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Increase by oestrogen of calcium entry and calcium channel density in uterine smooth muscle

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1 The effect of *in vivo* oestrogen treatment on both ^{45}Ca influx in myometrial strips and the kinetics of [^{3}H)-nitrendipine binding in the isolated plasma membrane (PM) was studied.

2 After four days of continuous oestrogen treatment of ovariectomized rats, ^{45}Ca influx in both resting (unstimulated) and K-stimulated myometrium was substantially increased.

3 Oestrogen treatment significantly increased the density in PM of nitrendipine binding sites and also caused a slight and insignificant increase in the affinity for nitrendipine.

4 The data suggest that the increase in Ca entry following oestrogen treatment prol ably results from an increase in calcium channel density.

Introduction

It is well known that oestrogen treatment in many species, including rat, rabbit and man, increases the excitability and motility of the uterus (Bozler, 1941). Since calcium plays a key role in the excitation, and excitation-contraction-coupling processes, it would be logical to assume that calcium permeability following oestrogen treatment is altered. Some preliminary evidence for an increase in uterine calcium uptake *in vitro* after oestrogen treatment in rats has previously been presented (Batra & Sjögren, 1983). One mechanism by which oestrogens could increase calcium entry into the myometrium cell is by inducing the formation of new calcium channels in the myometrial membrane. The influx of extracellular calcium through membrane calcium channels is considered to be of major importance for the initiation of contraction in smooth muscle. Data on the characterization of membrane channels for calcium entry in the rat myometrium, using nitrendipine as the radioligand, were recently presented (Batra, 1985). In the present study the effect of oestrogen treatment on both net influx of calcium in the intact uterine smooth muscle and density of calcium channels, in the isolated membranes of ovariectomized rats, was examined.

Methods

Animals and treatment

Female rats weighing between 150 and 200 g were bilaterally ovariectomized. Untreated rats were used as such after two weeks whereas treated rats were given a single injection of polyoestradiol phosphate (Estradurin, Leo, Sweden) dissolved in saline solution 1 mg kg^{-1} i.m. With this treatment steady and relatively high levels of oestradiol in the blood are maintained for at least 10 days (Batra *et al.*, 1978). The rats were killed 4 days after the injection and uterine horns removed. Generally, uteri from 11–12 rats were collected. After trimming excess fat and connective tissue the uterine horn was cut open and endometrium removed by thorough scraping.

Preparation of subcellular fractions

The various subcellular fractions were isolated by a method essentially similar to that described by Jaqua-Steweart *et al.* (1979) with some modifications (Batra, 1985). The tissue was homogenized in about 4 volumes of sucrose-HEPES (sucrose 0.25 M and HEPES 10 mM) buffer with a Polytron homogenizer (PGA 10–35) for 2 s at one half speed. The homogenate was centrifuged at 200 *g* for 3 min. The supernatant fluid was removed with a Pasteur pipette and kept. The residue was resuspended in the original volume of buffer, homogenized and centrifuged again as above. This process of residue homogenization and centrifugation was repeated six times and the supernatant fluid after each centrifugation was kept. The pooled supernatant was filtered through two layers of gauze and the filtrate centrifuged at 175,000 *g* for 20 min in a Beckman L-5-65 centrifuge. The supernatant was discarded

and the pellet, which represented crude membrane preparation, was suspended in 20 ml of buffer and subjected subsequently to centrifugation on a discontinuous sucrose gradient as described previously (Batra, 1985). Bands containing plasma membrane (PM), endoplasmic reticulum (ER) and mitochondria (M) were successively aspirated from the top of the centrifuge tube. The residue at the bottom, containing nuclei myofibrills and cell debris, was discarded. The collected PM, ER and M bands were then centrifuged at 175,000 *g* for 30 min. The final pellet from each was suspended in sucrose-HEPES buffer to give a protein concentration of approximately 1 mg ml^{-1} and stored frozen at 70°C until used for binding experiments. The protein concentration was determined by the method of Lowry *et al.* (1951). Specific binding of [^{3}H]-nitrendipine to subcellular fractions was measured at 25°C as described previously (Batra, 1985). Data of preliminary experiments showed that nitrendipine binding to plasma membrane at 37°C was lower than that measured at 25°C.

Tritiated nitrendipine with a specific activity of 79 Ci $mmol^{-1}$ was purchased from New England Nuclear Corporation. The radiochemical purity of [^{3}H]-nitrendipine was checked by h.p.l.c. Over 95% of the radioactivity was obtained in a single peak. Nitrendipine was protected from light during use and storage and it was checked periodically for purity.

Calcium influx

The increase in the influx of calcium into the uterine smooth muscle following K^+-depolarization was estimated by measuring the increase at 37°C in ^{45}Ca content of the uterine strip before and after depolarization. Lanthanum, which has been shown to displace extracellular calcium while having no effect on intracellular calcium was used to remove ^{45}Ca in the extracellular space (Van Breemen *et al.*, 1972; Godfraind, 1976). The method by which only the net calcium influx was measured in uterine tissue has been described in detail previously (Batra, 1985). The washing of tissue in ice-cold $LaCl_3$ containing solution ensured removal of extracellular calcium as well as inhibition of the active extrusion of intracellular calcium.

Myometrial strips (3 × 3 mm) were equilibrated for 60 min in a physiological Na-HEPES solution (composition, mM: NaCl 135, KCl 4.6, $MgCl_2$ 1.2, $CaCl_2$ 1.5, glucose 11, HEPES 10) maintained at 37°C and gassed with 100% O_2. After this preincubation period the strips were incubated for 2 min in Na-HEPES solution containing ^{45}Ca to allow exchange of extracellular calcium with the tracer. Thereafter the strips were incubated additionally for 2 min in ^{45}Ca-containing Na-HEPES solution (unstimulated ^{45}Ca uptake) or depolarizing K-HEPES solution (composition, mM: NaCl 4.6, KCl 135, $MgCl_2$ 1.2, $CaCl_2$ 1.5, glucose 11, HEPES 10). This short period of exposure to ^{45}Ca and K^+ stimulation ensured that only the net calcium influx was being measured. The tissues were then taken out and rinsed quickly (about 5 s) in a large volume (150 ml) of La-HEPES solution (composition, mM: NaCl 125, KCl 4.6, $MgCl_2$ 1.2, $LaCl_3$ 10, glucose 10, HEPES 10) maintained at 2°C. Uterine strips were then washed for 45 min in 5 ml of ice-cold (2°C) La-HEPES solution. After washing, the tissues were lightly blotted with a filter paper, placed in scintillation vials and weighed. After the addition of 10 ml Instagel to the vial, radioactivity of the samples was counted in a liquid scintillation counter. The radioactivity was related to the apparent tissue content of calcium (μmol g^{-1} wet weight).

Results

The data presented in Figure 1 show that calcium entry following oestrogen treatment in the resting (unstimulated) as well as K-stimulated uterine smooth muscle was substantially increased. The difference between control and oestrogen-treated for both unstimulated and K-stimulated tissues was significant ($P < 0.005$). Figure 2 shows data on the specific binding of [^{3}H]-nitrendipine to isolated uterine subcellular fractions from ovariectomized rats. Binding to PM was at least six times higher than that in ER or M fractions.

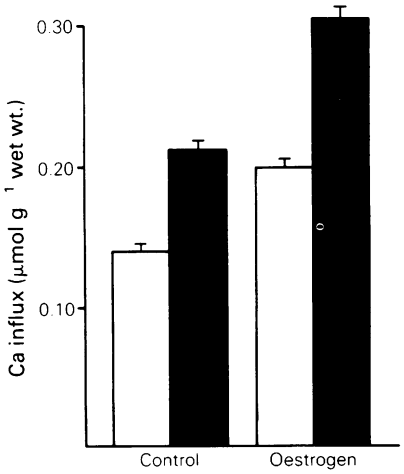

Figure 1 Effect of *in vivo* oestrogen treatment on calcium influx in the rat isolated myometrium. The influx of ^{45}Ca was measured at 37°C in both unstimulated (open columns) and K-stimulated (solid columns) myometrial strips. Columns represent the mean of 5 separate determinations and vertical lines show s.e.mean.

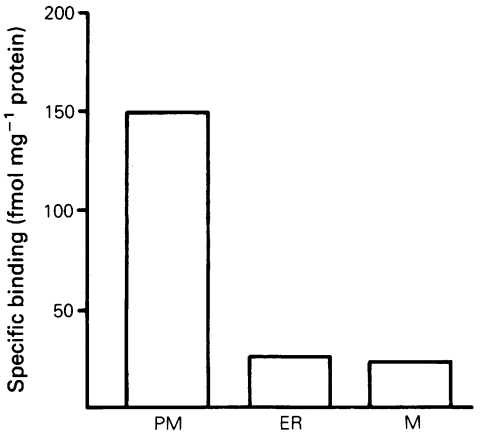

Figure 2 Specific binding of [^{3}H]-nitrendipine in plasma membrane (PM), endoplasmic reticulum (ER) and mitochondrial (M) fractions of the rat myometrium from ovariectomized untreated rats. Subcellular fractions were incubated with 0.5 nM [^{3}H]-nitrendipine for 30 min at 25°C. Non-specific binding was measured by the addition of 0.25 μM nitrendipine.

The time course of association of nitrendipine binding to PM preparation from untreated rats shown in Figure 3 was very similar to that found earlier in oestrogen-treated rats (Batra, 1985). The binding kinetics, as analysed by Scatchard plot, for both untreated and oestrogen-treated rats are shown in Figure 4. The density of binding sites in the myometrial PM from untreated and oestrogen-treated rats was 168 ± 16.2 and 329 ± 30.4 fmol mg^{-1} protein, respectively ($n = 5$), and the difference between the two was significant ($P < 0.01$). Although the mean value for the dissociation constant (K_D) was lower in the oestrogen-treated (168 pM) than that in the untreated group (290 pM), the difference was not statistically significant.

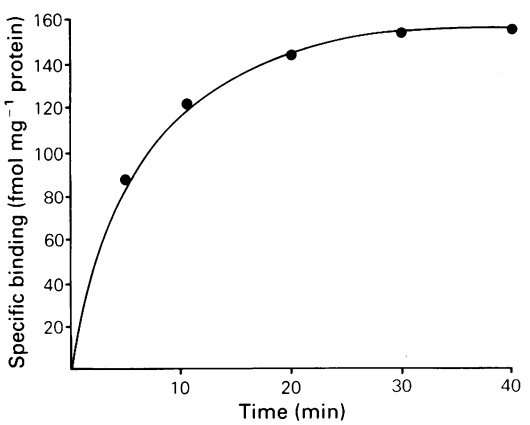

Figure 3 Time course of association of [^{3}H]-nitrendipine binding to plasma membrane (PM) fraction. PM was incubated with 0.5 nM nitrendipine at 25°C for various time intervals as indicated. Each point is the mean of four determinations.

Discussion

Oestrogen treatment has previously been shown to increase adrenoceptors in the uterine and urethral smooth muscle (Williams & Lefkowitz, 1977; Larsson *et al.*, 1984), oxytocin receptors in the uterus (Fuchs *et al.*, 1983) and muscarinic cholinoceptors in the urinary bladder (Levin *et al.*, 1980). The data of the present study show for the first time that oestrogen also increases the density of membrane calcium channels. The relative binding of nitrendipine to various subcellular fractions was very similar to that found previously in oestrogen-treated rat uteri (Batra, 1985), indicating the predominance of binding sites in PM. The time course of nitrendipine binding was also very similar to that found previously in oestrogen-treated rat uteri.

Published data on the effect of various calcium channel blockers seem to suggest that blocking of calcium channels by these agents is use-dependent. Although the state of the channels, activated or non-activated, in the broken cell membranes used in this study is not known, the present data clearly showed an increase in both calcium entry (Figure 1) and calcium

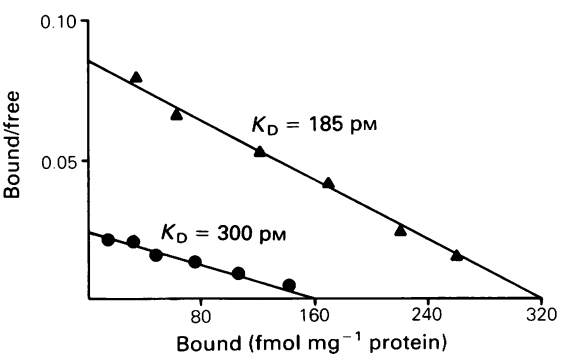

Figure 4 Scatchard analysis of specific binding of [^{3}H]-nitrendipine to plasma membrane (PM) fraction of the rat myometrium from untreated (●) and oestrogen-treated (▲) ovariectomized rats; PM was incubated for 30 min at 25°C with various concentrations (0.02–0.5 nM) of [^{3}H]-nitrendipine.

channel density (Figure 4) after *in vivo* oestrogen treatment.

In a previous study where we measured total calcium uptake after one hour of incubation instead of net influx of calcium as determined in the present study, we found that following oestrogen treatment calcium uptake was increased by more than two fold (Batra & Sjögren, 1983). In the present study the net influx of calcium following oestrogen treatment increased by about 50% in both unstimulated and K-stimulated myometrial strips.

Since oestrogens when applied *in vitro* inhibit calcium uptake (Batra & Bengtsson, 1978), it would appear that the increased calcium entry following *in vivo* treatment with oestrogen was probably a result of formation of new channels. Data from our previous study also showed that one hour after oestrogen treatment the increase in calcium uptake was not significant, but it was significant after 24 h and tended to increase thereafter with longer duration of treatment (Batra & Sjögren, 1983). This, together with the data presented here suggests that oestrogen induced the formation of new calcium-channels in myometrial membrane through a genomic mechanism. The effect of oestrogen treatment on calcium uptake appears to be specific for the uterus, a target-organ for oestrogen, since recent data from this laboratory indicated the absence of such an effect in female urethra and urinary bladder (Batra, 1986). These observations further suggest that the present action of oestrogen is mediated through genomic activation (Batra, 1980). Whether it is strictly regulated by mechanisms involving an intracellular oestrogen receptor is not known, but it offers a most intriguing area for future studies.

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