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Dehydroepiandrosterone with other neurosteroids preserve neuronal mitochondria from calcium overload

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Abstract

This current study was designed to test whether the dehydroepiandrosterone (DHEA) and other neurosteroids could improve mitochondrial resistance to ischemic damage and cytoplasmic Ca^{2+} overload. To imitate these mechanisms at mitochondrial level we treated the saponin permeabilized neurons either with the respiratory chain inhibitor, 1-methyl-4-phenylpyridinium or raised free extra-mitochondrial [Ca^{2+}]. Loss of mitochondrial membrane potential (as an indicator of loss of function) was detected by JC-1. The results demonstrate that DHEA partly prevented Ca^{2+} overload induced loss of mitochondrial membrane potential but not the loss of potential induced by the inhibitor of the respiratory chain. A similar effect was observed in the presence of other neurosteroids, pregnenolone, pregnanolone and allopregnanolone. DHEA inhibited also the Ca^{2+} accumulation to the mitochondria in the presence of Ca^{2+} efflux inhibitors. Thus, in the present work we provide evidence that DHEA with several other neurosteroids protect the mitochondria against intracellular Ca^{2+} overload by inhibiting Ca^{2+} influx into the mitochondrial matrix.
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Keywords: Mitochondrial membrane potential; Neurosteroids; Dehydroepiandrosterone; Neuroprotection

1. Introduction

Dehydroepiandrosterone (DHEA), together with its sulphated form (DHEAS; collectively DHEA(S)), is the major secreting product of the adrenal gland. DHEA(S) is also produced in the central nervous system. DHEA appears to have memory enhancing, anxiolytic, and anti-aggression effects in animals and may be helpful for major depression in humans. In addition, accumulating evidence suggests that DHEA(S) is neuroprotective in a variety of paradigms [1,2]. It was recently shown that DHEA(S) is neuroprotective in in vitro [3,4] and in vivo [3,5] models of brain ischemia. Moreover, concentration of DHEA(S) decrease dramatically with age in humans and it has been proposed that this may contribute to increased vulnerability of the ageing or stressed human brain to ischemia [3]. Nevertheless, the mechanisms through which DHEA(S) exerts these protective effects are still not unravelled. No specific receptors in the plasma membrane, nucleus or cytoplasm for DHEA(S) have been found. It has been shown that DHEA and its sulphate modulate GABAergic and glutamatergic neurotransmission. However, while the neuroprotective effect of DHEAS might be attributed to its γ-aminobutyric acid (GABA(A)) receptor-modulating properties [6], DHEA itself is not interacting with the $GABA_A$ receptor [7]. The neuroprotective effects of DHEA seem not to be related with its *N*-methyl-D-aspartate (NMDA) or sigma receptor-activating properties as well [8,9]. It has been shown that DHEA opposes the action of glucocorticoids, antagonizing corticosterone neurotoxicity in neuronal cultures and in hippocampus [10], but the mechanism of such an interaction is unclear. In earlier works, DHEA(S) have also shown to affect the mitochondrial function [11,12]. Morin et al. recently demonstrated that DHEA partly preserves the function of isolated mitochondria altered by anoxia-reoxygenation [13]. We have shown previously that DHEAS preserves the loss of mitochondrial activity following exposure of neurons to ischemic insult [4]. Several other steroids have been shown to preserve mitochondrial transmembrane potential after exposure of cells to toxic insults [14,15]. However, the latter studies are unable to prove the direct link between neurosteroids and mitochondria: neurosteroids may protect neurons by other mechanisms and the mitochondrial potential could be preserved because of that neuroprotection. To find an answer to that question we decided to study the effect of DHEA, as well as other neurosteroids, on membrane potential of neuronal mitochondria in their

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natural surroundings using the permeabilization technique, a method that have been extensively taken into use when studying the mitochondrial function in the heart [16–18].

2. Materials and methods

2.1. Cerebellar granule cell cultures

Primary cultures of cerebellar granule cells were prepared according to the method of Gallo et al. [19], with minor modifications [4]. In brief, the cerebelli were dissected from 8-day-old Wistar rats (Kuopio Animal Center, Finland) and cells were dissociated by mild trypsinization (0.025% trypsin) at 35 °C for 15 min followed by trituration in a 0.004% DNAse solution containing 0.05% soybean trypsin inhibitor. The cells were re-suspended in basal Eagle medium with Earle's salts containing 10% foetal bovine serum, 25 mM KCl, 2 mM glutamine and 100 ng/ml gentamicin. The cell suspension was seeded in dishes with a culture area of 8.8 cm^2 for toxicity experiments or 8-well Lab-Tek™ II Chambered Coverglass (Nunc) with a culture area of 0.9 cm^2 per well for fluorescence imaging. Both were pre-coated with poly-(L-lysine) at a density of $(1.3 \pm 1.4) \times 10^6$ cells/ml. The cell cultures were grown for 8 days in a humidified 5% CO_2/95% air atmosphere at 37 °C. After at least 24 h of incubation 10 mM cytosine arabinoside was added to prevent the proliferation of non-neuronal cells.

2.2. Permeabilization of neurons

Previous investigations of mitochondrial function have been mainly carried out either in living cells or in isolated mitochondria. However, both approaches have serious drawbacks, at least for this current study. Visualization of mitochondrial membrane potential in living cells reflects rather the extent of cellular damage than the specific effect of the compound at mitochondrial level. On the other hand, the isolation procedure damages the connections between the cytoskeleton and mitochondria [16]. Moreover, the phase of active respiration of the isolated mitochondria is rather short for experiments aimed to use actively respiring mitochondria for monitoring the changes in membrane potential. To overcome these difficulties we used the permeabilization technique [16–18], giving the opportunity to study mitochondrial function in situ, in their natural surrounding where the active phase of respiration is not any more restricted due to the presence of cellular ATPases. In brief, specific permeabilization of the plasmalemma was obtained by incubating the cultures for 20 min at 4 °C in an *intracellular solution* at pCa 7 containing additionally 50 μg/ml saponin. This *solution* contained 10 mM ethylene glycol-bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 60 mM *N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES, pH 7.1), 5.69 mM MgATP, 1 mM free Mg^{2+}, 20 mM taurine, 3 mM K_2HPO_4, 0.5 mM dithiothreitol and the ionic strength was adjusted to 160 mmol/l with potassium methanesulfonate. The desired pCa was obtained by varying CaK_2EGTA/K_2EGTA.

2.3. Measurement of mitochondrial membrane potential

$\Delta\psi_m$ was estimated in permeabilized neurons loaded with the $\Delta\psi_m$ sensitive fluorescent dye JC-1 (Molecular Probes, USA). The permeabilized cultures were first incubated for 10 min at room temperature on a slowly 3D rotating rocker with the intracellular solution at different pCa containing mitochondrial substrates, 5 mM glutamic, 2 mM malic acid and neurosteroids or other compounds of interest. Stock solutions (100 mM) of DHEA, DHEAS, pregnenolone sulfate, androstenedione and 17β-estradiol were made in DMSO, and cholesterol, pregnenolone, allopregnanolone and pregnanolone in ethanol. Final concentrations of these solvents never exceeded 0.3% and similar concentrations of solvents were also present in control preparations. At the end of incubation, 3 μM JC-1 was added and incubation was continued for the next 10 min. The dishes were then mounted on the stage of a laser scanning confocal microscope MRC 1024 (BioRad). Random fields of neurons (∼300 cells) were illuminated with the 488-line of an Ar/Kr laser and emission at 522DF35 and 605DF32 nm was monitored. The fluorescent emission wavelength of JC-1 depends on the aggregation of the JC-1 molecules in mitochondria that in turn depends on the $\Delta\psi_m$ [20]. Relative changes in $\Delta\psi_m$ were assessed by monitoring the ratio of JC-1 fluorescence at 605 nm (aggregate) and 522 nm (monomer).

2.4. Measurement of intra-mitochondrial Ca^{2+}

For quantitative measurement of mitochondrial Ca^{2+} signals, permeabilized neurons were loaded at room temperature for 20 min with 3 μM X-rhod-5F-AM (Molecular Probes, USA) in an *intracellular solution* and incubated at room temperature for 30 min to allow hydrolysis of X-rhod-5F-AM trapped in mitochondria. This was followed by a 20 min incubation in an *intracellular solution* containing compounds of interest at different pCa after which the dishes were mounted on the stage of a laser scanning confocal microscope and illuminated with the 568-line of an Ar/Kr laser and emission at 605DF32 nm was monitored. Mitochondria were prepared as described previously [17].

2.5. Statistical analysis

The mean (±S.E.M) values were compared using one-way ANOVA and Bonferroni post hoc analysis.

3. Results

Neurons were skinned by 50 μg/ml saponin that specifically removes plasmalemma, but leaves intracellular

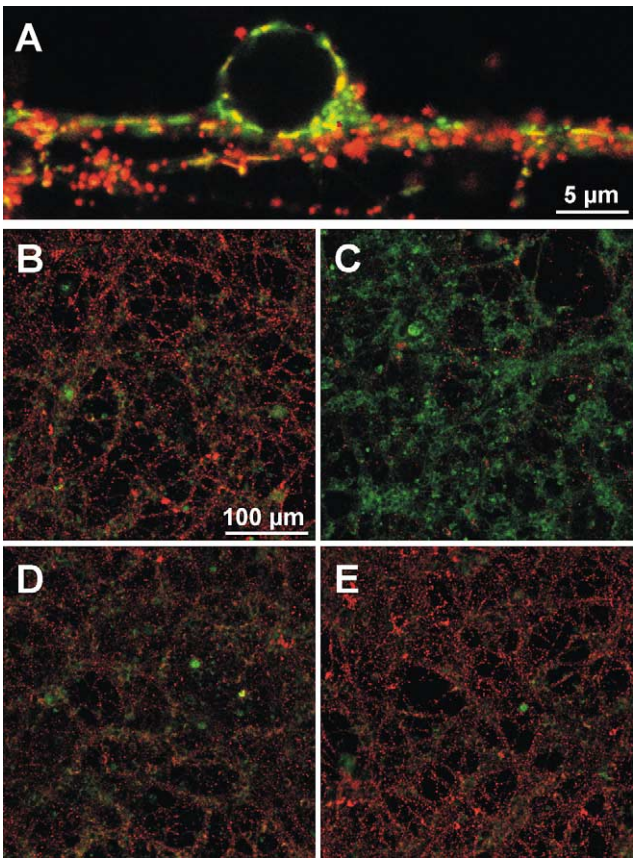

Fig. 1. (A) Mitochondrial membrane potential in saponin skinned neuron as stained by JC-1 (green, emission at 522DF35 nm—low potential; red, emission at 605DF32 nm—high potential). Field of neurons (B) at control conditions or at elevated intracellular calcium (pCa 5.5): (C) in the absence or (D) presence of 100 μM DHEA or (E) 10 μM ruthenium red.

structures completely intact and well exposed to the extracellular medium [17,18]. This is shown in Fig. 1(A) where neurons have been labelled with the membrane potential sensitive dual emission probe JC-1. As shown in the confocal image, in the presence of mitochondrial substrates (glutamate and malate) and ATP, most of the mitochondria were functional as they maintained a high membrane potential.

Ischemia-reperfusion has two immediate consequences at the mitochondrial level: oxygen deficiency hampering the respiratory chain and cytoplasmic Ca^{2+} overload leading to accumulation of Ca^{2+} in the mitochondrial matrix (see reviews in [21,22]). To imitate these mechanisms we treated the skinned neurons either with a respiratory chain inhibitor, 1-methyl-4-phenylpyridinium (MPP^+) or raised free extra-mitochondrial [Ca^{2+}] and detected the ratio of red/green fluorescence of JC-1 after 10 min of incubation. As expected, both applications lowered the red/green fluorescence ratio significantly, indicating a fall of mitochondrial membrane potential. The 50% inhibition was observed at 2 mM MPP^+ or at 2 μM free [Ca^{2+}]. In the next experiments, the effect of DHEA (100 μM, added at the start of incubation with MPP^+ or high [Ca^{2+}]) against the 5 mM MPP^+ or 3.2 μM free [Ca^{2+}] induced loss of

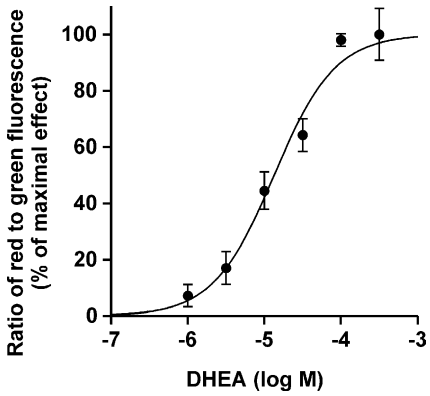

Fig. 2. Dose response curve of DHEA. Skinned neurons incubated at pCa 5.5 and at different concentrations of DHEA were stained with JC-1. Relative $\Delta\psi_m$ was assessed by monitoring the ratio of JC-1 fluorescence intensities at 605DF32 and 522DF35 nm. Values are expressed as percentage of maximal effect of DHEA ($n = 5$–8).

mitochondrial membrane potential was studied. In cells treated with MPP^+, DHEA had no effect on mitochondrial membrane potential (data not shown). Cells exposed to high [Ca^{2+}] in the presence of DHEA, however, showed significantly higher red to green fluorescence ratio of JC-1 than the ones with the absence of DHEA (Fig. 1(B)–(D)). The effect of DHEA was concentration dependent. As can be seen from Fig. 2, the maximal response was obtained at 100 μM concentration with EC_{50} 15 μM. A similar effect was observed when the cells were treated with other neurosteroids (100 μM) pregnenolone, pregnanolone, allopregnanolone and to a lesser extent also 17β-estradiol but not with DHEAS, pregnenolone sulphate or androstenedione (Table 1). 100 μM cholesterol was ineffective. We also tested the possibility whether the mixture of neurosteroids in

Table 1
Effect of different steroids on mitochondrial membrane potential

Treatment	Mean ± S.E.M.
pCa 7.0	100 ± 7
pCa 5.5	36 ± 2
pCa 5.5 + DHEA 100 μM	63 ± 7[a]
pCa 5.5 + DHEAS 100 μM	29 ± 3
pCa 5.5 + pregnenolone 100 μM	81 ± 4[a]
pCa 5.5 + pregnenolone sulphate 100 μM	32 ± 3
pCa 5.5 + pregnanolone 100 μM	64 ± 7[a]
pCa 5.5 + allopregnanolone 100 μM	51 ± 5[a]
pCa 5.5 + 17 β-estradiol 100 μM	43 ± 2[a]
pCa 5.5 + androstenedione 100 μM	40 ± 5
pCa 5.5 + cholesterol 100 μM	38 ± 2
pCa 5.5 + mixture of neurosteroids[b]	50 ± 2[a]

Relative $\Delta\psi_m$ was assessed by monitoring the ratio of JC-1 fluorescence intensities at 605DF32 and 522DF35 nm and is expressed as a percentage of control at pCa 7.0.

[a] Difference compared with pCa 5.5 group, $P < 0.05$, $n = 7$–27.

[b] Mixture of neurosteroids contained 150 nM DHEA, 200 nM DHEAS, 900 nM pregnenolone, 100 nM pregnenolone sulphate and 4 nM pregnanolone.

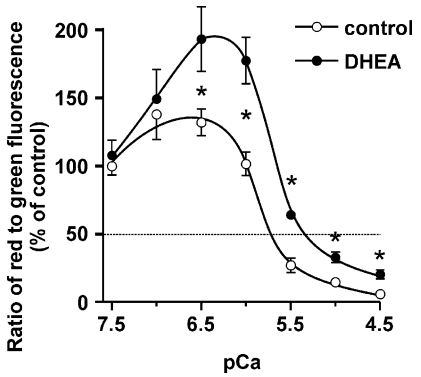

Fig. 3. Effect of DHEA on mitochondrial membrane potential at different free Ca^{2+} concentrations. Skinned neurons incubated at different free Ca^{2+} concentrations in the presence or absence of 100 μM DHEA were stained with JC-1. Relative $\Delta\psi_m$ was assessed by monitoring the ratio of JC-1 fluorescence intensities at 605DF32 and 522DF35 nm and is expressed as a percentage of control at pCa 7.5. Asterisk signifies difference between groups, $P < 0.05$, $n = 6$–14.

a range of physiological concentrations in the human brain [23] could be protective in our model. As can be seen from Table 1, the mixture of DHEA, DHEAS, pregnenolone, pregnenolone sulphate, pregnanolone protected mitochondrial membrane potential from cytoplasmic Ca^{2+} overload. It should also be noted here that neurosteroids themselves had no effect on the fluorescence spectra of JC-1.

The effect of DHEA depended on the extra-mitochondrial [Ca^{2+}] used. As demonstrated by Fig. 3, the increasing of external Ca^{2+} (up to 32 μM of free ion, pCa 4.5) produced a bell-shaped curve: the initial small increase of mitochondrial membrane potential was followed by a decrease when pCa exceeded 6.5. In the presence of 100 μM DHEA, however, the initial rise was significantly higher and the whole curve for membrane potential was shifted to the right. Thus, while in control preparations the membrane potential fell to half of the initial value at pCa 5.7 (2 μM), the same level (50% of control value) was achieved significantly later, at pCa 5.3 (5 μM) in the DHEA treated group.

It should be noted here that a similar shift, however, more potent, was observed in the presence of the inhibitor of mitochondrial Ca^{2+} influx, ruthenium red, e.g. 10 μM ruthenium red led to an increase in membrane potential at pCa 7.0 and was also protective at higher Ca concentrations between pCa 5.5 and 4.5 (Fig. 1(E)). At the same time CGP-37157 or cyclosporin A, inhibitors of mitochondrial Ca^{2+} efflux pathways, e.g. Na^+/Ca^{2+} exchanger and mitochondrial permeability transition pore, respectively, did not reverse the protective effect of DHEA. Both compounds alone had no effect on membrane potential suggesting that DHEA do not activate the Ca^{2+} efflux pathways but rather inhibit the influx of Ca^{2+} into the mitochondrial matrix.

To determine if these effects of DHEA on mitochondrial membrane potential are associated with a mitochondrial [Ca^{2+}] we carried out experiments to directly measure free [Ca^{2+}] in the mitochondrial matrix. Mitochondrial calcium sequestration was examined by imaging the fluorescent calcium indicator recently developed fluorinated analogue of rhod-2, acetoxymethyl ester of X-rhod-5F. The acetoxymethyl ester of X-rhod-5F is cationic and membrane-permeant but possesses no detectable calcium-dependent fluorescence. When added to the extra-mitochondrial media, it accumulates in the mitochondria because of the mitochondrial membrane potential. Once de-esterified by mitochondrial esterases, however, X-rhod-5F is retained in the mitochondrial matrix, regardless of mitochondrial membrane potential. Compared with rhod-2 X-rhod-5F has a lower Ca^{2+} binding affinity (K_d: 0.6 and 1.6 μM, respectively) and saturates at higher free $[Ca^{2+}]_m$ making it more suitable for monitoring intra-mitochondrial Ca^{2+} overload. To verify that X-rhod-5F and it's loading protocol led to a specific loading of the mitochondria we carried out control experiments where we also loaded the cells with MitoTracker Green, a mitochondrial specific dye. The results (not shown) indicated that X-rhod-5F and MitoTracker Green were loaded into the same cellular compartment with a characteristic distribution similar to that seen with mitochondrial potential sensitive dye JC-1 (Figs. 1A and 4A). To avoid impact of

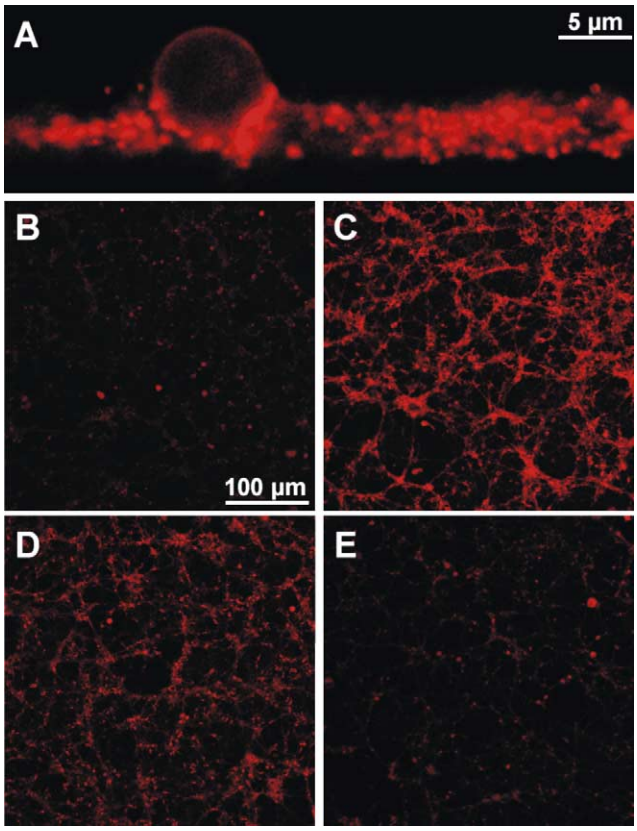

Fig. 4. (A) Free [Ca^{2+}] in mitochondrial matrix of saponin skinned neuron as stained by X-Rhod-5F-AM (emission at 605DF32 nm). Field of neurons at (B) control conditions or at elevated intracellular calcium (pCa 5.5): (C) in the absence or (D) presence of 100 μM DHEA or (E) 10 μM ruthenium red.

different membrane potentials on loading, all preparations were loaded at high membrane potential after which the experiment was started.

First we measured the X-rhod-5F response following the increase in extra-mitochondrial [Ca^{2+}] up to pCa 5.5 in the presence of Ca efflux inhibitors, CGP-37157 and cyclosporin. As shown in Fig. 4(B) and (C), this led to an increase in the X-rhod-5F fluorescence suggesting calcium accumulation into the mitochondrial matrix. However, this accumulation was partially inhibited in the presence of 100 μM DHEA. As shown in Fig. 4(D), addition of 100 μM DHEA induced a drop in the high extra-mitochondrial [Ca^{2+}] induced X-rhod-5F fluorescence. No Ca^{2+} accumulation at all was observed when extra-mitochondrial Ca^{2+} was raised in the presence of a potent inhibitor of mitochondrial Ca^{2+} uniporter, ruthenium red (Fig. 4(E)). For quantitative analysis, the mean intensity of X–rhod–5F fluorescence was determined in 8–20 random microscopy fields (200–300 neurons per field). The results show that high extra-mitochondrial [Ca^{2+}] led to a three-fold increase of fluorescence intensity over the control values from which 30% was reduced by DHEA (Fig. 5).

Similar results were obtained when isolated mitochondria were used. Mitochondria were loaded with X-Rhod-5F, incubated at different pCa for 20 min and after that the intra-mitochondrial free Ca^{2+} was detected fluorimetrically. In this case, treatment with DHEA at the same concentration as in situ experiments (100 μM) lowered significantly the sensitivity of mitochondrial Ca^{2+} accumulation to extra-mitochondrial [Ca^{2+}] (from 0.5 ± 0.1 to 0.9 ± 0.2 μM, $P < 0.01$) without affecting the maximal Ca^{2+} concentration in the matrix (Fig. 6). From these results we conclude that DHEA inhibits partly the Ca^{2+} influx into the mitochondrial matrix.

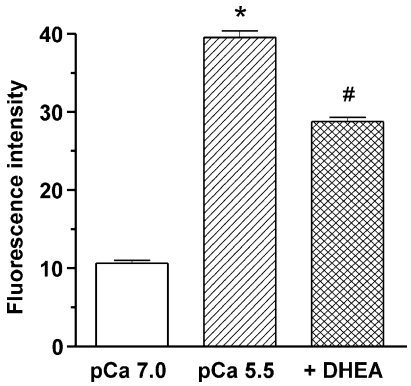

Fig. 5. Measurement of free [Ca^{2+}] in the mitochondrial matrix. Skinned neurons stained with X-Rhod-5F-AM were incubated at pCa 7.0 as a control or at pCa 5.5 in the absence or presence of 100 μM DHEA. All experiments were performed in the presence of 10 μM CGP-37157 and 10 μM cyclosporin to block the mitochondrial Ca^{2+} efflux pathways. Relative free [Ca^{2+}] in the mitochondrial matrix was assessed by monitoring the X-Rhod-5F fluorescence intensity at 605DF32 nm. Asterisk indicates significant difference vs. control and cross vs. pCa 5.5, $P < 0.01$, $n = 8$–20.

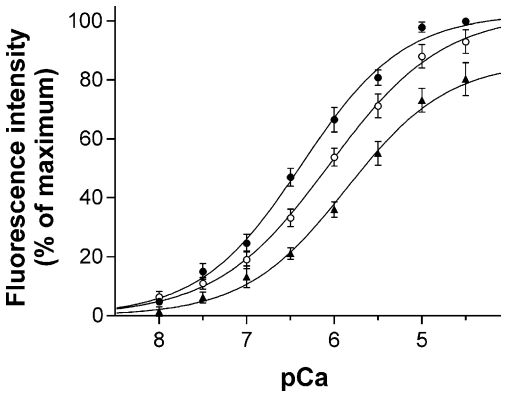

Fig. 6. Measurement of free [Ca^{2+}] in the mitochondrial matrix. Isolated mitochondria loaded with X-Rhod-5F were incubated at pCa: (closed circles) in the absence or (open circles) presence of 100 μM DHEA or (triangles) 10 μM ruthenium red. Results are expressed as a percentage of maximal fluorescence intensity obtained at control conditions.

4. Discussion

The present study is the first report, to the best of our knowledge, demonstrating that DHEA and as well the other neurosteroids inhibit Ca^{2+} influx into the mitochondria. The mitochondrial calcium uptake pathway is electrogenic and is expected to cause a depolarization of the mitochondrial potential. Inhibition of Ca^{2+} influx, on the other hand, should have an opposite effect and support the mitochondrial membrane potential. Indeed as is demonstrated in Fig. 3, in the presence of DHEA, suggested to inhibit that pathway, and at free Ca^{2+} concentrations higher than 0.32 μM, the mitochondrial membrane potential was always higher than in the control group. On the other hand, the effect of DHEA either on membrane potential or Ca^{2+} accumulation was not modulated by the inhibitors of mitochondrial Ca^{2+} efflux pathways suggesting that the observed effect was due because of inhibiting the influx and not because of the activating of efflux pathways. Moreover, the observed effect of DHEA was also evident at conditions of Ca^{2+} overload (at pCa 6–5). The latter allows the suggestion that in the presence of DHEA the mitochondria could be more resistant to cytoplasmic Ca^{2+} overload.

What could be the physiological or pharmacological significance of that phenomenon? Cytoplasmic Ca^{2+} overload and subsequent Ca^{2+} accumulation in the mitochondrial matrix are among the most devastating events of ischemic damage. Resultant loss of mitochondrial membrane potential and the concomitant decline in energy production are associated with the increased open probability of the mitochondrial permeability pore. Opening of the latter is a catastrophic event leading to the “death” of mitochondrion that will initiate pathways to cell death [21,22]. Thus, any compound that could block the mitochondrial Ca^{2+} influx reducing thus the mitochondrial [Ca^{2+}], preserves the mitochondrial membrane potential and thereby protects cells from the initiation of death cascades. Ruthenium red, the

well-known and widely used inhibitor of mitochondrial Ca^{2+} influx [24], has been shown to be protective against excitotoxic damage of neuronal cells [25–27]. Such a neuroprotectivity of ruthenium red demonstrates the importance of the mitochondrial Ca^{2+} overload in the pathogenesis of ischemia/reperfusion injury and therefore proposes antagonism of the mitochondrial Ca^{2+} uniporter as an attractive therapeutic target. However, ruthenium red, as well as other known inhibitors, has poor cell penetration and several non-specific effects (as blocking of the Ca^{2+} release channel in endoplasmic reticulum [28] and binding to several Ca^{2+} sequestering proteins [29,30]) that may lead to intracellular Ca^{2+} dishomeostasis resulting in excitotoxicity [31,32].

In our experiments, the protective effect of DHEA became evident at micromolar concentrations and achieved 50% level at 15 μM concentration. This is in the same range as observed in some in vitro experiments showing that DHEA protects hippocampal cells or hippocampal neurons against oxidative stress or excitotoxic damage at μM concentrations [33,34]. However, it should be noted that in other experiments the optimal protection against kainic acid induced toxicity was achieved already at 0.1 μM concentration [3]. Blood levels of DHEA(S) range within 1–10 μM but only a very small fraction of it circulates as free steroid [1,2]. Although the free form dominates in the brain, its concentration does not reach to micromolar levels [35]. This makes it unlikely that DHEA alone could affect the mitochondrial Ca^{2+} homeostasis in physiological conditions. However, our finding, that also other neurosteroids, pregnenolone, pregnanolone, allopregnanolone and also estradiol had a similar effect, suggests this effect to be not specific to DHEA but rather to several neurosteroids. This fits well with findings showing the neuroprotective effect of β-estradiol [36] as well of allopregnanolone [4]. It should be noted here that, at physiological concentrations, estrogens protect mitochondria also from high calcium-induced release of cytochrome *c* [37]. Structural requirements for steroid interaction with the mitochondria seem to involve α-hydroxyl at C3 of the steroid A ring. At the same time, cholesterol alone was ineffective suggesting that the above-mentioned effect is not common for all steroid structures.

Considering that the concentration of free neurosteroids is much higher in the brain than in blood and reaching to the micromolar level [23,35,38,39], it cannot be excluded that the pool of endogenous neurosteroids in the brain acts as a gatekeeper for mitochondrial Ca^{2+} influx, regulating thus the mitochondrial response to cytoplasmic Ca^{2+} overload. This is supported by our results showing that the mixture of neurosteroids at concentration range observed in the human brain [23] protected mitochondrial membrane potential from cytoplasmic Ca^{2+} overload. In summary, this current report provides evidence that DHEA with several other neurosteroids protect the mitochondria against intracellular Ca^{2+} overload by inhibiting Ca^{2+} influx into the mitochondrial matrix.

Acknowledgements

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