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EFFECT OF SOME ESTROGENS AND PROGESTERONE ON CALCIUM UPTAKE AND CALCIUM RELEASE BY MYOMETRIAL MITOCHONDRIA

SATISH C. BATRA

Department of Obstetrics and Gynecology and the Department of Pharmacology, University of Lund, Lund, Sweden

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Abstract—Uptake of Ca by human myometrial mitochondria was inhibited by as low as 4 μM of diethyl stilbestrol (DES) in the medium, and 20 μM inhibited Ca uptake almost completely. Ethinyl estradiol and estradiol-17β also inhibited Ca uptake but the inhibition was smaller than DES and in the order DES $\gg$ ethinyl estradiol > estradiol-17β. None of the agents had an effect on the mitochondrial ATPase in concentrations that inhibited Ca uptake. Progesterone did not affect either the Ca uptake or the ATPase activity. DES was also able to release Ca stored by mitochondria in concentrations that inhibited Ca uptake. Addition of supernatant fraction in the medium afforded protection from DES inhibition of Ca uptake. These findings together with those reported previously lend support to the argument for a role of mitochondria in contraction and relaxation of human myometrium.

THE MECHANISM by which estrogens influence the contractile activity of the uterus is not understood. Recent studies on the binding of estrogens to uterine tissue indicated that estrogens penetrate the uterine cells rapidly.[1,2] In previous studies from this laboratory[3,4] it was demonstrated that mitochondria and microsomes isolated from the human myometrium were able to take up Ca from the incubation medium in the presence of ATP. The large Ca binding capacity of the mitochondria suggested that they may play a significant role in the contraction and relaxation of the uterus by controlling the intracellular Ca concentration. In a more recent report[5] it was shown that diethyl stilbestrol in very low concentrations inhibited the uptake of Ca by mitochondria but had little effect on microsomal Ca uptake. Mitochondria from human myometrium were more sensitive to this effect of diethyl stilbestrol than mitochondria from the rat heart. It was therefore of interest to study the effects of this synthetic estrogen, natural estrogen and natural progesterone on the inhibition of Ca uptake, in the hope of elucidating their respective modes of action.

METHODS

Pieces of myometrium were removed by an operation, usually cesarean section, and collected in Krebs–Ringer bicarbonate solution.[6] They were cut into strips 2–3 mm wide and homogenized in ice-cold sucrose (0·25 M) and histidine (5 mM) solution. Mitochondria were isolated as previously described.[5] The supernatant was obtained after centrifugation at 110,000 *g* for 1 hr.

Unless otherwise stated, the standard medium for studying the uptake of Ca consisted of 0·125 M sucrose, 2 mM histidine, 5 mM ATP, 5 mM $MgCl_2$, 0·1 mM $CaCl_2$

with 1·5 μCi^{45}Ca, 50 mM Tris buffer (pH 7·2) and 0·2–0·3 mg mitochondrial protein in a total volume of 1 ml. At specified time intervals, 0·4 ml of the incubation medium was filtered through Millipore filters (0·45 μdiam.) as previously described.[4,5] All experiments were done at 30°. Ca uptake was determined by measuring the radioactivity in the filtrate by a Packard Tri-Carb liquid scintillation counter. Diethyl stilbestrol (DES), estradiol-17β and ethinyl estradiol were purchased from Sigma Chemical Company. Progesterone was generously given by Schering AG, Berlin. Stock solutions of these compounds were prepared in absolute ethanol. In the control experiments ethanol was added to obtain a concentration of 1% in the final reaction mixture. This concentration of ethanol did not affect the Ca uptake by the mitochondria.

When Ca release was studied, the mitochondria were allowed to take up Ca for 15 min and then 10 μl of the appropriate stock solution of DES was added. Ca release was followed for the next 30 min. Appropriate controls were run simultaneously. ATPase activity was usually assayed simultaneously with Ca uptake experiments by measuring Pi-content of the filtrates according to the method of Lecocq and Inesi.[7] Protein concentration was determined by the method of Lowry *et al.*[8] using bovine serum albumin as a standard.

RESULTS

The effect of three different concentrations of DES on the rate and the capacity of Ca uptake by mitochondria is shown in Fig. 1. The amount of Ca taken up by mitochondria is higher than reported previously because greater care was taken to reduce

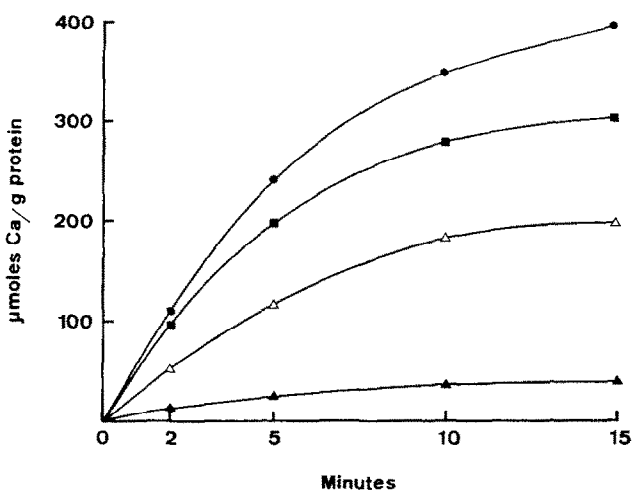

FIG. 1. Effect of three different concentrations of DES on Ca uptake by human myometrial mitochondria. Control (●), DES 4 μM (■), DES 10 μM (△) and DES 20 μM (▲).

to a minimum the time between the isolation of the mitochondria and Ca uptake measurements. Occasionally values for Ca uptake as high as 600 μmoles Ca/g protein were obtained. Ca uptake was inhibited by 24 per cent with the lowest concentration (4μM) of DES used; 10 μM DES in the medium resulted in 50 per cent inhibition and there was almost complete inhibition with 20 μM DES.

Ca uptake was also inhibited by estradiol and ethinyl estradiol, but to a smaller degree than by DES. The amount of Ca uptake after 5 and 15 min of incubation in the presence of these compounds and also in the presence of progesterone is shown in Table 1. The uptake of Ca after 15 min was inhibited by 17 and 27 per cent by 20 μM estradiol and ethinyl estradiol, respectively. With the same concentration of progesterone in the medium there was slight but insignificant inhibition of Ca uptake.

TABL 1. EFFECT OF DIETHYL STILBESTROL (DES), ESTRADIOL, ETHINYL ESTRADIOL (E. ESTRL.) AND PROGESTERONE (PROG.) ON THE UPTAKE OF Ca BY MITOCHONDRIA FROM HUMAN MYOMETRIUM

Time (min)	Control	Ca uptake (μmoles/g protein) DES	Estradiol	E. Estrl.	Prog.
5	242·98 ± 7·25	23·71 ± 0·71	194·07 ± 5·79	181·87 ± 6·07	229·76 ± 6·86
15	396·28 ± 13·86	40·04 ± 2·10	327·80 ± 11·46	290·59 ± 11·42	373·45 ± 13·06

Mean values of six experiments ± S. E. M.
The amount of Ca taken up was measured after 5 and 15 min incubation at 30°. Concentration of the above compounds was 20 μM.

In order to investigate whether the observed inhibition of Ca uptake by estrogen was a consequence of an effect on mitochondrial ATPase activity, the amount of Pi split from ATP in the absence and presence of the compounds was determined. The results are shown in Table 2. With none of the compounds used was there a significant inhibition of ATP splitting by the mitochondria.

TABLE 2. ATP SPLITTING IN THE PRESENCE OF 20 μM DES OR STEROIDS

Time (min)	Control	Pi liberated, μmoles/mg protein DES	Estradiol	E. Estrl.	Prog.
5	1·40 ± 0·05	1·28 ± 0·10	1·37 ± 0·08	1·42 ± 0·06	1·29 ± 0·11
15	3·13 ± 0·16	2·99 ± 0·13	3·24 ± 0·16	3·47 ± 0·10	3·13 ± 0·32

Mean values of six experiments ± S. E. M.
Incubations were carried out as described in Table 1.

The next step was to see whether DES, the most potent inhibitor in the present results, was able to release Ca stored by the mitochondria. Two concentrations of DES were tested. There was 33 and 72 per cent release of Ca after 30 min by 10 and 20 μM of DES, respectively, added at the time shown (Fig. 2), while the control preparation continued to accumulate Ca.

There are recent reports in the literature showing the binding of estrogen in the cytoplasmic fraction of the myometrium and endometrium of animals and man.[1,2] It was therefore of interest to see whether the inhibition by DES is influenced with the

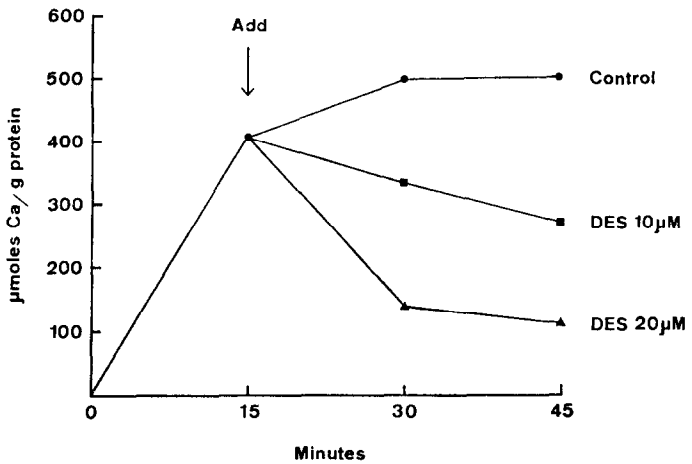

FIG. 2. Ca release by mitochondria after the addition of DES in the incubation medium.

addition of supernatant in the medium for Ca uptake. As seen in Table 3, the inclusion of a small quantity (0·24 mg protein/ml) of supernatant from the same tissue from which the mitochondria were obtained, lowered the inhibition by DES by 40 per cent.

TABLE 3. EFFECT OF THE ADDITION OF SUPERNATANT ON DES INHIBITION OF Ca UPTAKE BY MITOCHONDRIA

Fraction	Ca uptake, µmoles/g protein		
	Control	DES	Control (%)
Mitochondria	422·18	216·24	51·22
Mitochondria + supernatant	440·76	384·44	91·06

The supernatant used was obtained after centrifugation at 110,000 *g*.

The concentration of supernatant added was 0·24 mg protein/ml of the incubation medium.

Concentration of DES in the medium was 10 µM and Ca uptake was measured after 15 min at 30°.

DISCUSSION

The present results on DES inhibition of Ca uptake are in agreement with those reported previously.[5] The inhibition by an equal concentration of other estrogens was smaller and in the order DES ≫ ethinyl estradiol > estradiol-17β. This may be due to a difference in the binding affinity of these compounds to the mitochondrial components. DES binding to the estrogen receptors in the uterus has been found to be several times higher than that of estradiol.[9,10]

The present results showing an inhibition of mitochondrial Ca uptake by estrogens may be of some functional importance. The general conclusion drawn from studies on

the effect of estrogens on human uterus *in vivo* is that estrogens have some oxytocic effect in pregnant women near term.[11] Studies on myometrial strips *in vitro* have shown that estrogens are able to potentiate the responses to oxytocin and norepinephrine.[11,12] The mechanism by which estrogens influence the contractile activity of the uterus is not understood. It is now generally accepted that the intracellular concentration of Ca ions determines the contractile activity of the muscle. A concentration of cytoplasmic Ca greater than 10^{-7} M is considered to cause contraction and a reduction in concentration to initiate relaxation.[3,13–15] Thus, a decrease in the intracellular binding of Ca or a release of bound Ca would be expected to enhance contractile activity of the muscle. It is therefore not unreasonable to speculate that one of the mechanisms by which estrogens increase contractile responses of myometrium to oxytocin and other drugs may be due to their inhibition of Ca uptake by mitochondria. The large Ca binding capacity of human myometrial mitochondria and the selective inhibition of Ca uptake by estrogens[5] would seem to support this view.

It should, however, be pointed out that a greater part of estrogen entering the uterine cell would be expected to bind to the cytoplasmic or nuclear proteins (estrogen receptors) as shown by several investigators[1,2] and indirectly shown in the present results (Table 3). It would therefore leave only a fraction of the total estrogen to be available to act on mitochondria. This fractional amount would be determined by the number of estrogen receptors, the total amount of estrogen and the number of mitochondria in a given cell volume. There is no definite information yet available on these parameters. Furthermore, it is important to know the effects of estrogens on the cell membrane, particularly their influence on Ca permeability, before a meaningful interpretation of their effect on mitochondrial Ca uptake in terms of myometrial contractility can be made.

The mechanism by which estrogens lower Ca binding by mitochondria is not related to the splitting of ATP (Table 2). However, an effect on the utilization of energy from ATP splitting cannot be ruled out. On the other hand, a direct effect on the mitochondrial membrane causing it to be "leaky" would be a preferred explanation in view of the results showing a release of stored Ca by DES.

The protection from DES inhibition by the supernatant fraction may simply be explained by its binding to the supernatant. Considerable evidence exists for the binding of estrogens to the cytoplasmic proteins.[1,2]

In contrast to estrogens, progesterone, which is generally claimed to have an inhibitory effect on myometrial excitability and contractibility,[11] did not affect Ca uptake by mitochondria in the present experiments. However, the inhibitory effect of progesterone on the myometrium is considered to be due primarily to its hyperpolarizing effect on the cell membrane.[11] An inhibition by progesterone of human myometrium actomyosin ATPase has also been observed.[16]

There are several reports in the literature showing that steroids inhibit oxidation of a number of substrates and are also able to uncouple oxidative phosphorylation by rat liver mitochondria.[17,18] An inhibition of mitochondrial ATPase by progesterone has also been observed.[19] However, in all these studies much higher concentrations of these compounds were used to demonstrate the above effects. Recently, reports on the effect of steroids on erythrocyte membranes, lysosomes and liposomes have appeared.[20–22] In erythrocyte membranes and lysosomes, steroids in low concentrations (10^{-6}–10^{-4} M) have a stabilizing action but, this is reversed to a labilizing action with

higher concentrations of the steroids.[20,21,23] Both progesterone and stilbestrol in the concentration range used in the present experiments stabilized erythrocyte membranes.[23] In liposomes both estrogen and progesterone caused an increased permeability to potassium, which was slightly greater with progesterone than that obtained with estrogen.[22] It is clear therefore, that the action of steroids on different membranes is not only concentration-dependent but varies with the origin of the membranes. This is not surprising in view of the fact that different membranes show a wide variation in their lipid and protein components.[24,25] Further evidence in support of this is provided by a previous report from this laboratory[5] showing a marked inhibition by diethyl stilbestrol of mitochondrial Ca uptake but little effect on microsomal Ca uptake. In addition, myometrial mitochondria were considerably more sensitive to this effect of diethyl stilbestrol than cardiac mitochondria.[5]

There appears to be a single report in the literature[26] showing the effect of estrogen on Ca binding by mitochondria. This study, however, was performed by administering the steroids to rats and comparing Ca uptake by isolated liver mitochondria with uptake by mitochondria from control animals. There was a higher Ca uptake by mitochondria from the estrogen-treated animals. If estrogen was able to release Ca from mitochondria *in vivo* as it did in the present experiments *in vitro*, the mitochondria isolated from the treated animals would be depleted of Ca, and thus should be able to take up more Ca than the untreated animals.

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