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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

(Fig. 1B). Based on our preliminary results, we investigated this effect under the simultaneous control of the Ca^{2+} response of the mitoBK$_{Ca}$. The different FA- or Ca^{2+}-containing solutions were added in a specific order. The experiment was divided into seven parts, each lasting 7 min (Fig. 1C). If possible, blockers of the mitoBK$_{Ca}$ were added at the end of the experiments. The location of the patch pipette with the attached mitoplast inside the pipe of the perfusion system is shown in Fig. 2A. Not surprisingly, increased concentrations of FAs (10 μM, 30 μM, and 100 μM) decreased the electrical resistance of the mitochondrial membrane as demonstrated in Fig. 2B. This decrease in the resistance occurred almost linearly (Fig. 2C). Occasionally, the highest FA concentrations caused the loss of the patch. Because FAs are known to be oxidized by oxygen, the stock solutions of all of the FAs and the daily preparations of the isotonic solutions were bubbled with nitrogen. Fig. 2D shows that 10 min of nitrogen bubbling applied to the isotonic solutions had no effects on the channel activity.

3.2. mitoBK$_{Ca}$ sensitivity to arachidonic acid, n-3 PUFA

As the first step of the experiments, we examined the effect of arachidonic acid on the mitoBK$_{Ca}$ activity. It is known that AA is released from the membranes during ischemia and can interact with plasma membrane K-channels ([20,33]; depicted in Fig. 3A). To study whether Ca^{2+} ions play a role in the effect of AA on the mitoBK$_{Ca}$, AA was tested in isotonic solutions with either low or high Ca^{2+} concentrations. The application of 10 μM AA in an isotonic solution containing 100 μM Ca^{2+} did not significantly alter the channel conductance (data not shown), but it increased the P_o (Fig. 3B). This

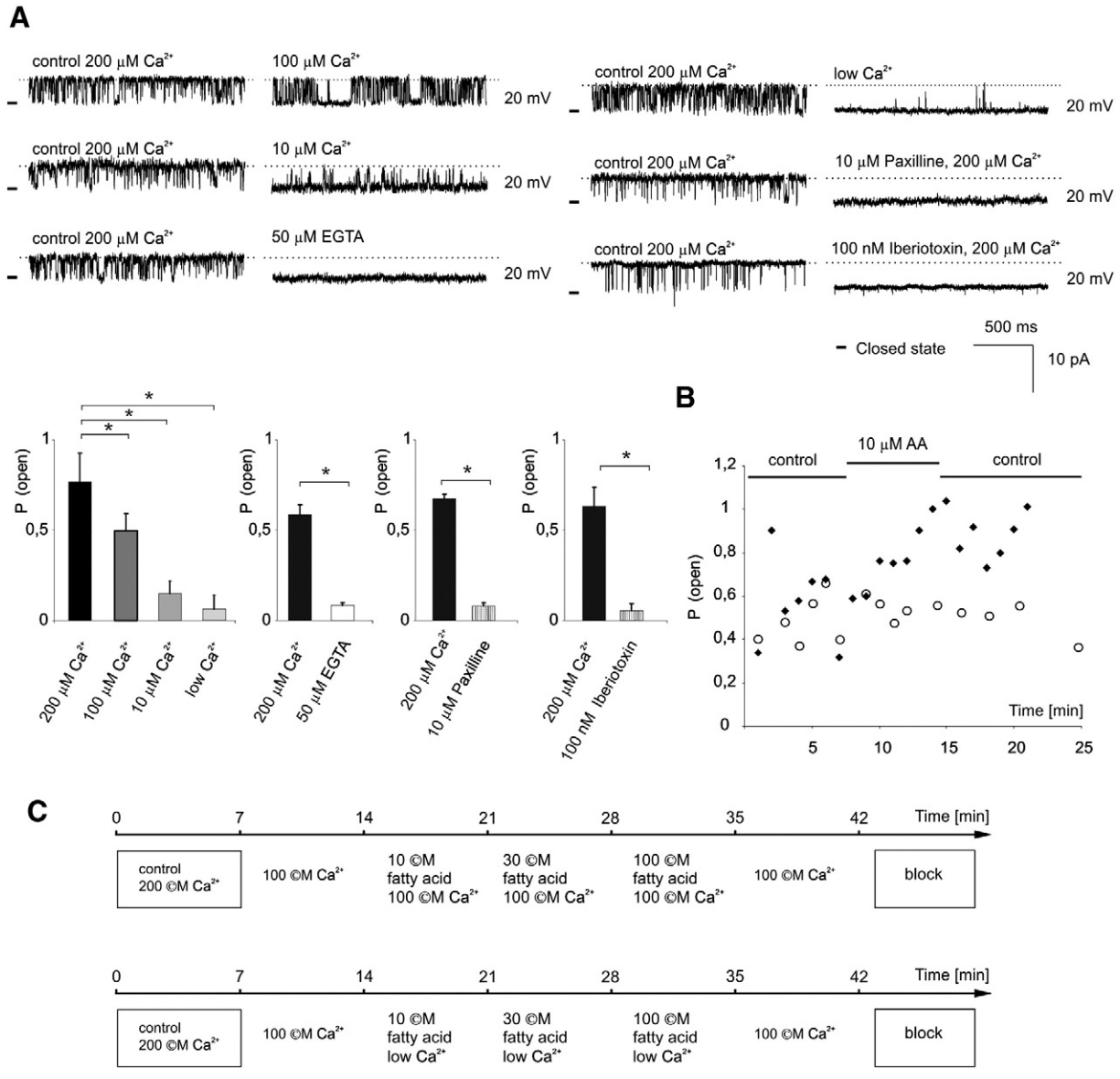

Fig. 1. Identification of the mitoBK$_{Ca}$ channel by single-channel recording in mitoplasts from human astrocytoma cells and schematic description of the experiments with the use of fatty acids. (A) Left panel shows Ca^{2+}-dependence and sensitivity to iberiotoxin and paxilline, inhibitors of the mitoBK$_{Ca}$. Symmetrical (150/150 mM KCl) solutions containing 200 μM Ca^{2+} (control) and, thereafter, 100 μM Ca^{2+}, 10 μM Ca^{2+}, 50 μM EGTA, low Ca^{2+} concentration (without added Ca^{2+}), 10 μM paxilline and 100 nM iberiotoxin. Dashes indicate the closed state, dotted lines the fully open state, $E_h = +20$ mV. Recordings are from one representative out of three independent experiments. Right panel shows mean open probability (P_o) (±SEM, n = 14). (B) Time course of P_o of the mitoBK$_{Ca}$ reveals that arachidonic acid slowly increased P_o. Control: isotonic solution (100 μM Ca^{2+}). AA: control plus 10 μM arachidonic acid. Recordings are from one representative out of three independent experiments. (C) Patch-clamp experiments were performed in seven parts of 7 min each. For control of Ca^{2+} dependence of the channel we started in isotonic solution (200 μM Ca^{2+}) then switching to isotonic solution (100 μM Ca^{2+} or low Ca^{2+}). Next, we applied test solutions with increasing concentrations of fatty acids (10 μM, 30 μM, and 100 μM) and finally tested washout of fatty acids in control solution. In sufficiently stable patches the channels were inhibited by specific blockers (paxilline, iberiotoxin) thereafter.

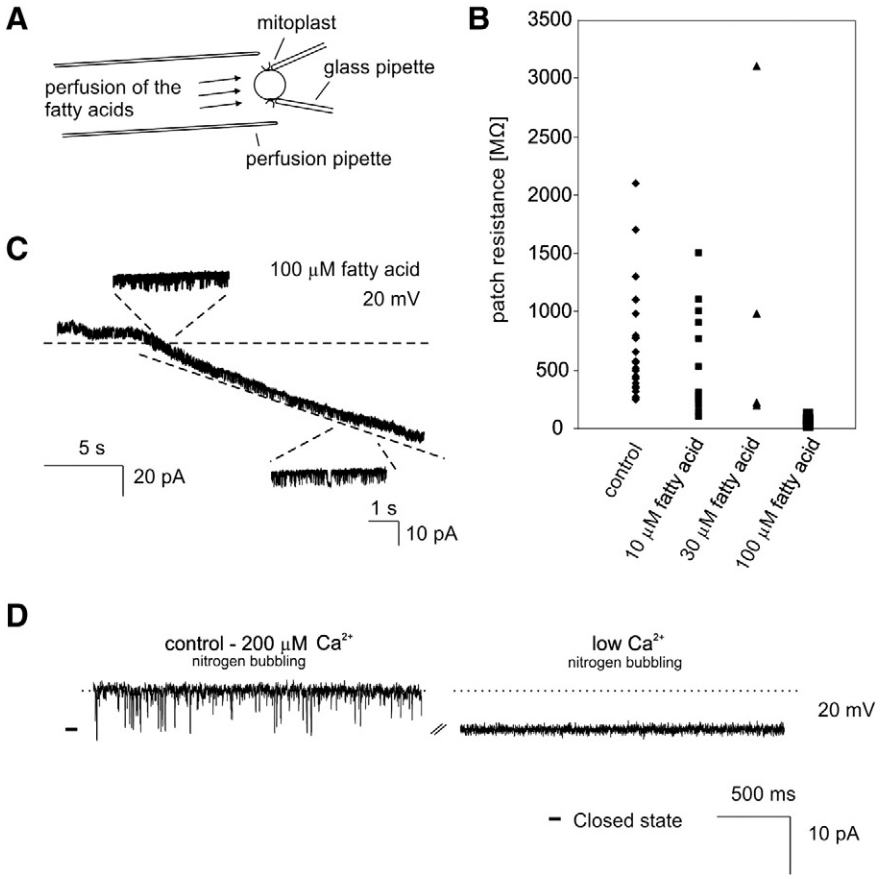

Fig. 2. Stability of the patch during the external perfusion with diluted fatty acids proves the integrity of the membrane. (A) Sketch of a patch pipette with a mitoplast in the mitoplast-attached mode. It was moved into different pipes of the flow system. (B) Patch resistance after adding 10 μM, 30 μM and 100 μM fatty acids. Data from twenty-one independent experiments. Resistance was determined using the pClamp10 software in the Ω_0-mode. (C) Single-channel recordings at +20 mV in symmetric (150/150 mM KCl) solution containing 100 μM fatty acid. Recordings are demonstrating the decreasing resistance of the patch (declining baseline) after application of the fatty acid (example after treatment of AA). An unchanged current amplitude of the channel indicates stable E_h. (D) Representative current traces before and after application of isotonic solution bubbled with nitrogen for 10 min.

increase was statistically significant and reversible ($p < 0.05$, $n = 4$; Fig. 3D, left panel). We also tested the AA action in an isotonic solution with a low Ca^{2+} concentration. We found that the addition of 10 μM or 30 μM AA did not alter the current amplitude (data not shown) or the P_o (Fig. 3D, right panel). An increase of the P_o appeared only after the addition of 100 μM AA. These data suggest that the action of AA is Ca^{2+} dependent. AA can affect ion channels either directly or by its enzymatic or non-enzymatic metabolites. The non-enzymatic metabolites of AA can be produced by oxygenation. To exclude the possibility that superoxide anions are responsible for the activation of AA, we examined the effect of AA in the presence of superoxide dismutase (SOD). SOD added alone (data not shown) or in parallel with AA changed neither the channel amplitude nor the conductance (Fig. 3C, D). To analyze the second possibility of AA action via its enzymatic products, we decided to use a non-metabolizable analog of AA, eicosatetraynoic acid (ETYA). ETYA has four triple bonds in the 20-carbon chain and it is also an inhibitor of cyclooxygenase and of lipoxygenase. The application of 10 μM ETYA did not affect the mitoBK$_{Ca}$. With the control solution and a solution containing 10 μM ETYA, the single-channel current traces were comparable (Fig. 4A). The channel amplitude was unchanged (data not shown), and the P_o at $E_h = +20$ mV was not significantly changed (Fig. 4B). Additionally, we made the same routine control as in experiments with the used of AA. The co-application of SOD with ETYA, as shown in Fig. 4C, had the same effect as application of ETYA alone. In summary, the results from the experiments suggest that AA cause reversibly mitoBK$_{Ca}$ channel activation and the oxidative products of AA do not play a role in this activation. Moreover mitoBK$_{Ca}$ channel is insensitive to ETYA, a fatty acid known as AA non-metabolizable analog.

3.3. mitoBK$_{Ca}$-sensitivity to 4-hydroxynonenal

To investigate whether the effect of AA on the mitoBK$_{Ca}$ depends on some products of AA metabolism, we conducted experiments using the final product of AA peroxidation. The primary products of these reactions are reactive aldehyde species, such as trans-4-hydroxy-2-nonenal (4-HNE). Fig. 5A presents the general peroxidation products of PUFAs and the biological effects of these products. Fig. 5B depicts current records showing that the application of 10 μM 4-HNE did not mimic the channel amplitude. Additionally, the data summarized in Fig. 5C show that the open probability at $E_h = +20$ mV was not significantly affected. In summary, our results suggest that the AA-induced stimulation of the mitoBK$_{Ca}$ is not mediated by the final product of AA peroxidation.

3.4. mitoBK$_{Ca}$-sensitivity to docosahexaenoic acid and eicosapentaenoic acid, n-6 PUFAs

After demonstrating AA effect on the mitoBK$_{Ca}$ channel, we examined the effect of other PUFAs. First, we used the docosahexaenoic acid (DHA). The application of 10 μM, 30 μM, and 100 μM DHA in an isotonic solution containing 100 μM Ca^{2+} did not significantly alter the P_o or the current amplitude of the mitoBK$_{Ca}$ (Fig. 6A). Fig. 6A depicts channel recordings of the control with 100 μM Ca^{2+} and after application of 10 μM and 30 μM DHA. In an isotonic solution without Ca^{2+}, but under otherwise unchanged conditions, the P_o of the channel increased in a dose-dependent manner after the application of 10 μM, 30 μM, and 100 μM DHA (Fig. 6B). This effect was reversible and statistically

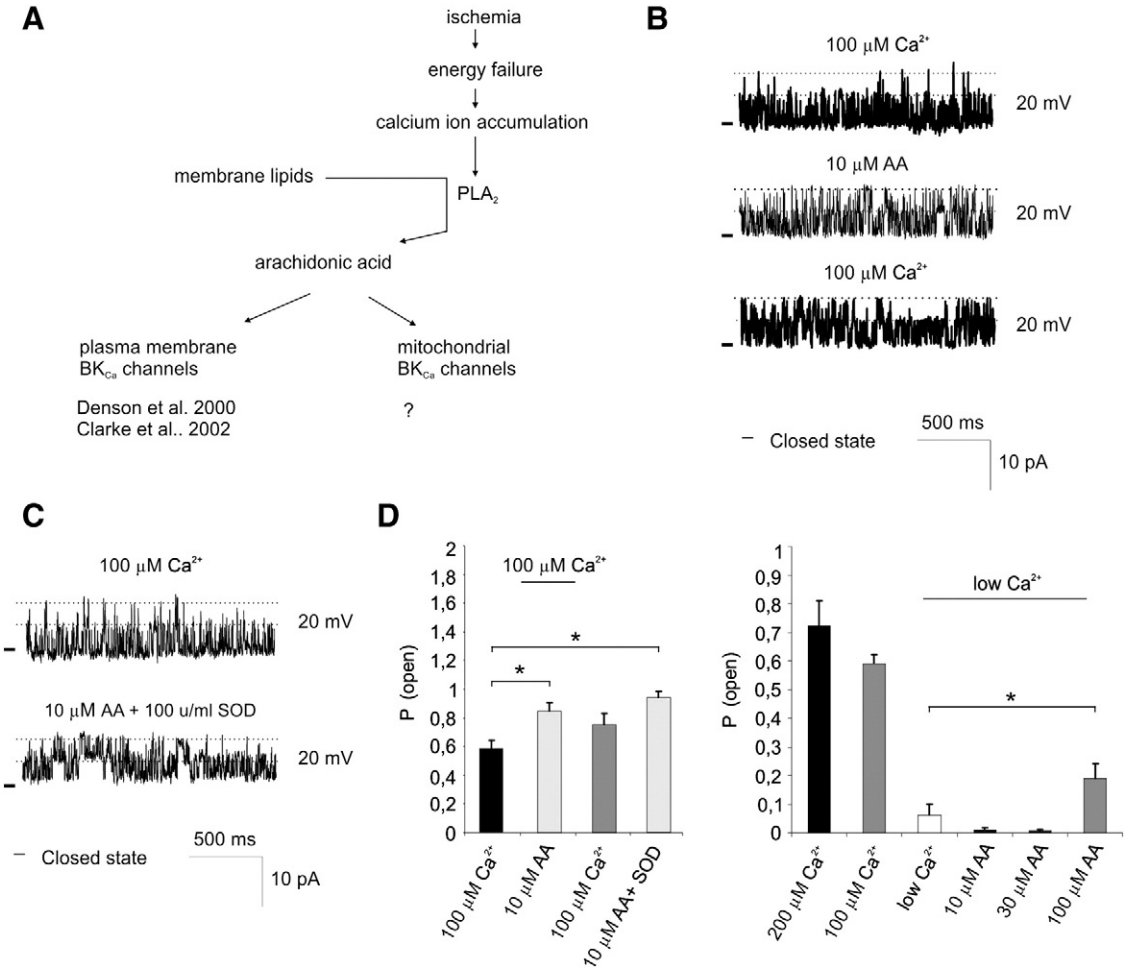

Fig. 3. The n-3 PUFA-arachidonic acid (AA) increases P_o of the mitoBK$_{Ca}$ with and without Ca^{2+} ions. (A) Mechanism of AA release from the plasma membrane during ischemia. Ischemia promotes intracellular Ca^{2+} accumulation and activation of phospholipases (PLA_2). PLA_2 promotes the release of AA from the membranes which then interact with ion channels. (B) Recordings at +20 mV in symmetrical (150/150 mM) KCl solutions (control, 100 μM Ca^{2+}), after application of 10 μM AA, and after washout without AA. Dashes indicate closed state. Dotted lines indicate current amplitudes of two mitoBK$_{Ca}$ channels present in this patch. Recordings are from one representative out of four independent experiments. (C) Current traces before and after application of 10 μM AA plus 100 U/ml superoxide dismutase (SOD). Recordings obtained from one representative out of three independent experiments. (D) Bar graphs summarizing the effect of AA on the mitoBK$_{Ca}$. Changes of the P_o of the mitoBK$_{Ca}$ after adding increasing concentrations of AA in the absence (n = 4) and in the presence of Ca^{2+} (n = 3). Mean values of independent experiments are indicated (±SEM), *$p < 0.05$.

significant ($p < 0.05$, n = 3). In the last step, we studied the effect of eicosapentaenoic acid (EPA). Our preliminary experiments in isotonic solutions with 100 μM Ca^{2+} produced the same results as those obtained with DHA; specifically, no channel activation or inhibition was observed (data not shown). Surprisingly, after the EPA application in a low-Ca^{2+} solution, the mitoBK$_{Ca}$ channel responded in two ways. In half of the experiments, 10 μM EPA increased the P_o of the channel significantly and reversibly (Fig. 7A) ($p < 0.05$, n = 3), and in the other half of the experiments, there was no significant response (Fig. 7B).

4. Discussion

Our studies are the first to reveal the presence of a PUFA-regulated BK$_{Ca}$ in the inner mitochondrial membrane of human astrocytoma cells. The major findings of our investigation are as follows: (1) the mitoBK$_{Ca}$ is sensitive to 10 μM AA in isotonic solutions with high Ca^{2+} concentrations (at low Ca^{2+} concentrations, only the addition of 100 μM AA is sufficient to activate the channel); (2) the mitoBK$_{Ca}$ is insensitive to 10 μM ETYA and 4-HNE (10 μM to 100 μM); (3) the oxidative products of AA do not play a role in the activation of the mitoBK$_{Ca}$; and (4) the mitoBK$_{Ca}$ is activated by DHA and EPA only at low Ca^{2+} concentrations.

PUFAs can modulate a variety of K-channels from the plasma membrane. These PUFAs may act via directly binding to a channel protein or to an auxiliary protein that regulates the channel. Another possibility is that PUFAs alter the mechanical properties of the lipid bilayer after their insertion into the membrane. This latter type of regulation and the regulation of K-channels via the metabolic products of FAs are indirect forms of regulation. Based on the literature, there does not appear to be a general mechanism of PUFA action on the K-channels of the plasma membrane.

Because the effects of the FAs on mitoBK$_{Ca}$ channel were observed in our experiments only after 3–4 min, we could speculate that the effect depends on the indirect mechanism. FAs with different potencies to change the membrane structure should be evaluated using the patch-clamp technique to test their ability to modulate the mitoBK$_{Ca}$. Based on research involving GH_3 cells [27], it has been concluded that there is no correlation between the ability of a FA to change membrane fluidity and its ability to alter the activity of the BK$_{Ca}$. Following the application of ETYA, a smaller change in membrane fluidity was observed than that observed following the application of AA. However, the change in the P_o values was not in accordance with this effect [27].

The next problem is that FAs can flip across the plasma membrane; thus, it is difficult to localize the site of their action. The BK$_{Ca}$ channels from airway smooth muscle cells [34] and from rabbit pulmonary

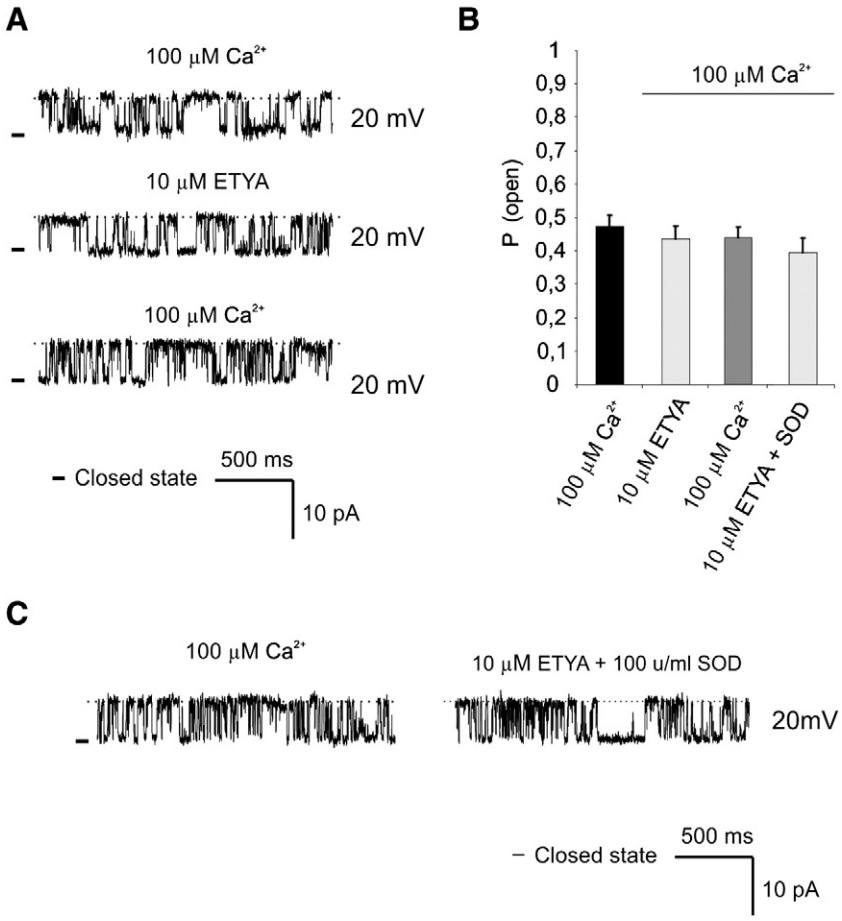

Fig. 4. Non-metabolizable analog of arachidonic acid, eicosatetraynoic acid (ETYA) has no significant effect on the mitoBK$_{Ca}$ in the presence of Ca^{2+}. (A) Single-channel recordings at $E_h = +20$ mV in symmetrical (150/150 mM) KCl solutions containing 100 μM Ca^{2+} (control), in isotonic solution containing 100 μM Ca^{2+} plus 10 μM ETYA, and in control with 100 μM Ca^{2+} but without ETYA. Dashes indicate closed state. Dotted lines indicate current amplitude of the fully open state. Recordings are from one representative out of four independent experiments. (B) Bar graph demonstrating that ETYA has no effect on the P_o of the mitoBK$_{Ca}$ in the presence of Ca^{2+}. Mean values of independent experiments are indicated (±SEM, n = 4). (C) Representative current traces recorded before and after the application of 10 μM ETYA plus 100 U/ml SOD. Recordings from one representative out of three independent experiments.

arteries [28] are known to be sensitive to some FAs when the FAs are applied to the extracellular side of the membrane. In GH_3 cells, the opposite phenomenon has been observed; specifically, the BK_{Ca} was active when the AA co-enzyme was applied from the cytosolic side [27]. This latter effect was also observed with the small-conductance K-channel from the smooth muscle cells of the toad stomach [35]. Such

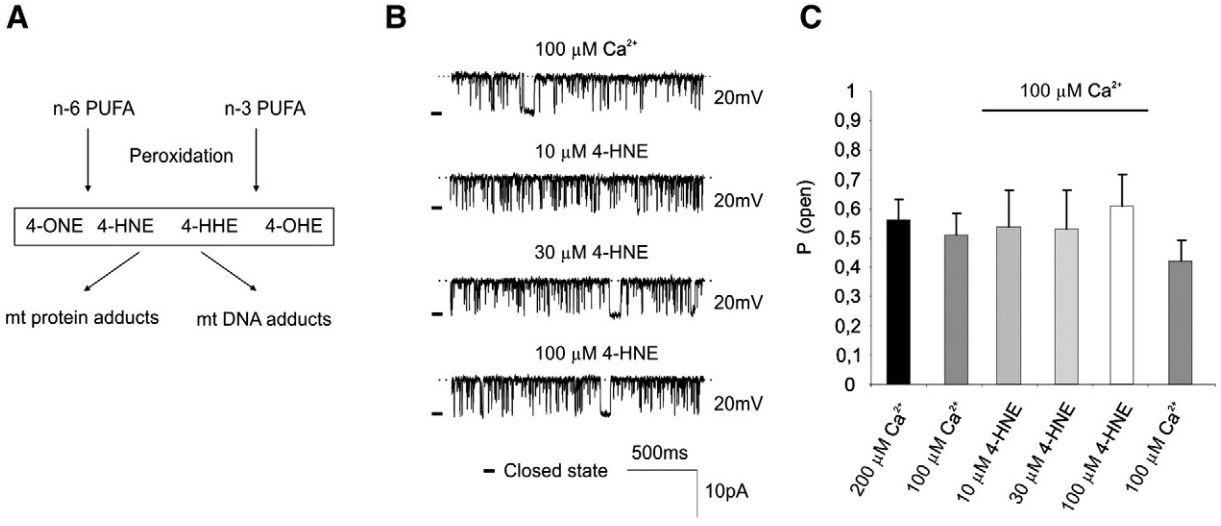

Fig. 5. The last metabolic product of arachidonic acid, 4-hydroxynonenal (4-HNE) has no significant effect on the mitoBK$_{Ca}$ in the presence of Ca^{2+}. (A) The lipid peroxidation product 4-HNE can diffuse into the cytoplasm resulting in protein modification that causes altered protein activity. Additionally, 4-HNE can form adducts with DNA, resulting in mispairing. (B) Single-channel current of the mitoBK$_{Ca}$ with 100 μM Ca^{2+} plus different concentrations of 4-HNE. Recordings are from one representative out of four independent experiments. (C) Bar graph demonstrating missing effect of different 4-HNE concentrations on the P_o after adding 10 μM, 30 μM, and 100 μM 4-HNE. Mean values of independent experiments are indicated (±SEM, n = 4).

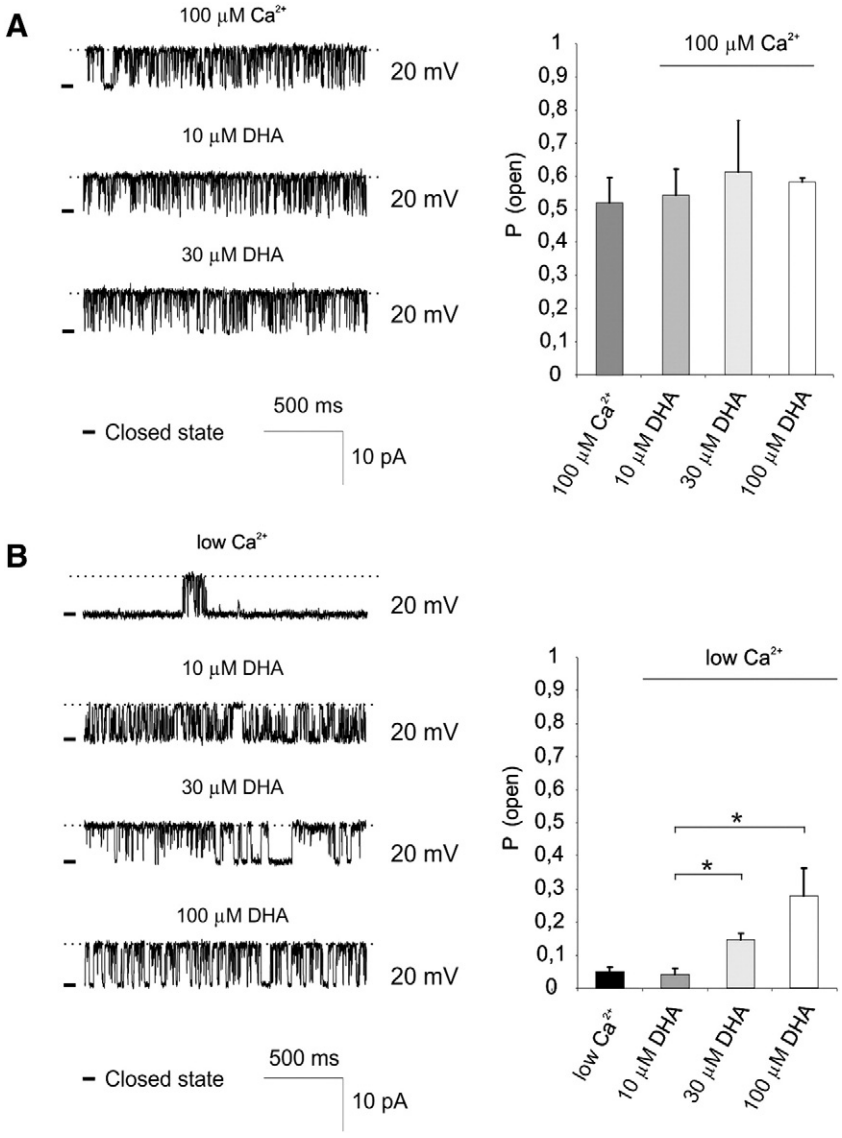

Fig. 6. The n-6 PUFA-docosahexaenoic acid (DHA) increases the P_o of the mitoBK$_{Ca}$ only in the absence of Ca^{2+}. (A) Left panel shows recordings in symmetrical (150/150 mM KCl) solution (control, +100 μM Ca^{2+}), and in isotonic solutions (+100 μM Ca^{2+}) containing 10 μM and 30 μM DHA. $E_h = +20$ mV. Recordings are from one representative out of three independent experiments. Right panel shows mean P_o (±SEM, n = 3). (B) Left panel shows recordings under the same conditions as in A but without added Ca^{2+}, and after application of isotonic solutions with 10 μM, 30 μM, and 100 μM DHA. Recordings from one representative out of three independent experiments. Right panel shows mean P_o (±SEM, n = 3). Closed state in A and B indicated by dashes; dotted lines indicate current amplitude of the fully open state. *$p < 0.05$.

discrepancies may indicate a more direct mechanism of FA action rather than a nonspecific change in the membrane environment. Finally, the action of PUFAs may depend on the Ca^{2+} concentration. Due to the strong negative membrane potential the mitochondrial matrix physiologically contains many more Ca^{2+} ions than the intermembrane space does. The Ca^{2+} binding site of the mitoBK$_{Ca}$ is located on the inner site of the membrane. As Fig. 6 shows a less pronounced DHA effect at high [Ca^{2+}] a direct modulation of the mitoBK$_{Ca}$ by PUFAs appears to be more likely.

Conversely, certain structural features of the FAs enable the FAs to interact with the plasmalemmal BK$_{Ca}$. These structural requirements include the length of the carbon chain, the number of unsaturated bonds, the protein conformation, and the charge [27,28]. Horimoto et al. observed that FAs with more than two double bonds are able to activate K-channels from cortical neurons [36]. Similar observations were described for the BK$_{Ca}$ from gastric myocytes [37]. Collectively, these results again support the idea of the direct regulation of the channel by FAs.

K-, Na-, Ca-, and Cl-channels are all modulated either by AA directly or by one of the metabolites of AA [33]. Our results indicate for the first time that the mitoBK$_{Ca}$ is sensitive to AA. The addition of ETYA, which is a non-metabolizable AA analog, did not cause a significantly different effect on the channel. This should incline us to the conclusion that AA effect is indirect, but it is too far-reaching conclusion and needs further evidence.

We also considered the fact that the effect of the PUFAs may be due to the formation of reactive oxygen species. Based on a review of the literature, we know that the skeletal muscle BK$_{Ca}$, when incorporated into artificial membranes or expressed in oocytes, displays a decreased number of active channels and a reduced P_o when exposed to hydrogen peroxide [38]. Thus, we used a free radical scavenger together with AA or ETYA (Figs. 4, 5). Our data suggest that the oxidation products of AA are not involved in the activation of the mitoBK$_{Ca}$.

To further test the hypothesis that the action of the mitoBK$_{Ca}$ is modulated by the metabolic products of AA, we performed experiments using 4-HNE. ROS produced by the mitochondrial respiratory chain and the products of the metabolism of AA by LOX can react with the double bonds of the FAs to produce peroxides, such as unsaturated aldehydes [39]. 4-HNE is a toxic product of the peroxidation of membrane

PUFAs, mainly AA. The interactions of 4-HNE with the PTP [40] and cytochrome c oxidase [41] can induce mitochondrial uncoupling via the uncoupling protein, UCP [42]. This mild uncoupling limits the production of ROS in the mitochondria. Therefore, we attempted to determine whether this mild uncoupling may also be due to the interaction of FAs with mitochondrial K-channels. Neither the addition of 10 μM 4-HNE nor the addition of higher concentrations of 4-HNE (30 μM and 100 μM) altered the P_o of the mitoBK$_{Ca}$. Since mitoBK$_{Ca}$ was insensitive to the final peroxidation product of AA metabolism, the effect of AA must be either direct or connected with other products of AA metabolism.

The other FAs that we analyzed are the cell-protective n-3 PUFAs, such as DHA and EPA. Several reports describe the protective effect of DHA in ischemia and spinal cord injury. Unfortunately, the protective mechanism is not yet understood. Treatment with DHA reduced the total infarct volume and potentiated the synthesis of neuroprotectin D1 (NPD1) in the penumbra following focal cerebral ischemia [21]. The administration of DHA also reduced the occurrence of brain

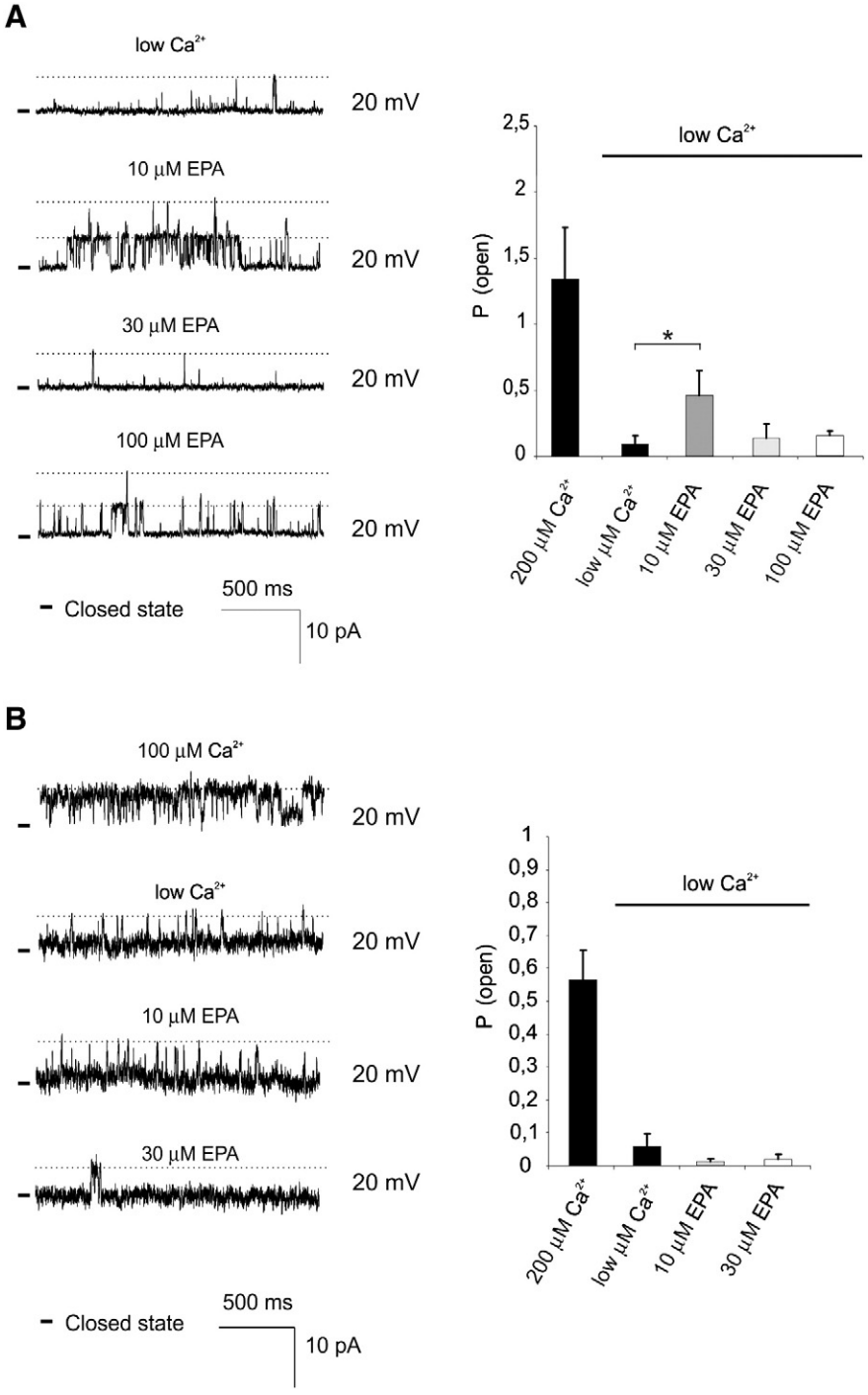

Fig. 7. The n-6 PUFA - eicosapentaenoic acid (EPA) inhibits the P_o of the mitoBK$_{Ca}$ in the presence of a low Ca^{2+}. (A) Left panel shows single-channel recordings in symmetric (150/150 mM) KCl solution (control) with low Ca^{2+}-concentration, and in solutions with 10 μM and 30 μM EPA. $E_h = +20$ mV. Recordings are from one representative out of five independent experiments. Right panel shows mean P_o (±SEM; n = 5) under the same conditions. (B) Left panel shows recordings under the same conditions described in A but with a low Ca^{2+} and in isotonic solutions with 10 μM, 30 μM, and 100 μM EPA. Recordings from one representative out of three independent experiments. The right panel shows the P_o (±SEM, n = 3) under the same conditions. Dashes in A and B indicate closed state. Dotted lines indicate amplitude of the fully open state(s). *$p < 0.05$.

infarction and edema. Treatment of the hippocampal tissue with DHA has a neuroprotective effect [21]. DHA activates the BK_{Ca} current in rat coronary arterial smooth muscle cells [43]. EPA provides protective effects following brain ischemia, inhibits the inflammatory reaction and oxidative damage, and prevents memory impairment [25]. Denson and co-workers demonstrated that EPA and all FAs containing *cis* double bonds are able to activate the BK_{Ca} from the GH_3 cell line. Following the application of 10 μM to 100 μM DHA, the open probability of the $mitoBK_{Ca}$ increased in a dose-dependent manner. Interestingly, the application of EPA caused $mitoBK_{Ca}$ activation in half of the experiments and had no effect in the other experiments.

The results presented here may have implications under certain pathophysiological conditions. Deregulated lipid metabolism in the central nervous system was observed in patients with various neurological disorders. Membrane lipid degradation was shown to be an important factor contributing to cell damage. Furthermore, the cells that undergo ischemia were found to have an elevated level of free FAs. There is also a strong link between mitochondrial function and the presence of PUFAs. Therefore, we investigated the influence of FAs on cell-protective mitochondrial K-channels.

Collectively, our experiments indicate that the observed large-conductance calcium-activated potassium channel from human astrocytoma U87 MG cells is sensitive to arachidonic acid or enzymatic metabolites of arachidonic acid. Furthermore, the channel is sensitive to eicosapentaenoic acid and docosahexaenoic acid.

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