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POSSIBLE ROLE OF INTRACELLULAR Ca^{2+} IN THE TOXICITY OF PHENFORMIN

STEPHEN D. GETTINGS,* JOHN E. REEVE and LAURENCE J. KING†
Division of Pharmacology and Toxicology, Department of Biochemistry, University of Surrey, Guildford, Surrey GU2 5XH, U.K.

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Abstract—Selective use of various mitochondrial Ca^{2+} transport inhibitors indicated that significant Ca^{2+} redistribution may occur during the isolation of mitochondria. Exposure of guinea-pig liver mitochondria to phenformin (β-phenethylbiguanide) during the isolation procedure resulted in decreased mitochondrial Ca^{2+}. Novel isolation conditions were developed to determine liver mitochondrial calcium content considered to reflect that *in vivo*. Administration of phenformin to rats and guinea-pigs resulted in decreased mitchondrial Ca^{2+}. Decreased liver mitochondrial Ca^{2+} correlated inversely with raised blood lactate concentrations in the guinea-pig; 2-oxoglutarate, but not succinate oxidation, was inhibited in these mitochondrial preparations. A mechanism of action for phenformin-associated lactic-acidosis, attributable to impaired mitochondrial function arising from inactivation of Ca^{2+}-sensitive, NAD^+-dependent mitochondrial dehydrogenases (e.g. 2-oxoglutarate dehydrogenase) due to alteration in mitochondrial calcium content, is proposed.

The history, pharmacodynamics and therapeutic use of biguanides have been reviewed by Schafer [1]. The association between biguanide therapy and clinical lactic acidosis is well documented [2]; the mechanism of toxicity may be considered as an extension of therapeutic action (particularly enhanced peripheral anaerobic glycolysis and inhibition of hepatic and renal gluconeogenesis) at high drug concentrations [3]. Recent evidence would suggest that the extra-hepatic splanchnic bed (gastro-intestinal tract) is a major source of lactate production in biguanide-associated lactic acidosis [4–6]. Despite an apparent multi-site action of biguanides on metabolic function, the concept of an underlying mechanism at the molecular level has been addressed by Schafer [7, 8]. Under physiological conditions, alkyl-biguanides such as phenformin (β-phenethylbiguanide) exist as mono-protonated, resonance-stabilised cations [9–11] which adsorb strongly to membrane interfaces via the alkyl side-chain, the hydrocarbon moiety oriented towards the hydrophobic phase and the guanidinium cation exposed to the aqueous phase [12]. *In vitro* experiments suggest that biguanides are ineffective against isolated, solubilised enzymes but act only on biological processes associated with membranes, and on mitochondrial membranes in particular [8]. Generation of a positive shift in mitochondrial inner membrane potential due to the contribution of firmly fixed positive charge by guanidinium cations is associated with concentration-dependent inhibition of oxidative phosphorylation [13], which may explain the metabolic effects of phenformin and other hypoglycaemic biguanides *in vivo* [8]. The low binding affinity of 4-hydroxy-phenformin (primary metabolite of phenformin) in comparison to the parent drug, correlates with a lowered inhibitory action on mitochondrial oxidative phosphorylation *in vitro* and weak anti-diabetic action *in vivo* [14].

Cook *et al.* [15] have concluded that phenformin has no direct effect on gluconeogenesis but suggest that intracellular conditions may be altered in some way as to effectively inhibit one or more gluconeogenic enzymes. Implicit in the model of biguanide action proposed by Schafer [8] is an alteration in ion flux and metabolite distribution between cytosol and mitochondria as a result of changes in mitochondrial proton gradient and membrane potential. Schafer [14] has shown that there exists a direct interaction between biguanides and the migration of cations across the inner mitochondrial membrane; in particular, modulation of surface charge by *n*-octylbiguanide caused a reduction in the rate constant of mitochondrial Ca^{2+} uptake, whilst magnitude remained unaltered. Inhibition of Ca^{2+} movements is of especial interest since this cation is of potential importance in the regulation of oxidative metabolism in mammalian mitochondria via the activation of pyruvate, isocitrate and 2-oxoglutarate dehydrogenases [16]. Evans *et al.* [17] have suggested that inhibition of NAD^+-dependent mitochondrial dehydrogenases may be related to the impairment of gluconeogenesis by phenformin. The objective of our investigations was to test the hypothesis that the hypoglycaemic and hyperlactataemic effects of phenformin may arise from an inactivation of Ca^{2+}-sensitive, NAD^+-dependent mitochondrial dehydrogenases as a result of alteration in mitochondrial calcium content.‡

* Current address: Department of Biochemistry, University of Louisville School of Medicine, Louisville, Kentucky 40292, U.S.A.
† To whom correspondence should be addressed.
‡ The terms "mitochondrial calcium content" and "mitochondrial Ca^{2+}" are used synonymously for the purpose of this communication.

MATERIALS AND METHODS

Chemicals. Phenformin hydrochloride and 4-hydroxyphenformin hydrochloride were donated by Sterling-Winthrop Research and Development, Alnwick, Northumberland, U.K. Metformin hydrochloride was a gift from Rona Laboratories Ltd. (Hitchin, Hertfordshire, U.K.). Disodium succinate and 2-oxoglutarate (monopotassium salt) were supplied by Sigma (London) Chemical Co. Ltd., U.K., and were neutralised with 4 M potassium hydroxide before use. Mersalyl (MERS) and Ruthenium Red (RR) were purchased from Sigma. RR was used as supplied, without further purification; where indicated, concentrations are of actual dye content (45%). Sodium pentobarbitone solution (Sagatal) was obtained from May & Baker Ltd. (Dagenham, Essex, U.K.). All other reagents were obtained from British Drug Houses Ltd. (Poole, Dorset, U.K.) or from Sigma, and were of analar grade.

Animals and administration of compounds. Adult male animals were used in all experiments. Starved animals were deprived of food for 24 hr; water was allowed *ad libitum*. Wistar/Albino rats (approximately 250 g) were obtained from a derived strain (University of Surrey); Dunkin–Hartley albino guinea-pigs (approximately 300 g) were purchased from Graystone Guinea-pigs (Ringwood, Hampshire, U.K.). Phenformin, 4-hydroxyphenformin and metformin hydrochlorides were dissolved in isotonic saline (0.9% w/v) such that the volume administered by intraperitoneal injection was approximately 0.5 ml; control animals received equivalent volumes of saline.

Determination of blood lactate and plasma glucose. Blood samples were withdrawn from the inferior vena cava of anaesthetised animals (sodium pentobarbitone (Sagatal); 60 mg/kg, i.p.); aliquots were subsequently analysed for lactate (de-proteinised whole-blood supernatant) or glucose (plasma) on a Cobas Bio centrifugal analyser (Roche Products Ltd., Welwyn Garden City, Hertfordshire, U.K.) using Boehringer (Lactate; Cat. No. 124842), (Boehringer Corp. (London) Ltd., Lewes, East Sussex, U.K.) and Roche (Glucose; List No. 0711004) assay kits.

Isolation of mitochondria. Isolation of mitochondria was based upon the procedure of Chappell and Hansford [18] using a manually operated Dounce homogeniser with a loose-fitting pestle. Isolation medium (0.25 M sucrose) contained 2 mM EGTA, 5 mM succinate, 10 μM mersalyl and 5 μM Ruthenium Red as indicated. All solutions were prepared in deionised water and adjusted to pH 7.6 before use. Washed mitochondrial fractions were carefully resuspended (2 g original tissue/ml) in 0.25 M sucrose, 3.4 mM Tris HCl, pH 7.4 (enzyme studies) or de-ionised water (determination of mitochondrial Ca^{2+}). Electron microscopic and marker-enzyme analysis of isolated mitochondrial fractions suggested only minimal contamination by subcellular organelles other than lysosomes.

Determination of mitochondrial Ca^{2+}. Mitochondrial Ca^{2+} was determined by the method of McDonald *et al.* [19]. Mitochondrial preparations were frozen in sterilised plastic tubes prior to analysis. Aliquots (1.0 ml) were solubilised with 10 M NaOH at 75°, incubated (5 min, 75°) with 1.0 ml solution containing 0.01 M lanthanum chloride, 0.025 HCl, 0.02 M EDTA and 0.08 M NaOH, allowed to cool and subsequently analysed by atomic absorption spectroscopy. Samples were processed in sterilised plastic tubes to minimise contamination from extraneous Ca^{2+}.

Protein was determined by the method of Lowry *et al.* [20]. Determinations of mitochondrial Ca^{2+} (nmol Ca^{2+}/mg protein) reported here may be slightly lower than true values (nmol Ca^{2+}/mg mitochondrial protein) since protein measurements were not corrected for the small contribution by protein of non-mitochondrial origin.

Determination of 2-oxoglutarate and succinate dehydrogenase activities in isolated mitochondria. Incubations were performed at 30° in the reaction vessel of a Clark-type oxygen-electrode (Rank Brothers, Bottisham, Cambridge, U.K.). Incubations contained approximately 4 mg mitochondrial protein in a total volume of 2.0 ml buffer. ADP and substrates were prepared in buffer such that 10 μl additions gave the indicated concentrations in 2.0 ml. 2-Oxoglutarate dehydrogenase activity was determined in the presence of 1 mM malate: malate facilitates mitochondrial uptake of 2-oxoglutarate [21, 22] and itself causes very little oxygen consumption. Reactions were started by the addition of substrate. State III respiration rates, determined as: (reaction rate with ADP and substrate)-(reaction rate with substrate, ADP fully utilised) are reported in all examples.

Analysis of kinetic data. Deviation from normal hyperbolic kinetics was investigated by fitting data to equations of the type: $v = V_{max}/\{1 + K_m/[S]^{nH}\}$. Derived values of K_m and V_{max} were determined by the method of Wilkinson [23] using a computer programme developed by Dr R. Gray (University of Louisville).

RESULTS

Effect of systematic alteration of isolation conditions on the total calcium content of liver mitochondria

Mitochondria were prepared from the livers of control and treated rats (phenformin HCl, 120 mg/kg; i.p.) and guinea-pigs (phenformin HCl, 30 mg/kg; i.p.) in isolation media containing combinations of mitochondrial Ca^{2+} transport inhibitors and EGTA. The effect of systematically altering isolation conditions on total calcium content of rat and guinea-pig mitochondria (Table 1) indicates that significant Ca^{2+} movements occur during tissue disruption and subsequent isolation procedures. In both the rat and the guinea-pig, administration of phenformin *in vivo* appeared to have no significant effect on mitochondrial Ca^{2+} in preparations isolated in sucrose only. Although mitochondrial Ca^{2+} appeared to be lower in the livers of phenformin-treated rats, differences did not achieve significance at $P < 0.05$. In contrast, mitochondrial Ca^{2+} was significantly decreased following the administration of phenformin, in guinea-pig liver mitochondria isolated in media containing Ca^{2+} transport inhibitors and EGTA. Addition of EGTA to the sucrose isolation

Table 1. Effect of systematic alteration of isolation conditions on guinea-pig liver mitochondrial Ca^{2+} after phenformin administration *in vivo*

Additions to isolation medium	Mitochondrial calcium content (nmol/mg protein)			
	Rat liver		Guinea-pig liver	
	Control	Treated	Control	Treated
Tris/Sucrose (pH 7.6)	10.6 ± 1.71 (8)	9.9 ± 1.92 (8)	14.6 ± 2.96 (4)	13.0 ± 1.39 (4)
+EGTA	5.5 ± 1.19 (8)	5.6 ± 1.51 (4)	7.9 ± 0.94 (4)	4.6 ± 1.42 (3)**
+EGTA/RR	2.8 ± 0.49 (8)	2.4 ± 0.34 (8)	4.0 ± 0.75 (4)	2.2 ± 0.73 (4)**
+EGTA/RR/MERS/Succinate	2.1 ± 0.26 (4)	1.8 ± 0.24 (4)	5.6 ± 0.56 (4)	2.1 ± 0.46 (4)***

Male rats (approximately 250 g) and male guinea pigs (approximately 300 g) received phenformin HCl (120 mg/kg; i.p. and 30 mg/kg; i.p., respectively). Control animals received equivalent volumes of saline. Animals were killed 2.5 hr (rats) and 4 hr (guinea pigs) after dosing. Results are expressed as means ± SD; number of animals shown in parenthesis. Statistical significance of differences between control and treated groups; ** $P < 0.02$, *** $P < 0.001$.

medium decreased the Ca^{2+} content of rat liver mitochondria by 52%: a combination of Ruthenium Red and EGTA reduced Ca^{2+} content to 26% of values obtained for the isolation of mitochondria in sucrose alone; similar results were obtained in preparations of isolated guinea-pig liver mitochondria. Reinhart *et al.* [24] reported a 40% decrease in mitochondrial calcium content after isolation in 1.6 μM Ruthenium Red and 10 μM EGTA. These values would suggest confirmation that a combination of Ruthenium Red and EGTA remove all external Ca^{2+} and inhibit mitochondrial Ca^{2+} uptake [25, 26].

Bernardi and Azzone [27] have suggested that membrane potential may regulate phosphate-stimulated mitochondrial Ca^{2+} efflux; in addition, mersalyl has been shown to inhibit the parallel efflux of Ca^{2+} and phosphate in energised rat liver mitochondria. Addition of succinate (energising substrate) and mersalyl to isolation medium containing EGTA and Ruthenium Red increased the mitochondrial calcium content of isolated control guinea-pig mitochondria by 40%; addition of succinate and mersalyl to the isolation medium had no effect on rat liver mitochondrial Ca^{2+}, indicating that Ca^{2+} efflux from rat liver mitochondria may be very low under these conditions. Control values for rat liver mitochondrial Ca^{2+} determined under these conditions (2.1 ± 0.26 nmol Ca^{2+}/mg protein) are in close agreement with those reported by Reinhart *et al.* [24] of 2–3 nmol Ca^{2+}/mg protein for rat liver mitochondria isolated with 10 mM succinate, 10 μM EGTA, 1.6 μM Ruthenium Red and 0.4 mM nupercaine, and claimed to be representative of calcium content *in vivo*; corresponding values for guinea-pig liver mitochondrial Ca^{2+} (Table 1) were 5.6 ± 0.16 nmol Ca^{2+}/mg protein.

Under the conditions considered by us to be optimal for the measurement of mitochondrial Ca^{2+}, i.e. in the presence of EGTA, Ruthenium Red, mersalyl and succinate, rat liver mitochondrial Ca^{2+} was marginally decreased (non-significant) by 14.3% from 2.1 to 1.8 nmol Ca^{2+}/mg protein; guinea-pig liver mitochondrial Ca^{2+} was decreased by 62.5% from 5.6 to 2.1 nmol Ca^{2+}/mg protein ($P < 0.001$).

Concentration-dependent variation in guinea-pig liver mitochondrial Ca^{2+} after exposure to phenformin in the isolation medium

Exposure of control guinea-pig liver mitochondria to increasing concentrations of phenformin (0–10 mM) during the isolation procedure resulted in a concentration-dependent decrease in mitochondrial Ca^{2+}, apparently reaching a plateau (65% of control value) between 5–10 mM phenformin (Fig. 1).

Comparison of the effects of in vivo *administration of phenformin, 4-hydroxyphenformin and metformin on guinea-pig liver mitochondrial Ca^{2+}*

In contrast to phenformin (30 mg/kg; i.p.) which

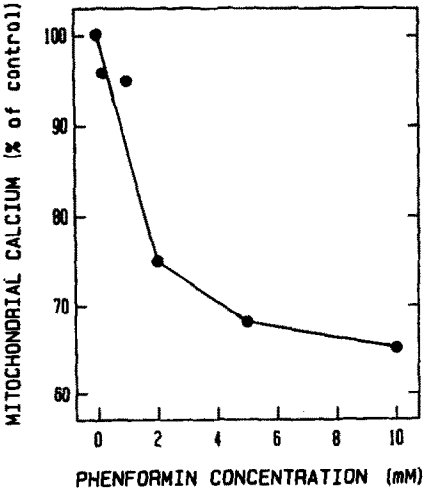

Fig. 1. Alteration in calcium content of guinea-pig liver mitochondria following inclusion of phenformin in the isolation medium. Livers were removed from untreated guinea-pigs. A portion of each liver was used to isolate control mitochondria in Tris/sucrose medium (see Materials and Methods); the remainder were isolated in Tris/sucrose medium containing phenformin HCl (0.2–10 mM). Mitochondrial Ca^{2+} was determined in each fraction: results are expressed as percentage of corresponding control (20.8–24.1 nmol Ca^{2+}/mg protein); duplicates gave identical values.

Table 2. Guinea-pig liver mitochondrial Ca^{2+} following *in vivo* administration of 4-hydroxyphenformin, phenformin or metformin

Treatment	Mitochondrial calcium content (nmol/mg protein)	
	Control	Treated
Phenformin	5.7 ± 0.55 (4)	2.1 ± 0.45 (4)****
4-Hydroxyphenformin	5.2 ± 1.71 (4)	4.3 ± 0.94 (4)
Metformin	4.0 ± 0.83 (4)	2.1 ± 0.78 (4)**

Male guinea-pigs (approximately 300 g) received 4-hydroxyphenformin HCl (32 mg/kg; i.p.), phenformin HCl (30 mg/kg; i.p.) or metformin (500 mg/kg; i.p.); control animals received equivalent volumes of saline. Animals were killed after 3 hr (metformin treated group) or 4 hr, livers removed and mitochondria isolated in 0.25 M sucrose, pH 7.6, containing 2 mM EGTA, 5 mM succinate, 10 μM MERS and 5 μM RR. Values are means ± SD; number of animals shown in parenthesis. Statistical significance of differences between control and treated groups; ** $P < 0.02$, **** $P < 0.001$.

caused a 37% decrease ($P < 0.001$) in mitochondrial Ca^{2+} after 4 hr, a molar equivalent dose of 4-hydroxyphenformin (32 mg/kg; i.p.) had no significant effect on the calcium content of subsequently isolated guinea-pig liver mitochondria (Table 2). Guinea-pigs given metformin (500 mg/kg; i.p.) showed distinct clinical signs of hypoglycaemia after 3 hr; mitochondrial Ca^{2+}, as measured in subsequently isolated mitochondria, was significantly decreased ($P < 0.02$) in treated guinea-pigs (2.1 ± 0.78 nmol Ca^{2+}/mg protein) compared to controls (4.0 ± 0.83 nmol Ca^{2+}/mg protein). Guinea-pigs given metformin at a lower dose-level (150 mg/kg; i.p.) showed no superficial signs of toxicity and values for mitochondrial Ca^{2+} were not significantly different from controls (data not shown).

Comparison of the effects on liver mitochondrial Ca^{2+} *with metabolic effects* in vivo *following intraperitoneal administration of phenformin to guinea-pigs*

In an attempt to compare the effects of phenformin on liver mitochondrial Ca^{2+} with metabolic effects *in vivo*, terminal blood samples were taken from anaesthetised control and treated guinea-pigs for the analysis of lactate and glucose (Table 3). Values for liver mitochondrial Ca^{2+} determined in either control or phenformin-treated guinea-pigs were not significantly different from those in similar experiments in which animals were killed by cervical dislocation (results not shown).

In contrast to the hyperglycaemia (14.6 ± 7.05 mM) in treated animals killed after 2 hr, plasma glucose concentrations fell to well below control values (6.6 ± 1.44 mM) and showed a marked hypoglycaemia (0.2 ± 0.23 mM) after 4 hr. Blood lactate concentrations were significantly increased ($P < 0.001$) after 2 hr (5.7 ± 1.71 mM) but were not significantly different from values in treated animals killed after 4 hr (5.9 ± 0.92 mM). Although not statistically significant, there was a tendency towards decreased liver mitochondrial Ca^{2+} after 2 hr in guinea-pigs treated with phenformin (30 mg/kg; i.p.). Mean values were decreased from 5.2 ± 1.38 nmol Ca^{2+}/mg protein in controls to 3.9 ± 0.32 nmol Ca^{2+}/mg protein in treated animals at 2 hr, and further decreased (1.52 ± 0.51 nmol Ca^{2+}/mg protein) in treated animals killed after 4 hr. Data plotted as blood lactate concentration (abscissa) versus liver mitochondrial Ca^{2+} (ordinate) (Fig. 2), suggest a close correlation ($r = 0.69$; $P < 0.001$) between the two effects of phenformin *in vivo*; similar treatment of plasma glucose concentration versus mitochondrial Ca^{2+} reveals no such relationship (data not shown).

Table 3. Variation in guinea-pig liver mitochondrial Ca^{2+}, plasma glucose and blood lactate with time following administration of phenformin *in vivo*

Time (post-dose) (hr)	Mitochondrial Ca^{2+} content (nmol Ca^{2+}/mg protein)	Glucose (mM)	Lactate (mM)
0†	5.2 ± 1.38	6.6 ± 1.44	1.4 ± 0.62
2	3.9 ± 0.32	14.6 ± 7.05	5.7 ± 1.71****
4	1.5 ± 0.51****	0.2 ± 0.23****	5.9 ± 0.92****

Male guinea-pigs (approximately 300 g) received phenformin HCl (30 mg/kg; i.p.); control animals (†, zero time) received equivalent volumes of saline. Treated and control animals received pentobarbitone (60 mg/kg; i.p.) at the times indicated; blood samples were withdrawn from the inferior vena cava after onset of anesthesia. Liver mitochondria were isolated in 0.25 M sucrose, pH 7.6, containing 2 mM EGTA, 5 mM succinate, 10 μM MERS, 5 μM RR. Values are means ± SD for between 4 and 7 animals. Statistical significance of differences between control (zero time) and treated groups; **** $P < 0.001$.

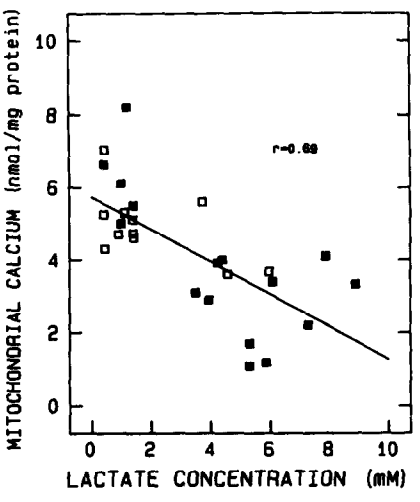

Fig. 2. Correlation between blood lactate concentration and mitochondrial calcium content following administration of phenformin *in vivo*. Male guinea-pigs (approximately 300 g) received phenformin HCl (30 mg/kg; i.p.) (■), or equivalent volumes of saline (□). Animals were killed between 2 and 4 hr after dosing. Blood samples were withdrawn from the inferior vena cava whilst still under anesthesia (phenobarbitone, 60 mg/kg, i.p.). Mitochondria were isolated in 0.25 M sucrose, pH 7.6, containing 2 mM EGTA, 5 mM succinate, 10 μM MERS and 5 μM RR.

Effect of phenformin administration in vivo *on liver mitochondrial* Ca^{2+} *in starved guinea-pigs*

Fasted animals are more sensitive to the metabolic effects of phenformin *in vivo* [28, 29]. Starvation significantly decreased ($P < 0.001$) liver mitochondrial Ca^{2+} in control guinea-pigs from mean values of 4.7 ± 0.64 nmol Ca^{2+}/mg protein to 2.9 ± 0.48 nmol Ca^{2+}/mg protein (Table 4). Corresponding plasma glucose concentrations were similarly decreased ($P < 0.05$) from 8.0 ± 1.16 nM in fed animals to 6.1 ± 0.94 mM in starved guinea-pigs; blood lactate concentrations were unaltered. Phenformin (30 mg/kg, i.p.) further decreased ($P < 0.001$) mitochondrial Ca^{2+} in the livers of starved guinea-pigs; mean values (1.3 ± 0.27 nmol Ca^{2+}/mg protein) were lower than those in mitochondria isolated from fed animals treated with phenformin (2.0 ± 0.86 nmol Ca^{2+}/mg protein), although the difference was not significant at $P < 0.05$. Glucose concentrations were lowered ($P < 0.001$) and lactate concentrations increased ($P < 0.02$) compared to starved controls. Administration of phenformin resulted in lower plasma glucose concentrations in starved guinea-pigs (0.1 ± 0.01 mM) than in fed animals (0.3 ± 0.2 mM), although the difference was not statistically significant. Blood lactate concentrations were higher ($P < 0.05$) in starved guinea-pigs (8.6 ± 2.16 mM) than in fed animals treated with phenformin (5.2 ± 1.25 mM).

Effect of phenformin administration in vivo *on the oxidation of 2-oxoglutarate and succinate in isolated guinea-pig liver mitochondria*

Mitochondria isolated in 0.25 M sucrose, pH 7.6, containing 2 mM EGTA, 5 mM succinate, 10 μM mersalyl and 5 μM Ruthenium Red utilised oxygen at an appreciable rate which remained constant for at least 30 min (results not shown). Respiratory control was apparent upon addition of ADP; similar rates of oxygen reduction, progressively inhibited by increasing concentrations of disodium malonate, were observed in mitochondria from both treated and control animals. Mitochondria isolated in the presence of EGTA and mitochondrial transport inhibitors, but in the absence of succinate exhibited hyperbolic kinetics (nH = 1) with respect to both 2-oxoglutarate (Fig. 3a) and succinate (Fig. 3b) as added substrate. Mitochondrial Ca^{2+} determined under these conditions was significantly lower ($P < 0.02$) in the livers of treated animals (4.9 ± 1.40 nmol Ca^{2+}/mg protein; N = 3) than in controls (8.4 ± 1.18 nmol Ca^{2+}/mg protein; N = 4).

At all concentrations of 2-oxoglutarate investigated respiration was markedly lower in mitochondria isolated from phenformin-treated animals than in corresponding controls (Fig. 3a). Although the range of V_{max} values was broad (34.4–17.1 nmol

Table 4. Effect of phenformin administration *in vivo* on liver mitochondrial Ca^{2+}, plasma glucose and blood lactate in starved guinea-pigs

	Mitochondrial Ca^{2+} (nmol/mg protein)		Glucose (mM)		Lactate (mM)	
	Control	Treated	Control	Treated	Control	Treated
Fed	4.7 ± 0.64	2.0 ± 0.86	8.0 ± 1.16	0.3 ± 0.20	2.1 ± 1.69	5.2 ± 1.25
Fasted	2.9 ± 0.48***	1.3 ± 0.27***	6.1 ± 0.94*	0.1 ± 0.01****	2.5 ± 1.31	8.6 ± 2.16**

Male guinea-pigs (approximately 300 g) were randomized in 3 groups. Where indicated, fasted animals were deprived of food for 24 hr but were allowed water *ad libitum*. Treated animals received phenformin HCl (30 mg/kg; i.p.); control animals received equivalent volumes of saline. Test and control animals received pentobarbitone (60 mg/kg; i.p.) after 4 hr; blood samples were withdrawn from the inferior *vena cava* after onset of anesthesia. Liver mitochondria were isolated in 0.25 M sucrose, pH 7.6, containing 2 mM EGTA, 5 mM succinate, 10 μM MERS and 5 μM RR. Values are means ± SD for between 4 and 7 animals. Statistical significance of differences between fed and fasted animal groups, * $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$, **** $P < 0.001$. Differences between control and treated groups were significant ($P < 0.001$) for each of the parameters.

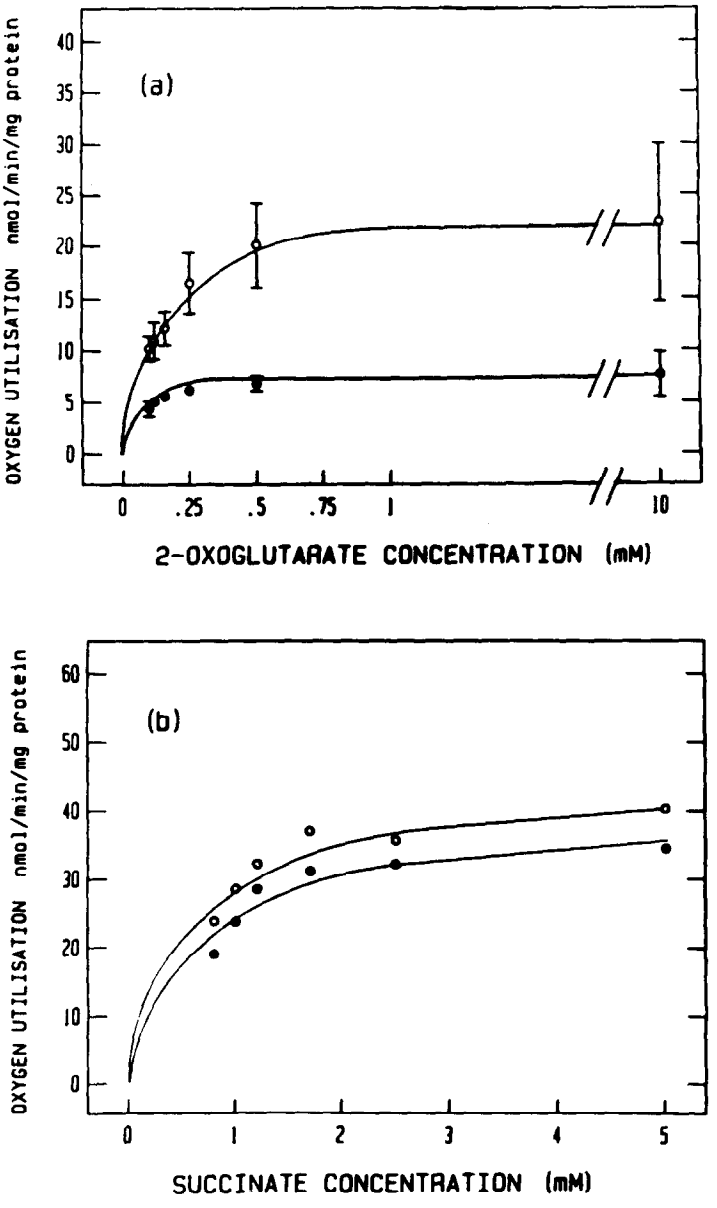

Fig. 3. Effect of phenformin *in vivo* on the oxidation of 2-oxoglutarate (a) and succinate (b) by mitochondria isolated with EGTA, Ruthenium Red and mersalyl. Male guinea pigs (approximately 300 g) received phenformin (30 mg/kg; i.p.) or an equivalent volume of saline. Animals were killed 3–4 hr later. Mitochondria were isolated in sucrose-based medium (containing 2 mM EGTA, 10 μM MERS, 5 μM RR; pH 7.6) resuspended (approximately 4 mg) in 2.0 ml medium (see Materials and Methods) and incubated at 30° with the indicated concentrations of substrate (+1 mM malate in (a)) and 0.1 mM ADP. Values for oxygen consumption (State III respiration) are means (±SD in (a)) for determinations in preparations from 2–5 control (○) and phenformin-treated guinea-pigs (●). Individual values were determined in duplicate; variation between duplicates was less than 10%.

O_2 reduced/min/mg protein; mean value 23.2 ± 6.87 nmol O_2 reduced/min/mg protein, N = 5), corresponding values in mitochondria from treated animals (8.6 and 6.0 nmol O_2 reduced per min per mg protein) were considerably lower. Similarly, values for K_m were also decreased in mitochondria from treated animals (0.03 ± 0.01 mM; N = 2) compared to controls (0.12 ± 0.05 mM, N = 5). Acceptor control ratios (5 mM 2-oxoglutarate) were higher in mitochondria from control animals (range, 3.2–4.9) than in mitochondria isolated from treated guinea-pigs (2.1 and 2.2). In a single experiment with uncoupled mitochondria (5 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP), data not shown) incubation with 2-oxoglutarate resulted in values of K_m (0.12 and 0.04 mM) and V_{max} (24.5 and 11.1 nmol O_2 reduced/min/mg protein; from control and phenformin-treated animals, respectively) which

were similar to corresponding values in coupled mitochondria. Respiration with respect to succinate as substrate (Fig. 3b) was essentially similar in both test (K_m, 0.67 ± 0.19 mM; V_{max}, 40.4 ± 3.48 nmol O_2 reduced/min/mg protein) and control mitochondria (K_m, 0.58 ± 0.15 mM; V_{max}, 45.5 ± 3.22 nmol O_2 reduced/min/mg protein); acceptor control ratios (5 mM succinate) were also similar (mean value 3.2 ± 0.31).

DISCUSSION

Addition of phenformin to guinea-pig liver mitochondria *in vitro* has been shown to inhibit Ca^{2+} uptake at concentrations of 1 mM and above [30] but enhanced the initial rate of uptake at concentrations below 50 μM [31]. Administration of phenformin *in vivo* significantly decreased guinea-pig liver mitochondrial Ca^{2+} in subsequently isolated mitochondria (Table 1) under all isolation conditions except sucrose alone. The difference was most significant (62.5%) under the conditions claimed to assess calcium content *in vivo*; Table 1 clearly shows how altered Ca^{2+} homeostasis *in vivo* may be masked by Ca^{2+} redistribution *in vitro*. Similarly, the addition of phenformin to isolation medium results in a concentration-dependent decrease in guinea-pig liver mitochondrial Ca^{2+} (Fig. 1). Our results suggest that the inclusion of Ca^{2+} transport inhibitors in mitochondrial isolation media prevent the accumulation of intra-mitochondrial Ca^{2+} during preparative procedures, and that phenformin does the same. In addition, our findings would suggest a persistent effect on the mitochondrial Ca^{2+} content of isolated mitochondria following interaction with phenformin *in vivo*.

Differences in the effect of hyperlactataemic and hypoglycaemic doses of phenformin on rat and guinea-pig liver mitochondrial Ca^{2+}, may suggest a different mechanism of action of the drug in the two species since guinea-pigs are more susceptible than rats to low doses of phenformin. Following administration of phenformin at concentrations which give rise to maximum elevated lactate concentrations in the rat after 2 hr [31], liver mitochondrial Ca^{2+} determinations were lower in controls, but differences were not significant (Table 1). Guest [32] demonstrated that blood lactate concentrations in the guinea-pig reached a maximum 4–5 hr after receiving phenformin, at which time we have demonstrated that liver mitochondrial Ca^{2+} is significantly decreased (Table 2). Administration of phenformin to anaesthetised guinea-pigs results in a massive hyperglycaemia, with peak effect after 2 hr, followed by severe hypoglycaemia after 3–4 hr [32]. In unanaesthetised animals phenformin caused a time-dependent decrease in hepatic mitochondrial Ca^{2+} (Table 3). Our data would suggest a closer correlation between hepatic mitochondrial Ca^{2+} and blood lactate (Fig. 2), rather than with glucose. In addition, 4-hydroxyphenformin has little or no effect on blood lactate and glucose concentrations *in vivo* and had no significant effect on liver mitochondrial Ca^{2+} (Table 2). The ability of the less lipophilic biguanide, metformin, to decrease mitochondrial Ca^{2+} only at high dose levels (Table 2) may suggest that a relationship exists between an effective biguanide concentration *in vivo* and altered mitochondrial function.

Experimental evidence suggests that fasted animals are more sensitive to the hypoglycaemic effects of phenformin. In fasted animals blood sugar is maintained almost exclusively by gluconeogenesis [33]; inhibition of gluconeogenesis from various precursors by biguanides is a well established observation [15, 17, 34–42]. Starvation caused a 37% reduction in liver mitochondrial Ca^{2+} in guinea-pigs (Table 4); administration of phenformin caused a further decrease (57%) compared to untreated, starved controls. Although plasma glucose concentrations were not significantly different from fed guinea-pigs after phenformin, corresponding blood lactate concentrations at 4 hr were greater than those determined in similarly treated fed animals, suggesting a possible relationship between glucose metabolism and intracellular Ca^{2+} homeostasis. Alteration of Ca^{2+} concentrations in both the mitochondrial and cytoplasmic compartments may produce significant changes in the activities of a number of regulatory enzymes and in the response to hormones [43]. Although there is no clear evidence that changes in Ca^{2+} homeostasis are involved in the direct mechanism of glucagon action in the liver [43], Davidoff [44] has suggested that the glucose lowering effect of low concentrations of phenformin may be due to limitation of glucagon-dependent gluconeogenesis through a mechanism involving intracellular Ca^{2+} distribution.

In the absence of further exogenous substrate, mitochondria isolated in the presence of succinate utilised oxygen at an appreciable rate which was enhanced by the addition of ADP. Abolition of respiratory control and of endogenous respiration by malonate, a specific inhibitor of succinate dehydrogenase [45, 46] indicated that mitochondria isolated under these conditions were massively loaded with succinate. In mitochondria isolated under similar conditions, but in the absence of succinate, administration of phenformin *in vivo* had little or no effect on the oxidation of succinate added subsequently (Fig. 3b); in contrast, administration of phenformin *in vivo* resulted in marked inhibition of 2-oxoglutarate oxidation across the concentration range investigated (Fig. 3a). 2-Oxoglutarate oxidation in mitochondria from treated animals showed little variation with concentration of substrate and most values were near V_{max}. Under these conditions it is difficult to determine "true" values for K_m and any apparent values will be very low. However, the similarity of K_m values for 2-oxyglutarate oxidation under either coupled or uncoupled conditions for both test and control mitochondria, suggests that the dehydrogenase enzyme is the rate-limiting factor in mitochondrial substrate oxidation. The absence of any significant effect on succinate oxidation in mitochondria from phenformin-treated animals suggests no major impairment of oxidative phosphorylation by interference with either proton re-uptake through ATP synthetase, or with P_i and nucleotide transport. Similarly, impairment of 2-oxoglutarate oxidation in both coupled and uncoupled mitochondria, suggests that prior administration of phenformin does not

interfere with mitochondrial proton extrusion in subsequently isolated mitochondria. McCormack and Denton [47] reported that the concentration of extramitochondrial Ca^{2+} had no effect on the activity of succinate dehydrogenase in uncoupled mitochondria from brown adipose tissue of cold-adapted rats. In contrast, Ca^{2+} has been shown to increase the activity of 2-oxoglutarate dehydrogenase in uncoupled brown adipose tissue mitochondria [47] and in coupled rat heart mitochondria [38, 48, 49]. In particular, Ca^{2+} greatly diminished the K_m for oxoglutarate, with no effect on V_{max} [48]. Although the lack of effect on succinate dehydrogenase activity in mitochondria from treated animals, i.e. lowered mitochondrial Ca^{2+}, is in accord with the findings of McCormack and Denton [47], the pattern of inhibition of 2-oxoglutarate dehydrogenase activity (apparently decreased K_m, decreased V_{max}) is not as predicted (increased K_m, little or no effect on V_{max}): these results may suggest that the response of 2-oxoglutarate dehydrogenase to intra-mitochondrial Ca^{2+} in guinea-pig liver is different to the activation of the enzyme in rat heart or adipose tissue; alternatively, phenformin may exert multiple effects *in vivo*, e.g. possible inhibition of mitochondrial 2-oxoglutarate transport, which persist *in vitro*.

In conclusion, the results of these investigations suggest that Ca^{2+} may have a role in the toxicity of phenformin: hyperlactataemic and hypoglycaemic effects *in vivo* may be attributable to impaired ATP production arising from inactivation of Ca^{2+}-sensitive, NAD^+-dependent mitochondrial dehydrogenases due to alteration in mitochondrial calcium content by interaction of the drug with mitochondrial membranes. The postulated mechanism suggests that elevated blood lactate concentrations are a consequence of the hypoglycaemic effect. A specific locus for the action of phenformin at 2-oxoglutarate dehydrogenase as a result of decreased mitochondrial Ca^{2+} has not been elucidated; the mechanism by which phenformin exerts a clear inhibitory effect on 2-oxoglutarate oxidation is unknown, and may or may not involve modulation of 2-oxoglutarate dehydrogenase activity by alteration in the concentration of intramitochondrial Ca^{2+}.

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