CMLS, Cell. Mol. Life Sci. 58 (2001) 636–644
1420-682X/01/040636-09 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Article

Binding of estradiol to synaptosomal mitochondria: physiological significance

A. Horvat*, G. Nikezić, S. Petrović and D. T. Kanazir

Laboratory of Molecular Biology and Endocrinology, Vinča Institute of Nuclear Sciences, P.O.B. 522, 11001 Belgrade (Yugoslavia), Fax +381 11 455 561, e-mail: ahorvat@rt270.vin.bg.ac.yu

Received 10 August 2000; received after revision 5 February 2001; accepted 7 February 2001

Abstract. The subsynaptosomal distribution and specific binding of 17β-estradiol in vitro to mitochondria isolated from presynaptic nerve endings of female rat brain were examined. 17β-Estradiol is (i) distributed unequally in synaptosomes and mitochondria posses the highest capacity to bind estradiol with respect to the available amount of the hormone. (ii) Estradiol binds specifically to isolated synaptosomal mitochondria. A Michaelis-Menten plot of specific binding was sigmoidal within a concentration range of 0.1–5 nM of added estradiol, with a saturation plateau at 3 nM. Binding of higher estradiol concentrations demonstrated an exponential Michaelis-Menten plot, indicating non-specific binding to mitochondria. V_{max} and K_m for the sigmoidal-shape range were estimated as 46 ± 6 fmol of estradiol/mg of mitochondrial proteins and 0.46 ± 0.07 nM free estradiol respectively. (iii) Esstradiol binding is not affected by the removal of ovaries. The results show that inhibition of Na-dependent Ca^{2+} efflux from mitochondria by estradiol occurs according to an affinity change of the translocator for Na^+, at the same estradiol concentrations that show specific binding to mitochondrial membranes. These data imply that physiological concentrations of estradiol, acting on mitochondrial membrane properties, extragenomically modulate the mitochondrial, and consequently the synaptosomal content of Ca^{2+}, and in that way exert a significant change in nerve cell homeostasis.

Key words. Rat brain; mitochondria; estradiol binding; calcium transport.

Steroid hormones can modulate various processes in nervous tissues. Thus, steroid hormones applied in vivo or in vitro [1–12] affect Ca^{2+} transport in various cells and tissues. Our previous investigations demonstrated that estradiol 17β-benzoate and progesterone in vivo [4, 5], and estradiol in vitro [6, 7], modulate both voltage- and Na-dependent Ca^{2+} transport in rat brain synaptosomes. The described effects of the steroids, or some of them, appear to be non-genomic and mediated via plasma membrane-binding sites [11, 13–17].

Steroid hormones are also known to affect various processes in mitochondria. Estrogen may prevent neuronal death (apoptosis) by decreasing the accumulation and production of mitochondrial reactive oxygen radicals, thereby preserving mitochondrial function, through receptor-dependent and receptor-independent mechanisms [18]. An estradiol effect has been seen on mitochondrial membrane potential changes (reduction) in cultured osteoblasts [19], F0F1-ATPase activity in rat brain [20], as well as on cytochrome c oxidase activity from brown adipose tissue [21]. Diethylstilbestrol has also been found to be a potent F0-directed inhibitor of F0F1-ATPase of rat liver mitochondria, and synthetic glucocorticoid inhibits both ATPase and ATP-dependent proton translocation activities of purified and membrane-bound enzyme [22]. Various steroid hormones may influence energy coupling

* Corresponding author.

[23], such as Ca^{2+} transport in mitochondria [24–28], but the mechanism of action is not quite clear. Some of these effects seem to be mediated via the mitochondrial membrane and are devoid of cytosolic hormone receptor. Thus, specific binding of various steroid compounds was found on non-synaptosomal mitochondria [29]. Estradiol has been reported to bind to a specific protein in rat pancreatic acinar cells which is localized in the rough endoplasmatic reticulum and mitochondria [30]. Specific binding sites have also been found for deoxycorticosterone, deoxycortisol, corticosterone, and progesterone in beef adrenal cortex mitochondria [31], and for corticosterone in rat liver mitochondria [32].

To our knowledge, there is no evidence of estradiol binding to mitochondria isolated from synaptosomes. Thus, this study, besides evaluating the intrasynaptosomal distribution of estradiol, contributes the estimation of steroid-specific binding to mitochondria with the aim of establishing whether physiological concentrations of hormone contribute to the modulation of mitochondrial Ca^{2+} transport *via* membrane-binding site(s), in that way regulating mitochondrial homeostasis and cytosolic Ca^{2+} concentrations by changing Ca^{2+} sequestration and release.

Materials and methods

Mature, cycling and chronically (3 weeks prior to use) ovariectomized (ovx) female Wistar rats were used in the experiments. The animals were maintained under constant conditions (lights on: 05.00–17.00 hours, and temperature 24 °C) and had free access to food and water.

(2,4,6,7-^{3}H)Estradiol (specific activity 84 Ci/mmol) and $^{45}CaCl_2$ (specific activity 68 mCi/nmol) were purchased from Amersham and New England Nuclear, respectively. 17β-Estradiol, and other chemicals were purchased from Sigma or Calbiochem-Boehring. Cellulose nitrate filters (pore size 0.45 µm) were purchased from Whatman.

Preparation of synaptosomes

Animals were sacrificed by cervical dislocation and whole brains were homogenized in ice-cold buffered sucrose (0.32 M sucrose, 5 mM Tris-HCl, pH 7.4). The synaptosomes were isolated and purified from pools of brain tissue (six brains/pool). Purified synaptosomes were obtained using a Ficoll gradient: 7.5% and 13% resolved in isolation medium containing 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, 50 µM K-EDTA, according to the method of Gray and Whittaker [33] modified by Cotman and Matheus [34]. Purified synaptosomes were lysed by suspension in 5 mM Tris-HCl and freezing at –20 °C. Synaptosomal lysate was used for mitochondria preparation.

Preparation of synaptosomal mitochondria

Synaptosomal mitochondria used for Ca^{2+} transport measurement and estradiol binding were prepared from synaptosomal lysate according to the procedure of Lai and Clark [35]. Isolated synaptosomal mitochondrial pellets were suspended in 0.3 M mannitol and kept at –20 °C until use. To our knowledge and that of our colleagues (working on mitochondrial SOD), mitochondria stored this way stay undisrupted and are suitable for the examinations we performed. Protein concentration was determined by the method of Lowry et al. [36] as modified by Markwell et al. [37].

Intrasynaptosomal estradiol distribution

Isolated synaptosomes (1 mg/ml) were preincubated in physiological buffer containing (in mM): 4 KCl, 142 NaCl, 2 $MgCl_2$, 10 sucrose, 25 Tris-HCl, pH 7.4 for 10 min at 37 °C. Incubation was started by adding 50 nM (^{3}H)estradiol and lasted 5–30 min. After incubation, synaptosomes were washed from unbound estradiol by centrifuging for 5 min at 1000 × g and recentrifuging with the addition of physiological buffer (5 ml). Pelleted synaptosomes were lysed by adding 5 ml of 5 mM Tris-HCl, pH 7.4, and stored at –20 °C overnight. Thawed lysate was homogenized and applied on a discontinuous sucrose gradient (in M: 1.5, 1.2, 1.0, 0.8, 0.6, and 0.4). Intrasynaptosomal organelles were separated by centrifugation at 90,000 × g for 120 min.: synaptic vesicles (0.4 M sucrose), microsomes (0.6 M sucrose), plasma membranes (interface between 0.8 M and 1.0 M sucrose), and mitochondria (interface between 1.2 M and 1.5 M sucrose). Twenty-microliter aliquots of each sucrose fraction were applied on filters and radioactivity was counted. Concentrations of estradiol calculated from radioactivity in each fraction were defined as available estradiol. Aliquots of 1 ml were vacuum filtered and washed with 5 ml of physiological buffer containing 1 mM $CaCl_2$. Concentrations of estradiol retained in structures were calculated from the radioactivity detected on filters corrected for a filter blank defined from radioactivity after filtration of (^{3}H)estradiol in washing buffer. All measurements were performed in triplicate.

Binding assay

Binding of 2,3,4,6(^{3}H)estradiol was measured in a medium containing (in mM): 300 mannitol, 10 KCl, 1 maleate, 5 glutamate, 10 Tris-HCl, pH 7.4, in a final volume of 200 µl where mitochondrial respiration (coupling) was provided. Binding of estradiol was investigated in mitochondria obtained from synaptosomes isolated from brain of cycling (intact) and OVX rats with respect to mitochondrial protein concentrations (0.05–1.25 mg/ml) and time of incubation (1–60 min). After preincubation for 10 min at 22 °C in incubation medium without hormone, mitochondria (0.65 mg/ml)

were incubated with (^{3}H)estradiol (0.1–100 nM) in a total volume of 200 µl for an additional 10 min for total hormone binding. Nonspecifically bound estradiol was determined by incubating identical aliquots of mitochondria with the labelled estradiol as above, and a 100-fold excess of unlabelled estradiol. At the end of the incubation, the mitochondria were harvested by vacuum filtration (celluloso-nitrate filters pore size 0.45 µm), and after being washed twice with 3 ml of ice-cold 0.25 M sucrose and 5 mM EDTA (to remove unbound steroid) were transferred into scintillation vials for radioactivity counting. Specific hormone binding was calculated by subtracting non-specific bound from total bound estradiol. Counts of appropriate mitochondria-free filter blanks were subtracted. The total estradiol concentration was determined in each incubation assay by counting radioactivity of 20 µl applied on filters without vacuum filtration.

Enzyme assay

F0F1-ATPase activity was assayed according to Martinez et al. [38]. Enzyme activity was determined by measuring the inorganic phosphate (P_i) liberated from ATP hydrolysis. A typical incubation lasting 10 min for F0F1-ATPase activity measurement contained (in mM): 10 Tris-HCl, pH 8.0, 2 $MgCl_2$, 2 ATP, and 130 µg of mitochondrial proteins in a final volume of 0.5 ml. The mixture was preincubated for 10 min at 30 °C with or without ATPase inhibitors. The enzyme assay was started by addition of 2 mM ATP and stopped by adding 55 µl of ice-cold 3 M perchloroaceticic acid and cooling at 0 °C for 15 min. Concentrations of P_i were measured according to Pennial [39]. The inhibitors used were 1 mM ouabain, as a specific inhibitor of plasma membrane Na,K-ATPase; 1 mM NaF/theophylline, as an inhibitor of non-specific phosphatases, and oligomycin (75 µg/mg protein) as a specific inhibitor of mitochondrial F0F1-ATPase.

Ca^{2+} transport

After preincubationo at 22 °C for 10 min in medium containing (in mM): 300 mannitol, 10 KCl, 1 maleate, 5 glutamate, 10 Tris-HCl, pH 7.4, in the absence (for Ca^{2+} loading in efflux monitoring) and presence of 5 pM–1 µM estradiol (for influx monitoring), uptake of Ca^{2+} to synaptosomal mitochondria was initiated by adding 0.2 mM $CaCl_2$ (0.6 µCi $^{45}CaCl_2$) and lasted 5 min. Uptake of Ca^{2+} was stopped by ruthenium red (17.5 µg/mg protein), a specific inhibitor of the mitochondrial Ca^{2+}-uniporter, and 1-ml aliquots were vacuum-filtered on 0.45-µm-pore-size cellulose-nitrate filters. After being washed twice with 3 ml of 0.2 M sucrose, the Ca^{2+} retained in mitochondria was calculated from radioactivity counting (corrected for a blank without mitochondria and for non-specific binding). For Ca^{2+} efflux monitoring, mitochondria were loaded with 0.2 mM $CaCl_2$ (0.6 µCi $^{45}CaCl_2$) for 5 min and after adding ruthenimured were incubated with estradiol (5 pM–1 µM or 0.5 nM for Ca^{2+} efflux at various Na^+ concentrations) for 10 min. Efflux of Ca^{2+} was initiated by adding NaCl (20 mM) and 0.2 mM EDTA and lasted 5 min. Aliquots of 1 ml were vacuum-filtered and washed as previously. Na-dependent Ca^{2+} efflux was calculated by subtracting the Ca^{2+} concentration retained in mitochondria after addition of Na/EDTA from the Ca^{2+} concentration in mitochondria after addition of ruthenium red (no Na/EDTA). Final concentrations of ethanol in the test and control media were 0.1%.

Results

Intrasynaptosomal estradiol distribution

Figure 1 shows the (^{3}H)estradiol distribution in the sucrose fraction (available) and its retention in appropriate subsynaptosomal structures during various incubation times. Estradiol was retained in the subsynaptosomal structures in the following order of decreasing efficiency: synaptic vesicles > microsomes > plasma membranes > mitochondria. However, estimating the percentage of retained estradiol with respect to the hormone available for the synaptosomal structures (table 1) shows that mi-

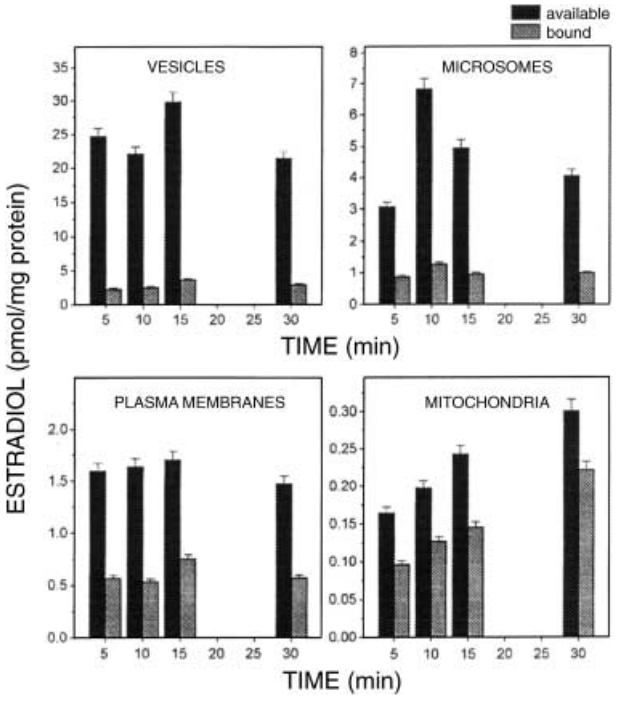

Figure 1. Intrasynaptosomal estradiol distribution. Synaptosomes (1 mg/ml) were incubated with 50 nM (^{3}H)estradiol. After the appropriate time, the amount of available and retained (after filtration on nitrocellulose filters of pore size 0.45 µm) estradiol were determined in subsynaptosomal structures: synaptic vesicles, microsomes, plasma membrane, and mitochondria (see Materials and methods). Results represent the mean + SEM of three experiments performed in triplicate.

Table 1. Intrasynaptosomal distribution of retained estradiol.

Structure	Retained estradiol[a] (% ± SEM with respect to available estradiol[b])			
	5 min	10 min	15 min	30 min
Synaptic vesicles	12.65 ± 0.63	10.27 ± 0.5	10.40 ± 0.20	10.26 ± 0.30
Microsomes	28.40 ± 1.21	21.31 ± 1.20	21.21 ± 0.34	17.84 ± 0.24
Synaptosomal plasma membranes	33.00 ± 1.10	45.00 ± 1.70	40.25 ± 1.10	42.00 ± 1.90
Mitochondria	50.50 ± 1.90	62.75 ± 1.30	56.00 ± 2.30	55.00 ± 1.94

[a] Amount of estradiol detected on filters after vacuum filtration of corresponding sucrose band.
[b] Amount of estradiol detected in sucrose band for appropriate structure.

tochondria retained the highest percentage of the hormone during the entire time investigated. Mitochondria thus appear to possess the highest affinity and/or more binding sites for estradiol than the other subsynaptosomal structures investigated.

Purity of mitochondrial preparation

To check the purity of isolated synaptosomal mitochondria, the activity of F1F0-ATPase was examined in the presence of inhibitors. The results are presented in table 2 as the percentage of ATPase activity compared to control (without inhibitors) activity. As can be seen, 70% of the detected activity can be attributed to mitochondrial ATPase (inhibited by oligomycin a specific inhibitor of mitochondrial ATPase). On the basis of these results, we concluded that the preparation of mitochondria was satisfactory for further work.

Estradiol binding to synaptosomal mitochondria

The possibility that endogenous ovarian hormones affect binding of estradiol to mitochondria was checked by comparing results obtained from OVX and intact (in proestrus) animals. The values of hormone binding were identical for OVX and intact animals, showing that this type of binding is independent of endogenous ovarian hormones (data not shown). Therefore, intact animals were used in further experiments aimed to estimate the kinetic parameters of estradiol binding to mitochondria from rat brain synaptosomes. In addition, following time dependence experiments of estradiol binding, highest binding was found to be after 10 min of incubation (fig. 2).

Figure 3 illustrates specific estradiol binding to mitochondria relative to hormone concentration. Estradiol

Table 2. Activity of F0F1-ATPase of mitochondrial preparations in the presence of various inhibitors.

Inhibitors	F0F1-ATPase activity	
	µmol Pi/mg protein/min	% control
None	0.210 ± 0.005	
Ouabain (0.2 mM)	0.183 ± 0.004	12.36 ± 3.19
NaF/theophilline (1/1 mM)	0.202 ± 0.002	3.76 ± 1.25
Oligomycin (75 µg/mg protein)	0.057 ± 0.001	72.25 ± 2.37

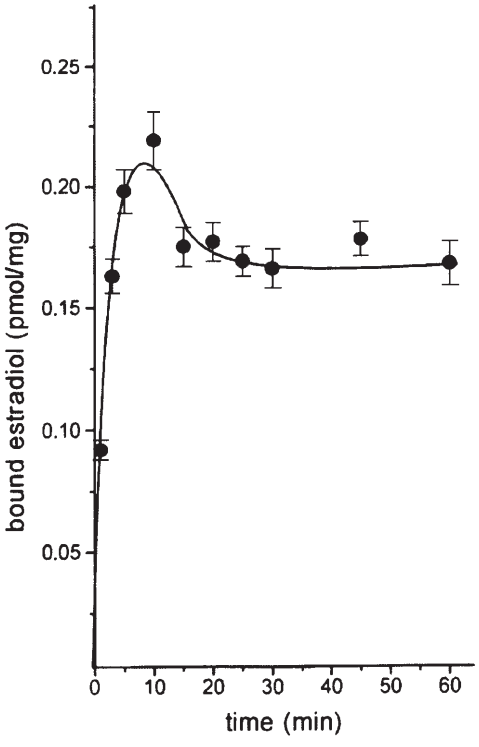

Figure 2. Effect of incubation time on estradiol binding to synaptosomal mitochondria. Mitochondria from synaptosomes were isolated and incubated in the presence of 1 nM (^{3}H)estradiol at 22 °C for various periods of time. Bound estradiol was calculated from radioactivity counting retained on nitrocellulose filters (pore size 0.45µm) after vacuum filtration, corrected for a filter blank. Results are presented as the mean ± SEM of three experiments performed in triplicate.

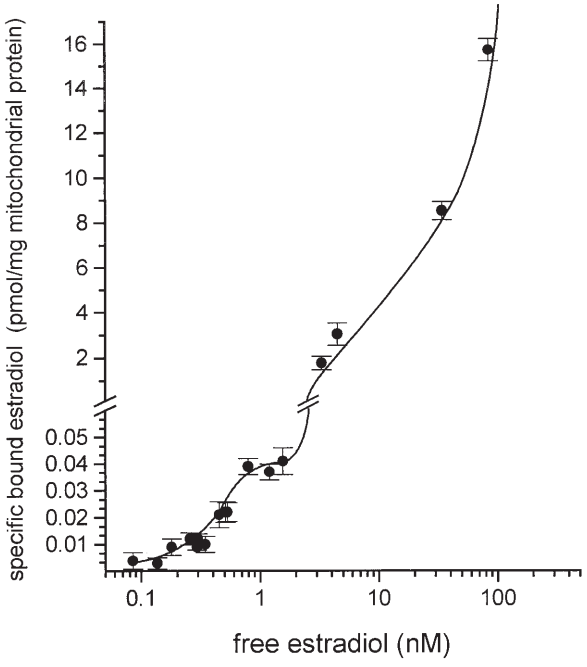

Figure 3. Dose dependence of specific (^{3}H)estradiol binding to synaptosomal mitochondria. Mitochondria (0.65 mg/ml) were incubated for 10 min with various concentration of labelled estradiol, for total binding, and with labelled and 100-fold higher unlabelled estradiol, for non-specific binding, at 22 °C. Specific binding was calculated by subtracting the non-specific binding from total bound hormone. Results represent the mean ± SEM of four separate experiments (triplicate determinations).

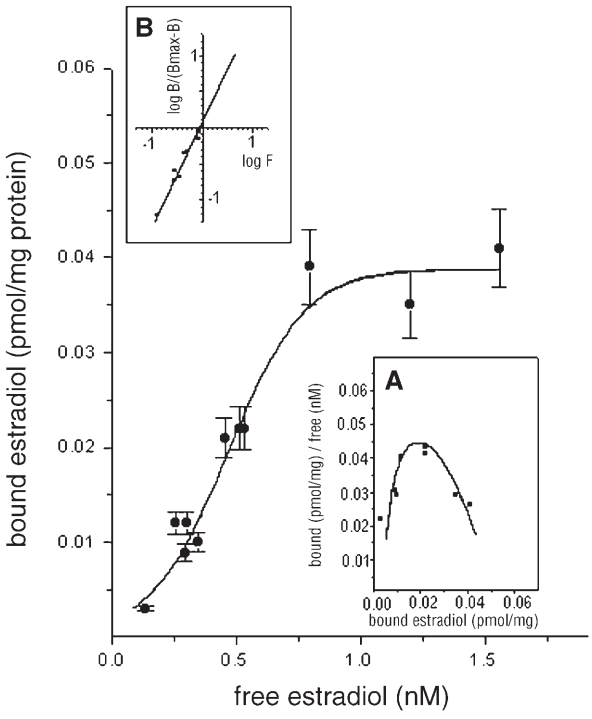

Figure 4. Michaelis-Menten plot of specific (^{3}H)estradiol binding to synaptosomal mitochondria. Mitochondria (0.65 mg/ml) were incubated for 10 min with various concentrations of labelled estradiol, for total binding, and with labelled plus 100-fold higher unlabelled estradiol, for non-specific binding, at 22 °C. Specific binding was calculated by subtracting the non-specific binding from total bound hormone. Results represent the mean ± SEM of four experiments (triplicate determinations). Insets represent Scatchard (*A*) and Hill (*B*) plots of obtained data.

binding reaches one plateau at 1–2 nM estradiol and exhibits an exponential shape at concentrations higher than 2 nM. A Michaelis-Menten plot (fig. 4) of specific estradiol binding to mitochondria in the presence of up to 2 nM of estradiol demonstrates a sigmoidal shape. The estimated V_{max} and K_m were 46 ± 5 fmol/mg protein and 0.46 ± 0.07 nM of free estradiol, respectively. A Scatchard plot (concave upward) and Hill coefficient (n = 1.5) (fig. 4 insets A, B) indicate the existence of positive cooperativity.

Effect of estradiol on Ca^{2+} transport

When comparing mitochondrial Ca^{2+} transport in the presence (5–100 nM) and absence of estradiol there were no changes in either Ca^{2+} influx through the ruthenium red-sensitive uniporter (fig. 5) or in the affinity of the uniporter for Ca^{2+} (data not shown). However, Na-dependent Ca^{2+} efflux is affected by estradiol and the dose-dependency curve shows a biphasic effect of the hormone on Ca^{2+} efflux (fig. 5). Concentrations of estradiol near physiological (up to 5 nM) decrease Ca^{2+} efflux up to 63%.

The dependence of Ca^{2+} release on the external concentration of Na^+ in the presence and absence of 0.5 nM estradiol (fig. 6) and estimation of V_{max} and K_m for Na^+ indicate that estradiol decreased the K_m value by 28% relative to the control value (control K_m was 43.7 ± 2.6 mM Na^+ and in the presence of estradiol the K_m was 31.86 ± 0.42 mM Na^+), whereas it had no influence on V_{max}.

Discussion

In this study, *in vitro* distributions of the hormone were determined in synaptosomes from whole rat brain and 0.7% of (^{3}H)estradiol added to a synaptosomal preparation was detected in mitochondria. This finding is in agreement with results on rat liver cells where a similar distribution of dexamethasone and cortisol was found [40–41]. The percentage of retained hormone (table 1) was highest in mitochondria, indicating that mitochondria possess the highest affinity or a higher number of binding sites for estradiol relative to other organelles, in agreement with the results of Moats and Ramirez [17]. In liver, adrenals, and spleen, these authors found over 75% of BSA-conjugated 17β-estradiol associated with mitochondrial and lysosomal membranes. Mitochondrial localization of steroid hormones was also found in rat pancreatic acinar tumor cells [42], and in *Xenopus laevis*

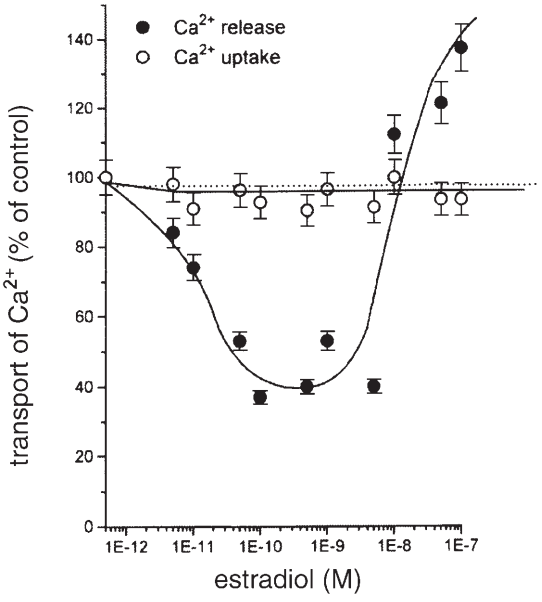

Figure 5. Effect of estradiol in vitro on mitochondrial Ca^{2+} uptake and release. Mitochondria (0.5 mg/ml) were preincubated in the presence of estradiol for 10 min at 22 °C and uptake of Ca^{2+} (200 µM of $CaCl_2$, 0.6 µCi) was determined after 5 min (open symbols). For determination Ca^{2+} release, preloaded mitochondria with 200 µM of $CaCl_2$ (0.6 µCi), after addition of ruthenium red (10 µM) were incubated for 10 min in the presence of various concentrations of estradiol. Na-dependent release of Ca^{2+} was initiated by 20 mM NaCl and 0.2 mM EDTA (solid symbols). Uptake and release of Ca^{2+} are presented as a percentage of the respective control values (10.56 ± 0.54 and 0.43 ± 0.02, respectively). Results represent the means ± SEM from six experiments (triplicate determination) (dashed line represents control values).

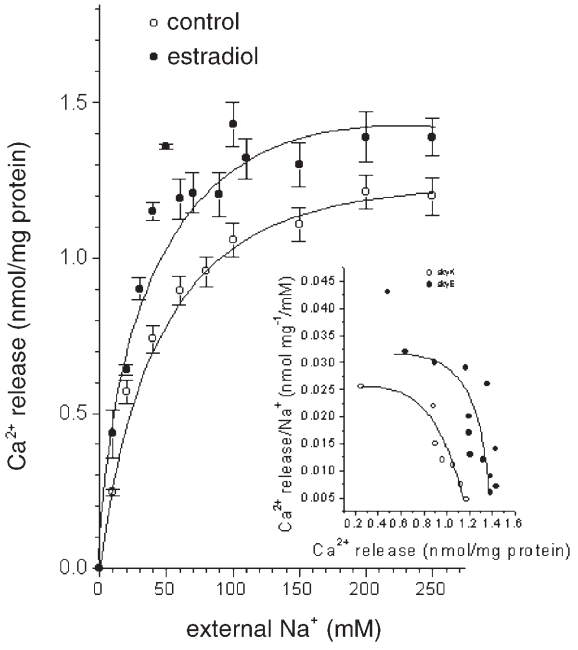

Figure 6. Mitochondrial Na-dependent Ca^{2+} release in the absence or presence of estradiol. Ca^{2+}-preloaded mitochondria as indicated in the legend to figure 4 were incubated in the absence (open symbols) or presence (solid symbols) of 0.5 nM estradiol for 10 min, and the release was measured after addition of various concentrations of external NaCl and 0.2 mM EDTA. Amounts of released Ca^{2+} were estimated as indicated in Materials and methods. Results are represented as means ± SEM from five experiments (triplicate determinations). Insert represents Scatchard plot of data.

oocytes where the intracellular pattern of progesterone, estradiol, and corticosterone were highly specific and correlated with the rhodamine 123 pattern, suggesting the involvement of mitochondria [43].

The lack of differences between estradiol binding to mitochondria isolated from intact and OVX rat brain synaptosomes (data not shown) indicates that estradiol binding to mitochondria is not under hormonal regulation. It also shows that mitochondrial samples are not contaminated with cytoplasmatic receptors, which are known to be steroid hormone inducible [44]. Similar results were obtained for rat brain synaptic plasma membranes [15]. Furthermore, binding results on intact animals (in proestrus) confirm the existence of mitochondrial-specific binding site(s) for estradiol in the concentration range up to 10^{-9} M (figs. 3, 4). Binding of the hormone at concentrations higher than 3 nM exibits an exponential binding profile indicating probable nonspecific binding (fig. 3). The existence of binding sites for cortisol on mitochondria as well as the plasma membrane in amphibian synaptosomes was proposed by Orchinik et al. [45]. Specific binding to mitochondria of deoxycorticosterone and corticosterone in beef adrenal cortex as well corticosterone [46] and estradiol [17] in liver and human hepatoblastoma cells [47] have also been demonstrated.

In the past few years, the identity and function of steroid membrane receptors involved in non-nuclear effects have been extensively investigated. A putative progesterone membrane-binding protein has recently been indentified in sperm [48] and expression of a membrane progesterone receptor cDNA leads to increased microsomal progesterone binding. A novel functional 29-kDa estrogen receptor on the sperm surface has been identified [49] which is involved in the rapid increase in intracellular calcium concentrations. Specific and saturable estradiol-binding sites of high affinity have been detected in uterine microsomes at a higher concentration than in the cytosol [50] and the existence of novel estradiol membrane-binding proteins was confirmed using monoclonal antibodies against nuclear estrogen receptor. Non-nuclear membrane effects of steroid receptors have been confirmed in endothelial cells [51–53] and breast cancer cells [54].

In our previous work [15], we found that estradiol specifically binds to synaptic plasma membranes from rat forebrain with a B_{max} of 2 pmol/mg proteins and K_m of 40 nM free estradiol. The estimated affinity of estradiol binding to the synaptosomal mitochondria isolated from

whole brain in this paper was about 100-fold higher than for synaptic plasma membranes with an about 40-fold lower capacity for estradiol (which is 10 times higher than for the cytoplasmic estradiol receptor). These findings explain the results for subsynaptosomal estradiol distribution and confirm the proposition that the higher percentage of hormone in the mitochondria compared to other structures is a consequence of higher mitochondrial affinity for estradiol.

Differences in affinity and capacity for estradiol binding between synaptic plasma membranes and mitochondria might have physiological significance in their hormonal concentration environment. Since no effect of estradiol on mitochondrial Ca^{2+} uptake, which occurs through the ruthenium red-sensitive uniporter [27], was seen (fig. 5), one might conclude that estradiol does not affect the characteristics of this protein. On the other hand, there is evidence that estradiol increases synaptic plasma membrane voltage-dependent uptake of Ca^{2+} [6, 11] and that augmentation in cytosolic Ca^{2+} results in an increase in matrix Ca^{2+} [55]. On the basis of these data, we may conclude that estradiol indirectly, acting at the plasma membrane level [6, 8, 11], increases the matrix concentration of Ca^{2+}.

The almost exponential enhancement of mitochondrial Ca^{2+} release by estradiol et concentrations of 10 nM and above (fig. 5) could be explained by non-specific effects of estradiol, which may act as a detergent to solubilize membrane proteins [7, 56, 57], and as a consequence of ion leakage. This non-specific effect on Ca^{2+} release is in agreement with the finding that estradiol at these concentrations binds non-specifically to mitochondria (fig. 3). Similar results were obtained with the Na/Ca exchanger of synaptic plasma membranes [6] and membrane vesicles [7], where estradiol exerts an exponential augmentation at a concentration where the hormone binds non-specifically to synaptic plasma membranes [15]. On the other hand, estradiol at concentrations up to 1 nM significantly inhibits the efflux of Ca^{2+} (fig. 5). Given that inhibition of Ca^{2+} efflux was detected at the same estradiol concentration at which the hormone bound specifically to mitochondria, estradiol may exert its effect *via* this transporter protein. In favour of this proposal is the finding that estradiol at concentrations at which it inhibits efflux of Ca^{2+} augments the affinity of the exchanger for Na^+ (fig. 6). The inhibitory effect of estradiol on Ca^{2+} efflux may be realized by changing the affinity of the exchanger for Na^+. The possibility that estradiol exerts its effect on the mitochondrial Na^+/Ca^{2+} exchanger, changing its affinity for Na indirectly through some other mechanism(s), cannot be excluded. Thus, Zheng and Ramirez [29] found specific binding of a 17β-estradiol-BSA conjugate to brain mitochondria (crude mitochondrial preparation) on the OSCP subunit of F0F1-ATPase/synthase. The same authors [20] have also demonstrated the inhibition of F0F1-ATPase activity in the presence of pharmacological concentrations of estrogen (higher then 10^{-4} M). The effects of nanomolar concentrations of estradiol on mitochondrial function (Ca^{2+} efflux) that we have established indicate the existence of another binding protein in the mitochondrial membrane beside the OSCP proposed by Zheng and Ramirez, or different effects of estradiol and BSA conjugate hormone [58] on non-synaptosomal and synaptosomal mitochondria.

Scheller and coworkers [59] showed that mitchondria of HeLa and Hep-2 cells were the site of glucocorticoid receptor localization and that the receptor was located within the inner space of the mitochondria, suggesting a direct pathway of hormonal regulation of mitochondrial gene transcription. Our results indicate that estradiol possesses a membrane effect independent of a receptor in the mitochondria since the effect on Ca^{2+} transport was for a short period of time and there was no difference in estradiol binding between intact and OVX animals. Furthermore, an effect of estradiol on the Na/Ca exchanger affinity for Na^+, which is opposite to the matrix side of the mitochondrial membrane, excludes an intramitochondrial receptor-mediated effect.

Estradiol (up to 1 nM) by decreasing mitochondrial Ca^{2+} efflux will also increase the mitochondrial concentration of Ca^{2+}. Elevation of mitochondrial Ca^{2+} is known to increase the activity of Ca-sensitive dehydrogenases, as has been seen for heart and other tissues [55, 60–62]. Consequently, respiration is activated and protons are extruded. An elevated proton flux may stimulate ATP-synthase activity and ATP synthesis [55, 62], which is the mode of action of some hormones [63, 64]. In addition, Ca^{2+} may control F0F1-ATPase/synthase activity, possibly mediated through the ATPase inhibitor protein [63].

Estradiol modulation of Na-dependent Ca^{2+} transport of synapsomal mitochondria, by increasing the affinity of the exchanger for Na^+, as shown in this study, in addition to the finding that estradiol modulates synaptosomal Na-dependent Ca^{2+} transport [6, 7] possibly *via* membrane-binding sites for estradiol [15], implicate estradiol as a modulator of Ca^{2+} transport mechanisms in the rat brain independent of the genome. The identity of binding sites in synaptosomal mitochondria will be a subject of our future investigations.

Acknowledgement. This research was supported by the Ministry of Science and Technology of the Republic of Serbia, project No. 03E24.

1 Batra S. (1986) Effect of estrogen and progesterone treatment on calcium uptake by the myometrium and smooth muscle of the lower urinary tract. Eur. J. Pharmacol. **127:** 37–42

2 Blackmore P. F., Beebe S. J., Danforth D. R. and Alexander N. (1990) Progesterone and 17α-hydroxyprogesterone: novel

stimulators of calcium influx in human sperm. J. Biol. Chem. **265:** 1376–1380
3 Foresta C., Rossato M. and Di Virgilio F. (1993) Ion fluxes through the progesterone-activated channel of the sperm plasma membrane. Biochem. J. **294:** 279–283
4 Nikezić G., Horvat A., Milenković L. and Martinović J. V. (1988) Ca^{2+} uptake by rat brain region synaptosomes following in vivo administration of ovarian steroids. Mol. Cell. Endocrinol. **57:** 77–80
5 Horvat A., Nikezić G., Milenković L. and Martinović J. V. (1991) Evidence for suppresion of Na-dependent Ca^{2+} efflux from rat brain synaptosomes by ovarian steroids in vivo. Experientia **47:** 627–631
6 Nikezić G., Horvat A., Nedeljković N. and Martinović J. V. (1996) 17β-Estradiol in vitro affects Na-dependent and depolarization-induced Ca^{2+} transport in rat brain synaptosomes. Experientia **52:** 217–220
7 Nikezić G., Nedeljković N., Horvat A., Kanazir D. T. and Martinović J. V. (1997) Estradiol in vitro modulates Na^{+}-dependent Ca^{2+} uptake by synaptic plasma membrane vesicles of rat brain regions. J. Endocrinol. Invest. **20:** 664–668
8 Benten W. P., Lieberherr M., Giese G. and Wunderlich F. (1998) Estradiol binding to cell surface rais cytosolic free calcium in T cells. FEBS Lett. **6:** 349–353
9 Benten W. P., Lieberherr M., Stamm O., Wrehlke C., Guo Z. and Wunderlich F. (1999) Testosterone signaling through internalizable surface receptors in androgen receptor-free macrophages. Mol. Biol. Cell. **10:** 3113–3123
10 Okabe K., Inoue Y. and Soeda H. (1999) Estradiol inhibits Ca^{2+} and K^{+} channels in smooth muscle cells from pregnant rat myometrium. Pharmacology **376:** 101–108
11 Han H. J., Lee Y. H. and Park S. H. (2000) Estradiol-17beta-BSA stimulates Ca(2+) uptake through nongenomic pathway in primary rabbit kidney proximal tubule cells: involvement of cAMP and PKC. J. Cell Physiol. **183:** 37–44
12 Lyng F. M., Jones G. R. and Rommerts F. F. (2000) Rapid androgen action on calcium signaling in rat sertoli cells and two human prostatic cell lines: similar biphasic responses between 1 picomolar and 100 nanomolar concentrations. Biol. Reprod. **63:** 736–747
13 Duval D., Durant S. and Homo-Delarche F. (1983) Interactions of steroid molecules with membrane structures and functions. Biochim. Biophys. Acta **773:** 409–442
14 Kanazir D. T., Trajković D. P., Ribarac-Stepić N., Popić S. D. and Metlaš R. (1978) Cortisol dependent acute metabolic responses in rat liver cells. J. Steroid Biochem. **9:** 467–469
15 Horvat A., Nikezić G. and Martinović J. V. (1995) Estradiol binding to synaptosomal plasma membranes of rat brain regions. Experientia **51:** 11–15
16 Nemere I. and Farach-Carson M. C. (1998) Membrane receptors for steroid hormones: a case for specific cell surface binding sites for vitamin D metabolites and estrogens. Biochem. Biophys. Res. Commun. **30:** 443–449
17 Moats R. K. 2nd and Ramirez V. D. (1998) Rapid uptake and binding of estradiol-17beta-6-(O-carboxymethyl)oxime: 125I-labeled BSA by female rat liver. Biol. Reprod. **58:** 531–538
18 Mattson M.P., Robinson N. and Guo Q. (1997) Estrogens stabilize mitochondrial function and protect neuronal cells against pro-apoptotic action of mutant presenilin-1. Neuro Rep. **8:** 3817–3821
19 Troyan M. B., Gilman V. R. and Gay C. V. (1997) Mitochondrial membrane potential changes in osteoblasts treated with parathyroid hormone and estradiol. Exp. Cell Res. **15:** 274–280
20 Zheng J. and Ramirez V. D. (1999) Rapid inhibition of rat brain mitochondrial proton F0F1-ATPase activity by estrogens: comparison with Na^{+},K^{+}-ATPase of porcine cortex. Eur. J. Pharmacol. **368:** 95–102
21 Puerta M., Rocha M., Gonzales-Covaleda S., McBennett S. M. and Andrews J. F. (1999) Changes in cytochrome oxidase activity in brown adipose tissue during oestrus cycle in the rat. Eur. J. Endocrinol. **139:** 433–437
22 McEnery M. W. and Pedersen P. L. (1986) Diethylstilbestrol: a novel F0-directed probe of the mitochondrial proton ATPase. J. Biol. Chem. **261:** 1745–1752
23 Starkov A. A., Simonyan R. A., Debukhova V. I., Mansurova S. E., Palamarchuk L. A. and Skulachev V. P. (1997) Regulation of the energy coupling in mitochondria by some steroid and thyroid hormones. Biochim. Biophys. Acta **16:** 173–183
24 Kodavant P. R. S., Ward T. R., McKinney J. D. and Tilson H. A. (1996) Inhibition of microsomal amd mitochondrial Ca-sequestration in rat cerebellum by polychlorinated biphenyl mixtures and congeners. Arch. Toxicol. **70:** 150–157
25 Rottenberg H. and Marbach M. (1992) The effect of alcanols on Ca transport in brain mitochondria. Cell Calcium **13:** 41–47
26 Cox D. A., Conforti L., Sperelakis N. and Matlib M. A. (1993) Selectivity of inhibition of Na-Ca exchange of heart mitochondria by benzothyazepine CGP-37157. J. Cardiovasc. Pharmacol. **21:** 595–599
27 Rottenberg H. and Marbach M. (1990) Regulation of Ca transport in brain mitochondria. I. The mechanism of spermine enhancement of Ca uptake and retention. Biochim. Biophys. Acta **1016:** 77–86
28 Horvat A., Marko M., Nikezić G., Nedeljković N. and Kanazir D. T. (2000) Activity of F0F1-ATPase in mitochondria isolated from rat brain synaptosomes. Arch. Biol. Sci. (Belgrade) **51:** 181–188
29 Zheng J. and Ramirez V. D. (1999) Purification and identification of an estrogen binding protein from rat brain: oligomycin sensitive-confering protein (OSCP), a subunit of mitochondrial F0F1-ATP synthase/ATPase. J. Steroid Biochem. Mol. Biol. **68:** 65–75
30 Beaudoin A. R., Grondin G., St Jean P., Pettengill O., Longnecker D. S. and Grossman A. (1991) Marked differences in immunocytological localization of (^{3}H)estradiol-binding protein in rat pancreatic acinar tumor cells compared to normal acinar cells. Endocrinology **128:** 1617–1622
31 Satre M. and Vignais V. (1974) Steroid 11beta-hydroxylation in beef adrenal cortex mitochondria: binding affinity and capacity for specific ^{14}C steroids and for (^{3}H) metyrapol, an inhibitor of the 11β-hydroxylation reaction. Biochemistry **13:** 2201–2209
32 Leshchenko M. V. and Sergeev P. V. (1987) Nature of steroid hormone binding to mitochondria. Farmacol. Toksikol. **50:** 60–63
33 Gray G. E. and Whittaker P. V. (1962) The isolation of nerve endings from brain: an electron-microscopic study of cell fragments derived by homogenization and centrifugation. J. Anat. **96:** 79–88
34 Cotman C. W. and Matheus D. A. (1971) Synaptic plasma membranes from rat brain synaptosomes: isolation and partial characterisation. Biochim. Biophys. Acta **192:** 380–394
35 Lai J. C. K. and Clark J. B. (1970) Preparation of synaptic and nonsynaptic mitochondria from mammalian brain. Methods Enzymol. **55:** 51–59
36 Lowry O. H., Rosebrough A., Farr A. L. and Randall R. J. (1951) Protein measurements with the Folin-phenol reagent. J. Biol. Chem. **193:** 265–275
37 Markwell M. A., Hass S. M., Bieber L. L. and Tolbert N. E. (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87:** 206–210
38 Martinez F., Meaney A., Espinosa-Garcia M. T., Pardo J. P., Uribe A. and Flores-Herrera O. (1996) Characterization of the F0F1-ATPase and tightly-bound ATPase activities in submitochondrial particles from human term placenta. Placenta **17:** 345–350

39 Pennial R. (1966) An improved method for the determination of inorganic phosphate by the isobutanol-benzene extraction procedure. Anal. Biochem. **14:** 87–90
40 Grote H., Ioannou I., Voigt J. and Sekeris C. E. (1993) Localization of the glucocorticoid receptor in rat liver cells: evidence for plasma membrane bound receptor. Int. J. Biochem. **25:** 1593–1599
41 Beato M., Biesewig D., Braendle W. and Seketis C. E. (1989) On the mechanism of hormone action. 15. Subcellular distribution of 1,2-(^{3}H)cortisol in rat liver. Biochem. Biophys. Acta **192:** 494–507
42 Baudoin A.R., Grondin G., St Jean P., Pettengill O., Longnecker D. S. and Grossman A. (1991) Marked differences in immunocytological localzation of (^{3}H)estradiol-binding protein in rat pancreatic acinar tumor cells compared to normal acinar cells. Endocrinology **128:** 1617–1622
43 Bronson D. D. and Stumpf W. E. (1991) Autoradiography of progesterone and model compound entry and distribution in *Xenopus laevis* oocytes. Prog. Histochem. Cytochem. **22:** 1–59
44 MacLusky N. J., Lieberburg I., and McEwen B. S. (1979) The development of estrogen receptor systems in the rat brain: perinatal development. Brain Res. **178:** 129–142
45 Orchinik M., Murray T. F. and Moore F. L. (1991) A corticosteroid receptor in neuronal membranes. Science **252:** 1848–1851
46 Leshchenko M. V. and Sergeyev P. V. (1985) Character of steroidal hormone binding to mitochondria. Pharmakologija **3:** 60–63
47 Moats R. K. II and Ramirez V. D. (2000) Electron microscopic visualization of membrane-mediated uptake and translocation of estrogen-BSA:colloidal gold by Hep G2 cells. J. Endocrinol. **166:** 631–647
48 Falkenstein E., Heck M., Gerdes D., Christ M., Weigel M., Buddhikot M. et al. (1999) Specific progesterone binding to a membrane protein and related nongenomic effects on Ca^{2+}-flux in sperm. Endocrinology **140:** 5999–6002
49 Luconi M., Muratori M., Forti G. and Baldi E. (1999) Identification and characterization of a novel functional estrogen receptor on human sperm membrane that interferes with progesterone effects. J. Clin. Endocrinol. **84:** 1670–1678
50 Monje P. and Boland R. (1999) Characterization of membrane estrogen binding protein from rabbit uterus. Mol. Cell. Endocrinol. **147:** 75–84
51 Shaul W. P. (1999) Rapid activation of endothelial nitric oxide synthase by estrogen. Steroids **64:** 28–34
52 Rubio-Gayosso I., Sierra-Ramirez A., Garcia-Vazquez A., Martinez-Martinez A., Munoz-Garcia O., Morato T. et al. (2000) 17Beta-estradiol increases intracellular calcium concentration through a short-term and nongenomic mechanism in rat vascular endothelium in culture. Cardiovasc. Pharmacol. **36:** 196–202
53 Chambliss K. L., Yuhanna I. S., Mineo C., Liu P., German Z., Sherman T. S. et al. (2000) Estrogen receptor alpha and endothelial nitric oxide syntase are organized into a functional signaling module in caveolae. Circ. Res. **87:** E44–E52
54 Importa-Brears T., Whorton A. R., Codazzi F., York J. D., Meyer T. and McDonnell D. P. (1999) Estrogen-induced activation of mitogen-activated protein kinase requires mobilization of intracellular calcium. Proc. Natl. Acad. Sci. USA **96:** 4686–4691
55 Garcia C., Pardo J. P. and Sanchez R. M. (1996) Control of oxidative phosphorylation supported by NAD-like substrates in rat brain mitochondria. Biochem. Arch. **12:** 157–176
56 Metlaš R., Nikezić G., Horvat A. and Trajković D. (1994) Cortisol stimulates membrane-bound adenylate cyclase activity. Jugoslav. Physiol. Pharmacol. Acta **30:** 83–88
57 Deliconstantinos G. (1985) Cortisol effect on (Na^++K^+)-stimulated ATPase activity and on bilayer fluidity of dog brain synaptosomal plasma membranes. Neurochem. Res. **10:** 1605– 1613
58 Stevis P. E., Deecher D. C., Suhadolnik L., Mallis L. M. and Frail D. E. (1999) Different effects of estradiol and estradiol-BSA conjugates. Endocrinology **140:** 5455–5458
59 Scheller K., Sekeris C. E., Krohne G., Hock R., Hansen I. A. and Scheer U. (2000) Localization of glucocorticoid hormone receptors in mitochondria of human cells. Eur. J. Cell. Biol. **79:** 299–307
60 Denton R. M. and McCormack J. G. (1990) Ca as second messenger within mitochondria of the heart and other tissues. Annu. Rev. Physiol. **52:** 451–466
61 Hansford R. G. (1991) Dehydrogenase activation by Ca in cells and tissues. J. Bioenerg. Biomembr. **23:** 823–854
62 Aprille J. R. (1988) Regulation of mitochondrial adenine nucleotide pool size in liver: mechanism and metabolic role. FASEB J. **2:** 2547–2556
63 Hoek J. B., Faber J. L., Thomas A. P. and Wang X. (1995) Calcium ion-dependent signalling and mitochondrial dysfunction: mitochondrial calcium uptake during hormonal stimulation in intact liver cells and its implication for the mitochondrial permeability transition. Biochim. Biophys. Acta **1271:** 93–102
64 Mc Cormack J. G., Halestrap A. P. and Denton R. M. (1990) Role of calcium ions in regulation of mammalian intramitochondrial metabolism. Physiol. Rev. **70:** 391–425