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Interference of the sulphonylurea antidiabeticum gliquidone with mitochondrial bioenergetics in the rat under *in vitro* conditions[1]

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The hypoglycaemic sulphonylurea gliquidone and glibenclamide exerted a partial uncoupling effect on mitochondrial respiration of liver under *in vitro* conditions using various citrate cycle intermediates as substrates. Besides the uncoupling effect, gliquidone and glibenclamide caused a direct inhibition of ATP – as well as DNP – stimulated oxigen consumption. Both phenomena proved to be dose dependent. Respiratory control ratio decreased progressively with increasing concentrations of sulphonylureas mainly through the inhibition of ADP-stimulated respiration. Basal and DNP-stimulated ATP-ase activity of isolated mitochondria changed similarly to the respiratory parameters. Changes in membrane permeability of mitochondria and the inhibition of substrate uptake further support the assumption of structural and functional alteration of mitochondria by the hypoglycaemic compounds tested.

Keywords: sulphonylurea antidiabetica, gliquidone, glibenclamide, mitochondrial bioenergetics, respiration, ATP-ase, mitochondrial swelling, substrate uptake

In the last decades several sulphonylurea antidiabetic drugs have been synthetized which effectively lowered the level of blood glucose, inhibited the hepatic gluconeogenesis and influenced the peripheral glucose utilization [6, 9, 10, 14, 15, 27, 34–36].

[1] This paper is dedicated to the memory of Professor Tibor Kovács (1929–1994)

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Most peroral antidiabetica have a lipophyl character, thus they may cause disturbances in the structure and function of biomembranes. These compounds, depending on their concentration applied either *in vitro* or *in vivo*, may influence the mitochondrial oxidative processes and the energy household of the cells [3, 8, 12, 21, 23].

The widely used sulphonylurea, glibenclamide inhibited the pyruvate carboxylase in liver mitochondria [12] and this effect was associated with the reduction of mitochondrial ATP-ase activity [8]. The sulphonylureas could also interfere with the mitochondrial substrate uptake processes [23].

The side-effects mentioned above have been already described in the case of most sulphonylurea antidiabetica including glibenclamide. Interestingly, no data are available, whether the newer sulphonylurea compound gliquidone might similarly influence the mitochondrial bioenergetic processes. This question seems to be important, since the effective dose of gliquidone is at least 5 times higher, than that of glibenclamide [5, 36].

The present study was designed to examine a presumable interference of gliquidone with mitochondrial bioenergetics. The widely known hypoglycaemic agent, glibenclamide served as a reference substance.

Materials and Methods

Animals

Male CFY rats weighing 150–180 g (LATI, Gödöllő, Hungary) were used for experiments. The animals were appropriately housed and fed by corresponding laboratory diet supplemented with vitamin premix and water *ad lib*.

Chemicals

Gliquidone (1-Cyclohexyl-3-[<p-[2-(3,4-dihydro-7-methoxy-4,4-dimethyl-1,3-dioxo-2(1H)-isochinolyl)ethyl] phenyl>sulphonyl]-urea) and glibenclamide (N-4-[2-(5-chloro-2-methoxybenzmido)-ethyl]-phenyl-sulphonyl-N-cyclohexylurea) were obtained from Dr. Karl Thomae GmbH, Biberach an der Riss, Germany. L-(U^{14}C)-malic acid (40 mCi/mmol) was purchased from Amersham, England, and (1-4^{14}C)-succinic acid (40 mCi/mmol) was obtained from New England Nuclear, Dreieich, Germany. All other chemicals and biochemical products were of the highest purity commercially available.

Preparation of liver mitochondria

Mitochondria from rat livers were isolated by the method of Schneider [29] with minor modifications. Briefly, the livers were washed in preparation solution (0.25 M sucrose, 0.005 M Tris-HCl, 0.001 M EGTA, pH 7.2), cleaned and minced. Homogenization was carried out by 6 to 10 strokes in a glass/teflon homogenizer. Nuclei and non-disrupted cells from the 10 per cent homogenate were sedimented by 500×g for 10 min. The supernatant was centrifuged for 20 min at 4000×g. The pellet was resuspended in the preparation solution, homogenized and recentrifuged at 4000×g for 15 min. This washing procedure was repeated once more. The final pellet was resuspended in a minimal volume of preparation solution. After determination of protein content (20), the final concentration was adjusted to 60 mg protein/ml. Only

those preparations were used, where the respiratory control ratio reached the minimal value of 6 with glutamate + malate as respiratory substrates.

Measurement of the mitochondrial respiration

O_2 uptake was measured polarographically using a Clark type oxygen electrode. The registration was carried out in 2.0 ml of standard medium (80 mM KCl, 20 mM Tris-HCl 5 mM KH_2PO_4, 1 mM EGTA, pH 7.2) previously equilibrated with air. 2.5 mg mitochondrial protein was pipetted into the glass chamber which was kept in thermostat at 37 °C. Further additions were performed by microsyringes through the central hole of the stopper in a minimal volume. The following substrates were used in the experiments: 5 mM glutamate + 1 mM malate, 5 mM pyruvate + 1 mM malate; 5 mM succinate with 1.5 μM rotenone. ADP and DNP were added at final concentrations of 0.4 mM and 40 μM, respectively. Gliquidone and glibenclamide were dissolved in dimethylsulphoxide (DMSO), if not otherwise indicated 10 μl of dissolved drugs or DMSO alone were added to the glass chamber.

Determination of ATP-ase activity of mitochondria

ATP-ase activity was measured in 2.0 ml standard respiratory medium without KH_2PO_4 supplemented with 5 mM $MgCl_2$ and 5 mM Na-ATP, pH 7.2 and 40 μM DNP if indicated. The reaction was initiated by the addition of 0.5 mg mitochondrial protein. After 10 min incubation at 37 °C 1.0 ml 20 per cent TCA was added and the P_i liberated was estimated in the protein free filtrate according to Lohmann and Jendrassik [19].

Measurement of mitochondrial swelling

Mitochondrial osmotic volume changes were estimated by measuring the apparent absorbance changes at 540 nm spectrophotometrically. The measurements were carried out in 3.0 ml of appropriate isoosmotic media at 25 °C as indicated in the legends to the Figures.

Measurement of substrate uptake

Mitochondria were suspended in a loading medium containing 225 mM mannitol, 75 mM sucrose, 20 mM Tris-HCl pH 7.4, 0.5 mM EDTA, 5 mM malonate. The suspensions were preincubated for 20 min at 0 °C. After centrifugation with 4500×g for 10 min the pellets were suspended in the above-mentioned medium at a concentration of 50 mg/ml. The substrate uptake of mitochondria was measured in 0.3 ml reaction volume containing 0.27 ml incubating solution (15 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, 50 mM Tris-HCl pH 7.4, 1 μM Na_2 arsenite, 0.2 μM mersalyl and 1 μM rotenone), 30 μl mitochondrial suspension (1.5 mg protein), 10 μl of 30 mM ^{14}C-malate or ^{14}C-succinate and 3 μl glibenclamide or gliquidone dissolved in dimethylsulphoxide. After a 5 or 20 s incubation at 0 °C, 2.7 ml incubating medium (containing 25 mM phenylsuccinate) was added to the mixtures and the media were filtered through Whatman glass GF/F filters and the radioactivity of the filters was measured by liquid scintillation. Substrate uptake was calculated from the difference of radioactivity between the 20 s and 5 s values.

Results

The influence of gliquidone and glibenclamide on mitochondrial respiration capacity

To test whether gliquidone as well as glibenclamide influence the mitochondrial ATP generation and therefore the energy dependent metabolic pathways of the cells, their effects were investigated on respiration capacity of isolated rat liver mitochondria.

Under *in vitro* conditions both gliquidone and glibenclamide exerted a dual effect on mitochondrial respiration. As it is shown in Fig. 1 after the addition of the drugs at increasing concentrations, the basal respiration was progressively enhanced in a concentration dependent manner, under the same condition the ADP – as well as DNP – stimulated O_2 uptake was gradually decreased reaching the value of the basal respiration when glutamate and malate substrates were used. Although the basal respiration by 50 μM gliquidone was only slightly affected, gliquidone at this concentration caused an approximately 50 per cent inhibition of ADP-stimulated respiration. 100 μM gliquidone produced more than 70 per cent inhibition of ADP-dependent O_2 uptake, and increased the basal respiration threefold. These changes indicated a certain uncoupling of the mitochondrial oxidative phosphorylation besides the direct inhibition of the respiratory capacity. As a result, the respiratory control ratio (RCR) also progressively decreased, causing a 50 per cent reduction at about 50 μM gliquidone (Fig. 1). The ADP/O quotient decreased similarly (data not shown). Glibenclamide elicited a similar, but somewhat more pronounced effect on the respiration parameters than gliquidone. 100 μM glibenclamide caused a total uncoupling of oxidation from phosphorylation. Similar gliquidone – as well as glibenclamide – induced alterations were observed when the oxidation of pyruvate + malate (Fig. 2) and that of succinate (in the presence of rotenone) was measured (Fig. 3).

Effect of gliquidone and glibenclamide on the mitochondrial ATP-ase activity

Gliquidone and glibenclamide induced similar changes of the ATP-ase activity of liver mitochondria to those of respiration.

Under *in vitro* conditions both gliquidone and glibenclamide exerted a dual effect on mitochondrial ATP-ase activity. After the addition of the drugs at increasing concentrations, the basal ATP-ase activity was progressively enhanced in a concentration dependent manner, under the same conditions the DNP-stimulated ATP-ase activity decreased gradually reaching the basal value. These changes supported further a potential damage of structural integrity of mitochondria due to sulphonylureas investigated. No significant difference was observed between gliquidone and glibenclamide in their inhibitory actions upon mitochondrial ATP-ases (Fig. 4).

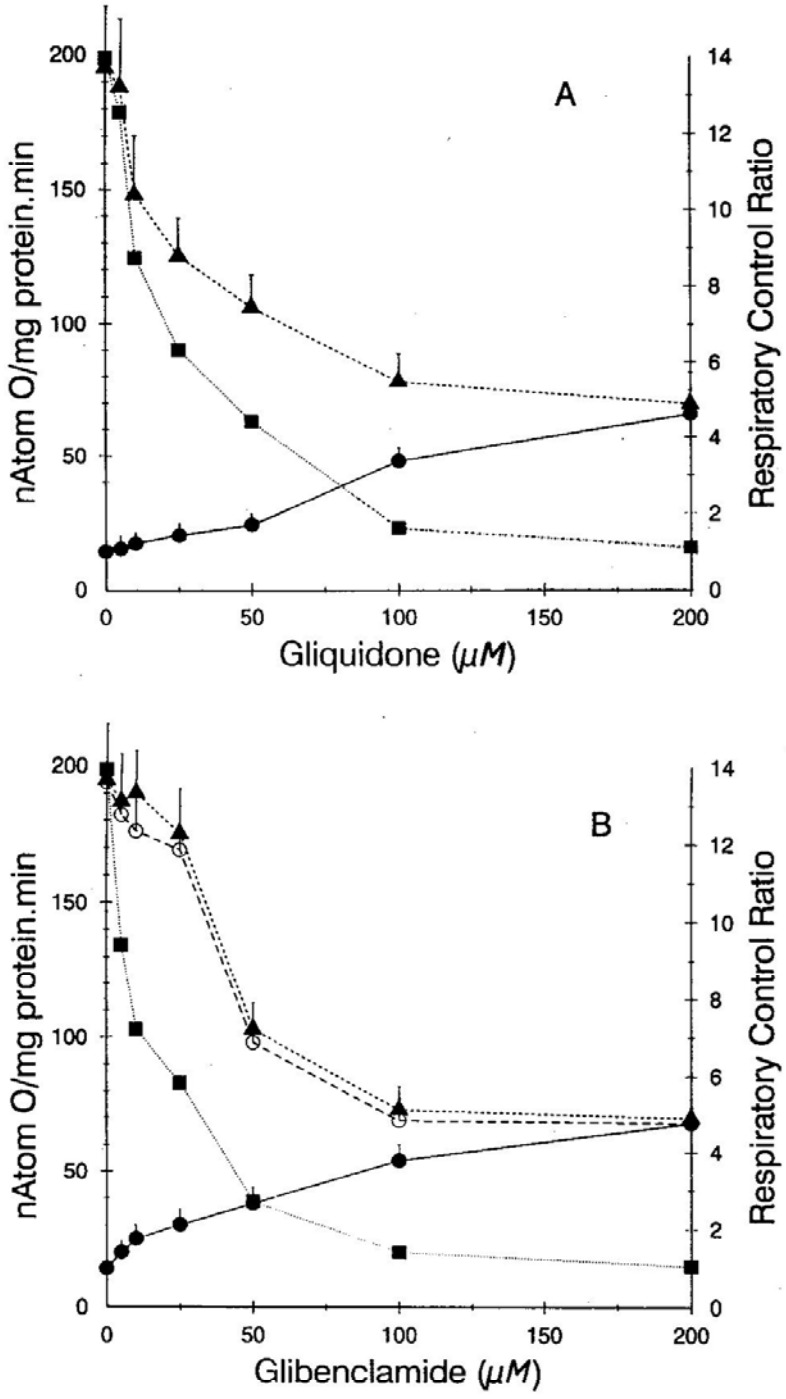

Fig. 1. Effect of gliquidone and glibenclamide on the mitochondrial respiration in the presence of glutamate + malate. A: effect of gliquidone; B: effect of glibenclamide; Basal respiration •---•; ADP-stimulated respiration ▲---▲; DNP-stimulated respiration o --- o ; respiratory control ratio (RCR) ■---■; The details of the experimental conditions were as described in **Methods**

Effects of gliquidone and glibenclamide on mitochondrial swelling

Both sulphonylurea compounds investigated caused a slight but measurable progressive swelling of isolated mitochondria in isoosmotic KNO_3 indicating an enhanced K^+-permeability. However, this swelling was not comparable to the effect of the K^+-ionophore valinomycin (Fig. 5).

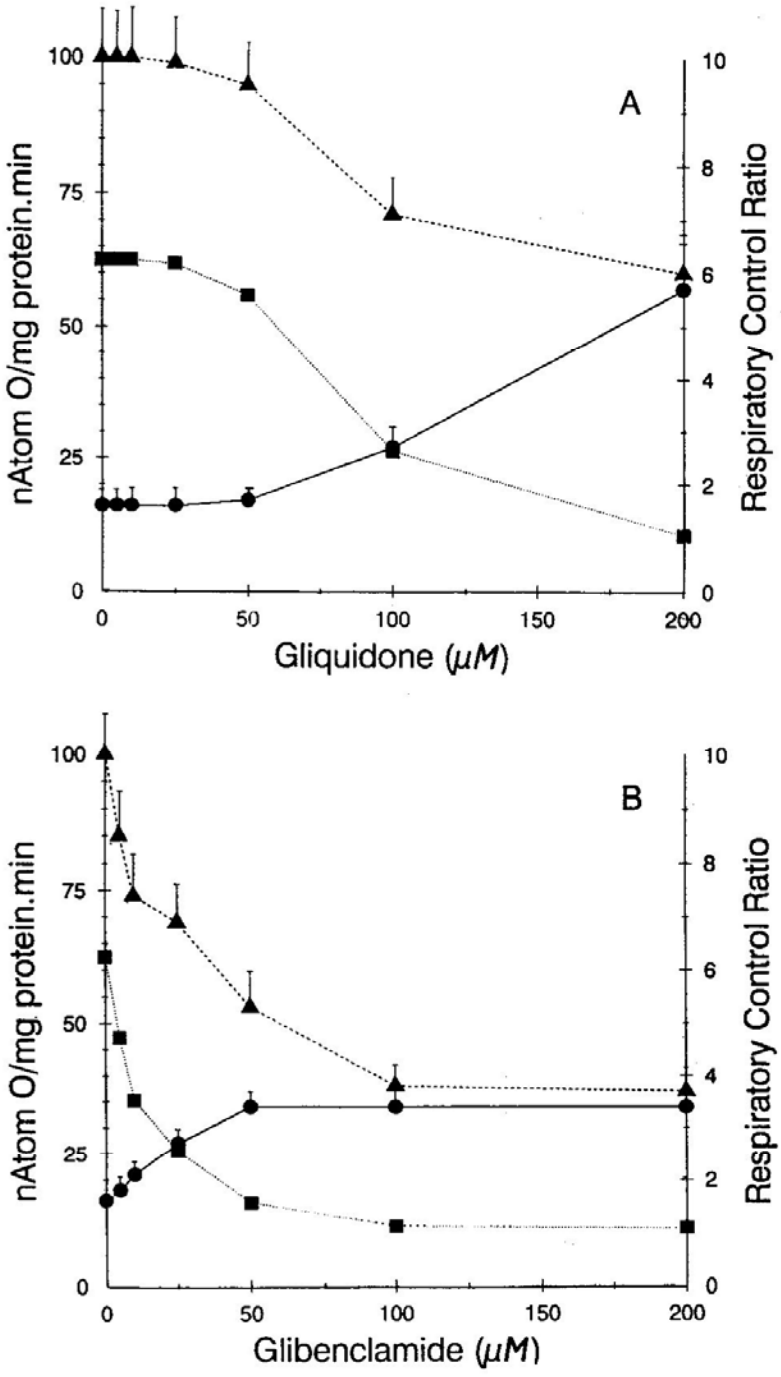

Fig. 2. Effect of gliquidone and glibenclamide on the mitochondrial respiration in the presence of pyruvate + malate. For symbols and experimental details see the legend of Fig. 1

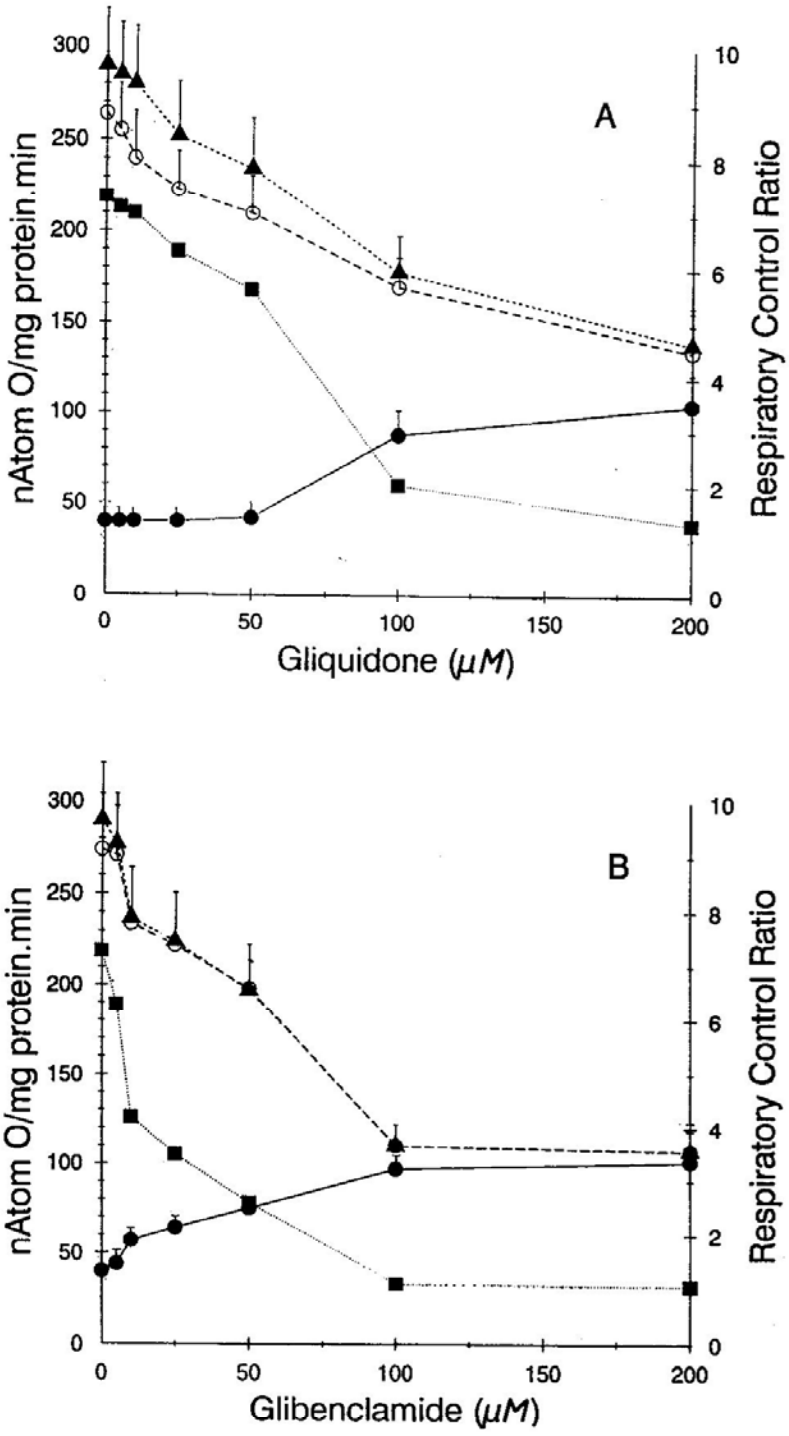

Fig. 3. Effect of gliquidone and glibenclamide on the mitochondrial respiration in the presence of succinate with rotenone. For symbols and experimental details see the legend of Fig. 1

When the mitochondria were suspended in isoosmotic $NaNO_3$ no significant swelling could be observed (not shown). Mitochondrial swelling can be also observed when the mitochondria are suspended in isotonic ammonium phosphate. NH_3 can

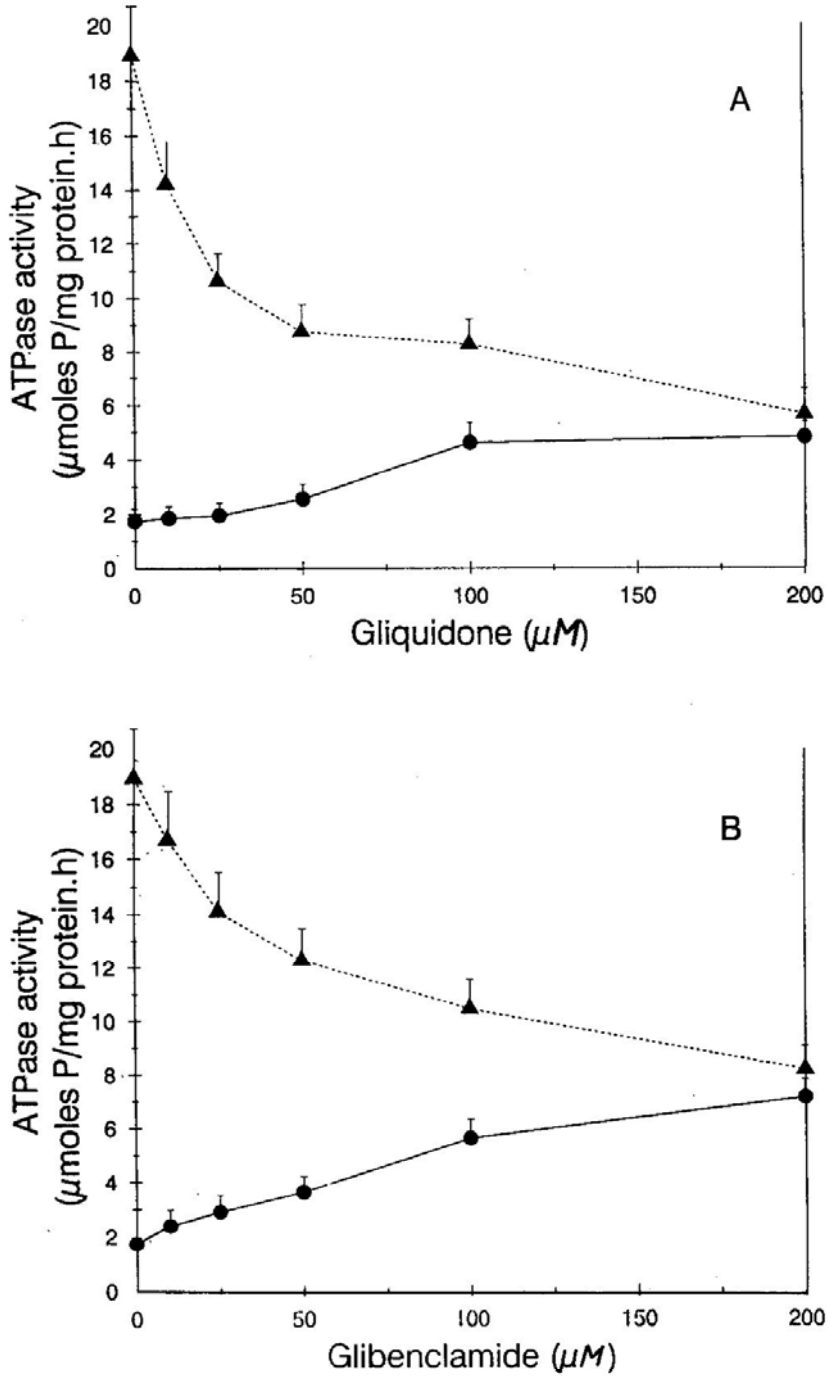

Fig. 4. Influence of gliquidone and glibenclamide on the mitochondrial ATP-ase activity. A: effect of gliquidone; B: effect of glibenclamide; basal ATP-ase activity •—•; DNP-stimulated ATP-ase activity ▲—▲; The experimental details were as described in **Methods**

penetrate the membrane freely, and $H_2PO_4^-$ will be transported together with a proton via the phosphate carrier system [11]. When the phosphate carrier is blocked by mercurials or N-ethylmaleimide (NEM) no osmotic volume change can occur. Gliquidone and glibenclamide caused only a partial inhibition of the swelling in ammonium phosphate medium indicating a depressed penetration of phosphate into mitochondria which may also contribute to the decreased activity of oxidative phosphorylation (Fig. 6).

Effect of gliquidone and glibenclamide on the substrate uptake of mitochondria

Ten μM gliquidone caused an approximately 10 per cent inhibition of malate and succinate uptake into mitochondria. Fifty per cent inhibition was produced by 50 μM gliquidone while 70 per cent inhibition was observed in the presence of 100 μM. 0.5 mM gliquidone practically abolished the substrate uptake into mitochondria. No significant difference was found in the gliquidone sensitivity of malate and succinate uptake (Fig. 7). Glibenclamide resulted in similar changes in substrate uptake processes as gliquidone (Fig. 8).

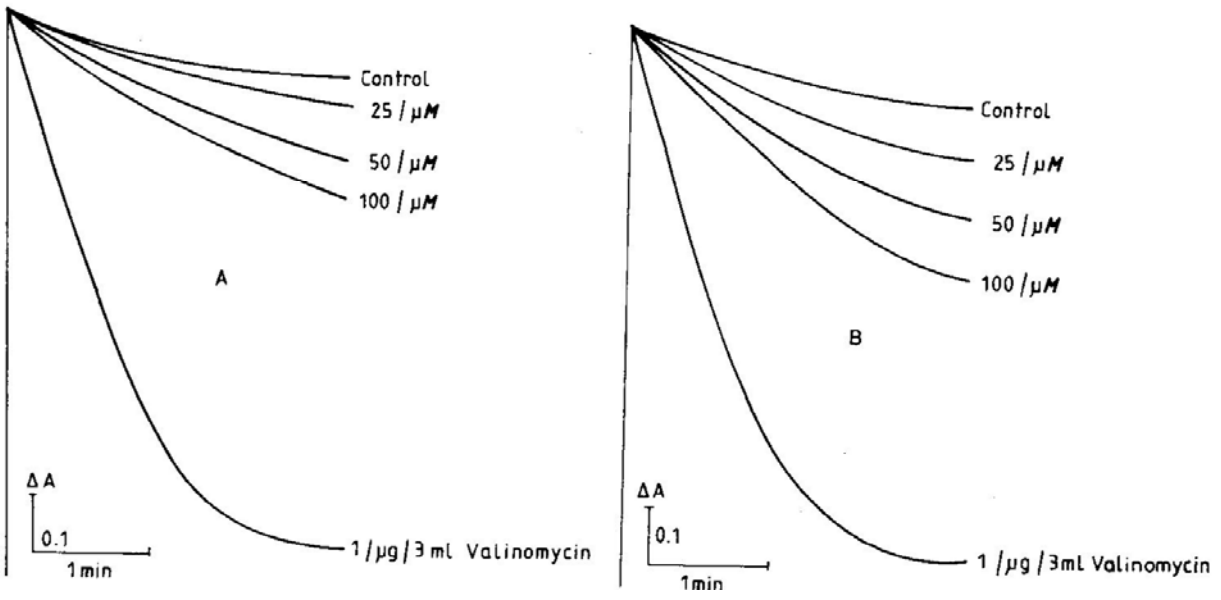

Fig. 5. Influence of gliquidone and glibenclamide on the mitochondrial swelling in isoosmotic KNO_3 medium A: effect of gliquidone; B: effect of glibenclamide; mitochondria (1 mg protein) were suspended in 3 ml medium containing 5 mM Hepes, 135 mM KNO_3, 0.1 mM EDTA, and 2 μM rotenone (pH 7.2). All other experimental details were as described in **Methods**

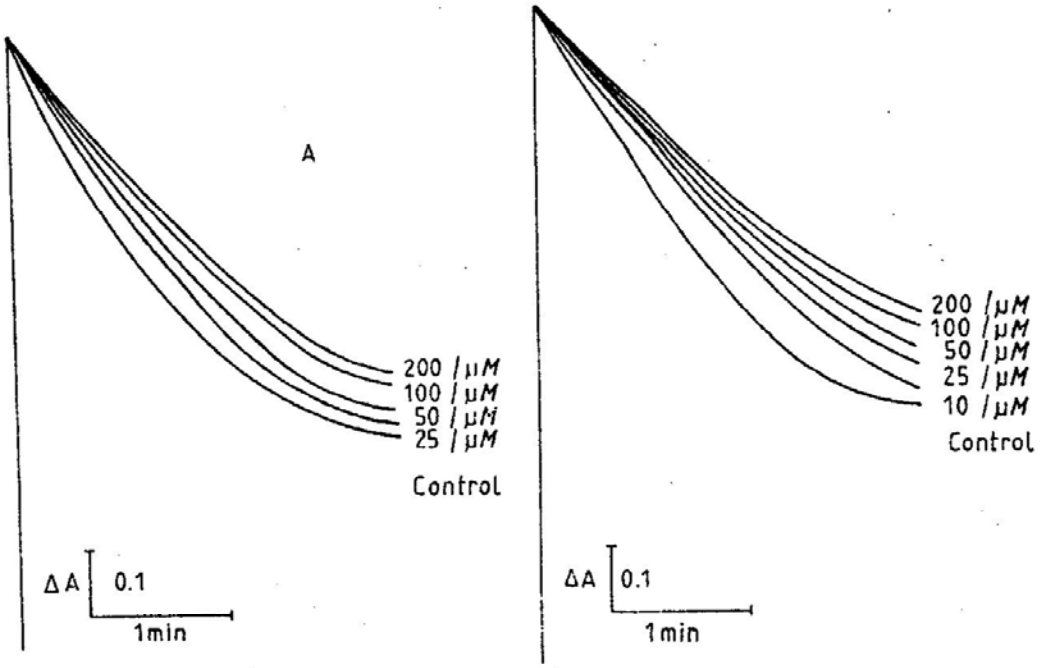

Fig. 6. Influence of gliquidone and glibenclamide on mitochondrial swelling in isoosmotic ammoniumphosphate medium. A: effect of gliquidone; B: effect of glibenclamide; mitochondria (1 mg protein) were suspended in 3 ml medium containing 5 mM Hepes, 135 mM $(NH_4)_3PO_4$, 0.1 mM EDTA and 2 μM rotenone (pH 7.2). All other experimental details were as described in **Methods**

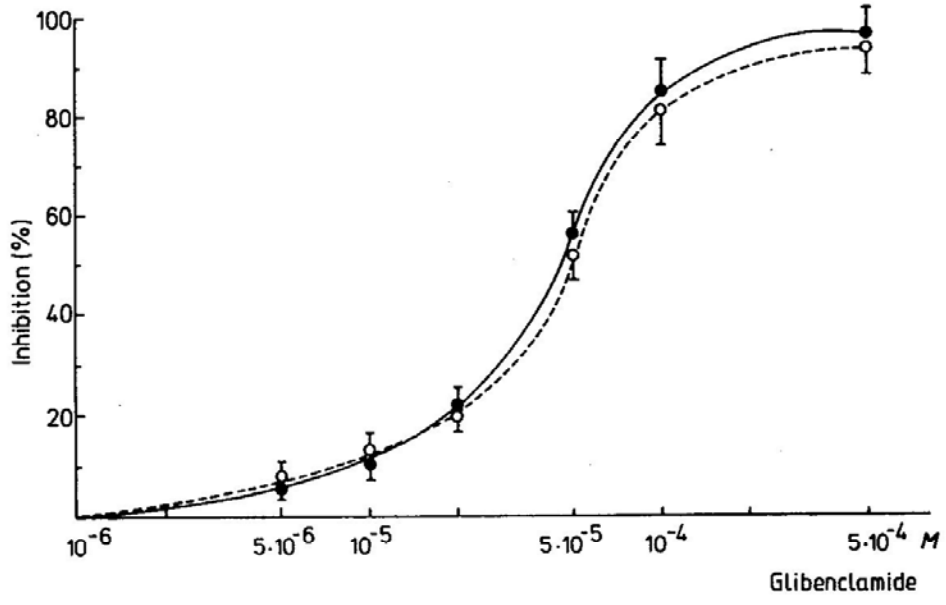

Fig. 7. Effect of glibenclamide on succinate and malate uptake into mitochondria; succinate •---•; malate o --- o ; The experimental details were as described in **Methods**

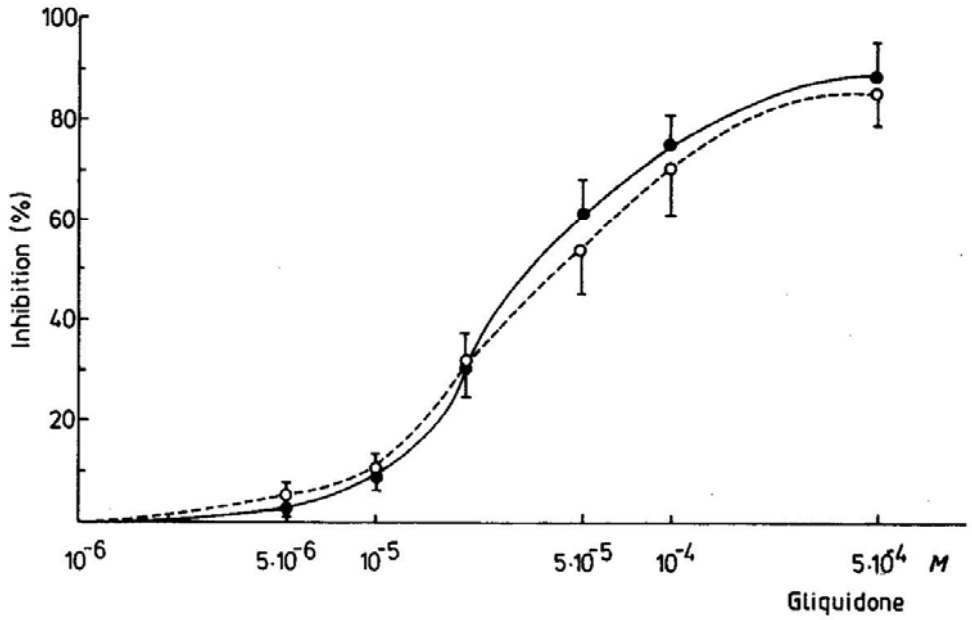

Fig. 8. Effect of gliquidone on succinate and malate uptake into mitochondria; For symbols and experimental details see the legend of Fig. 7

Discussion

In spite of many detailed investigations, the exact mechanism of the antidiabetic action of sulphonylurea compounds remains to be clarified. Besides the effects on beta-cells of pancreas [5, 13, 16, 19], different extrapancreatic actions of sulphonylurea compounds were also demonstrated in various tissues [2, 17, 18, 28, 31]. Among these effects the potentiation of insulin action and the reduction of glucose production of liver seem to be undoubtedly the most important [1, 4, 7, 16]. Since the gluconeogenesis is a highly energy consuming process, it can be supposed that the reduction of gluconeogenesis by sulphonylureas may be partially due to the available energy sources [30]. In this connection the inhibitory action of antidiabetic sulphonylureas on the bioenergetic processes in the liver should be taken into consideration.

The antidiabetic sulphonylurea compounds tested in this study caused a direct inhibition of ADP-stimulated respiration and exerted a partial uncoupling effect on oxidative phosphorylation in liver under *in vitro* conditions using various citrate cycle intermediates as substrates. In this respect not only the damage of citrate cycle but the inhibition of the oxidation of long chain fatty acids by the sulphonylurea compounds seems to be also important [26]. In an earlier study we could detect glibenclamide in liver and kidney mitochondria even one hour after intra-peritoneal administration of the compound [32]. The mitochondrial effects of sulphonylureas seem not to be very specific, because they are not limited to the electron–transport chain. These hypoglycaemic agents can interfere with mitochondrial substrate uptake processes [23]. In this study we have also demonstrated the inhibition of substrate uptake process by

gliquidone and glibenclamide. Furthermore, changes in the K^+-permeability of mitochondrial inner membrane under influence of both sulphonylureas produce further evidence that due to their lipophylic character these compounds may cause disturbances in the structure and functions of the mitochondria.

The question arises whether the alterations in the mitochondrial functions described here could take place also under *in vivo* conditions. In this connection the chronic treatment with hypoglycaemic sulphonylureas should be taken into consideration especially in the case of liver damage. In the forthcoming paper we try to give an appropriate answer to this problem [33].

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