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NEUROACTIVE STEROIDS MODULATE *IN VIVO* THE Mg^{2+}/Ca^{2+} - ATPase ACTIVITY IN RAT CORTICAL AND CEREBELLAR SYNAPTOSOMAL MEMBRANES

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Summary: Estradiol and pregnenolone sulfate administered *in vivo* inhibited the Mg^{2+}/Ca^{2+} - ATPase activity in synaptosomal membranes from rat cortex by 20% and 30%, respectively. In cerebellum, estradiol decreased the activity up to 43%. The calmodulin - stimulated activity declined in cortex after treatment with estradiol and pregnenolone sulfate, but significantly increased in cerebellar membranes. Dehydroepiandrosterone sulfate had influence neither on enzyme activity, nor on stimulation by calmodulin in both examined rat brain regions.

Recent findings of several laboratories indicated that neurosteroids may interact with some effector systems in neuronal membranes, including G-proteins - coupled receptors or ligand-gated ion channels [1for rev.]. Allosteric modulation by progesterone and pregnenolone of drug - membrane interactions, from positive to negative, were observed for GABA A, NMDA, glycine or nicotinic receptors. Moreover, DHEAS in micromolar concentration rapidly and reversibly suppressed voltage-gated Ca^{2+} currents in hippocampal neurons [2]. The reduced metabolites of progesterone revealed also a potent anesthetic effect [3]. Another membrane effector, Na^{+}/K^{+}- ATPase pump, was significantly inhibited by estradiol and progesterone, but stimulated by testosterone treatment [4]. The neurosteroids, besides their genomic effects, may also alter the structure of lipid bilayer. 17-β-estradiol has been shown to enhance the phosphatidylinositol hydrolysis in cerebellar neurons [5].

Mg^{2+}/Ca^{2+}- ATPase (PMCA) is the plasma membrane Ca^{2+} transporter important for the maintenance of the low resting Ca^{2+} level in the nerve terminal.

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Abbreviations : **E**, estradiol; **PreS**, pregnenolone sulfate; **DHEAS**, dehydroepiandrosterone sulfate; **CaM**, calmodulin; **PMCA**, plasma membrane Ca^{2+}-ATPase.

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One peculiar property of the PMCA is the multiplicity of its regulatory mechanisms [6]. It is also the only transport ATPase regulated directly by calmodulin [7]. Three main isoforms designated PMCA 1, PMCA 2, and PMCA 3 were found in rat. Using *in situ* hybridization Stahl *at al.* [8] demonstrated nonuniform distribution of their mRNAs in different regions of brain. In addition, several alternatively spliced variants of PMCA isoforms exist.

The purpose of the present study was to determine, if the neurosteroids treatment *in vivo* modulated the plasma membrane Mg^{2+}/Ca^{2+}- ATPase activity. We have also tested whether CaM - stimulation of enzyme changed after hormone administration.

Materials and Methods

All reagents used were of analytical grade. Dehydroepiandrosterone sulfate (DHEAS), pregnenolone sulfate (PreS), 17-β-estradiol (E) were purchased from Sigma. Calmodulin was from Calbiochem. .

Animals. 3 months old Wistar female rats (6 in each group) were used for experiments. Steroids were injected interperitoneally (1μg/g) for 5 days. Control group of rats was treated with 0.9% NaCl. 12 hours after the last injection the rats were sacrificed without anesthetics. Synaptosomal membranes from cerebral cortex and cerebellum were prepared by the method of Booth and Clark using Ficoll gradient centrifugation [9]. All membranes were stored frozen at -80^0 C.

Protein assay. The concentration of protein in the synaptosomal membranes was measured by UV absorbance (280 nm), using Bluprot reagent (Electrophoresis Technics, Poland), based on Bradford dye-binding procedure [10] with bovine serum albumin as a standard.

Enzyme activity assay. Mg^{2+}/Ca^{2+}-ATPase activity was measured by colorimetric determination of Pi hydrolyzed from ATP, by the methods of Lin and Morales [11]. Calcium stimulated activity was determined by subtracting the activity in the absence of calcium from the activity in the presence of Ca^{2+}. 5μg of synaptosomal membranes were incubated for 15 min. at 37^0 C in the reaction mixture containing 50 mM Tris-HCl, pH 7.4, 130 mM KCl, 3 mM MgCl2, 3 mM ATP, 0.1 mM ouabain, 1 mM EGTA, and 1.040 mM CaCl2 in total volume 200 μL. Calmodulin when present was 72nM. The data presented in the figures are representative of at least four similar experiments performed in duplicate. The statistic calculations were carried out by Student's t-test ($P<0.05$).

Results

The activity of Mg^{2+}/Ca^{2+}- ATPase in synaptosomal membranes from rat cortex and cerebellum was examined after *in vivo* treatment with estradiol, pregnenolone sulfate and dehydroepiandrosterone sulfate. The enzyme activity of the animals treated with neurosteroids was compared with the group of rats receiving 0.9% NaCl. The basal activity of enzyme in control synaptosomal membranes was 1.89 ± 0.12 μmoles Pi/mg/h for cortex, and 1.28 ±0.10 μmoles Pi/mg/h for cerebellum. The effect of neuroactive steroids on cortical enzyme is shown in Fig.1. E and PreS decreased Mg^{2+}/Ca^{2+}- ATPase activity by 20% and 30%, respectively.

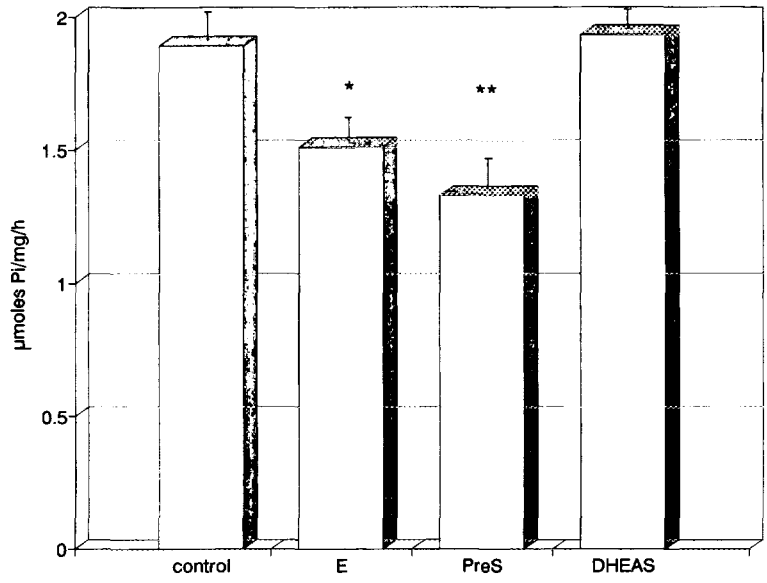

Figure 1. The effect of neurosteroids on the Mg^{2+}/Ca^{2+}-ATPase activity in rat cortical synaptosomal membranes. The results are expressed as the means ± SD of duplicate determinations with n = 4. The statistical significance: * $p<0.05$, ** $p<0.001$.

The DHEAS treatment was without a significant effect on the enzyme. The results summarized in Fig. 2 show that in PreS - and DHEAS - treated rats, the cerebellar Mg^{2+}/Ca^{2+}-ATPase activity was comparable to the control value. Only with estradiol we observed the inhibition of ATPase activity up to 43 %.

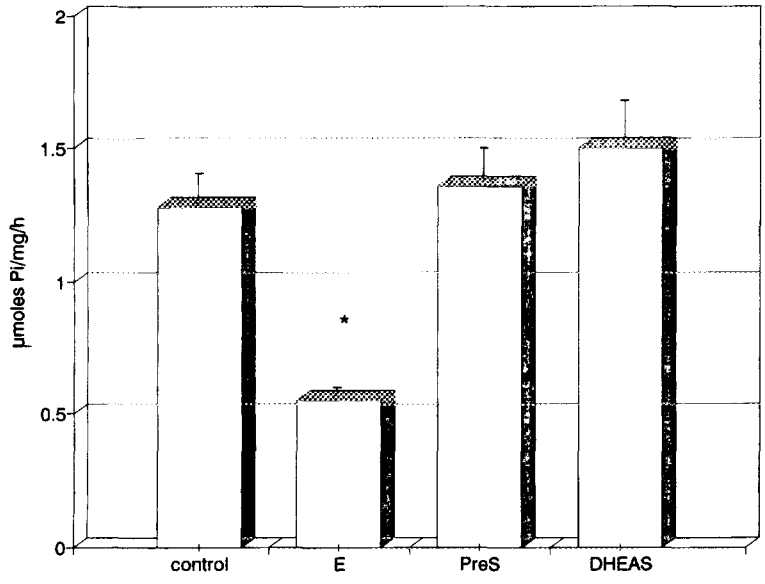

Figure 2. The effect of neurosteroids on the Mg^{2+}/Ca^{2+}-ATPase activity in rat cerebellar synaptosomal membranes. The results are expressed as the means ± SD of duplicate determinations with n = 4. The statistical significance : * $p<0.001$.

To reveal the relationship between neurosteroids modulation of the Mg^{2+}/Ca^{2+}- ATPase activity and stimulation of enzyme by calmodulin, the assay was also performed in the presence of 72 nM CaM (Fig.3). In control membranes the basal activity was stimulated by factor 1.7 for both examined regions. In cortical membranes after administration of E and PreS, the activity for hydrolysis of ATP was considerably less enhanced by CaM, and was approximately 30% - 20% lower than in the control group. In contrast, in cerebellum, the activity of enzyme with CaM increased 2.4-fold after E treatment, and 2-fold after treatment with PreS. It is important to note that DHEAS had no effect on CaM- stimulation in both examined rat brain regions.

Discussion

Mg-dependent Ca^{2+}-ATPase, a crucial membrane enzyme controlling intracellular calcium concentration, is modulated by several regulatory mechanisms. The present our study indicates that neuroactive steroids alter the ATPase activity when administered *in vivo*. Estradiol decreased the enzyme activity in both brain regions, while pregnenolone sulfate altered the activity only in cortical membranes. E and PreS revealed an opposite effect on stimulation by CaM in cortex and cerebellum . Neither ATPase activity, nor CaM-stimulation were changed after DHEAS treatment.

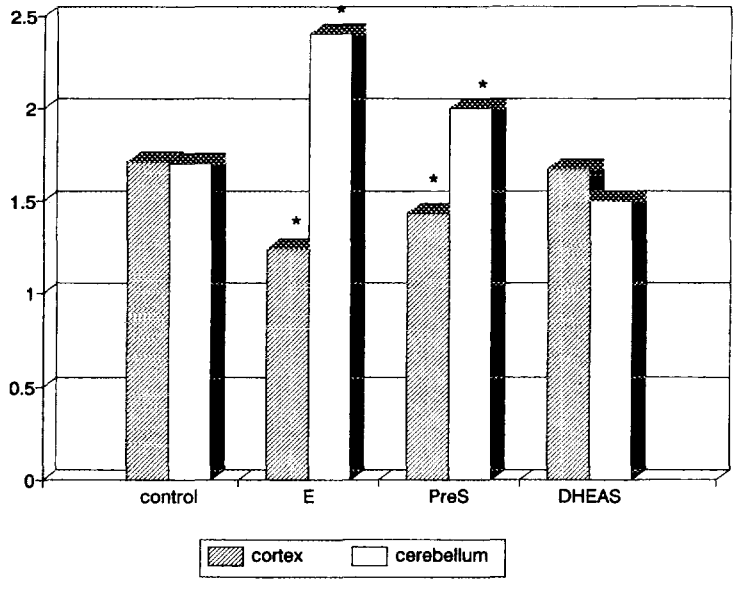

Figure 3. Effect of calmodulin on Mg^{2+}/Ca^{2+}-ATPase activity. The ability of CaM to stimulate enzyme was expressed as an activation factor (f). f=Vc/Vo, where Vc is the activity in the presence of CaM, and Vo is the activity without CaM. The statistical singnificance : * $p<0.001$.

Pregnenolone sulfate was reported to inhibit GABA- and glycine-evoked Cl^- currents and to potentiate NMDA-induced currents [12,13] . Moreover, Irvin et al. [14] have shown that PreS enhanced NMDA-induced increase in intracellular Ca^{2+} concentration. Our results suggest that the increase of calcium ions inside the nerve terminals in cortex could be also achieved by inhibition of Mg^{2+} - dependent Ca^{2+}-ATPase activity.

The synthesis of DHEA and its sulfate ester in brain is not well established yet, but the concentration of these steroids may exceed concentration in the blood [15]. Their accumulation in brain is apparently independent of adrenal and gonadal sources . The binding of DHEAS to purified synaptosomal membranes has been reported, and the dissociation constant was estimated to be 0.8 μM [16]. The sulfate esters of neurosteroids, as amphipathic molecules, could also become the integral components of cell membranes. In our experiments DHEAS was completely ineffective on either ATPase activity or CaM-stimulation, although we observed a significant increase of Mg^{2+}-ATPase activity in both tested membranes (data not shown). Mg-ATPase in synaptosomal membranes has externally oriented active sites, and can be classified as an ecto-enzyme [17,18]. The neurosteroids have been described to interact with a site located on the extracellular part of the membrane [19] . In conclusion, these and our findings may suggest that rather Mg^{2+}-ATPase can be a 'target site' for DHEAS action in synaptosomal membranes, but this hypothesis needs further investigation.

In rat brain astrocyte culture Na^+/K^+ - ATPase was inhibited by 32% after estradiol treatment [4]. In addition, estradiol has been shown to inhibit the Na^+/K^+ - ATPase in many tissues [ref. in 4]. This membrane enzyme belongs to the same P-type ion pumps as Mg^{2+}-dependent Ca^{2+}-ATPase, thus the inhibitory effect of estradiol on ATPase activity observed in cortex and cerebellum may suggest a similar mechanism of E action.

The PMCA 1-3 isoforms showed a different distribution in brain, and relative proportion between them is region-dependent. In cerebral cortex mRNAs of PMCA isoforms are equally distributed on intermediate level. A very high level of PMCA 2 expression was found only in cerebellum. PMCA 1 and PMCA 3 in this region were 10-fold less frequent than PMCA 2 [8]. Although the PMCA 4 mRNA was also detected in rat piriform cortex and neocortex, it was suggested that PMCA 4 is not a housekeeping form of the Ca^{2+}-ATPase [20]. The localization of particular isoforms of enzyme in brain may result in their different regulation. Additionally, the susceptibility of Ca^{2+}-ATPase for the regulation can also depend on specific protein/lipid milieu of neuronal membranes. Calmodulin increases the affinity for Ca^{2+} and the maximal rate of Ca^{2+} pumping [6]. Without calmodulin, the CaM-binding domain interacts with another part of the ATPase molecule, and shields the Ca^{2+} binding domain [7]. The opposite effect of PreS and E on CaM-stimulation in cortex and cerebellum can reflect the region-specific distribution of PMCA isozymes, but also the possibility that the neurosteroids in the absence of CaM, could stabilize

the less active conformation of enzyme. This might explain the discrepancy in neurosteroid effects observed by us in both regions of rat brain.

The importance of these results is of great interest, as the presence of neurosteroids in neurons, and the synthesis of some of them in brain has been reported [16,21]. Up to date, we cannot explain whether the observed effects of neurosteroids on Mg^{2+}/Ca^{2+}-ATPase activity are genomic and/or non-genomic. Moreover, direct interaction between membrane enzyme and steroids hormones cannot be excluded. However, our results provide initial evidence that neuroactive steroids can affect the Mg^{2+}/Ca^{2+} - ATPase activity, and it may represent a new regulatory mechanism of synaptosomal enzyme. Experiments in progress with purified enzyme are directed toward the elucidation of this possibility.

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