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INHIBITION OF MITOCHONDRIAL Na^+-DEPENDENT Ca^{2+} EFFLUX BY 17β-ESTRADIOL IN THE RAT HIPPOCAMPUS

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Abstract—Our results, as well as those of others, have indicated that 17β-estradiol (E2) exerts its nongenomic effects in neuronal cells by affecting plasma membrane Ca^{2+} flux. In neuronal cells mitochondria possess Ca^{2+} buffering properties as they both sequester and release Ca^{2+}. The goal of this study was to examine the rapid non-genomic effect of E2 on mitochondrial Ca^{2+} transport in hippocampal synaptosomes from ovariectomised rats. In addition, we aimed to determine if, and to what extent, E2 receptors participated in mitochondrial Ca^{2+} transport modulation by E2 *in vitro*. E2-specific binding and Ca^{2+} transport was monitored. At physiological E2 concentrations (0.1–1.5 nmol/L), specific E2 binding to mitochondria isolated from hippocampal synaptosomes was detected with a $B_{max.}$ and K_m of 37.6±2.6 fmol/mg protein and 0.69±0.14 nmol/L of free E2, respectively. The main mitochondrial Ca^{2+} influx mechanism is the Ruthenium Red-sensitive uniporter driven by mitochondrial membrane potential. Despite no effect of E2 on Ca^{2+} influx, a physiological E2 concentration (0.5 nmol/L) protected mitochondrial membrane potential and consequently Ca^{2+} influx from the uncoupling agent carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (1 μmol/L). In neuronal cells the predominant mitochondrial Ca^{2+} efflux mechanism is the Na^+/Ca^{2+} exchanger. E2 caused Ca^{2+} efflux inhibition (by 46%) coupled with increased affinity of the Na^+/Ca^{2+} exchanger for Na^+. Using E2 receptor (ERα and ERβ) antagonists and agonists, we confirmed ERβ's involvement in E2-induced mitochondrial membrane potential protection as well as Ca^{2+} efflux inhibition. In summary, our results indicate that the non-genomic neuromodulatory role of E2 in rat hippocampus is achieved by affecting mitochondrial Ca^{2+} transport via, in part, mitochondrial ERβ.

Key words: mitochondria, Ca^{2+} transport, estradiol, estradiol receptors, rat hippocampus.

Intracellular divalent calcium (Ca^{2+}) is an important component of signal transduction pathways in a variety of cell types, especially excitable cells. In neurons, Ca^{2+} serves as a universal messenger to modulate many specific activities such as rapid channel functions, neurotransmitter release and synaptic plasticity (Smith and Augustine, 1988; Miller, 1991) as well as many physiological processes during cell metabolism, differentiation and cell death (Kirichok et al., 2004; Yano et al., 2004). Neuronal Ca^{2+} homeostasis is maintained by controlling Ca^{2+} movement across the plasma membrane (Blaustein and Ector, 1976) and/or by Ca^{2+} sequestration within the endoplasmic reticulum and mitochondria (McGraw et al., 1980).

Transient changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in neurons are largely determined by mitochondria due to their large Ca^{2+} storage capacity. Mitochondrial Ca^{2+} influx is driven by the mitochondrial membrane potential ($\Delta\psi_m$) and occurs at the threshold of $[Ca^{2+}]_i$, followed by slow efflux leading to a net accumulation of mitochondrial calcium ($[Ca^{2+}]_m$) and transient changes in physiological $[Ca^{2+}]_i$ (Kann and Kovács, 2007). Mitochondrial dysfunction often leads to deregulation of Ca^{2+} homeostasis and downstream effects, including further mitochondrial damage and initiation of the mitochondrial spiral that is associated with multiple CNS disorders (Blass, 2000).

The mitochondrial Ca^{2+} flux in neuronal cells mainly depends on the activity of two distinct components: Ca^{2+} influx by the Ruthenium Red (RR)-sensitive uniporter and Ca^{2+} efflux by the Na^+/Ca^{2+} exchanger. The RR-sensitive uniporter, a specific Ca^{2+} channel in the inner mitochondrial membrane, permits Ca^{2+} to flow into the mitochondrial matrix down its steep electrochemical gradient, and its activity enables mitochondria to act as temporary Ca^{2+} sinks after depolarisation-induced $[Ca^{2+}]_i$ elevation in neurons (Colegrove et al., 2000). In addition, a low basal $[Ca^{2+}]_m$ in neurons is predominantly maintained by Na^+/Ca^{2+} exchanger activity, which is the main Ca^{2+} efflux mechanism in neurons (Gunter and Pfeiffer, 1990; Cox et al., 1993). Due to their Ca^{2+} transport mechanisms, mitochondria are organelles critical for Ca^{2+} buffering in neuronal cells. The $[Ca^{2+}]_m$ regulates the rate of respiration and ATP production, generation of reactive oxygen species, occurrence of $\Delta\psi_m$ collapse, and is a critical trigger for the opening of the permeability transient pore and

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Abbreviations: $[Ca^{2+}]_i$, intracellular calcium concentrations; $[Ca^{2+}]_m$, mitochondrial calcium concentration; CGP, 7-chloro-5-(2-chlorophenyl)-1,5-dihyhdro-4,1-benzothiazepin-2(3*H*)-one; DPN, 2,3-*bis*(4-hydroxyphenyl)-propionitrile; E-BSA, 17β-estradiol conjugated to bovine serum albumin; ECL, enhanced chemiluminescence; EDTA, ethylenediamine tetraacetic acid; E2, 17β-estradiol; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; ICI 182,780, 7α,17β-[9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol; OVX, bilateral ovariectomy; PPT, 4,4′,4″-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl) trisphenol; PVDF, polyvinylidene fluoride; RH 123, rhodamine 123; RR, Ruthenium Red; $\Delta\psi_m$, mitochondrial membrane potential.

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therefore for apoptosis (Rutter and Rizzuto, 2000; Rizzuto et al., 2003).

The steroid hormone, estradiol (E2) can modulate various processes in the brain. The hippocampus is an important structure responsible for attentional functions, learning, memory and mood and behavioral modulation, and is therefore a likely target for hormonal influences (Foy, 2001). Estrogens may exert their potent neuroprotective effects among the others via mitochondrial mechanisms. Xiao-Wu Xu and colleagues (Xu et al., 2008) found that estrogen (and phytoestrogen) could reverse the alteration of mitochondrial ultrastructure and ATP content in the hippocampal CA1 region caused by ovariectomy. Consistent with this observation was the report that estrogen therapy increased ATP in hippocampal neurons (Birge, 1997). In human neuroblastoma, estrogen protects mitochondrial function from hydrogen peroxide-mediated toxicity by preserving mitochondrial membrane potential, attenuating ATP depletion and ablating mitochondrial Ca^{2+}overloading (Wang et al., 2006).

Our previous work using synaptosomal mitochondria isolated from whole rat brains indicated that E2 affected mitochondrial Ca^{2+} transport (Horvat et al., 2000). In this work, we sought to determine if E2, in a non-genomic manner, could influence mitochondrial Ca^{2+} transport in the hippocampus. To examine the mechanism(s) of E2 action, we analysed (*i*) specific E2 binding to mitochondria isolated from hippocampal synaptosomes and its contribution to the modulation of mitochondrial Ca^{2+} transport and (*ii*) using an impermeable E2 conjugate to bovine serum albumin and ERs antagonist and specific agonists, the contribution of ERα and/or ERβ in Ca^{2+} transport modulated by E2. The observed effects on the protection of mitochondrial membrane decline as well as on mitochondrial Ca^{2+} sequestration may be a consequence of membrane binding sites and/or mitochondrial estradiol receptors (ERα and/or ERβ) activation.

Our results have shed light on the pathways through which E2 regulates mitochondrial Ca^{2+} sequestration and consequently modulation of global cell Ca^{2+} homeostasis in hippocampal neurons.

EXPERIMENTAL PROCEDURES

Mature, chronically (3 weeks prior to use) ovariectomised (OVX) female Wistar rats were used in the experiments. The animals were derived from special animal breeding unit of our laboratory and were maintained under constant conditions (light on: 05.00–17.00 h, temperature 24 °C, free access to food and water). All procedures were approved by the ethics committee of the Serbian Association for the Use of Animals in Research and Education and are in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

(2,4,6,7-^{3}H)estradiol (specific activity 83.7 Ci/mmol) and $^{45}CaCl_2$ (specific activity 16.8 mCi/mg) were purchased from PerkinElmer (Massachusetts, USA).

Rhodamine 123 (RH 123) was purchased from Molecular Probes (Eugene, OR, USA). Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3*H*)-one (CGP), 7α,17β-[9[(4,4,5,5,5-pentafluoropentyl) sulfinyl]nonyl] estra-1,3,5(10)-triene-3,17-diol (ICI 182,780), 4,4′,4″-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl) *tris*phenol (PPT) and 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) were purchased from Tocris Cookson Ltd. (Ellisville, MO, USA). Diltiazem, nifedipine, 17β-estradiol (E2), 17β-estradiol conjugated to bovine serum albumin (E-BSA) and other chemicals were purchased from Sigma, Chemical Co. (St Louis, MO, USA). E-BSA was filtered before use to eliminate free E2.

Cellulose nitrate filters (pore size, 0.45 μm) were from Whatman International (Kent, ME, USA).

Preparation of synaptosomal mitochondria

Rats were sacrificed by cervical dislocation and their brains were removed on ice. Hippocampi were isolated and homogenised in ice-cold buffered sucrose (0.32 mol/L sucrose and 5 mmol/L Tris–HCl, pH 7.4). Synaptosomes were isolated and purified from pools of hippocampi (10 hippocampi/pool). Purified synaptosomes were obtained using Ficoll gradient: 7.5% and 13% resolved in medium containing 0.32 mol/L sucrose, 10 mmol/L Tris–HCl, pH 7.4 and 50 μmol/L potassium-EDTA, according to the method of Gray and Whittaker (1962) modified by Cotman and Matthews (1971). Purified synaptosomes were lysed by resuspension in 5 mmol/L Tris–HCl and freezing at −20 °C overnight.

Synaptosomal mitochondria used for Ca^{2+} transport measurements and E2 binding were prepared from synaptosomal lysate according to the procedure of Lai and Clark (1970). A discontinuous gradient consisting of 4.5% and 6% Ficoll was used. Isolated synaptosomal mitochondrial pellets were resuspended in 0.3 mol/L mannitol and kept at −20 °C. Our experience has shown that mitochondria stored this way stay suitable for subsequent manipulations and analyses. Protein was determined by the method of Lowry et al. (1951) as modified by Markwell et al. (1978).

Estradiol binding assay

The E2 binding was examined in isolated synaptosomal mitochondria obtained from hippocampi of OVX rats. The assay was performed in same conditions as already published for synaptosomal mitochondria isolated from whole rat brain (Horvat et al., 2001). The medium contained: 300 mmol/L mannitol, 10 mmol/L KCl, 1 mmol/L maleate, 5 mmol/L glutamate and 10 mmol/L Tris–HCl, pH 7.4 in a final volume of 200 μl in which mitochondrial respiration (coupling) was present. After pre-incubation for 10 min at 23 °C, mitochondria (0.2 mg protein/ml) were incubated with (2,4,6,7-3H)estradiol (0.05–7.5 nmol/L) in a total volume of 200 μl for an additional 10 min for total hormone binding. Non-specifically bound E2 was determined by incubating identical aliquots of mitochondria with the labelled E2 as mentioned earlier and a 100-fold excess of unlabelled E2. At the end of the incubation, the mitochondria were harvested by vacuum filtration (cellulose-nitrate filters pore size, 0.45 μm). After being washed twice with 3 ml of ice-cold 0.25 mol/L sucrose and 5 mmol/L EDTA (to remove unbound hormone), filters were transferred into scintillation vials for radioactivity counting. Specific hormone binding was calculated by subtracting non-specific bound from total bound E2. Counts of appropriate mitochondria-free filter blanks were subtracted. The total E2 concentration was determined in each incubation assay by counting radioactivity in 20 μl directly applied to filters without subsequent vacuum filtration.

Mitochondrial Ca^{2+} transport

Ca^{2+} transport in hippocampal synaptosomal mitochondria was performed in same conditions as already published for synaptosomal mitochondria isolated from whole rat brain (Horvat et al., 2000, 2001). After pre-incubation at 23 °C for 10 min in a medium containing: 300 mmol/L mannitol, 10 mmol/L KCl, 1 mmol/L maleate, 5 mmol/L glutamate and 10 mmol/L Tris–HCl, pH 7.4 in the