Eur J Clin Pharmacol (1992) 43: 149–155

European Journal of Clinical Pharmacology

The direct effects of diethylstilboestrol and nifedipine on the contractile responses of isolated human and rat detrusor muscles

R. A. Elliott, C. M. Castleden, A. Miodrag, and P. Kirwan

University Departments of Medicine for the Elderly and Gynaecology, Leicester General Hospital, Leicester, UK

Received: November 6, 1991/Accepted in revised form: March 17, 1992

Summary. We have studied the direct effect of 2 $\mu mol \cdot l^{-1}$ diethylstilboestrol on isolated rat and human detrusor muscles. Diethylstilboestrol significantly reduced the amplitude of the contractile response of rat detrusor muscle to stimulation with acetylcholine, carbachol, electrical field stimulation, and 5-hydroxytryptamine. In isolated human bladder it also significantly reduced contractions stimulated with acetylcholine, carbachol, and electrical field stimulation. In depolarized rat detrusor muscle stimulated with different concentrations of calcium ions, the contractile responses were significantly reduced by the addition of diethylstilboestrol. Diethylstilboestrol also significantly reduced the amplitude of contractile response to potassium chloride. The inhibitory action of diethylstilboestrol was enhanced by the reduction of extracellular calcium ions, the maximum contractile response to acetylcholine, carbachol, and electrical field stimulation being reduced by a further 32%, 23%, and 45% respectively. Diethylstilboestrol did not have a significant effect on carbachol-induced contractions in depolarized rat detrusor muscle suspended in a calcium-free environment. Diethylstilboestrol was effective in blocking rat and human detrusor muscle contraction. The likely mechanism is a reduction of the influx of calcium ions into the cell during contraction rather than an effect on intracellular calcium release. These results give support for treating incontinent patients with drugs that block calcium ion uptake, and may suggest a further beneficial effect of oestrogen therapy in postmenopausal women.

Key words: Oestrogen, Nifedipine, Muscle contraction; bladder; acetylcholine; carbachol; 5-hydroxytryptamine

Urinary incontinence affects between 5 and 10% of old people in the community and up to 50% in institutions. The prevalence is considerably higher in women than in men [1]. Most patients with urinary incontinence have detrusor instability, in which powerful detrusor muscle contractions overcome urethral sphincter closure pressure [2]. Current pharmacological intervention is therefore aimed at blocking or suppressing these contractions with anticholinergic drugs, antispasmodics, and calcium antagonists [3].

A line of therapy hitherto unexplored scientifically, although widely used in some countries, is oestrogen therapy [4]. Such treatment is commonly used for atrophic urethritis and stress incontinence [5], but there is little information on its potential use in detrusor instability. Studies in rabbits have shown that there are oestrogen receptors in detrusor muscle, and that treatment with oestrogens can reduce muscarinic receptor density [6, 7]. It has also been shown in these animals that oestrogens shift the carbachol dose-response curve to the right. Preliminary studies in man have confirmed that there are oestrogen receptors in detrusor muscle [8, 9], but such studies clearly needed to be extended before this potentially beneficial treatment could be used rationally in man. We have therefore compared the direct effects of an oestrogen, diethylstilboestrol, on detrusor muscles in rat and man.

Materials and methods

Rat experiments

Virgin female Wistar rats (150–200 g) in the dioestrus phase, as judged from vaginal smears, were killed by a blow on the head and exsanguinated. The bladders were removed and two strips per bladder were placed in Krebs solution (see below). After the removal of fat and serosa, strips of muscle (7 mm × 4 mm) were suspended in a 50 ml organ-bath chamber containing Krebs solution at 37°C, aerated with 95% oxygen and 5% carbon dioxide.

The base of the muscle strip was fixed to a hook in the chamber and the apex was attached by a thread 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 before the addition of any drugs. Acetylcholine (10^{-8} to 2×10^{-4} $mol \cdot l^{-1}$), carbachol (10^{-8} to 10^{-4} $mol \cdot l^{-1}$) or potassium chloride (KCl) (10 $mmol \cdot l^{-1}$ to 60 $mmol \cdot l^{-1}$) were each injected into the bath in a cumulative manner to obtain dose-response curves. 5-hydroxytryptamine (10^{-8} to 10^{-5} $mol \cdot l$) was injected at 5 min intervals and the preparation was washed between doses to avoid tachyphylaxis.

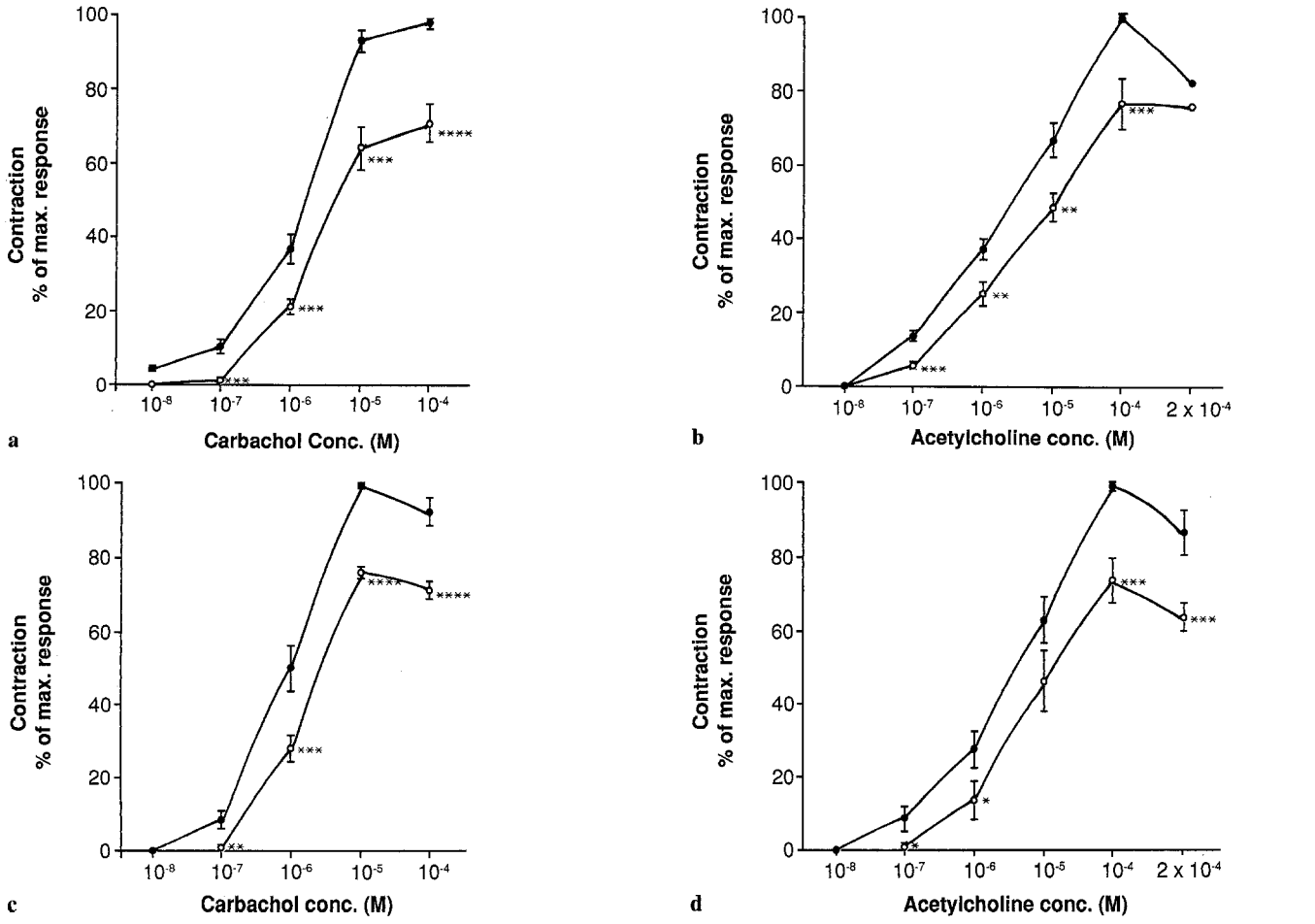

Fig. 1 a–d. The effect of 2 μmol·l^{-1} of diethylstilboestrol on the carbachol (**a**) and acetylcholine (**b**) dose-response curves of isolated rat detrusor muscle, and on the carbachol (**c**) and acetylcholine (**d**) dose-response curves of isolated human detrusor muscle. ● = control, ○ = after the addition of diethylstilboestrol. Vertical bars represent the standard error of the mean ($n = 5$). **** = $P < 0.001$, *** = $P < 0.01$, ** = $P < 0.05$; * = $P < 0.10$

When consistent dose-response curves had been obtained, diethylstilboestrol 2 μmol·l^{-1} was added to the bath. This concentration was used because the concentrations produced in the organ-bath chamber could have been produced pharmacologically in women. When dose-response curves were repeated an incubation time of 10 min was allowed between them in every case.

Human detrusor muscle

Bladder muscle biopsies were obtained from women with healthy bladders undergoing routine gynaecological operations. Muscle strips (7 mm × 4 mm) were removed from the fundus of the bladder at the time of abdominal hysterectomy. These women had not taken any oestrogens before surgery. Full informed consent was obtained. The local ethics committee gave permission for the study.

Biopsy samples were immediately placed into Krebs solution, and taken to the laboratory. They were mounted in the organ-bath chamber and treated in the same way as the rat samples.

Electrical field stimulation

For these experiments the bladder strips were passed through two parallel circular electrodes connected to a Digitimer stimulator. The stimulator delivered 1–80 pulses per second at 4–6 volts with a 1 msec pulse width in 10 s trains at 2 min intervals. A frequency response curve was obtained by stimulating the tissue with 1, 5, 10, 20, 40, 60, and 80 pulses per second. When consistent curves were obtained, diethylstilboestrol 2 μmol·l^{-1} was injected into the bath and the frequency-response curves were repeated.

Depolarized preparations

After equilibration the samples were stimulatetd with acetylcholine 10^{-4} mol·l^{-1} at 10 min intervals until consecutive responses were almost the same. This was taken to be the maximum contractile response. The tissues were depolarized by placing them into calcium-free potassium-rich Krebs solution containing 127 mmol·l^{-1} KCl and 1.2 mmol·l^{-1} EGTA to reduce the concentration of free calcium ions in the external medium. This resulted in an initial contraction followed by relaxation.

After 90 min of equilibration, during which the preparation was washed twice, the strips were stimulated with increasing concentrations of calcium ions (0.1 mmol·l^{-1} to 1.5 mmol·l^{-1}), to obtain a dose-response curve. The preparations were then washed in calcium-free potassium-rich Krebs solution for a further 15 min and the procedure was repeated 10 min after the addition of diethylstilboestrol 2 μmol·l^{-1}.

For preparations suspended in a low-calcium medium, the concentration of calcium chloride in Krebs solution was reduced to 0.3 mmol·l^{-1}. In depolarized and low-calcium experiments, the ef-

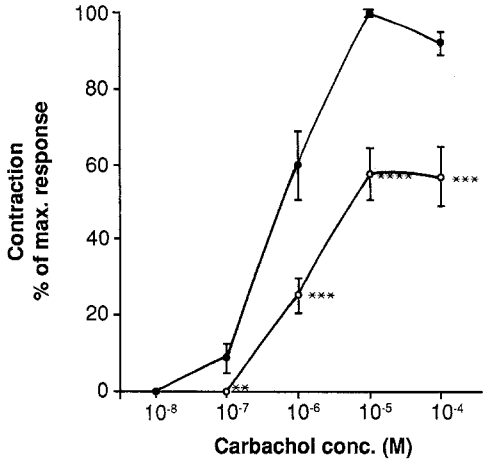

Fig. 2. The effect of 0.03 μmol·l^{-1} nifedipine on the carbachol dose-response curve of isolated human detrusor muscle. ● = control, ○ = after the addition of nifedipine. Vertical bars represent the standard error of the mean ($n = 5$). **** = $P < 0.001$, *** = $P < 0.01$, ** = $P < 0.05$

fects of the calcium antagonist nifedipine (0.03 μmol·l^{-1}) were also investigated and compared with those of diethylstilboestrol. This concentration reflected those in the plasma after therapeutic doses.

Solutions and chemicals

Krebs solution contained (mmol·l^{-1}): NaCl 119, KCl 4.4, $NaHCO_3$ 20, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5, glucose 11.

Calcium-free potassium-rich Krebs solution contained (mmol·l^{-1}): KCl 127, $NaHCO_3$ 20, NaH_2PO_4 1.2, $MgCl_2$ 1.2, EGTA 0.01, glucose 11.

Low-calcium Krebs solution contained (mmol·l^{-1}): NaCl 119, KCl 4.6, $NaHCO_3$ 20, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 0.3, glucose 11.

Acetylcholine chloride (Sigma), carbamylcholine chloride (Sigma), and 5-hydroxytryptamine (Sigma) were all dissolved in distilled water and made up on the day of the experiment. Diethylstilboestrol (Sigma) and nifedipine (Sigma) were dissolved in ethanol. The concentration of ethanol in the organ bath chamber did not exceed 3 mmol·l^{-1}.

Nifedipine was kept in a darkened container.

For each experiment the results are the means of five different bladder samples. Different bladders were used for each agonist. Statistical analyses were carried out using Student's *t*-test. The individual dose-response curves for acetylcholine and carbachol were drawn by hand and the EC_{50} values were calculated graphically by determining the concentration of agonist required to produce a 50% response for each dose-response curve. These concentrations were read from the graphs as log concentrations. Individual values were meaned and the SEM established statistically. Comparisons of the EC_{50} values were carried out using Student's *t*-test. In the presence of diethylstilboestrol the values were 50% of the maximum response; all curves were scaled on their own maxima.

Results

Effects of diethylstilboestrol and nifedipine on carbachol- and acetylcholine-induced responses

Rat detrusor muscle showed rhythmic spontaneous contractions when set up in Krebs solution. This activity was maintained for up to 7 h. Diethylstilboestrol 2 μmol·l^{-1} did not alter these contractions, but 20 μmol·l^{-1} totally abolished them. Carbachol and acetylcholine produced a rapidly developing contraction which was dose-dependent. Diethylstilboestrol 2 μmol·l^{-1} caused a 30% reduction in the maximum contractile response for carbachol ($P < 0.001$) and a 25% reduction for acetylcholine ($P < 0.01$). The carbachol dose-response curve, but not that for acetylcholine, showed a significant shift to the right, with an increase in the mean log EC_{50} from 1.8 (SD 0.5) × 10^{-6} mol·l^{-1} to 5.4 (1.6) × 10^{-6} mol·l^{-1} ($P < 0.01$) (Fig. 1 a, b).

Human bladder muscle did not exhibit spontaneous contractile activity, and its response to stimulation with carbachol and acetylcholine was less rapid than that of rat detrusor muscle. However, 2 μmol·l^{-1} of diethylstilboestrol inhibited the human detrusor contractile response. The dose-response curve for carbachol, but not for acetylcholine, showed a significant shift to the right, with an increase in the mean log EC_{50} from 1.3 (0.5) × 10^{-6} mol·l^{-1} to 4.8 (0.6) × 10^{-6} mol·l^{-1} ($P < 0.001$). The maximum contractile response was reduced by 26% to acetylcholine and by 23% to carbachol ($P < 0.001$) (Fig. 1 c, d).

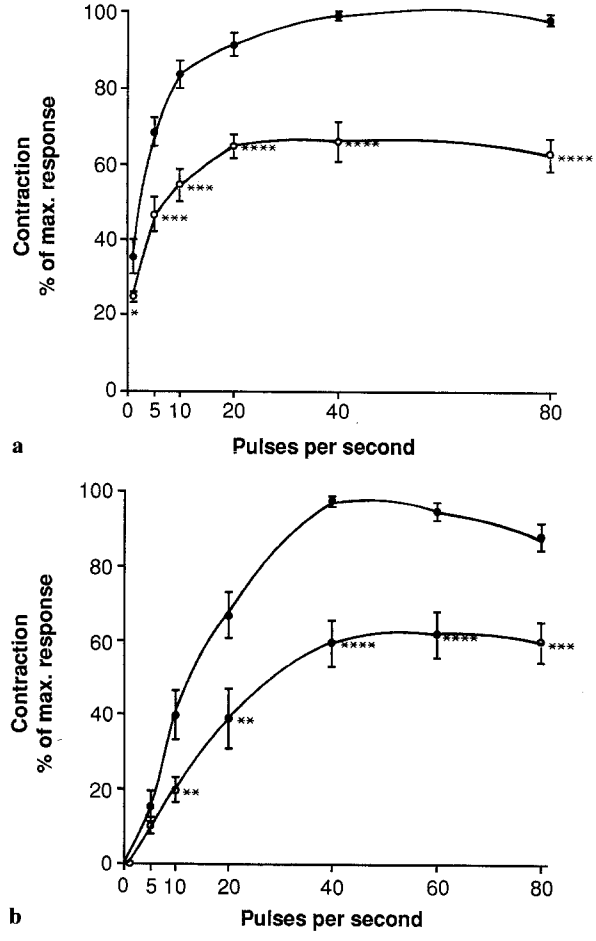

Fig. 3 a, b. The effect of 2 μmol·l^{-1} of diethylstilboestrol on the electrically-induced frequency dose-response curve of isolated rat (**a**) and human (**b**) detrusor muscle. ● = control, ○ = after the addition of diethylstilboestrol. Vertical bars represent the standard error of the mean ($n = 5$). **** = $P < 0.001$, *** = $P < 0.01$, ** = $P < 0.05$; * = $P < 0.10$

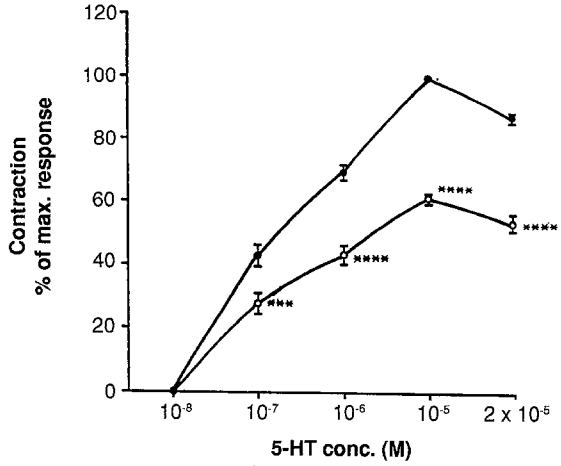

Fig. 4. The effect of 2 μmol·l^{-1} of diethylstilboestrol on the 5-HT-induced dose-response curve of isolated rat detrusor muscle. ● = control, ○ = after the addition of 2 μmol·l^{-1} of diethylstilboestrol. Vertical bars represent the standard error of the mean ($n = 5$). **** = $P < 0.001$, *** = $P < 0.01$

The effect of nifedipine, 0.03 μmol·l^{-1}, on carbachol-induced responses in human detrusor samples was similar to that of diethylstilboestrol, with a 39% reduction in the maximum response ($P < 0.001$) (Fig. 2).

Effects of diethylstilboestrol on contraction in response to electrical field stimulation

Rat detrusor muscle showed frequency-dependent contractile responses to electrical stimulation. The responses were rapid and relaxation was immediate on withdrawal of stimulation. Diethylstilboestrol 2 μmol·l^{-1} significantly reduced rat detrusor muscle contraction in response to electrical field stimulation ($P < 0.001$). The maximum contractile response was reduced by 33% (Fig. 3 a).

Human bladder muscle showed similar inhibition, with a reduction in the maximum contractile response of 32% ($P < 0.001$) (Fig. 3 b).

Effect of diethylstilboestrol on 5HT stimulation

Rat detrusor muscle had a slowly developing phasic response to stimulation with 5-hydroxytryptamine. The maximum response was much less than with cholinergic or electrical stimulation. The inhibitory action of diethylstilboestrol was more potent on 5HT-induced contractions than on cholinergic or neuronally-evoked responses, with a reduction in the maximum contractile response of 40% ($P < 0.001$) (Fig. 4).

Effect of diethylstilboestrol and nifedipine on calcium-induced contractions

When rat detrusor muscle was placed in calcium-free potassium-rich Krebs solution there was an immediate contraction followed by relaxation. The rhythmic spontaneous contractions normally seen in rat detrusor preparations were also abolished. The contractile response to the readdition of calcium reached a maximum at a concentration of 1.5 mmol·l^{-1}. The addition of 2 μmol·l^{-1} of diethylstilboestrol to the external medium resulted in inhibition of these contractions. The maximum response was reduced by 40% ($P < 0.001$) (Fig. 5 a). Nifedipine 0.03 μmol·l^{-1} completely abolished the rat detrusor response to calcium stimulation and a concentration of 0.01 μmol·l^{-1} reduced the maximum response by 72% (Fig. 5 b).

Effect of diethylstilboestrol and nifedipine on carbachol- and acetylcholine-induced contractions in a low-calcium medium

Rhythmic spontaneous contractions in the rat detrusor muscle were maintained in a low-calcium (0.3 mmol·l^{-1}) Krebs solution. However, the frequency and magnitude of this activity were slightly reduced when compared with tissue exposed to an external medium of normal calcium content (2.5 mmol·l^{-1}). The inhibitory effect of 2 μmol·l^{-1} of diethylstilboestrol on carbachol- and ace-

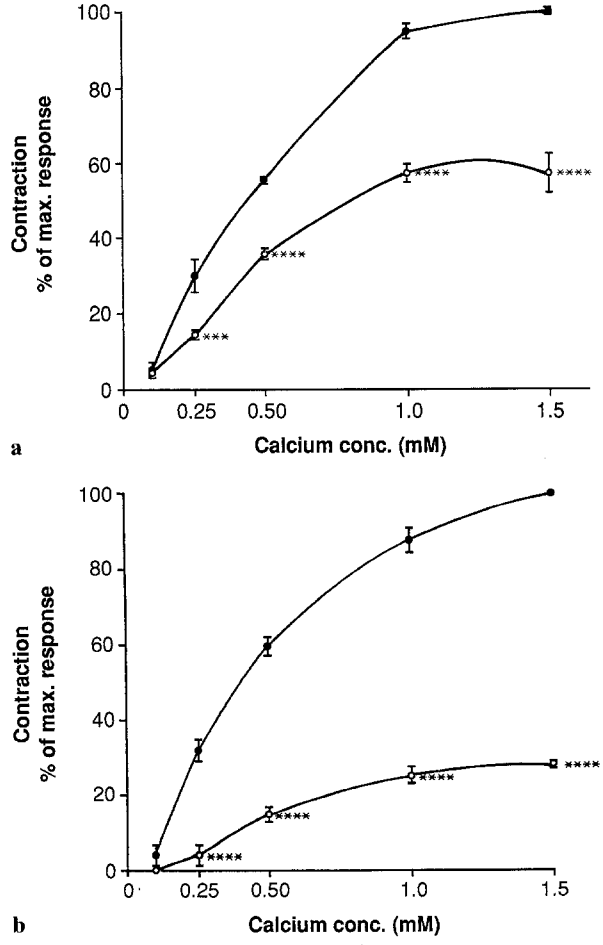

Fig. 5 a, b. The effect of 2 μmol·l^{-1} of diethylstilboestrol (**a**) and 0.01 μmol·l^{-1} of nifedipine (**b**) on the calcium-induced dose-response curve of depolarized rat detrusor muscle. ● = control, ○ = after the addition of diethylstilboestrol or nifedipine. Vertical bars represent the standard error of the mean ($n = 5$). **** = $P < 0.001$, *** = $P < 0.01$

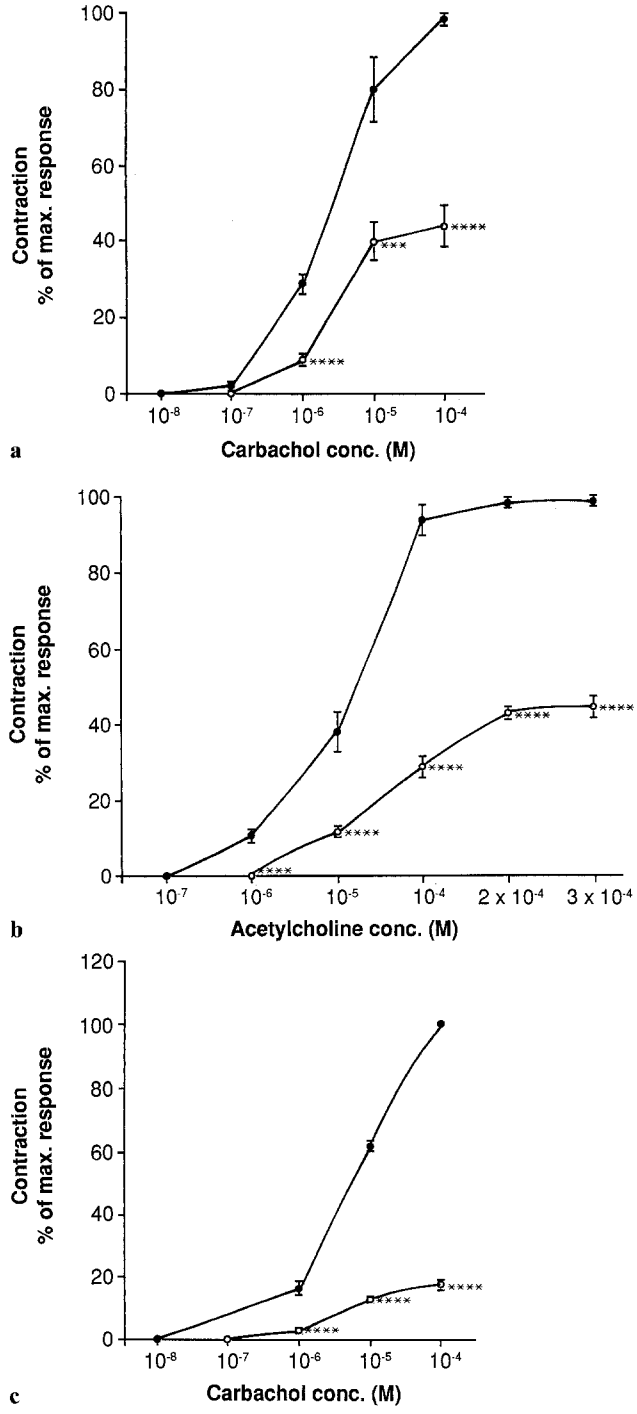

Fig. 6a–c. The effect of 2 μmol·l^{-1} of diethylstilboestrol on (**a**) carbachol and (**b**) acetylcholine dose-response curves, and (**c**) the effect of 0.03 μmol·l^{-1} nifedipine on the carbachol-induced dose-response curve of isolated rat detrusor muscle suspended in a low-calcium medium. ● = control, ○ = after the addition of 2 μmol·l^{-1} of diethylstilboestrol or nifedipine. Vertical bars represent the standard error of the mean, ($n = 5$). **** = $P < 0.001$, *** = $P < 0.01$

tylcholine-induced contractions was increased when the calcium content of the external medium was reduced. The dose-response curves for these agonists also showed a 53% reduction in the maximum response for carbachol ($P < 0.01$) (Fig. 6a) and a 55% reduction for acetylcholine ($P < 0.001$) (Fig. 6b).

For comparison, the effect of a calcium antagnist, nifedipine 0.03 μmol·l^{-1}, on the carbachol dose-response curve in low-calcium medium was investigated. The results were similar to those of the diethylstilboestrol experiments. However, nifedipine had a more potent inhibitory effect, with an 80% reduction in the maximum response (Fig. 6c).

Effect of diethylstilboestrol on electrical field induced contraction in a low calcium ion medium

Diethylstilboestrol 2 μmol·l^{-1} had a striking inhibitory effect on electrical field-induced contractions in a low-calcium environment. The maximum response was reduced by 78% ($P < 0.001$) (Fig. 7). The control contractile responses to electrical stimulation were also reduced in a low-calcium medium, but only by 17%.

Effect of diethylstilboestrol on KCl-induced contractions

The cumulative dose-response curves to KCl showed a significant reduction in the maximum response after the addition of 2 μmol·l^{-1} of diethylstilboestrol ($P < 0.01$). The maximum response was reduced by 30%. This inhibitory effect was observed at low and high KCl concentrations (Fig. 8).

Effect of diethylstilboestrol on carbachol-induced contractions in depolarized rat detrusor muscle

Before the addition of each dose of carbachol, the bladder tissues were primed with a low concentration of calcium (0.3 mmol·l^{-1}) for 10 min to replace released calcium and then washed with calcium-free Krebs. After 5 min carbachol (10^{-4} mol·l^{-1}) was injected into the bath and the response was recorded. The response before and after the addition of diethylstilboestrol were not significantly different.

Discussion

These preliminary in vitro studies support a possible role of oestrogens in the treatment of female patients with motor-urge incontinence secondary to detrusor instability. The results also corroborate previous work on the likely beneficial use of nifedipine in this condition [3]. It is clear that diethylstilboestrol had a significant inhibitory effect on rat and human detrusor muscle contractions when added directly to the bathing solution.

Our results suggest that diethylstilboestrol affected the movement of extracellular calcium ions into detrusor muscle cells. Nifedipine is known to inhibit calcium influx into smooth muscle cells from the surrounding medium [10]. Our results strongly suggest that diethylstilboestrol had a similar, although less potent, effect. In the presence of a high external potassium concentration, to open potential-dependent calcium channels, diethylstilboestrol re-

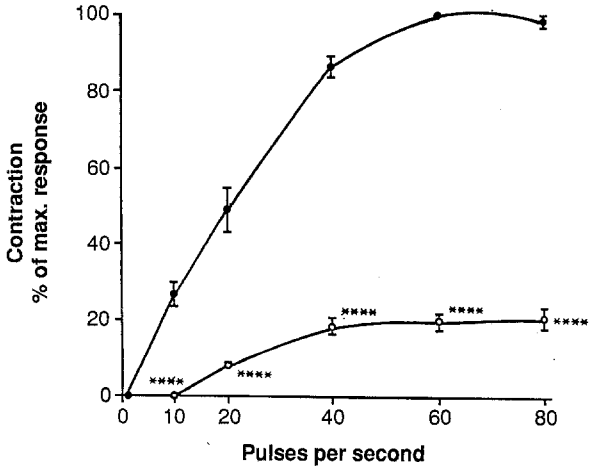

Fig. 7. The effect of 2 μmol·l^{-1} of diethylstilboestrol on the electrically-induced frequency-response curve of isolated rat detrusor muscle suspended in a low-calcium medium. ● = control, ○ = after the addition of diethylstilboestrol. Vertical bars represent the standard error of the mean ($n = 5$). **** = $P < 0.001$

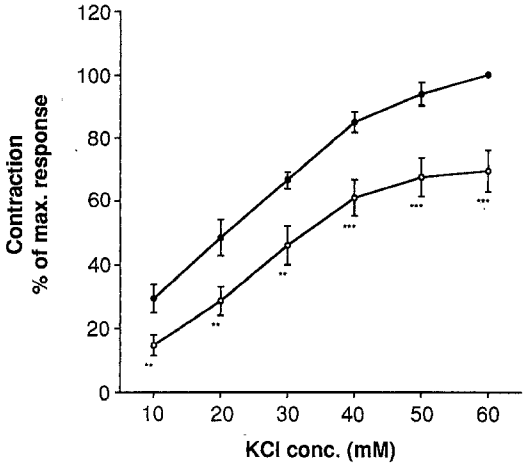

Fig. 8. The effect of 2 μmol·l^{-1} of diethylstilboestrol on the KCl dose-response curve of isolated rat detrusor muscle. ● = control, ○ = after the addition of diethylstilboestrol. Vertical bars represent the standard error of the mean ($n = 5$). *** = $P < 0.01$, ** = $P < 0.05$

duced the maximum response to increasing concentrations of calcium. Furthermore, the effects on carbachol, acetylcholine, 5-HT, and electrical field stimulation were more marked if the calcium concentration of the surrounding medium was kept low. Finally, the inhibitory effect of diethylstilboestrol on KCl-induced contractions suggests a selective effect on calcium influx, since extracellular calcium is the sole source of calcium ions for these contractions in human bladder muscle [11]. Batra & Bengtsson [12] came to similar conclusions on the action of diethylstilboestrol on rat uterine muscle, although they used concentrations which were ten times higher. It is possible that a higher concentration of diethylstilboestrol might have had a greater inhibitory effect on detrusor muscle stimulation, since 20 μmol·l^{-1} of diethylstilboestrol abolished spontaneous activity in the rat detrusor muscle.

Although all the stimulatory mechanisms used in these experiments might have involved cholinergic receptors [13, 14, 15, 16], it is unlikely that diethylstilboestrol had its effect on them. Electrical field stimulation produces its effect through both cholinergic and non-cholinergic neurotransmission in the rat, the latter almost certainly being via ATP [13]. However, in man there appears to be little non-cholinergic effect of electrical field stimulation [14, 15], and so a far greater effect would have been seen in man than in rat if diethylstilboestrol influenced muscarinic receptors. However, the effects were similar in the two species. We found no evidence that diethylstilboestrol altered the activity of cholinesterase, since its effects on acetylcholine and carbachol were similar.

We did not find evidence of inhibition by diethylstilboestrol of calcium release from intracellular stores, because when the detrusor muscle samples were depolarized and primed with a low concentration of calcium, diethylstilboestrol did not affect the contractile response to carbachol. Mostwin [17] has previously shown that muscarinic receptor activation by carbachol can release calcium ions from the intracellular calcium ion store in detrusor smooth muscle. Thus, carabchol can contract the detrusor muscle despite the inactivation of external calcium ion transport mechanisms [11].

Acknowledgements. We should like to thank Jenny Rees, Steve Brice, and Linda Scrimshire for carrying out the vaginal smears, and we are also very grateful to Professor S. Nahorski (University of Leicester) for his patient help and advice.

References

1. McGrother CW, Castleden CM, Duffin HM and Clarke M (1986) Provision of services for incontinent elderly people at home. J Epid Com Health 40: 134–138
2. Castleden CM, Duffin HM and Asher MJ (1981) Clinical and urodynamic studies in 100 elderly incontinent patients. Br Med J 282: 1103–1105
3. Battcock TM and Castleden CM (1990) Pharmacological treatment of urinary incontinence. Br Med Bull 46: 147–155
4. Molander U, Milsomn I, Ekelund P and Mellstrom D (1990) An epidemiological study of urinary incontinence and related urological symptoms in women. Maturitas 12: 51–60
5. Miodrag A, Castleden CM and Vallance TR (1989) Sex hormones and the female urinary tract. Drugs 36: 491–504
6. Shapiro E (1986) Effect of oestrogens on the weight and muscarinic receptor density of the rabbit bladder and urethra. J Urol 135: 1084–1087
7. Batra S and Andersson KE (1989) Oestrogen-induced changes in muscarinic receptor density and contractile responses in the female rabbit urinary bladder. Acta Physiol Scand 137: 135–141
8. Iosif CS, Batra S, Anders EK and Birger A (1981) Oestrogen receptors in the human female lower urinary tract. Am J Obstet Gynecol 141: 817–820
9. Ingelman-Sundberg A, Rosen J, Gustafsson A and Carlstrom K (1981) Cytosol estrogen receptors in the urogenital tissues in stress incontinent women. Gynaecol Scand 60: 585–586
10. Zar MA, Iravani MM and Luheshi GN (1990) Effect of nifedipine on the contractile response of the isolated rat bladder. J Urol 143: 835–839
11. Maggi CA, Patacchini SGR, Turini D, Barbanti G, Giachetti A and Meli A (1989) Multiple sources of calcium for contraction of the human urinary bladder muscle. Br J Pharmacol 98: 1021–1031

12. Batra S and Bengtsson B (1978) Effects of diethylstilboestrol and ovarian steroids on the contractile responses and calcium movements in rat uterine smooth muscle. J Physiol 276: 329–342
13. Brading AF and Williams JH (1990) Contractile responses of smooth muscle strips from rat and guinea-pig urinary bladder to transmural stimulation: Effects of atropine or alpha, beta-methylene ATP. Br J Pharmacol 99: 493–498
14. Sibley GNA (1985) A comparison of spontaneous and nerve-mediated activity in bladder muscle from man, pig and rabbit. J Physiol 354: 431–443
15. Kinder RB and Mundy AR (1985) Atropine blockade of nerve-mediated stimulation of the human detrusor. Br J Urol 57: 418–421
16. Chen HI (1990) Evidence for the presynaptic action of 5-hydroxytryptamine and the involvement of purinergic innervation in the rabbit lower urinray tract. Br J Pharmacol 101: 212–216
17. Mostwin JL (1985) Receptor operated intracellular calcium stores in the smooth muscle of the guinea pig bladder. J Urol 133: 900–902

Dr. C. M. Castleden
University Department of Medicine for the Elderly
Leicester General Hospital
Gwendolen Road
Leicester, LE5 4PW, UK