Arch. Biol. Sci., Belgrade, 61 (2), 171-177, 2009 DOI:10.2298/ABS0901171P

INHIBITION OF MITOCHONDRIAL Na-DEPENDENT Ca^{2+} EFFLUX FROM RAT BRAIN STEM BY 17β-ESTRADIOL

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Abstract — The role of membrane-bound estradiol in modulation of mitochondrial Ca^{2+} flux in nerve endings isolated from rat brain stem was examined. Physiological concentrations of 17β-estradiol bind specifically to isolated mitochondria (Bmax 33.8 ± 2.5 fmoles estradiol/mg of protein, Km 0.185 ± 0.006 nmoles/l free estradiol). At concentrations ranging from 1 x 10-10 to 2 x 10-9 moles/l, estradiol significantly (by 23-28%) decreases mitochondrial Na-dependent calcium efflux. Decreased calcium efflux was associated with increased affinity of the Na^+/Ca^{2+} exchanger for Na^+ and decreased capacity of the exchanger to extrude Ca^{2+}. Calcium ion efflux modulation and mitochondrial ion retention may be the way that 17β-estradiol exerts its role in nerve cell homeostasis.

Key words: Mitochondria, estradiol, Ca^{2+} influx, Ca^{2+} efflux

UDC 577.25

INTRODUCTION

The maintaining of Ca^{2+} ion homeostasis is of great importance for normal functioning of cells, especially the excitable ones. In nerve cells, Ca^{2+} is vital for neuronal excitability control, neurotransmitter release, nerve impulse passing, and other specialized cell functions. All of those activities are associated with changes in cytosolic free Ca^{2+} concentration (Smith and Augustine, 1988; Miller, 1991). Any cytosolic Ca^{2+} overload, including the Ca^{2+} elevation during normal physiological events, has to be cleared quickly because of its toxic nature and involvement in cell damage and consequent apoptotic or necrotic cell death (Trump and Berezesky, 1995). In nerve cells, excessive cytosolic Ca^{2+} is removed by extrusion across the plasma membrane by activity of either Ca^{2+}-ATPase or the Na^+/Ca^{2+} exchanger (Gill et al., 1984; Pekovic et al., 1986). On the other hand, cytosolic Ca^{2+} may be decreased by sequestration in intracellular organelles such as the endoplasmatic reticulum and mitochondria (White and Reynolds, 1995).

Mitochondria, organelles critical for Ca^{2+} buffering in neurons, can rapidly accumulate Ca^{2+} when its concentration is greatly increased in the cytoplasm. Because the outer mitochondrial membrane seems to be readily permeable to small molecules, the machinery for Ca^{2+} transport lies primarily at the inner mitochondrial membrane (Babcock and Hille, 1998). The influx of Ca^{2+} into the matrix occurs through a uniporter system that is specifically inhibited by the glycoprotein stain ruthenium red. Activity of the uniporter is dependent on the electrochemical gradient across the inner membrane. This gradient (up to - 180 mV, negative to the cytosol) is developed and maintained primarily through proton extrusion by the electron transport chain (Duchen, 2000). Thus, disturbance of mitochondrial respiration and collapse of the mitochondrial membrane potential could prevent mitochondrial Ca^{2+} influx. The Ca^{2+} efflux from brain mitochondria is predominantly an Na-dependent, electroneutral process mediated by an antiporter, the Na^+/Ca^{2+} exchanger (Gobbi et al., 2007). The exchanger's activity, which couples uphill Ca^{2+} extrusion to downhill Na^+ influx, has a crucial role in maintaining a low intramitochondrial Ca^{2+} concentration (Duchen, 2000).

Steroid hormones can modulate various pro-

cesses in mitochondria of nervous tissues (Mattson et al., 1997). Some of these effects seem to be mediated via the mitochondrial membrane. In our previous work, specific binding of 17β-estradiol (E2) to mitochondria isolated from nerve cell endings from whole rat brain was found (Horvat et al., 2001). At concentrations that specifically bind to the mitochondrial membrane, estradiol was found to contribute to modulation of mitochondrial Ca^{2+} transport. In that way estradiol regulates mitochondrial homeostasis and cytosolic Ca^{2+} concentrations by changing sequestration and release of those ions (Horvat et al., 2001).

In order to examine relations between effects on mitochondrial Ca^{2+} flux and binding to the mitochondrial membrane in a specific brain area, the brain stem, specific binding sites and concentration-dependent modulation of influx and efflux of Ca^{2+} in the presence of E2 were quantified in the present study.

MATERIALS AND METHODS

Ovariectomized three weeks prior to use, mature female Wistar rats were used in the experiments. The animals were kept in constant conditions (light on for 8-18 h and temperature of 24°C) and had free access to food and water. All procedures were approved by the Ethical Committee of the Serbian Association for the Use of Animals in Research and Education.

(2,3,4,6-^{3}H)-Estradiol (specific activity of 94 Ci/mmole) and $^{45}CaCl_2$ (specific activity of 70 mCi/nmole) were purchased from Amersham and PerkinElmer, respectively. 17β-Estradiol and other chemicals were purchased from Sigma or Calbiochem-Boehring. Cellulose nitrate filters (with pore size of 0.45 μm) were obtained from Whatman.

Preparation of brain stem synaptosomes and synaptosomal mitochondria

Animals were sacrificed by cervical dislocation and whole brains were rapidly removed. Brain stems (BS) were separated and homogenized in ice-cold buffered sucrose (0.32 moles/l sucrose, 5 mmoles/l Tris-HCl, pH 7.4). Purified synaptosomes were obtained according to the method of Gray and Whittaker (Gray and Whittaker, 1962) using a discontinuous (7.5 and 13%) Ficoll gradient. Synaptosomes were lyzed by suspension in 5 mmoles/l Tris-HCl and frozen at -20°C. The synaptosomal lyzate was used for preparation of mitochondria according to the method of Lai and Clark (Lai and Clark, 1970) using a discontinuous (4.5 and 6%) Ficoll gradient. Isolated synaptosomal mitochondrial pellets were suspended in 0.3 moles/l mannitol and kept at -20°C until use. Protein concentration was determined by the Lowry method (Lowry et al., 1951).

Binding assay

Binding of (2,3,4,6-^{3}H)-estradiol was measured in a medium (MUM) containing (in mmoles/l): 300 mannitol, 10 KCl, 1 maleate, 5 glutamate, and 10 Tris-HCl, pH 7.4, in a final volume of 200 μl, where mitochondrial respiration (coupling) was provided for. After preincubation for 10 min at 22°C in medium without hormone, mitochondria (0.2 mg/ml) were incubated with (2,3,4,6-^{3}H)-estradiol (3 x 10^{-11}-1.5 x 10^{-9} moles/l) for an additional 10 min for total hormone binding. Nonspecifically bound estradiol was determined by incubating identical aliquots of mitochondria with labeled estradiol as above and a hundred-fold excess of unlabelled estradiol. At the end of incubation, the mitochondria were harvested by vacuum filtration (using cellulose nitrate filters), and after being washed twice with 3 ml of ice-cold 0.25 moles/l sucrose and 5 mmoles/l EDTA (to remove unbound steroid) were transferred to scintillation vials for radioactivity counting. Specific hormone binding was calculated by subtracting non-specifically bound from total bound estradiol.

Ca^{2+} influx and efflux

Synaptosomal mitochondria were preincubated for 10 min at 22°C in the same medium (MUM) as for monitoring of hormone binding. The influx of Ca^{2+} was initiated by adding 0.2 mmoles/l $CaCl_2$ (0.6 μCi of $^{45}CaCl_2$) to the incubation mixtures, lasted for 5 min, and was stopped by ruthenium red (17.5 μg/mg of protein) and 2 ml of 0.25 moles/l sucrose. Aliquots of 1 ml were vacuum-filtered through cel-

lulose-nitrate filters. Calcium ions retained in the mitochondria were calculated from radioactivity counting and presented as nmoles of Ca^{2+}/mg of protein. For Ca^{2+} efflux monitoring, mitochondria were loaded with calcium in the same way and after adding ruthenium red the Ca^{2+} efflux was initiated by adding NaCl (10^{-1} moles/l) and 2 x 10^{-4} moles/EDTA and lasted for 5 min. The Ca^{2+} efflux dependence on sodium concentrations was determined by incubating preloaded mitochondria with or without 5 x 10^{-10} moles/l E2 for 10 min, and efflux was initiated by addition of NaCl (0-3 x 10^{-1} moles/l) and 2 x 10^{-4} moles/l of EDTA. The E2 effects on mitochondrial Ca^{2+} influx and efflux were examined by incubating mitochondria in the presence of 5 x 10^{-12}-5 x 10^{-8} moles/l of 17β-estradiol during preincubation.

RESULTS AND DISCUSSION

In this study, E2 binding and calcium ion movement through the BS mitochondrial membrane were monitored in order to explore *in vitro* the effect of E2 on the mitochondrial Ca^{2+} flux. The BS is the region of interest because of permanent intensive neuronal activity and intensive ion movements (especially Ca^{2+} movements) in that structure, which serves to connect the forebrain and the spinal cord (Fig. 1).

As presented in Fig. 1, E2 specifically binds to synaptosomal mitochondria isolated from the BS, and this binding reaches a plateau in the presence of E2 concentrations higher than 4 x 10^{-10} moles/l. A Michaelis-Menten plot of specific estradiol binding to mitochondria indicates one binding site with estimated B_{max} of 33.8 ± 2.5 fmoles E2/mg of mitochondrial protein and K_m of 0.185 ± 0.006 nmoles/l free estradiol. An upwardly concave Scatchard plot and the Hill coefficient (n = 1.5) estimated from the Hill plot (Fig. 1, insets A and B, respectively) indicate the existence of positive cooperativity in E2 binding to mitochondria. Comparing the characteristics of E2 binding on mitochondria presented in this work with our previous results (Horvat et al., 1995) on synaptic plasma membranes isolated from the BS revealed that: i) two binding sites are present on plasma membranes, but one on mitochondria; ii) binding characteristics of membrane binding sites were B_{max1} of 0.3 pmoles/mg and K_{m1} of 26 nmoles/l free E2 for the high-capacity/low-affinity site and B_{max2} of 60 fmoles/mg and K_{m2} of 4 nmoles/l free E2 for the low-capacity/high-affinity site; and iii) on mitochondria, the latter binding site possessed two-fold lower binding capacity with 20-fold higher affinity compared to the plasma membrane low-capacity/high-affinity site. These binding characteristics indicate that different proteins (receptors) are responsible for specific E2 binding to synaptosomal plasma membrane and mitochondria isolated from the BS.

To explore the possible physiological significance of specific estradiol binding to synaptosomal mitochondria, transport of calcium ions through the mitochondrial membrane in the presence of estradiol was examined. In comparing mitochondrial Ca^{2+} influx through the ruthenium red-sensitive uniporter in the presence (5 x 10^{-12}-5 x 10^{-8} moles/l) and absence of E2, no statistically significant changes were found (Table 1). On the basis of these results, it might be concluded that E2 has no effect on the characteristics of this protein. On the other hand, there is evidence that E2 increases synaptic plasma membrane voltage-dependent uptake of Ca^{2+} (Nikezic et al., 1996) and that augmentation of cytosolic Ca^{2+} results in an increase of matrix Ca^{2+} (Nilsen and Brinton, 2003). On the basis of these data, it could be supposed that E2 indirectly, acting

Table 1. Dose-dependent effect of estradiol in vitro on mitochondrial Ca^{2+} flux presented as % of inhibition (-) or stimulation (+) of control value (1.92 nmoles of Ca^{2+}/mg of protein for influx; 1.64 nmoles of Ca^{2+}/mg of protein for efflux).

E2 concentration (moles/l)	Ca^{2+} influx (%)	Ca^{2+} efflux (%)
5 x 10^{-12}	-8	-13
1 x 10^{-11}	+10	-14
5 x 10^{-11}	0	-13
1 x 10^{-10}	0	**-23**
2.5 x 10^{-10}	0	**-28**
5 x 10^{-10}	-10	**-24**
1 x 10^{-9}	-5	**-27**
2 x 10^{-9}	+10	**-26**
5 x 10^{-9}	-5	-11
1 x 10^{-8}	+10	+5.6
5 x 10^{-8}	0	+10

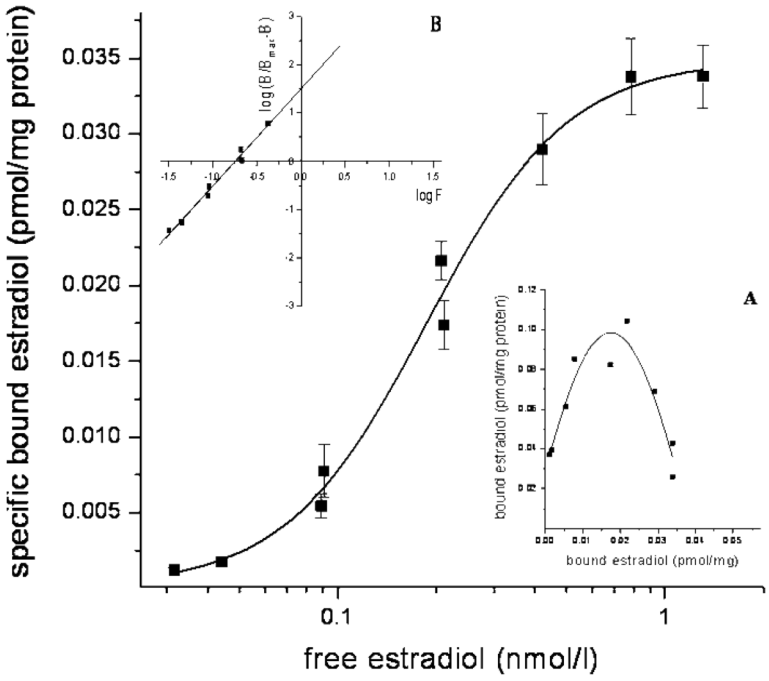

Fig. 1. Specific estradiol binding to synaptosomal mitochondria isolated from brain stem. Mitochondria (0.2 mg/ml) were incubated for 10 min with various concentrations of labelled (2,3,4,6-3H)-estradiol for total binding plus hundred-fold higher concentrations of unlabelled estradiol for non-specific binding at 22°C. Specific binding was calculated by subtracting non-specific binding from the total bound hormone. Results represent means ± SEM of three experiments (triplicate determinations). Inserts represent Scatchard (A) and Hill (B) plots of the obtained data.

at the plasma membrane level, increases mitochondrial Ca^{2+} (Table 1).

In the case of the Na-dependent Ca^{2+} efflux, E2 exerts a dose-dependent effect (Table 1). Estradiol at concentrations up to 5 x 10^{-11} moles/l exerted modest and statistically insignificant inhibition of Ca^{2+} efflux, while concentrations between 1 x 10^{-10} and 2 x 10^{-9} moles/l, concentrations at which specific estradiol binding was detected, significantly decreased Ca^{2+} efflux in BS mitochondria (by 23-28%). This result is in accordance with our earlier findings on synaptosomal mitochondria isolated from the rat brain (Horvat et al., 2001) and nucleus caudatus and hippocampus (Petrovic et al., 2005), in which estradiol decreased calcium efflux from mitochondria at similar concentrations as in the BS. Since calcium efflux inhibition was detected at the same estradiol concentrations at those at which the hormone binds specifically to mitochondria, the possibility that estradiol exerts its effect by changing properties of the Na^+/Ca^{2+} exchanger protein was explored (Fig. 2).

The dependence of Na^+/Ca^{2+} exchanger activity on the external concentration of Na^+ (5 x 10^{-3}-3 x 10^{-1} moles/l) in the presence or absence of 5 x 10^{-10} moles/l estradiol was evaluated by measuring Ca^{2+} efflux. From the Na-dependent Ca^{2+} efflux curve (Fig. 2), it was obvious that Ca^{2+} efflux was decreased in the presence of estradiol. The values of V_{max} for Ca^{2+} efflux (V_{max} 1.85 ± 0.051 nmoles of Ca^{2+}/mg of protein and 1.23 ± 0.021 nmoles of Ca^{2+}/mg of protein in the control and after estradiol treatment, respectively) indicate that estradiol decreased exchanger capacity by 35%. Estimated values of K_m pointed to increase in affinity of the exchanger for Na^+ in the presence of estradiol (control K_m was

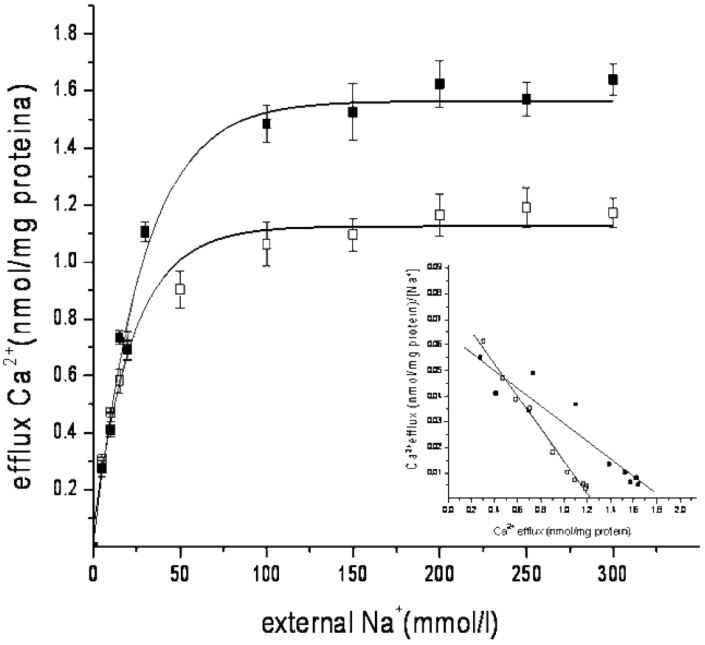

Fig. 2. Mitochondrial Na-dependent Ca^{2+} efflux in the absence (▪) and presence (□) of estradiol. Ca^{2+}-preloaded mitochondria were incubated in the absence or presence of 5 x 10-10 moles/l E2 for 10 min. The efflux was measured after addition of various concentrations of external NaCl and 2 x 10-4 moles/l EDTA. Amounts of released Ca^{2+} were estimated as indicated in Materials and Methods. Results represent means ± SEM of three experiments (triplicate determinations). The insert represents a Scatchard plot of the obtained data.

29.03 ± 4.2 mmoles/l Na^+, while K_m in the presence of estradiol was 15.74 ± 2.3 mmoles/l Na^+) by about 50%. The inhibitory effect of estradiol on Ca^{2+} efflux can be realized through alteration of either Ca^{2+} capacity or Na^+ affinity of the exchanger. Similar results were obtained on synaptosomal mitochondria isolated from the whole brain (Horvat et al., 2001) and from the nucleus caudatus and hippocampus (unpublished data).

Estradiol (up to 2 x 10^{-9} moles/l) by acting on Ca^{2+} retention and enhancement in the mitochondrial matrix, could indirectly increase the activity of Ca^{2+}-sensitive dehydrogenase, as has been seen for brain and other tissues (Garcia et al., 1996). As a consequence, a change occurs in the citric acid cycle, respiration is activated, and protons are extruded. An elevated proton flux may stimulate ATP-synthase activity and ATP synthesis (Garcia et al., 1996), which is a way of connecting mitochondrial energy production and cellular energy demand. The involvement of estradiol as a modulator of Ca^{2+} transport mechanisms in mitochondria may be the way it exerts its role in nerve cell homeostasis and function.

CONCLUSIONS

Transport of Ca^{2+} in rat brain stem mitochondria can be modulated by estradiol. While the influx of Ca^{2+} was unchanged, estradiol decreases capacity of the Na^+/Ca^{2+} exchanger and inhibits Ca^{2+} efflux by increasing the exchanger's affinity for Na^+. At the same concentrations as those at which it binds specifically to mitochondria, estradiol decreases Na^+/Ca^{2+} exchanger activity, possibly acting via mitochondrial membrane binding sites.

Acknowledgment — This study was supported by the Serbian Ministry of Science (Project No. 143044).

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17β-ЕСТРАДИОЛ ИНХИБИРА Na-ЗАВИСНИ ИЗЛАЗАК Ca^{2+} ИЗ МИТОХОНДРИЈА МОЖДАНОГ СТАБЛА ПАЦОВА

СЊЕЖАНА ПЕТРОВИЋ, МАЈА МИЛОШЕВИЋ, ИВАНА СТАНОЈЕВИЋ, НАТАША ВЕЛИЧКОВИЋ, ДУЊА ДРАКУЛИЋ и АНИЦА ХОРВАТ

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У раду је испитивана физиолошка улога естрадиола у митохондријама изолованим из можданог стабла пацова. Испитивано је специфично везивање естрадиола за митохондријалне мембране и *in vitro* ефекат физиолошких концентрација 17β-естрадиола на митохондријални транспорт јона калцијума. Нађено је да се 17β-естрадиол специфично везује за изоловане митохондрије (B_{max} 33.8 ± 2.5 fmol естрадиола/mg proteina, K_m 0.185 ± 0.006 nmol/l слободног естра-

диола). У условима *in vitro*, присуство естрадиола у концентрацијама све до 5 x 10^{-8} mol/l нема утицаја на улазак јона Ca^{2+} у митохондрије преко рутенијум ред осетљивог канала. Истовремено, естрадиол у концентрацијама од 1x10^{-10}-2x10^{-9} mol/l доводи до значајног (23-28 %) смањења Na-зависног изласка јона Ca^{2+} из митохондрија. Смањени излазак јона Ca^{2+} је повезан са повећањем (50 %) афинитета Na^{+}/Ca^{2+} измењивача за Na^{+} јоне и смањењем капацитета измењивача да избацује јоне Ca^{2+}. Један од начина деловања 17β-естрадиола на функцију митохондрија се остварује специфичним везивањем за мембрану и утицајем на излазак Ca^{2+} јона и њихово задржавање у митохондријама, чиме естрадиол утиче и на хомеостазу нервних ћелија.