

# Glibenclamide Interferes with Mitochondrial Bioenergetics by Inducing Changes on Membrane Ion Permeability

Maria A. S. Fernandes,<sup>1</sup> Maria S. Santos,<sup>1</sup> António J. M. Moreno,<sup>1</sup> Gunars Duburs,<sup>3</sup> Catarina R. Oliveira,<sup>4</sup> and Joaquim A. F. Vicente<sup>2</sup>

<sup>1</sup>Departamento de Zoologia, Universidade de Coimbra, 3004-517 Coimbra, Portugal; E-mail: [mfer@ci.uc.pt](mailto:mfer@ci.uc.pt)

<sup>2</sup>Departamento de Botânica da Faculdade de Ciências da, Universidade de Coimbra, 3004-517 Coimbra, Portugal

<sup>3</sup>Latvian Institute of Organic Synthesis, 21 Aizkraukles Street, Riga, LV-1006, Latvia

<sup>4</sup>Serviço de Bioquímica, Faculdade de Medicina, Universidade de Coimbra, 3004-504 Coimbra, Portugal

Accepted 4 April 2004

**ABSTRACT:** The interference of glibenclamide, an antidiabetic sulfonylurea, with mitochondrial bioenergetics was assessed on mitochondrial ion fluxes ( $H^+$ ,  $K^+$ , and  $Cl^-$ ) by passive osmotic swelling of rat liver mitochondria in K-acetate,  $KNO_3$ , and KCl media, by  $O_2$  consumption, and by mitochondrial transmembrane potential ( $\Delta\psi$ ). Glibenclamide did not permeabilize the inner mitochondrial membrane to  $H^+$ , but induced permeabilization to  $Cl^-$  by opening the inner mitochondrial anion channel (IMAC).  $Cl^-$  influx induced by glibenclamide facilitates  $K^+$  entry into mitochondria, thus promoting a net  $Cl^-/K^+$  cotransport,  $\Delta\psi$  dissipation, and stimulation of state 4 respiration rate. It was concluded that glibenclamide interferes with mitochondrial bioenergetics of rat liver by permeabilizing the inner mitochondrial membrane to  $Cl^-$  and promoting a net  $Cl^-/K^+$  cotransport inside mitochondria, without significant changes on membrane permeabilization to  $H^+$ . © 2004 Wiley Periodicals, Inc. *J Biochem Mol Toxicol* 18:162–169, 2004; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jbt.20022

**KEYWORDS:** Antidiabetics; Cerebrocrast; 1,4-Dihydropyridine Derivatives; Bioenergetics; Glibenclamide; Mitochondria; Sulphonylureas; Toxicity; Type 2 diabetes

## INTRODUCTION

Sulfonylureas, such as glibenclamide, glipizide, and tolbutamide, are oral hypoglycemics widely used in the treatment of type 2 diabetes to stimulate insulin release from pancreatic  $\beta$ -cells [1]. Although the molecular mechanism of action of antidiabetic sulfonylureas is not fully understood, it is believed that the therapeutic effect of these drugs results primarily from their binding to high-affinity receptors (SUR) in the plasma membrane of pancreatic  $\beta$ -cells [2]. The pancreatic  $\beta$ -cell SUR was cloned [1] and identified as an element composing, together with a  $K^+$  pore, the functional ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channel [3]. Binding of sulfonylureas to the SUR causes a closure of the  $K_{ATP}$  channel, leading to  $\beta$ -cell membrane depolarization, opening of voltage-dependent  $Ca^{2+}$  channels, and, ultimately, increase in the exocytosis of insulin [2].

A  $K^+$  channel with properties similar to those of the  $K_{ATP}$  channel from the plasma membrane of pancreatic  $\beta$ -cells was described in the inner membrane of rat liver and beef heart mitochondria, and designated as the mitochondrial ATP-sensitive  $K^+$  channel (mito $K_{ATP}$  channel) [4,5]. Equilibrium binding studies using [ $^3H$ ]-labelled glibenclamide revealed a single class of low-affinity binding sites for glibenclamide in intact rat liver mitochondria (mitochondrial sulfonylurea receptor, mitoSUR) with a  $K_d$  of 4  $\mu M$  [6]. In beef heart mitochondria, the  $K_d$  for glibenclamide binding is much lower: 300 nM [6]. The use of [ $^{125}H$ ]glibenclamide led to the identification of the mitoSUR as a 28-kDa polypeptide [7], which may represent part of a more complex mito $K_{ATP}$  channel. Glibenclamide binding to mitochondria is modulated by SH reagents such as *N*-ethylmaleimide and mersalyl [8].

Correspondence to: Maria A. S. Fernandes.

Contract Grant Sponsor: Fundação para a Ciência e Tecnologia (FCT), Portugal.

Contract Grant Sponsor: D13 COST action (E.U.).

© 2004 Wiley Periodicals, Inc.

The effects of antidiabetic sulfonylureas on mitoK<sub>ATP</sub> channel have been observed both in intact mitochondria [4,9] and in proteoliposomes reconstituted with partly purified mitoK<sub>ATP</sub> channel [5,10]. This channel is blocked by glibenclamide [4,5] after being opened by Mg<sup>2+</sup>, ATP, and physiological activators such as GTP, or the K<sup>+</sup> channel opener diazoxide [10]. In such an induced open state of the mitoK<sub>ATP</sub> channel, glibenclamide inhibited the channel activity with a K<sub>1/2</sub> value of 1 to 6 μM [10]. In intact mitochondria, the electrogenic transport of potassium (K<sup>+</sup> uniport) induced by Mg<sup>2+</sup> depletion is also blocked by glibenclamide in a concentration-dependent manner, with an IC<sub>50</sub> of 20 μM [11]. However, a more recent study showed that 50 μM glibenclamide induced, by itself, K<sup>+</sup> influx inside mitochondria [12].

Additionally, glibenclamide interferes with mitochondrial bioenergetics, as shown by inhibition of uncoupled respiration in both liver (IC<sub>50</sub> = 70 ± 2 μM) and heart mitochondria (IC<sub>50</sub> = 5.3 ± 0.5 μM) [10], dissipation of mitochondrial membrane potential and proton gradient (IC<sub>50</sub> = 70 ± 7 μM) [13], and lowering of mitochondrial ATP content in rat liver mitochondria [14]. However, the mechanism by which glibenclamide interferes with mitochondrial bioenergetics is not completely understood. Some investigators reported that it may be related with the ability of glibenclamide to increase the proton conductance of the inner mitochondrial membrane [13], while others report that it may be related with K<sup>+</sup> influx inside mitochondria [12].

The mitochondrial inner membrane possesses an anion channel (IMAC) mediating the electrophoretic transport of a wide variety of anions, and it is believed to be an important component of the mitochondrial volume homeostasis mechanism [15,16].

In order to clarify the mechanism by which glibenclamide interferes with mitochondrial bioenergetics, the influence of glibenclamide on mitochondrial ion fluxes (H<sup>+</sup>, K<sup>+</sup>, and Cl<sup>-</sup>) was assessed by passive osmotic swelling of rat liver mitochondria in K-acetate, KNO<sub>3</sub>, and KCl media, by mitochondrial transmembrane potential ( $\Delta\psi$ ), and by O<sub>2</sub> consumption in state 4 respiration.

## MATERIALS AND METHODS

### Animals

Male Wistar rats (250–350 g), housed at 22 ± 2°C under artificial light for a 12-h light/dark cycle and with access to water and food ad libitum, were used throughout the experiments. The experiments were carried out in accordance with the National Requirements for Vertebrate Animal Research and European Convention for

the Protection of Animals used for Experimental and other Scientific Purposes.

### Isolation of Rat Liver Mitochondria

Rat liver mitochondria were isolated from male Wistar rats (6 weeks) by differential centrifugation according to conventional methods [17]. The pellet was gently resuspended in washing medium, at a protein concentration of about 50 mg/mL. Protein content was determined by the biuret method [18], using bovine serum albumin as standard.

### Mitochondrial Swelling

Mitochondrial osmotic volume changes were measured by the apparent absorbance changes at 520 nm with a suitable spectrophotometer-recorder setup. Mitochondria (1 mg) were suspended in 2.5 mL of the required medium (K<sup>+</sup>-acetate, KNO<sub>3</sub>, or KCl) supplemented with 2 μM rotenone, at 30°C. Each medium contained 55 mM potassium salts, 5 mM HEPES (pH 7.1), and 0.1 mM EDTA.

### Measurement of Respiratory Activities

Oxygen consumption was monitored polarographically at 30°C with a Clark oxygen electrode, in a closed chamber with magnetic stirring. The reaction medium consisted of 250 mM sucrose, 10 mM HEPES (pH 7.2), 20 mM KCl, 5 mM potassium phosphate, and 2 mM MgCl<sub>2</sub>. Mitochondria (1 mg protein) were incubated in 1 mL of medium containing 250 mM sucrose, 10 mM HEPES (pH 7.2), 20 mM KCl, 5 mM potassium phosphate, and 2 mM MgCl<sub>2</sub>, supplemented with 2 μM rotenone and energized with 10 mM succinate. State 4 respiration was achieved after phosphorylation of 50 nmol adenosine diphosphate (ADP). State 4 respiration rate was calculated considering that the saturation oxygen concentration was 232 nmol O<sub>2</sub>/mL in the medium at 30°C, and the values are expressed in nmol O<sub>2</sub> (mg protein)<sup>-1</sup> min<sup>-1</sup>.

### Measurement of Mitochondrial Transmembrane Potential ( $\Delta\psi$ )

The mitochondrial transmembrane potential ( $\Delta\psi$ ) was measured indirectly based on the activity of the lipophilic cation tetraphenylphosphonium (TPP<sup>+</sup>) using a TPP<sup>+</sup>-selective electrode in combination with an Ag/AgCl-saturated reference electrode, as previously described [19]. Mitochondria (1 mg protein) were incubated in 1 mL of medium containing 250 mM sucrose,

10 mM HEPES (pH 7.2), 20 mM KCl, 5 mM potassium phosphate, and 2 mM MgCl<sub>2</sub>, supplemented with 2  $\mu$ M rotenone and 3  $\mu$ M TPP<sup>+</sup> and energized with 10 mM succinate. No correction was made for the "passive" binding of TPP<sup>+</sup> to the mitochondria membranes because the purpose of the experiments was to show relative changes in potential rather than absolute values. As a consequence, we can anticipate some overestimation for the  $\Delta\psi$  values. Glibenclamide, and the other compounds added (propranolol, cerebrocrast, nigericin, and dinitrophenol), did not affect TPP<sup>+</sup> binding to mitochondria membranes or the electrode response.

## Chemicals

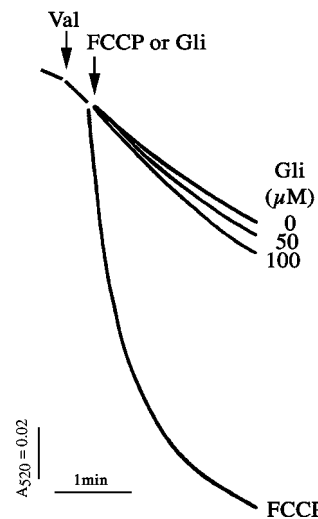
All chemicals were obtained from Sigma Chemical Company (St Louis, MO, USA) except cerebrocrast (IOS-1.1212; 2-propoxyethyl ester of 2,6-dimethyl-4-[2-difluoromethoxyphenyl]-1,4-dihydropyridine-3,5-dicarboxylic acid), which was synthesized at the Latvian Institute of Organic Synthesis, 21 Airzkraukles Street, Riga, LV-1006, Latvia. Cerebrocrast and glibenclamide were dissolved in absolute dimethyl sulfoxide (DMSO). Pure solutions of DMSO were added to controls at the highest volume of glibenclamide DMSO solutions used [0.1% (v/v) of the experiments final volume], having no effects on the measured activities.

## RESULTS

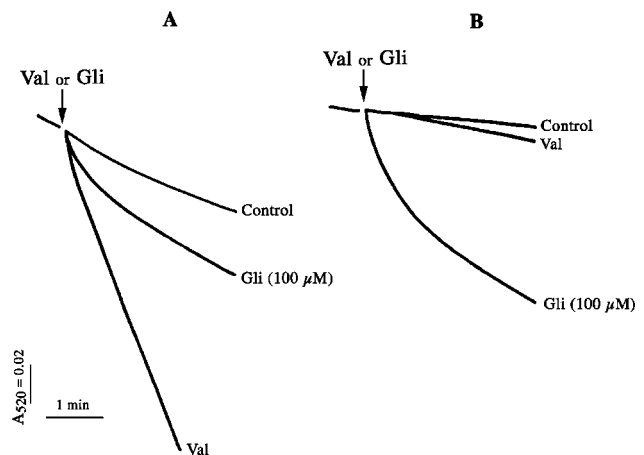
### Effects of Glibenclamide on Nonrespiring Mitochondria

The effect of glibenclamide on inner mitochondrial membrane permeabilization to H<sup>+</sup> was evaluated by swelling of nonrespiring mitochondria suspended in potassium acetate medium (Figure 1). Protonated acetate can cross the inner mitochondrial membrane and dissociate to acetate anion and H<sup>+</sup> into the mitochondrial matrix producing a proton gradient which inhibits swelling. When valinomycin is present, swelling occurs if the proton gradient is dissipated by the addition of a protonophore. Glibenclamide, at concentrations up to 100  $\mu$ M, leads to a very small increase on valinomycin-dependent mitochondrial swelling, indicating that the proton conductance of the inner mitochondrial membrane is almost not affected.

The effect of glibenclamide on the inner mitochondrial membrane permeabilization to K<sup>+</sup> was evaluated by swelling of nonrespiring mitochondria suspended in KNO<sub>3</sub> medium (Figures 2A and B). Inner mitochondrial membrane freely permeabilizes nitrate (NO<sub>3</sub><sup>-</sup>), but swelling is observed only in conditions of K<sup>+</sup> influx. In the absence of valinomycin, mitochondria swell



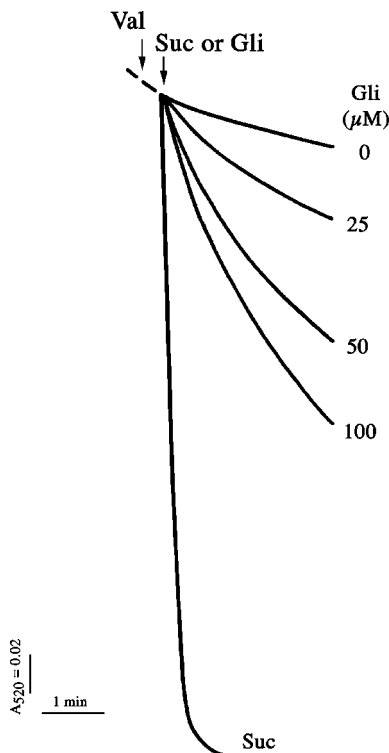
**FIGURE 1.** Effect of glibenclamide on permeabilization to H<sup>+</sup> by inner membrane of rat liver mitochondria. Assays were performed in swelling of K-acetate. Mitochondria (1 mg) suspended in 2.5 mL of K-acetate medium [54 mM K-acetate, 5 mM HEPES (pH 7.1), 0.1 mM EGTA, and 0.2 mM EDTA] supplemented with 2  $\mu$ M rotenone were incubated at 30°C. At the end of 30 s of incubation, valinomycin (Val) (1  $\mu$ g/mL) was added, followed by 1  $\mu$ M carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCCP) or glibenclamide (Gli) (50 and 100  $\mu$ M) 30 s later. Data represent typical recordings of experiments obtained from six different mitochondrial preparations.



**FIGURE 2.** Comparative effects of glibenclamide on permeabilization to K<sup>+</sup> by inner membrane of rat liver mitochondria, as dependent on the presence of the anion nitrate (A) or chloride (B). Swelling assays were performed in KNO<sub>3</sub><sup>-</sup> and KCl media, respectively. Mitochondria (1 mg) suspended in 2.5 mL of the appropriate medium [containing 5 mM HEPES (pH 7.1), 0.1 mM EGTA, and 0.2 mM EDTA] supplemented with 2  $\mu$ M rotenone were incubated at 30°C. At the end of 30 s of incubation, valinomycin (Val) (1  $\mu$ g/mL) or glibenclamide (Gli) (100  $\mu$ M) were added. Control denotes assays in the absence of additions. Data represent typical recordings of experiments obtained from six different mitochondrial preparations.

slowly because of the low permeability of the inner mitochondrial membrane to  $K^+$ . Maximum swelling was observed by adding valinomycin to provide a uniport to  $K^+$ . Addition of glibenclamide (100  $\mu\text{M}$ ), instead of valinomycin, leads to a small increase on swelling, indicating that the  $K^+$  conductance of the inner mitochondrial membrane is not greatly affected (Figure 2A). However, if  $\text{Cl}^-$  instead of  $\text{NO}_3^-$  is present as the representative anion, the  $K^+$  conductance of the inner mitochondrial membrane greatly increases, suggesting that  $K^+$  entry is dependent on the presence of  $\text{Cl}^-$  (Figure 2B).

The effect of glibenclamide on the inner mitochondrial membrane permeabilization to  $\text{Cl}^-$  was evaluated by valinomycin-dependent swelling of nonrespiring mitochondria suspended in KCl medium (Figure 3). After addition of valinomycin to provide a uniport for  $K^+$ , mitochondria swell slowly because of the low permeability of the inner mitochondrial membrane to  $\text{Cl}^-$  [20,21], as compared with maximum swelling induced by addition of succinate. Succinate, by promoting mito-



**FIGURE 3.** Effect of glibenclamide on permeabilization to  $\text{Cl}^-$  by inner membrane of rat liver mitochondria. Swelling assays were performed in KCl medium. Mitochondria (1 mg) suspended in 2.5 mL of KCl [54 mM KCl, 5 mM HEPES (pH 7.1), 0.1 mM EGTA, and 0.2 mM EDTA] supplemented with 2  $\mu\text{M}$  rotenone were incubated at 30°C. At the end of 30 s of incubation, valinomycin (Val) (1  $\mu\text{g}/\text{mL}$ ) was added, followed by 2 mM succinate (Suc) or glibenclamide (Gli) (25, 50, and 100  $\mu\text{M}$ ) 30 s later. The traces are typical recordings of experiments obtained from six different mitochondrial preparations.

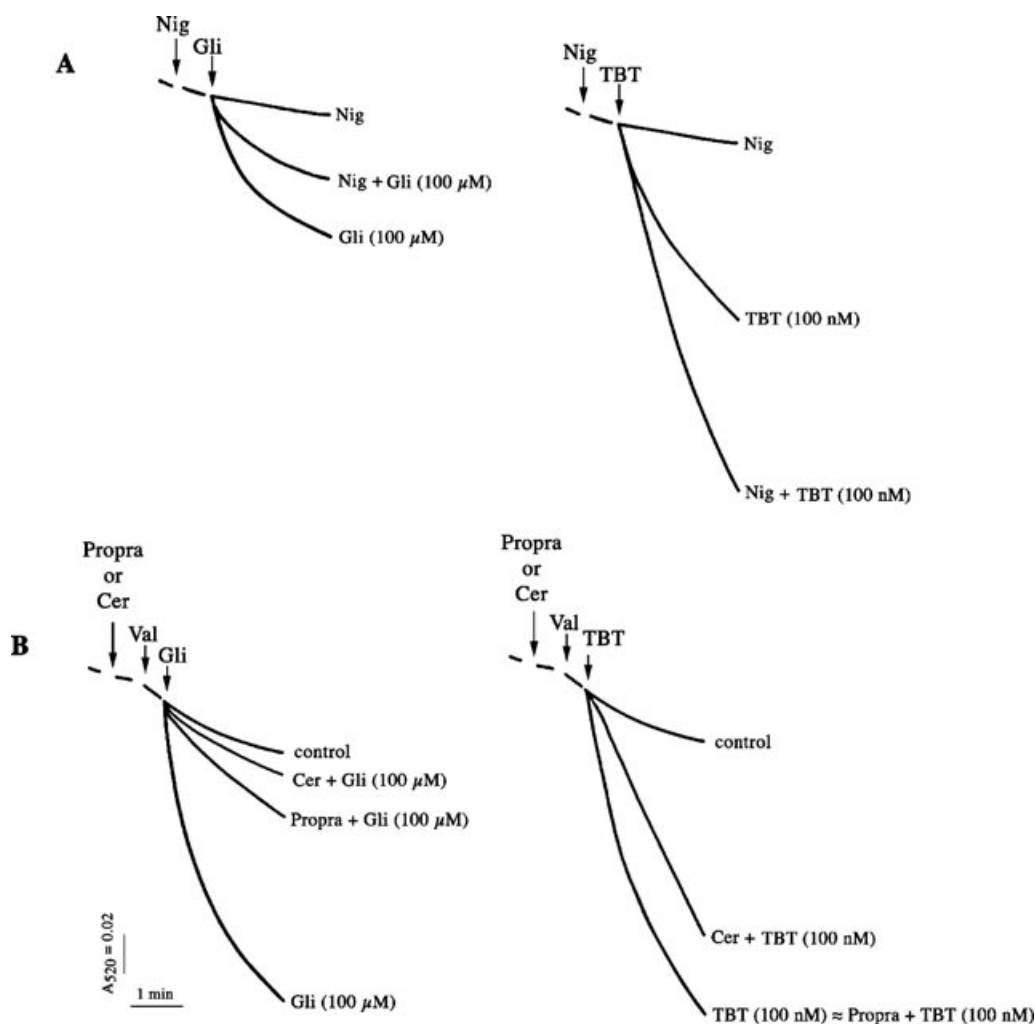
chondrial respiration, induces mitochondrial matrix alkalization and IMAC opening, thus permitting maximal permeabilization to  $\text{Cl}^-$ . Addition of glibenclamide (25, 50, and 100  $\mu\text{M}$ ), instead of succinate, induced a significant increase on mitochondrial swelling in a concentration-dependent manner. These findings indicate that glibenclamide promotes the  $\text{Cl}^-$  influx into the mitochondrial matrix, probably by opening IMAC.

Transport of  $\text{Cl}^-$  into mitochondria can be electroneutral ( $\text{Cl}^-/\text{OH}^-$  exchange) or electrogenic ( $\text{Cl}^-$  uniport) via the inner mitochondrial anion channel (IMAC) [20]. To discern between these two possibilities, the effect of glibenclamide on the swelling of nonrespiring mitochondria suspended in KCl medium was compared with that of tributyltin (TBT), a well-known  $\text{Cl}^-/\text{OH}^-$  exchanger [21–23] (Figure 4). Nigericin was used to provide  $\text{K}^+/\text{H}^+$  antiport, favoring TBT action, while propranolol and cerebrocrast were used as inhibitors of the  $\text{Cl}^-$  entry into mitochondrial matrix via IMAC [15,24]. In the absence of valinomycin, glibenclamide (100  $\mu\text{M}$ ) and TBT (100 nM) increased the swelling of nonrespiring mitochondria in KCl medium (Figure 4A). Under these conditions, nigericin (1  $\mu\text{M}$ ) stimulated the swelling induced by TBT, but it inhibited the swelling induced by glibenclamide (Figure 4A). In the presence of valinomycin, glibenclamide (100  $\mu\text{M}$ ) and TBT (100 nM) also increased the swelling of nonrespiring mitochondria in KCl medium (Figure 4B). Under these conditions, the swelling induced by TBT was not affected by propranolol (200  $\mu\text{M}$ ) and slightly inhibited by cerebrocrast (25  $\mu\text{M}$ ), while the swelling induced by glibenclamide was strongly inhibited by both propranolol and cerebrocrast (Figure 4B). Controls in the presence of propranolol (200  $\mu\text{M}$ ) and cerebrocrast (25  $\mu\text{M}$ ) were also performed. Their traces overlap the control trace, and they are not represented in Figure 4B. In contrast to TBT, which promotes  $\text{Cl}^-$  entry by  $\text{Cl}^-/\text{OH}^-$  exchange across the inner mitochondrial membrane, these findings indicated that the  $\text{Cl}^-$  transport promoted by glibenclamide is mainly via IMAC.

### Effects of Glibenclamide on Succinate-Respiring Mitochondria

As mitochondria are respiring in normal physiological conditions, the effects of glibenclamide described above for nonrespiring mitochondria were also evaluated by valinomycin-induced volume changes of succinate-respiring mitochondria in KCl medium (Figure 5), and  $\Delta\Psi$  detection in association with  $\text{O}_2$  consumption (Figure 6).

Figure 5 (A and B) shows that addition of valinomycin to succinate-respiring mitochondria causes a

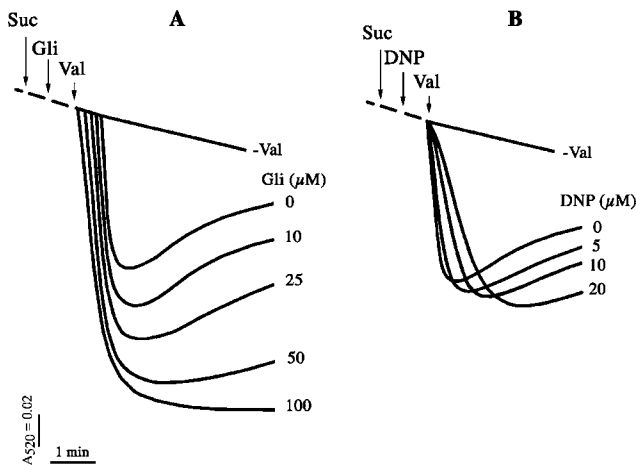


**FIGURE 4.** Comparative effects of glibenclamide and tributyltin (TBT) on permeabilization to  $\text{Cl}^-$  by inner membrane of rat liver mitochondria. (A), swelling recordings of glibenclamide and TBT in the presence and absence of nigericin; (B), swelling recordings to compare propranolol and cerebrocrast actions on the effects of  $\text{Cl}^-$  permeabilization induced by TBT and glibenclamide. Swelling assays were performed in KCl medium. Mitochondria (1 mg) suspended in 2.5 mL of KCl [54 mM KCl, 5 mM HEPES (pH 7.1), 0.1 mM EGTA, and 0.2 mM EDTA] supplemented with 2  $\mu\text{M}$  rotenone were incubated at 30°C. The additions were performed as indicated in the recordings. Nig, TBT, Gli, Val, Propra and Cer denote nigericin (1  $\mu\text{M}$ ), tributyltin (100 nM), glibenclamide (100  $\mu\text{M}$ ), valinomycin (1  $\mu\text{g}/\text{mL}$ ), propranolol (200  $\mu\text{M}$ ), and cerebrocrast (25  $\mu\text{M}$ ), respectively. Control denotes swelling in the presence of valinomycin alone. The traces are typical recordings of experiments obtained from six different mitochondrial preparations.

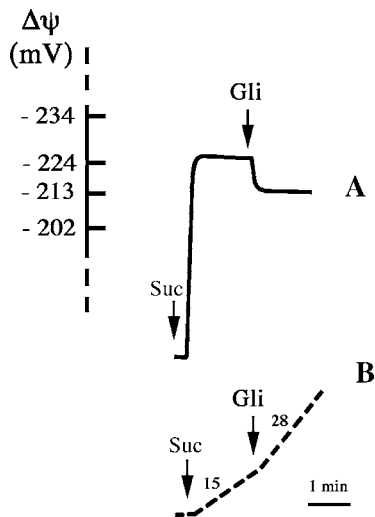
rapid swelling followed by contraction. The swelling phase reflects passive electrophoretic uniport of  $\text{K}^+$ , and  $\text{Cl}^-$  entry inside mitochondria, while the contraction phase reflects the efflux of internal  $\text{K}^+$  in exchange for external protons ( $\text{K}^+/\text{H}^+$  exchange) driven by the pH gradient generated by the respiratory chain  $\text{H}^+$  pumping [15]. Addition of glibenclamide, at concentrations up to 100  $\mu\text{M}$ , did not affect the initial rate of swelling, but increased its amplitude (Figure 5A). Addition of dinitrophenol (DNP), instead of glibenclamide, decreased the initial rate of swelling, without significant effect on its amplitude (Figure 5B). DNP also inhibited the rate and amplitude of mitochondrial con-

traction. The data indicated that, in contrast with DNP, glibenclamide did not affect  $\text{Cl}^-$  influx promoted by respiration of rat liver mitochondria. However, 100  $\mu\text{M}$  inhibited the  $\text{K}^+/\text{H}^+$  exchange, like DNP. The effects of DNP are related with its ability to dissipate the  $\Delta\text{pH}$  component of the electrochemical proton gradient, as a consequence of its protonophoric action. Hence, glibenclamide, at concentrations up to 100  $\mu\text{M}$ , has no significant protonophoric action on respiring mitochondria.

The effects of glibenclamide on mitochondrial  $\Delta\Psi$  and respiration, developed by mitochondria upon succinate oxidation, are shown in Figure 6. After succinate addition, mitochondria develop a  $\Delta\Psi$  of about



**FIGURE 5.** Comparative effects of glibenclamide (A) and dinitrophenol (DNP) (B) on permeabilization to  $\text{Cl}^-$  by inner membrane of rat liver mitochondria. Swelling assays were performed in KCl medium. Mitochondria (1 mg) suspended in 2.5 mL of KCl medium [54 mM KCl, 5 mM HEPES (pH 7.1), 0.1 mM EGTA, and 0.2 mM EDTA] supplemented with 2  $\mu\text{M}$  rotenone were incubated for 1 min at 30°C, and energized by addition of 2 mM succinate. Glibenclamide (10, 25, 50, and 100  $\mu\text{M}$ ) or DNP (5, 10 and 20  $\mu\text{M}$ ) was added 30 s after energization, followed by valinomycin 30 s later. Suc denotes succinate; Gli, glibenclamide; Val, valinomycin (1  $\mu\text{g}/\text{mL}$ ); DNP, dinitrophenol; and -Val, assays in the absence of valinomycin. Data represent typical recordings of experiments obtained from six different mitochondrial preparations.



**FIGURE 6.** Effects of glibenclamide on mitochondrial transmembrane potential ( $\Delta\psi$ ) (A) and state 4 respiration (B) of rat liver mitochondria. Assays were performed with mitochondria (1 mg) incubated, for 1 min at 30°C, in 1 mL of medium containing 250 mM sucrose, 10 mM HEPES (pH 7.2), 20 mM KCl, 5 mM potassium phosphate, and 2 mM  $\text{MgCl}_2$ , supplemented with 2  $\mu\text{M}$  rotenone and 3  $\mu\text{M}$   $\text{TPP}^+$ , and energized with 10 mM succinate (Suc). State 4 respiration was reached after phosphorylation of 50 nmol adenosine diphosphate (ADP) (not represented in the records). Glibenclamide (Gli) 100  $\mu\text{M}$  was added to mitochondria respiring in state 4. Values indicated are in  $\text{nmol O}_2 (\text{mg protein})^{-1} \text{min}^{-1}$ . The traces are typical recordings of experiments obtained from six different mitochondrial preparations.

-224 mV (negative inside). The respiratory rate of state 4 was 15  $\text{nmol O}_2 (\text{mg protein})^{-1} \text{min}^{-1}$ . Addition of glibenclamide (100  $\mu\text{M}$ ) depressed  $\Delta\psi$  to -213 mV, increasing state 4 respiration rate to 28  $\text{nmol O}_2 (\text{mg protein})^{-1} \text{min}^{-1}$ , thus indicating that glibenclamide interferes with mitochondrial bioenergetics. These effects of glibenclamide are compatible with permeabilization of the inner mitochondrial membrane to cations such as  $\text{H}^+$  or  $\text{K}^+$ , but the results presented in this study favor permeabilization to  $\text{K}^+$  (Figures 2 and 3), rather than to  $\text{H}^+$  (Figures 1 and 5).

## DISCUSSION

The results presented in this study clearly showed that glibenclamide has no significant effect on inner mitochondrial membrane permeabilization to  $\text{H}^+$  (Figure 1), permeabilizing it to  $\text{Cl}^-$ , in a concentration-dependent manner (Figure 3). Permeabilization to  $\text{K}^+$  was dependent on  $\text{Cl}^-$  influx induced by glibenclamide (Figures 2 and 3), thus promoting a net  $\text{Cl}^-/\text{K}^+$  co-transport (Figure 3),  $\Delta\psi$  dissipation, and stimulation of state 4 respiration rate in rat liver mitochondria (Figure 6).

Glibenclamide-induced membrane permeabilization to  $\text{Cl}^-$  was inhibited by specific inhibitors of IMAC, namely propranolol and cerebrocrast [15,24] (Figure 4B). It was also inhibited in the presence of nigericin, an exogenous  $\text{K}^+/\text{H}^+$  antiporter, which favors the  $\text{Cl}^-/\text{OH}^-$  exchange by tributyltin (Figure 4A). The data suggested that  $\text{Cl}^-$  influx induced by glibenclamide in rat liver mitochondria is mainly via IMAC rather than  $\text{Cl}^-/\text{OH}^-$  exchange. Permeabilization of anions by IMAC only occurs under low  $\text{Mg}^{2+}$  concentrations and/or alkaline matrix conditions [15]. The possibility of glibenclamide to induce IMAC opening by depletion of matrix  $\text{Mg}^{2+}$  by exchange with  $\text{H}^+$ , like A23187 [15], is excluded, because glibenclamide, in contrast with A23187, did not increase the transmembrane potential ( $\Delta\psi$ ) of succinate-respiring mitochondria in a free- $\text{Mg}^{2+}$  reaction medium (results not shown). IMAC opening by glibenclamide may be related with its ability to induce mitochondrial matrix alkalization [12]. This is the first study presenting evidence that glibenclamide permeabilizes the inner mitochondrial membrane to  $\text{Cl}^-$  by opening IMAC. However, activation of  $\text{Cl}^-$  channels by glibenclamide in the membrane of insulin secretory granules of pancreatic  $\beta$  cells has been reported [24–28].

Comparison of the effects of glibenclamide on the swelling of nonrespiring mitochondria in KCl and  $\text{KNO}_3$  media showed that glibenclamide stimulates swelling in KCl to a greater extent than in  $\text{KNO}_3$  medium (Figures 2 and 3), indicating that membrane

permeabilization to  $K^+$  by glibenclamide is dependent on the presence of  $Cl^-$ , thus promoting a net  $Cl^-/K^+$  cotransport inside rat liver mitochondria.

Valinomycin-induced swelling of succinate-respiring mitochondria in KCl shows that, in contrast with DNP, glibenclamide did not affect the initial rate of swelling, but increased its amplitude (Figure 5), thus confirming that glibenclamide has no significant protonophoric action (Figure 1). Therefore, the  $\Delta\Psi$  dissipation and stimulation of state 4 respiration rate induced by glibenclamide (Figure 6) [13,14] may be related with its ability to permeabilize the inner mitochondrial membrane to  $K^+$  and to promote a net  $Cl^-/K^+$  cotransport inside mitochondria rather than with a protonophoric action.

In summary, glibenclamide interferes with mitochondrial bioenergetics of rat liver by permeabilizing the inner mitochondrial membrane to  $Cl^-$  and promoting a net  $Cl^-/K^+$  cotransport inside mitochondria, without significant changes on membrane permeabilization to  $H^+$ .

## REFERENCES

- Aguilar-Bryan L, Nichols CG, Wechsler SW, Clement JP IV, Body AE III, González G, Herrera-Sosa H, Nguy K, Bryan J, Nelson DA. Cloning of the  $\beta$  cell high-affinity sulfonylurea receptor: A regulator of insulin secretion. *Science* 1995;268:423–426.
- Szewczyk A. Intracellular targets for antidiabetic sulfonylureas and potassium channel openers. *Biochem Pharmacol* 1997;54:961–965.
- Inagaki N, Gono T, Clement JP IV, Namba N, Inazawa J, Gonzales G, Aguilar-Bryan L, Seino S, Bryan J. Reconstitution of IKATP: An inward rectifier subunit plus the sulfonylurea receptor. *Science* 1995;270:1166–1170.
- Inoue I, Nagase H, Kishi K, Higuti T. ATP-sensitive  $K^+$  channel in the mitochondrial inner membrane. *Nature* 1991;352:244–247.
- Paucek P, Mironova G, Mahdi F, Beavis AD, Woldegiorgis G, Garlid KD. Reconstitution and partial purification of the glibenclamide-sensitive, ATP-dependent  $K^+$  channel from rat liver and beef heart mitochondria. *J Biol Chem* 1992;267:26062–26069.
- Szewczyk A, Piluka S, Nalecz MJ. Effects of inhibitors and activators of ATP-regulated  $K^+$  channel on mitochondrial potassium uniport. *Biochem Mol Biol Internat* 1996;38:477–484.
- Szewczyk A, Wójcik G, Lobanov NA, Nalecz MJ. The mitochondrial sulfonylurea receptor: Identification and characterization. *Biochem Biophys Res Commun* 1997;230:611–615.
- Szewczyk A, Wójcik G, Lobanov NA, Nalecz MJ. Modification of the mitochondrial sulfonylurea receptor by thiol reagents. *Biochem Biophys Res Commun* 1999;262:255–258.
- Belyaeva EA, Szewczyk A, Mikolajek B, Nalecz MJ, Wojtczak L. Demonstration of glibenclamide-sensitive  $K^+$  fluxes in rat liver mitochondria. *Biochem Mol Biol Int* 1993;31:493–500.
- Jaburek M, Yarov-Yarovoy V, Paucek P, Garlid KD. State-dependent inhibition of the mitochondrial KATP channel by glyburide and 5-hydroxydecanoate. *J Biol Chem* 1998;272:13578–13582.
- Szewczyk A, Pikula S, Wojcik G, Nalecz MJ. Glibenclamide inhibits mitochondrial  $K^+$  and  $Na^+$  uniports induced by magnesium depletion. *Int J Biochem Cell Biol* 1996;28:863–871.
- Balakirev MY, Zimmer G. Gradual changes in permeability of inner mitochondrial membrane precede the mitochondrial permeability transition. *Arch Biochem Biophys* 1998;356:46–54.
- Szewczyk A, Czyz A, Nalecz MJ. ATP-regulated potassium channel blocker, glibenclamide, uncouples mitochondria. *Pol J Pharmacol* 1997;49:49–52.
- White CW, Rashed HM, Patel TB. Sulfonylureas inhibit metabolic flux through rat liver pyruvate carboxylase reaction. *J Pharmacol Exp Ther* 1988;246:971–974.
- Beavis AD. Properties of the inner membrane anion channel in intact mitochondria. *J Bioenerg Biomembr* 1992;24:77–90.
- Garlid D, Paucek P. The mitochondrial potassium cycle. *IUBMB Life* 2001;52:153–158.
- Gazotti P, Malmström K, Crompton MA. Laboratory manual on transport and bioenergetics. In Carafoli E, Semenza G, editors. *Membrane Biochemistry*. New York: Springer Verlag; 1979. pp 62–69.
- Gornall G, Bardawill CJ, David MM. Determination of serum proteins by means of the biuret reaction. *J Biol Chem* 1949;177:751–766.
- Kamo N, Muratsugu M, Hongoh R, Kobatake N. Membrane potential of mitochondria measured with an electrode sensitive to tetraphenylphosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state. *J Membr Biol* 1979;49:105–121.
- Garlid KD, Beavis AD. Evidence for the existence of an inner membrane anion channel in mitochondria. *Biochem Biophys Acta* 1986;853:187–204.
- Beavis AD, Garlid KD. The mitochondrial inner membrane anion channel. Regulation by divalent cations and protons. *J Biol Chem* 1987;262:15085–15093.
- Selwyn MJ, Dawson AP, Stockdale M, Gains N. Chloride-hydroxide exchange across mitochondrial, erythrocyte and artificial lipid membranes mediated by trialkyl- and triphenyltin compounds. *Eur J Biochem* 1970;14:120–126.
- Watling AS, Selwyn MJ. Effects of diphenyleneiodonium and trialkyltin compounds on photophosphorylation and chloride-hydroxide exchange in isolated chloroplasts. *Biochem J* 1972;128:86p–87p.
- Fernandes MAS, Santos MS, Vicente JAF, Moreno AJM, Veleno A, Duburs G, Oliveira CR. Effects of 1,4-dihydropyridine derivatives (cerebrocrast, gammapyrone, glutapyrone, and diethone) on mitochondrial bioenergetics and oxidative stress: A comparative study. *Mitochondrion* 2003;3:47–59.
- Barg S, Renström E, Berggren P-O, Bertorello A, Bokvist K, Braun M, Eliasson L, Holmes WE, Köhler M, Rorsman P, Thévenod F. The stimulatory action of tolbutamide on  $Ca^{2+}$ -dependent exocytosis in pancreatic  $\beta$  cells is mediated by a 65-kDa mdrl-like

- P-plycoprotein. *Proc Natl Acad Sci USA* 1999;96:5539–5544.
26. Barg S, Huang P, Eliasson L, Nelson DJ, Obermüller S, Rorsman P, Thévenod F, Renström E. Priming of insulin granules for exocytosis by granular  $\text{Cl}^-$  uptake and acidification. *J Cell Sci* 2001;114:2145–2154.
  27. Renström E, Barg S, Thévenod F, Rorsman P. Sulfonylurea-mediated stimulation of insulin exocytosis via an ATP-sensitive  $\text{K}^+$  channel-independent action. *Diabetes* 51 2002;(Suppl 1):S33–S36.
  28. Barg S. Mechanisms of exocytosis in insulin-secreting-B-cells and glucagon-secreting A-Cells. *Pharmacol Toxicol* 2003;92:3–13.