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Effects of ATP-sensitive potassium channel activators diazoxide and BMS-191095 on membrane potential and reactive oxygen species production in isolated piglet mitochondria

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Abstract

Mitochondrial ATP-sensitive potassium (mitoK$_{ATP}$) channel openers protect the piglet brain against ischemic stress. Effects of mitoK$_{ATP}$ channel agonists on isolated mitochondria, however, have not been directly examined. We investigated the effects of K$_{ATP}$ channel openers and blockers on membrane potential and on the production of reactive oxygen species (ROS) in isolated piglet mitochondria. Diazoxide and BMS-191095, putative selective openers of mitoK$_{ATP}$, decreased the mitochondrial membrane potential ($\Delta\Psi_m$). On a molar basis, diazoxide was less effective than BMS-191095. In contrast, diazoxide but not BMS-191095 increased ROS production by mitochondria. Since diazoxide also inhibits succinate dehydrogenase (SDH), we examined the effects of 3-nitropropionic acid (3-NPA), an inhibitor of SDH. 3-NPA failed to change the $\Delta\Psi_m$ but increased ROS production. Inhibitors of K$_{ATP}$ channels did not affect resting $\Delta\Psi_m$ or ROS production, but glibenclamide and 5-hydroxydecanoate (5-HD) blocked effects of diazoxide and BMS-191095 on $\Delta\Psi_m$ and diazoxide effects on ROS production. We conclude that BMS-191095 has selective effects on mitoK$_{ATP}$ channels while diazoxide also increases ROS production probably via inhibition of SDH.

Keywords: Diazoxide; BMS-191095; Potassium channels; Mitochondria; Succinate dehydrogenase; Ischemia; Inwardly rectifying potassium channel; Sulfonylurea receptors

1. Introduction

ATP-sensitive potassium (K$_{ATP}$) channels with different pharmacological properties are present on the plasmalemmal membrane and on the inner mitochondrial membrane [1,2]. Considerable interest has been generated recently from studies in which activation of mitochondrial (mito)K$_{ATP}$ channels has been shown to be protective against anoxic and chemical stresses in brain. In the first publication on this topic, we showed that application of diazoxide, the prototypic opener of mitoK$_{ATP}$ channels, provided immediate protection of neurons against ischemic stress in neonatal pigs [3]. Protective effects of diazoxide were reversed by co-administration of 5-hydroxydecanoate (5-HD) which has been suggested to be a selective antagonist of mitoK$_{ATP}$ channels or at least a selective antagonist of the actions of diazoxide [6,7,9]. Additional studies in cultured brain cells [11–13,18,21,22,24] in the neonatal pig [4] or in the rat brain [10,19,20] in vivo have further established the protective effects of diazoxide. Protective effects of diazoxide apparently involve both mitoK$_{ATP}$ channel opening and production/actions of reactive oxygen

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species (ROS) [11,15,18]. Whether the production of ROS by diazoxide is secondary to mitoK$_{ATP}$ opening or is due to an inhibitory action of diazoxide on succinate dehydrogenase (SDH) is unknown [11].

The purpose of this study was to examine whether the effects of diazoxide on mitochondrial membrane potential ($\Delta\Psi_m$) and ROS production were due to the activation of mitoK$_{ATP}$ channels or other effects, such as SDH inhibition. In isolated piglet mitochondria, we compared effects of diazoxide with a newly developed mitoK$_{ATP}$ channel opener, BMS-191095, which does not increase ROS production in cultured neurons [12] and apparently does not inhibit SDH. Additionally, we administered 3-nitropropionic acid (3-NPA), a specific inhibitor of SDH [23], which appears to have no direct effect on mitoK$_{ATP}$ channels.

2. Methods

2.1. Mitochondrial isolation

All procedures were approved by the Animal Care and Use Committee at Wake Forest University Health Sciences. We used brains from neonatal pigs (1–7 days of age) to isolate mitochondria as previously described [13,16]. The piglets were over-anesthetized with sodium thiopental (100 mg/kg, i.p.) and the brain removed. Isolated mitochondria preparations were collected by discontinuous percoll gradient purification. Slices of tissue were removed from the cortex and placed in cold isolation buffer. Pieces of cortex were added on a 10% (w/v) to isolation buffer, homogenized, and centrifuged at 500 × *g* for 3 min at 4 °C. The supernatant was collected and resuspended with 1:1 volume 24% percoll, layered on top of a discontinuous percol gradient (24;40%), and centrifuged at 28000 × *g* for 5 min. The layer containing the purified mitochondria was collected and diluted 1:4 with isolation buffer, and centrifuged at 15,000 × *g* for 12 min. The upper 2/3 of the supernatant was removed gently and the pellet was resuspended in 1 ml isolation buffer and centrifuged in a microfuge at 11,000 × *g* for 10 min. The final pellet was resuspended in isolation buffer containing 2% BSA to obtain a concentration of 3–5 mg/ml of mitochondrial protein. Freshly isolated mitochondria were dispersed in buffer containing 125 mmol/l KCl, 2 mmol/l K_2HPO_4, 5 mmol/l $MgCl_2$, 10 mmol/l HEPES, 10 μmol/l EGTA at pH 7.0 and plated on poly-D-lysine coated cover slips for confocal microscopy (Zeiss 510, Jena, Germany).

2.2. Determination of reactive oxygen species (ROS) generation

Reactive oxygen species generation was assessed using the oxidation-sensitive dye dyhydroethidum (HEt, Molecular Probes, Eugene, OR, USA), which is oxidized to the fluorescent ethidium by free radicals [11,12]. Advantages of HEt over other oxidation-sensitive dyes include its relative resistance to both auto- and photo-oxidation and the increasing intensity of the dye fluorescence seen after intercalation of ethidium within DNA. In the case of isolated mitochondria, the target for ethidium will be mitochondrial DNA. The HEt stock was prepared at a concentration of 5 mM in DMSO and stored at −20 °C. Mitochondrial suspensions were loaded in the dark at 22 °C with 5 μM HEt in phosphate-buffered saline and placed on the stage of the confocal microscope without washing the mitochondria. Fields of mitochondria were randomly selected under differential interference contrast optics. Diazoxide (50, 100, 250, 500 μM), 3-NPA (10, 20, 100 μM), BMS (10, 20, 30 μM), vehicle (DMSO, 1:1000), or 5-hydroxydecanoate (5-HD) (1 mM) were applied into the medium and fluorescent images were obtained using excitation λ = 488 nm and emission λ > 560 nm (560 nm long-pass filter). Images were recorded every 20 s for 10 min after drug application, and the average pixel intensity in individual mitochondria was determined using the software supplied by the manufacturer (Zeiss).

2.3. Determination of $\Delta\Psi_m$

Mitochondrial membrane potential was monitored using the $\Delta\Psi_m$-sensitive dye, tetramethylrhodamine ethyl ester (TMRE, Molecular Probes, Eugene, OR, USA) [12]. Fluorescent images (λ_{ex} = 543 nm, λ_{em} > 560 nm) were recorded every 20 s for 5 min after application of vehicle, diazoxide (50, 100, 500 μM), BMS (10 μM), and 3-NPA (100 μM). Glibenclamide (25 μM) and 5-HD (50 μM) were also co-applied with BMS and diazoxide as well as alone. The average pixel intensity in individual cell bodies was determined using the software supplied by the manufacturer (Zeiss).

2.4. Statistics

ANOVA was used for comparisons between and among the different treatment groups. Differences with $P < 0.05$ were considered statistically significant. All results are expressed as mean ± S.E.M.

3. Results

3.1. Effects of agonists and antagonists on $\Delta\Psi_m$

DMSO (Figs. 1 and 3), glibenclamide (13 ± 24%), and 5-HD (7 ± 11%) failed to significantly affect membrane potential of isolated mitochondria under baseline conditions. Application of diazoxide depolarized mitochondria in a dose-dependent manner for 50 and 100 μM (Figs. 1 and 3). However, while diazoxide at 500 μM also depolarized mitochondria, effects were reduced compared to the lower doses of diazoxide (data not shown). Similarly, 10 μM BMS-191095 depolarized mitochondria to a degree similar to 100 μM diazoxide (Figs. 1–3). Diazoxide- and BMS-191095-induced depolarization was reversed completely by