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Biochimie Open 4 (2017) 78–87

http://www.journals.elsevier.com/biochimie-open

Phosphorylation of purified mitochondrial Voltage-Dependent Anion Channel by c-Jun N-terminal Kinase-3 modifies channel voltage-dependence

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Received 5 December 2016; revised 2 March 2017
Available online 10 March 2017

Abstract

Voltage-Dependent Anion Channel (VDAC) phosphorylated by c-Jun N-terminal Kinase-3 (JNK3) was incorporated into the bilayer lipid membrane. Single-channel electrophysiological properties of the native and the phosphorylated VDAC were compared. The open probability versus voltage curve of the native VDAC displayed symmetry around the voltage axis, whereas that of the phosphorylated VDAC showed asymmetry. This result indicates that phosphorylation by JNK3 modifies voltage-dependence of VDAC.
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Keywords: Phosphorylation; Voltage-Dependent Anion Channel; c-Jun N-terminal Kinase-3; Bilayer electrophysiology

1. Introduction

Post-translational modifications like phosphorylation, nitrosylation etc. regulate the activity of ion channels in cells [1]. The effect of phosphorylation on the gating of ion channels purified from tissues can be checked *in vitro* by incorporating them on the artificially prepared bilayer lipid membranes (BLM) by two different ways. One way is to carry out the *in vitro* phosphorylation reaction and then study the electrophysiological properties of the phosphorylated channel by incorporating it into the BLM [2,3]. The other is to first incorporate the ion channel into the BLM and then carry out the phosphorylation reaction [4–7]. The latter has been studied by us where we have reported that phosphorylation of rat brain purified outer mitochondrial membrane Voltage-Dependent Anion Channel (VDAC) by c-Jun N-terminal Kinase-3 (JNK3) enzyme leads to closure of the channel [7]. However, the former, we believe, is important as it would help understanding the ion channel structure–function relationship at the molecular level [2,3].

VDAC is a porin which is present at the nuclear envelope, endosomes, plasma membrane, at the sarcoplasmic/endoplasmic reticulum membrane and at the outer mitochondrial membrane in cells [8]. The structure of VDAC consists of an N-terminal α-helix and a β-barrel cylinder which forms its lumen or pore [8]. At the outer mitochondrial membrane, it controls the transport of ions, adenine nucleotides like ATP and energy related metabolites between the mitochondria and the cytosol by voltage-dependent gating. At voltages $\leq \pm 20$ mV VDAC remains in open-state and favors anion transport, whereas at voltages >20 mV it preferably displays steady lower-conductance closed state(s) (VDAC closed state(s) are not fully closed and are also known as sub-states) which favor cation transport. Also, VDAC has been shown to

Abbreviations: Phospho-VDAC, Phosphorylated Voltage-Dependent Anion Channel; JNK3, c-Jun N-terminal Kinase-3; MAPK, mitogen activated protein kinase; BLM, bilayer lipid membrane.

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http://dx.doi.org/10.1016/j.biopen.2017.03.002
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be important in promoting cytochrome *c* release during mitochondrion-mediated apoptosis by different proposed mechanisms [9]. JNKs are cytosolic mitogen activated protein kinases (MAPKs) which regulate normal physiological functions like immune responses, cell and tissue morphogenesis and also they are involved in pathological processes [10,11]. JNKs are activated by phosphorylation and after activation translocate towards mitochondrion in cells. There are indirect evidences which suggest that activation of JNKs by phosphorylation during mitochondrial apoptosis can lead to phosphorylation of VDAC. A parallel increase in the levels of phosphorylated VDAC, phosphorylated JNKs and cytosolic cytochrome *c* has been reported during mitochondrion-mediated apoptosis in renal ischemia-reperfusion injury [10]. Furthermore, in cervical cancer cells, arsenic oxide treatment has been shown to result in induction of mitochondrion-mediated apoptosis with JNK1/2 activation and homodimerization of VDAC [11]. JNK3 isoform of JNKs has been shown to get activated in brain cells during neurodegeneration [12]. After activation JNK3 translocates toward mitochondrion and results in biochemical modulation of outer mitochondrial membrane proteins leading to apoptosis [12]. In the present work JNK3 phosphorylated purified mitochondrial VDAC has been incorporated into the BLM and its single-channel electrophysiological properties have been studied.

2. Materials and methods

2.1. Purification of VDAC

VDAC was purified from rat brain mitochondria by standard method [7,13]. Purified mitochondria were allowed to swell by resuspending them in a chilled hypotonic solution containing 10 mM Tris−HCl (pH 7.4) and 1 mM K^+-EDTA for 15 min. Meanwhile, mitochondrial concentration (mg/ml) was determined. Mitochondrial suspension was centrifuged at 27,000*g* for 10 min. The swollen mitochondrial pellet was gently resuspended in a solution containing 10 mM Tris−HCl, 1 mM K^+-EDTA and 3% (v/v) Triton X-100 detergent (pH 7.4) at a final concentration of 5 mg/ml. Triton X-100 gently solubilizes outer mitochondrial membrane proteins and VDAC only forms Triton X-100-Lipid-VDAC micelles at 5 mg/ml concentration. The suspension was centrifuged at 44,000*g* for 30 min. Supernatant was loaded on a hydroxyapatite (0.1 g/mg mitochondria):Celite (2:1 w/w) dry column and pure VDAC was eluted from the column in the initial 1 ml fractions. Permission for this experiment was obtained from the Committee for the Purpose of Control and Supervision of Experiments on Animals, India.

2.2. Phosphorylation of VDAC

Phosphorylation of purified VDAC by JNK3 was carried out and checked using Pro-Q Diamond dye method as standardized in our laboratory [7,14]. Pro-Q Diamond dye binds to the phosphate groups non-specifically and thus identifies phosphorylated proteins distinctly from the unphosphorylated ones. Initial fractions obtained from the hydroxyapatite:celite column containing high amounts of purified VDAC were chosen for the phosphorylation reaction. Triton X-100 shields VDAC in purified preparations which would reduce VDAC phosphorylation by JNK3 but it is necessary to maintain the solubility of VDAC. VDAC (600 μl) was equally divided and added into three eppendorf tubes corresponding to Negative Control (VDAC + Mg^{2+}ATP), Experimental Sample (VDAC + JNK3 + Mg^{2+}ATP) and Positive Control [VDAC + JNK3 + Mg^{2+}ATP + Alkaline Phosphatase (added later)]. 0.32 μl (0.5 mg) dually phosphorylated His-JNK3 enzyme (Enzo Life Sciences, USA), ATP (Final concentration 100 mM) and $MgCl_2$ (Final concentration 10 mM) were added to the respective tubes. Phosphorylation reaction was carried out by incubating the cocktail at 30 °C for 30 min. In the positive control, 0.5 μl (0.1 mg) of calf intestinal alkaline phosphatase prepared in 10 mM HEPES−KOH buffer (pH 7.4) was added and incubated for another 30 min at 30 °C. The samples were precipitated using chloroform:methanol mixture. Protein pellets were air dried and dissolved in 1× sample buffer. All the tubes were boiled for 5 min at 100 °C along with the peppermint stick phosphoprotein molecular weight standards (Molecular probes, Inc., Eugene, OR, USA), 2 μl in 12 μl of 1× sample buffer. Samples were resolved on 12.5% SDS-PAGE at a constant 100 V and the gel was fixed in 50% Methanol and 10% Acetic acid for 30 min on a shaker. Fixative was replaced with fresh one and left overnight. Next day, gel was washed thrice with Milli-Q water for 15 min each. It was stained using fluorescent Pro-Q Diamond phosphoprotein gel stain (Molecular probes, Inc., Eugene, OR, USA) for 2 h and then de-stained with Pro-Q Diamond phosphoprotein destaining solution (Molecular probes, Inc., Eugene, OR, USA) for 1.5 h thrice for 30 min each time in a dark room on the shaker. The gel was washed thrice for 5 min each with Milli-Q water and visualized on FLA-9000 phosphoimager (Fuji Fim Inc., Tokyo, Japan). After visualization, the gel was silver stained to show VDAC was loaded equally in all the wells.

2.3. Single-channel bilayer electrophysiological recordings of native and phosphorylated VDAC

Native and Phosphorylated VDAC were incorporated into the BLM as standardized in our laboratory [5−7,14]. The apparatus consisted of a perfusion BLM cup (Warner Instruments Corp., Hamden, CT) made up of polystyrene with a thin wall separating two aqueous compartments (cis and trans) of BLM chamber (Warner Instruments) containing BLM buffer [1 M KCl, 10 mM $MgCl_2$, 10 mM HEPES (pH 7.4)]. The BLM cup had a circular aperture with a diameter of 150 μm. Aqueous compartments were connected to an amplifier (Axopatch 200B, Molecular devices, CA, USA) through a pair of matched Ag/AgCl electrodes. The voltage in the trans-compartment was held at virtual ground by the amplifier and the cis compartment was connected to the headstage for applying the desired voltage. Lipid mixture containing DPhPE (1,2-DiPhytanoyl-*sn*-glycero-3-PhosphoEthanolamine) and DPhPC (1,2-DiPhytanoyl-*sn*-glycero-3-PhosphatidylCholine)

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