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Relaxant Effects of Estradiol through Non-Genomic Pathways in Male and Female Pig Bladder Smooth Muscle

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Key Words
Smooth muscle · Urinary bladder · Estradiol · Overactive bladder · Estrogen receptor antagonist

Abstract
The precise effect of low estrogen levels on urinary bladder contractility remains controversial. The present study was designed to analyze the effect of 17β-estradiol in bladder smooth muscle contractility and the involvement of specific estrogen receptor stimulation in this effect. Castrated male and female pig detrusor strips were mounted for tension recording in an organ bath, superfused with Krebs solution at 37°C and stimulated electrically and pharmacologically. In order to verify the acute effect of 17β-estradiol on muscle contractility, the strips were incubated with different concentrations of the hormone. Muscle contractions were induced by potassium chloride, acetylcholine chloride and electrical field stimulation. The involvement of the estrogen receptor in the effects of 17β-estradiol was assessed by incubation of some strips with the selective estrogen receptor antagonist ICI 182.780 before estradiol was applied. Estradiol at a dose of 30 μmol/l elicited a lower amplitude of contractions induced by EFS, Ach and KCl in female as well as in castrated male pig bladder smooth muscle strips. The effects of 17β-estradiol were stronger in contractions induced by potassium chloride than those induced by other forms of stimulation. Pre-treatment with the pure estrogen receptor antagonist had no effect on 17β-estradiol-induced inhibition of muscle contractility. These observations suggest that 17β-estradiol induces lower amplitude of contraction of female as well as castrated male pig detrusor which is not mediated by the classic estrogen receptor. Furthermore, we can conclude that estradiol has a stronger inhibitory effect on the depolarization of muscle cell membrane compared to a muscarinic receptor-induced contraction.

Introduction

Numerous epidemiological observations, clinical mechanistic studies, and basic laboratory studies have suggested that the incidence of urinary symptoms increases with menopause and that hormone replacement therapy, or more specifically, estrogen replacement therapy is associated with beneficial effects on the lower urinary tract in post-menopausal women [1–3]. Estrogen has a multitude of biological effects that may account for its apparent benefits on the urinary tract (which remain to be proved in randomized clinical trials) including favorable effects on smooth muscle of both bladder and urethral

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vasculature [1, 2]. However, there are considerable contradictory data published in the literature on the specific effects of estrogen administration on bladder contractility [1, 4].

Steroids hormones are recognized as producing their major long-term effects on cell structure and function via intracellular receptors acting on the expression of genes. There are currently new reports of studies that have concentrated on the genomic or morphological manifestations of steroids on receptor expression, transmitter synthesis and morphology [5, 6]. However, there is increasing evidence that steroids also affect the surface of cells and alter ion permeability, as well as release of neuronal hormones and neurotransmitters. For example, estrogen has been shown to immediately alter the tone of vascular and other smooth muscle, probably, through steroid receptors located in the cell membrane which are not inhibited by classic estrogen receptor (ER) antagonist [7]. Other steroids, such as adrenal steroids and naturally produced and synthetic analogs of steroids, also show membrane effects. Whereas there is no doubt that the distinction between genomic and non-genomic mechanisms has considerable validity, it does not go far enough in addressing the variety of mechanisms that steroid hormones use to produce their effects on cells.

The purpose of the present study was to examine the acute effects of 17β-estradiol on castrated male and female bladder contractile function, as well as to determine whether ERs are necessary for mediation of the effect of estradiol on muscle contractility.

Materials and Methods

Tissue Preparation

Experiments were performed on adult female and castrated male pig urinary bladders obtained from the slaughterhouse approximately 30 min after slaughter. Each group of experiments was performed in six male and six female pig bladders. Strips of 2 × 2 cm were taken, dissected from the dorsal side of the bladder dome and transported to the laboratory in oxygenated Krebs solution (NaCl 118 mmol/l; KCl 4.7 mmol/l; $NaHCO_3$ 25 mmol/l; KH_2PO_4 1.2 mmol/l; CaCl 1.8 mmol/l; $MgSO_4$ 1.2 mmol/l; glucose 11 mmol/l; pH 7.4; aerated with 95% O_2/5% CO_2). The mucosa and the submucosal fat layer were removed using a binocular microscope, and strips of a comparable size, 0.3 mm diameter and length between 1 and 2 mm, were excised. To facilitate diffusion, the thin layer covering the muscle fiber was opened and for the greater part removed. Care was taken that the muscle fibers were running longitudinally.

Organ Bath

Each strip was positioned horizontally in our custom-made organ bath, which contained a volume of 0.23 ml (IDEE, University Maastricht) between two tweezers of which one was attached to a KG4 force transducer connected to the BAM4C amplifier (Scientific Instruments, Heidelberg, Germany). Another tweezer was connected to a translation stage that was regulated by a controller (translation stage M111, 1DG; Mercury Controller C860; Physic Instrumente). An electrical field was generated between two platinum electrodes by a HM8130 Function Generator from Hameg Instruments. Flow in the organ bath was regulated with separate in- and outflow syringe pumps (Vickers Medical, IP4). Temperature was kept at 37°C using infrared radiation from a halogen lamp (Philips, 12 V, 20 W, 6°) and controlled using a 200-μm diameter thermocoupler (Omega ChAl/005).

Experimental Protocol

The muscle strips were placed in the organ bath and incubated in Krebs. We determined L_0 at 37°C; this is the length at which maximum isometric force is developed. The strips were stimulated at the length at which maximum isometric force was developed. The strips were stimulated in a random order: once electrically, once with acetylcholine (10 μmol/l) and once with potassium (100 mmol/l). Electrical field stimulation (EFS) was given for 10 s with alternating amplitude of 7.5 V, a pulse duration of 5 ms and a frequency of 100 Hz. When a muscle strip developed <100 μN force it was excluded from further measurements. There was an interval of 10 min between each stimulation. To examine the concentration-dependent effects on muscle contractility, each strip was exposed to 17β-estradiol in three different concentrations (1, 10 and 30 μmol/l). After 20 min of incubation with a concentration of the agent, the strips were stimulated again in a random order with electrical field, acetylcholine and potassium. After washing out the hormone for 20 min, a last EFS and pharmacological stimulation was applied before the next concentration of estradiol was applied to the organ bath. The control group with six muscle strips underwent the same stimulation protocol with dimethylsulfoxide in order to see whether it induced muscle relaxation.

The involvement of ERs in the effects of 17β-estradiol were assessed by incubating six castrated male and six female strips with the selective ER antagonist ICI 182.780 (at the same concentrations of estradiol) for 45 min before the three different concentrations of 17β-estradiol were added to the organ bath. ICI 182.780 is a type of receptor antagonist that is specific to intracellular ER-β.

Compounds

Acetylcholine hydrochloride (Ach), potassium chloride (KCl) and 17β-estradiol were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). ER antagonist (ICI 182.780) was purchased from Tocris Cookson, Inc. (Ellisville, Mo., USA). All solutions were freshly prepared. 17β-Estradiol and ICI 182.780 were dissolved in dimethylsulfoxide (DMSO) and diluted in Krebs and others were dissolved and diluted in Krebs. The final concentration of DMSO in bathing solution was <0.03%.

Data Analysis

All isometric contractions were sampled at a rate of 100 Hz with a Pentium® 133 MHz type (AD conversion DAS 800) computer and stored for further analysis. Phase plots, which represent the first derivative of force as a function of the force itself, were calculated. Normally these phase plots of isometric smooth muscle contractions can be characterized by a straight line where F is the measured force, F_{iso} is the maximum extrapolated isometric force, t is time, and C (see formula) is the negative reciprocal of the time constant for iso-

Table 1. Values of maximal force of contractions induced by electrical field stimulation (100 Hz), acetylcholine (10 μmol/l) and potassium (100 mmol/l) in isolated smooth muscle strips from castrated male and female pig bladders in Krebs solution (without any treatment)

	Maximal force of contractions, μN	
	mean in 6 female pigs	mean in 6 male pigs
Electrical field stimulation	579.7 (498.1–602.1)	550.6 (478.0–588.8)
Acetylcholine hydrochloride	550.8 (512.7–613.4)	514.3 (458.4–568.4)
Potassium chloride	470.3 (415.5–498.6)	439.8 (412.3–487.4)

Values are described in microNewton (μN).

metric force development. The time constant is an indicator of the rate-limiting process in the excitation-contraction coupling and tells when 66% of the maximum force saturation level is reached [8]. The smaller the value of C, the faster the rate of force development:

$$F = F_{iso} [1 - e^{-(t/C)}]$$

where C represents the rate-limiting constant in the excitation-contraction coupling process.

Artifacts due to movement of fluid could be excluded from further analysis because they were recognized as irregular spikes. The value of EFS at L_0 was seen as the optimal stimulation and therefore as reference for F_{iso} and time constant. This is maximum force development without treatment with estradiol. When the strips were incubated with estradiol, we calculated the percentage of reduction of initial force development. These percentages were averaged for all six muscle strips from each group. Changes in rate of force development compared to the baseline measurements were determined. All calculations were processed in Matlab® 12.1.

Statistical Evaluation

Data are expressed as means ± SEM, when appropriate. Differences of maximal force and time constant between pre- and post-treatment were evaluated with SPSS 8.0 for Windows using Student's paired t test. The level of significance chosen was 95% ($p < 0.05$). It was not necessary to normalize the values of maximal force and time constant of the strips because care was taken that the strips were very similar in order to reduce variability.

Results

Characteristics of Contractions of Isolated Detrusor Strips Induced by Electrical and Pharmacological Stimuli in Krebs Solution

The maximal force of contractions induced by EFS and Ach was higher than those induced by KCl under normal conditions in female as well as male bladder smooth muscle. However, these results were not statistically significant ($p > 0.05$). The values of maximal force of contractions induced by EFS, Ach and KCl are described in the table 1.

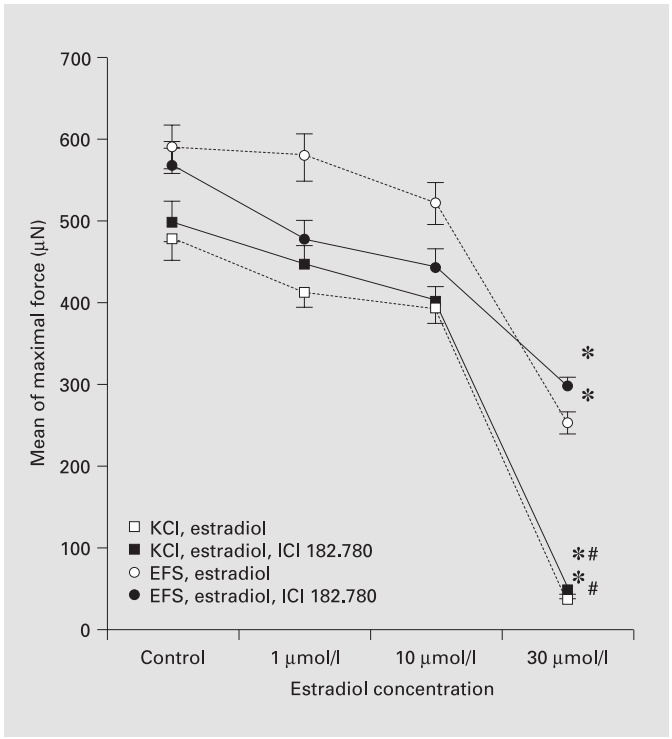
Effects of Vehicle Control

The vehicle control for estradiol and ER antagonist, DMSO (0.03%), had no effect on the muscle contractility (results have not been shown).

Effects of 17β-Estradiol on Muscle Contractility

17β-Estradiol at a dose of 30 μmol/l elicited a reversible lower amplitude of contractions induced by EFS, Ach and KCl in female as well as in castrated male pig bladder smooth muscle strips (fig. 1–3) ($p < 0.05$). In contractions induced by KCl a reduction was seen to 10% of the maximal force in female bladders and 11% in male bladders after incubation with estradiol (30 μmol/l). Contractions induced by EFS reduced to 53 and 40% after estradiol has been added to the organ bath, in female (fig. 4) and male bladders, respectively. Incubation of the strips with estradiol reduced the maximal force of contractions induced by Ach to 38 and 34% in female and male bladder strips, respectively. The decrease of the maximal contractile response of bladder strips after KCl-induced contractions was more than after EFS- and Ach-induced contractions ($p = 0.03$). The data above show that the relaxation was similar in female and male pig bladders.

There was not any statistically significant difference shown in the rate of force development among contractions induced by EFS, Ach and KCl in female and castrated male strips that were pre-treated with estradiol in comparison with contractions induced in normal conditions (Krebs solution) ($p > 0.05$).



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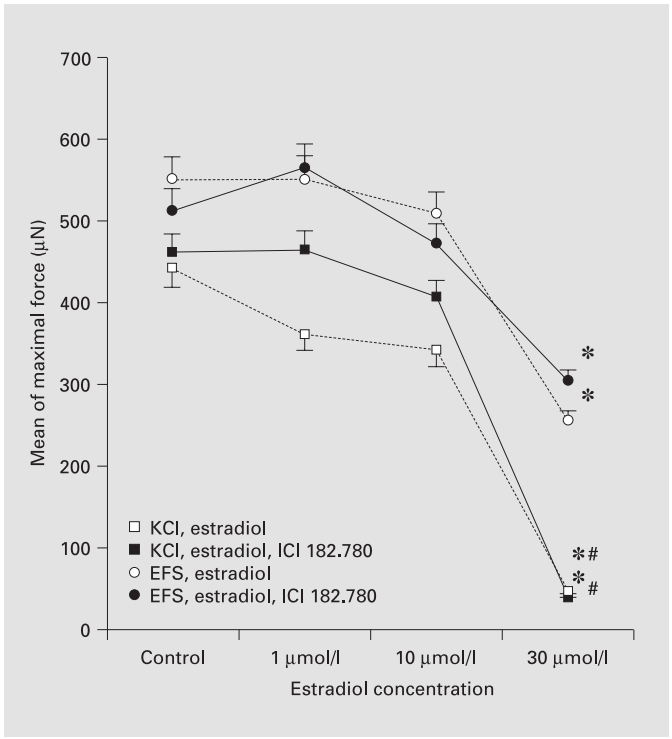

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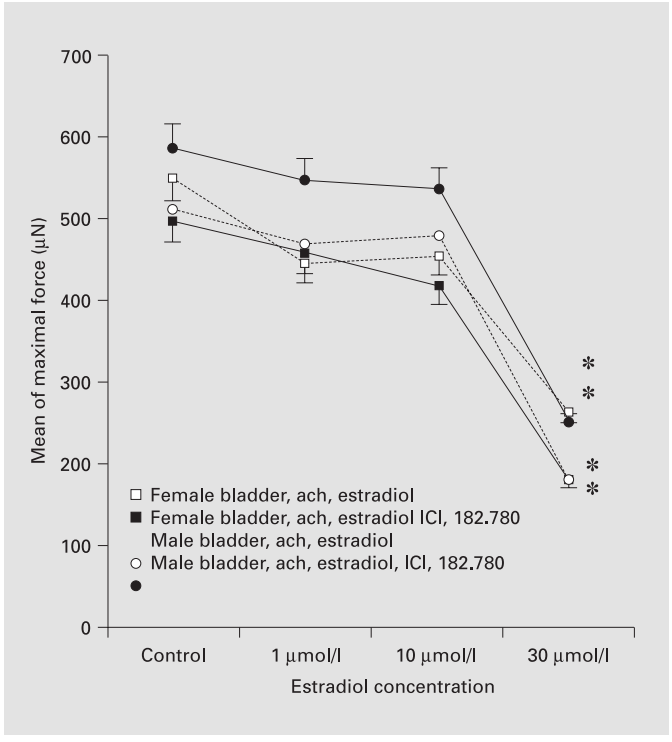
Fig. 1. Effects of pre-incubation of detrusor strips from the female pig with different concentrations of 17β-estradiol with and without pre-treatment with ER antagonist on the maximal force of contractions induced by EFS and KCl (100 mmol/l). The values are means ± SEM from 6 animals in each group. * $p < 0.05$ vs. control group. # $p < 0.05$ vs. EFS responses at 30 µmol/l of estradiol.

Fig. 2. Effects of pre-incubation of detrusor strips from the castrated male pig with different concentrations of 17β-estradiol with and without pre-treatment with ER antagonist on the maximal force development contractions induced by EFS and KCl (100 mmol/l). The values are means ± SEM from 6 animals in each group. * $p < 0.05$ vs. control group. # $p < 0.05$ vs. EFS responses at 30 µmol/l of estradiol.

Fig. 3. Effects induced by 17β-estradiol in control conditions and during pre-incubation with the selective ER antagonist ICI 182.780 on maximal force of contractions induced by Ach (10 µmol/l). Values are mean ± SEM from 6 bladder strips in each group. * $p < 0.05$ vs. control.



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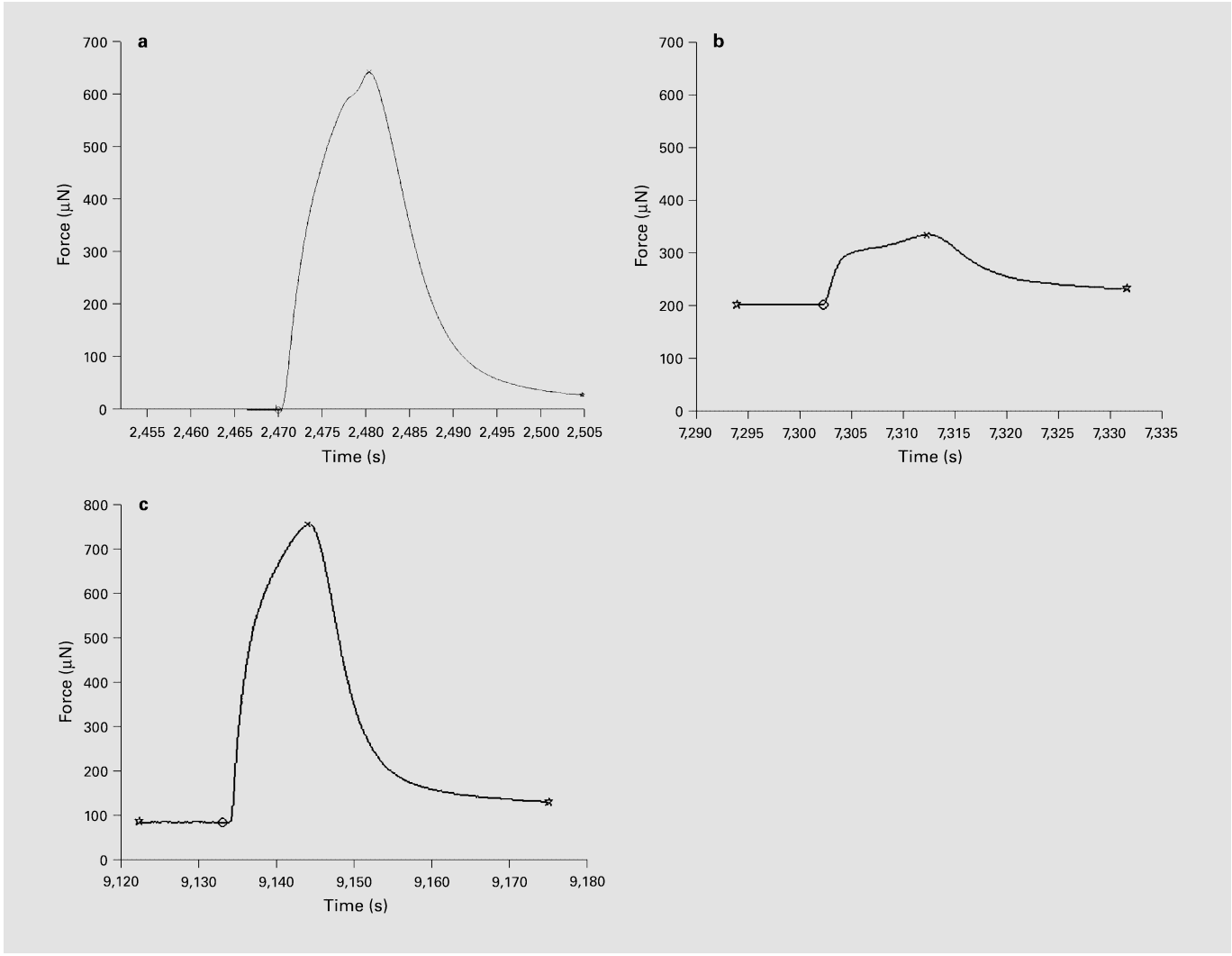

Fig. 4. Maximal force of a muscle contraction induced by EFS (100 Hz) in a female bladder: (**a**) muscle contraction induced in normal conditions (Krebs solution); (**b**) muscle contraction induced after 20 min of incubation of the muscle strip with 17β-estradiol at 30 µmol/l (the contraction reduced to 23% of the maximal force after incubation with estradiol), and (**c**) muscle contraction obtained after removal of estrogen from the bathing solution.

Effects of the ER Antagonist

As summarized in the figures 1–3, the preceding incubation with the ER antagonist ICI 182.780 did not modify the inhibitory effects of 17β-estradiol at 30 µmol/l on muscle contractility after contractions induced by electrical field and pharmacological stimuli ($p > 0.05$).

Discussion

Since its discovery and recognition as a 'female' sex hormone, estradiol has been studied for its effects on the female reproductive tissues as well as its actions in the urinary system, and most recently for its specific effect on bladder function. Results from the present study indicate that 17β-estradiol at high concentrations causes inhibition of contraction in adult female and castrated male pig bladder. The observation that estradiol can act on female and male smooth muscle is in general agreement with previous studies [7].

The majority of estradiol effects are mediated by estradiol receptors that act as ligand-activated transcription factors [9, 10]. Estradiol binding to specific receptors causes translocation of the receptor-hormone complex from the cytosol to the nucleus where the estradiol-receptor complex binds in a highly specific manner to the estradiol-response element of a promoter region of specific genes that results in an altered pattern of gene expression. Two types of intracellular ERs – ER-α and ER-β – have been described in the literature [10]. Measurements of mRNA for ER-α and ER-β reveal distributions in the body that differ quite markedly from each other, with high expression of ER-β in the lower urinary tract of both male and female rats [11]. These effects of estradiol are delayed in onset and prolonged in duration, and are called 'genomic' effects. However, estradiol can also have effects that can be rapid in onset and short in duration, they are called 'non-genomic' effects [12].

Several lines of evidence argue against the possibility that smooth muscle relaxation induced by estradiol is mediated by genomic mechanisms involving the nuclear ERs: the concentrations of 17β-estradiol required to evoke smooth muscle lower amplitude of contraction are several orders of magnitude higher than those required for genomic actions [13]; the rapid onset of action of 17β-estradiol is inconsistent with the time course of a response requiring gene transcription and the selective nuclear ER antagonist did not suppress vascular smooth muscle relaxation by estradiol [14]. The relatively rapid changes in muscle contractility observed in the present study and the observation that the pure ER antagonist ICI 182.780 did not inhibit the relaxant effects of 17β-estradiol in smooth muscle, are clearly not compatible with the classic genomic mechanism for the action of 17β-estradiol, which involves translocation of receptors to the nucleus and protein synthesis [15]. Furthermore, micromolar concentrations of 17β-estradiol were necessary to induce lower contractions of pig detrusor and those concentrations are clearly higher than physiological serum levels of the estrogen (around 100 pg/ml, that is, in the nanomolar range). However, White et al. [16] suggested that protein (steroid hormone binding globulin)-bound hormone could act in vivo as a free fraction due to a rapid dissociation of bound steroid hormones. Under our conditions, the bathing solution did not contain steroid binding protein, thus higher concentrations of free hormone would be necessary to mimic in vivo physiological conditions. These data, taken together, support the hypothesis that estrogen may affect bladder smooth muscle contractility through a rapid non-genomic mechanism. However, the physiological importance of this acute inhibition by estradiol on bladder contraction is not clear.

A change in intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$ is a crucial signal for diverse cellular functions. Previous research has shown that calcium release from intracellular stores by an agonist and by inositol 1,4,5-trisphosphate (IP_3) does not appear to be modulated by acute administration of estradiol in vascular smooth muscle [17]. Therefore, it was suggested that the ovarian steroid hormone relaxes smooth muscle predominantly by inhibiting the entry of $[Ca^{2+}]_i$ into the cells [18]. In a previous study from our laboratory [unpubl. data], we have demonstrated that an active IP_3 pathway was necessary for development of a contraction with a fast rate of force development. Thus, the specific inhibition of this pathway by Xestospongin C induced a much slower contraction in pig urinary bladder smooth muscle. In this present study, the rate of force development of contractions was slower than rates during specific IP_3 stimulation and not affected by estradiol pre-incubation. This result is consistent with the conclusion that estradiol does not appear to modulate the IP_3 pathway and also that the mechanisms of estradiol induced-inhibition of muscle contractility do not affect the duration of bursts of intracellular calcium concentration per se.

Ogata et al. [19] suggested that estrogen acts on the cell membrane receptors rather than on cytosolic receptors because its action appeared to be very quickly and current was restored rapidly after removal of estrogen from the bathing solution. In our experiments, the inhibitory action of estradiol on EFS-, Ach- and KCl-induced contractions was observed after 20 min of incubation of the muscle strips with the hormone, and this inhibition could easily be removed by washout. It is possible that these plasma membrane receptors are also involved in $[Ca^{2+}]_i$ regulation by estrogens and mediate some of the effects on muscle contractility observed in the present study.

Herrera et al. [15] have shown that different types of potassium channels are present in the guinea-pig detrusor, whose modulation offers a potential mechanism for affecting bladder contractility and function. Our results show that estradiol (30 μmol/l) was more potent in decreasing maximal force of contractions induced by KCl compared to contractions evoked by EFS and acetylcholine. Thus, these findings suggest that estradiol has a stronger inhibitory effect on the depolarization of the muscle cell membrane than on the muscarinic receptor.

In conclusion, we speculate that under our conditions estrogen may act through a non-genomic pathway, most likely via cell membrane receptors which are not inhibited

by ICI 182.780. Furthermore, the possible role of K(+) channel deactivation in the inhibition of contraction induced by 17β-estradiol in pig bladder provides a new approach to further research into the treatment of detrusor overactivity.

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