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Research Article

MgATPase Activity May be Dispensable for the Opening of Mitochondrial Katp Channel by Diazoxide

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Abstract

It is generally accepted that activation of mKATP channels by diazoxide and others KATP channels openers (KCOs), similarly to sKATP channels, require MgATPase activity of the receptor SUR subunit, and the presence of MgATP as the prerequisite for the channel activation. However, literary data obtained on isolated mitochondria were controversial in this regard and numerous studies showed activation of mitochondrial K+ uptake by KCOs in the absence of MgATP. Recently we have shown high sensitivity of ATP-sensitive K+ transport of rat liver mitochondria to DZ in the absence of MgATP. Considering that KATP channels of different cell types may exhibit different sensitivity to DZ, the purpose of this work was to examine possible tissue specificity of this phenomenon and compare the effect of DZ on ATP-sensitive K+ transport in brain and liver mitochondria in the absence and the presence of MgATP. On isolated mitochondria, we have found that full activation of K+ transport of both liver and brain mitochondria occurred at < 0.5 μM of DZ. MgATP shifted the sensitivity of K+ transport to DZ to micromolar concentration scale. Without MgATP, activation of ATP-sensitive K+ transport was verified by the blockage with MgATP, glibenclamide, and 5-hydroxydecanoate, which allowed for its attribution to mKATP channels activity. Based on the experiments, we hypothesized that MgATP may be dispensable for the activation of native mKATP channel by DZ, and that mKATP channels might comprise high affinity sites for DZ binding in the absence of Mg·ATP. Obtained results reveal novel aspects of mKATP channels activation by DZ.

Keywords: *KATP Channels; Mitochondria; Brain; Liver; K+ Transport; Diazoxide*

Introduction

KATP channels of plasma membrane (sKATP channels) and mitochondria (mKATP channels) are ubiquitously present in the living organism, where they accomplish several cell-specific functions. In cardiovascular and central nervous systems cytoprotective effects of both sKATP and mKATP channels opening and their involvement in the well known phenomenon of preconditioning were widely described in the literature [1-5]. KATP channels are octameric complexes, which possess four K^+ conductant (Kir6.x in sarcolemmal KATP channels) and four receptor subunits (SUR). KATP channels opening by pharmacological openers (diazoxide, pinacidil and others) require the binding of the drugs to the receptor SUR subunit of KATP channels. Diazoxide (DZ), which is much more specific for mKATP than sKATP channel, is a promising drug for the treatment of pathophysiological states and conditions in cardiovascular, CNS and other systems of the organism [4-6,8].

However, in the endocrine system diversity of the effects of KATP channels opening, and particularly with DZ, was shown [10-14]. It is known that under diabetes, which is one of the most common and most grave endocrine disorders, sKATP channels opening contributes

to the impaired insulin secretion in β-cells and overactivity of sKATP channels due to Kir6.2 polymorphism predisposes to the risk of type 2 diabetes [12,13]. Also, overactivity of sKATP channels was shown to be associated with impaired insulin release and enhanced insulin sensitivity in adults with normal glucose tolerance [11].

On the contrary, the opening of mKATP channels by DZ in β-cells was reported to produce bioenergetic effects usually considered as cytoprotective, and similar to those observed in other cell types [14]. So, in the endocrine system, DZ still may be useful, based on different sensitivity of sKATP and mKATP channels to this drug. Extensive studies still are required to assess cell-specific functions of mKATP channels and the mechanisms of their pharmacological regulation. However, till recently this task was largely prevented by the unknown molecular composition of mKATP channels. Also, still unknown is molecular mechanism of mKATP channels opening by DZ and others mKATP channels openers. Till the discovery of molecular composition of mKATP channels [15], the knowledge about their functions was obtained only by indirect methods.

Thus, for about 30 years since their discovery these channels could be assessed mainly on a functional basis. In isolated mitochondria only the studies of ATP-sensitive K^+ transport, its biochemical properties and bioenergetic effects were accessible. The role of mKATP channels in the regulation of mitochondrial functions was evaluated based on a measure of bioenergetic effects produced by mKATP channels openers (diazoxide, pinacidil, nicorandil) and blockers (glibenclamide, 5-hydroxydecanoate), on the rate of respiration, membrane potential, Ca^{2+} transport, ATP synthesis and ROS production [16-20]. A correlation could be observed between the abundance of mKATP channels in a certain cell types (e.g. neurons) and depolarizing effect of mKATP channels opener diazoxide, as it was shown in brain mitochondria [19].

Disclosure of the molecular composition of mKATP channels in 2019 [15] started new wave in mKATP channels research and opened new prospective in the assessment of their properties and physiological functions on molecular level. However, at present still much controversy remains about the properties of mKATP channel in isolated mitochondria. Rather striking was that the properties of mKATP channel known from the literature largely depended on the preparations used to study mKATP channels (isolated mitochondria, mitoplasts, proteoliposomes, and isolated K^+ conductant subunit [21,22]). Especially, this is related to the sensitivity of mKATP to DZ and the requirement of MgATP for the activation of ATP-sensitive K^+ transport by DZ and other KCOs.

From the studies on sarcolemmal KATP channels, it is known that SUR subunit possesses intrinsic MgATPase activity [23,24]. So, in numerous studies on isolated mitochondria, it was generally assumed that the presence of Mg^{2+} and ATP was required for mKATP channel opening by KCOs [21,22,25]. However, literary data obtained on isolated mitochondria in this regard were controversial, and multiple evidences in different tissues were obtained about the activation of ATP-sensitive K^+ transport in the absence of MgATP [26-29].

Recently we have shown that native liver mKATP channel was activated by diazoxide on sub-micromolar scale in the absence of MgATP, which implies that MgATPase activity was not a prerequisite for mKATP channel activation by diazoxide [30].

Aim of the Study

The aim of this work was to examine possible tissue-specificity of this effect and to compare the effects of DZ on the ATP-sensitive K^+ transport in isolated brain and liver mitochondria in the absence and the presence of MgATP.

Materials and Methods

Mitochondrial preparations: The work has been carried out in accordance with "Guide for the Care and Use of Laboratory Animals" 8th ed. Washington, DC: National Research Council of the National Academies: The National Academic Press, 2011 approved by the Ethics Commission on Animal Experiments of A.A. Bogomoletz Institute of Physiology, NAS of Ukraine. Adult Wistar-Kyoto female rats with 180 -

200g mean body weight were used. Brain and liver were washed by cold 0.9% KCl solution (4˚C), minced and homogenized in 1:5 volume of the isolation medium: 250 mM sucrose, 1 mM EDTA, 1 mg/ml BSA, 20 mM Tris-HCl buffer, 4˚C (pH 7.2). Mitochondria were isolated by centrifugation for 7 min at 1000 x *g* (4˚C) and after the pellets have been discarded; supernatants were centrifuged again for 15 min at 12000 x *g* (4˚C). Final pellets were resuspended in a small volume of isolation medium without EDTA and stored on ice. The protein content was determined by the Lowry method.

Oxygen consumption assay: Oxygen consumption was studied polarographically in 1 cm^3 closed termostated cell at 26˚C with platinum electrode at constant stirring in standard incubation medium: 120 mM KCl, 0.5 mM EDTA, 5 mM sodium glutamate, 1 mM KH_2PO_4, 5 mM Tris-HCl buffer (pH 7.4), oligomycin (1 µg/mg protein). Dependent on the conditions, $MgCl_2$ (1 mM), ATP (0.3 mM), glibenclamide (5 µM) and 5-HD (100 µM), were added. Diazoxide was added to standard incubation medium at concentrations required. In the presence of Mg^{2+} EDTA was replaced by EGTA. Mitochondria were added at 1.5 - 2.0 mg/ml protein.

Potassium transport assay: The effects of mKATP channels openers on potassium transport were assessed based on light scattering of mitochondrial suspensions. Light scattering is known to decrease because of matrix swelling due to obligatory water uptake in the course of potassium transport [25]. Initial rates of potassium transport (V_0) were found by monitoring light scattering at 520 nm excitation/emission wavelengths in 1 cm^3 cell starting from the addition of mitochondria at 0.3 mg/ml in standard incubation medium.

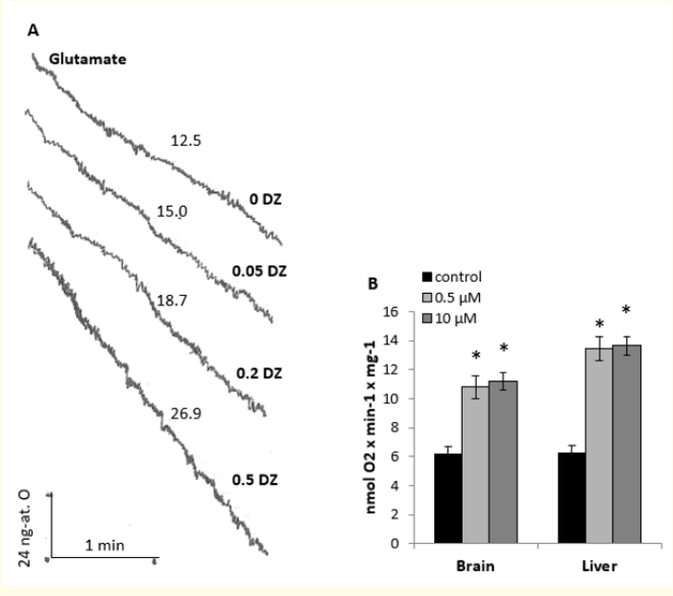
Chemicals: All reagents were from Sigma-Aldrich, USA. Deionized water was used for medium preparations.

Statistical analysis: The data were expressed as mean ± S.E. of 4 - 6 independent experiments. Statistical analysis was performed using paired Student's t-test; $P < 0.05$ was taken as the level of significance.

Results

The effect of diazoxide on mitochondrial respiration and matrix volume: The effects of DZ on K^+ transport were assessed indirectly based on the oxygen consumption and light scattering of mitochondrial suspensions. As it is known, oxygen consumption is coupled to K^+ transport by stoichiometric proportions, and under proper conditions increase in the rate of respiration is proportional to the rate of K^+ uptake. For NADH-dependent substrates K^+: O ratio constitutes 10:1 [31]. Also, because of obligatory water uptake, the rate of matrix swelling as well reflects the rate of K^+ uptake [32].

As showed representative polarographic records and swelling assays, DZ increased the rate of oxygen consumption and the amplitude of matrix swelling, which reflected increase in K^+ uptake. This occurred in a concentration-dependent way with maximum at < 0.5 µM (Figure 1A and 1B). The rate of oxygen consumption, as well as swelling of brain mitochondria did not increase with the increase of DZ concentration above 0.5 µM (Figure 1B). As for liver mitochondria, recently we have shown that DZ was capable of non-specific activation of K^+ transport, which was abolished in the presence of Mg^{2+} [30]. So, to remove this non-specific effect and assess the activation effect of DZ on ATP-sensitive potassium transport, we studied the dependence of mitochondrial swelling on diazoxide concentration in the presence of 1 mM Mg^{2+} (Figure 2).



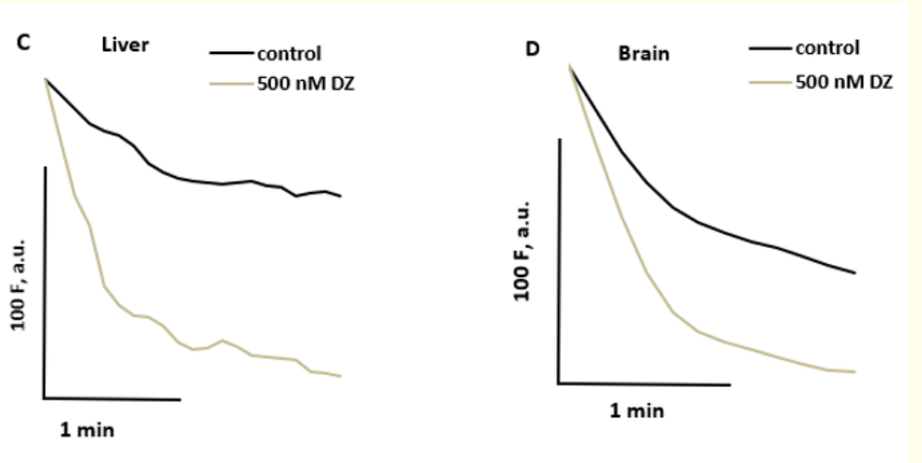


Figure 1: *The effect of diazoxide on mitochondrial respiration and matrix volume in the absence of MgATP: A: Scanned oxygen consumption records of rat liver mitochondria in the presence of different concentrations of DZ; B: The effect of DZ on mitochondrial respiration; C-D: The effect of DZ on the light scattering of mitochondrial suspensions in standard incubation medium. M ± m, n = 4; * - P < 0.05.*

The sensitivity of K^+ transport to DZ: As showed normalized concentration dependences of the initial rates of matrix swelling (V_0), in the absence of MgATP half activation of K^+ transport of both liver and brain mitochondria occurred on sub-micromolar scale. EC_{50} for DZ activation of K^+ transport constituted ~150 nM and ~180 nM in liver and brain mitochondria (Figure 2A). No activation was observed in the presence of mKATP channels blocker glibenclamide (Figure 2A and 2B). Also, we did not observe any activation in the presence of another blocker of mKATP channel, 5-HD (not shown). As for liver mitochondria, EC_{50} obtained in the presence of Mg^{2+} was close to the value of ~140 nM obtained earlier in the absence of Mg^{2+} and ATP [30]. So, Mg^{2+} at concentration 1 mM had no effect on the activation of K^+ transport in native mitochondria by DZ.

In the presence of MgATP, conventional shift of EC_{50} towards much higher concentrations was observed, with full activation around 1 - 3 μM for liver and brain mitochondria respectively (Figure 2B). In native mitochondria the activation of K^+ transport by nanomolar concentrations of DZ was not observed in the presence of either MgATP (Fig. 2A), or glibenclamide (Figure 2A and 2B), which indicated identical sensitivity of the activation effect to the blockers of mKATP channel. In the presence of MgATP, glibenclamide blocked K^+ transport activated by 30 μm DZ (Figure 2B), which agreed with the literature [25]. The same we obtained using 5-HD (not shown).

As we have shown earlier in liver mitochondria, Mg^{2+} completely blocked ATP-insensitive component of K^+ transport activated by DZ, so that in the presence of Mg^{2+} activation effect was related solely to ATP-sensitive K^+ transport and respectively was blocked by ATP. Accordingly, in the presence of ATP no activation of K^+ transport by sub-micromolar concentrations of DZ was observed under the conditions of experiments (Figure 2B). The data obtained in brain mitochondria were close to the results obtained on liver mitochondria.

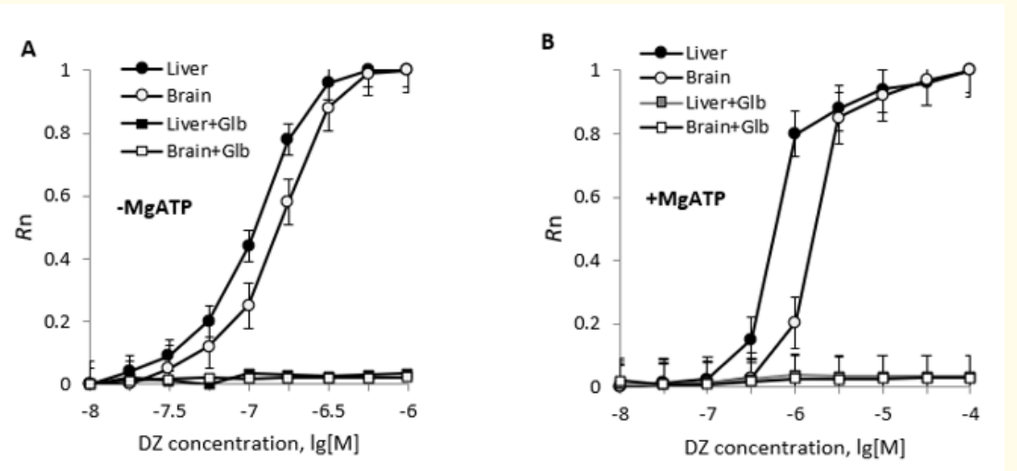
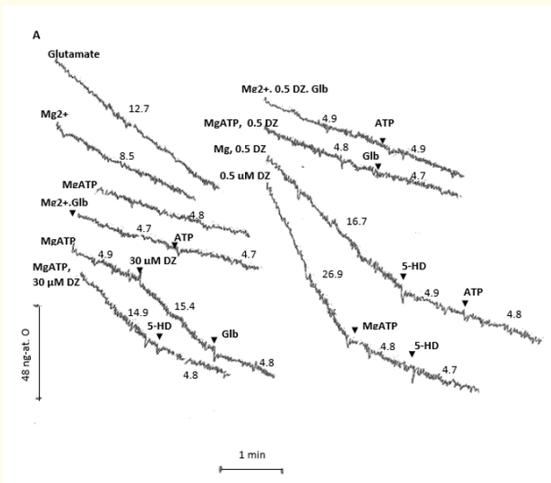


Figure 2: *The effects of DZ on the normalized rates of matrix swelling of liver (black squares and circles) and brain mitochondria (grey squares and circles) in the absence (A) and the presence (B) of MgATP. In the lines with squares glibenclamide was added to the incubation medium at 10 μM. M ± m, n = 4.*

So, without MgATP, we obtained strong evidence of high sensitivity of ATP-sensitive K^+ transport to diazoxide with full activation at < 0.5 μM of the drug. MgATP shifted it to micromolar concentration level, which agreed with literary data. By our earlier estimations [33], in native brain mitochondria V_0 of ATP-sensitive K^+ transport constituted ~40 nmol $K^+ \cdot min^{-1} \cdot mg^{-1}$. In native liver mitochondria V_0 of ATP-sensitive K^+ transport constituted ~40 nmol $K^+ \cdot min^{-1} \cdot mg^{-1}$, and ~65 nmol $K^+ \cdot min^{-1} \cdot mg^{-1}$ upon activation by DZ [30]. As we have found in this work, in brain mitochondria as well, DZ increased this value by ~1.5-2 times, so that V_0 of ATP-sensitive K+ transport activated by DZ by our estimate could reach up to ~80 nmol $K^+ \cdot min^{-1} \cdot mg^{-1}$.

Pharmacological identification of ATP-sensitive K^+ transport: One crucial point of our study was attribution of ATP-sensitive K^+ transport studied in our work to mKATP channels activity. Generally, identification of mKATP channel, especially after its novel discovery [15], requires genetic approaches. However, in this study we made an attempt to identify ATP-sensitive K^+ transport on pharmacological level using DZ and different blockers of KATP channels. To ascertain the activation of mKATP channel, we assumed that if certain unidentified K^+ transport sensitive to MgATP and mKATP channels blockers, but not related to mKATP channel activity, was activated by DZ in the absence of MgATP, then the effect of sequential blocking of total K^+ transport by glibenclamide and MgATP should exceed the effect of glibenclamide alone, in case if glibenclamide was not capable of blocking mKATP channel without MgATP [21,22,25]. So, in this work we conducted a set of experiments based on monitoring of oxygen consumption, which allowed sequential addition and the assessment of separate effects of the blockers of mKATP channel.

As showed original oxygen consumption records, in the presence of Mg^{2+} ATP-sensitive K^+ transport, either native, or activated by DZ, was evenly blocked by glibenclamide or ATP (Figure 3A). After the blocking by glibenclamide, no additional effect of MgATP was observed. Similar observations were made with other mKATP channel blocker, 5-HD (Figure 3A). And *vice versa*, after the blocking of mKATP channel with MgATP, no additional effects of either glibenclamide or 5-HD were observed. Part of respiration related to K^+ transport and blocked by glibenclamide equaled to K^+ transport blocked by MgATP and reactivated by 30 μM DZ, which was generally attributed to mKATP channel activity (Figure 3A and 3B). Similar results were obtained using 5-HD (Figure 3A and 3B). This strongly indicated the identity of ATP-sensitive K^+ transport studied in this work with mKATP channels activity and showed its sensitivity to the activation by DZ on nanomolar scale without MgATP and independent of MgATPase activity of the receptor subunit of mKATP channel. By the way, our data indicated the ability of glibenclamide and 5-HD to block ATP-sensitive K^+ transport as well without MgATP, which confirmed our earlier work on liver mitochondria where we have shown that glibenclamide blocked K^+ transport, either native or activated by DZ, in the absence of MgATP [34].



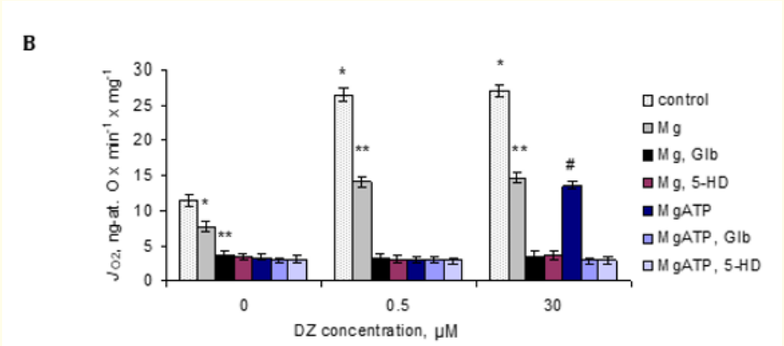


Figure 3: *The effect of DZ on the oxygen consumption of rat liver mitochondria: A: Scanned oxygen consumption records of rat liver mitochondria in the presence of different additions (additions and the rates of respiration in ng-at. O· min-1· mg-1 are shown on the figures); B: the effect of mKATP channels blockers (ATP, glibenclamide and 5-HD) on the rate of respiration of liver mitochondria at different concentrations of DZ; the results of sequential additions of the blockers are shown. M ± m, n = 4; * - P < 0.05 as compared to control (no DZ); ** - P<0.05 as compared to Mg2+; # - P<0.05 as compared to MgATP.*

Discussion and Conclusion

As it was shown in the literature, the properties of mKATP channel much differ, dependent on the preparation (mitoplasts, isolated mitochondria, lipid bilayers [21,22,35]). Unfortunately, there are no means now to assess the properties of mKATP channel in isolated mitochondria using biophysical methods, such as patch clamp. So, one of the most useful approach to study mKATP channel directly in mitochondria is based on the monitoring mitochondrial swelling using light scattering or absorbance technique. Monitoring of oxygen consumption in addition to light scattering assays is useful for quantification of K^+ transport in mitochondria. Also, an advantage of polarographic method is that it allows sequential addition of the modulators of mKATP channel activity, which is close to the conditions *in situ*. In this work, using oxygen consumption and light scattering assays, we established that high sensitivity to DZ is a common property of ATP-sensitive K^+ transport in isolated mitochondria. Also, by using combined blocking of ATP-sensitive K^+ transport by the blockers of mKATP channel we obtained strong evidence that ATP-sensitive K^+ transport studied in our work represented mKATP channel activity. While in sKATP channels MgATPase activity is required for the channel opening by DZ, our data on liver ([30] and this work) and brain mitochondria showed that MgATPase activity was dispensable for mKATP channels activation by this drug.

One critical issue of our work was molecular identification of mitochondrial ATP-sensitive K^+ transport by pharmacological means. From the studies on sarcolemmal [24] and mitochondrial KATP channels [21,25], it is known that native mKATP channel is blocked by MgATP and activated mKATP channel is blocked by KATP channels blockers (glibenclamide and 5- hydroxydecanoate) in the presence of MgATP. So, to prove identity of mKATP channel on pharmacological level, we used combined blocking of ATP sensitive K^+ transport by MgATP and specific blockers of KATP channels glibenclamide and 5-HD. As we observed with MgATP, DZ activated ATP-sensitive K^+ transport in native mitochondria. In the presence of MgATP, no additional blocking of both native and activated ATP-sensitive K^+ transport by either glibenclamide or 5-HD was observed. When ATP-sensitive K^+ transport was activated by DZ either in the absence or the presence of MgATP, the same (by absolute value) activation effect was observed, as well as the same blocking effect of glibenclamide and 5-HD in the presence of MgATP, which proved pharmacological identity of ATP-sensitive K^+ transport with mKATP channel activity. Thus, the properties of ATP-sensitive K^+ transport studied in this work could be ascribed to the same molecular entity known as 'mKATP channel'.

Based on our experiments we came to the following conclusions: 1) high sensitivity to DZ with full activation at < 0.5 μM independent of MgATPase activity is one common property of native mKATP channels exhibited in isolated mitochondria; 2) Mg^{2+} alone could not affect the mKATP channels affinity to DZ, but the presence of MgATP shifted it to much higher micromolar concentration level; 3) the results of our work allowed us assume that native mKATP channel might comprise the sites with high affinity to diazoxide, possibly screened by the binding of MgATP. Obtained results indicated novel common features in the mechanism of native mKATP channel activation by DZ.

Molecular identification of mKATP channel in 2019 answered numerous unresolved questions, but put still novel ones. One of the most intriguing issues is physiological relevance of KATP channels subunits found in mitochondria by earlier researches, such as Kir6.1 and 6.2, SUR1 and SUR2, and different ROMK isoforms. Based on the present knowledge, mitochondria can possess more than one type of ATP-sensitive K^+ conductance [15,24], not counting earlier work showing channel properties of a multiprotein complex containing succinate dehydrogenase [36]. So, it is tempting to hypothesize a multiplicity of ATP-sensitive K^+ channels in these organelles. Extensive studies are required now to assess their properties and physiological functions. We believe that the results of our work contribute to the understanding of basic mechanisms of the regulation of mKATP channels activity by DZ in isolated mitochondria.

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