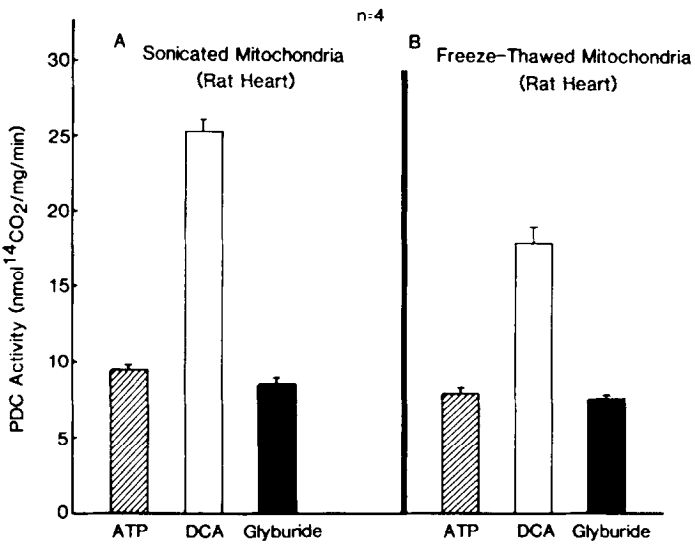

Figure 3: The effect of glyburide on the PDC of disrupted mitochondria. Heart mitochondria were disrupted by sonication (panel A) or freeze-thawing (panel B) and incubated in the presence or absence of 2 x 10^{-4}M glyburide for 10 minutes at 30°C. PDC activity was then assessed as previously described. Values represent the mean ± S.E. of triplicate samples.

DISCUSSION

Sulfonylureas are reported to influence myocardial energy metabolism. Using the perfused rat heart, Kramer et al (11) demonstrated a tolbutamide-mediated increase in the contribution of glucose metabolism to ATP synthesis. Evidence presented by these investigators (11) as well as by others (21) implicates a possible role of sulfonylurea on the PDC in stimulating glucose oxidation. However, to our knowledge, the present report is the first demonstration of a direct influence of glyburide on the PDC. Furthermore, the stimulation occurs with therapeutic levels of glyburide and in the absence of insulin.

The mechanism(s) underlying glyburide-mediated stimulation of pyruvate decarboxylation are unknown but may involve kinase-phosphosphatase-mediated interconversion of the pyruvate dehydrogenase enzyme from its inactive (phosphorylated) to active (dephosphorylated) form. Our observation that in a cell-free mitochondrial system glyburide stimulates activity of the PDC suggests an effect of these agents on the phosphorylation state of the enzyme. The requirement of

an intact mitochondrial membrane for glyburide-induced activation of the enzyme (Fig. 3) further suggests an effect of glyburide on the intramitochondrial levels of one or more of the metabolic factors known to regulate the activation state of the enzyme, i.e., AcetylCoA, CoA, ATP, ADP, NADH, NAD+, mono- and divalent cations, disulfides and pyruvate (22-28). Studies are currently in progress to investigate the mechanism of action of glyburide on the myocardial PDC.

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Address reprint requests to Dr. Abbas E. Kitabchi, 951 Court Ave., Rm. 335M, Memphis, Tennessee 38138 USA.

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