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Direct and Off-Target Effects of ATP-Sensitive Potassium Channels Opener Diazoxide

Olga V Akopova*

Circulation Department, Bogomoletz Institute of Physiology, NAS of Ukraine, Kiev, Ukraine

***Corresponding author:** Dr. Olga V Akopova, Circulation Department, Bogomoletz Institute of Physiology, NAS of Ukraine, Bogomoletz Street 4, 01601, Kiev, Ukraine, Tel: +38 044 256 2496; E-mail: olga.akopova01@mail.ru

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Abstract

Diazoxide (DZ) is a well-known cardioprotective drug capable of mimicking ischemic preconditioning. Being primarily a pharmacological opener of mitochondrial ATP-sensitive potassium channels (mK_{ATP} channels), DZ is known to produce multiple side effects because of its interactions with different cellular targets (such as plasma membrane K_{ATP} channels, F_0F_1 ATP synthase, succinate dehydrogenase and others), capable of confounding an understanding of direct bioenergetic effects of mK_{ATP} channels opening in mitochondria. In this review direct and off-target effects of DZ were discussed. The emphasis was made on molecular basis of DZ interaction with K_{ATP} channels and different K_{ATP} channels isoforms sensitivity to this drug. The present knowledge on DZ interaction with mK_{ATP} channels is outlined as well as DZ interactions with other molecular targets affecting mitochondrial functions and bioenergetics. Conclusion was reached that high sensitivity of mK_{ATP} channel to DZ allows for avoiding off-target effects of this drug in studies on isolated mitochondria, which makes it a useful tool in an appraisal of diverse functional effects of mK_{ATP} channel opening.

Keywords: Diazoxide; K_{ATP} channels; Sulfonylurea receptors; Mitochondria; mK_{ATP} channels; Potassium cycle; Bioenergetics

Abbreviations:

DZ: Diazoxide; KCO: K^+ Channels Opener; Kir: Inward Rectifier Potassium Channel; NBD: Nucleotide Binding Domain; PKC: Protein Kinase C; SDH: Succinate Dehydrogenase; SUR: Sulfonylurea Receptor

Introduction

Diazoxide (DZ) is an effective cardioprotective drug capable of mimicking ischemic preconditioning via the opening of mitochondrial ATP-sensitive potassium channels (mK_{ATP} channels) [1-3]. K_{ATP} channels are ubiquitously expressed in plasma membrane (sK_{ATP} channels) and mitochondria (mK_{ATP} channels), and different isoforms of these channels differ in their sensitivity to DZ [4]. Being preferably mK_{ATP} channels opener, DZ is capable of interactions with sK_{ATP} channels isoforms sensitive to this drug within circulation, CNS, endocrine, and other systems of a living organism [2,5,6].

Based on the activation constants ($K_{1/2}$) [7], mK_{ATP} channels, most sensitive to this drug, are supposed to be one of the primary cellular targets of DZ. This implies that DZ administration should result in the modulation of mitochondrial functions, ensuing from mK_{ATP} channel opening and affect mitochondrial bioenergetics as it was shown in the heart, brain, liver, and pancreatic beta-cells [8-10]. But while the opening of mK_{ATP} channel is generally thought to be cytoprotective, appraisal of bioenergetic and functional effects of mK_{ATP} channel opening in mitochondria using DZ as pharmacological tool, is complicated by several side effects of this drug [2,11]. Bioenergetic effects of DZ (modulation of the oxygen consumption, mitochondrial uncoupling, ATP synthesis and ROS production) have been widely described in the literature. However main concern is that direct bioenergetic effects of DZ at mitochondria level do not much differ from indirect ones, thereby hindering an understanding of physiological significance of mK_{ATP} opening with respect to mitochondrial functions and metabolism.

DZ is a benzothiadiazine derivative, 7-chloro-3-methyl-2H-1,2,4-benzothiadiazine 1,1-dioxide ($C_8H_7ClN_2O_2S$), and ionizable sulfonyl group makes it extremely lipid soluble. Being a highly hydrophobic compound, DZ easily binds to and penetrates cellular membranes [12]. This ability implies several cellular targets of DZ, and thus far several side effects of DZ on mitochondrial functions have been reported, such as protonophoric properties at high micromolar concentrations [13,14]; inhibition of succinate dehydrogenase (SDH) and succinate-driven respiration [15-17]; inhibition of F_0F_1 ATP synthase [18,19]; flavoprotein oxidation and the interference with ROS detection using the fluorescent probe Dichlorofluorescein (DCF) [16,20]; at last, direct activation of protein kinases (C and B) and other signaling pathways [2,21,22].

Specific effect of DZ as mK_{ATP} channel opener implies the existence of high-affinity binding sites of this drug within molecular structure of the channel. However, the main complexity in studying of DZ interaction with K_{ATP} channels, especially, mK_{ATP} channels, arises not only from the multiplicity of their subunit isoform distribution in different cells, but from the absence of reliable data on the channel structure in mitochondria. For this reason, molecular basis of DZ action on mK_{ATP} channels is not sufficiently clear, and the data on the mechanisms of DZ interactions with its other molecular targets likewise are rather scarce. So, the aim of this work was a brief outline of the present knowledge on DZ interactions with its cellular targets needed for better understanding of the direct and off-target effects of DZ modulating mitochondrial functions and bioenergetics.

K_{ATP} Channels as Cellular Targets of Diazoxide

Subunit composition and cell-specific subunit distribution of K_{ATP} channels

K_{ATP} channels are octameric protein complexes composed of four pore-forming Kir (potassium inward rectifier) subunits and four SUR (sulfonyl urea receptor) subunits [23,24]. SURs are receptors for physiological ligands (Mg^{2+}, ATP, and ADP) and pharmacological modulators of channels activity, potassium channels openers (KCOs) and blockers. Both Kir (Kir6.1 and 6.2) and SUR (SUR1, SUR2A and SUR2B) subunits participate in metabolic regulation of channel activity [23-25]. While plasmalemmal K_{ATP} channels (sK_{ATP}) are sufficiently well studied, little is known on the subunit composition and tissue distribution of mitochondrial K_{ATP} channel (mK_{ATP}). So, the notions on the sK_{ATP} channel subunit composition and pharmacological regulation were generally extrapolated on mK_{ATP} channel. This helped in understanding the key mechanisms of mK_{ATP} channel regulation by physiological and pharmacological ligands, but multiple concerns still remain.

Similar to sK_{ATP} channels, mK_{ATP} channels are thought to be composed of a combination of Kir and SUR subunits. SUR1 and SUR2 (A and B isoforms) belong to the so-called ATP-binding cassette proteins (ABC proteins) containing two Nucleotide Binding Domains (NBD) forming an ATP-binding pocket in which one lysine and one aspartate residues are critical for ATP binding and hydrolysis [25]. SUR subunits of K_{ATP} channels lack the transport properties, but similar to other ABC proteins, possess intrinsic Mg ATPase activity that is important in regulating channel conductance and the activation by KCOs.

As it is known of sK_{ATP} channels, ATP binding to Kir closes the channel, whereas Mg·ADP binding (or Mg·ATP binding and hydrolysis) to SUR enhances the channel activity [24-27]. ATP binding pocket is formed of amino acid residues of both N- and C-terminus of Kir subunits [25]. Co-expression of Kir and SUR subunits enhances the potency of ATP block several fold [28].

From the studies on sK_{ATP} channels it is known that KCOs binding requires a conformational change induced by ATP hydrolysis in NBDs of SUR, and C-terminus of SUR affects KCOs affinity in NBD domains [26,29,30]. Requirement for ATP was shown for KCOs binding to SUR1, SUR2A and SUR2B [29]. Besides, the activation of SUR2A-containing channels required ADP presence, while the activation of channels formed by SUR1 and SUR2B did not [24,27,30]. Similar to other KCOs, DZ required intrinsic MgATPase activity to elicit full stimulatory response [29,31]. While the above notions were obtained for sK_{ATP} channels, it was helpful for understanding of the basic mechanisms of pharmacological regulation of mK_{ATP} channel. However, several unresolved questions, primarily arising from the unknown structure of mK_{ATP} channel, still exist.

Sensitivity of sK_{ATP} channels to DZ was shown to be cell-specific and based on their subunit isoform distribution. The studies of the last decade improved our knowledge on tissue distribution of mK_{ATP} channels too, but notions on mK_{ATP} channel are scarce. *So, SUR1 comprising channels were shown to be expressed in cardiac tissues [32,33],* pancreatic beta cells (Kir6.2/*SUR1 [4,10]), liver and liver mitochondria* (Kir6.1/SUR1 [34]). *SUR2A* showed relatively high expression in cardiac and skeletal muscle cells [24,32-34], whereas *SUR2B* was more broadly expressed in smooth muscle cells and brain [35,36]. In cardiac tissues SUR and Kir subunit distribution showed cell-specificity. Thus, in rodent heart, the predominant combination of mK_{ATP} channel subunits found in ventricle was Kir6.2 and SUR2A [24], whereas mK_{ATP} channel in atria mainly consisted of Kir6.2 and SUR1 [32-34]. K_{ATP} channel of brain mitochondria was shown to comprise both Kir6.1 and Kir6.2, together with ~55 kDa splice variant of SUR2A, "mitoSUR" [37-39]. However, despite that expression of Kir6.2 and SUR2A isoforms in cardiac mitochondria was firmly established [24], it was doubted whether these subunits might constitute functionally active mK_{ATP} channel based on the lack of evidence confirming the participation of Kir6.2 in the response of mK_{ATP} channel to DZ [40].

As an alternative to Kir6.x, Kir1.1 (primarily known as renal outer medullary potassium channel, ROMK) was recently proposed to be a pore-forming mK_{ATP} channel subunit [41]. This hypothesis was supported by the expression of different ROMK isoforms found in heart, liver and brain mitochondria [41]. At functional level, this was confirmed by the sensitivity of mK_{ATP} channel to ROMK blocker, honeybee venom tertiapin Q. Meanwhile, pharmacological properties of ROMK differ of those known of K_{ATP} channels, and consistent with literary data, in heart mitochondria none of the ROMK isoforms (Kir1.1, 3.1, or 3.4) [32] were responsible for DZ-evoked swelling and/or potassium uptake (routinely used to test mK_{ATP} channel activity). Besides, low abundance of ROMK in mitochondria reported in the literature [42] questions its ability to produce significant bioenergetic effects. Thus, molecular entity of Kir subunits of mK_{ATP} channels, even in the best studied heart mitochondria, largely remains unknown. The studies are still more complicated because of the absence of antibodies quite specific for Kir proteins [43] and possible expression of several splice variants of K_{ATP} channel with different biophysical properties [35-38].

Molecular background of KATP channels sensitivity to DZ

Different Kir/SUR isoforms of K_{ATP} channels differ in their sensitivity towards DZ. While SUR1 comprising channels (e.g. Kir6.1/SUR1 expressed in liver mitochondria [39] and Kir6.2/SUR1 expressed in beta cells) showed marked sensitivity to DZ, SUR2 comprising channels generally were unaffected by this drug. So, EC_{50} for DZ activation of Kir6.1/SUR1 constituted ~10 μM [4], and K_{ATP} currents of Kir6.2/SUR1 were activated by DZ at ~100-300 μM [4,27]. Meanwhile, DZ was ineffective in opening Kir6.1/SUR2A, Kir6.2/SUR2A and Kir6.2/SUR2B isoforms of K_{ATP} channel [4]. In brain, "mitoSUR" K_{ATP} of brain mitochondria, shown to represent a splice variant of SUR2A, was reported to be markedly insensitive to pharmacological K_{ATP} channels modulators, DZ, pinacidil, and glibenclamide, and was 70-fold less sensitive to block by ATP compared with the SUR2A K_{ATP} channel [38].

Based on the published data, the difference in the sensitivity between SUR1 and SUR2 isoforms to DZ was manifested in the requirement of Mg·ADP for their activation [27]. Thus, in the presence of Mg·ADP SUR2A comprising channels, otherwise insensitive to this drug, were fully activated by ~300 μM of DZ [27]. Unlike this, SUR1 and SUR2B comprising channels did not require Mg·ADP for DZ activation [24,27].

The sensitivity of K_{ATP} channels to DZ can be explained on the basis of molecular composition of both SUR and Kir subunits. Within SUR subunit, DZ affinity was shown to be greatly dependent on NBDs in C-terminus of SUR [24,27,30,31]. So, critical lysine and aspartate residues localized in NBDs of SUR1 and responsible for ATP binding [26] were shown to be responsible for DZ sensitivity too, and mutations in these

residues abolished SUR1 sensitivity to DZ [27]. According to the works [26,31], markedly different diazoxide sensitivity between SUR1 and SUR2A subunit isoforms was dependent on a single lysine residue in nucleotide binding domain of SUR1, which was essential for DZ ability to activate sK_{ATP} currents. Thus, a single amino acid substitution of 1369 serine to a lysine in the SUR1 subunit markedly decreased its sensitivity to DZ and caused both MgATPase activity and diazoxide pharmacological profiles to resemble those of channels containing the SUR2A subunit [31]. The difference in DZ sensitivity between "DZ-insensitive" SUR2A and SUR2B too was shown to depend on C-terminal tail of respective subunits [24].

While there was no experimental evidence on the direct interaction of Kir subunit with DZ, clinical studies allow for assumption that some of the critical amino acid residues within Kir subunit, responsible for ATP sensitivity of K_{ATP} channel could be responsible for DZ and sulfonylureas sensitivity as well. Thus, K_{ATP} channel sensitivity to DZ [6] was shown to be dependent on E23K (i.e., Glu23Lys) polymorphism in the Kir6.2 subunit of the K_{ATP} channel, which is a recognized risk factor for the development of type II diabetes associated with decreased channel sensitivity to ATP [44-46].

These data, primarily relevant to sK_{ATP} channels, imply tissue-specific diversity in sensitivities of both plasmalemmal and mitochondrial K_{ATP} channels to DZ and other KCOs, dependent on the K_{ATP} channel isoforms expression in different cell types. For this reason clinical application of DZ and sulfonylureas might produce diverse therapeutic effects, especially under diabetes treatment [6,10,47]. Thus the knowledge about tissue-specific mK_{ATP} channels subunits composition is important for the drug design aimed at selective modulation of mK_{ATP} channels activity. However, the studies are much complicated by the expression of several splice variants of K_{ATP} channel, and by the possibility of random assembly of Kir/SUR subunits forming functionally active K_{ATP} channels [38,39,48].

mKATP channels as the target of diazoxide

While the above findings mainly refer to sK_{ATP} channels, and help shed light on molecular background of the sensitivity of sK_{ATP} channels to DZ, molecular basis of DZ interaction with mK_{ATP} channel still remains elusive. Because of the absence of a reliable data on channel subunit composition and distribution in mitochondria of different tissues, a disclosure of molecular structure of the site(s) responsible for DZ binding and the activation of potassium flux to mitochondrial matrix was not yet achieved.

Although this issue still remains unanswered, the sensitivity of mK_{ATP} channels to pharmacological modulators was studied extensively. Already in early works of Garlid et al. high sensitivity of liver and cardiac mK_{ATP} channel to DZ (EC_{50} ~3 μM) as opposed to cardiac sK_{ATP} channel (EC_{50} ~900 μM), was found [7]. This discrepancy in DZ sensitivities was well explained later by the data of Liu et al., who showed different sensitivity of SUR1 and SUR2 isoforms to DZ and established EC_{50} for DZ activation of Kir6.1/SUR1 comprising channel expressed in HEK293 cells to be of the order of ~10 μM, which resembled the so-called "mK_{ATP} channel" [4]. Much later studies found the expression of Kir6.1/SUR1 channel in liver mitochondria [35], which was consistent with high sensitivity of liver mK_{ATP} channel to DZ observed experimentally [7]. Meanwhile, in ventricle myocyte mitochondria predominant expression of "DZ-insensitive" Kir6.2/SUR2A was established [32,40], although heart and liver mitochondria were shown to not much differ in their sensitivities to DZ [7]. Thus, existing knowledge on the expression of mK_{ATP} channels subunit isoforms in mitochondria is insufficient for the explanation of mK_{ATP} channels interaction with DZ. Moreover, in spite of the progress reached in disclosure of subunit composition of cardiac mK_{ATP} channels, it still remains questionable which subunit combination might constitute functionally active channels responsive to DZ in heart mitochondria, and which of a Kir subunits (Kir6.2, 1.1, 3.1, or 3.4) could be responsible for DZ-evoked potassium transport and swelling [32,40].

The properties of mK_{ATP} channels and their sensitivity to physiological and pharmacological ligands were studied in different preparations, such as giant mitoplasts obtained from liver mitochondria where mK_{ATP} channel currents were first observed directly [49], the preparations of mK_{ATP} channel isolated and reconstituted in proteoliposomes and lipid bilayer membranes [7,50], and mitochondrial preparations using indirect methods (for the most part light scattering and fluorescent probes to monitor potassium transport [7,40,51]). Pioneering works of Garlid's group contributed much to the studies of biophysical and biochemical properties of mK_{ATP} channels and their sensitivities to physiological and pharmacological modulators.

Based on Garlid's works, native mK_{ATP} channels similarly to sK_{ATP} channels, were activated by DZ, and the requirement for Mg·ATP too was shown for the channel activation by KCOs and blockage by glibenclamide and 5-HD in cardiac, liver, and brain mitochondria [7,50,51]. Resembling sK_{ATP} channels, both Kir and SUR subunits were required for the effective block of mK_{ATP} channel by ATP, while Kir subunit alone (isolated as ~55 kDa protein) was blocked by ATP with low affinity [50]. Lacking SUR subunit, mitochondrial Kir channel was insensitive to the openers (DZ, cromakalim) and blockers (glibenclamide and 5-HD) [50].

The sensitivities of mK_{ATP} channels towards ATP and pharmacological modulators were greatly dependent on the preparations used for monitoring of mK_{ATP} channel activity. Thus, $K_{1/2}$ for ATP inhibition of mK_{ATP} channel was found to be ~1 μM (isolated mitochondria), ~20-30 μM (the channel reconstituted in liposomes) [50], and ~800 μM (giant mitoplasts [49]), which was similar to low ATP sensitivity of isolated Kir channel (600-800 μM) [50]. Mg^{2+} was indispensable for ATP inhibition of native (isolated mitochondria) and reconstituted mK_{ATP} channel (lipid bilayers), while ATP inhibition of isolated Kir channel did not require the presence of Mg^{2+} [50], similarly to mK_{ATP} channel in giant mitoplasts [49]. DZ activation likewise showed dependence on mK_{ATP} channels preparations. Thus, $K_{1/2}$ for DZ activation of reconstituted mK_{ATP} channel was as low as ~370 nM, while DZ affinity of native mK_{ATP} channel was ten times lower ($K_{1/2}$ ~3-4 μM) [7]. From these data it could be inferred that membrane environment plays an important role in the regulation of mK_{ATP} channel affinity to physiological and pharmacological ligands.

Meanwhile, several sets of data showed susceptibility of mK_{ATP} channel to activation by KCOs and the blockage by glibenclamide and 5-HD in the absence of Mg·ATP [51-54]. So, using indirect methods cardiac mK_{ATP} channel activation by KCOs (pinacidil, cromakalim) was shown without Mg·ATP [52] and in the presence of Mg^{2+} alone (pinacidil, DZ [53,54]), while the channel blockage by glibenclamide and 5-HD similarly did not require Mg·ATP [52,54].

Based on this knowledge, we raised a doubt that Mg·ATP was indispensable for mK_{ATP} channel activation by DZ. With liver mitochondria, we not only have shown the activation of mK_{ATP} channel by DZ in the absence of Mg·ATP, but observed high sensitivity

of the channel to this opener within nanomolar concentration range. Using indirect methods (polarography and light absorbance to monitor the oxygen consumption and mitochondrial volume changes), we have shown full activation of native liver mK_{ATP} channel elicited by ≤500 nM of DZ [55]. Likewise, with the same methods we observed that channel blockage by glibenclamide and 5-HD did not require the presence of Mg·ATP in isolated liver and brain mitochondria [55,56].

Our observations allow us hypothesize that, different of sK_{ATP} channel, mK_{ATP} channel might comprise the site(s) accessible to pharmacologic modulators and responsible for the channel activation by DZ and the blockage by glibenclamide and 5-HD in the absence of Mg·ATP, and moreover, that Mg·ATP binding might screen some putative high affinity binding sites of DZ within mK_{ATP} channel. Not only SUR (which is SUR1 in liver mitochondria), but possibly Kir subunit interaction with DZ and the blockers needs to be taken into account, however, it still remains to be answered, which of a Kir subunits found thus far (Kir6.1 [34], ROMK isoforms [41], or else) might be responsible for the effects of this K_{ATP} channel opener.

Nonspecific Cellular Targets of Diazoxide

Functional and bioenergetic consequences of mK_{ATP} channel opening by DZ were extensively studied in different cell types. The main complexity in these studies arises from the findings of different cellular targets of DZ within cytoprotective concentration range. The ability of DZ to bind to ATP-binding proteins implies multiple nonspecific targets of this drug with several ensuing off-target effects affecting mitochondrial functions and bioenergetics. Indeed, the interaction of DZ with F_0F_1 ATP synthase [15,18,19], Ca^{2+}, Mg^{2+}-ATPase(s) [19], and ADP/ATP carrier (ANT) [57] was shown. Other well-known targets of DZ are succinate dehydrogenase (SDH) [15-17] and protein kinases, especially protein kinase C epsilon [2,21,22].

F_0F_1 ATP synthase: In several works down modulation of F_0F_1 ATP synthase activity by DZ, either resulting [18,58], or not resulting from mK_{ATP} channel opening [15,19] was reported. As the side effect of DZ, the direct binding of the drug to this enzyme was shown [19]. It was established that DZ could directly interact with F_1 fragment, thereby promoting the binding of F_0F_1 ATP synthase inhibitor protein to beta-subunit. Similar to SUR subunits of K_{ATP} channels, DZ binding occurs in NBD domain of beta subunit of the enzyme, and binding requires the presence of Mg·ATP. Mg·ADP exerts a stabilizing effect on DZ binding [59]. DZ binding results in F_1 conformation favorable for the binding of inhibitor protein, thereby leading to the inhibition of ATP synthesis [19,59]. Direct interaction with F_0F_1 ATP synthase makes it difficult to discriminate the effects on ATP synthesis ensuing from mK_{ATP} channel opening from those ensuing from the direct binding of DZ to the enzyme. However, regardless of specific mechanism of action, DZ was shown to inhibit both the synthesis and hydrolysis of ATP [15,18], which appeared to be cardioprotective under ischemia because of preventing cellular ATP levels of severe depletion due to mitochondrial ATPase activity [15].

ATP/ADP carrier (ANT): One of the well-known effects of DZ is respiratory uncoupling, which can occur due to protonophoric properties of this drug at high concentration (≥50 μM) [13,14]. However, in rat heart mitochondria respiring on Complex I substrates (glutamate/malate) both DZ- and pinacidil-induced uncoupling was suppressed by the ANT inhibitors (carboxyatractiloside and bongkrecic acid) [57]. The authors came to the conclusion that ANT could participate in the regulation of potassium flux via mK_{ATP} channel, but from their data it could be inferred as well that ANT inhibition might interfere with protonophoric uncoupling of mitochondria by DZ, and proton flux caused by high micromolar DZ concentrations used in this work (58.8-1348.3 μM) was mediated by ANT. While no data are known on the direct DZ interaction with ANT, a published molecular docking study has shown the ability of mK_{ATP} channel blockers, glibenclamide and 5-HD to bind to this carrier [60].

ATPases: Different cellular compartments possess multiple ATPases activity, such as Na^+, K^+-ATPase of plasma membrane, ATPase of actomyosine complex, Ca^{2+}-ATPase of plasma membrane and sarcoplasmic reticulum, which all are ATP-consuming enzymes. Mitochondrial ATP synthase too can hydrolyze ATP to maintain mitochondrial energy state under certain conditions, such as ischemia, and DZ was shown to inhibit ATP hydrolysis by this enzyme [15,18]. While the effect of DZ on cellular ATPases was not yet studied in detail, the suppression of total Ca^{2+}, Mg^{2+}-ATPase activity by ~30% in heart mitochondria homogenates was shown [15]. Together with the inhibition of F_0F_1 ATP synthase activity, this helped to preserve cellular ATP level under myocardial ischemia [15,18].

Succinate Dehydrogenase (SDH), a Complex II of the respiratory chain, is well-established mitochondrial target of DZ [15-17]. SDH is a FAD-dependent enzyme, and mitochondrial ROS production generated from Complex-II greatly depends on flavoprotein redox state and the functioning of a coenzyme Q cycle. Although SDH was proposed to be a component of a multiprotein complex, which after isolation and reconstitution exhibited K_{ATP} channel properties [60,61], of pharmacological mK_{ATP} channel openers only DZ was known to inhibit SDH activity. Important physiological consequence of this inhibition is a suppression of SDH-derived ROS production shown to be important risk factor under several pathological states, including cancer and diabetes [17]. Accordingly, similar protective effects of DZ and SDH inhibitor malonate in ischemic preconditioning were observed, in no way dependent on mK_{ATP} channel activity [17].

Protein Kinase C (PKC-epsilon isoform) is one more known cellular target of DZ [2,21]. Thus, PKC-epsilon activation by DZ was shown to indirectly activate the mK_{ATP} channel by translocation of PKC-epsilon from the cytosol to mitochondria [21]. This indirect mK_{ATP} channel activation caused flavoprotein oxidation that was similarly reversed either by the PKC inhibition, or mK_{ATP} channel block by 5-HD [21]. It was shown too that PKC activation could promote import of Kir6.2-containing sK_{ATP} channels to mitochondria, and the enrichment of mitochondria by Kir6.2/SUR2A comprising channels was shown to result from PKC activation [62]. The question remains, whether the mechanism described is relevant for other subunit isoforms of K_{ATP} channel. In turn, cardiac mK_{ATP} channel opening resulted in PKC-epsilon activation ensuing from the elevation of hydroperoxide production in mitochondrial matrix [63]. Thus a reciprocal dependence appears between PKC-epsilon and mK_{ATP} channel activation, which likely can be amplified by DZ with consequent bioenergetic effects in mitochondria.

Interestingly, all described off-target effects of DZ seem to be cytoprotective. So, suppression of ATP hydrolysis due to the down modulation of F_0F_1 ATP synthase [19] and the inhibition of cellular ATPases seem to be helpful in preventing cellular ATP depletion under ischemia [15,18], while the suppression of ATP synthesis likely could help to keep K_{ATP} channels in highly active state in order to afford cytoprotection via mild uncoupling, SDH inhibition, suppression of ROS overproduction [8,17,20,64] and the inhibition of permeability

transition pore activity [8,63]. At last, PKC activation was shown to exert several cytoprotective actions via cell-signaling pathways [63,65].

In addition to cytoprotective effects well documented in the literature, DZ was shown to represent a promising pharmacological intervention against cytotoxicity of the drugs known to cause severe impairment of mitochondria, such as isoproterenol and doxorubicin [8,66]. Moderation of ROS production ensuing from DZ administration [8,64,66] was shown to be a part of cytoprotective action. However, no direct evidence for the involvement of mK_{ATP} channel in cytoprotection was yet obtained in living organism. Moreover, from the above said it follows that known bioenergetic effects of DZ observed *in vivo* could well be the consequences of the side effects of this drug, having nothing in common with potassium transport at all. But although the side effects of DZ appear to be beneficial in living organism and afford multiple positive therapeutic effects, they are hindering in proper understanding of physiological role of mK_{ATP} channel and ATP-sensitive potassium transport.

All known side effects of DZ were shown to be concentration-dependent [2], however *in vivo* it seems quite impossible to choose a "safe" dose of this drug to prevent its off-target actions. It appears that lowering of commonly used pharmacological doses of DZ and other KCOs [67] would help discriminate between therapeutic effects of mK_{ATP} channel opening and those caused by off-target effects of DZ

Nevertheless, under appropriate conditions bioenergetic effects produced by DZ on isolated mitochondria could be ascribed to the opening of mK_{ATP} channel showing DZ to be a suitable pharmacological tool to assess physiological diversity of mK_{ATP} channel functions.

Assessment of the Bioenergetic Consequences of mK_{ATP} Channels Opening in Mitochondria

Direct bioenergetic effects of mK_{ATP} channel opening

Surprisingly, direct bioenergetic effects of DZ as mK_{ATP} channels opener are very similar to indirect ones. It is well established that ATP-sensitive potassium entrance into matrix results in mitochondrial swelling due to obligatory water uptake [68]. The dilution of a matrix milieu due to the increased matrix water content decreases matrix Mg^{2+} concentration and leads to the dissociation of Mg^{2+} from the Mg^{2+} binding sites, releasing a "Mg^{2+} bracket" from K^+/H^+ exchanger and unmasking its activity [68]. Simultaneous work of K^+ uptake via several mitochondrial potassium channels and K^+/H^+ exchanger results in activation of so-called "potassium cycle", i.e., cyclic potassium transport across mitochondrial inner membrane [69]. Potassium uptake "phase" of potassium cycle is an energy dissipating process, because it requires a membrane potential, built up by the work of the respiratory chain, thus an activation of potassium cycle ensuing from mK_{ATP} channel opening eventually results in mitochondrial uncoupling [69].

One direct consequence of energy dissipation because of potassium cycling is the uncoupling of most important physiologically energy-dependent processes, such as Ca^{2+} uptake [70] and ATP synthesis [52,58]. Besides, increase in oxygen consumption and the waste of free energy generated by the respiratory chain on potassium cycle diminishes a redox-potential of the sites being a primary source of ROS in the respiratory chain within the Complexes I, II and III [17,71], thereby diminishing ROS production. While the effects of mK_{ATP} channels opening on ROS production known thus far were rather ambiguous, in several works including our own, decrease of ROS production ensuing of mK_{ATP} opening and mild mitochondrial uncoupling was observed [8,17,20,56].

It worth mention that off-target effects of DZ outlined above were observed at high DZ concentrations, above ~50 μM [16,19,21], and could be easily circumvented by lowering concentration of this drug [13]. Another requirement for the discrimination between the direct and side effects of DZ is the strong dependence of the direct effects on potassium transport, since indirect ones were observed as well in potassium-free media. Because these requirements could be met only *in vitro*, the studies on isolated mitochondria remain the shortest way to assess the direct functional effects of ATP-sensitive potassium transport ensuing from the opening of mK_{ATP} channel by DZ. Besides, as showed literary and our own studies, using isolated mitochondria to assess functional effects of DZ as mK_{ATP} channel opener allowed for the quantitative estimation of the contribution of mK_{ATP} channel to overall potassium transport, oxygen consumption and oxygen consuming processes.

Appraisal of functional consequences of K_{ATP} channels opening in mitochondria

As it was generally supposed, the beneficial bioenergetic effects of mK_{ATP} channels opening by DZ depend on the activation of potassium cycle, K+ uptake and K+/H+ exchange [69]. However, conventional use of Mg·ATP complex for monitoring mK_{ATP} channel opening hinder the direct studies of DZ effect on K+ cycle because of severe suppression of K+/H+ exchanger by Mg2+ ions [68,72]. Indeed, based on our observations as well, Mg2+ and Mg·ATP completely inhibited K +/H+ exchanger, while Mg2+ chelating with EDTA permitted to observe the activation of K+/H+ exchange by DZ in liver mitochondria in the same concentration range where full activation of mK_{ATP} channel was reached (≤500 nM) [55]. So, using combined polarographic and light absorbance studies, in our works we found high sensitivity of liver mK_{ATP} channel to DZ in the absence of Mg·ATP, and developed an approach to the direct study of bioenergetic effects ensuing from the activation of potassium cycle avoiding both off-target effects of high concentrations of this mK_{ATP} channel opener and the suppression of K+-cycle by Mg·ATP.

But although in liver mitochondria we obtained a reliable evidence on full mK_{ATP} channel and K+-cycle activation by DZ within nanomolar concentration range, when using DZ without Mg·ATP, another novel non-specific side effect of this drug was observed, i.e., the activation of ATP-insensitive potassium transport in line with ATP-sensitive one [55]. Unlike mK_{ATP} channel, ATP-insensitive potassium transport in our work was suppressed by Mg2+, and could not be restored by DZ. While the nature of this ATP-insensitive potassium transport remained unclear, for the estimation of bioenergetic effects of mK_{ATP} channel opening in mitochondria (mitochondrial uncoupling, depolarization, suppression of ATP synthesis and ROS production), we applied polarographic approach based on the estimation of a partial share of mK_{ATP} channel in total potassium transport, using stoichiometric ratios between the cation transport and state 4 oxygen consumption. Thus we established partial shares of both ATP-sensitive and ATP-insensitive constituents in potassium transport, and have shown that respiratory stimulation by DZ in liver mitochondria directly ensued from the activation of potassium cycle, eventually resulting in mitochondrial uncoupling [55].

Because mitochondrial membrane potential and the uncoupling of the respiratory chain (assessed as RCR ratio) were both dependent on the state 4 oxygen consumption, this allowed us to estimate the share of mK_{ATP} channel in bioenergetic effects of DZ and total potassium transport regardless of the presence of ATP-insensitive component [55,56]. Thus we obtained a reliable appraisal of the share of mK_{ATP} channel in potassium transport and mitochondrial uncoupling by DZ in liver mitochondria [55]. Using DZ as pharmacological tool, we also established the share of mK_{ATP} channel in membrane depolarization and suppression of potential-dependent ROS production in brain mitochondria, which was confirmed independently by using K_{ATP} channel blockers, glibenclamide and 5-HD [56].

Conclusion

The studies on K_{ATP} channels reviewed in this work show different tissue-specific sensitivities of K_{ATP} channels to DZ and other KCOs, dependent on the diversities of K_{ATP} channels expression in different cell types. This and multiple cellular and mitochondrial targets of DZ are capable of producing confounding results in the attempts to assess functional and bioenergetic effects of mK_{ATP} channel opening. It is even tempting to speculate that cytoprotective actions afforded by off-target effects of DZ might prevail over those afforded by mK_{ATP} channel opening alone. For the same reason the application of DZ as pharmacological tool to study physiological significance of mK_{ATP} channel opening *in vivo* is rather restricted. Thus till the definitive discovery of molecular entity of mK_{ATP} channels permitting the design of highly selective pharmacological modulators, the studies on isolated mitochondria remain the shortest way to appraise direct bioenergetic consequences of mK_{ATP} channels functioning (respiratory uncoupling, inhibition of ATP synthesis, modulation of ROS production and Ca^{2+} transport).

Based on the results of our studies in liver mitochondria, we came to the conclusion that DZ within nanomolar concentration range well met the requirement of an effective pharmacological activator of mK_{ATP} channel and potassium cycle. Avoiding off-target effects of high concentrations of DZ and the blockage of K^+/H^+ exchanger by Mg^{2+} ions, our approach allowed direct estimation of the share of mK_{ATP} channel in bioenergetic effects of DZ. So, we found this drug to be a useful pharmacological tool for the direct *in vitro* study of functional consequences of mK_{ATP} channels opening in mitochondria.

The present knowledge is lacking in the understanding of the true bioenergetic effects of mK_{ATP} channels opening in a living organism. Numerous off-target effects of DZ resulting in modulation of mitochondrial functions and different tissue-specific sensitivity of K_{ATP} channels to this drug complicate its use *in vivo* as specific modulator of mK_{ATP} channel activity, which is required for the treatment of several diseases where selective mK_{ATP} channels opening is supposed to afford cytoprotective effects. Thus the knowledge about tissue-specific distribution of different mK_{ATP} channel isoform subunits, the structural data on the sites of KCOs binding, and better understanding of the regulatory mechanisms involved in binding of physiological and pharmacological ligands are important for the development of therapeutic strategies and drug design aimed at selective modulation of mK_{ATP} channels activity with consequent modulation of mitochondrial functions and bioenergetics.

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