The effect of *in vivo* oestrogen pretreatment on the contractile response of rat isolated detrusor muscle

Ruth A. Elliott, ¹C.M. Castleden & A. Miodrag

Department of Medicine for the Elderly, Leicester General Hospital, Gwendolen Road, Leicester LE5 4PW

- 1 The effect of oestradiol pretreatment was investigated on the response of rat isolated detrusor muscle to cholinergic, electrical and 5-hydroxytryptamine (5-HT) stimulation with and without diethylstilboestrol (DES) (2 μ M) in the organ bath.
- 2 Virgin female Wistar rats were injected subcutaneously for 8 days with oestradiol benzoate 150 μ g kg⁻¹. Control rats received no injections or injection only with the vehicle, ethyl oleate.
- 3 Detrusor muscle from treated rats showed a decreased sensitivity to acetylcholine (ACh) and carbachol-induced contractile responses. The dose-response curves to these agonists showed a 44% reduction in maximum contractile response for ACh ($P < 0.001$), and a 38% reduction in maximum contractile response for carbachol ($P < 0.05$). The addition of 2 μ M DES to the bathing medium further significantly reduced the maximum contractile response by 56 and 57% of control respectively.
- 4 Electrically stimulated detrusor muscle from treated rats showed a significant 49% reduction in the maximum contractile response ($P < 0.001$). The addition of 2 μ M DES to the bathing medium further significantly reduced the maximum contractile response by 66% of control. The tetrodotoxin resistant responses were smaller in pretreated rats, suggesting a reduced sensitivity of the smooth muscle to direct electrical stimulation.
- 5 The response to 5-HT stimulation by detrusor muscle samples from oestradiol-treated rats showed a non-significant reduction in maximum contractile response, but the addition of 2 μ M DES to the bath chamber resulted in a 67% reduction in the response ($P < 0.001$).
- 6 Oestradiol pretreatment did not affect the potassium dose-response curve.
- 7 Oestradiol pretreatment reduced the rat detrusor muscle sensitivity to the blocking effect of atropine on the response to electrical field stimulation. Pretreatment also reduced the potentiating effect of physostigmine on the same response.
- 8 These results suggest that oestradiol pretreatment had a modulating effect on cholinergic responses. The addition of oestrogen to the tissue environment enhances this inhibitory effect.

Keywords: Oestrogen; bladder; carbachol; 5-hydroxytryptamine

Introduction

Oestrogens have been used for a number of years to treat urinary symptoms especially those associated with the lower urinary tract such as atrophic urethritis. Their place in the management of motor urge incontinence has never been established (Miodrag *et al.*, 1988) despite the fact that high affinity oestradiol receptors have been isolated in human (Iosif *et al.*, 1981; Batra & Iosif, 1983), rabbit (Urner *et al.*, 1983; Shapiro, 1986; Batra & Andersson, 1989) and baboon detrusor muscle (Weaker *et al.*, 1983). Treatment with oestradiol decreased the muscarinic receptor density in rabbits (Shapiro, 1986) but the muscarinic response to carbachol and the cholinergic neurogenic response following electrical field stimulation was not greatly decreased despite a markedly decreased density in muscarinic receptors (Batra & Andersson, 1989).

Earlier experiments in our laboratories have shown that diethylstilboestrol (DES) added directly to the organ bath had a profound effect on the contractile response following cholinergic, 5-hydroxytryptamine (5-HT), calcium ion, potassium and electrical field stimulation of rat and human detrusor muscle. This was probably due to a reduction in calcium ion uptake by the detrusor muscle cells rather than an effect on intracellular calcium release (Elliott *et al.*, 1992). If *in vivo* treatment with oestradiol had similar effects on muscarinic receptors in rats as in rabbits (Shapiro, 1986) then pretreatment and direct oestrogen would have different mechanisms

of action and thus a summation of effects might be seen on cholinergic stimulation. Because there appear to be no reports illustrating the effects of pretreatment and direct oestrogen treatment on the same detrusor muscle, the present experiments were performed.

Methods

Virgin female wistar rats were injected subcutaneously with oestradiol benzoate 150 μ g kg⁻¹, twice a day for 8 days. Treatment was initiated when the rats were in the dioestrus phase, as judged by vaginal smears. The 8 day treatment regimen covered two cycles, after which the rats were killed by a blow to the head. The bladders were removed and dissected free of fat and serosa. Strips of bladder muscle 7 mm by 4 mm were suspended in a 50 ml organ bath chamber containing Krebs solution at 37°C and aerated with 95% oxygen and 5% carbon dioxide. The bladder base was attached to a fixed hook in the chamber and the apex by a thread attached to an isometric transducer connected to a two channel Washington oscillograph. The tissues were allowed to equilibrate for 1 h under a tension of 10 mN.

After equilibration, acetylcholine (ACh) (10^{-8} M– 2×10^{-4} M), carbachol (10^{-8} M– 10^{-4} M) or potassium chloride (KCl) (10 mM–60 mM) was injected cumulatively into the bath chamber to obtain dose-response curves. 5-Hydroxytryptamine (5-HT) (10^{-8} M– 10^{-5} M) was injected at 5 min intervals and samples were washed between doses to avoid tachyphylaxis. For electrical field stimulation, muscle strips were

¹ Author for correspondence.

passed through two parallel circular electrodes connected to a Digitimer stimulator. The stimulator delivered 1–80 pulses s^{-1} at 4–6 V and a 1 ms pulse width in 10 s trains at 2 min intervals. Frequency-response curves were obtained by stimulating the tissue with 1, 5, 10, 20, 40, 60, 80 pulses per second. The effect of oestradiol pretreatment plus the presence of $2 \mu M$ DES in the external medium was investigated by repeating the dose-response curves after the addition of DES to the bath chamber. Effects of bath-applied DES were not easily reversible. Even after several washes the response did not return to pre-applied levels.

Tetrodotoxin (TTX, $1.6 \times 10^{-6} M$) was used to distinguish between nerve-mediated contractile responses, and those due to direct muscle stimulation in controls and pretreated samples.

Different bladder muscle samples were used for each agonist. Control dose-response curves were obtained from rat bladder muscle taken from untreated rats in the dioestrus phase. Samples were also taken from rats injected only with the vehicle ethyl oleate. Dose-response curves for the comparison of bladders from rats pretreated with oestradiol and controls are presented as concentration of agonist against the increase in tension, and not percentage of maximum response. This is to demonstrate the absolute decrease in response obtained after oestradiol treatment which would not be apparent when calculating percentage of maximum response if the same bladder samples were used as its own control.

Solutions and chemicals

Krebs solution contained (mM): NaCl 119, KCl 4.4, $NaHCO_3$ 20, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5 and glucose 11.

ACh chloride (Sigma), carbamylcholine chloride (Sigma), atropine sulphate (Sigma), physostigmine (Sigma) and 5-HT (Sigma) were all dissolved in distilled water and made up on the day of the experiment. Diethylstilboestrol (DES) (Sigma) was dissolved in ethanol and the concentration of ethanol in the organ bath chamber did not exceed 3 mM. Oestradiol benzoate (Paines & Byrne) was supplied in vials containing $5 mg ml^{-1}$. TTX (Sigma) was made up in distilled water and stored at $-20^\circ C$ in 1 ml aliquots.

For each experiment the results were the mean of 5 different bladder muscle samples, unless otherwise stated. Statistical analysis was carried out with Student's *t* test.

Results

Following 8 days oestradiol treatment the rat uterus showed marked hypertrophy compared to non-treated animals. This was taken as an indication of oestrogenisation. The bladders removed from treated rats were also hypertrophic (mean weight $94.32 \pm 8.60 mg$) compared to the non-treated animals (mean weight $66.65 \pm 2.54 mg$) ($P < 0.05$). The response of the detrusor muscle in control rats and those treated with vehicle only did not differ.

Effect of oestrogen on electrical field stimulation

The spontaneous contractions normally exhibited by rat detrusor muscle were markedly reduced in frequency and amplitude in samples taken from oestradiol treated rats. The frequency dose-response curve of the detrusor muscle of such rats to electrical field stimulation showed a 49% reduction in maximum response compared to control ($P < 0.001$). When $2 \mu M$ DES was added to the surrounding medium, the result was a further significant reduction of maximum response (66%, Figure 1). The maximum response obtained by electrical field stimulation was 61.4% of the maximum response obtained with $10^{-4} M$ carbachol in controls and 61.5% in pretreated rats.

Figure 1 Dose-response curves to electrical field stimulation in rat isolated detrusor muscle: (●) control; (○) after 8 days oestradiol treatment; (Δ) after 8 days oestradiol treatment plus $2 \mu M$ diethylstilboestrol in the organ bath ($n = 5$). Vertical bars represent s.e. mean. *** $P < 0.01$; **** $P < 0.001$.

Figure 2 Frequency-response curves showing the effect of oestradiol pretreatment on the tetrodotoxin-resistant response to electrical field stimulation in rat isolated detrusor muscle: (●) control; (○) control after TTX; (□) after 8 days treatment; (Δ) after 8 days treatment and TTX, ($n = 5$). Vertical bars represent s.e. mean. ** $P < 0.05$.

Effect of oestrogen on tetrodotoxin sensitivity

Tetrodotoxin blocked about 50% of the maximum contractile response to electrical field stimulation at our parameters. At lower frequencies (10 pulses s^{-1}) about 75% of the contractile response was blocked. The TTX-resistant responses were significantly smaller in rats pretreated with oestradiol than in control rats ($P < 0.05$, Figure 2).

Effect of oestrogen on acetylcholine and carbachol response

The contractile response of detrusor muscle to ACh stimulation was reduced in amplitude in oestradiol-treated rats compared to controls. The dose-response curve showed a 44% reduction in maximum response ($P < 0.001$). The addition of $2 \mu M$ DES to the water bath resulted in a further significant

reduction in maximum response (56%, Figure 3a). Oestradiol pretreatment resulted in similar effects on carbachol-induced contractions with a reduction of 38% in maximum response being obtained ($P < 0.05$), which was reduced further with the addition of $2 \mu\text{M}$ DES (57%, Figure 3b).

Effect of oestrogen on 5-hydroxytryptamine response

Rat detrusor muscle response to 5-HT stimulation was phasic and much reduced in amplitude compared to cholinergic and electrical field stimulation responses. Detrusor muscle from oestradiol-treated rats showed a non significant reduction in maximum response of 22%, but with the addition of $2 \mu\text{M}$ DES to the bath chamber this response was significantly reduced by 67% ($P < 0.001$), Figure 4.

Effect of oestrogen on atropine sensitivity

Detrusor muscle from control rats, stimulated electrically, showed a 19% reduction in maximum contractile response in the presence of $10 \mu\text{M}$ atropine (Figure 5a). Atropine exerted its inhibitory effect mainly on high frequency induced contractile responses.

Figure 3 (a) Dose-response curves to acetylcholine stimulation in rat isolated detrusor muscle: (●) control ($n = 8$); (○) after 8 days oestradiol treatment; (△) after 8 days oestradiol treatment plus $2 \mu\text{M}$ diethylstilboestrol (DES) in the organ bath ($n = 9$). (b) Dose-response curves to carbachol stimulation in rat isolated detrusor muscle: (●) control; (○) after 8 days oestradiol treatment; (△) after 8 days oestradiol treatment plus $2 \mu\text{M}$ DES in the organ bath ($n = 5$). Vertical bars represent s.e.mean. * $P < 0.10$; ** $P < 0.05$; *** $P < 0.01$; **** $P < 0.001$.

Figure 4 Dose-response curves to 5-hydroxytryptamine (5-HT) stimulation of rat isolated detrusor muscle: (●) control; (○) after 8 days oestradiol treatment; (△) after 8 days oestradiol treatment plus $2 \mu\text{M}$ diethylstilboestrol in the organ bath ($n = 5$). Vertical bars represent s.e.mean. **** $P < 0.001$.

Figure 5 (a) Dose-response curves to electrical field stimulation in rat isolated detrusor muscle from untreated rats ($n = 4$). (b) Dose-response curves to electrical field stimulation in rat isolated detrusor muscle from rats pretreated with oestradiol for 8 days: (●) control; (○) after $10 \mu\text{M}$ atropine ($n = 5$). Vertical bars represent s.e.mean. ** $P < 0.50$; *** $P < 0.01$; **** $P < 0.001$.

The sensitivity of the detrusor muscle to blockade of the cholinergic component of electrical stimulation was almost totally abolished in bladder muscle samples taken from oestradiol-treated rats. The dose-response curve showed no significant difference before and after the addition of atropine $10 \mu\text{M}$ (Figure 5b).

Effect of oestrogen on physostigmine potentiation

Control detrusor muscle samples showed potentiation of electrically induced contractile responses in the presence of $0.01 \mu\text{M}$ physostigmine. The maximum contractile response was increased by 30% ($P < 0.001$) (Figure 6a).

Bladder muscle from oestradiol pretreated rats had lost its sensitivity to physostigmine. The electrically induced contractile responses were not significantly different in the presence or absence of the drug (Figure 6b).

Effect of pretreatment on KCl response

Oestradiol pretreatment did not affect the KCl dose-response curve.

Figure 6 (a) Dose-response curves to electrical field stimulation in rat isolated detrusor muscle from untreated rats ($n = 5$). (b) Dose-response curves to electrical field stimulation in rat isolated detrusor muscle from rats pretreated with oestradiol for 8 days ($n = 5$): (●) control; (○) after the addition of $0.01 \mu\text{M}$ physostigmine. Vertical bars represent s.e.mean. *** $P < 0.01$; **** $P < 0.001$.

Discussion

Previous results from our laboratory have shown that the addition of DES to the organ bath had a profound effect on the contractile response of rat and human detrusor muscle. The results suggested that DES affected the movement of extracellular calcium ions into the muscle cells (Elliott *et al.*, 1992). If previous studies were plotted on the present dose- and frequency-response curves, the resultant curve would be situated between the control and pretreatment curves.

The present results showed that there was a significant decrease in the contractile response to muscarinic receptor stimulation after pretreatment with oestradiol and that this reduction in contractility could be further enhanced by the direct addition of DES to the tissue environment. Since there was no difference between the effect of oestradiol on the responses to ACh and carbachol, pretreatment is unlikely to have affected cholinesterase activity. The decrease in contraction following electrical stimulation was due predominantly to an effect on the cholinergic component since pretreatment with oestradiol almost abolished the atropine sensitive component of electrical field stimulation. The enhancing effect of physostigmine on this response was also greatly reduced after oestradiol pretreatment. In man it is likely that the effect of oestradiol pretreatment will be even greater as the contraction following electrical field stimulation is mainly cholinergic (Sjögren *et al.*, 1982; Sibley, 1984; Kinder & Mundy, 1985). Pretreatment did not affect the KCl response and it is unlikely therefore that pretreatment is affecting calcium ion permeability. This is in contrast to the direct effect of DES (Elliott *et al.*, 1992). The results with TTX show that, with our parameters of stimulation, the nerve-mediated response was 50% of the contractile response to electrical field stimulation at high frequencies and that 50% was direct muscle action. This is true for both control and pretreated rats. The TTX-sensitive response was reduced after pretreatment with oestradiol, suggesting that oestradiol decreases the sensitivity of detrusor muscle to direct electrical stimulation. Brading & Williams (1990) have clearly shown that the predominant mechanical response to intrinsic nerve stimulation of rat and guinea-pig detrusor was through non-muscarinic receptors and that contractile responses resistant to atropine are most clearly seen in the early response to electrical field stimulation. Conversely neostigmine (Brading & Mostwin, 1989) and physostigmine potentiated electrical field stimulation at low although less than at high frequencies. There is now good evidence that the non-cholinergic transmitter is adenosine 5'-triphosphate (ATP) (Brading & Mostwin, 1989; Brading & Williams, 1990; Parija *et al.*, 1991), but whether pretreatment affects this mechanism is unknown at present.

Pretreatment with oestradiol in the rat also had no effect on the contractile response to 5-HT. Chen (1990) has shown that there is a cholinergic component to 5-HT stimulation in the rabbit in addition to non-adrenergic, non-cholinergic excitatory neurotransmission. The present results would suggest that this cholinergic component was relatively unimportant following 5-HT stimulation in the rat. The addition of DES to the tissue environment caused a significant reduction in contractile response to 5-HT possibly due to changes in cell membrane permeability to calcium ions (Elliott *et al.*, 1992).

The most likely explanation for the inhibitory effect of pretreatment with oestradiol on the cholinergic response was that there was down-regulation of muscarinic receptors, although there may have been a minor effect on the sensitivity to direct electrical stimulation. There was no overall decrease in contractility of the pretreated detrusor muscles in the present study, despite considerable hypertrophy of the bladder. As early as 1977, Roberts *et al.* showed that pretreatment with oestradiol could increase α -adrenoceptor densities in the rabbit uterus. Larsson *et al.* (1984) reported that this increase in α -adrenoceptors could also be induced in the female rabbit urethra. They clearly showed that the increase

in receptors was not proportional to the increase in the weight of the tissue as it was in the uterus. Shapiro (1986) found that pretreatment with oestradiol decreased the muscarinic cholinergic receptor density in the rabbit bladder; this was despite a marked increase in the weight of the bladder body. Batra & Andersson (1989) also using the rabbit confirmed previous work that muscarinic receptor density was reduced following oestradiol treatment but were unable to show that contractile responses to electrical field stimulation and carbachol were significantly decreased. The difference between their results and the present ones could represent species differences, but is unlikely to be due to inadequate oestrogen-treatment since Batra & Andersson (1989) produced a reduction in muscarinic receptor density of 90% after 4 weeks. Anderson & Marks (1982) showed that only a small fraction of the cholinergic receptors needed to be occupied to produce contractile responses, and thus there was a large receptor reserve. They further argued that the rate limiting step for

regulation of the contractile response to carbachol was neither muscarinic receptor occupation nor membrane calcium channel opening but the intracellular mechanisms which regulate the responsiveness of the myofibrils to calcium ions.

In conclusion, pretreatment with oestradiol in rats significantly reduced muscarinic receptor-stimulated contractions of detrusor muscle although the exact mechanism by which this was brought about is uncertain. The addition of DES directly to the organ bath in pretreated animals caused a further decrease in contractile response. This combined effect of oestradiol has not been shown before but if confirmed in man would clearly mimic the situation in women given oestradiol therapy long-term for over-activity of the detrusor muscle. In such women detrusor contractions are associated with urge incontinence and thus pretreatment with oestradiol may have a very significant clinical role in the control of urinary incontinence.

References

- ANDERSON, G.F. & MARKS, B.H. (1982). Spare cholinergic receptors in the urinary bladder. *J. Pharmacol. Exp. Ther.*, **221**, 598–603.
- BATRA, S. & ANDERSSON, K.-E. (1989). Oestrogen-induced changes in muscarinic receptor density and contractile responses in the female rabbit urinary bladder. *Acta Physiol. Scand.*, **137**, 135–141.
- BATRA, S.C. & IOSIF, C.S. (1983). Female urethra: a target for oestrogen action. *J. Urol.*, **129**, 418–420.
- BRADING, A.F. & MOSTWIN, J.L. (1989). Electrical and mechanical responses of guinea-pig bladder muscle to nerve stimulation. *Br. J. Pharmacol.*, **98**, 1083–1090.
- BRADING, A.F. & WILLIAMS, J.H. (1990). Contractile responses of smooth muscle strips from rat and guinea-pig urinary bladder to transmural stimulation: effects of atropine and α , β -methylene ATP. *Br. J. Pharmacol.*, **99**, 493–498.
- CHEN, H.I. (1990). Evidence for the presynaptic action of 5-hydroxytryptamine and the involvement of purinergic innervation in the rabbit lower urinary tract. *Br. J. Pharmacol.*, **101**, 212–216.
- ELLIOTT, R.A., CASTLEDEN, C.M., MIODRAG, A. & KIRWAN, P. (1992). The effects of diethylstilboestrol and nifedipine on the contractile response of isolated human and rat detrusor muscle. *Eur. J. Clin. Pharmacol.*, (in press).
- IOSIF, S.C., BATRA, S., ANDERS, E.K. & ASTEDT, B. (1981). Oestrogen receptors in the human female lower urinary tract. *Am. J. Obstet. Gynecol.*, **141**, 7, 817–820.
- KINDER, R.B. & MUNDY, A.R. (1985). Atropine blockade of nerve-mediated stimulation of human detrusor. *Br. J. Urol.*, **57**, 418–421.
- LARSSON, B., ANDERSSON, K.-E., BATRA, S., MATTIASSEN, A. & SJÖGREN, C. (1984). Effects of estradiol on norepinephrine-induced contractions, alpha adrenoceptor number and norepinephrine content in the female rabbit urethra. *J. Pharmacol. Exp. Ther.*, **229**, 557–563.
- MIODRAG, A., CASTLEDEN, C.M. & VALLANCE, T.R. (1988). Sex hormones and the female urinary tract. *Drugs*, **36**, 491–504.
- PARIJA, S.C., RAVIPRAKASH, V. & MISHRA, S.K. (1991). Adenosine and α , β -methylene ATP-induced differential inhibition of cholinergic and non-cholinergic neurogenic responses in rat urinary bladder. *Br. J. Pharmacol.*, **102**, 396–400.
- ROBERTS, J.M., INSEL, P.A., GOLDFIEN, R.D. & GOLDFIEN, A. (1977). Alpha adrenoreceptors but no beta adrenoreceptors increase in rabbit uterus with oestrogen. *Nature*, **270**, 624–625.
- SHAPIRO, E. (1986). Effect of oestrogens on the weight and muscarinic cholinergic receptor density of the rabbit bladder and urethra. *J. Urol.*, **135**, 1084–1087.
- SIBLEY, G.N.A. (1984). A comparison of spontaneous and nerve-mediated activity in bladder muscle from man, pig, and rabbit. *J. Physiol.*, **354**, 431–443.
- SJÖGREN, C., ANDERSSON, K.-E., HUSTED, S., MATTIASSEN, A. & MOLLER-MADSEN, B. (1982). Atropine resistance of transmurally stimulated isolated human bladder muscle. *J. Urol.*, **128**, 1368–1371.
- URNER, F., WEIL, A. & HERRMAN, W.L. (1983). Estradiol receptors in the urethra and the bladder of the female rabbit. *Gynecol. Obstet. Invest.*, **16**, 307–313.
- WEAKER, F.J., HERBERT, D.L. & SHERTDAN, P.J. (1983). Autoradiographic demonstration of binding sites of oestradiol and dihydrotestosterone in the urinary tract of male and female baboons. *Urol. Res.*, **11**, 127–130.

(Received January 2, 1992

Revised June 8, 1992

Accepted July 16, 1992)