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Inhibitory effect of glybenclamide on mitochondrial chloride channels from rat heart

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ABSTRACT

Glybenclamide is used as a pharmacological tool in studies of mitochondrial functions supposing its main role to block ATP-dependent potassium (K_{ATP}) channel. The aim of this study was to test whether glybenclamide might interact with the mitochondrial chloride channels. Mitochondrial membranes, isolated from rat heart muscle, were incorporated into lipid bilayer membrane and single chloride channel currents were measured in 250/50 mM KCl *cis*/*trans* solutions. The observed chloride channels ($N = 11$) with mean conductance 120 ± 14 pS were sensitive to glybenclamide, which decreased the open probability ($IC_{50} = 129$ μM) and affected the channel gating kinetics ($IC_{50} = 12$ μM) by perturbing its open state. It did not influence the channel conductance or reversal potential. These results indicate that glybenclamide interacts with chloride channels what should be taken into consideration, when glybenclamide is used as a specific inhibitor of K_{ATP} channels.

1. Introduction

The sulfonylurea derivative glybenclamide (GLYB) is used as oral hypoglycemic agent to treat non-insulin dependent diabetes mellitus [1,2]. The antidiabetic sulfonylureas in general bind to high affinity sulfonylurea receptors, which are the structural elements of ATP-sensitive potassium (K_{ATP}) channels, and so inhibit the channels [3,4]. The property of GLYB to inhibit K_{ATP} channel is widely used as a pharmacological tool in numerous studies including mitochondria [3].

Effect of GLYB on mitochondrial function is complex. It activates cyclosporine A-sensitive mitochondrial permeability transition, induces swelling of mitochondria, increases calcium efflux, inhibits K+ and Na+ uniports, decreases the mitochondrial membrane potential, inhibits respiration and interferes with mitochondrial bioenergetics or reduced intracellular ATP level [4–8]. The complexity of the GLYB effects indicates that more than one molecular mechanism is involved.

Since it was observed that GLYB inhibited cystic fibrosis transmembrane regulator (CFTR), swelling-activated, and Ca^{2+}-activated Cl^- channels in cardiac myocytes and plasma membrane of cultured cells [9–11] it was of interest to know whether GLYB interacts with mitochondrial chloride channels what might contribute to understand its complex mitochondrial effect.

We used the lipid bilayer technique to examine the effects of GLYB on the activity of chloride channels derived from rat heart mitochondria at single channel level. We found that GLYB inhibits these channels by open-channel block mechanism.

2. Material and methods

2.1. Chemicals

Lipids were from Avanti Polar Lipids (Alabaster, AL, USA). Protease inhibitors were from Roche Diagnostics GmbH (Mannheim, Germany). All other chemicals including GLYB were purchased from Sigma–Aldrich (Germany). GLYB was prepared as 100 mM stock solution in DMSO. The final concentration of DMSO in the bath solutions was <0.5%, which, by itself, did not affect chloride currents.

2.2. Isolation of mitochondrial membrane vesicles

Mitochondria from the hearts of male Wistar rats were isolated as described in details in our previous study [12]. All procedures were approved by the State VET and Nutritive Administration of Slovak Republic. In brief, the hearts were excised after thoracotomy. The ventricles were separated and homogenized. The tissue suspension was processed in several steps of differential centrifugation until the final membrane fraction was obtained. The membrane fraction was exposed to sonication at 35 kHz. The purity of this fraction was analyzed as described in [12]. The final membrane fraction consisted mostly of outer and inner mitochondrial

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membranes. The membrane fraction was aliquoted and stored at −80 °C until use. All procedures were performed at 4 °C, and isolation buffers contained a mixture of protease inhibitors (1 μM leupeptin, 1 μM pepstatin, 1 mM benzamidine, 1 μM aprotinin, and 0.2 mM Pefabloc SC).

2.3. Bilayer lipid membrane (BLM) measurement

The native vesicle was fused into BLM formed across an aperture (diameter approx. 0.1 mm) separating the *cis* and *trans* chambers as described in our previous study [12]. The composition of the solutions (in mM) was: *trans*: 50 KCl, 2 $MgCl_2$, 0.4 $CaCl_2$, 1 EGTA, 10/5 HEPES/Tris, 7.4 pH (intracellular side), and *cis*: 250 KCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 10/5 HEPES/Tris, 7.4 pH (matrix side). The single channel current was measured by the bilayer clamp amplifier (BC-525C, Warner Instrument, Hamden, CT, USA), filtered at 1 kHz cut off frequency and the data were analyzed by Clamfit10 software (Axon Instrument, USA). All voltages reported refer to the *trans* side; the *cis* side was grounded. Under our conditions, the positive current amplitude that increased at the application of positive voltages means a flux of chloride anions from the *cis* to the *trans* side. All procedures were carried out at room temperature (22 °C).

The channel conductance (G), the reversal potential (E_{rev}), calculated from the current–voltage relationship, and a relative ionic Cl^-/K^+ selectivity was evaluated as in our previous study [12]. The single channel open probability (P_{open}) was determined from 3 min recordings at control and each concentration of GLYB. The data are shown as either mean ± standard deviation (conductance, reversal potential, current amplitude) or median and interquartile range: lower quartile – Q1 and upper quartile – Q3 (P_{open}, mean open time τ_{open}, mean closed time τ_{closed}).

Chloride channels were readily distinguished from K^+ channels using *cis*/*trans* solutions 250/50 mM KCl. The theoretical E_{rev} for K^+, presuming 100% permeability to K^+ and zero permeability for other ions, is +40.7 mV. In our experimental conditions we controlled purity of Cl^- current by applying voltage of ±30 mV.

3. Results

3.1. Single channel properties of mitochondrial chloride channels

After fusion of a vesicle containing ion channel into BLM we observed the activity of chloride channels with the mean single channel chloride current amplitude at 0 mV equal 2.8 ± 0.4 pA ($N = 11$). All chloride channels were highly active under control conditions, with median P_{open} equal 0.5970 (0.4948–0.7826; Q1–Q3). The mean conductance of the chloride channels was $G = 120 \pm 14$ pS ($N = 6$) and the mean reversal potential was $E_{rev} = -22.4 \pm 2.2$ mV ($N = 6$), giving the selectivity Cl^-/K^+ ratio of 4.2. The chloride channels were under control conditions characterized by median of mean open time $\tau_{open} = 14.14$ ms (7.98–16.50; Q1–Q3) and of mean closed time $\tau_{closed} = 4.04$ ms (2.25–4.83; Q1–Q3). The voltage dependence of P_{open} was bell-shaped and consistent with [13].

3.2. Effect of glybenclamide on mitochondrial chloride channels behaviour

To quantify the effect of GLYB on the activity of the chloride channels, an average chloride current, P_{open}, single channel amplitude histogram, conductance, reversal potential and mean open and close times were measured and evaluated. GLYB was added to the *cis* compartment (matrix side in our experimental conditions). In all experiments ($N = 11$) GLYB had concentration

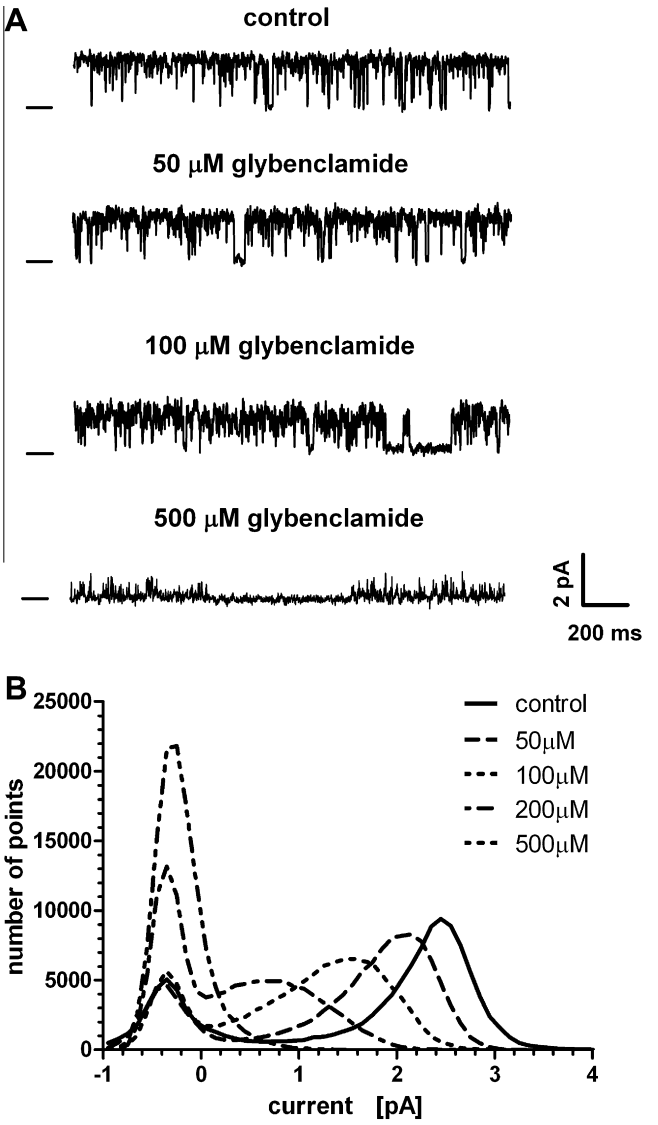

Fig. 1. (A) Effect of GLYB on single channel chloride current. These representative current traces show the decrease of current amplitude with increasing concentration of GLYB applied to the *cis* side at 0 mV. At 500 μM concentration, the openings were to short to be detected with the sampling rate of 4 kHz. The channels open upwards; black line on the left indicated the closed level. (B) Amplitude histogram showing the decrease of current amplitude at different GLYB concentrations. Both decrease of current amplitude and increase of open level noise (increase of peak width) as function of GLYB concentration is seen in the graph.

depended inhibitory effect on the chloride channels (Figs. 1 and 2). An apparent decrease of the single channel current amplitude was also observed (Fig. 1B). However, it is likely that the decreased current amplitude was the consequence of faster gating than 1 kHz cut off filter frequency, because we did not observe any clear open level after application of ⩾100 μM GLYB.

GLYB decreased P_{open} in a concentration dependent manner Fig. 2. The logarithm of GLYB concentration for half-maximal inhibition of activity is equal to 2.11 ± 0.04 (mean, SE; log (μM)), which gives the value of $IC_{50} = 129$ μM (95% confidence interval from 86 to 191 μM, $R^2 = 0.9961$).

To check whether GLYB affects the channel gating, we monitored the mean open time and mean closed time values. These are obtained by fitting the dwell time histograms with single exponential function. Fig. 3A shows the changes of mean open time τ_{open} as function of increasing GLYB concentration. The value of τ_{open} significantly decreased (Kruskal–Wallis test, $P = 0.0011$). Even

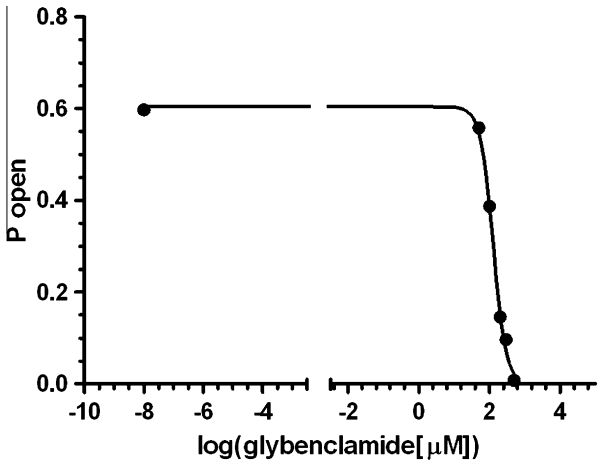

Fig. 2. Dose-response relationship of open probability on the concentration of GLYB. The fit of the data by sigmoidal function resulted in IC_{50} value 129 μM. The control data point was set to 10^{-8} μM GLYB in order to allow the fit in logarithmic scale.

at the first applied concentration (50 μM), the median of τ_{open} fell from 14.14 ms (7.98–16.50; Q1–Q3) to 2.97 ms (2.68–5.25; Q1–Q3). The mean closed time (τ_{closed}) increased slightly but insignificantly (Kruskal–Wallis test, $P = 0.3808$) with up to 300 μM GLYB from control value to 8.58 ms (4.04–8.99; Q1–Q3); at 500 μM concentration it attained median values above 200 ms, as the channels were mostly closed at this concentration (Fig. 3B). When the mean open times were fitted with dose–response curve, the IC_{50} value was approximately 10× smaller than for the P_{open}, $IC_{50} = 12.45$ μM (95% confidence interval from 5.86 to 26.44 μM). The application of glybenclamide did not affect the reversal potential of the chloride channels.

When applied to *trans* side, GLYB had similar effect on the channel as observed from the *cis* side ($N = 2$; data not shown). The inhibitory effect of GLYB was reversible ($N = 2$), after wash-out of GLYB from the *cis* compartment, P_{open} was restored along with kinetics and, consequently, the current amplitude (Fig. 4).

4. Discussion

The single channel properties of the observed mitochondrial chloride channels were different from reported voltage dependent anion channel (VDAC) of the mitochondrial outer membrane. The VDAC has conductance 4 nS in 1 M KCl, ionic selectivity 2:1 for Cl^- over K^+. The VDAC closes to subconductance states when either positive or negative potentials are applied [14]. On contrary, we measured chloride channels with lower conductance, higher selectivity for Cl^- over K^+ and channels did not close to subconductance states when either positive or negative voltage was applied. Our observed chloride channels share some characteristics with the centum pS channels like the response to ATP or conductance [12,15].

It is now recognized that activation of mitochondrial KATP channels in cardiac myocytes is an important and potent cardioprotective mechanism [16–18]. However, recently it was proposed that chloride channels also contribute to ischemic preconditioning in the myocardium [19,20]. The effect of ischemic preconditioning was abolished in CFTR knock-out mice [20]. It has been proposed that anion efflux through mitochondrial inner membrane anion channel (IMAC) may be a safeguard against excessive matrix swelling. Mitochondrial anion efflux has also recently been implicated in regulation of mitochondrial membrane potential. Inhibition of chloride flux by 4′-chlorodiazepam is beneficial for the recovery from ischemia/reperfusion process and it prevents from the collapse of mitochondrial membrane potential during oxidative stress [21,22].

GLYB is a sulfonylurea drug that is widely used to treatment of diabetes mellitus of type 2 [23]. At nanomolar concentrations it binds to the sulfonylurea receptor in the plasma membrane of pancreatic β-cells to cause the inhibition of KATP channels and promote insulin secretion [24]. Higher (μM) concentrations of GLYB inhibit KATP channels in other tissues [25], including skeletal muscle [26] or rat ventricular myocytes [27]. GLYB blocks pancreatic β-cells KATP and cardiac KATP channels with high affinity; but only for cardiac KATP channels was this block reversible [28]. The sulfonylureas can bind to various intracellular organelles including mitochondria [3]. Its effect on mitochondrial KATP channel have been observed both in patched mitochondria [29] and in proteoliposomes with partly purified mitochonodrial KATP channel reconstituted into bilayer lipid membrane [29,30]. This channel is blocked by GLYB [30,31] with a IC_{50} value below 100 nM [31].

Other membrane currents are affected as well. Some studies have provided evidence that sulfonylureas, in addition to blocking KATP channels, also inhibit chloride and calcium channels. GLYB has been shown to almost inhibit the current generated by Na^+–K^+ pumps in a concentration-dependent manner (with $IC_{50} \approx 100$ μM) and to decrease the current through L-type

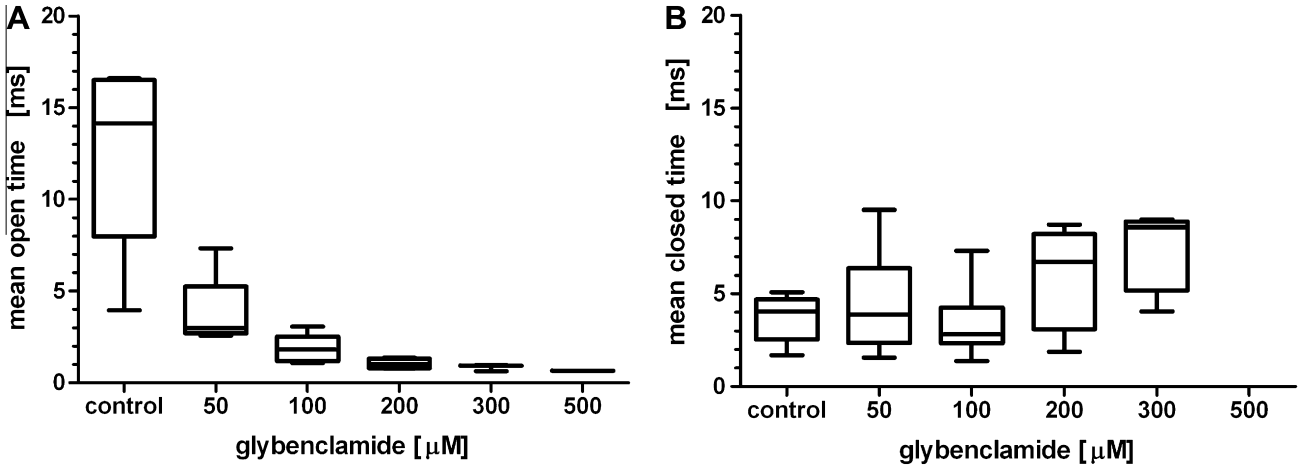

Fig. 3. (A) Changes of open dwell time in response to GLYB. Mean open dwell time significantly decreased with GLYB concentration increasing in *cis* compartment. Similar results were observed also from *trans* side. The mean dwell time markedly dropped already at 50 μM GLYB. (B) Effect of GLYB on mean closed time. There was no significant change of mean closed dwell times up to 300 μM concentration of GLYB. At 500 μM GLYB, the channels were mostly closed and the closed time was above 200 ms, this point is out of the shown range.

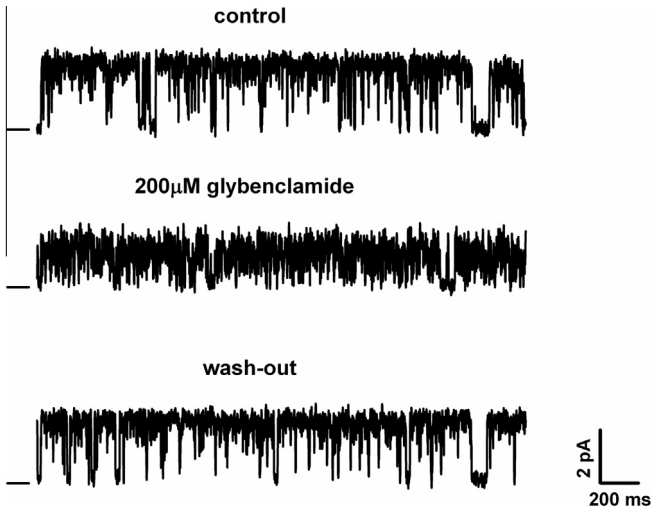

Fig. 4. Reversibility of the effect of GLYB. The current traces show the restoration of both current amplitude and slower kinetics after wash-out of GLYB from the *cis* compartment ($N = 2$). This effect indicates the mechanism of open-channel block. The channel opens upwards; black line marks the closed level.

calcium channels in cardiac myocytes [32]. GLYB markedly inhibited cystic fibrosis transmembrane regulator chloride currents in a voltage-independent manner, with IC_{50} value of 12.5 μM at +50 mV. The outwardly rectifying swelling-activated Cl^- current in atrial cells was less sensitive to GLYB and the block was voltage-dependent [9]. Macroscopic chloride currents that are activated by an increase of intracellular Ca^{2+} were also markedly inhibited by GLYB in a voltage-independent manner, with IC_{50} - ≈ 62 μM at +50 mV [9].

In our experiments, GLYB inhibited the activity of single chloride channels from vesicles of mitochondrial membranes isolated from rat heart muscle. Half-maximal inhibition of activity (IC_{50}) with the data fitted by a sigmoidal function was achieved at GLYB concentration of 129 μM. This value is of the same order as is the IC_{50} for outwardly rectifying swelling-activated Cl^- current in atrial cells (193 μM at +50 mV) and twice the IC_{50} for Ca^{2+}-dependent Cl^- current [9]. GLYB affected the channels already at concentration range where the activity was not yet decreased at this concentration, in Fig. 3 can be seen the effect on mean open time τopen. The IC_{50} value for the shortening of τopen is in range of tens of μM GLYB. With only slow changes of mean closed time, this indicates that GLYB might act like an open-channel blocker. This mechanism of action is consistent with the observed decrease of current amplitude. The increase of mean closed time at 500 μM concentration of GLYB was also due to the low amplitude of the very short events, thus, many events did not achieve the 50% threshold for the detection of opening. Inhibition by GLYB was reversible, after wash out of GLYB away from solution the activity of chloride channel was fully restored and the open level could be discerned properly again. Open-channel block by GLYB was also observed on both whole-cell current through volume-sensitive outwardly rectifying chloride channels [33] and at single channel level [10,11]. Our findings could be in line with the work of Ovide-Bordeaux et al., who observed the lack of effect of either GLYB or KATP channel opener – diazoxide in absence of exogenous ATP. In presence of exogenous ATP, 100 μM GLYB inhibited ADP-stimulated respiration, an effect which was not connected to the activity of mitochondrial KATP channel because it was not reversed by diazoxide [34]. As they discussed, GLYB might have other targets. One of them seems to be also the mitochondrial chloride channel, whose inhibition was shown to have cardioprotective effect, similarly as the activation of mitochondrial KATP channel [35].

We show on single channel level that sulfonylurea GLYB, blocker of KATP channel, can also reversibly inhibit mitochondrial chloride channels by mechanism of open-channel block. This study indicates that, when using GLYB in order to target mitochondrial KATP channels and study the effect of GLYB on mitochondria, one should also consider its interaction with mitochondrial chloride channels in range from tens of μM concentration.

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