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Modulation of the mitochondrial large-conductance calcium-regulated potassium channel by polyunsaturated fatty acids

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ABSTRACT

Polyunsaturated fatty acids (PUFAs) and their metabolites can modulate several biochemical processes in the cell and thus prevent various diseases. PUFAs have a number of cellular targets, including membrane proteins. They can interact with plasma membrane and intracellular potassium channels. The goal of this work was to verify the interaction between PUFAs and the most common and intensively studied mitochondrial large conductance Ca^{2+}-regulated potassium channel ($mitoBK_{Ca}$). For this purpose human astrocytoma U87 MG cell lines were investigated using a patch-clamp technique. We analyzed the effects of arachidonic acid (AA); eicosatetraynoic acid (ETYA), which is a non-metabolizable analog of AA; docosahexaenoic acid (DHA); and eicosapentaenoic acid (EPA). The open probability (P_o) of the channel did not change significantly after application of 10 μM ETYA. P_o increased, however, after adding 10 μM AA. The application of 30 μM DHA or 10 μM EPA also increased the P_o of the channel. Additionally, the number of open channels in the patch increased in the presence of 30 μM EPA. Collectively, our results indicate that PUFAs regulate the BK_{Ca} channel from the inner mitochondrial membrane.

1. Introduction

Polyunsaturated fatty acids (PUFAs) are integral, structural components of the phospholipid bilayer of cell membranes. Conditions that promote the accumulation of PUFAs, such as brain ischemia, increase the level of extracellular and intracellular PUFAs [1]. One such critical metabolic event during ischemia is the activation of PLA_2 due to an increased concentration of intracellular Ca^{2+} ions [2]. This activation results in the hydrolysis of membrane phospholipids and the release of free fatty acids, particularly arachidonic acid (AA).

A growing body of evidence suggests that ischemic preconditioning, short episodes of ischemia that increase tissue tolerance to lethal insults, could be mimicked by the administration of openers of mitochondrial K-channels [3,4]. The transport of K^+ ions into the mitochondrial matrix can trigger the protection of the injured cardiac and neuronal tissues. It has been suggested this transport causes changes in the volume of the mitochondrial matrix [4], the inner membrane potential [5], the rate of generation of reactive oxygen species [6], and the influx of Ca^{2+} [7]. These changes most likely occur to protect the cell from death [8]. The ATP-regulated K-channels and the $mitoBK_{Ca}$ are among the best-described channels in the inner mitochondrial membranes and also play roles in cytoprotection. The $mitoBK_{Ca}$ was initially described in human glioma cells [9]. Later, it was identified in inner mitochondrial membranes of guinea pig ventricular cells [10], skeletal muscles [11], rat astrocytes [12], and, recently, in neuronal cells [13].

Neuronal and glial cells respond to ischemic injury in different ways. We have focused on astrocyte-type cells, which are less vulnerable to ischemic brain damage than the neighboring neurons are [14]. One of the protective functions of astrocytes during ischemia is the uptake of glutamate, thus limiting the excitotoxic injury to the neighboring neurons [15]. Astrocytes also provide neurons with antioxidants, such as glutathione. The neurons are thought to be the cells that are most vulnerable to ischemia. This conclusion is based primarily on the observation that astrocyte cultures exhibit greater resistance to some ischemia-like insults than neurons do [16].

One target of ischemic injury is the mitochondrion. Interestingly, mitochondrial dysfunction in astrocyte cells reduced their ability to protect neurons against glutamate toxicity. The inhibition of astrocyte mitochondria by fluorocitrate has been shown to increase the neuronal sensitivity to ischemia [17].

The role of the mitochondria in ischemic preconditioning has been studied in the heart and in the brain. During ischemia degradation of phospholipids occurs in the mitochondrial membranes [18]; therefore, one action of PUFAs or their metabolites, namely, the regulation of intracellular ion channels, was investigated within these membranes.

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The aim of this project was to elucidate the mechanism by which fatty acids (FAs) are modulating the mitoBK$_{Ca}$ activity. We examined the influence of PUFAs on mitochondrial channels for the following reasons: (1) differences in the PUFA levels have been found in patients with various neurological disorders, especially during brain ischemia [19]; (2) PUFAs may display cell-protective properties [20,21]; (3) PUFAs are able to modulate mitochondrial functions, such as oxygen consumption [22], mitochondrial swelling [23], reactive oxygen species production [24], cytochrome c release [25], and permeability transition [26]; and (4) FAs are able to interact with the BK$_{Ca}$ from the plasma membrane [27,28].

2. Materials and methods

2.1. Human astrocytoma U87MG cell line

The short tandem repeat (STR) profiling technique was performed according to the guidelines published recently [29,30]. Briefly, our cells were expanded and frozen at 90% confluence during the exponential growth phase and sent for STR profiling analyses to the German Collection of Microorganisms and Cell Cultures [Leibniz Institut Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany]. STR DNA profiling was carried out using fluorescent PCR in combination with capillary electrophoresis as described previously [31]. Using different alternate colors, the PowerPlex VR 1.2 system (Promega, Mannheim, Germany) was modified in order to run a two-color DNA profiling allowing the simultaneous single-tube amplification of eight polymorphic STR loci and Amelogenin for gender determination. STR loci of CSF1PO, TPOX, TH01, vWA and Amelogenin were amplified by primers labeled with the Beckman/Coulter dye D3 (green; Sigma-Aldrich, Munich, Germany), while the STR loci D16S539, D7S820, D13S317 and D5S818 were amplified using primers labeled with D2 (black). All the loci except the Amelogenin gene in this set are true tetranucleotide repeats. All primers are identical to the PowerPlex VR 1.2 system except the fluorescent color. Data were analyzed with the CEQ 8000 software (Beckman-Coulter, Krefeld, Germany), which enables an automatic assignment of genotypes and automatic export of resulting numeric allele codes into the reference DNA database of the DSMZ [32].

2.2. Preparation of mitochondria from human astrocytoma U87MG cell line

Human astrocytoma cells were cultured in DMEM as described elsewhere [12]. After loosening the cells by trypsin, the solution was gently removed, and the cells were washed with CMF (Ca^{2+}-Mg^{2+}-free solution). The loosened cells were dispersed in DMEM and centrifuged at 800 ×g for 10 min. The pellet was resuspended in a preparation solution (250 mM sucrose, 5 mM HEPES, pH 7.2) and homogenized. The homogenate was centrifuged at 9200 ×g for 10 min, resuspended in the preparation solution, and centrifuged at a low speed (790 ×g) for 10 min to separate the fraction of purified mitochondria. The preparation solution containing sucrose was removed by two fast centrifugations (9200 ×g for 10 min) in the storage solution (150 mM KCl, 10 mM HEPES, pH = 7.2). The mitoplasts (mitochondria without the outer membrane) were prepared from the mitochondria by the addition of a hypotonic solution (5 mM HEPES, 100 μM $CaCl_2$, pH = 7.2) to induce swelling, which was followed by the rupture of the outer membrane. Isotonicity was restored by adding a hypertonic solution (750 mM KCl, 30 mM HEPES, 100 μM $CaCl_2$, pH = 7.2).

2.3. Patch-clamp measurements

An isotonic solution (150 mM KCl, 10 mM HEPES, and 0.1 mM $CaCl_2$; pH = 7.2) was used as the control solution for all of the experiments. The external Ca^{2+} concentration of the mitoplast (i.e., inside the patch pipette) was 0.1 mM in the isotonic solution. The isotonic bath solution had a Ca^{2+} concentration of 0.2 mM or 0.1 mM. The inward current always deflects downward, and the holding potentials (E_h) at the inner side of the membrane are reported. Stock solutions of arachidonic acid, eicosatetraynoic acid, docosahexaenoic acid and eicosapentaenoic acid were made in 99% ethanol. The oxygen contained in the stock solutions was removed by nitrogen bubbling. Before running each experiment concentrated stock solutions were diluted in nitrogen-bubbled isotonic solution to a final concentration (test solution). Air bubbles were removed from the isotonic bath solution, isotonic solution in the measured pipette and test solutions pumped by a peristaltic pump-driven capillary-pipe system.

2.4. Data analysis

Data were analyzed using the pClamp10 software package. Events shorter than 0.5 ms were ignored. The conductance was calculated from the current–voltage characteristics. The open probability of the channel (P_o) was determined using the single-channel mode of the Clampfit10 software. The data are reported as the mean ± SEM (standard error of the mean). Student's *t* test was used to evaluate the significant differences between two groups, and $p < 0.05$ was considered statistically significant (in figures marked by asterisk).

3. Results

3.1. Patch-clamp experimental conditions and controls

The mitoplast-attached patches obtained from human astrocytoma mitochondria were examined to determine the kinetic properties of the mitoBK$_{Ca}$ after application of the FAs. First, the patches were exposed to isotonic solutions with different Ca^{2+} concentrations ranging from a low level (i.e., without added Ca^{2+} or Ca^{2+} chelators, which means μM concentrations due to impurities from the other chemicals and the glassware) to a maximum of 200 μM. We observed channel features similar to those previously reported in astrocytes [12], which are characteristic of a large-conductance calcium-activated K-channel (mitoBK$_{Ca}$). Patches with an appropriate seal resistance that exhibited mitoBK$_{Ca}$ activity were obtained 53 times in 41 mitochondrial preparations. In some cases, we recorded two or three mitoBK$_{Ca}$ channels within a single patch. The single-channel recordings obtained at $E_h = +20$ mV are presented in Fig. 1A.

To exclude the possibility that the measured current was caused by another type of mitochondrial K-channel, we used inhibitors that are commonly known to block mitoBK$_{Ca}$, such as iberiotoxin and paxilline (Fig. 1A). Occasionally, the measured channel exhibited a smaller current than that of the fully open state. Due to both, the behavior observed in the blocking experiments and the voltage dependence of the current observed for these channels, we identified this current as a substate of the fully open mitoBK$_{Ca}$. We also controlled the Ca^{2+} dependence of the channel by replacing the isotonic solution containing 200 μM Ca^{2+} with an isotonic solution containing either 100 μM Ca^{2+} or no added Ca^{2+} ions (referred to as low Ca^{2+} in the manuscript). Fig. 1A shows the controls that were prepared at the start of the experiments. The application of an isotonic solution containing either 100 μM Ca^{2+} or 10 μM Ca^{2+} decreased the P_o of the channel. Similarly, single-channel recordings performed under control conditions (200 μM Ca^{2+}) and low-Ca^{2+} conditions revealed the dependence of the channel activity on the Ca^{2+} concentration. Moreover, we demonstrated that the complete removal of Ca^{2+} ions by the addition of 50 μM EGTA to the external isotonic solution blocked the channel activity completely but reversibly.

After characterizing the mitoBK$_{Ca}$ properties, we studied the effect of FAs on the channel in the presence and absence of Ca^{2+} ions. To investigate the possible effects of FAs on the mitoBK$_{Ca}$, we first determined how fast the channel responded to the external application of FAs. The effects of the FAs appeared after 3–4 min

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