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Acute relaxation of mouse duodenun by estrogens Evidence for an estrogen receptor-independent modulation of muscle excitability

Mario Díaz[a,b,*], Cristina M. Ramírez[a,c], Raquel Marin[b,c], Jorge Marrero-Alonso[a] Tomás Gómez[a,b], Rafael Alonso[b,c]

[a]*Departamento de Biología Animal, Laboratorio de Fisiología Animal, Facultad de Biología, Universidad de La Laguna, 38206 Tenerife, Spain*
[b]*Instituto de Tecnologías Biomédicas, Universidad de La Laguna, 38206 Tenerife, Spain*
[c]*Departamento de Fisiología, Laboratorio de Neurobiología Celular, Facultad de Medicina, Universidad de La Laguna, 38206 Tenerife, Spain*

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Abstract

17-β-Estradiol, the stereoisomer 17-α-estradiol and the synthetic estrogen diethylstilbestrol (DES), all caused a rapid (<3 min) dose-dependent reversible relaxation of mouse duodenal spontaneous activity, reduced basal tone and depressed the responses to $CaCl_2$ and KCl. The steroidal antiestrogen 7α-[9-[(4,4,5,5,5,-pentafluoropenty)sulphinyl]nonyl]-estra-1,3,5(19)-triene-3,17β-diol (ICI182,780) failed to either mimic or prevent the effect of 17-β-estradiol. The effect of estrogens was unrelated to activation of nitric oxide (NO), mitogen-activated protein kinase (MAPK), protein kinase A (PKA), protein kinase G (PKG) or protein kinase C (PKC). Estrogen-induced relaxation was partially reversed by 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-pyridine-3-carboxilic acid methyl ester (BAY-K8644), depolarization, or by application of tetraethylammonium or 4-aminopyridine, but not by glibenclamide, apamin, charybdotoxin, paxilline or verruculogen. The effects of BAY-K8644 and K^+ channel blockers were synergistic, and allowed relaxed tissues to recover spontaneous activity and basal tone. We hypothesize that the rapid non-genomic spasmolytic effect of estrogens on mouse duodenal muscle might be triggered by an estrogen-receptor-independent mechanism likely involving activation of tetraethylammonium- and 4-aminopyridine-sensitive K^+ channels and inhibition of L-type $Ca2^+$ channels on the smooth muscle cells.

Keywords: Rapid estrogen effect; Intestinal smooth muscle; K^+ channel; Voltage-dependent Ca^{2+} channel; Spontaneous activity

1. Introduction

Evidence accumulated over the last two decades have unambiguously demonstrated that, besides their ability to activate transcriptional processes, estrogens can also exert many actions by non-genomic mechanisms (reviewed in Falkenstein et al., 2000; Kelly and Levin, 2001; Nadal et al., 2001). Often these effects are initiated by interacting with membrane and/or cytoplasmic targets, and the effects are fully achieved within minutes or even seconds after exposure to the hormone via transcriptional- and translational-independent mechanism(s) (Falkenstein et al., 2000). Among the different organs where these effects have been demonstrated, vascular tissues have proven to be particularly responsive to estrogens. Hence, it seems now clear that estrogen reduces vascular tone not only by activating nitric oxide generation at the endothelial lining (Farhat et al., 1996; Shaul, 1999), but also by directly modulating ion channel activity at the smooth muscle cells themselves (Geary et al., 1998; Nakajima et al., 1995; Valverde et al., 1999; Brenner et al., 2000). Likewise, in vitro studies on uterine and urinary bladder smooth muscles have unveiled acute effects of estrogens and phytoestrogens (Fernández et al., 1993; Gutiérrez et al., 1998; Ratz et al., 1999; Sheldon

* Corresponding author. Tel.: +34 922 318343; fax: +34 922 318311.
E-mail address: madiaz@ull.es (M. Díaz).

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and Argentieri, 1995). However, although the ability of estrogens to acutely modify smooth muscle activity appears to be a widespread phenomenon, it is noteworthy that similar effects have not been observed for intestinal muscle cells to date. This is particularly relevant considering that gastrointestinal smooth muscle is regulated by complex mechanisms involving most molecular targets reported to be rapidly modulated by estrogens, these including nitric oxide (Rosenfeld et al., 2000; Shaul, 1999), intracellular messengers (Aronica et al., 1994; Farhat et al., 1996; Ropero et al., 1999), protein kinases (Doolan et al., 2000; Murad, 1994; Ropero et al., 1999), phospholipases (Picotto et al., 1999; Sylvia et al., 2000) and ion channels (Gu and Moss, 1998; Ruehlmann et al., 1998; Valverde et al., 1999). Therefore, the aim of the present work was to assess the hypothesis that intestinal contractile activity might be acutely modified by estrogens. In addition, the putative mechanisms responsible for such rapid effects were also explored.

2. Materials and methods

2.1. Experimental procedure

Male mice weighing 25–30 g were killed by decapitation following ether anaesthesia. Animal procedures comply, and were approved, by the Animal Care and Use Committee of Universidad de La Laguna. Only male tissues were used to avoid influence of estrogen cycling. Longitudinal strips of duodenal smooth muscle were dissected, cleaned and immediately placed in cold physiological salt solution (PSS), containing (in mM) NaCl, 120; KCl, 4.7; $MgSO_4.7H_2O$, 1.2; $CaCl_2$, 1.6; KH_2PO_4, 1.2; $NaHCO_3$, 25.0 (adjusted to pH 7.4); glucose 12.0. Ca^{2+}-free solutions were prepared by removing all $CaCl_2$ from the standard PSS and supplemented with 250 μM EGTA. Strips were placed in a 25-ml organ bath at 37 °C and bubbled continuously with a 95% O_2 and 5% CO_2 mixture. Tissues were equilibrated for 30 min at the optimal resting tension of 1 g. Bath solution was replaced every 15–20 min. Isometric tension developed by duodenal strips was measured using an isometric force transducer (TRI110, Letica, Spain). At the beginning of each experiment, the maximal contraction produced by 50 mM KCl using the standard PSS, and the minimal tension recorded under free-Ca^{2+} conditions were measured for each muscle strip to obtain the relative contractile activity range (100% RA). Voltage signals were amplified using a DC amplifier and the output relayed to both, the A/D interface (LabPC+, National Instruments, Austin, USA) and the multichannel polygraph (S460, Goerz Metrawatt, Germany). Signals were digitised at 20 Hz, stored on-line onto the computer hard disk, and visualised on the computer screen using a data acquisition and analysis program (PHYSCAN) written by one of the authors (MD). Data were low-pass filtered at 10 Hz and analysed using computer routines included in the acquisition software. Routinely, 20% of the relative range (20% RA) was used as reference bars in the illustrated figures.

2.2. Effects of estrogens on spontaneous activity

Tissues were incubated in PSS and the spontaneous activity was recorded for 5 min after a 30-min equilibration period. Estrogens were then added in small volumes (5–30 μl) directly to the bath solution, and the time-course of muscle activity was recorded. In some experiments, duodenal tissues were allowed to preincubate for additional 20–30 min with different inhibitors before the exposure to estrogens.

2.3. Effect of estrogens on $CaCl_2$-induced contraction

Duodenal preparations were incubated in Ca^{+2}-free solutions for 5 min and then 3 mM $CaCl_2$ was added to the bath. The resulting peak contraction was used as a control value for subsequent effects. Afterwards, the solution was replaced with a Ca^{2+}-free solution and let to stabilise for 5 min. Tissues were then exposed to different concentrations of estrogens for another 5 min. At the end of this period, the peak contraction elicited by a second application of 3 mM $CaCl_2$ was measured. Bath solutions were then replaced, and tissues washed three times with fresh PSS and left for additional 10 min in the Ca^{2+}-free solution.

2.4. Effect of estrogens on KCl-induced contraction

Duodenal tissues were incubated in PSS for 5 min, and the amplitude of peak contraction induced by addition of 33 mM KCl was used as a control value for subsequent effects. Afterwards, bath solution was replaced with fresh PSS three times and then exposed to different concentrations of estrogens for additional 5 min. At the end of this period, the peak amplitude elicited by a second application of 33 mM KCl was measured. Bath solutions were then replaced, washed three times with fresh PSS and left for additional 20 min in PSS until the next assay.

2.5. Western blot analyses

Aortic and duodenal muscles from male mice were dissected in Ringer's solution and homogenized in SDS lysis buffer (0.075 M Tris–HCl, pH 6.8, 2% [w/v] SDS, 10% [v/v] glycerol and 5% [w/v] β-mercaptoethanol). After centrifugation at 13,000×g to remove tissue debris, protein extracts were transferred to clean tubes and boiled at 95 °C for 5 min. An aliquot of each extract was preserved for protein quantification using a commercial DC protein assay kit (Bio-Rad, Hercules, CA). Equal protein amounts (100 μg) of each sample were electrophoresed on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to Hybond-P transfer membranes. Immunodetection on the Western blots was carried out by

first incubating with the different primary antibodies used in this study. The two specific anti-estrogen receptor α (ERα) antibodies were rat monoclonal H226 that recognizes the DNA binding domain of ERα, and H-151 antibody that recognizes the hinge region of this receptor. Both antibodies were diluted 1:50 in Blotto. Mouse monoclonal anti-Maxi-K^+ channel α subunit antibody and goat polyclonal anti-Maxi-K^+ β1 subunit antibody were also diluted 1:50 in Blotto. As a control of protein extraction and transferring, membranes were re-blotted with a mouse monoclonal antibody directed to caveolin-1 (1:500). After incubation with the different primary antibodies for 3 h at room temperature, membranes were washed three times for 10 min in phosphate-buffered saline with 0.05% Tween 20, and incubated for 3 h at room temperature with anti-mouse, anti-rat or anti-goat horseradish peroxidase-linked secondary antibody (Bio-Rad) diluted 1:5000 in Blotto. Membranes were washed (three times, 10 min each), and specific bands obtained were visualized with the Amersham enhanced chemiluminescence kit.

2.6. Statistics and mathematical analyses

One-way analysis of variance (ANOVA) and Student–Newman–Keuls *t*-test were used to determine differences between sample means. Results are expressed as mean± S.E.M. Dose–response curves were fitted to logistic equation using nonlinear regression analysis tools provided in SigmaPlot software (Jandel Scientific, San Rafael, CA). Frequency analysis was assessed using the fast Fourier transform (FFT) algorithm implemented in the acquisition software. Analyses were performed on 512 data segments taken from data windows of 1.70–3.41 min from the steady-state phases of each experimental condition. Linear trends were removed from each data segment. The spectral coefficients of the power spectra were smoothed to reduce

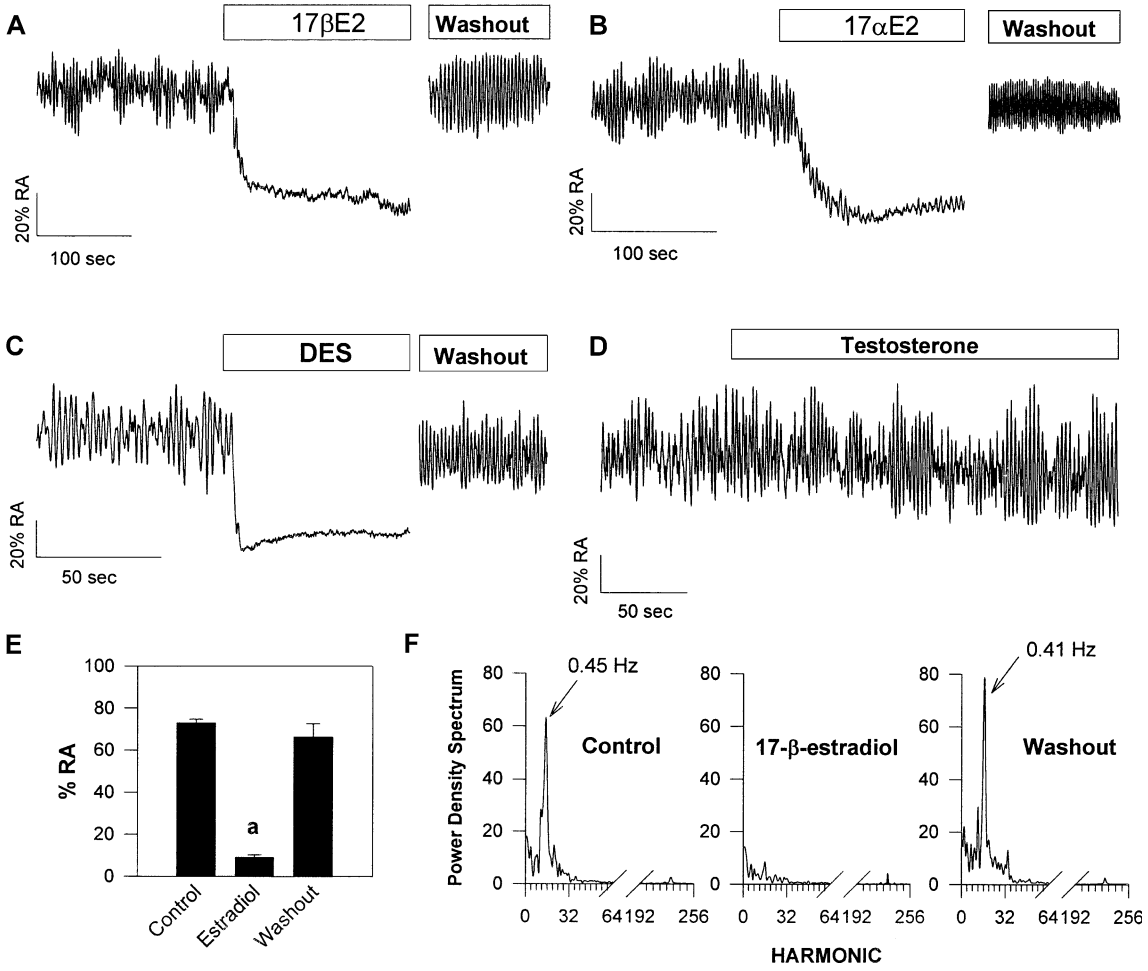

Fig. 1. Estrogens rapidly relax mouse duodenal muscle. (A–D) Effects of 17-β-estradiol (17βE2, 10 μM), 17-α-estradiol (17αE2, 30 μM), diethylstilbestrol (DES, 10 μM) and testosterone (10 μM) on duodenal spontaneous activity. Washout traces were obtained 3–5 min upon replacement of tested molecules from the bathing solution. (E) Comparison of mean isometric tension in response to 17-β-estradiol (10 μM). (F) Fourier analysis of duodenal spontaneous activity determined under control conditions (left panel), in the presence of 17-β-estradiol (middle panel) and after replacement of 17-β-estradiol with normal PSS (right panel). Ordinates represent the power spectral density (PSD) and abscise the harmonic components. Abscises have been scaled to illustrate frequencies associated with contractile activity. Results are representative of, at least, 10 different experiments. [a]$P<0.01$ compared to control value.

their variance and averaged to obtain the ensemble power spectrum (Díaz, 2002).

2.6.1. Drugs

17-β-Estradiol, 17-α-estradiol, diethylstilbestrol, acetylcholine, carbachol, tetraethylammonium chloride, EGTA, bovine serum albumin, 4-aminopyridine, *N*-nitro-L-arginine methyl ester (L-NAME) and 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-pyridine-3-carboxilic acid methyl ester (BAY-K8644) were obtained from Sigma (Biosigma, Spain). The steroidal antiestrogen 7α-[9-[(4,4,5,5,5,-pentafluoropenty) sulphinyl]nonyl]-estra-1,3,5 (19)-triene-3,17β-diol (ICI182,780) was provided by Astra Zeneca Pharmaceuticals (Madrid, Spain). Chelerethryne, PD98059, forskolin, 8-Br-cyclic-GMP and KT5823 were purchased from Calbiochem (Bionova, Spain). Charybdotoxin, apamin, paxilline and verruculogen were purchased from Alomone Labs (Jerusalem, Israel). Mouse monoclonal primary anti-estrogen receptor α (ERα) antibody H-151 was from Stressgen Biotechnologies (Victoria, Canada), and rat monoclonal H226 anti-ERα antibody was a kind gift from Dr. Geoffrey L. Greene (University of Chicago, USA). Monoclonal antibody generated from human Maxi-K^+ channel α subunit was a gift from Dr. Enrico Stefani (UCLA, USA), and goat polyclonal antibody raised against Maxi-K^+ β1 subunit of human origin was purchased from Santa Cruz Biotechnology (Sta. Cruz, CA, USA). Mouse monoclonal antibody directed to caveolin-1 was provided by BD Transduction Laboratories (Madrid, Spain). 17-β-Estradiol, 17-α-estradiol, diethylstilbestrol and ICI182,780 were dissolved in ethanol and stored at 4°C as 1-20 mM stock solutions. Solvent (ethanol or dimethyl sulfoxide, DMSO) concentrations in the bath never exceeded 0.1%.

3. Results

3.1. Effects of estrogens on spontaneous duodenal activity

Under control conditions, mouse duodenal muscle developed a rhythmic spontaneous activity that lasted several hours as long as the extracellular conditions remained optimal. Spontaneous activity was unaffected by addition of vehicles, ethanol or DMSO, at the maximal volume used in this study (30 μl, not shown). Application of 17-β-estradiol (5–30 μM) reduced spontaneous activity and basal tone within 0.5–3 min after exposure to the hormone (Fig. 1A). This relaxant effect was reproduced by the steroisomer 17-α-estradiol (17αE2, Fig. 1B) and also by the synthetic estrogen diethylstilbestrol (DES, Fig. 1C). The inhibition of spontaneous activity induced by estrogens was completely reversed upon washout, and basal tone was restored to control values within few min after estrogen removal (Fig. 1E). Interestingly, the relaxing effects of estrogens were not reproduced by either testosterone (10 μM, Fig. 1D) or progesterone (10 μM, not shown), indicating a certain degree of specificity in the relaxant effect of estrogens. At this stage, we could not detect any gender differences, that is, female tissues were also readily and reversibly relaxed by estrogens. A quantitative assessment of contractile activity by frequency analysis using the FFT algorithm (Díaz, 2002) revealed the presence of discrete frequency components under control conditions that vanished in the presence of estrogens but reappeared without significant variations in the density spectrum following washout. An illustrative example of this type of analyses is shown in Fig. 1F for the experiment depicted in Fig. 1A using 17-β-estradiol (17βE2). As it can be observed, the significant principal frequency component around 0.41 Hz that dominates the activity bandwidth under control conditions becomes considerably reduced during the relaxation induced by estradiol, but finally recurs to nearly the same value upon ~3-min washout.

3.2. Involvement of classical estrogen receptors on the effect of estradiol

The data shown above suggested that the duodenal smooth muscle constitutes a putative target for estrogens. Consequently, we have explored the presence of estrogen receptors in this tissue. Using two antibodies directed to different epitopes of the canonical estrogen receptor α, we demonstrated by immunoblotting on duodenal protein extracts, the presence of a band migrating at 65 kDa corresponding to estrogen receptor α (ERα, Fig. 2A). This band was also

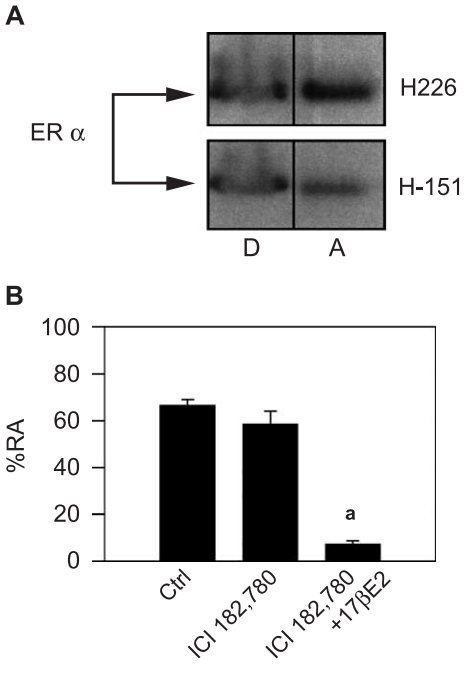

Fig. 2. Involvement of classic estrogen receptors in the response to 17-β-estradiol. (A) Western blots assays with two different anti-estrogen receptor α (ERα) antibodies (H-226 and H-151) on mouse duodenal (D) and aortic (A) extracts. A single 65-kDa protein corresponding to ERα was identified. (B) Effects of pure steroidal antiestrogen ICI182,780 (10 μM) on 17-β-estradiol (17βE2, 10 μM)-induced inhibition of duodenal spontaneous activity. [a]$P<0.01$ compared to control and ICI182,280 values.

observed in mouse aortic muscle with both anti-ERα antibodies (Fig. 2A). These results were noteworthy since they provided the first indication for the existence of classical estrogen-receptors in male mouse small intestine.

Nonetheless, the pure steroidal antiestrogen ICI182,780 (10 μM) was unable, not only to mimic, but also to antagonise the effect of 17-β-estradiol (17βE2, Fig. 2B). Together with the fast time-course of the estrogen-induced relaxing effect as well as its rapid reversibility, these findings ruled out the participation of classical estrogen-receptor-triggered transcriptional activation, and indicated that the spasmolytic effect of estrogens on mouse duodenum was presumably estrogen-receptor-independent.

3.3. Effects of estrogens on duodenal $CaCl_2$-induced contractions

In order to provide a quantitative assessment of these rapid estrogenic effects, we first evaluated the effects of preincubation with estrogens on the $CaCl_2$ (3 mM)-induced contraction. This procedure has been previously reported to provide an accurate and reproducible method to work out the dose–response parameters in mouse duodenum (Díaz, 2002). As stated in Materials and methods, tissues were first incubated under Ca^{2+}-free conditions and the peak contraction in response to a brief pulse of $CaCl_2$ was measured as 100% activity. Afterwards, tissues were returned back to Ca^{2+}-free conditions and incubated in the presence of different concentrations of selected estrogens for 5 min, followed by a second pulse of Ca^{2+} (Fig. 3A). The percentage of activity with regards to the control value was plotted against estrogen concentration (Fig. 3B). The analysis of the dose–response curves showed that 17-β-estradiol and diethylstilbestrol were nearly equipotent reducing Ca^{2+}-induced contraction, with IC50 values of 0.39 and 2.71 μM, respectively. On the other hand, the non-physiological steroisomer 17-α-estradiol (17αE2), which was only capable to affect contractile activity at concentrations above 5 μM, was found to be about two orders of magnitude less potent that 17-β-estradiol (IC50=27.3 μM).

3.4. Effects of carbachol and KCl on estrogen-relaxed tissues

In order to ascertain the possible locus for the relaxant effects of estrogens, we performed experiments aimed at characterizing the response of estrogen-relaxed tissues to agents known to alter the excitation–contraction coupling. We initially explored the response to the muscarinic agonist carbachol, which would lead to Ca^{2+} release from intracellular stores in response to cholinergic-induced IP_3 generation (Sims and Jansen, 1993). Results showed that estrogen-relaxed tissues were capable to respond to carbachol (CCH, 1 μM), with an initial transient contraction of duodenal tissues, often followed by a sequence of oscillations and, eventually, by the restoration of the relaxed state (Fig. 4A–C). This response was identical to that obtained using Ca^{2+}-free solutions in either the presence or the

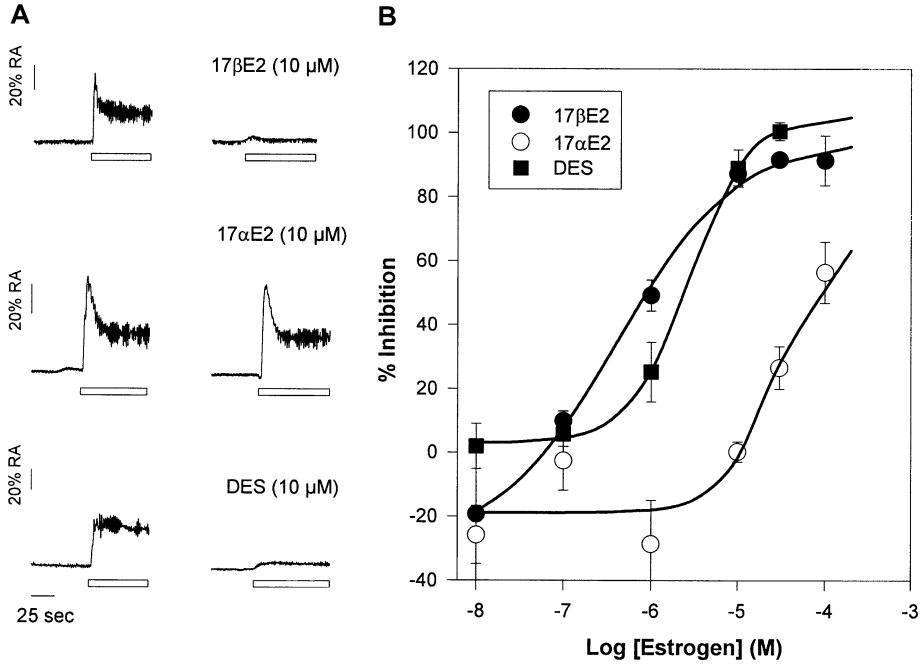

Fig. 3. Estrogens cause a dose-dependent inhibition of $CaCl_2$-induced contraction. (A) Representative traces showing the inhibitory effects of 17-β-estradiol (17βE2, 10 μM), 17-α-estradiol (17αE2, 10 μM) and diethylstilbestrol (DES, 10 μM) on $CaCl_2$-induced contractions in isolated duodenal muscle strips. Control and test recordings were obtained in the same tissue. (B) Cumulative concentration–response curves for 17-β-estradiol (17βE2), 17-α-estradiol (17αE2), diethylstilbestrol (DES). Values are expressed as mean±S.E.M. for percentage of reduction compared to 100% maximal tension in the test pulse (left traces in panel A). At least five different preparations were used for each concentration.

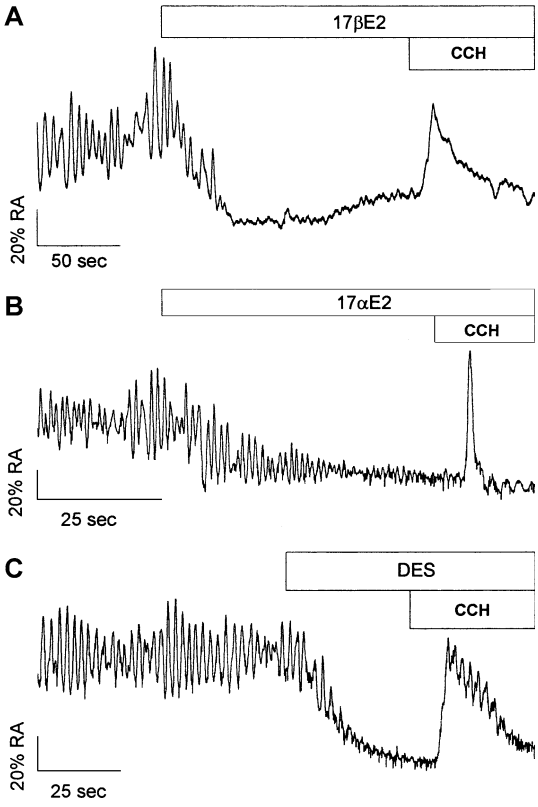

Fig. 4. Effects of carbachol on estrogen-relaxed tissues. (A–C) Representative traces showing the effect of carbachol (CCH, 1 μM) on the relaxant effect of 17-β-estradiol (17βE2, 10 μM), 17-α-estradiol (17αE2, 60 μM) and diethylstilbestrol (DES, 10 μM). Recordings are illustrative of five different duodenal preparations.

absence of estrogens (not shown). These results strongly indicated that the contractile machinery in estrogen-relaxed tissues retained its ability to initiate the contractile cascade upon induction of the Ca^{2+} surge within the smooth muscle cells.

The next set of experiments was conducted to assess the response to changes on membrane potential using KCl to induce an artificial depolarization of duodenal smooth muscle. It is well known that both K^+ channel blockade or depolarization by high extracellular K^+ cause a considerable increase in contraction force generated by gastrointestinal muscles (Meiss, 1987). As can be observed in the results presented in Fig. 5A–C, irrespective of the estrogenic molecule used, estrogen-relaxed tissues responded to KCl (33 mM) with a transient increase of isometric tension. In order to quantitatively assess the depolarization-induced response of duodenal tissues to the different estrogen molecules, we performed dose–response experiments on tissues preincubated with different concentrations (10 nM–30 μM) of estrogens for 5 min before the application of a second pulse of KCl. The results summarized in Fig. 5D show that estrogens provoked a concentration-dependent reduction of KCl-induced peak contractions, thus indicating that estrogens were also able to depress the sensitivity of duodenal tissues to depolarization. Analyses of dose–response curves gave IC50 values of 14.3, 44.5 and 1.05 μM for 17-β-estradiol, 17-α-estradiol and diethylstilbestrol, respectively. Interestingly, at the largest concentrations used here (30 μM), none of the three estrogenic compounds were capable of completely abolishing the response to depolarization (Fig. 5D).

3.5. Effects of protein kinase C and mitogen-activated protein kinase inhibitors on the response to estradiol

A number of studies have demonstrated that some of the rapid effects of estrogens observed in different systems, including differentiated smooth muscle cells, are due to activation of mitogen-activated protein kinases (MAPK) or protein kinase C (PKC) (Condliffe et al., 2001; Doolan and Harvey, 2003; Kelly et al., 1999; Migliaccio et al., 1996; Watters et al., 1997). Activation of either kinase has been associated with the modulation of smooth muscle contractile state, and to affect the contractile responses to agonists and KCl (reviewed in Horowitz et al., 1996). In order to ascertain whether the relaxing effects on duodenal tissues observed here were linked to activation of MAPK or PKC pathways, we have performed experiments using the specific inhibitors PD98059 (20 μM) and chelerethryne (1 μM) at concentrations known to inhibit MAPK and PKC activities, respectively. Duodenal tissues were preincubated for 20 min with the inhibitors and then exposed to 17-β-estradiol. The results shown in Table 1 demonstrate that inhibition of MAPK or PKC did not substantially affect spontaneous activity nor did prevent the 17-β-estradiol-induced relaxation (Table 1). Thus, at this stage we may conclude that none of these conventional pathways seemed to be involved in the relaxation induced by estrogens. Therefore, we decided to explore the participation of other potential intracellular modulators transducing the estrogenic effect on mouse duodenal tissues.

3.6. Effects of intracellular cyclic nucleotide modulators on duodenal contractile activity

Several studies have shown that the intracellular accumulation of cyclic nucleotides, i.e. cyclic-AMP or cyclic-GMP, are responsible for the estrogen receptor-independent estrogen-induced acute effects observed in different biological preparations, including mammalian intestine (Aronica et al., 1994; Doolan et al., 2000; Kelly et al., 1999; Ropero et al., 1999). Therefore, we considered worthwhile to explore the possibility that estrogenic response of duodenal muscle could be initiated by generation of cyclic nucleotides. Initially, as a first approach, we explored the effects of adenylate cyclase activation with forskolin, and the artificial increase of intracellular cyclic GMP on the contractile activity of duodenal tissues. Our data show that stimulation of cAMP generation with forskolin (10–20 μM) or application of the membrane-permeable analogue 8-Br-cyclic-GMP (100–500 μM) rapidly reduced basal tone and

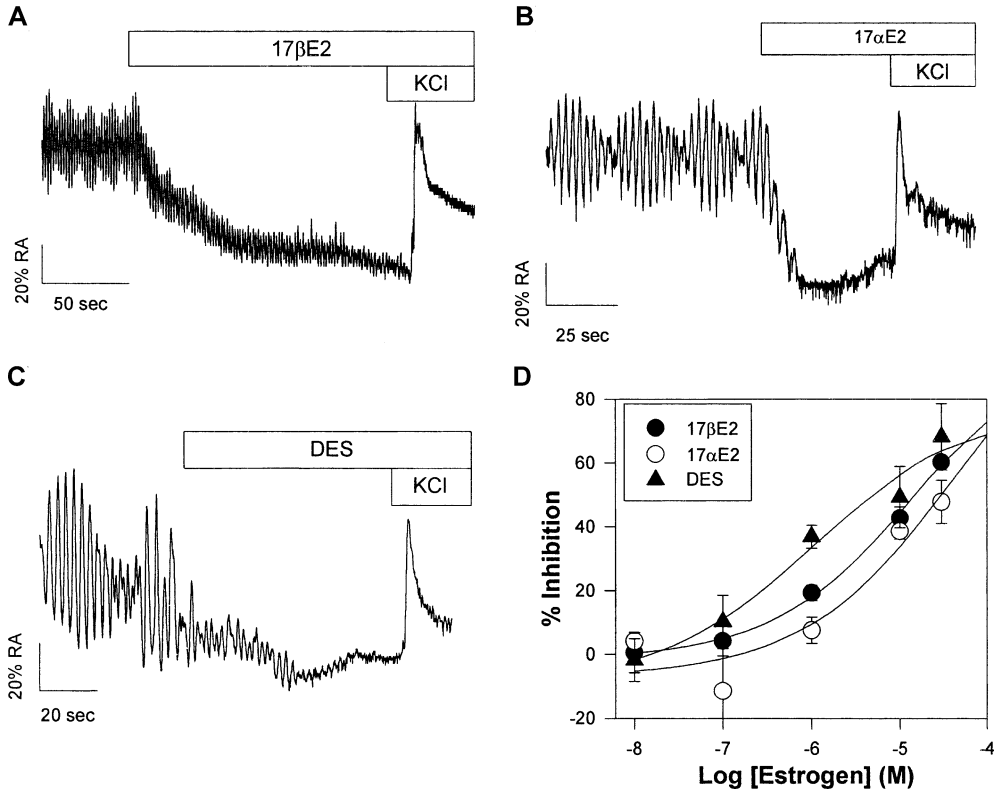

Fig. 5. Effects of KCl-induced depolarization on estrogen-relaxed tissues. (A–C) Original recordings showing the effects of KCl (33 mM)-induced depolarization on estrogen-relaxed duodenal tissues. Estrogen concentrations were 15, 30 and 10 μM for 17-β-estradiol (17βE2), 17-α-estradiol (17αE2) and diethylstilbestrol (DES), respectively. (B) Concentration–response curves for 17-β-estradiol (17βE2), 17-α-estradiol (17αE2), diethylstilbestrol (DES). Values are expressed as mean±S.E.M. for percentage of reduction compared to 100% maximal tension in the control response to KCl. At least five different preparations were used for each concentration.

depressed spontaneous activity (Fig. 6A,B; Table 2A,C). However, only in the case of 8-Br-cyclic-GMP, relaxed tissues could respond to KCl (Fig. 6B, Table 2C) or carbachol (not shown), therefore mimicking the effect of estrogens. In line with these results, blockade of protein kinase A (PKA) with the specific inhibitor KT5720 (0.5 and 5.0 μM) failed to prevent the effect of estrogen (Table 2B).

Table 1
Effect of PKC and MAPK inhibitors on estrogen-relaxed duodenal tissues

(A)	
Control	57.52±4.34
Chelerethryne (1μM)	49.54±2.90
17-β-Estradiol+chelerethryne	7.84±0.90[a]
(B)	
Control	47.56±4.40
PD98059 (20 μM)	41.73±2.90
17-β-Estradiol+PD98059	8.24±1.12[a]

Tissues were preincubated for 20 min with either the general PKC inhibitor chelerethryne (A) or the specific MAPK inhibitor PD98056 (B) before addition of 17-β-estradiol (10 μM).
Results are expressed as mean±S.E.M. of relative isometric tension for three different experiments in each case.
[a] Statistically significant with $P<0.01$ compared to both control condition and preincubation with inhibitors.

These findings were particularly interesting because they suggested that the rapid generation of cyclic GMP induced by estrogens could underlie their relaxing effects on duodenal muscle.

3.7. Effects of cyclic GMP transducers on the duodenal response to estradiol

Estrogen-induced relaxation of vascular smooth muscle has been associated with the generation of nitric oxide (NO) in adjacent endothelial cells and the initiation of a NO-induced process involving activation of guanylyl cyclase (Geary et al., 1998; Murad, 1994; Rosenfeld et al., 2000). The possibility that a similar mechanism being responsible for the relaxant effects of estrogens on intestinal muscle was tested using the general nitric oxide synthase (NOS) inhibitor L-NAME. Our results show that preincubation with L-NAME (300 μM) for 20 min did not prevent the relaxation induced by estrogens (Table 2D, Fig. 6C), which indicated that this effect was not due to activation of nitrergic neurons at the myenteric plexus. Though the results of these experiments rule out the participation of NOS, activation of membrane or soluble guanylate cyclase by estrogens was still conceivable. Thus, we tested for the possibility that protein kinase G (PKG) activation may

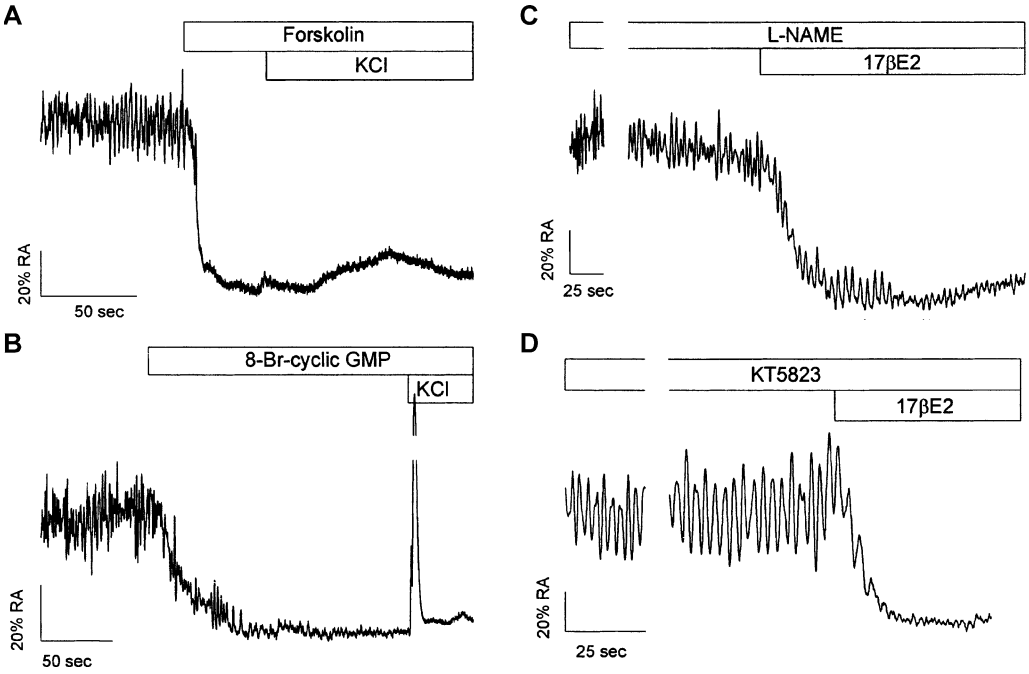

Fig. 6. Effects of different cyclic nucleotide modulators on duodenal contractile activity. Effect of putative effector inhibitors on estrogen-induced relaxation. (A–B) Representative experiments showing the effects of the cyclic AMP-elevating agent forskolin (20 μM) and 8-Br-cyclic GMP (100 μM) on duodenal spontaneous activity. Relaxed tissues were submitted to depolarization with KCl (33 mM) at the time indicated in the horizontal bars. (C) Effects of preincubation with the general NOS inhibitor L-NAME (300 μM) for 20 min before addition of 17-β-estradiol (17βE2). (D) Original recording of similar experiments showing the effect of preincubation with the specific PKG inhibitor KT2823 (333 nM) for 20 min before addition of 17-β-estradiol (17βE2). The concentration of 17-β-estradiol used was 10 μM in both cases. Traces are representative of another three experiments using different concentrations of inhibitors. Illustrated recordings were obtained from different animals.

constitute the final step in the response to estrogens using the specific inhibitor KT5823. We observed that preincubation with KT5823 for 20 min (Table 2E, Fig. 6D), at a concentration (333 nM or 1.0 μM) known to prevent PKG-induced activation of K^+ channels and inhibition of Ca^{2+} channels (White et al., 1995; Ziolo et al., 2003), did not affect the rapid induction of estrogen effects (Table 2E, Fig. 6D). From these experiments, it was concluded that despite the similarities between the responses to estradiol and 8-Br-cyclic GMP, the molecular processes underlying both effects differ from the mechanistic point of view.

3.8. Effects of K^+ channel blockers and Ca^{2+} channel activators on estrogen-relaxed tissues

In view of the lack of experimental evidence indicating the involvement of intracellular signalling pathways accounting for the relaxant effect of estrogens on the mouse duodenum, we have assessed other possible alternative mechanisms. Several studies have shown that estrogens may alter smooth muscle cell excitability by directly modulating ion channel activity, including K^+ and Ca^{2+} channels (Kelly et al., 2002; Martínez et al., 2001; Nakajima et al., 1995; Ruehlmann et al., 1998; Valverde et al., 1999; Zhang et al., 1994). Among the best characterized channels modulated by estrogens, large-conductance Ca^{2+}-sensitive K^+ channels (BK), small-conductance Ca^{2+} activated K^+ channels (SK) and ATP-dependent K^+ channels (K_{ATP}) have been involved in the relaxant effect of estrogens in different types of smooth muscle. Clearly, activation of K^+ channels would lead to the hyperpolarization of smooth muscle cells and, consequently, would move the resting membrane potential to values below the activation threshold for Ca^{2+} channels. Therefore, we have used a pharmacological approach to assess the possible involvement of K^+ channels in the relaxation induced by estrogens. Given the number of reports demonstrating the special sensitivity of Maxi- K^+ channels to estrogens (Dick et al., 2001; Valverde et al., 1999), we thought that these Ca^{2+} and voltage-dependent channels were the most attractive candidates to underlie the effects observed here. The rationale for this hypothesis is that they are engaged with the Ca^{2+}-dependent repolarization phase during the rhythmic electrical changes in the intestinal smooth muscle cells. Initially, we explored the effect of different Maxi-K^+ channel blockers on the relaxation induced by 17-β-estradiol. Surprisingly, we found that neither charybdotoxin (33–100 nM) nor verrucologen (100–500 nM) or paxilline (100–500 nM) elicited any macroscopic effect on the isometric tension recorded from estrogen-relaxed tissues (Table 3, Fig. 7A–C). In line with these results, we also tested for the presence of Maxi-K^+ α and β1 subunits in mouse smooth muscles by Western blotting using specific antibodies directed to either Maxi-K^+ α or Maxi-K^+ β1 subunits. Our data show that, both mouse

Table 2
Effects of cyclic nucleotide modulators and inhibitors on duodenal contractile activity

(A)	
Control	76.53±6.9
FSK (20 μM)	17.28±7.9[a]
FSK+KCl (4)	20.90±6.0[a]
(B)	
Control	42.93±4.8
KT5720 (0.5 μM)	49.66±4.5
17-β-Estradiol+KT5720 (3)	6.52±7.9[b]
Control	52.63±14.0
KT5720 (5.0 μM)	41.13±3.2
17-β-Estradiol+KT5720 (3)	3.05±4.1[b]
(C)	
Control	54.36±7.1
8-Br-cGMP (100 μM)	9.64±2.0[a]
8-Br-cGMP+KCl (3)	70.22±14.1[b]
(D)	
Control	73.07±9.7
L-NAME (300 μM)	75.97±7.1
17-β-Estradiol+L-NAME (3)	15.24±3.6[b]
(E)	
Control	59.02±5.2
KT5823 (333 nM)	61.88±4.7
17-β-Estradiol+KT5823 (3)	17.01±7.6[b]
Control	66.39±4.2
KT5823 (1 μM)	57.99±8.7
17-β-Estradiol+KT5823 (3)	8.85±8.6[b]

Results are expressed as mean±S.E.M. of relative isometric tension for the number of experiments indicated in parentheses.
FSK- and 8-Br-cGMP-relaxed tissues were submitted to depolarization with KCl (33 mM).
Inhibitors KT5720 (0.5 and 5.0 μM), L-NAME (300 μM) and KT5823 (333 nM and 1.0 μM) were preincubated for 20 min before addition of 17-β-estradiol (10 μM).
[a] Statistically different from control condition with $P<0.01$.
[b] $P<0.01$ compared to modulators or inhibitors alone.

duodenal and aortic muscles express detectable amounts of Maxi-K^+ α, whereas Maxi-K^+ β1 subunit was only detected in mouse aortic tissues (Fig. 7D). These findings were striking since Maxi-K^+ β1 subunit has been demonstrated to be responsible for the sensitivity of different types of smooth muscles to estrogens, but are in good agreement with the lack of effect of charybdotoxin, paxilline and verruculogen on the estrogen-induced relaxation demonstrated herein.

Likewise, we found that application of glibenclamide (GLB, 10–100 μM) or apamin (1 μM) failed to affect the relaxed state of duodenal tissues (Table 3, Fig. 7E,F), providing evidence that activation of neither K_{ATP} nor small-conductance Ca^{2+}-sensitive K^+ channels underlie the relaxing effect of 17-β-estradiol. On the contrary, application of barium (2 mM) or tetraethylammonium (TEA, 2–5 mM) rapidly restored contractile activity and increased basal tone (Table 3, Fig. 7G,H). Furthermore, application of the Kv channel inhibitor 4-aminopyridine (0.5–3 mM) induced a concentration-dependent transient increase of isometric tension in 17-β-estradiol-relaxed tissues which was followed by a partial recovery of spontaneous activity, featured by rhythmic contractions of smaller amplitude when compared to control recordings obtained before application of estrogens (Table 3, Fig. 7I).

The analysis of the responses of 17-α-estradiol- and diethylstilbestrol-relaxed tissues to K^+ channel blockers (Fig. 8A) revealed a similar pharmacological profile to that demonstrated for 17-β-estradiol, suggesting that a common mechanism to activate K^+ channel and to relax duodenal muscle is shared by all estrogenic molecules studied here. Also, these data led us to define a pharmacological profile for the putative channel which is consistent with Kv channels (Cook, 1990).

The fact that tetraethylammonium and 4-aminopyridine only partially restored basal tone, and that estrogens induced a dose-dependent reduction of $CaCl_2$-induced and KCl-induced contractions of duodenal tissues, suggested that, in synergy with K^+ channel activation, a reduction of Ca^{2+} influx might also be involved in the relaxing effect of estrogens. Changes on Ca^{2+} channel activities have been reported for other smooth muscle cell paradigms in response to estrogens (Jiang et al., 1991; Nakajima et al., 1995; Ruehlmann et al., 1998; Salom et al., 2002; Zhang et al., 1994). Moreover, using this same preparation, we have previously observed that the non-genomic inhibitory action of tamoxifen was due to a Ca^{2+} influx antagonism, and that the relaxing effect could be totally reversed by exposure of duodenal muscle to the L-type Ca^{2+} channel agonist BAY K8644 (Díaz, 2002). Likewise, application of BAY K8644 (1 μM) to estrogen-relaxed tissues partially restored spontaneous activity and increased basal tone in the continuous presence of estrogens, suggesting that estrogens have also inhibited Ca^{2+} channels (Table 3, Fig. 8B). The reversing effect of BAY K8644 was completely blocked by verapamil (100 μM, not shown). Interestingly, we could observe that subsequent addition of tetraethylammonium or 4-aminopyridine to BAY K8644-treated tissues brought about a further increase on basal tone and restored spontaneous activity to control levels (Figs. 9 and 10).

Frequency analyses using Fast Fourier Transforms of contractile activity in controls and after addition of 17-β-estradiol (or diethylstilbestrol), BAY K8644 and tetraethylammonium, revealed that the main frequency (harmonic) component detected under control conditions vanished in the presence of estradiol, but reappeared following addition of BAY K8644 and tetraethylammonium (TEA, Figs. 9C and 10C). Thus, the frequency spectrum obtained under control conditions revealed that spontaneous activity was consistent with one to two main harmonic components corresponding to frequencies between 0.30 and 0.50 Hz. Addition of either 17-β-estradiol or diethylstilbestrol altered the power density spectrum and abolished all significant

Table 3
Effects of K^+ channel inhibitors and Ca^{2+} channel modulators on estrogen-relaxed duodenal tissues

Verruculogen (VCG)		Paxilline (PAX)		Charybdotoxin (ChTx)	
Control	73.85±12.01	Control	48.32±15.69	Control	77.72±19.23
17-β-Estradiol	20.89±4.31[a]	17-β-Estradiol	8.31±2.56[b]	17-β-Estradiol	31.52±11.63[b]
+VCG (100 nM)	16.29±8.21	+PAX (100 nM)	10.11±4.56	+ChTx (33 nM)	26.32±11.65
Control	82.00±6.39	Control	65.07±11.69	Control	81.93±18.70
17-β-Estradiol	32.25±11.98[b]	17-β-Estradiol	14.29±6.13[a]	17-β-Estradiol	43.09±24.15
+VCG (500 nM)	15.26±3.25	+PAX (500 nM)	11.55±3.44	+ChTx (100 nM)	21.75±15.57
Glibenclamide (GLB)		**Tetraethylammonium (TEA)**		**4-Aminipyridine (4-AP)**	
Control	66.12±8.12	Control	58.21±8.66	Control	75.55±16.53
17-β-Estradiol	12.36±6.68[a]	17-β-Estradiol	12.63±2.58[a]	17-β-Estradiol	20.56±8.35[b]
+GLB (10 μM)	15.23±6.32	+TEA (2 mM)	25.36±2.36[c]	+4AP (0.5 mM)	32.36±6.35
Control	81.98±18.70	Control	70.05±19.10	Control	60.72±14.75
17-β-Estradiol	29.87±13.60[b]	17-β-Estradiol	17.93±6.60[b]	17-β-Estradiol	10.18±5.22[a]
+GLB (100 μM)	14.86±9.05	+TEA (5 mM)	43.19±13.54[d]	+4AP (3 mM)	34.82±8.23[c]
Apamin		**Barium**		**BAY K8644**	
Control	80.90±19.11	Control	65.00±13.52	Control	81.00±14.21
17-β-Estradiol	26.69±4.37[b]	17-β-Estradiol	18.90±7.78[b]	17-β-Estradiol	13.28±5.47[a]
+Apamin (1 μM)	19.96±7.15	+Barium (2 mM)	35.68±7.01[d]	+BAY K8644 (1 μM)	36.42±7.64[c]

Results are expressed as mean±S.E.M. of relative isometric tension for, at least, three different experiments.
Potassium channel inhibitors were added immediately after the steady-state of 17-β-estradiol-induced relaxation (10–30 μM).
[a] Statistically different from control condition with $P<0.01$.
[b] Statistically different from control condition with $P<0.05$.
[c] Different from 17-β-estradiol with $P<0.05$.
[d] Different from 17-β-estradiol with $P<0.1$.

frequencies (Figs. 9C and 10C). Surprisingly, sequential addition of BAY K8644 and tetraethylammonium gave raise to the generation of significant frequency components which clearly fell in the bandwidth of control periods. Interestingly, the power density spectrum in the presence of BAY K8644 alone (i.e., obtained before the addition of tetraethylammonium or 4-aminopyridine) showed frequency components (0.6–0.75 Hz), clearly faster than those observed in either the combined presence of BAY K8644 and tetraethylammonium or in control periods (Figs. 9C and 10C). The existence of faster components in response to the L-type Ca^{2+} channel agonist may be interpreted in terms of a faster repolarization due to activation of K^+ channels in response to estrogens. Similar results were obtained using 4-aminopyridine instead of tetraethylammonium (data not shown).

4. Discussion

The data in the present work show that the gonadal hormone 17-β-estradiol, the physiologically inactive steroisomer 17-α-estradiol and the synthetic estrogen diethylstilbestrol, all inhibit spontaneous activity, Ca^{2+}-induced and depolarization-induced contractions, and reduce basal tone in mouse duodenal muscle. 17-β-Estradiol and diethylstilbestrol were nearly equipotent reducing Ca^{2+}-induced contraction with IC50 values of 0.39 and 2.71 μM, respectively. In contrast, the non-physiological steroisomer 17-α-estradiol was found to be nearly two orders of magnitude less potent than 17-β-estradiol and incompletely inhibited Ca^{2+}-induced contractions at concentrations as high as 100 μM. The inhibitory effect on duodenal activity was rapidly achieved ($t_{0.5}<1$ min), and was specific for estrogens since it was not mimicked by other steroidal hormones, i.e. progesterone or testosterone. In all cases, the relaxing effects of estrogenic molecules assayed here were readily reversible upon washout. To our knowledge, although acute effects of estrogens have been reported for different types of smooth muscle studied in vitro, including vascular (Farhat et al., 1996; Mugge et al., 1993; Shaul, 1999; Shaw et al., 2000), uterine (Fernández et al., 1993; Kostrzewska et al., 1993) and urinary (Ratz et al., 1999), the present results provide the first indication that intestinal muscle activity may be acutely modulated by estrogens. Interestingly, the concentration range for the effects reported here fell within the range of values published for the relaxant effect of estrogens on both, endothelium-denuded vascular tissues and A7r5 smooth muscle cells (Mugge et al., 1993; Nakajima et al., 1995; Ruehlmann et al., 1998; Salom et al., 2002; Zhang et al., 1994).

We have also found that the steroidal antiestrogen ICI182,780, which has been long recognised as a pure antiestrogen interacting with estrogen receptors, failed to prevent the effect of 17-β-estradiol. This is in agreement with the observations that vascular responsiveness to estrogens in endothelium-denuded aortic rings and coronary and pressurized mesenteric arteries was ICI182,780 insensitive

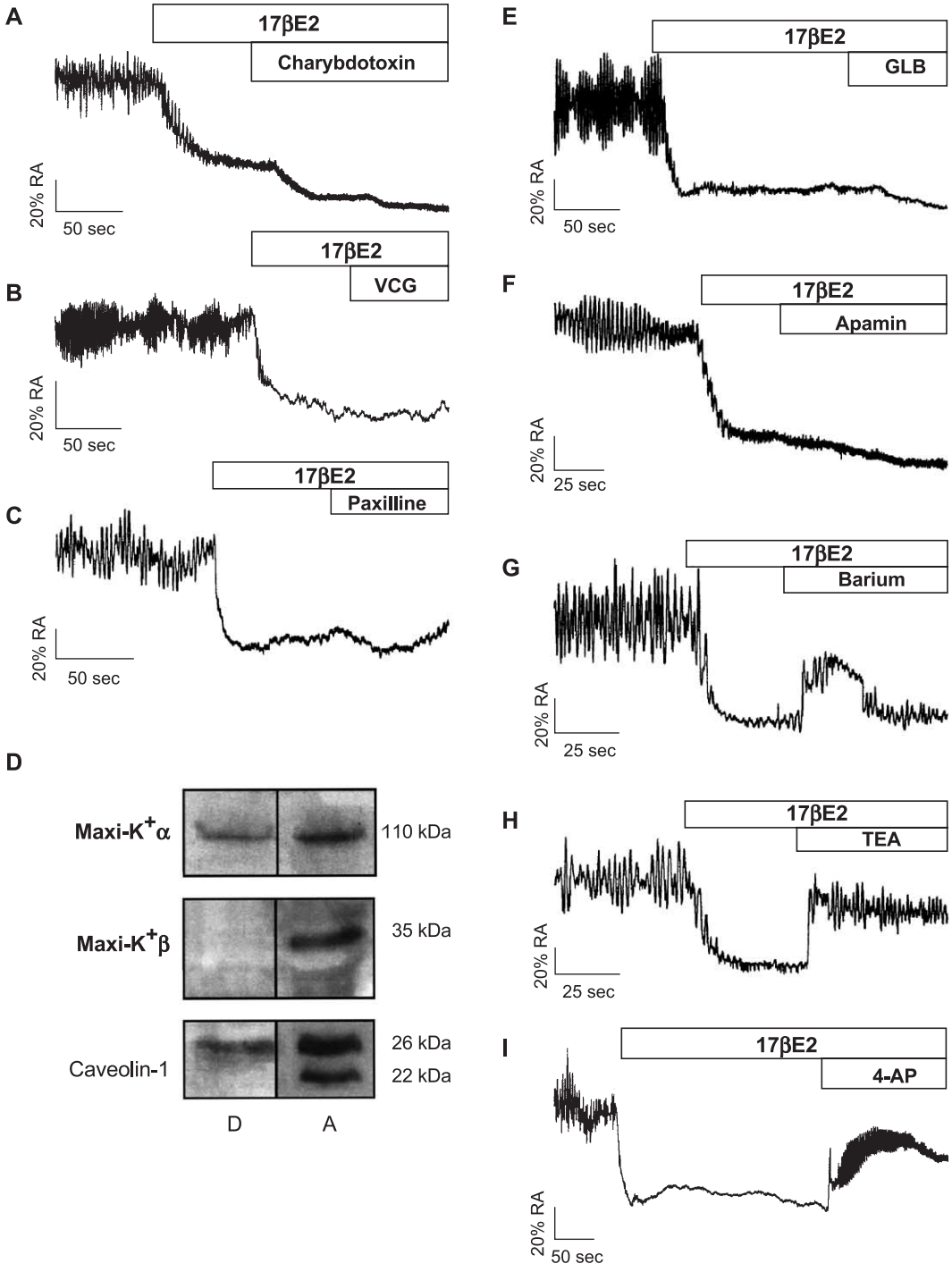

Fig. 7. Effects of different K^+ channel blockers on estradiol-relaxed duodenal tissues. (A–C) Representative traces showing the effects of charybdotoxin (100 nM), verruculogen (VCG, 100 nM) and paxilline (500 nM) on the relaxation induced by 17-β-estradiol (17βE2, 10 μM). Traces are representative of, at least, three different preparations. (D) Western blot analyses for Maxi-K^+ α and β1 subunits from mouse duodenal (D) and aortic (A) smooth muscle extracts obtained from the same animal. Caveolin-1 was used as experimental control. Unlike Maxi-K^+ α, Maxi-K^+ β1 subunit was only detected in aortic extracts. Assays were repeated three times for each type of tissue. (E–I) Recordings of isometric tension showing the effects of glibenclamide (GLB, 100 μM), apamin (1 μM), barium (2 mM), tetraethylammonium (TEA, 5 mM) and 4-aminopyridine (4-AP, 3 mM) on the relaxation induced by 17-β-estradiol (17βE2, 10–30 μM). Traces are representative of at least three different preparations.

(Shaw et al., 2000; Teoh et al., 2000). Therefore, although we have demonstrated for the first time in the male mouse duodenal muscle extracts the expression of estrogen receptor α protein, the involvement of classic genomic pathways for the estrogen effects reported here can be ruled out. Thus, the rapid onset of estrogen-induced relaxation, its

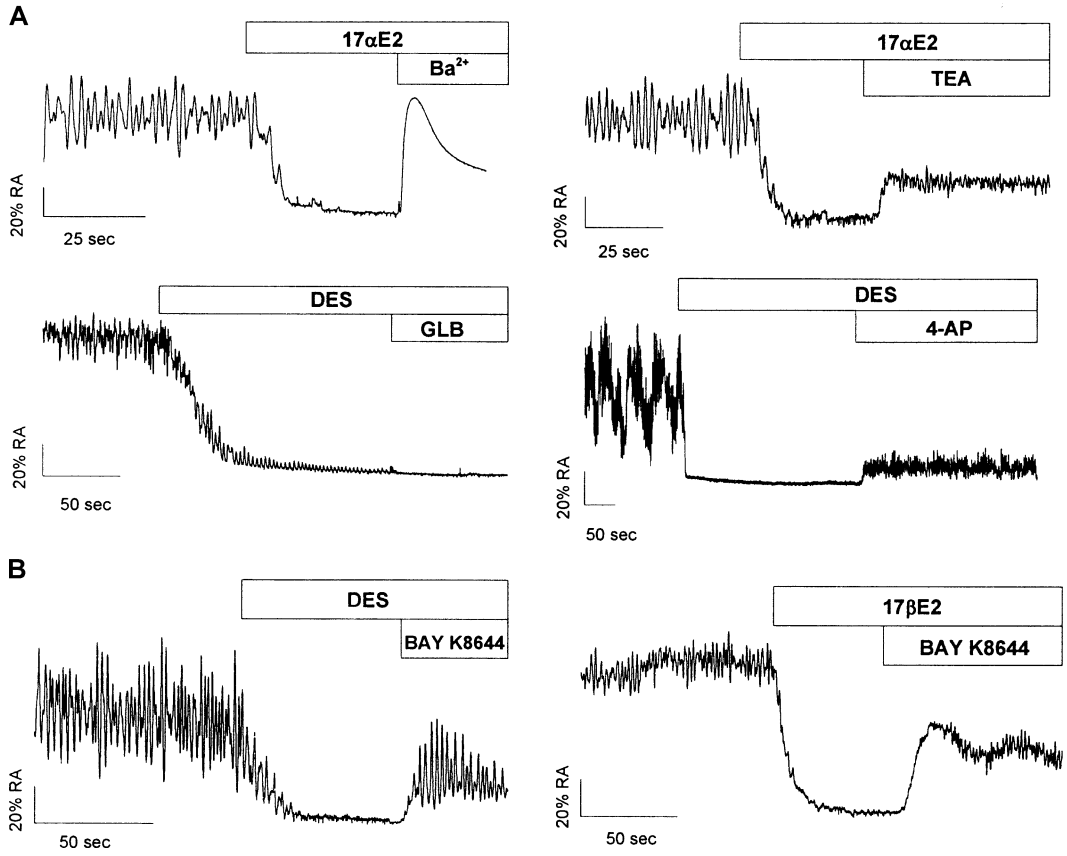

Fig. 8. (A) Effects of different K^+ channel blockers on the relaxant effect of 17-α-estradiol (17αE2) and diethylstilbestrol (DES). Traces show the effects of Ba^{2+} (2 mM) and tetraethylammonium (TEA, 5 mM) on 17-α-estradiol-relaxed duodenal tissues, and the effects of glibenclamide (GLB, 100 μM) and 4-aminopyridine (4-AP, 1 mM) on diethylstilbestrol-relaxed duodenal tissues. Traces are representative of at least five different preparations. The concentrations of 17-α-estradiol (17αE2) and diethylstilbestrol (DES) used in these experiments were 60 M and 10 μM, respectively. (B) Recordings of isometric tension showing the effects of the L-type Ca^{2+} channel agonist BAY K8644 (1 μM) on the relaxation induced by diethylstilbestrol (DES, 10 μM) and 17-β-estradiol (17βE2, 10 μM). Traces are representative of at least three different preparations.

rapid reversibility and the absence of effects of ICI182,780 suggest that a non-classical alternative mechanism of action might account for the estrogen-induced relaxation of duodenal tissues (Falkenstein et al., 2000; Kelly and Levin, 2001; Nadal et al., 2001). However, at this stage, it is not possible to ascertain whether a plasma membrane ICI182,780-insensitive estrogen receptor-related binding site could participate in the rapid response triggered by estrogens in the duodenal tissues, as it has been shown for the acute modulation of acethylcholine-induced Ca^{2+} transients in GT1-7 cells by estradiol (Morales et al., 2003), or for the rapid insulinotropic effect of estrogens in pancreatic β cells (Nadal et al., 1998).

In an attempt to further characterize the mechanism(s) underlying these relaxant effects, we have explored the possible molecular intracellular targets for estrogens. In the gastrointestinal muscle syncytium, the rhythmic electrical activity underling synchronous muscle contraction is supported by slow waves in membrane potential consisting on depolarization–repolarization phases with superimposed action potentials (Szurszewski, 1987). These depolarization–repolarization phases reflect a balance between the activity of voltage-sensitive Ca^{2+} channels (VSCC) and Ca^{2+}-activated K^+ channels (Lee et al., 1999). Once cytosolic Ca^{2+} ions are available, activation of the Ca^{2+}-calmodulin complex triggers a Ca^{2+}-calmodulin-dependent phosphorylation of the regulatory myosin light chain (MLC_{20}) by myosin light chain kinase (MLCK), leading to the initiation of the contractile process (Horowitz et al., 1996). In the isolated rabbit femoral artery, 17-β-estradiol has been shown to reduce force and MLC_{20} phosphorylation by means of a non-genomic mechanism (Kitazawa et al., 1997). The possibility that a similar mechanism could be responsible for the estradiol action on duodenal muscle was assessed here by dissecting the excitation and contraction processes using the muscarinic agonist carbachol. This procedure would lead to Ca^{2+} release from intracellular stores irrespective of rhythmic changes of membrane potential and extracellular Ca^{2+} influx. Results showed that estrogen-relaxed tissues were capable of responding to carbachol with a transient contraction that was indistinguishable from that obtained in the absence of extracellular Ca^{2+}. On the other hand, we have observed in parallel experiments (not shown) that inhibition of duodenal tissues with wortmannin (50 μM), a powerful MLCK inhibitor, failed to respond to KCl (33 mM), tetraethylammonium (2

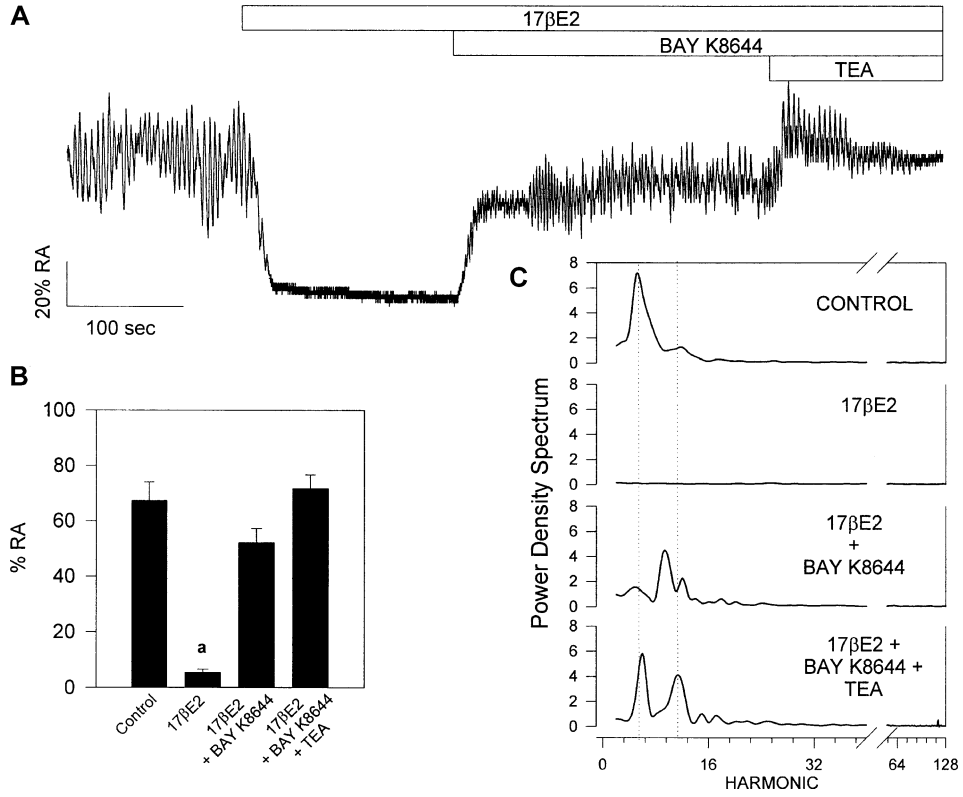

Fig. 9. The relaxing effect of 17-β-estradiol can be reversed by blocking K^+ channels and activating L-type Ca^{2+} channels. (A) Effects of sequential addition of BAY K8644 (1 μM) and tetraethylammonium (TEA, 2 mM) on the contractile activity of 17-β-estradiol-relaxed tissues (17βE2). The concentration of 17-β-estradiol used in this experiment was 15 μM and the experiment was repeated in four different tissues. (B) Measurement of basal tone from different conditions as illustrated in (A). Ordinates represent percentage of 100% control value and are expressed as mean±S.E.M. $^aP<0.001$ vs. Control. (C) Fourier analysis of duodenal spontaneous activity computed on 1.70 min segments from the different conditions shown in A. All computations were performed on the steady-state phases of each experimental condition, except for the case of tetraethylammonium where analysis was performed at the onset of the effect. Ordinates represent the power spectral density and abscise the harmonic components. Abscises have been scaled to illustrate frequencies (harmonic components) associated with contractile activity.

mM) or BAY K8644 (1 μM). These observations were crucial since they ruled out the possibility that the action of estrogen was due to the inactivation or inhibition of contractile machinery in the smooth muscle, i.e. reduction of MLC_{20} phosphorylation. Consequently the site(s) for the inhibitory effect had to be located upstream the contractile process, likely on the excitation phase.

The hypothesis that the locus for the relaxant effect of estrogens was located within the depolarization–repolarization phases of slow waves in duodenal smooth muscle cells was demonstrated by assessing the response to depolarization with KCl. We observed that depolarization of estrogen-relaxed tissues caused a transient contraction followed by a reversible inactivation of contractile activity, suggesting that estrogens relax duodenal muscle by hyperpolarizing smooth muscle cells through alteration of K^+ channel conductances. It is well established that K^+ channels play an important role in regulating the excitability of gastrointestinal syncytium by shaping the electrical activity of smooth muscle, so that opening of K^+ channels is associated with restoration of the resting potential and inhibition of contractile activity (see Vogalis, 2001 for an excellent review). Therefore, positive modulation of K^+ channels activity by estrogens would elicit an outward current that, eventually, would displace the resting membrane potential towards more negative values resulting in a hyperpolarization of smooth muscle cells. The large conductance BK type Ca^{2+}-activated K^+ channel (Maxi-K^+) has been shown to play a key role leading to hyperpolarization of the smooth muscle cell membrane, which causes deactivation of voltage-dependent Ca^{2+} channels and relaxation (Patterson et al., 2002). Recently, estrogen has been shown to bind to the β1 subunit of the BK channel in vitro, and to activate Maxi-K^+ channels when coexpressed with the pore-forming α subunit (Valverde et al., 1999). Moreover, clinically relevant concentrations of estradiol (0.1 to 10 μM), as those reported here, have been shown to increase the open probability and Ca^{2+} sensitivity of BK channel in canine colonic myocytes, perhaps by acting upon β1 subunit (Dick et al., 2001). These studies suggest that estradiol can activate BK channels via the accessory β1 subunit. Because β1 subunit is especially prevalent in smooth muscle cells from different origins, including gastrointestinal tract (Knaus et al., 1994; Toro et al., 1998), we explored the hypothesis of whether this molecular entity could underlie estrogen-induced inhibition of contractile activity using dual approach based on its well-

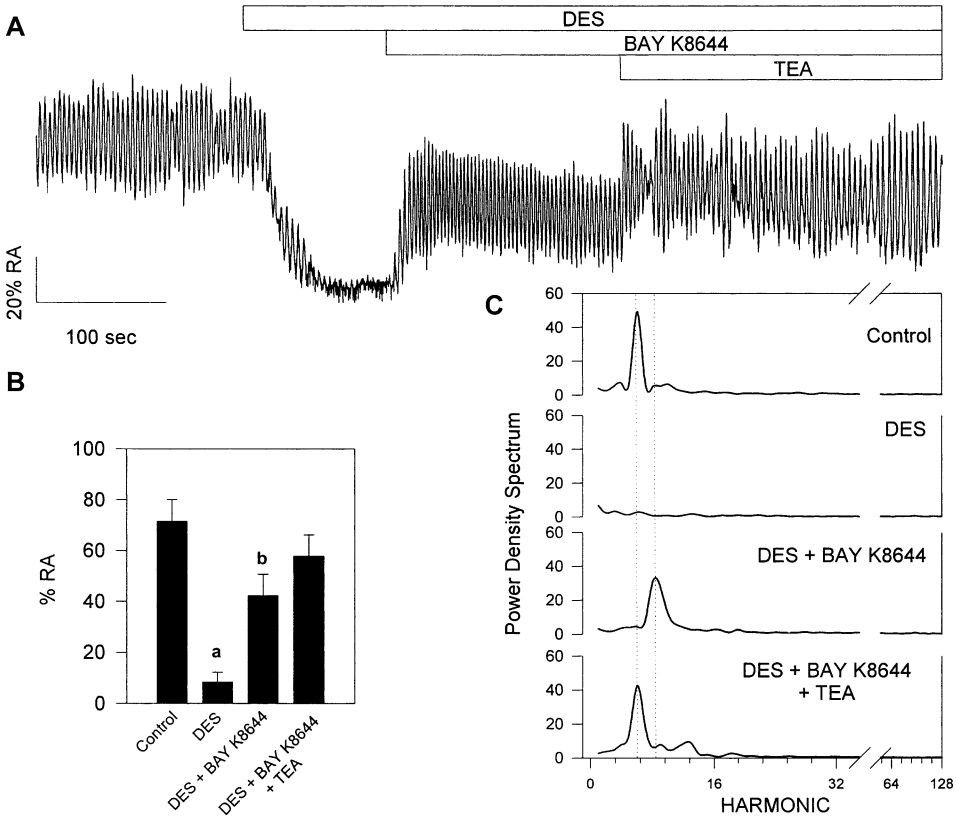

Fig. 10. The relaxing effect of diethylstilbestrol can be reversed by blocking K^+ channels and activating L-type Ca^{2+} channels. (A) Effects of sequential addition of BAY K8644 (1 μM) and tetraethylammonium (TEA, 2 mM) on the contractile activity of diethylstilbestrol-relaxed tissues. The concentration of diethylstilbestrol used in this experiment was 10 μM and the experiment was repeated in four different tissues. (B) Measurement of basal tone from different conditions as illustrated in (A). Ordinates represent percentage of 100% control value and are expressed as mean ± S.E.M. $^{a,b}P<0.001$ and $P<0.05$ vs. Control, respectively. (C) Fourier analysis of duodenal spontaneous activity computed. Details as in Fig. 9 legend.

known pharmacology and Western blotting. Strikingly, neither charybdotoxin nor verruculogen or paxilline, all specific inhibitors of Maxi-K^+ channels in different systems could minimally restore contractile activity. This absence of effect was further corroborated by Western blot analyses using a monoclonal antibody directed against the β1 subunit of Maxi-K^+. The results showed that, whereas the protein was satisfactorily immunodetected in mouse aorta, the protein levels were negligible in duodenum. Altogether, these data ruled out the participation of the Maxi-K^+ β1 subunit in the response to estrogen observed here.

Recently, Martínez et al. (2001) have demonstrated that the acute relaxation of rat aorta smooth muscle by diethylstilbestrol was mediated by K_{ATP} channels. Furthermore, K_{ATP} channel agonists have been demonstrated to cause both hyperpolarization and relaxation of mouse ileum (Sun and Benishin, 1994). On the other hand, activation of apamin-sensitive Ca^{2+}-activated K^+ (SK) channels by 17-β-estradiol has been associated with the rapid hyperpolarization of GnRH neurons, and to mediate the uncoupling of γ-amino-butyric-acid (GABA) and μ-opioid receptors to the G-protein-associated K^+-channel in gonadotropin releasing hormone (GnRH) and hypothalamic neurons (Kelly et al., 2002). Interestingly, it has been recently shown that activation of apamin-sensitive Ca^{2+}-dependent K^+ channels underlies the relaxing effect of low-frequency field stimulation of mouse isolated stomach (Mulè and Serio, 2003), and that purinergic stimulation of mouse ileum (Vogalis and Goyal, 1997) and colon (Koh et al., 1997) triggers the opening of apamin-sensitive SK channels. Therefore, given the fact that SK and K_{ATP} channels are expressed in mouse gastrointestinal tissues, we thought worthwhile to assess their possible involvement in the inhibitory response to estrogens. However, the lack of effects of glibenclamide and apamin in the duodenum precluded the possibility that activation of either of these channels may underlie the effect of estrogens in duodenal muscle. On the contrary, we have found that barium, tetraethylammonium and 4-aminopyridine were capable to, partially and rapidly, reverse the relaxation induced by all estrogen molecules used here. In addition, we have observed that, in most cases, application of low concentrations of tetraethylammonium and 4-aminopyridine effectively restored a rhythmic activity pattern in relaxed tissues (see Table 3 and Figs. 7 and 10). These findings strongly argue in favour of a primary action on the activation of a K^+ conductance, leading to the hyperpolarization of duodenal smooth muscle. Furthermore, the pharmacological profile of estrogen-relaxed tissues to the different inhibitors

points to the participation of voltage-dependent K^+ channels (Kv). tetraethylammonium- and 4-aminopyridine-sensitive Kv channels are expressed in all gastrointestinal smooth muscles studied so far, and appear to be minimally activated at the resting membrane potential. Hence, these channels make a finite contribution to the overall resting K^+ conductance, thereby playing a role in setting the resting potential (Vogalis, 2001). Interestingly, 4-aminopyridine-sensitive channels appear to be subjected to regulation by different modulators, including intracellular messengers and G proteins (Koh et al., 1996). Moreover, a recent report by Horinouchi et al. (2003) has demonstrated a primary role for 4-aminopyridine Kv channels in β3-adrenoceptor-mediated cAMP-independent relaxation of gastric fundus and duodenal smooth muscles. Therefore, in view of the data discussed up to now, it is tempting to conclude that activation of Kv channels sensitive to tetraethylammonium and 4-aminopyridine underlies the relaxing effect of estrogens on mouse duodenal tissues.

Our results also show that estrogen-induced inhibition of duodenal contractile activity could be partially reversed by application of the L-type Ca^{2+} channel agonist BAY K8644. In fact, application of the dihydropyridine derivative effectively restored spontaneous peristaltic activity and increased basal tone in estrogen-relaxed tissues. These findings strongly point to a dose-dependent negative modulation of L-type Ca^{2+} channels by estrogens. This is in agreement with several reports unequivocally demonstrating a direct inhibition of high threshold L-type Ca^{2+} channels in vascular smooth muscle by an endothelium- (and nitric oxide)-independent estrogen-induced mechanism (Jiang et al., 1991; Nakajima et al., 1995; Ruehlmann et al., 1998; Zhang et al., 1994). This effect leads to a diminished Ca^{2+} influx and, eventually, to a reduction in contractile activity (Jiang et al., 1991; Salom et al., 2002). In A7r5 aortic smooth muscle cells, 17-β-estradiol reduces the maximal conductance of L-type Ca^{2+} channels in a voltage-independent way, an effect that could be mimicked by diethylstilbestrol (Nakajima et al., 1995). Interestingly, this study showed that the potency of the inhibitory effect was 17-β-estradiol>diethylstilbestrol, and took place in the same range of concentrations reported in this work. Moreover, in the duodenal smooth muscle, such an inhibition of L-type Ca^{2+} channels would account for both, the reduction of basal tone and the response to depolarization, but also for the disappearance of spontaneous activity since these proteins provide the major source for Ca^{2+} entry from the extracellular space to support gastrointestinal rhythmic activity.

Although the precise site of estrogen action cannot be ascertained from the present data, several pieces of evidence suggest that the cellular targets are smooth muscle cells rather than Cajal's intersticial pacemaker cells. First, the fact that BAY K8644 partially restored basal tone and spontaneous activity shortly after exposure to the agonist, and that this effect was blocked by verapamil suggest the involvement of L-type Ca^{2+} channels being targeted by estrogens. It has been shown that, unlike inward Ca^{2+} currents in small intestine smooth muscle cells, the depolarization phase of Cajal's intersticial cells is insensitive to L-type Ca^{2+} channel blockers, and abolished by hyperpolarization (Lee et al., 1999). Second, the analysis of the power density spectrum in the presence of both BAY K8644 and tetraethylammonium (or 4-aminopyridine) showed that frequency components featuring rhythmic activity were very similar to those observed in the control phase of the same experiment. Third, the fact that the power density spectrum obtained in the presence of BAY K8644 exhibited a rightward shift to faster frequencies indicates a higher rate of repolarization, which must be attributable to the activation of an additional K^+ conductance (presumably the tetraethylammonium- and 4-aminopyridine-sensitive Kv channels discussed above). These findings can only be explained if the mechanisms responsible for generating the pace within the Cajal's intersticial cells remained unaltered by the presence of estrogens, but rather estrogens would modulate smooth muscle cell excitability. We could hypothesized a cellular scenario where estrogens, by activating a Kv channel conductance, would lead to a subtle hyperpolarization of the smooth muscle cell enough to keep voltage-dependent Ca^{2+} channels (also negatively modulated by estrogens) far from their activation threshold. The concomitant reduction of rhythmic Ca^{2+} influx, together with the reduction of membrane potential, would tend to keep BK channels in their closed state. When voltage-dependent Ca^{2+} channels are forced to open by the dihidropyridine agonist, then the dual effect of Ca^{2+} influx on ion concentration and membrane potential, would trigger the activation of high conductance K^+-selective BK channels. This would elicit an outward K^+ efflux that, in synergy with Kv channels, would initiate a rapid hyperpolarization of the smooth muscle cell, causing the fast rhythmic oscillation of contractile activity in the presence of the BAY K8644. Nevertheless, further electrophysiological experiments on isolated duodenal smooth muscle cells and Cajal's intersticial cells will be required to corroborate these hypotheses.

A number of studies in smooth muscle cells and other systems have demonstrated that some rapid effect of estrogens is due to the rapid generation of second messenger signalling pathways, including cyclic-AMP and cyclic-GMP (Aronica et al., 1994; Mugge et al., 1993; Ropero et al., 1999), modulation of different protein kinase activities, such as mitogen-associated protein kinases, ERK1 and ERK2 (Migliaccio et al., 1996; Watters et al., 1997), protein kinase C (Condliffe et al., 2001; Doolan and Harvey, 2003; Kelly et al., 1999), activation of nitric oxide formation (Geary et al., 1998; Rosenfeld et al., 2000), or stimulation of phospholipase C activity (Picotto et al., 1999; Sylvia et al., 2000). In addition, it has been well established that, in smooth muscle cells, both Kv channels and L-type Ca^{2+} channels are rapidly modulated by most of the aforementioned signalling molecules and transducers (reviewed in Horowitz et al., 1996; Vogalis, 2001). Therefore, we have explored the

possibility that estrogenic response in this tissue could have been triggered by any of these signal transduction systems, upon activation of a putative estrogen binding site. The results presented here using the MAPK inhibitor PD98059, the general PKC inhibitor chelerethryne and the general NOS synthase inhibitor L-NAME, demonstrated that pre-incubation with any of these compounds failed to prevent the acute relaxation induced by estrogens. On the contrary, stimulation of cyclic AMP generation with forskolin, or activation of PKG using the membrane-permeable analogue 8-Br-cyclic GMP, induced a rapid relaxation of duodenal tissues, thereby mimicking the effect of estrogens. However, none of these transducers seemed to underlie the action of 17-β-estradiol. On one side, forskolin-relaxed tissues failed to respond to depolarization and application of the PKA inhibitor KT5720, failed to prevent 17-β-estradiol effects, which rule out the involvement of cyclic AMP. On the other hand, in the case of cyclic GMP, notwithstanding relaxed tissues respond to KCl or tetraethylammonium, preincubation with the PKG inhibitor KT5823 did not preclude the rapid relaxation triggered by estrogens. It should be pointed out that cyclic GMP may itself modulate ion channel activity (including voltage-dependent K^+ channels) or Ca^{2+} sensitivity by PKG-independent mechanisms (Shimoda et al., 2002; Bonnevier et al., 2004). However, inhibition of K^+ channels by PKG-independent mechanisms (Shimoda et al., 2002) would induce an increase in isometric tension. Similarly, cGMP-induced PKG-independent desensitation (and inhibition) of contractile system (Bonnevier et al., 2004) in the ileum from PKG-deficient mice were only observed at 10- to 100-fold higher cGMP concentrations than those observed here (Bonnevier et al., 2004). Obviously, these arguments and observations cannot be put forward to explain our findings. Altogether, these results ruled out the active participation of conventional phosphorylation pathways, and pointed to either, a direct interaction of estrogens with the molecular entities providing the ionic channels, as it has been demonstrated for Maxi-K^+ channels (Valverde et al., 1999), or alternatively, by means of a short loop regulation mechanism involving interaction with heterotrimeric G proteins, as is the case of SK channels in GnRH neurons (Kelly et al., 1999), or estradiol-mediated potentation of kainate currents in hippocampal neurons (Gu and Moss, 1998). Additional experiments using appropriate pharmacological tools to explore G protein metabolism, together with electrophysiological recordings of Ca^{2+} and K^+ conductances, will aid to unravel the precise molecular mechanism responsible for the estrogens-induced relaxation in duodenal muscle.

In summary, the present study provides for the first time evidence for an acute effect of estrogens on gastrointestinal tissues at the organ level. The rapid macroscopic inhibition of contractile activity is consistent with a synergistic activation of a 4-aminopyridine- and tetraethylammonium-sensitive K^+ channel conductance and the inhibition of L-type Ca^{2+} channels at the plasma membrane of smooth muscle cells.

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