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Regular Article

Hemin inhibits the large conductance potassium channel in brain mitochondria: A putative novel mechanism of neurodegeneration

Bartłomiej Augustynek [a,b], Alexei P. Kudin [b], Piotr Bednarczyk [a,c], Adam Szewczyk [a,*], Wolfram S. Kunz [b]

[a] Laboratory of Intracellular Ion Channels, Department of Biochemistry, Nencki Institute of Experimental Biology, 3 Pasteur Street, 02-093 Warsaw, Poland
[b] Department of Epileptology, University of Bonn, 25 Sigmund-Freud Street, D-53105 Bonn, Germany
[c] Department of Biophysics, Warsaw University of Life Sciences, 159 Nowoursynowska Street, 02-776 Warsaw, Poland

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ABSTRACT

Intracerebral hemorrhage (ICH) is a pathological condition that accompanies certain neurological diseases like hemorrhagic stroke or brain trauma. Its effects are severely destructive to the brain and can be fatal. There is an entire spectrum of harmful factors which are associated with the pathogenesis of ICH. One of them is a massive release of hemin from the decomposed erythrocytes. It has been previously shown, that hemin can inhibit the large-conductance Ca^{2+}-regulated potassium channel in the plasma membrane. However, it remained unclear whether this phenomenon applies also to the mitochondrial large-conductance Ca^{2+}-regulated potassium channel. The aim of the present study was to determine the impact of hemin on the activity of the large conductance Ca^{2+}-regulated potassium channel in the brain mitochondria ($mitoBK_{Ca}$). In order to do so, we have used a patch-clamp technique and shown that hemin inhibits $mitoBK_{Ca}$ in human astrocytoma U-87 MG cell line mitochondria. Since opening of the mitochondrial potassium channels is known to be cytoprotective, we have elucidated whether hemin can attenuate some of the beneficiary effects of potassium channel opening. We have studied the effect of hemin on reactive oxygen species synthesis, and mild mitochondrial uncoupling in isolated rat brain mitochondria. Taken together, our data show that hemin inhibits $mitoBK_{Ca}$ and partially abolishes some of the cytoprotective properties of potassium channel opening. Considering the role of the $mitoBK_{Ca}$ in cytoprotection, it can be presumed that its inhibition by hemin may be a novel mechanism contributing to the severity of the ICH symptoms. However, the validity of the presented results shall be further verified in an experimental model of ICH.

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Introduction

Intracerebral hemorrhage is a type of intracranial bleeding that occurs within the brain tissue. It can either be caused by brain trauma or occur in hemorrhagic stroke. Stroke is the second most common cause of death worldwide (Donnan et al., 2008), and hemorrhagic strokes account for 10–15% of all stroke cases (Sudlow and Warlow, 1997). Mortality of hemorrhagic stroke is high, and has not fallen within the last years. According to different studies it varies from 31% at 7 days to more than 90% at 10 years (Flaherty et al., 2006; Fogelholm et al., 2005). In hemorrhagic stroke the rapture or leak of a blood vessel leads to the formation of hematoma which oppresses the surrounding tissue and hampers their supply in oxygen and nutrients. Cells within and adjacent to the hematoma die quickly and there is not much that can be done today to prevent it. However, there is a pool of cells that experience negative effects of hemorrhage, although do not die immediately. Such cells would be a suitable target for potential therapies. It is widely believed that one of the causes of cell loss in hemorrhagic stroke is the erythrocyte lysis that occurs in hematoma. Neurotoxic agents like hemoglobin, and its breakdown product hemin migrate from hematoma into surrounding tissue impairing the proper function of nervous cells (Chen-Roetling and Regan, 2006; Dang et al., 2011b). Hemin liberated from the hematoma can reach concentrations of as high as 10 mM (Robinson et al., 2009). Both astrocytes and neurons express heme carrier protein 1 (HCP1) in the plasma membrane, and hence can accumulate hemin upon its release from disintegrating erythrocytes (Dang et al., 2010, 2011b). Recent experimental data show rapid uptake of hemin by all brain resident cells, especially by microglia (Chen-Roetling et al., 2014). Additionally, hemin is a lipophilic compound that can intercalate into lipid bilayers, and cross them. However, one should expect that this phenomenon is rather of minor importance for hemin accumulation by cells. Still, it may play a role in hemin trafficking within the cell. Although neurotoxicity of hemin has been well documented, molecular basis of this phenomenon remains unclear. There are many processes and proteins which function can be impaired by this agent. One of them, the large-conductance Ca^{2+}-regulated potassium channel (BK_{Ca} channel) in the plasma membrane is inhibited by hemin (Tang et al., 2003).

* Corresponding author.
E-mail address: a.szewczyk@nencki.gov.pl (A. Szewczyk).

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The BK_{Ca} channel is ubiquitously expressed in the plasma membrane of both excitable and non-excitable cells. It is activated by the changes in membrane potential and/or the calcium ions. Although it was extensively studied mainly in the plasma membrane, its counterparts exist within several intracellular structures like mitochondria (Siemen et al., 1999; Szabò et al., 2012). MitoBK_{Ca} is one of the best described channels present in the inner mitochondrial membrane. It can be blocked by charybdotoxin (ChTx), iberiotoxin (IbTx) or paxilline (Pax) and stimulated by calcium ions, changes in membrane potential, or by certain synthetic agents like NS1619. The large conductance calcium regulated potassium channel was identified in mitochondria of several cell types, namely: human glioma cell line LN229 (Siemen et al., 1999), guinea pig ventricular cells (Xu et al., 2002), skeletal muscle (Skalska et al., 2008), endothelial cells (Bednarczyk et al., 2013a), astrocytes (Thiede et al., 2012), astrocytoma (Bednarczyk et al., 2013b) and neurons (Fahanik-Babaei et al., 2011; Piwonska et al., 2008; Skalska et al., 2009).

Although BK_{Ca} channels exist in many different locations throughout the cell, all of their forms share functional characteristics with their plasma membrane counterpart and all are encoded by a single gene: *Kcnma1*. Hence, it is widely believed that different types of BK_{Ca} channels are the results of alternative splicing of this gene. Although, the molecular identity of the mitoBK_{Ca} channel still remains to be elucidated, in 2013 Singh and co-workers have shown that a 50-aa C-terminal splice insert determines the mitochondrial location of mitoBK_{Ca} (Singh et al., 2013).

Potassium channels are notorious for their role in cytoprotection (Escande and Cavero, 1992; Garlid, 2000; Malinska et al., 2010). Such cytoprotection could be especially important for tissues with a poor endogenous antioxidant defense such as the cardiac muscle or the nervous system where undisturbed and continuous blood flow and oxygen supply are extremely important (Doré, 2002). It is broadly accepted that preconditioning with the potassium channel openers (KCOs) results in cytoprotection throughout activation of mitochondrial channels (Facundo et al., 2006). The basis of KCO cytoprotective properties still remains to be elucidated, although it is suspected that attenuation of ROS synthesis in mitochondria that is observed after KCO administration may play some role in this phenomenon. Under substrate conditions that allow reverse electron flow, matrix K^+ influx through the open mitoBK_{Ca} channel is believed to accelerate forward electron transfer and inhibit ROS production (Heinen et al., 2007a). Acceleration of ROS synthesis rate in turn is linked with several neurodegenerative diseases (Popa-Wagner et al., 2013). Another possible explanation of the cytoprotective properties of KCOs is mild uncoupling. The opening of mitochondrial potassium channels results in a modest dissipation of proton motive force by contributing inward current and following utilization of some of the proton gradient for K^+ ejection from the matrix through the K^+/H^+ exchanger (O'Rourke, 2004) leading to decreased ROS synthesis (Kulawiak et al., 2008).

The goal of this study was to elucidate whether hemin can inhibit the large conductance calcium regulated potassium channel in the mitochondrial inner membrane and if so, how does it alter some of the known beneficial effects of potassium channel openers on cell function.

Materials and methods

All standard chemicals along with 1,3-dihydro-1-[2-hydroxy5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one (NS1619), iberiotoxin (IbTx), charybdotoxin (ChTx), horseradish peroxidase (HRP) and hemin were purchased from Sigma-Aldrich. The uncoupler TTFB (4,5,6,7-tetrachloro-2-trifluoromethyl benzimidazole) was a kind gift from Prof. B. Beechey (Aberystwyth, UK). Catalase, digitonin and superoxide dismutase (SOD) were purchased from Serva, and bacterial proteinase from Fluka. All cell culture materials were purchased from Gibco.

Cell culture and preparation of astrocytoma mitochondria

Mitoplasts for patch-clamp experiments were prepared from the human astrocytoma U-87 MG cell line. The cell line identity was confirmed by the short tandem repeat (STR) profiling technique. This assay was performed according to the guidelines published by Masters et al. (2001). More details are in Bednarczyk et al. (2013b).

For the experiments, cells were cultured in DMEM medium (10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin) at 37 °C in a humidified atmosphere with 5% CO_2. The cells were fed and reseeded every third day. Once reached a confluence of about 70%, cells from five 75 cm^2 flasks were scraped and centrifuged at 800 $\times g$ for 10 min, resuspended in the preparation solution for the mitochondrial isolation (250 mM sucrose, 5 mM HEPES, pH = 7.2) and homogenized. First (9200 $\times g$, 10 min) and second (770 $\times g$, 10 min) centrifugation steps were performed to separate the fraction enriched in mitochondria. Sucrose was removed by two further fast centrifugation steps (9200 $\times g$, 10 min) in the storage solution (150 mM KCl, 10 mM HEPES, pH = 7.2). All procedures were performed at 4 °C.

Patch clamp experiments

Patch-clamp experiments on mitoplasts (spherical vesicles surrounded by the mitochondrial inner membrane) were performed as described previously (Bednarczyk et al., 2010, 2013b; Cheng et al., 2008; Toczyłowska-Mamińska et al., 2013). Briefly, a sample of purified human astrocytoma mitochondria was put into a hypotonic solution (5 mM HEPES, 200 μM $CaCl_2$, pH = 7.2) for about 1 min in order to induce swelling and disruption of the mitochondrial outer membrane. Then, the addition of a hypertonic solution (750 mM KCl, 30 mM HEPES, 200 μM $CaCl_2$, pH = 7.2) restored the isotonicity of the medium, and stopped further swelling. Mitoplasts were distinguished from the cellular debris present in preparation by their transparency, spherical shape, and characteristic 'cap' — remain of the outer membrane.

In each experiment, one free-floating mitoplast was attached to the patch-clamp pipette made of borosilicate glass filled with an isotonic solution (150 mM KCl, 10 mM HEPES, 200 μM $CaCl_2$, pH = 7.2). The isotonic solution was also used in control recordings for all presented data. Hemin and iberiotoxin (300 nM hemin and 2 nM IbTx in isotonic solution) were added from the back of the patch-clamp pipette through the peristaltic pump-driven capillary-pipe system. The experiments were carried out in patch-clamp inside-out mode. Reported voltages are those applied to the patch clamp pipette interior.

The current was recorded by the patch-clamp amplifier Axopatch 200B (Molecular Devices Corporation). The pipettes had a resistance of 10–20 MΩ and were pulled by a Flaming/Brown type P-100 puller (Sutter Instrument). The measured ion currents were low-pass filtered at 1 kHz and sampled at a frequency of 100 kHz. All experimental traces were recorded at the single-channel mode. The conductance was calculated from the current–voltage characteristics (data not shown). The probability of channel opening was determined using the single-channel search mode of the Axon™ pCLAMP® 10 Electrophysiology Data Acquisition & Analysis Software (Molecular Devices Corporation).

Isolation of rat brain mitochondria

Solutions used for: mitochondrial isolation: MSE solution (225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 5 mM HEPES, 1 mg/ml essential fatty acid free BSA, pH = 7.4); measurements: K^+ containing MTP medium (10 mM KH_2PO_4, 60 mM KCl, 60 mM Tris–HCl, 110 mM mannitol, 5 mM $MgCl_2$, 0.5 mM EDTA, pH = 7.4) and K^+ free MTP medium (10 mM NaH_2PO_4, 60 mM NaCl, 60 mM Tris–HCl, 110 mM mannitol, 5 mM $MgCl_2$, 0.5 mM EDTA, pH = 7.4).

Rat brain mitochondria were isolated according to the standard protocol described by Rosenthal et al. (1987), with a small modification

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