Non-genomic mechanism of 17β-oestradiol-induced inhibition of contraction in mammalian vascular smooth muscle

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1. 17β-Oestradiol (E2) at 0·1–10 μM directly inhibited various tonic and phasic smooth muscle contractions. The mechanism(s) of oestrogen-induced inhibition of contraction was studied using intact and permeabilized strips and isolated single cells of smooth muscle.

2. In endothelium-denuded vascular smooth muscle, E2 attenuated high K^+-induced force development and myosin light chain phosphorylation, and produced rapid and reversible relaxation. There were no significant differences in these inhibitory effects between tissue types (femoral artery *vs.* portal vein), species (rat *vs.* rabbit) or sexes.

3. The inhibitory potencies of several steroidal and non-steroidal oestrogen analogues were examined and their effects were for the most part stereo-specific. However, two steroids with negligible affinities for the nuclear oestrogen receptor also strongly inhibited high K^+-induced contraction.

4. Genomic modulators including a protein synthesis inhibitor, an RNA synthesis inhibitor, and oestrogen receptor antagonists did not affect the inhibitory actions of E2. Inhibitors of cyclic nucleotide-dependent protein kinases did not reduce the E2 effect.

5. Ca^{2+} release from intracellular stores by agonists and by inositol 1,4,5-trisphosphate (IP_3) does not appear to be modulated by E2. Neither pretreatment with ryanodine nor with thapsigargin affected the E2-induced inhibition of high K^+-induced contraction.

6. E2 had no effect on either normal or GTPγS-increased Ca^{2+} sensitivity of the regulatory and contractile apparatus.

7. E2 and its analogues rapidly inhibited voltage-dependent L-type Ca^{2+} channel currents in isolated smooth muscle cells. Repetitive stimulation was not required for E2-induced inhibition of the currents.

8. This study strongly suggests that at pharmacological concentrations oestrogen primarily reduces Ca^{2+} influx through inhibition of L-type Ca^{2+} channels in a non-genomic manner and decreases myosin light chain phosphorylation and contraction of smooth muscle.

Although coronary heart disease (CHD) is the leading cause of death in either sex, pre-menopausal women have a lower incidence of cardiovascular disease than men and post-menopausal women. After menopause, oestrogen deficiency appears to increase the risk of CHD (Barrett-Conner & Bush, 1991) while oestrogen supplementation decreases the risk, suggesting a protective effect of oestrogen on the development of CHD (for references see Farhat, Lavigne & Ramwell, 1996). Epidemiological studies indicate that the reduction in risk of CHD by oestrogen can be attributed, albeit only partly (25–50%), to its beneficial effects on the metabolism of lipids and lipoproteins (Barrett-Conner & Bush, 1991). However, mechanistically, little is known about other non-lipid effects of oestrogen.

Several reports indicate that administration of 17β-oestradiol (E2) attenuates the pressor response to several vasoconstrictors (Magness, Parker & Rosenfeld, 1993). *In vitro*, pharmacological concentrations of E2 and its analogues relax various smooth muscle types, including artery, vein, uterus and small intestine (Dawson & Robson, 1939; Batra & Bengtsson, 1978; Jiang, Sarrel, Poole-Wilson & Collins, 1992). These inhibitory effects are independent of the endothelium (Jiang, Sarrel, Lindsay, Poole-Wilson & Collins, 1991). During high K^+-treatment of smooth muscle, E2 shifted the relationship between Ca^{2+} and contraction to the right and also reduced the influx of $^{45}Ca^{2+}$ (Batra & Bengtsson, 1978; Jiang *et al.* 1991), suggesting the hormone possesses Ca^{2+}-antagonistic properties. In fact, E2 inhibits

voltage-dependent Ca^{2+} inward currents in cultured vascular smooth muscle cells (Shan, Resnick, Liu, Wu, Barbagallo & Pang, 1994; Zhang, Ram, Standley & Sowers, 1994; Nakajima, Kitazawa, Hamada, Hazama, Omata & Kurachi, 1995). Other interesting reports state that E2 increases cyclic nucleotide content, thus allowing for the possibility of a cyclic nucleotide-dependent mechanism in the relaxation response (Kuehl, Ham, Zanetti, Sanford, Nicol & Goldberg, 1974; Mugge, Riedel, Barton, Kuhn & Lichtlen, 1993).

In the present study, we first tested the hypothesis that the relaxing effect of E2 in smooth muscle is non-genomic and distinct from gene transcription through the classical nuclear oestrogen receptor. We then tested the idea that the inhibition of contraction is due to inhibition of voltage-dependent L-type Ca^{2+} channels. We also explored the potential effects on the contractile machinery and Ca^{2+} release from the intracellular stores. Furthermore, we report an E2-induced decrease in myosin light chain (MLC) phosphorylation associated with the relaxation, that is not mediated by cyclic nucleotide-dependent protein kinases. Preliminary results of some of these findings have been published in abstract form (Kitazawa, Nakajima, Hamada & Kurachi, 1994).

METHODS

Intact strips and measurement of isometric force

All animal procedures were approved by the Animal Care and Use Committee of Georgetown University. The rats and rabbits were killed by inhalation of CO_2 and halothane, respectively. The femoral, thoracic aorta, mesenteric and uterine arteries, portal vein and ileum were isolated from 250–300 g Sprague–Dawley male and female rats, and the femoral artery and portal vein from 2–3 kg New Zealand White male rabbits. Small strips of circular muscle from arteries and longitudinal smooth muscle from the portal vein and ileum were carefully dissected and freed of connective tissue with a small razor blade. The endothelium of the artery was removed by gentle rubbing with the razor blade. The size of the smooth muscle strips depended upon animal species, tissue type and the experimental purpose; they ranged from 30 to 80 μm in thickness, 200–700 μm in width and 1·5–3·0 mm in length. The largest strips (70–80 μm in thickness, 500–700 μm in width and 3 mm in length) were used when we needed to perform biochemical analysis of MLC phosphorylation (Kitazawa, Gaylinn, Denney & Somlyo, 1991*a*). To measure isometric force, strips were tied with monofilament silk to the fine tips of two tungsten needles, one of which was connected to a force transducer (AM801; SensoNor, Horten, Norway), and mounted in a well on a bubble plate to allow for rapid solution exchange by sliding the plate to an adjacent well, and for rapid freezing within a second for measurement of MLC phosphorylation.

The normal external solutions were neutralized with Tris to give a pH of 7·4 at 25 °C and contained (mM): NaCl, 150; KCl, 4; calcium methanesulphonate (Ca-Ms), 2; Mg-Ms, 2; glucose, 5·6; and Hepes, 5. To prevent the cells from swelling in depolarizing solutions where $[K^+]$ was high, these solutions were made by replacing NaCl in the normal external solution with an equimolar concentration of K-Ms, thereby maintaining the product of $[K^+]$ and $[Cl^-]$ constant. In Ca^{2+}-free external solutions, Ca-Ms was omitted and replaced with 2 mM EGTA.

E2 and most of its analogues were dissolved in 100% ethanol and added to the medium with a final ethanol concentration at 0·1%. Since this concentration of ethanol had a significant potentiating effect itself on high K^+-induced contraction in tonic smooth muscle, the control solutions without E2 or its analogues also contained 0·1% ethanol.

Permeabilization with *Staphylococcus aureus* α-toxin

The normal relaxing solutions for permeabilized preparations were neutralized to pH 7·1 with KOH at 20 °C and contained (mM): K-Ms, 74·1; Mg^{2+}, 2; MgATP, 4·5; EGTA, 1; creatine phosphate, 10; and piperazine-*N*,*N*′-bis(2-ethanesulphonic acid) (Pipes), 30. In the activating solutions, 10 mM EGTA was used, and specific amounts of Ca-Ms were added to yield a desired concentration of free Ca^{2+} ions. Free Ca^{2+} and Mg^{2+} concentrations were calculated on the basis of stability constant values for complexes in solutions, as described by Horiuti (1988). Creatine phosphate (10 mM) was used to maintain a constant intracellular ATP concentration since the permeabilized cells intrinsically had the creatine phosphokinase enzyme. An ionic strength of 0·2 M was achieved in activating and modified relaxing solutions by adjusting the concentration of K-Ms.

Smooth muscle strips were permeabilized for direct measurements of Ca^{2+} release from the sarcoplasmic reticulum (SR) and Ca^{2+} regulation of the contractile apparatus (Kitazawa *et al.* 1991*a*). Before permeabilizing the cells, we first measured contractions induced by high K^+ (154 or 64 mM) and by agonists (phenylephrine in vascular smooth muscle and carbachol in intestinal smooth muscle) at 30 °C to examine the integrity of the preparations. Then, the strips were incubated in relaxing solution for several minutes. For permeabilization with α-toxin, the strips from rabbit and rat were then treated for 30 min at 30 °C with, respectively, 5000 and 10000 units ml^{-1} of purified *Staphylococcus aureus* α-toxin (Gibco BRL) at pCa 6·7 buffered with 10 mM EGTA. This condition facilitated the high temperature dependence of permeabilization and simultaneously enabled us to examine the efficiency of the permeabilization. When the SR was depleted of Ca^{2+} and the cytoplasmic Ca^{2+} was held constant, strips were further treated with 10 μM of the Ca^{2+} ionophore A23187 for 20–25 min in the relaxing solution (Kitazawa *et al.* 1991*a*). Since the sensitivity of α-toxin was severalfold lower in rat vascular smooth muscle than in rabbit, we mainly utilized rabbit tissues for experiments using permeabilized strips.

Determination of myosin light chain phosphorylation

For determining the effect of oestrogen on MLC phosphorylation, rabbit femoral artery strips were pretreated with or without 5 μM E2 for 10 min in normal external solution, then rapidly frozen in liquid N_2-cooled liquid chlorodifluoromethane either directly after the pretreatment or 20 s after the addition of 64 mM high K^+ solution with or without E2. The MLC phosphorylation was measured as described previously (Kitazawa *et al.* 1991*a*). After incubation in acetone containing 10% trichloroacetic acid at −80 °C overnight, the frozen strips were gradually warmed up, washed first with fresh acetone and then with ether before being allowed to dry. The dried strips were thoroughly homogenized in glycerol sample buffer (containing 1% SDS, 10% glycerol, 20 mM dithiothreitol and 0·1 mg ml^{-1} bovine serum albumin (BSA)). First dimensional electrophoresis of an isoelectric focusing polyacrylamide-tube gel containing 5% pH ampholytes 4·5–5·4 (Pharmacia; Uppsala, Sweden) and 9·2 M urea ran overnight, until a steady current was obtained, to separate the proteins in the supernatant by charge. The second dimensional SDS polyacrylamide gel electrophoresis then separated the proteins by molecular weight; the appropriate portion of the first dimensional gel laid horizontally on the top of

the SDS stacking gel before it was run. Electrophoretic transfer was carried out from SDS polyacrylamide gels to nitrocellulose membranes for 5 h in 25 mM Tris, 194 mM glycine and 20% methanol. The membranes were washed with a phosphate buffer solution containing 0·3% Tween 20 overnight and then stained finally with colloidal gold (Bio-Rad; Hercules, CA, USA).

The colloidal gold-stained patterns of MLC were digitized with a colour scanner (Macintosh, Cupertino, CA, USA) and analysed with image processing software (Signal Analytics Co., Vienna, VA, USA) that permitted the subtraction of background obtained from regions adjacent to the focused proteins. The percentage of MLC phosphorylation was calculated by dividing the density of mono- and di-phosphorylated spots by the combined density of un-, mono-, and di-phosphorylated spots.

Isolation of smooth muscle myocytes

The longitudinal muscle layer was peeled from the underlying circular muscle layer of rabbit ileum and cut into small pieces in a low Ca^{2+} dissecting solution (pH 7·35) composed of (mM): NaCl, 136; KCl, 6; $CaCl_2$, 0·05; $MgCl_2$, 1; glucose, 11; and Hepes, 10. The pieces were placed into cell dispersion medium (the composition was essentially the same as the above low Ca^{2+} dissecting solution except for the addition of 2 mg ml^{-1} papain and 3 mg ml^{-1} BSA), and incubated for 20–30 min at 37 °C. Subsequent gentle pipetting and washing by centrifugation (1500 r.p.m. for 2 min) dispersed the cells which were then resuspended and kept at 5 °C in a high K^+, 0 Ca^{2+} solution (pH 7·35) that contained (mM): KCl, 85; K_2HPO_4, 30; Na_2ATP, 2; $MgCl_2$, 1; sodium pyruvate, 5; creatine, 5; taurine, 15; EGTA, 1; glucose, 10; and fatty acid-free BSA (1 mg ml^{-1}).

Viable cells from this suspension attached themselves to the bottom of small chambers, which rested on a microscope stage, and a 32–34 °C bath solution was perfused at 5 ml min^{-1}.

Whole-cell patch-clamp recording

Whole-cell membrane current was recorded using standard patch-clamp techniques (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The current amplifier used was an Axopatch-1C equipped with a CV-4 head stage. The current signals were filtered at 1 kHz, and digitized, stored and analysed using an IBM-AT personal computer with pCLAMP software (Axon Instruments). Patch pipettes had a resistance of 3–5 MΩ when filled with intracellular solutions. All currents measured were corrected for linear leakage resistance.

The bath perfusion solution (pH 7·4 with NaOH) for measurement of voltage-dependent Ca^{2+} current contained (mM): NaCl, 136; CsCl, 6; $BaCl_2$, 5; glucose, 11; and Hepes, 10. The pipette (intracellular) solution (pH 7·2 with CsOH) for measuring the Ca^{2+} current contained (mM): CsCl, 130; $MgCl_2$, 2; EGTA, 10; Na_2ATP, 3; Na_3GTP, 0·1; and Hepes, 10.

Statistics

All values are expressed as means ± S.E.M. Student's unpaired two-tailed t test was used for statistical analysis of the data and P values less than 0·05 were considered to be significant.

Materials

17β-Oestradiol and its analogues, testosterone, progesterone, cholesterol, corticosterone, tamoxifen, sodium nitroprusside, isoprenaline, quercetin, cycloheximide, phenylephrine, nifedipine and caffeine were purchased from Sigma. A23187, ryanodine, thapsigargin and IP_3 were from Calbiochem. GTPγS was from Boehringer Mannheim (Indianapolis, IN, USA). Rp-cAMPS and Rp-8Br-cGMPS were from BioLog (La Jolla, CA, USA). ICI 164,384 was a gift from Zeneca Pharmaceuticals (Wilmington, DA, USA).

RESULTS

E2 reversibly and rapidly inhibited smooth muscle contraction

E2 (5 μM) reversibly inhibited the development of high K^+- or excitatory agonist (10 μM phenylephrine)-induced contractions in endothelium-denuded rat femoral artery (Fig. 1*A* and *B*) and portal vein (not shown) smooth muscle. Addition of E2 also rapidly reduced the sustained component of contraction evoked by high K^+ or phenylephrine (PE) in rat portal vein (Fig. 1*C* and *D*) and femoral artery (not shown). We also found that the inhibitory effect of E2 was not restricted to rat femoral artery and portal vein; confirming previous results by different groups, similar inhibitory effects were observed in various types of smooth muscle in different animal species: rat thoracic aorta, mesenteric and uterine artery, ileum and uterus, rabbit portal vein, ileum and femoral artery, and porcine coronary artery (data not shown).

To determine how rapidly E2 affects contraction, we used thin strips (30–40 μm in thickness, 0·4 mm in width and 1·5 mm in length) of longitudinal smooth muscle from phasic rat portal vein that respond more rapidly to stimuli than arterial smooth muscle. During sustained contraction induced by high K^+ (64 mM) and PE (10 μM), addition of 5 μM E2 evoked a rapid relaxation as shown in Fig. 1*C* and *D*, respectively; the average half-time of relaxation in the high K^+ solution was 20 ± 2·6 s (Fig. 2*A*), slightly slower than that obtained by decreasing extracellular Ca^{2+} from 2 to 0·3 mM (10 ± 1·5 s). Pretreatment (half-time of 22 ± 1·8 s) of the portal vein strips with a normal Na^+ external solution containing E2 decreased the peak of high K^+-induced contraction (Fig. 2*B*). This time course of inhibition at rest was similar to that when E2 was applied during the membrane depolarization.

E2 depressed the maximum amplitude of contraction induced by various concentrations of K^+ with little shift in the K^+ concentration–contraction relationship to the right (Fig. 3).

Inhibitory effects on myosin light chain phosphorylation

High K^+-induced membrane depolarization of rabbit femoral artery smooth muscle significantly increased MLC phosphorylation from resting values (16 ± 5·0%) to 44 ± 1·9% ($n = 8$) of total MLC at 20 s after application of 64 mM K^+. Simultaneously measured force levels were also increased from 0 to 94 ± 1·2% ($n = 14$) of peak level of control contraction induced by the same concentration of K^+. Treatment of the strips with 5 μM E2 for 10 min before and during high K^+-induced contraction significantly inhibited high K^+-induced increases in both MLC phosphorylation (by 69 ± 2·6%; $n = 8$) and development of contraction (by 71 ± 3·7%; $n = 14$). The resting MLC phosphorylation values were not significantly affected by E2; 16 ± 5·0% ($n = 5$) for control *vs.* 16 ± 1·5% ($n = 7$) in the presence of E2.

Comparison of inhibitory effects of various steroidal and non-steroidal oestrogens on high K^+-induced contraction

E2 and its analogues dose dependently inhibited 64 mM K^+-induced contraction in rat femoral artery (Fig. 4*A*). The half-maximal inhibitory concentrations (IC_{50}) of E2, 2-methoxyoestradiol, 17-ethynyl β-oestradiol and diethylstilboestrol are, respectively, 2·2, 2·3, 1·4 and 0·35 μM. No inhibition was observed by β-oestradiol 17β-cypionate even at 30 μM. In rat portal vein, the IC_{50} of E2 is 3·6 ± 0·4 μM ($n = 4$), not significantly ($P > 0·05$) different from that of the femoral artery. Increasing solvent (ethanol) concentration from 0·1 to 0·5% did not modify the IC_{50} value for E2 (2·6 μM at 0·5% ethanol). Replacement of ethanol with DMSO did not affect the E2 effect. Serum albumin, having an affinity for the free form of steroidal hormones (Rosner,

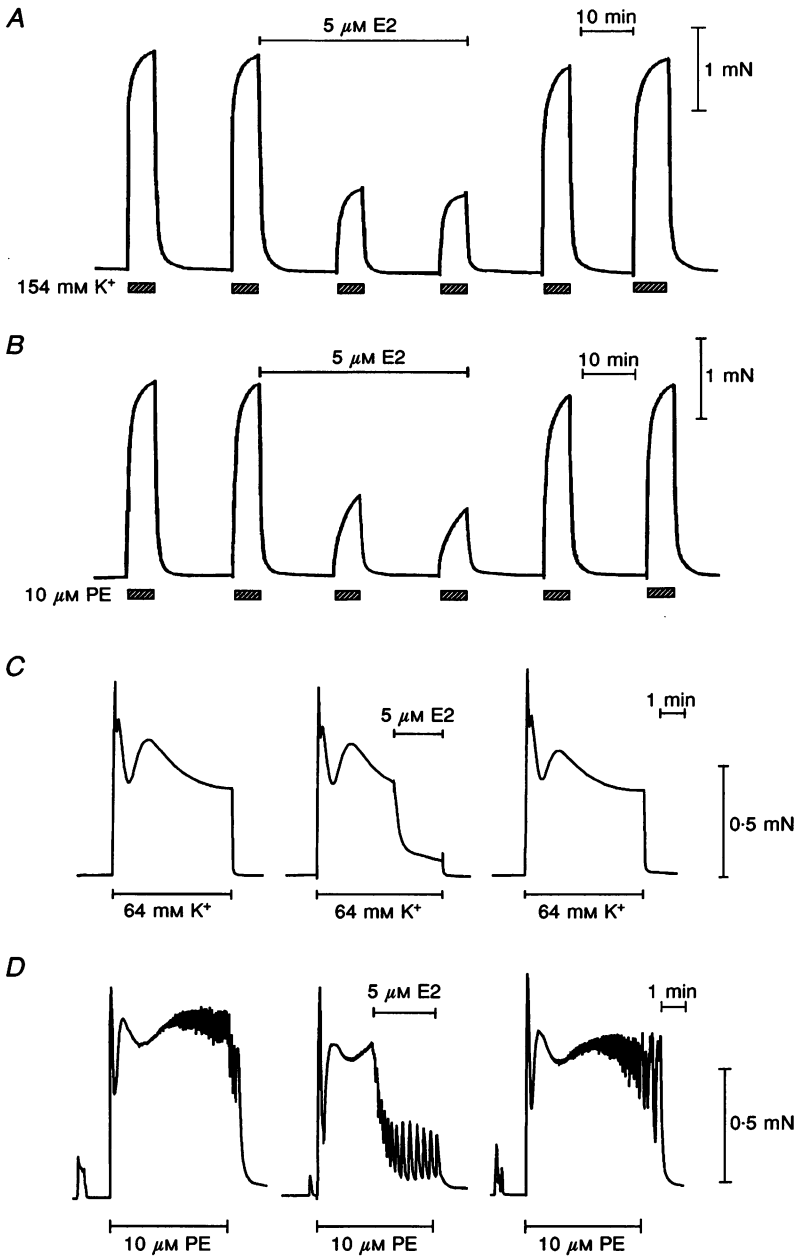


Figure 1. Effectiveness of E2 on contractile force in rat femoral artery (*A* and *B*) and portal vein smooth muscle (*C* and *D*)

A constant peak of contraction was obtained at 30 °C by repetitive 5 min stimulation with high K^+ (154 mM in *A* and 64 mM in *C*) or 10 μM phenylephrine (PE in *B* and *D*). E2 (5 μM) was added 15 min before and during contraction (*A* and *B*) or only during contraction (*C* and *D*) as indicated in the figure. Typical time courses of E2-induced relaxation can be seen in the high K^+-induced (*C*) and PE-induced contractions (*D*). The inhibitory effect was easily reversed by removal of E2. All solutions contained 0·1% ethanol. These traces are representative of 5–12 similar experiments.

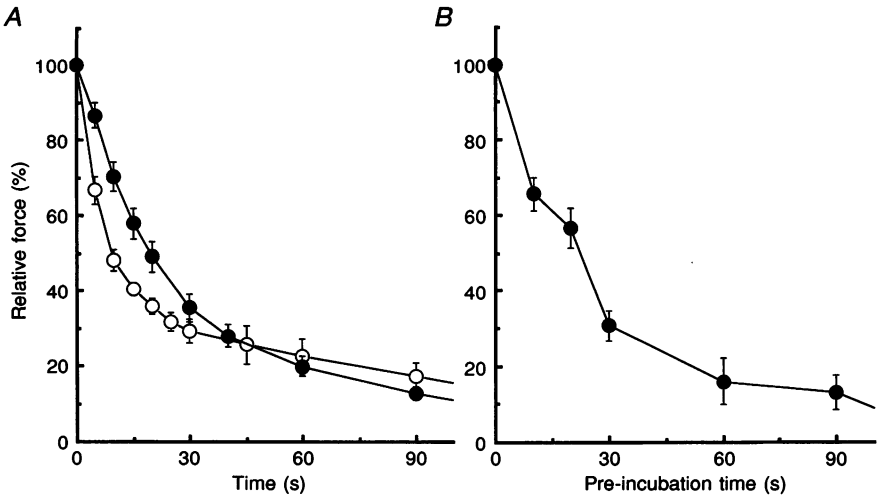


Figure 2. Time course of E2-induced inhibition of contraction

A, average time course of E2-induced relaxation in rat portal vein in comparison with that after a reduction in extracellular Ca^{2+}. E2 (5 μM) was added (●) and Ca^{2+} was reduced from 2 to 0·3 mM (○) during high (64 mM) K^+-induced contraction in rat portal vein as shown in Fig. 1*C*; $n = 5$. *B*, 64 mM K^+-induced contractions were reduced with an increase in the pretreatment time with 5 μM E2 in a normal Na^+ external solution. Rate of inhibition at rest (*B*) was similar to that during depolarization (*A*); $n = 4$.

1990), largely reduced the relaxant effect of the oestrogens; 5 μM E2 without the albumin inhibited the high (64 mM) K^+-induced contraction by 71 ± 3·9% ($n = 8$), but by only 31 ± 2·6% ($n = 4$) in the presence of 0·1% fatty acid-free BSA. Diethylstilboestrol at 5 μM, which completely inhibited high (64 mM) K^+-induced contraction, reduced the contraction only partially by 68 ± 5·6% ($n = 4$) in the presence of the same concentration of albumin. These results suggest that most of the oestrogen molecules dissolved freely in the solutions used.

There was no significant difference in the dose–response curve of E2-induced relaxation between femoral arteries from male and female rats (Fig. 4*B*), confirming and extending previous results (Jiang *et al.* 1991). We furthermore examined if progesterone has a synergistic action with E2. The presence of 1 μM progesterone did not significantly

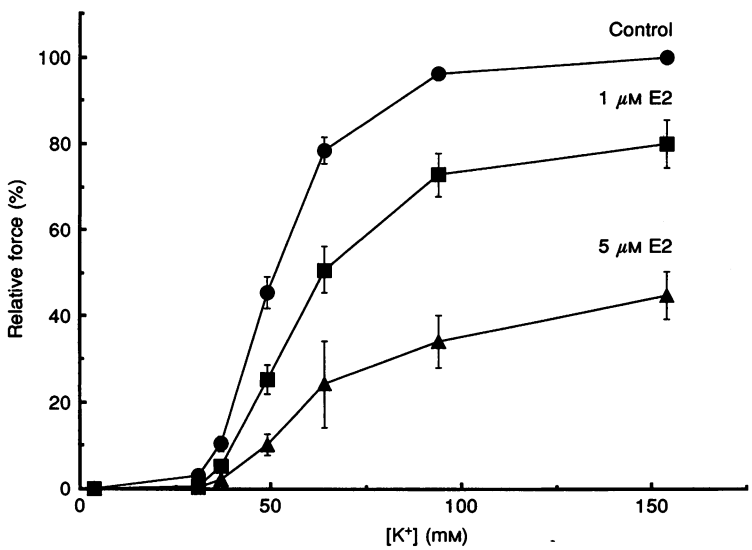
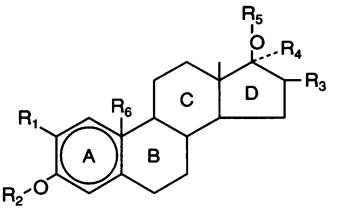


Figure 3. Effect of E2 on the relationship between K^+ concentration and contraction in rat femoral artery

For 15 min, strips were incubated in normal Na^+ external solution containing different concentrations of E2 and stimulated with various concentrations of high K^+ with the same E2. This protocol was repeated 3 times at each concentration. Peak contractions were measured relative to the 154 mM K^+-induced contraction in the absence of E2; $n = 5$.

Table 1. Inhibitory potencies of 17β-oestradiol and related structures at 5 μM on the 64 mM K^+-induced contractions of arterial smooth muscle



Trivial name	R_1	R_2	R_3	R_4	R_5	R_6	Relative force (%)	n*
17β-Oestradiol	-H	-H	-H	-H	-H	-H	26 ± 1·8	44
17α-Oestradiol	-H	-H	-H	-H	-H	-H	70 ± 1·8	5
2-Methoxyoestradiol	$-OCH_3$	-H	-H	-H	-H	-H	26 ± 3·9	6
2-Hydroxyoestradiol	-OH	-H	-H	-H	-H	-H	49 ± 5·4	5
β-Oestradiol 3-methyl ether	-H	$-CH_3$	-H	-H	-H	-H	15 ± 3·8	6
β-Oestradiol 3-benzoate	-H	$-COC_6H_5$	-H	-H	-H	-H	68 ± 2·7	4
β-Oestradiol 3-(β-D-glucuronide)	-H	$-C_5H_8O_4COONa$	-H	-H	-H	-H	100 ± 2·4	4
Oestriol	-H	-H	-OH	-H	-H	-H	83 ± 1·6	6
17-Ethynyl β-oestradiol	-H	-H	-H	-C≡CH	-H	-H	13 ± 2·4	4
β-Oestradiol 17β-acetate	-H	-H	-H	-H	$-COCH_3$	-H	2 ± 1·4	4
Oestrone	-H	-H	-H	-H	—	-H	80 ± 1·3	5
2-Methoxyoestrone	$-OCH_3$	-H	-H	-H	—	-H	79 ± 1·5	4
β-Oestradiol 17β-enanthate	-H	-H	-H	-H	$-CO(CH_2)_5CH_3$	-H	102 ± 2·6	6
β-Oestradiol 17β-cypionate	-H	-H	-H	-H	$-CO(CH_2)_2C_5H_9$	-H	101 ± 1·4	4
β-Oestradiol 17-(β-D-glucuronide)	-H	-H	-H	-H	$-C_5H_8O_4COONa$	-H	96 ± 1·1	4
Moestranol	-H	$-CH_3$	-H	-C≡CH	-H	-H	20 ± 6·0	4
β-Oestradiol 3, 17β-diacetate	-H	$-COCH_3$	-H	-H	$-COCH_3$	-H	2 ± 1·3	4
Testosterone	-H	—	-H	-H	-H	$-CH_3$	88 ± 2·1	4
Progesterone	-H	—	-H	-H	$-COCH_3$	$-CH_3$	79 ± 1·0	4
Others								
Diethylstilboestrol							0 ± 0·25	
Cholesterol							100 ± 0·44	
Corticosterone							98 ± 2·26	

*Number of rat femoral artery smooth muscle strips used.

affect the 1 μM E2-induced relaxation of high (64 mM) K^+-induced contraction, suggesting the lack of any synergistic effect on smooth muscle contraction.

There are many steroidal and non-steroidal oestrogens available. To determine their structure and activity relationship for inhibitory effects on smooth muscle contraction, we examined the efficacy of E2 as compared with twenty-one E2-related structures, each at 5 μM (Table 1). The relative potency for relaxing the high (64 mM) K^+-induced contraction of rat femoral artery smooth muscle is in the following order: diethylstilboestrol > β-oestradiol 17β-acetate, β-oestradiol 3, 17β-diacetate > 17-ethynyl β-oestradiol, β-oestradiol 3-methyl ether > moestranol, E2, 2-methoxyoestradiol ≫ 17 α-oestradiol, oestrone, oestriol. No effects were observed by β-oestradiol 3-(β-D-glucuronide), β-oestradiol 17-(β-D-glucuronide), β-oestradiol 17β-enanthate, β-oestradiol 17β-cypionate, or cholesterol even at 30 μM.

Effects of various antagonists and inhibitors on E2-induced inhibition of high K^+-induced contraction

None of the G protein-linked receptor antagonists, such as prazosin, atropine, propranolol and yohimbine, at 1 μM affected 5 μM E2-induced inhibition of high (64 mM) K^+-induced contraction in rat femoral artery (not shown). Tamoxifen and ICI 164,384 (Weatherill, Wilson, Nicholson, Davies & Wakeling, 1988) were used as specific antagonists for the classical cytosolic/nuclear high affinity oestrogen-binding receptor. At high concentrations (5–10 μM), these antagonists by themselves strongly inhibited the high K^+-induced contraction. Tamoxifen at a concentration of 5 μM reduced the control contraction by 99 ± 1·5% ($n = 4$) with a time course similar to that of E2, while 5 μM E2 decreased

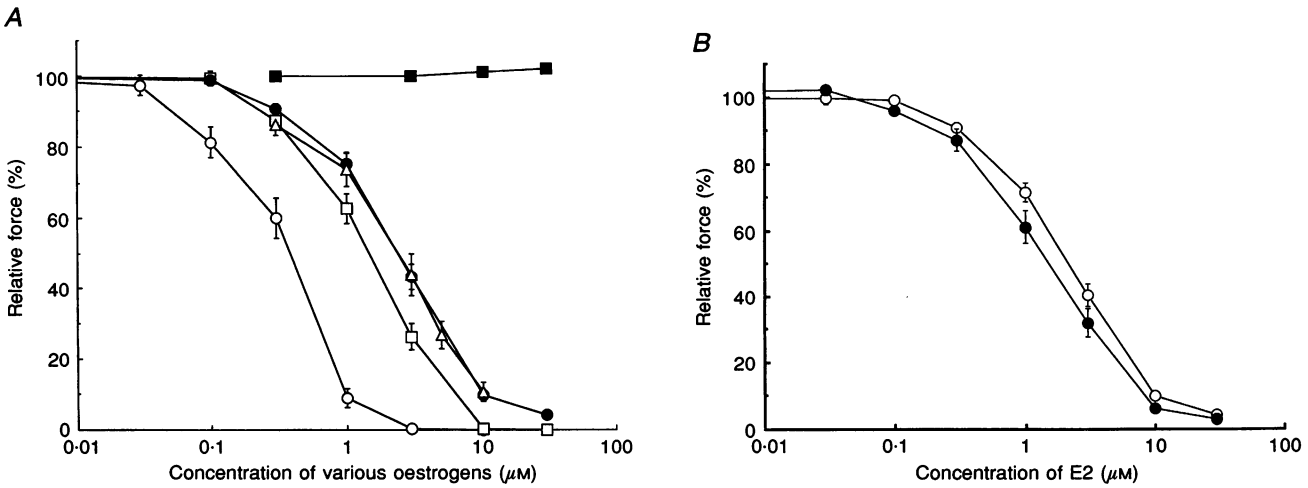


Figure 4. Dose–response curves of E2 and its analogues on high K^+-induced contraction (*A*) and no significant difference of E2 sensitivity between different sexes (*B*)

A, rat femoral artery smooth muscle strips were pre-incubated for 15 min in the normal Na^+ external solution containing various concentrations of E2 (●; $n = 19$), 2-methoxyoestradiol (△; $n = 4$), 17-ethynyl β-oestradiol (□; $n = 4$), diethylstilboestrol (○; $n = 4$) and β-oestradiol 17β-cypionate (■; $n = 4$). K^+ solution (64 mM) with the same concentration of oestrogens was then applied. *B*, femoral artery strips of similar size were dissected from either male (○; $n = 7$) or female (●; $n = 7$) rats with similar ranges of body weight (200–250 g).

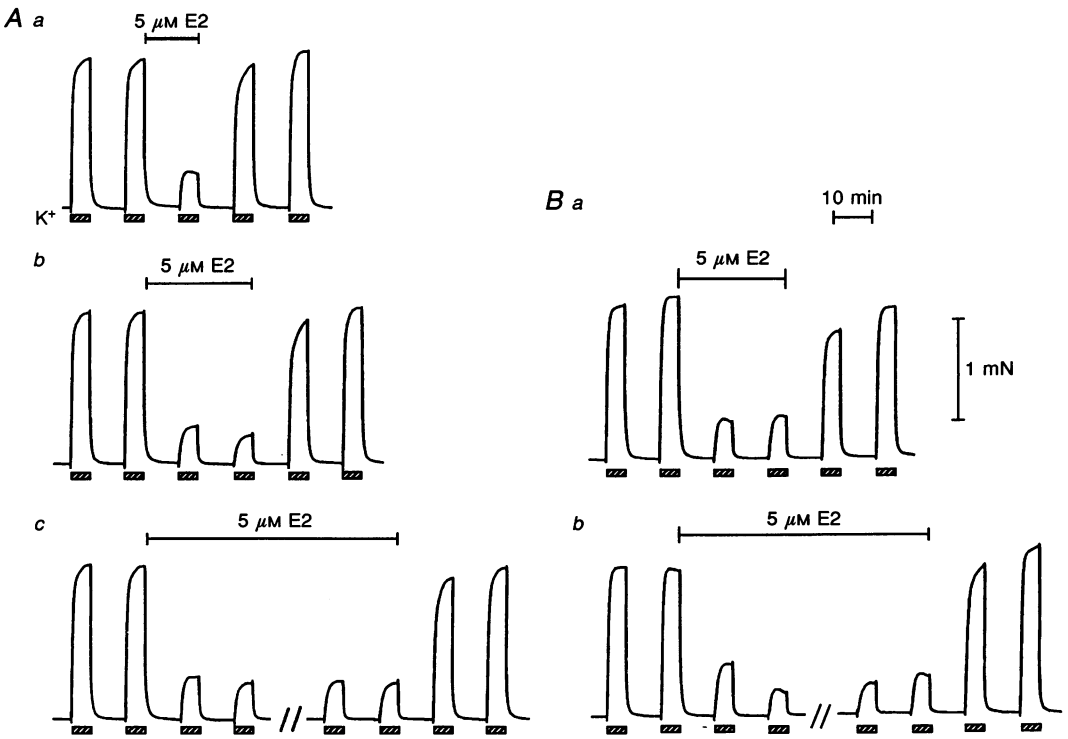


Figure 5. Effect of duration of E2 treatment (*A*) and effect of cycloheximide, a protein synthesis inhibitor (*B*), on E2-induced inhibition of contraction and on its recovery

Rat femoral artery smooth muscle strips were repetitively stimulated with 64 mM K^+ for 5 min at an interval of 15 min. E2 (5 μM) was applied for 15 (*A a*), 30 (*A b*) and 120 min (*A c*) in the absence of cycloheximide and for 30 (*Ba*) and 120 min (*Bb*) in the presence of the protein synthesis inhibitor (100 μM). These traces are representative of 5–7 similar experiments.

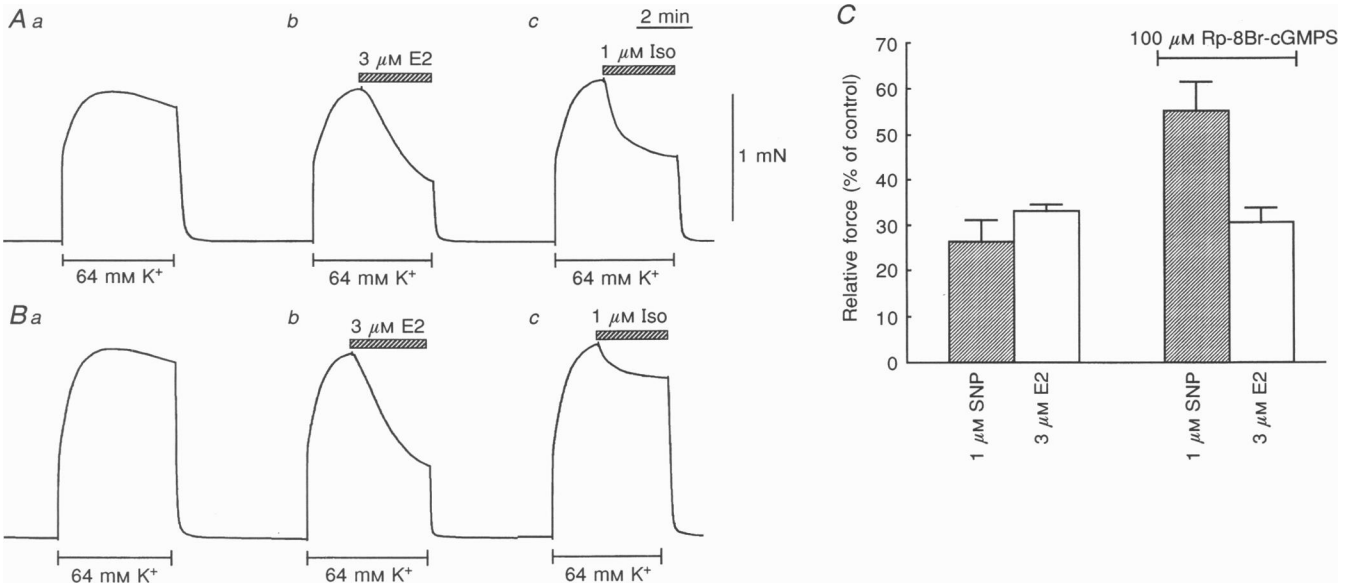


Figure 6. Effects of cyclic nucleotide-dependent protein kinase inhibitors on E2-induced relaxation

A a depicts a control time course of 64 mM K^+-induced contraction in rat femoral artery smooth muscle. The steady-state contraction was decreased by 3 μM E2 (*A b*) and 1 μM isoprenaline (*A c*; Iso). *B*, all solutions contained 200 μM Rp-cAMPS (R_P-isomer of adenosine-3′,5′-monophosphorothioate), a protein kinase A inhibitor. The isoprenaline-induced relaxation was significantly reduced, but the E2 response was not affected. These traces are representative of 4 similar experiments. *C*, 3 μM E2-induced relaxation was compared with that of 1 μM sodium nitroprusside (SNP). Rp-8Br-cGMPS (R_P-isomer of 8-bromo-guanosine-3′,5′-monophosphorothioate) at a concentration of 100 μM significantly reduced the SNP effect, but not the E2-induced relaxation ($n = 4$).

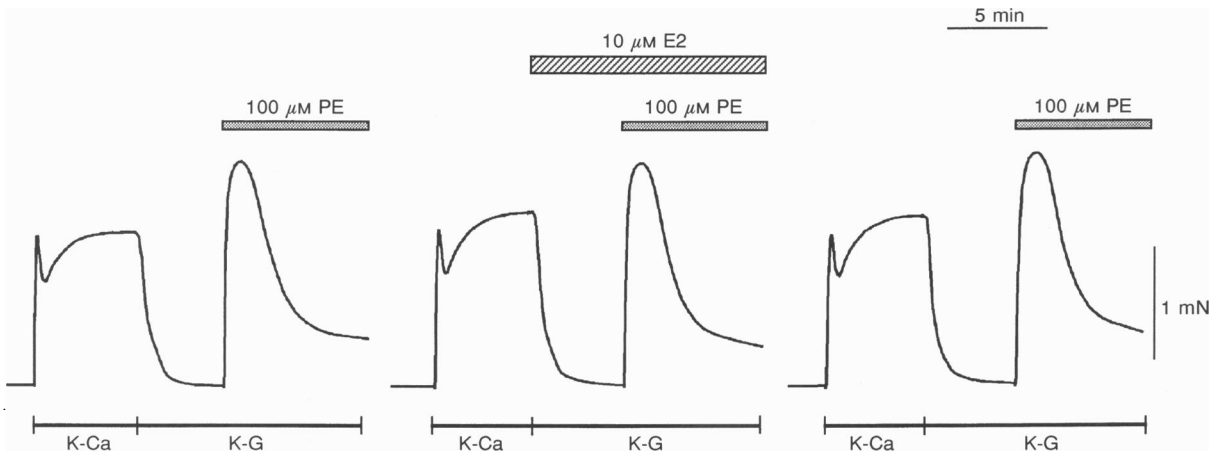


Figure 7. Effect of E2 on agonist-induced Ca^{2+} release from the SR

Before the traces shown, Ca^{2+} was depleted from the intracellular stores with a high (100 μM) concentration of phenylephrine in Ca^{2+}-free solution. Rabbit femoral artery smooth muscles were subsequently immersed in normal Ca^{2+}-containing extracellular solution for 10 min and contracted in the high (154 mM) K^+, Ca^{2+}-containing solution (K-Ca) for 5 min to load the intracellular calcium stores. The muscles were then relaxed by exposure to high K^+, Ca^{2+}-free (2 mM EGTA) solution (K-G) and exposed to 100 μM phenylephrine (PE). PE still evoked a large transient contraction in the absence of extracellular Ca^{2+}. In the middle trace, 10 μM E2 was added only after removal of Ca^{2+} so that the oestrogen did not affect Ca^{2+} loading of the SR during high K^+-induced contraction. The PE-induced contraction in the absence of Ca^{2+} was not affected by E2. These traces are representative of 4 similar experiments. The similar results were obtained in rat femoral artery and rabbit portal vein ($n = 4$ for each tissue).

contraction by 87 ± 3·2% ($n = 7$) in the same group of strips. ICI 164,384 at 10 μM also inhibited high K^+-induced contraction by 95 ± 3·8% ($n = 4$), but very slowly (the half-time was 36 ± 9 min). Lower concentrations (< 1 μM) of these antagonists did not alone markedly decrease high K^+-induced contraction, nor did they significantly ($P > 0·1$) affect the E2-induced inhibition of contraction. Quercetin is a potent, but not specific inhibitor of a low affinity oestrogen-binding site with a K_i of 30 nM (Gray, Biswas, Bashirelahi & Biswas, 1994). Pretreatment of rat femoral artery with 1 μM quercetin for 60 min decreased the control high K^+-induced contraction by 39 ± 7% ($n = 5$), but did not significantly affect the relative inhibition of contraction by 5 μM E2; 80 ± 1% ($n = 5$) inhibition for control and 78 ± 1% ($n = 5$) in the presence of quercetin.

An increase in the duration of 5 μM E2 treatment from 15 to 120 min affected neither the extent of inhibition of high K^+-induced contraction nor its recovery after washout of the hormones (Fig. 5*A*). Pretreatment for 120 min with cycloheximide (100 μM), a potent inhibitor of protein synthesis, did not affect the time course and extent of inhibition by E2 and its recovery (Fig. 5*B*). Pretreatment for at least 60 min with 4 μM actinomycin D, an inhibitor of RNA synthesis, did not block the E2-induced inhibition of contraction: 84 ± 1·5% ($n = 4$) inhibition by 5 μM E2 for control and 81 ± 4·0% ($n = 4$) in the presence of actinomycin D.

It is known that E2 increases cyclic AMP and cyclic GMP content to some extent in uterine and coronary artery smooth muscles (Kuehl *et al.* 1974; Mugge *et al.* 1993). To determine if these second messenger pathways are involved in the oestrogen-induced relaxation in smooth muscle, we used Rp-cAMPS (R_P-isomer of adenosine-3′,5′-cyclic monophosphorothioate) and Rp-8Br-cGMPS (R_P-isomer of 8-bromoguanosine-3′,5′-cyclic monophosphorothioate) to inhibit protein kinase A and G, respectively. Treatment for more than 60 min with solutions containing 200 μM Rp-cAMPS and 100 μM Rp-8Br-cGMPS significantly reduced, respectively, isoprenaline- (Fig. 6*A* and *B*) and sodium nitroprusside-induced relaxation (Fig. 6*C*), but neither significantly modified E2-induced relaxation.

Effect of E2 on Ca^{2+} release from the SR

To determine the effects of E2 on Ca^{2+} release from the intracellular calcium stores, i.e. the SR, three different protocols were used. First, we determined the effects of E2 on agonist-induced Ca^{2+}-release in intact femoral artery smooth muscle. As shown in Fig. 7, after treatment with 2 mM Ca^{2+}-containing high K^+ solution to load the SR with Ca^{2+} to a given level, the α_1-agonist, phenylephrine (100 μM) evoked a large contraction even under the Ca^{2+}-free, depolarized (154 mM K^+) conditions. Neither Ca^{2+} influx nor change in membrane potential can be expected under these conditions. This transient contraction was mainly due to Ca^{2+} release from the SR. Although the presence of 10 μM

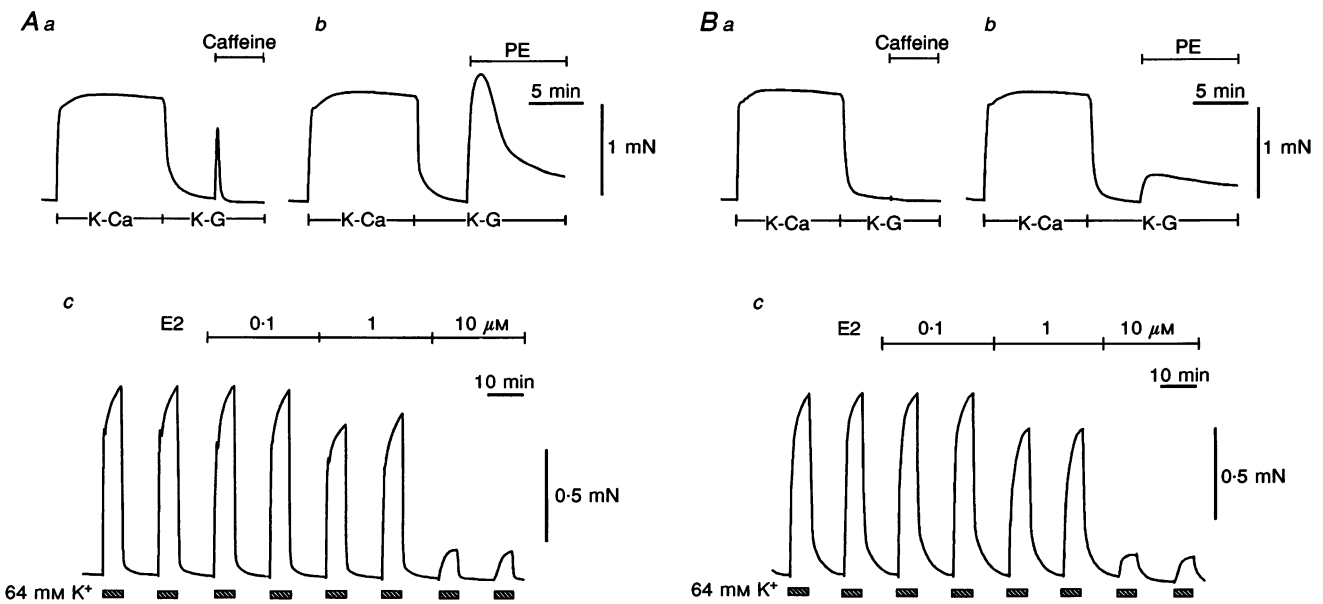


Figure 8. Effect of ryanodine treatment on E2-induced relaxation

Control contractile responses of rabbit femoral artery to 30 mM caffeine (*A a*) or 100 μM phenylephrine (PE; *A b*) in 154 mM K^+, Ca^{2+}-free solution (K-G), and the effect of various concentrations of E2 (*A c*) on 64 mM K^+-induced contraction were obtained before ryanodine treatment. The arterial strips were then treated with 10 μM ryanodine in the 154 mM K^+, Ca^{2+}-free solution containing 30 mM caffeine for 30 min to deplete the SR of Ca^{2+}. Subsequently, caffeine-induced contraction was completely abolished (*B a*) and the PE response was markedly reduced (*B b*), but the high (either 64 or 154 mM) K^+-evoked contractions were not affected. Dose dependence of E2-induced inhibition of K^+ contractions was not modified by ryanodine treatment (*A c vs. B c*). Similar results were obtained using 10 μM thapsigargin. These traces are representative of 4 similar experiments.

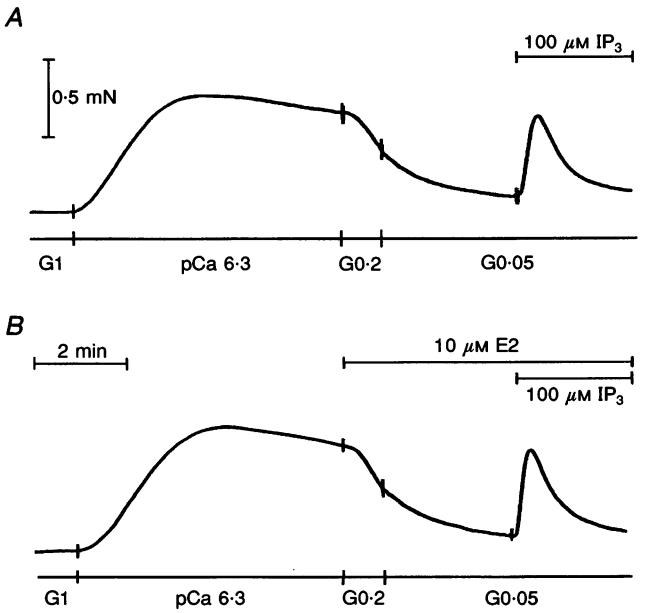


Figure 9. Effect on inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release

Strips of longitudinal smooth muscle layers of rabbit portal vein were permeabilized with 5000 units ml^{-1} of *Staphylococcus aureus* α-toxin at pCa 6·7 at 30 °C (see Methods) and incubated in the normal relaxing solution (G1). The solution with pCa 6·3 buffered with 10 mM EGTA was used to load the SR with Ca^{2+} for 6 min. Thereafter, free Ca^{2+} was removed and the EGTA concentration was reduced first to 0·2 mM (G0·2) and then to 0·05 mM (G0·05). IP_3 (100 μM) was added in the G0·05 in the absence (*A*) and presence of 10 μM E2 (*B*). The experiments were carried out at 20 °C. These traces are representative of 4 similar experiments.

E2 strongly inhibited the agonist-induced contraction in the presence of Ca^{2+} (Fig. 1*C*), it had no effect (99 ± 2% of control; $n = 4$) on the phenylephrine-induced contraction in the Ca^{2+}-free, depolarizing solution (Fig. 7).

Second, the effects of pretreatment with ryanodine or thapsigargin on the E2-induced inhibition of high K^+-induced contraction were examined in intact femoral artery smooth muscle. Ryanodine opens the Ca^{2+}-induced Ca^{2+} release channels of the SR (Meissner, 1986) and thapsigargin inhibits the SR Ca^{2+} pumps (Sagara & Inesi, 1991). Both deplete the SR of Ca^{2+}. These drugs did not markedly affect the development of high K^+-induced contraction in femoral artery smooth muscle and its relaxation, but completely blocked the caffeine-induced contraction (Fig. 8*Ba vs. A a*) and largely but not entirely inhibited agonist-induced contraction under Ca^{2+}-free conditions (Fig. 8*Bb vs. A b*). The latter small, but significantly sustained contractions induced by agonists after drug pretreatment are not evoked by Ca^{2+} release from the SR. Increased Ca^{2+} sensitivity of the regulatory/contractile apparatus by excitatory agonists

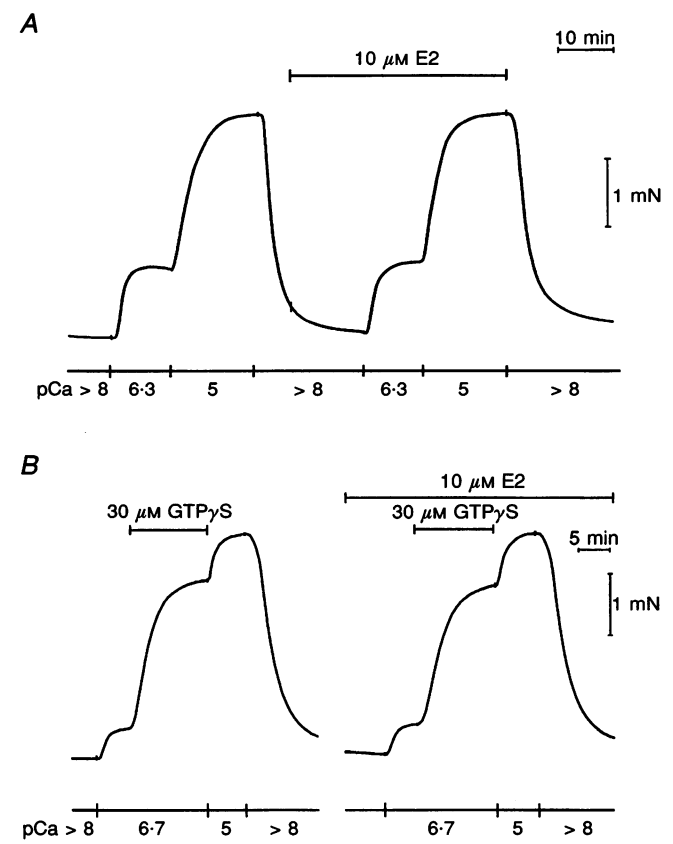


Figure 10. Effect on normal and GTPγS-increased Ca^{2+} sensitivity of the regulatory/contractile apparatus in α-toxin-permeabilized rabbit femoral artery smooth muscle

After permeabilization with α-toxin, femoral artery smooth muscle strips were treated with 10 μM A23187, a Ca^{2+} ionophore, to deplete the SR of Ca^{2+} (see Methods for details). *A*, lack of inhibition by 10 μM E2 of contractile response to submaximal (pCa 6·3) and maximal (pCa 5) Ca^{2+}. *B*, resistance to 10 μM E2 of the GTPγS-induced potentiation of contractile responses to pCa 6·7 at 20 °C. These traces are representative of 4 similar experiments for each experimental case.

is probably sufficient to account for this contraction as described previously (Himpens, Kitazawa & Somlyo, 1990). The inhibition of high K^+-induced contraction by various concentrations of E2 (Fig. 8*A c*) was not affected by 30 min pretreatment with 10 μM ryanodine (Fig. 8*Bc*); e.g. 10 μM E2 without ryanodine pretreatment reduced the contrations by 88 ± 1% and with the pretreatment by 87 ± 1% ($n = 4$). We also obtained similar results using 10 μM thapsigargin (not shown).

Third, we investigated the effect of E2 on Ca^{2+} release by inositol 1,4,5-trisphosphate (IP_3) from the SR in α-toxin-permeabilized rabbit portal vein smooth muscle (Fig. 9). The pCa 6·7 solution was used to load the SR with Ca^{2+} to a given level. Then, added Ca^{2+} was removed and the EGTA concentration was reduced to 0·2 and subsequently to 0·05 mM. The contractile IP_3 effect mediated by Ca^{2+} release from the SR was observed. When the Ca^{2+}-loading procedure was omitted or the strips were pretreated with ryanodine, the transient contractions evoked by IP_3 were not observed (not shown). The presence of 10 μM E2 after removal of Ca^{2+} (Fig. 9*B*) did not significantly affect the IP_3 responses; the peak of the IP_3-induced contraction was 105 ± 5% ($n = 4$) of control in the absence of E2.

Direct effect on regulatory/contractile apparatus

To examine the direct effect of E2 on the regulatory/contractile apparatus, we used α-toxin-permeabilized rabbit femoral artery smooth muscle further treated with 10 μM A23187 to deplete the SR of Ca^{2+}. These strips no longer responded to IP_3 or caffeine (not shown). The presence of E2 had no effect on the Ca^{2+}-activated contraction (Fig. 10*A*); the pCa 6·3 without E2 induced 35 ± 4% ($n = 8$) of maximum contraction at pCa 5 and 35 ± 3% ($n = 5$) with 10 μM E2.

Excitatory agonists and GTP or its analogues can increase the sensitivity of contraction and MLC phosphorylation to Ca^{2+} through inhibition of MLC phosphatase (Kitazawa, Masuo & Somlyo, 1991). As shown in Fig. 10*B*, a non-hydrolysable GTP analogue, GTPγS (30 μM) markedly enhanced a submaximal contraction induced by pCa 6·7 from 14 ± 3 to 80 ± 3% of maximum contraction at pCa 5 ($n = 4$). The enhanced contraction was not affected by 10 μM E2; GTPγS in the presence of 10 μM E2 potentiated the pCa 6·7 contraction from 13 ± 2 to 77 ± 4% ($n = 4$).

We obtained similar results using α-toxin-permeabilized rat femoral artery ($n = 4$).

Effects on voltage-dependent Ca^{2+} channel currents

To characterize the effects of oestrogens on voltage-dependent Ca^{2+} channel currents, smooth muscle myocytes were isolated from longitudinal muscle of rabbit ileum and the whole-cell membrane currents of voltage-clamped cells were measured. To block K^+ outward currents, the patch pipette contained 140 mM CsCl internal solution and the

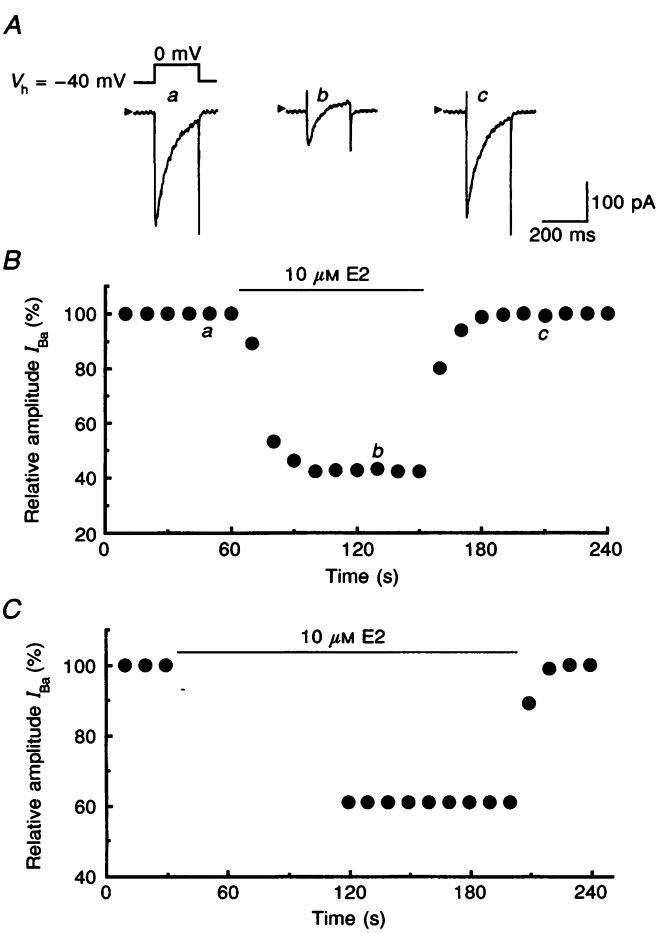


Figure 11. Inhibition of voltage-dependent L-type Ca^{2+} channel current by E2

Resting membrane potential (V_h) of smooth muscle myocytes was held at −40 mV and the command voltage pulses to 0 mV were applied every 10 s. The patch pipette contained CsCl internal solution and the bath was perfused with the solution containing 5 mM Ba^{2+}. The upper current traces (*a–c*) in *A* were recorded at the times indicated by the letters in *B*. *a*, control; *b*, in the presence of 10 μM E2; and *c*, after washout of E2. The zero current level in *A* is indicated by a triangle. The period of E2 perfusion is depicted by a bar illustrated in *B* and *C*. In *C*, E2 was added to the perfusing solution after the command pulse was ceased and 90 s after addition of E2 the repetitive command pulses were resumed. Relative amplitudes of Ba^{2+} currents (I_{Ba}) through L-type Ca^{2+} channels are plotted in *B* and *C*. These traces are representative of at least 5 similar experiments.

bath was perfused with 5 mM Ba^{2+}-containing external solution in the absence of Ca^{2+}. Inward Ba^{2+} currents (I_{Ba}) were observed when the membrane potential was depolarized from the holding potential (−40 mV) to 0 mV (Fig. 11*A a*). E2 (10 μM) rapidly decreased the magnitude of I_{Ba} by 44 ± 4·6% (Fig. 11*A b*). Removal of the oestrogen caused a rapid recovery of this reduced current (Fig. 11*A c*). Figure 11*B* shows the time course of inhibition of peak current by 10 μM E2. Steady-state inhibition and its full recovery were observed within 30 s. These I_{Ba} currents were completely blocked by 1 μM nifedipine, indicating L-type Ca^{2+} channel currents (not shown). This observation was further supported by the requirement of a high threshold voltage for channel opening (Fig. 12*A*).

Some of the organic Ca^{2+} channel blockers show use-dependent blocking, due to much higher affinity binding to the inactivated state of the Ca^{2+} channels subsequent to the open state than to the resting state (Bean, 1984). We examined whether inhibition by E2 required open-state channels leading to the inactivated state. The resting membrane potential was held at −40 mV and the 200 ms test pulse to 0 mV was applied every 10 s. After steady I_{Ba} was obtained in the absence of E2, depolarization pulses were discontinued and 10 μM E2 was applied. After a 90 s quiescent period, depolarizing test pulses were resumed. As shown in Fig. 11*C*, the amplitude of I_{Ba} during the first test pulse after resumption was already decreased and not further reduced by repetitive stimulation. Complete recovery of I_{Ba} by removal of E2 indicates that the inhibition of I_{Ba} during the first test pulse was not due to run-down cells.

E2 did not significantly shift the current–voltage relationship of I_{Ba}, but reduced the amplitude of the currents (Fig. 12*A*). A non-steroidal oestrogen analogue, diethylstilboestrol, at the same concentration (10 μM) more markedly inhibited I_{Ba} at various test voltages in a similar manner to E2. Oestrogen analogues such as 17-ethynyl β-oestradiol and diethylstilboestrol dose dependently inhibited smooth muscle I_{Ba}, but these drugs did not completely block the currents even at 30 μM (Fig. 12*B*).

To determine the effects of E2 on the gating properties of the Ca^{2+} channel, we compared, using a double-pulse protocol (Nakajima *et al.* 1995), the voltage dependence of its steady-state inactivation between the presence and absence of the oestrogen. As previously shown in cultured smooth muscle cells (Nakajima *et al.* 1995), 10 μM E2 reduced the amplitude of Ca^{2+} channel currents, but did not significantly affect the slope of the inactivation curve in isolated smooth muscle myocytes (not shown).

DISCUSSION

The well-known effects of steroidal sex hormones are mediated by binding to the cytosolic/nuclear receptors that modulate gene transcription in target cells. There is increasing evidence, however, that sex hormones have rapid effects and modulate intracellular Ca^{2+} signalling.

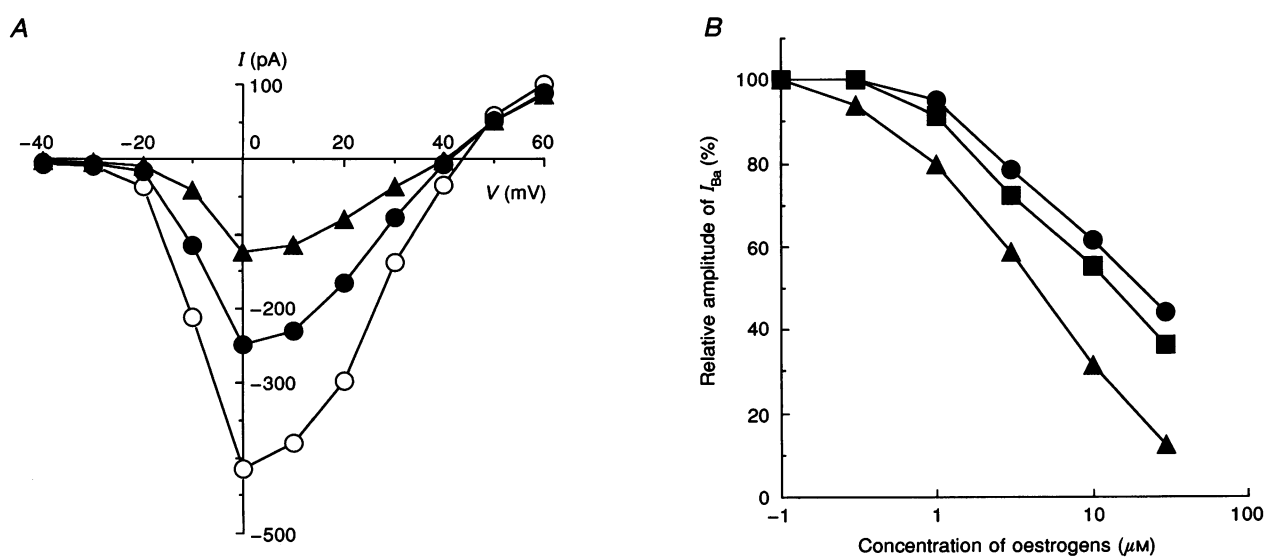


Figure 12. E2 and its analogues on the current–voltage relationship of L-type Ca^{2+} channel currents (*A*) and their dose–response (*B*)

A, inhibitory effect of 10 μM E2 (●) and diethylstilboestrol (▲) on the Ba^{2+} currents through L-type Ca^{2+} channels at different command voltages. The smooth muscle myocytes were held at −50 mV, at which the leakage currents were subtracted. 300 ms command voltage pulses were applied every 10 s. Open circles represent control values. In *B*, concentration-dependent inhibition of I_{Ba} by E2 (●), diethylstilboestrol (▲) and 17-ethynyl β-oestradiol (■). The myocytes were held at −40 mV and the command pulses to 0 mV were applied at every 10 s. The amplitude of I_{Ba} in the control was taken as 100%. These data are representative of 5–10 similar experiments.

Progesterone, even when immobilized by covalent linkage to serum albumin to prevent penetration of the plasma membrane, increases the Ca^{2+} influx and intracellular Ca^{2+} concentrations in sperm (Blackmore, Neulen, Lattanzio & Beebe, 1991). Testosterone also increases Ca^{2+} concentration through pertussis toxin-sensitive formation of IP_3 in male osteoblasts (Lieberherr & Grosse, 1994). In granulosa cells, oestrogens including oestrone and oestriol trigger a rapid release of Ca^{2+} from intracellular stores (Morley, Whitfield, Vanderhyden, Tsang & Schwartz, 1992). In maturing human oocytes, E2 and its membrane-impermeant conjugates, but not progesterone, develop $[Ca^{2+}]_o$-dependent Ca^{2+} oscillations (Tesarik & Mendoza, 1995). These steroidal effects appear to be evoked by non-genomic modulation of intracellular Ca^{2+} signalling through specific receptors in the plasma membrane. This suggestion is strongly supported by the demonstration of specific binding sites for steroidal sex hormones in the plasma membrane (Pietras & Szego, 1979).

The effect of oestrogen on smooth muscle contraction reported in this study is one of the examples cited above. Oestrogen-induced inhibition of contraction and Ca^{2+} inward current is consistent with a non-genomic mechanism possibly triggered by the binding of cell-surface receptors, rather than gene transcription, due to the following reasons. (1) Rapidity and reversibility: half-times of inhibition of both Ca^{2+} channel current and contraction by E2 and of their recovery were about 20 s with no significant delay, while the rapid onset of gene activation by E2 requires about 30 min (Orimo *et al.* 1993). (2) Effects of inhibitors and antagonists: various RNA/protein synthesis inhibitors and cytosolic/nuclear oestrogen receptor antagonists did not significantly affect the time course of oestrogen-induced inhibition of contraction. (3) Order of potency for E2 and its various analogues for the relaxation response: among only certain groups of oestrogens, the order of potency for inhibition of contraction is similar to the order of binding affinity to the nuclear oestrogen receptor, e.g. diethylstilboestrol > 17-ethynyl β-oestradiol > E2 > oestrone, oestriol, progesterone > testosterone (Notides, 1970). A substitution for one of the hydrogen atoms on the E2 phenolic A ring markedly reduces the binding affinity to the receptors. For example, 2-methoxyoestradiol and β-oestradiol 3-methyl ether have, respectively, more than 1000 times and 30 times less binding affinity than E2 (Martucci & Fishman, 1976; Katzenellenbogen, 1978). However, relaxing potencies of these catechol oestrogens were not decreased, but similar or rather stronger than that of E2 (Table 1). Taken together, these results strongly support the conclusion that the inhibitory effects of oestrogens on both contraction and L-type Ca^{2+} channel current in smooth muscle are not mediated through interactions with the cytosolic/nuclear oestrogen receptor. However, a plasma membrane oestrogen receptor(s) linked to a voltage-dependent Ca^{2+} channel remains to be identified.

As for Table 1, it is also important to mention that a hydroxylation of R1 of E2 did not markedly reduce the inhibitory potency and a further acylation of R2 of β-oestradiol 17β-acetate did not affect the degree of relaxation, while the substitutions to longer chains, e.g. β-D-glucuronide, markedly reduced the relaxation response. The substitution for a hydrogen atom on the D ring of E2 also markedly affected this response; replacement of hyrdogen at R5 with acetate potentiated the relaxation while its simple removal or substitution to enanthate, cypionate or β-D-glucuronide led to a loss of the relaxing response.

High doses of the steroidal hormones needed to induce rapid effects on Ca^{2+} channels, however, could be the result of a non-specific effect of the steroids on the plasma membrane, such as changes in the membrane fluidity and/or expansion. In fact, most of steroidal hormones are known to have some anaesthetic action (Selye, 1941) which is rapid and is evoked at the level of plasma membrane. Those anaesthetic steroids may non-specifically inhibit the activity of transmembrane ion channels and decrease ion fluxes. However, since oxygen in position 3, 17 and 20 of the steroid ring structure is necessary for the anaesthetic action, progesterone has much stronger anaesthetic potency than does E2. This order of potency is directly opposite to that of the inhibiting action on smooth muscle Ca^{2+} channel currents and contraction. 17α-oestradiol, an isomer of E2 that is thought to have similar physico-chemical properties, but with a much weaker potency than E2, inhibited Ca^{2+} channels (Zhang *et al.* 1994) and caused relaxation of smooth muscle (Salas *et al.* 1994). This indicates that the effect of E2 is stereo-specific, but not mediated through non-specific interaction with the plasma membrane. This point is also supported by the fact that E2 even at 30 μM has neither significant effect on endothelin-activated non-selective cation channel currents nor Ca^{2+}-activated K^+ currents in cultured smooth muscle cells (Nakajima *et al.* 1995) while the voltage-dependent Ca^{2+} channels are markedly inhibited.

Results from this and other studies clearly indicate that E2 and its analogues inhibit L-type Ca^{2+} channel currents and decrease intracellular Ca^{2+} concentrations during membrane depolarization (Shan *et al.* 1994; Zhang *et al.* 1994; Nakajima *et al.* 1995; this study). This results in decreased Ca^{2+}–calmodulin-dependent MLC phosphorylation and contraction induced by high K^+. The excitatory agonists can evoke smooth muscle contraction mainly through three distinct signalling pathways: Ca^{2+} influx through plasma membrane Ca^{2+} channels, IP_3-induced Ca^{2+} release from the SR and modulation of Ca^{2+} sensitivity of regulatory/contractile apparatus (Somlyo & Somlyo, 1994). E2 inhibits only the voltage-gated Ca^{2+} channel with no effect on the latter two pathways. Therefore, the maximal relaxing effect of E2 depends upon the relative contribution of Ca^{2+} influx through voltage-gated Ca^{2+} channels towards the contraction and thus varies with different conditions, agonists and

smooth muscle tissue types. For example, E2 significantly inhibited α_1-agonist-induced contraction under the normal conditions (Fig. 1), but did not affect the same agonist-induced contraction in the absence of external Ca^{2+} (Fig. 8). The increase in intracellular Ca^{2+} seen in granulosa cells (Morley *et al.* 1992) and maturing human oocytes (Tesarik & Mendoza, 1995) in response to oestrogens was not observed in smooth muscle cells. Interestingly, the Ca^{2+}-modulating effects of a sex hormone appear to vary depending on cell types.

The Ca^{2+} antagonistic effect for oestrogen was suggested by several groups. Collins, Rosano, Jiang, Lindsay, Sarrel & Poole-Wilson (1993) proposed that some of the cardiovascular benefits of oestrogen replacement therapy may be due to a long-term calcium antagonistic effect of oestrogen observed *in vitro*. According to our, and other, results, E2 appears to be a selective and reversible Ca^{2+} channel blocker without significant effects on intracellular Ca^{2+} stores or on the regulatory/contractile apparatus. It is, however, very difficult to establish a relationship between the acute *in vitro* Ca^{2+} antagonistic effect of the E2 and the *in vivo* CHD-protective effects over a long period of time. To begin with, the concentration range of E2 required for the inhibition of Ca^{2+} inward current and contraction is three orders of magnitude higher than the total plasma concentration of the hormones in premenopausal women. Since an increase in the solvent did not affect the IC_{50} of E2 and since the measured solubility of 17α-oestradiol and 17-ethynyl β-oestradiol in pure water are slightly higher than 10 μM (Hurwitz & Liu, 1977), this low efficacy could not be due to poor solubility of E2 in the experimental solutions. Secondly, most of the natural steroidal hormones including oestrogens circulate in the blood in complexes with steroid-binding globulin and albumin (Rosner, 1990); these bound forms of oestrogens were significantly less effective for the inhibition of contraction. The concentration of the effective free form of oestrogen in the circulation is much lower than the total. Thirdly, we have preliminarily examined the *ex vivo* effects of E2 on vascular smooth muscle contraction: slow-release pellets (placebo and 10 mg kg^{-1} of E2) were subcutaneously implanted for 2 weeks in ovariectomized 14-week-old rats. This chronic treatment with E2 markedly increased the uterine weight to severalfold that of the control ovariectomized rats, but this *in vivo* treatment affected neither the *in vitro* sensitivity of E2 to high K^+-induced contraction nor the sensitivity of the contraction to $[Ca^{2+}]_o$ (T. Kitazawa, T. Murahashi & A. K. M. Gaznabi, unpublished observations). This result is also supported by the fact that the sensitivities of female rat femoral artery smooth muscle to E2 and to $[Ca^{2+}]_o$ were not different from those of male. All these results indicate that the circulating levels of E2 required for its *in vitro* Ca^{2+} antagonistic effects could not be achieved under normal physiological conditions. It is conceivable, however, that the local tissue concentrations near the sources of oestrogen, e.g. ovaries and placenta, or the circulating plasma levels following E2 therapy under abnormal endocrine conditions, reach such high pharmacological values. The requirement for high concentrations of steroidal hormones might be due to, at least in part, extensive metabolism of the steroids near the plasma membrane.

E2 has been shown to increase both cAMP and cGMP contents in smooth muscle (Kuehl *et al.* 1974; Mugge *et al.* 1993). However, protein kinase A and G (PKA and PKG) inhibitors did not inhibit E2-induced relaxation even though they reduced the relaxing effect of isoprenaline and sodium nitroprusside, respectively. The addition of cAMP, cGMP, forskolin or sodium nitroprusside markedly decreases the Ca^{2+} sensitivity of the regulatory/contractile apparatus in the α-toxin-permeabilized smooth muscle (Nishimura & van Breemen, 1989) that was also used in this study, but E2 had no observable effects in these preparations. Thus, the PKA and PKG pathways do not appear to contribute to the relaxation response by E2 at least in rat and rabbit femoral artery and portal vein smooth muscles.

Taken together, these results suggest that at pharmacological concentrations oestrogen reduces primarily Ca^{2+} influx through the inhibition of L-type Ca^{2+} channels in a non-genomic manner and therefore decreases MLC phosphorylation and contraction of smooth muscle. The findings from the present study will hopefully open the gates that may lead to the discovery of a whole new class of selective Ca^{2+} entry blockers.

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Acknowledgements

The authors thank Matthew R. Lee for his insightful suggestions on the manuscript, Drs Peter Ramwell and Yuichiro Suzuki for their useful comments and Dr Yoshihisa Kurachi for his expert advice and for enabling E.H. to be involved in this study. This work was supported by National Institutes of Health RO1 HL 51824 to T.K.

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Received 12 September 1996; accepted 18 November 1996.