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Effects of 17β-oestradiol on rat detrusor smooth muscle contractility

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The aim of this study was to investigate the effect of 17β-oestradiol (E_2) on detrusor smooth muscle contractility and its possible neuroprotective role against ischaemic-like condition, which could arise during overactive bladder disease. The effect of E_2 was investigated on rat detrusor muscle strips stimulated with carbachol, KCl and electrically, in the absence or presence of a selective oestrogen receptor antagonist (ICI 182,780) and, by using confocal Ca^{2+} imaging technique, measuring the amplitude ($\Delta F/F_0$) and the frequency of spontaneous whole cell Ca^{2+} flashes. Moreover, the effect of 1 and 2 h of anoxia–glucopenia and reperfusion (A-G/R), in the absence or presence of the hormone, was evaluated in rat detrusor strips perfused with Krebs solution which underwent electrical field stimulation to stimulate intrinsic nerves; the amplitude and the frequency of Ca^{2+} flashes were also measured. 17β-Oestradiol exhibited antispasmogenic activity assessed on detrusor strips depolarized with 60 mM KCl at two different Ca^{2+} concentrations. 17β-Oestradiol at the highest concentration tested (30 μM) significantly decreased detrusor contractions induced by all the stimuli applied. In addition, the amplitude and the frequency of spontaneous Ca^{2+} flashes were significantly decreased in the presence of E_2 (10 and 30 μM) compared with control detrusor strips. In strips subjected to A-G/R, a significant increase in the amplitude of both spontaneous and evoked flashes was observed. 17β-Oestradiol was found to increase the recovery of detrusor strips subjected to A-G/R. The ability of E_2 to suppress contraction in control conditions may explain its ability to aid recovery following A-G/R.

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The urinary bladder functions to store and to expel the urine from the body. These roles are achieved through complex interactions between the autonomic nervous system, the sensory nerves and the urinary bladder smooth muscle. Normal bladder function depends on the integrity of these interactions and is maintained by an adequate supply of oxygen and nutrients via the circulation. Greenland *et al.* (2000) observed a period of ischaemia and hypoxia during normal micturition in pigs, and noted that partial bladder outlet obstruction increased the severity and duration of bladder wall hypoxia. The effects of hypoxia/anoxia have been studied in a variety of smooth muscles, and several mechanisms could potentially contribute to hypoxia-induced reduction in force. Such mechanisms fall in two categories. The first relates to energy limitation, viewing cellular ATP production under hypoxic conditions as being unable to support actin–myosin ATPase activity, hence, contractile activity (Obara *et al.* 1997). The second involves some form of oxygen sensing that subsequently leads to modulation of pathways involved in excitation–contraction coupling. Both Ca^{2+}-dependent mechanisms, involving hypoxia-induced changes in intracellular [Ca^{2+}], and Ca^{2+}-independent mechanisms, involving the Ca^{2+} sensitivity of the contractile apparatus, may contribute (Shimizu *et al.* 2000). In addition to contractile dysfunction, ischaemic injury to the mucosa causes increased mucosal permeability and activation of sensory nerves with subsequent detrusor overactivity (Azadzoi *et al.* 1996), which may be related to irritability symptoms such as urgency, frequency and urge incontinence (the components of overactive bladder syndrome). Overactive bladder affects 33 million adults in the United States, which is approximately 16.5% of the population (Stewart

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et al. 2003). Knowledge of how the detrusor responds to ischaemic conditions is necessary for the development of ways to treat this syndrome.

Oestrogens are steroids, named for their importance in the oestrous cycle, which function as the primary female sex hormone. The most potent naturally occurring oestrogen in humans is 17β-oestradiol (E_2). Oestrogens have widespread biological actions. They stimulate growth, blood flow and water retention in sexual organs and they also influence differentiation, maturation and function of various tissues throughout the body, including the peripheral and central nervous systems. Furthermore, oestrogens have been shown to have beneficial effects in cellular and molecular systems relevant to neurodegenerative disorders (Behl *et al.* 1997). 17β-Oestradiol is a vaso- and neuroprotective agent (Green & Simpkins, 2000; Roof & Hall, 2000). It has been demonstrated to inhibit lipid peroxidation and protects neurons against oxidative stress (Behl & Holsboer, 1999).

Recently, Pessina *et al.* (2007) have observed in guinea-pig urinary bladder that there is a higher resistance to the effects of anoxia–glucopenia and reperfusion (A-G/R) in females compared with males; it was argued that E_2 might be responsible for this difference. Moreover, E_2 might affect the intracellular Ca^{2+} concentration (Pozzo-Miller *et al.* 1999), reducing Ca^{2+} influx primarily through the inhibition of L-type Ca^{2+} channels in a non-genomic manner and therefore decreasing myosin light chain (MLC) phosphorylation and contraction of smooth muscle (Kitazawa *et al.* 1997). In addition, E_2 could activate Ca^{2+}-dependent molecules, such as protein kinase C and Ca^{2+}–calmodulin (Hayashi *et al.* 1994; Kelly *et al.* 1999).

Oestrogens have been used for several years to treat urinary symptoms, especially those associated with the lower urinary tract. The action of oestrogen on the continence mechanism is likely to be complex. Oestrogens may affect continence by any of the following mechanisms: (a) increasing urethral resistance; (b) raising the sensory threshold of the bladder; (c) increasing α-adrenoreceptor sensitivity in the urethral smooth muscle; and (d) promoting β-adrenoceptor-mediated relaxation of the detrusor muscle (Kinn & Lindskog, 1988; Busby-Whitehead & Johnson, 1998; Matsubara *et al.* 2002). However, contradictory effects of oestrogens on bladder contractility have been reported (Diep & Constantinou, 1999; Jackson *et al.* 2002).

The aim of the present study was to investigate the effect of 17β-oestradiol on detrusor smooth muscle contractility and its possible role as neuroprotective agent against damage resulting from A-G/R. The effects of E_2 on detrusor smooth muscle contraction were investigated using both contraction and confocal Ca^{2+} imaging.

Methods

Preparation of detrusor strips

All experiments were performed in strict compliance with the recommendations of the EEC (86/609/CEE) for the care and use of laboratory animals and were approved by the Animal Care and Ethics Committee of the University of Siena, Italy. Sixty Wistar male rats (Charles River, Calco, Italy; 250–400 g) were anaesthetized with a mixture of ketamine hydrochloride (30 mg kg^{-1}, I.P; Ketavet®, Gellini, Aprilia, Italy) and xylazine hydrochloride (8 mg kg^{-1}, I.P; Rompum®, Bayer, Wuppertal, Germany) and killed by cervical dislocation. The bladders were isolated, cleaned of external fat and connective tissue, and opened along the ventral surface. Strips of detrusor muscle measuring approximately 1.0 mm × 0.5 mm × 8 mm were dissected following the direction of the muscle bundles. Fine silk ligatures were tied to each end of the strips, which were mounted in small (0.2 ml) superfusion organ baths between two platinum electrodes 1 cm apart. Strips were continuously superfused with Krebs solution (composition in mM NaCl, 120; KCl, 5.9; $MgCl_2$, 1.5; $CaCl_2$, 2.5; $NaHCO_3$, 15.4; NaH_2PO_4, 1; glucose, 11.5; pH 7.4) pumped by a peristaltic pump (Watson-Marlow, Falmouth, UK) at a constant rate of 1.5 ml min^{-1}. Strips were placed under an initial tension of 10 mN and allowed to equilibrate for at least 60 min. Contractions were measured isometrically using mechanoelectrical transducers (Basile, Comerio, Italy) and recorded using a PowerLab 8/30 data acquisition system (ADInstruments, Basile, Comerio, Italy) connected to a notebook computer running Chart 5 software (ADInstruments). Electrical field stimulation (EFS; 0.05 ms pulse duration, 50 V, 10 Hz, in 5 s trains) was delivered via a digital stimulator (LE 12106, LETICA Scientific Instruments, Barcelona, Spain) every 30 min. From preliminary experiments, when tissues were pre-incubated with 3 μM TTX for 20 min, EFS responses were 4.7 ± 1.6% of control values ($n = 4$), demonstrating their neurogenic origin.

The effects of E_2 on strips subjected to ischaemia–reperfusion-like conditions (anoxia–glucopenia and reperfusion; A-G/R)

In order to mimic ischaemic conditions, a number of modifications were carried out to the organ bath apparatus. The Krebs solution at 37°C was replaced by a glucose-free Krebs solution (glucose was replaced isosmotically with NaCl; glucopenia) and the solution was gassed with 95% N_2 and 5% CO_2 (anoxia). After this A-G period, initial conditions were restored (reperfusion).

To test the oxygen tension in the bath during A-G conditions, oxygen was measured with a galvanic oxygen electrode (model MLT1115; ADInstruments, Chalgrove,

UK). The electrode was calibrated using a three-point calibration with glucose-free Krebs solution (equilibrated in a large reservoir for 3 h with 0%O_2–95%N_2–5%CO_2, air or 95%O_2–5%CO_2). The equivalent O_2 saturation of the hypoxic–glucopenic solution in the contraction bath was 0.7%. Assuming an atmospheric pressure of 760 mmHg, this implies a partial pressure of O_2 under anoxic–glucopenic conditions of 5 mmHg.

The response of intrinsic nerves to EFS was expressed as a percentage of the initial response in standard Krebs solution, taken as 100%.

In a first set of experiments, after a 60 min equilibration period, in which control responses to various stimuli were obtained, strips were subjected to 60 min of A-G conditions followed by 120 min of R. In a second set of experiments, the length of the A-G phase was extended to 120 min, followed by 180 min of R. Drugs (E_2 at 0.1, 1, 3, 10 or 30 μM) were added to the superfusing solution 60 min before applying A-G (pre-incubation, P-I), during A-G and during the first 30 min of R, while stimulating the strips every 30 min, as described in the previous subsection.

Effects of E_2 on detrusor strip contractility

Detrusor strips, placed in the organ bath with standard Krebs solution at 37°C and bubbled with 95% O_2–5% CO_2 gas mixture, were left to equilibrate for 60 min. Then, each strip was stimulated in a random order: electrically (pulses of 0.05 ms, 50 V and 10 Hz in 5 s trains), with 10 μM carbachol (CCh) or with high-potassium (60 mM) solution with 30 min intervals between each stimulus. To examine the concentration-dependent effects of E_2 on muscle contractility, each strip was exposed to a different concentration of the hormone (0.1, 1, 3, 10 or 30 μM) or to the solvent (ethanol), taken as control. After 20 min of incubation, strips were stimulated again in the presence of E_2 or ethanol.

The involvement of oestrogen receptors (ERs) in the effects of E_2 was assessed by incubating the strips for 20 min with the selective ER antagonist ICI 182,780 (at the same concentration of E_2) before addition of E_2 to the organ bath.

Antispasmogenic activity of E_2 on detrusor smooth muscle strips

The antispasmogenic effect of E_2 or nifedipine was assessed in strips in which contractions were elicited by depolarization with 60 mM K^+ in the presence of 0.5 or 5 mM Ca^{2+}. Krebs solution containing 60 mM K^+ was prepared by replacing NaCl with equimolar KCl. When the Ca^{2+} concentration was changed, Ca^{2+} was replaced isosmotically with NaCl. After a 60 min equilibration period with Krebs solution containing either 0.5 or 5 mM Ca^{2+}, strips were exposed to high K^+ for 4 min every 20 min, until responses were reproducible, and these were taken as control values. Drugs at increasing concentrations (E_2 at 0.1, 1, 3, 10 and 30 μM; nifedipine at 0.1, 1, 10 and 100 nM and 1 μM) were tested on successive responses to high K^+ (each compound on a different strip, repeated on strips from 4 animals). Drugs were applied 10 min before as well throughout the depolarizing period (Pessina *et al.* 2001). Results are expressed as percentage of inhibition with respect to control values. The pharmacological effect of each substance is described as the mean ± S.E.M. value of the pIC_{50}.

Calcium imaging and analysis

After subjecting tissues to ischaemic-like conditions, as previously described, each detrusor strip was exposed to 10 μM Oregon Green-488 1,2-bis(*O*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-1 acetoxymethyl ester (BAPTA-1 AM) in 1% dimethyl sulphoxide and 0.2% pluronic F-127 in standard Krebs solution for 90 min at 36°C. Each strip was then rinsed in Krebs solution, and bubbled with 95% O_2 and 5% CO_2 for at least 10 min. Tissues were then pinned flat, serosal side up, in a Sylgard® -lined organ bath and mounted on the stage of an upright confocal microscope.

The detrusor strips were continuously superfused with Krebs solution (bath temperature 33–34°C). Images were acquired with a Leica SP2 upright confocal microscope (Leica Microsystems, Milton Keynes, UK). Oregon Green-488 BAPTA-1 AM was excited with 488 nm laser light, and emission was collected through a prism and shutters set to pass wavelengths longer than 510 nm. A series of 100 frames was captured at approximately 5 Hz, to generate one image set. Such sets were acquired once every minute. Ten sets were generated for each region of the preparation, with at least three regions sampled per preparation (Young *et al.* 2007).

In the first set of experiments, image analysis was performed with Image SXM (http://www.liv.ac.uk/~sdb/ImageSXM/); to correct for lateral movements (particularly the movement generated by contraction), all images were automatically aligned to a template image using the 'Autoregister' function of Image SXM and custom-written macros. A region of interest was established which encompassed the portion of a smooth muscle cell that was consistently within the field of view. The fluorescence signal in this region was measured over time throughout the image set. Data were exported to Chart 5 software for measurement of spikes in the Ca^{2+} signal. The threshold for spike detection (based on the amplitude of the first derivative of the fluorescence signal) was manually chosen to match the sensitivity of manual detection for that cell (Zhu *et al.* 2008). The output from Chart 5 was exported to Excel (Microsoft, Redmond, WA, USA) for further analysis, including a calculation

of the frequency of spontaneous Ca^{2+} transients and the probability that a field stimulus would evoke a Ca^{2+} transient.

In a second set of experiments, image analysis was performed with an Image J (http://rsb.info.nih.gov/ij/download.html) plug-in written by R. J. Amos (Department of Pharmacology, University of Oxford, Oxford, UK) to detect increases in fluorescence of the Ca^{2+} indicator. All images were automatically aligned to a template image using the 'Stack reg' plugin of Image J. In the first frame of the image series, a region of interest was established which encompassed the portion of a smooth muscle cell visible within the confocal plane. The fluorescence signal in this region was measured over time throughout the image set. Data were exported to Chart 5 software to measure calcium spikes and then to Excel for further analysis.

Statistical analysis

Results are expressed as means ± S.E.M. The area under the response–time curve (AUC) was calculated by the trapezoidal rule with the software GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Statistical analysis of the data was performed using Student's *t* test for paired or unpaired samples, or by one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test for multiple comparisons. Values of $P < 0.05$ were considered significant. Values of IC_{50} were estimated by linear regression analysis. The number of the strips used corresponds to the number of the animals, unless stated otherwise.

Drugs: commercial sources and solutions

17β-Oestradiol, carbamylcholine chloride (carbachol), nifedipine, TTX and pluronic F-127 were purchased from Sigma-Aldrich (St Louis, MO, USA). Oregon Green-488 BAPTA-1 AM was purchased from Invitrogen (Paisley, UK) and ICI 182,780 from Tocris Bioscience (Bristol, UK).

Stock solutions (10 mM) were prepared by dissolving 17β-oestradiol and nifedipine in absolute ethanol, kept refrigerated at −20°C and used within 1 week. Stock solutions of ICI 182,780 (10^{-2} M) were made by dissolving the drug in DMSO. Aliquots of 10 μM Oregon Green-488 BAPTA-1 AM were obtained by dissolving the powder in 40 μl DMSO and 20% pluronic acid F-127, then adding 460 μl Krebs solution, shielded from light and stored at −20°C. Subsequent dilution, on the day of the experiment, yielded a 10 μM Oregon Green-488 BAPTA-1 AM solution in 1% DMSO and 0.2% pluronic acid. The DMSO and ethanol exerted no significant effects at the maximal concentration used in all the experiments.

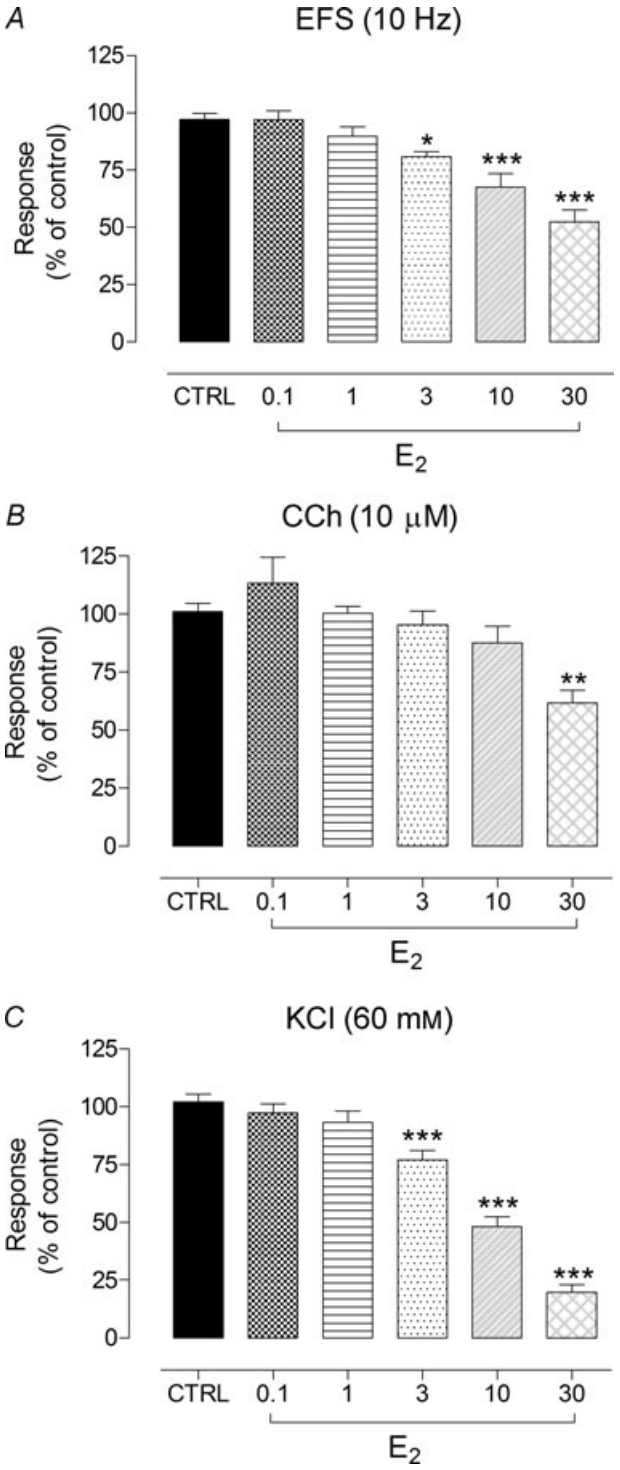

Figure 1. Effect of 17β-oestradiol (E_2) on the contractions induced by EFS (*A*), CCh (*B*) and KCl (*C*)
Results are expressed as a percentage of the control (CTRL) values obtained before applying E_2 (mean ± S.E.M. of 6 urinary bladders). Statistical analysis was performed using one-way ANOVA followed by Dunnett's *post hoc* test. Significant differences from control groups are indicated; $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Table 1. Effect of pre-incubation with ICI 182,780 on the contractions induced by electrical field stimulation (EFS), CCh and KCl

	1 μM E_2	1 μM (E_2 + ICI 182,780)	10 μM E_2	10 μM (E_2 + ICI 182,780)
10 Hz EFS	89.8 ± 6.3	80.4 ± 5.7	67.5 ± 5.9	69.7 ± 4.6
10 μM CCh	100.3 ± 2.9	95.6 ± 2.6	87.6 ± 7.2	82.4 ± 5.2
60 mM KCl	93.2 ± 4.9	88.8 ± 3.4	48.2 ± 4.3	48.5 ± 2.4

Effects of incubation with 1 and 10 μM E_2, with and without pretreatment with ICI 182,780 (at the same concentrations), on the contractions induced by EFS, CCh and KCl. Results are expressed as means ± S.E.M. (n = 6–10). Statistical analysis was performed using Student's t test. Any significant difference is detectable.

Results

Effects of E_2 on detrusor smooth muscle contractility

Detrusor strip responses to EFS (10 Hz), 10 μM carbachol (CCh) and 60 mM KCl in the absence (CTRL) or in the presence of E_2 at various concentrations are shown in Fig. 1. At 30 μM, E_2 significantly decreased detrusor contraction induced by all the stimuli applied (see also the original trace in Fig. 2). Both 10 and 3 μM E_2 significantly decreased EFS- and KCl-induced contractile responses, but at 0.1 and 1 μM E_2 did not exert any statistically significant effect on detrusor smooth muscle contractility evoked by any of the stimuli. 17β-Oestradiol had no effect on the spontaneous contraction of detrusor strips at any of the concentrations used. At 30 μM, E_2 significantly increased the resting tone by about 37% (Fig. 3).

Prior incubation with the ER antagonist ICI 182,780 at 1 or 10 μM did not modify the effects of E_2 on detrusor muscle contractility evoked either by EFS or by the two pharmacological stimuli (CCh and KCl; Table 1).

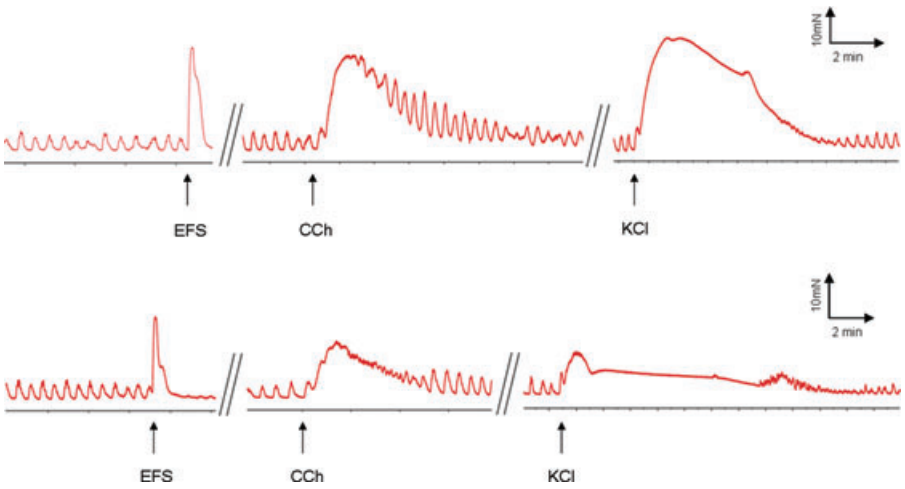

Figure 2. Original traces of contractions induced by EFS, CCh and KCl
Representative traces showing responses to EFS (0.05 ms pulse duration, 50 V, 10 Hz, 5 s trains), CCh (10 μM, 10 s application) and KCl (60 mM, 4 min application) from the same detrusor smooth muscle strip in the absence (upper traces) and in the presence of 30 μM E_2 (lower traces).

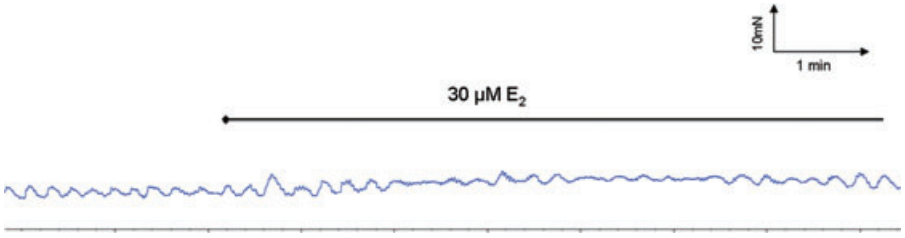

Figure 3. Effect of 30 μM E_2 on spontaneous activity and baseline of rat detrusor strip
Representative trace showing the baseline and the spontaneous activity of rat detrusor strip in the absence and in the presence of 30 μM E_2.

Table 2. Antispasmogenic effect of nifedipine and 17β-oestradiol (pIC_{50}) in detrusor strips superfused with Krebs solution at different Ca^{2+} concentrations and depolarized with 60 mM K^+

Compound	0.5 mM Ca^{2+}	5 mM Ca^{2+}
Nifedipine	9.56 ± 0.23	8.77 ± 0.11*
17β-Oestradiol	5.36 ± 0.05	4.86 ± 0.13**

Results are expressed as means $\pm$ S.E.M. ($n = 5$) of the pIC_{50}. Statistical analysis was performed using Student's *t* test. *$P < 0.05$, **$P < 0.01$ compared with 0.5 mM Ca^{2+}.

Antispasmogenic activity of E_2 on detrusor smooth muscle strips

In order to gain a better understanding of the inhibitory effect of E_2 on EFS-induced detrusor contraction, urinary bladder strips, equilibrated in Krebs solution containing 0.5 mM Ca^{2+}, were depolarized by Krebs solution containing 60 mM KCl to elicit contractions mediated by Ca^{2+} influx. The tension obtained was 8.3 ± 1.3 mN ($n = 10$ strips from 6 animals), which increased to 20.2 ± 2.7 mN ($n = 12$ strips from 6 animals) at a Ca^{2+} concentration of 5 mM. The Ca^{2+} antagonist activity of E_2 was assessed by comparing its antispasmogenic activity at the two Ca^{2+} concentrations. 17β-Oestradiol exhibited antispasmogenic activity, since the hormone showed the same behaviour as the well-known Ca^{2+} antagonist nifedipine; its pIC_{50} value significantly decreased as Ca^{2+} concentration increased (Table 2).

The effect of E_2 on the amplitude of global Ca^{2+} flashes of male rat detrusor strips

In order to gain a better understanding of the effect of E_2 on detrusor smooth muscle contraction, urinary bladder

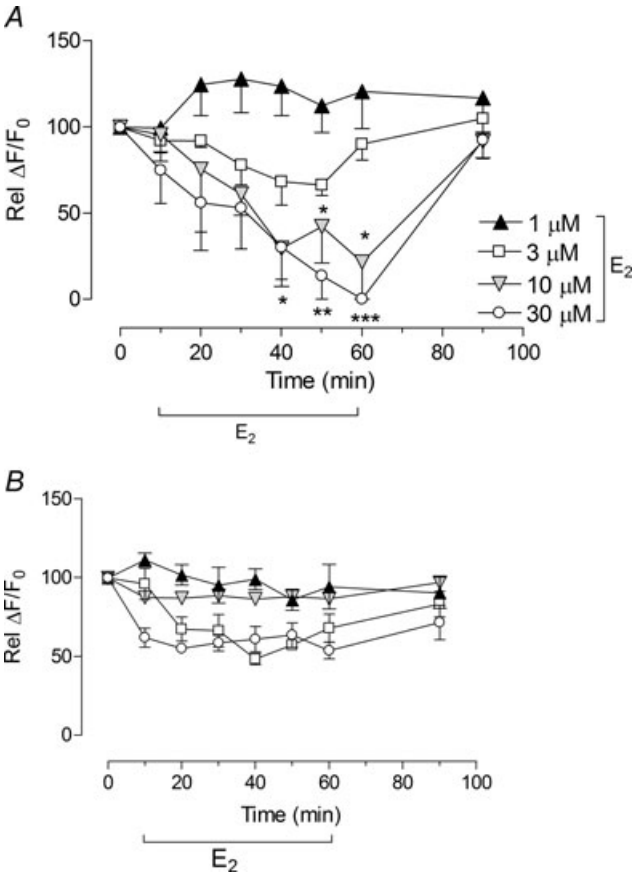

Figure 5. The effect of E_2 on the amplitude of spontaneous (*A*) and evoked global Ca^{2+} flashes (*B*)
Drugs were applied after 30 min of rest and kept for 60 min. The relative change in the fluorescence signal was measured before applying E_2 (time 0 min, control), during the 60 min of incubation and after 30 min of wash out. Results are expressed as means $\pm$ S.E.M. ($n = 5$–8). Statistical analysis was performed using one-way ANOVA followed by Dunnett's *post hoc* test. Significant differences are indicated; *$P < 0.05$, **$P < 0.01$ and *** $P < 0.001$ *versus* control.

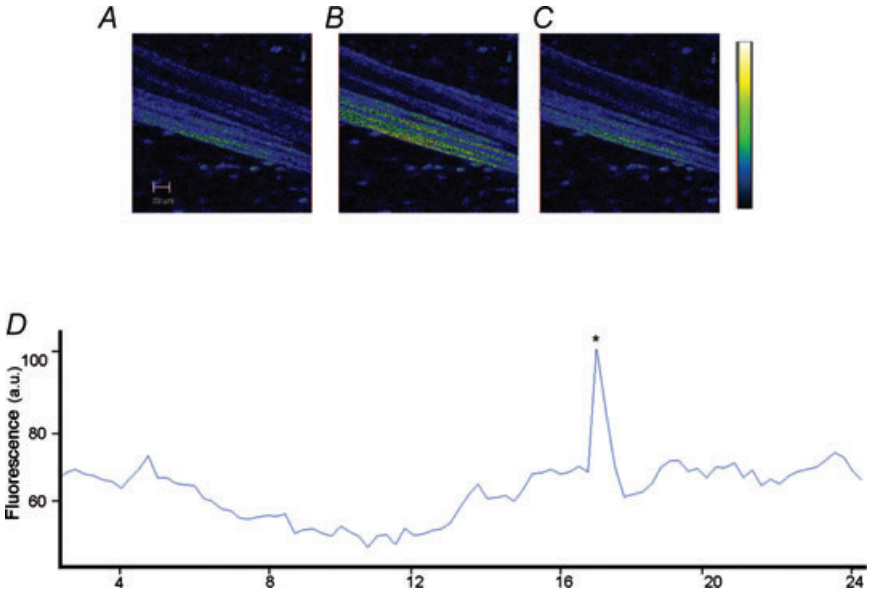

Figure 4. Global Ca^{2+} flashes in urinary bladder smooth muscle strip recorded on confocal microscope
A consecutive series of images of smooth muscle cells (running diagonally from upper left to lower right); some muscle cells are closely associated, forming smooth muscle bundles. Images were acquired at 5 frames s^{-1}. The images show the smooth muscle prior to (*A*), during (*B*) and after a spontaneous Ca^{2+} global flash (*C*). Note that Ca^{2+} concentration rises rapidly and synchronously in most cells in the field. Below, a typical trace (*D*) showing the fluorescent signal from a portion of a single cell; a single Ca^{2+} flash (*), detected automatically on the basis of a peak in dF/dt, is marked. a.u., arbitrary units.

smooth muscle (UBSM) strips were imaged with a laser scanning confocal microscope. Urinary bladder strips, loaded with Oregon Green-488 BAPTA-1 AM, appeared as light green, homogeneous bundles of cells when examined with laser scanning confocal microscopy. In a single field, several smooth muscle cells were often seen lying in parallel (Fig. 4). 17β-Oestradiol at different concentrations (1, 3, 10 and 30 μM) was added to the superfusing Krebs buffer on the confocal microscope stage for 1 h.

In control conditions, spontaneous whole cell Ca^{2+} flashes were observed. Smooth muscle cells displayed repetitive, large and rapid increase in the Ca^{2+} fluorescence that rose almost instantly in a single smooth muscle cell and spread quickly throughout bundle (Fig. 4). The amplitude of such spontaneous whole cell Ca^{2+} flashes was measured, and an increase in the fluorescence of the Ca^{2+} indicator relative to the fluorescence signal at rest ($\Delta F/F_0$) was calculated. In the presence of 30 μM E_2, a gradual decrease of the amplitude of spontaneous Ca^{2+} transient in UBSM strips (Fig. 5*A*) was observed, and after 1 h of treatment the flashes were totally abolished. However, after washing out the hormone, the amplitude of flashes recovered to their initial value. Similar results were obtained in the presence of 10 μM E_2; after 1 h, a reduction of the amplitude by 20% of the initial value was shown. 17β-Oestradiol at 1 and 3 μM did not have any effect on the amplitude of spontaneous Ca^{2+} flashes.

When UBSM strips were electrically stimulated, Ca^{2+} transients were intermittently evoked. The amplitude of Ca^{2+} transients was less affected than that of spontaneous flashes (not formally tested). In 30 μM E_2-treated strips the amplitude gradually decreased, reaching approximately 50% of the initial value; however, after washing out the hormone, the amplitude fully recovered. Similarly, in 10 μM E_2-treated strips the amplitude declined gradually to 60% of the control value. In contrast, at the lowest hormone concentrations tested (1 and 3 μM), the amplitude of evoked Ca^{2+} transients did not differ from the initial amplitude (Fig. 5*B*).

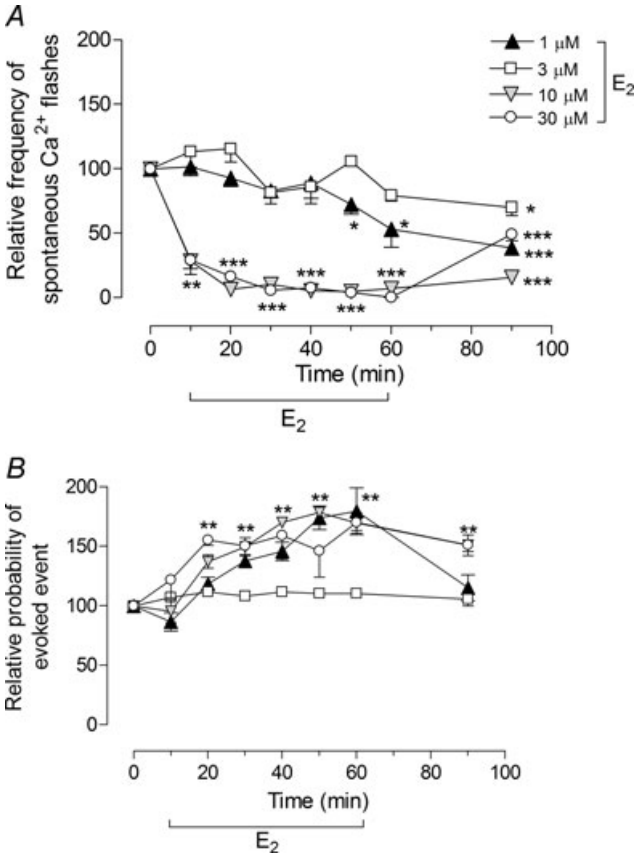

Figure 6. Effect of E_2 on the frequency of Ca^{2+} transients
Drugs were applied after 30 min of rest and kept for 60 min. The frequency of spontaneous Ca^{2+} flashes (*A*) and the probability that EFS evoked a Ca^{2+} transient (*B*) were measured before applying E_2 (time 0 min, control), during the 60 min of incubation and after 30 min of washing out. Results are expressed as means ± S.E.M. (n = 5–8). Statistical analysis was performed using one-way ANOVA followed by Dunnett's *post hoc* test. Significant differences are indicated; *$P < 0.05$, **$P < 0.01$ and *** $P < 0.001$ *versus* control.

The effect of E_2 on the frequency of global Ca^{2+} flashes of male rat detrusor strips

17β-Oestradiol significantly reduced the frequency of spontaneous Ca^{2+} transients at all the concentrations tested (Fig. 6*A*). Moreover, the effect of E_2 on the probability that a field stimulus would evoke a Ca^{2+} transient was also evaluated (Fig. 6*B*). This probability was significantly increased in strips incubated with 10 and 30 μM E_2, while at 1 and 3 μM E_2 there was no significant change in the probability that EFS would evoke a Ca^{2+} flash.

The effect of E_2 on response to EFS of male rat detrusor strips exposed to 1 h of A-G and 2 h of R

During A-G, the response to EFS in control strips gradually decreased, being abolished within an hour. On the contrary, the response to EFS of 0.1 μM E_2-treated tissues decreased much more slowly than in control strips, being significantly higher than that of control preparations ($P < 0.05$) at the end of A-G phase. Moreover, 0.1 μM E_2-treated strips showed a significantly higher recovery during R, compared with control strips. On the contrary, responses to EFS of 10 and 30 μM E_2-treated strips were significantly lower than those of control strips. At 1 and 3 μM, E_2 did not exert any significant effect (Fig. 7). Accordingly, the AUC (Fig. 7, inset) of 0.1 μM E_2-treated strips during the R phase was markedly increased (by 11.4%) compared with the control AUC, while the AUCs of 10 and 30 μM E_2-treated strips were significantly lower (21.9 and 52.1%, respectively) compared with control

tissues. Moreover, E_2 exerted some significant effects by itself on the response to EFS. In fact, at the highest E_2 concentrations used (30 μM) the AUC of the pre-incubation phase was significantly lower than that of control strips.

The effect of E_2 on response to EFS of male rat detrusor strips exposed to 2 h of A-G and 3 h of R

Figure 8 shows the effects of E_2 on EFS-induced contractile responses, when the length of exposure to A-G conditions was extended to 120 min. As described in the previous subsection, at the highest E_2 concentration tested (30 μM), the response to EFS was significantly decreased. However, during the R phase, only 1 μM E_2-treated strips showed a significantly higher recovery of response to EFS (about 22% higher than that of control strips) while in 30 μM E_2-treated strips, EFS recovery in the R phase was poor, being around 34% of the initial value.

Effect of E_2 on the amplitude of global Ca^{2+} flashes of detrusor strips subjected to 1 h of A-G and 2 h of R

After subjecting tissues to the contraction studies, as shown above, each detrusor strip was imaged with a laser scanning confocal microscope. In the tissues subjected to A-G/R (CTRL) the amplitude of the spontaneous (Fig. 9*A*) and evoked global Ca^{2+} flashes (Fig. 9*B*) was significantly higher than in strips not subjected to A-G/R. At the highest E_2 concentrations tested (10 and 30 μM), both in spontaneous and evoked whole Ca^{2+} flashes, the amplitude was significantly lower compared with control values.

Effect of E_2 on the frequency of global Ca^{2+} flashes of detrusor strips subjected to 1 h of A-G and 2 h of R

The frequency of spontaneous Ca^{2+} transients (Fig. 10*A*) and the probability that a field stimulus would evoke a Ca^{2+} transient (Fig. 10*B*) were also determined. There were no significant differences between tissues subjected and not subjected to A-G/R either in spontaneous or in evoked Ca^{2+} flashes. However, in E_2-treated strips the frequency of whole cell flashes (Fig. 10*A*) increased in a concentration-dependent manner, reaching a significant value above 1 μM E_2 ($P < 0.05$).

The probability of evoking a flash (Fig. 10*B*), however, was significantly decreased at the highest concentrations of E_2 used (10 and 30 μM), reaching about 68 and 71% of the control values, respectively.

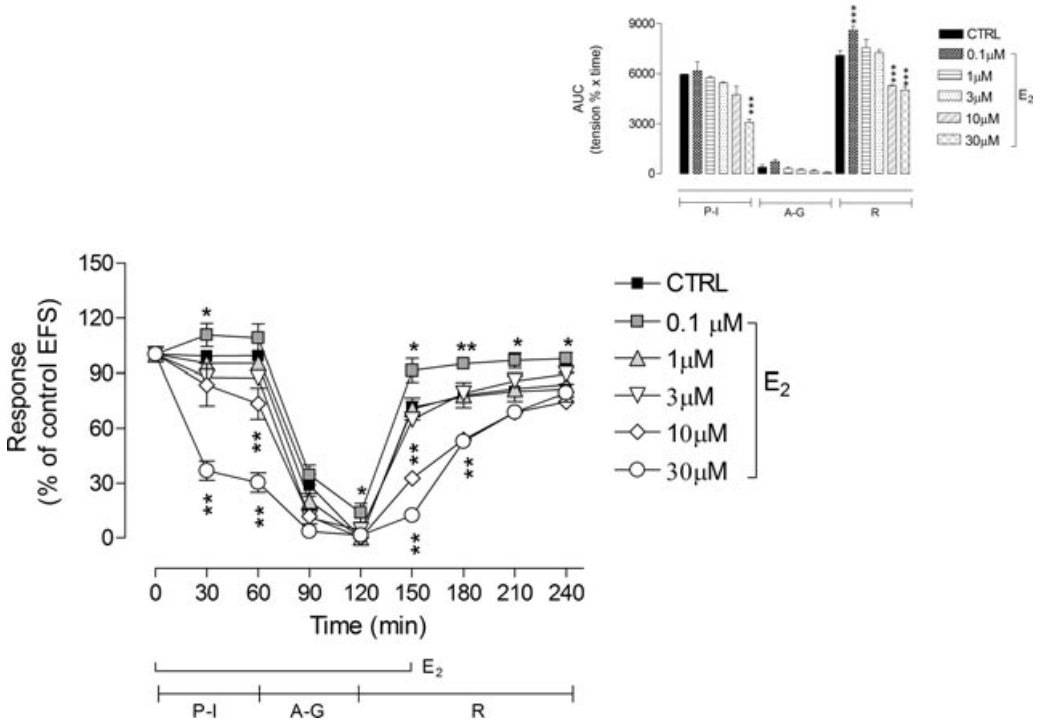

Figure 7. Electrical field stimulation-induced contractile responses of rat detrusor strips subjected to 1 h of A-G and 2 h of R

Experiments were carried out in presence or absence (CTRL) of E_2 at increasing concentrations. Drugs were applied for the first 60 min of the experiment, during A-G and for the first 30 min of R. Results are expressed as means ± S.E.M. ($n = 4$–9). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test. Inset shows results expressed as mean of AUC ± S.E.M. ($n = 4$–9), calculated in the P-I, A-G and R phases separately. Statistical analysis was performed using one-way ANOVA followed by Dunnett's *post hoc* test. Significant differences from the control group are indicated; $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Effect of E_2 on the amplitude and frequency of global Ca^{2+} flashes of detrusor strips subjected to 2 h of A-G and 3 h of R

Using the protocol of 2 h of A-G and 3 h of R, neither consistent effects on spontaneous and evoked Ca^{2+} flashes nor significant changes with E_2 treatments were seen. Similarly, there were no significant effects either on the frequency of Ca^{2+} transients or on the probability that EFS evoked them at all the concentrations of E_2 used (data not shown).

Discussion

In this study, the effect of E_2 on detrusor smooth muscle contractility was investigated, by using different excitatory stimuli to activate different pathways. To test the possible role of E_2 as a neuroprotective agent, urinary bladder smooth muscle strips were subjected to A-G/R conditions and the functionality of intrinsic nerves was assessed through EFS. Moreover, since the relaxing effect of E_2 seems to depend upon the relative contribution of Ca^{2+} influx through voltage-gated Ca^{2+} channels, the effects of E_2 on global calcium flashes, spontaneous and evoked, were studied.

Numerous epidemiological observations and clinical studies have suggested that oestrogen replacement therapy is associated with beneficial effects on the lower urinary tract in postmenopausal women (Suguita *et al.* 2000; Aikawa *et al.* 2003). However, there are several contradictory reports on the specific effects of oestrogen administration on bladder contractility in animal models (Diep & Constantinou, 1999; Jackson *et al.* 2002). Results from the present study indicate that E_2 at high concentrations reduces the contractility of male rat urinary bladders in response to either EFS or pharmacological stimuli (CCh and KCl). The effect of E_2 on the response to CCh, which acts through muscarinic receptors by activating IP_3-mediated release of calcium from intracellular stores (Mimata *et al.* 1997), was the weakest one compared with the other stimuli. Stimulation with high K^+, which acts by depolarizing the plasma membranes and triggering calcium entry through L-type calcium channels, elicited the strongest contractions compared with those evoked by EFS and CCh. Taken together, these results suggest that 17β-oestradiol preferentially inhibits pathways requiring depolarization of the muscle cell membrane.

Several lines of evidence argue against the idea that inhibition of contraction induced by E_2 is mediated by

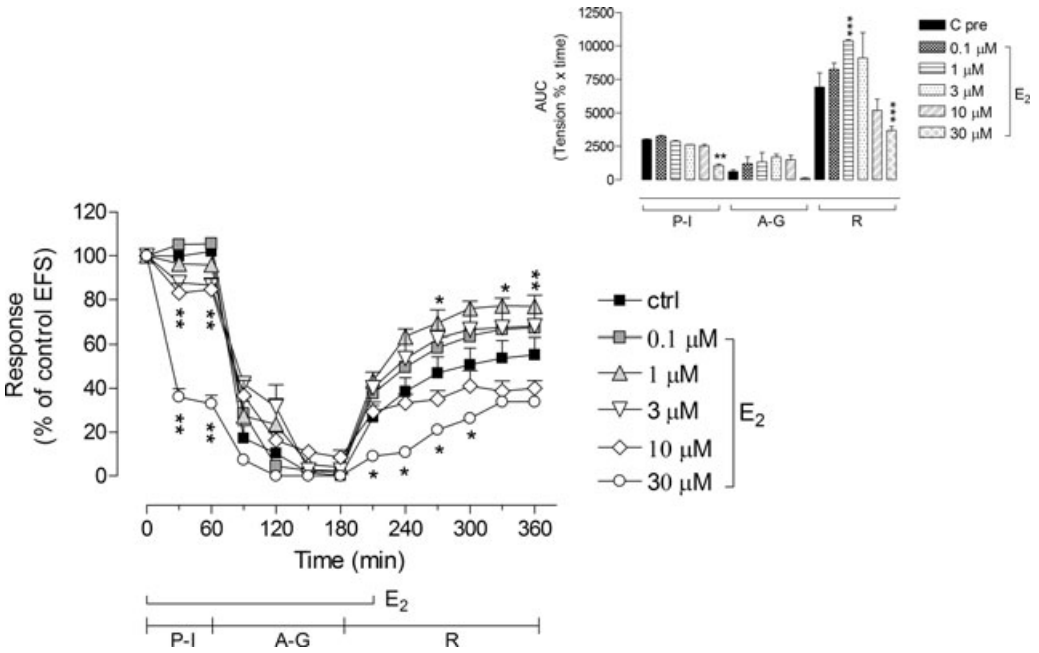

Figure 8. Electrical field stimulation-induced contractile responses of rat detrusor strips subjected to 2 h of A-G and 3 h of R

Experiments were carried out in presence or absence (CTRL) of E_2 at increasing concentrations. Drugs were applied for the first 60 min of the experiment, during A-G and for the first 30 min of R. Results are expressed as means ± S.E.M. ($n = 4$–7). Statistical analysis was performed using one-way ANOVA followed by Dunnett's *post hoc* test. Inset shows results expressed as mean of AUC ± S.E.M. ($n = 4$–7), calculated in the P-I, A-G and R phases separately. Statistical analysis was performed using ANOVA followed by Dunnett's *post hoc* test. Significant differences from the control group are indicated; $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

genomic mechanisms involving nuclear ERs, as follows: the concentrations of E_2 required to reduce smooth muscle contractility are several orders of magnitude higher than those required for genomic activation (McEwen, 1991); the rapid onset of action of E_2 is also inconsistent with the time course of responses requiring gene transcription; moreover, the selective nuclear ER antagonist did not suppress vascular smooth muscle relaxation by E_2 (Freay *et al.* 1997). In the present study, the relatively rapid changes in UBSM contractility (contractions were measured after 20 min of incubation with E_2) and the fact that ICI 182,780 did not inhibit the relaxant effect of E_2 are also not compatible with the genomic pathway. Furthermore, the lowest concentrations used were in the micromolar range, much higher than physiological plasma levels of E_2, which are in the nanomolar range. Moreover, Ogata *et al.* (1996) suggested that 17β-oestradiol acts on cell membrane receptors rather than on cytosolic receptors because its action appeared very quickly, taking place through reversible inhibition of voltage-dependent Ca^{2+} channels. In the present study, the Ca^{2+} antagonist effect of E_2 on rat UBSM is also likely. Firstly, E_2 caused a concentration-dependent decrease in the KCl-induced contractions, with an IC_{50} for contractile inhibition of 4.0 μM, in agreement with a previous study by Sheldon & Argentieri (1995) on guinea-pig detrusor strips, in

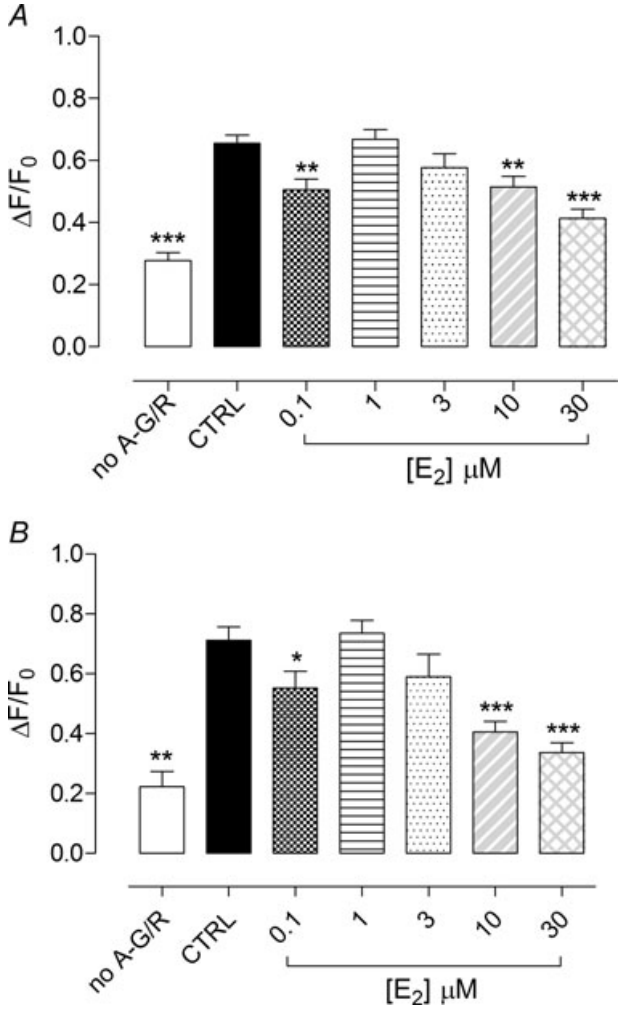

Figure 9. Effect of E_2 on the amplitude of spontaneous and evoked global Ca^{2+} flashes of strips subjected to 1 h of A-G and 2 h of R

Amplitude of spontaneous (*A*) and evoked global Ca^{2+} flashes (*B*) of rat detrusor strips subjected to 60 min of A-G and 120 min of R measured in the absence (CTRL) or presence of E_2. Results are expressed as means ± S.E.M. ($n = 6$) of $\Delta F/F_0$ (first derivative of the fluorescence signal). Statistical analysis was performed using one-way ANOVA followed by Dunnett's *post hoc* test. Significant differences are indicated; $^{**}P < 0.01$ and $^{***}P < 0.001$ *versus* CTRL.

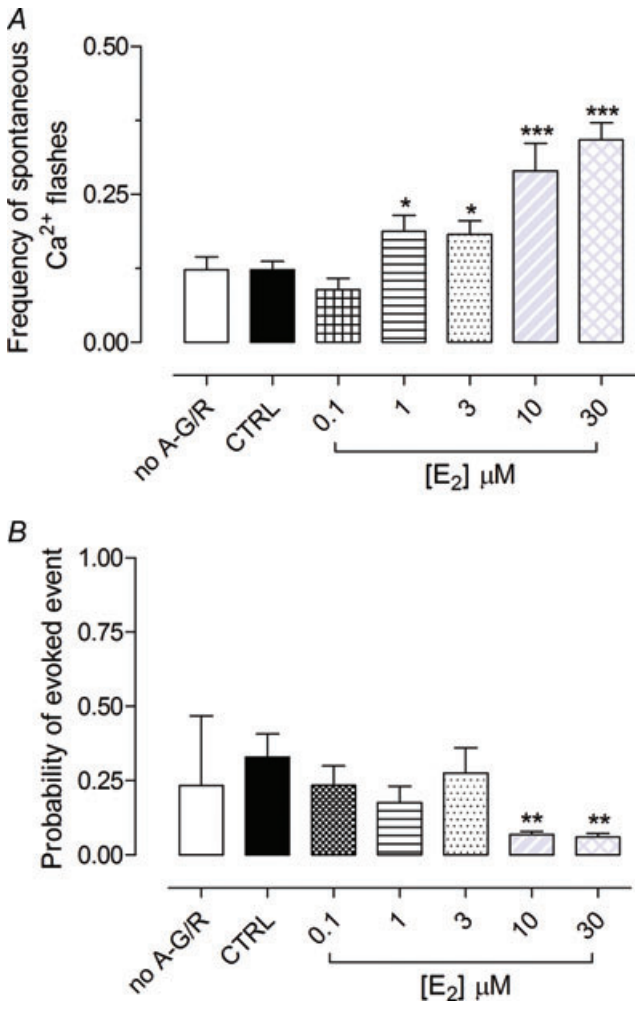

Figure 10. Effect of E_2 on the frequency of spontaneous and evoked global Ca^{2+} flashes of strips subjected to 1 h of A-G and 2 h of R

A, frequency of spontaneous Ca^{2+} flashes of detrusor strips subjected to 60 min of A-G and 120 min of R in absence (CTRL) or presence of E_2. *B*, probability that EFS evoked a Ca^{2+} event. Results are expressed as means ± S.E.M. ($n = 6$). Statistical analysis was performed using one-way ANOVA followed by Dunnett's *post hoc* test. Significant differences are indicated; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *versus* CTRL.

which E_2 had an IC_{50} of 1.7 μM. Secondly, whole cell Ca^{2+} flashes in UBSM, which are diltiazem sensitive and thus require Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Heppner *et al.* 2005), were decreased in amplitude by E_2 in both a concentration-dependent and a reversible manner. The requirement for smooth muscle action potentials is supported by the observation that action potentials occur spontaneously in UBSM (Heppner *et al.* 1997; Hashitani & Brading, 2003*a*,*b*; Meng *et al.* 2008), and simultaneous recordings of voltage and Ca^{2+} in the guinea-pig UBSM have revealed that each Ca^{2+} transient is associated with an action potential (Hashitani *et al.* 2004). Atropine is unable to affect the spontaneous electrical activity observed in mouse urinary bladder, although such spontaneous depolarizations are abolished when P2X receptors are blocked (Meng *et al.* 2008; Young *et al.* 2008). This suggests that spontaneous ACh release from parasympathetic nerve terminals, coreleased with ATP, is unable to affect the membrane potential. Hence, during brief trains of stimuli the smooth muscle Ca^{2+} transients may well be driven by release of ATP, rather than ACh, from nerve terminals driving smooth muscle action potential and Ca^{2+} influx.

It seems unlikely that an effect of E_2 on intracellular Ca^{2+} stores can explain the observed inhibition of contraction because: (a) E_2 reduces the amplitude of the Ca^{2+} flashes, and since these Ca^{2+} flashes depend on the opening of L-type Ca^{2+} channels during smooth muscle action potentials in this tissue (Meng *et al.* 2008; Young *et al.* 2008), inhibition of store release would not be expected to affect the amplitude of Ca^{2+} flashes; and (ii) the ability of E_2 to reduce KCl-induced contractions also argues against an obligate action on intracellular stores. However, we cannot be sure whether E_2 decreases electrical excitability, or more directly inhibits L-type Ca^{2+} channels.

In the present work, the slower recovery time of the amplitude of Ca^{2+} events, compared with the effect on their frequency, suggests that the mechanism driving action potential frequency is separate from that determining the amplitude, but that they are both affected by E_2.

When an obstruction is present or when there is overactivity of the bladder wall, a drop in blood flow and subsequent reduction of substrate and oxygen could occur, resulting in an ischaemic environment within the detrusor (Brading, 1997). The urinary bladder is therefore a good model for studying ischaemic injury. The neuronal damage caused by ischaemic insult plays an important role in the functional defects observed following partial outlet obstruction, nerve terminals being more vulnerable than smooth muscle (Pessina *et al.* 1997). Moreover, previous studies showed that both experimental ischaemia and partial outlet obstruction of the urinary bladder induce similar dysfunction with regard to the contractile responses to EFS (Zhao *et al.* 1997). Therefore, strips pre-incubated with E_2 were subjected to A-G/R, and whole cell Ca $^{2+}$ flashes of the same strips were investigated. These cell flashes represent a synchronous increase in the fluorescence of the indicator, hence of Ca^{2+}, throughout the visible portion of the smooth muscle cell. In strips subjected to A-G/R, a significant increase in the amplitude of both spontaneous and evoked flashes was observed. At higher concentrations of E_2, Ca^{2+} antagonist activity seemed to predominate, directly causing a decrease in amplitude of both spontaneous and evoked flashes. Moreover, a A-G/R-induced increase in the frequency of spontaneous Ca^{2+} transients was observed; the cause of this has not been identified. Furthermore, in strips subjected to A-G/R and incubated with high concentrations of E_2, there was a fall in the probability that a field stimulus evoked a response. 17β-Oestradiol may have decreased transmitter release from the nerves or, alternatively, the high frequency of spontaneous smooth muscle action potentials may have suppressed postjunctional excitability, through, for example, activation of BK channels. Yasay *et al.* (1995) have already demonstrated that E_2 possesses K^+ channel opening activity in guinea-pig urinary bladder smooth muscle, activating the Ca^{2+}-dependent large-conductance K^+ channels. Moreover, E_2, opening BK channels, significantly diminished action potential generation and spontaneous activity, providing negative feedback to limit Ca^{2+} influx (Tanaka *et al.* 2002).

In summary, E_2, at concentrations of 3 μM and above, suppresses the contractility of urinary bladder smooth muscle to nerve stimulation, consistent with a decrease in the amplitude of the Ca^{2+} response in the smooth muscles cells in conjunction with a decrease in the frequency of spontaneous smooth muscle action potentials. The ability of E_2 to suppress contraction in control conditions may explain its ability to aid recovery following anoxia–glucopenia, by reducing the metabolic load.

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