Original Article

Non-genomic vasorelaxant effects of 17 β -estradiol and progesterone on rat aorta are mediated by L-type Ca²⁺ current inhibition

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Aim: The sex hormones 17 β -estradiol (β ES) and progesterone (PRG) induce rapid non-genomic vasodilator effects which could be protective for the cardiovascular system. The purpose of this study was to analyze the mechanisms underlying their vasodilator effect in rat aortic smooth muscle preparations.

Methods: Endothelium-denuded aorta artery rings were prepared from male Wistar rats and incubated in an organ bath. The contractions of the preparation were recorded through isometric transducers. The effects of the hormones on K⁺ current and L-type Ca²⁺ current (LTCC) were analyzed by using the whole cell voltage-clamp technique in A7r5 cells.

Results: Both β ES and PRG (1–100 μ mol/L) concentration-dependently relaxed the endothelium-denuded aortic rings contracted by (–)-Bay K8644 (0.1 μ mol/L) or by KCl (60 mmol/L). The IC₅₀ values of the two hormones were not statistically different. The K_v channel blocker 4-aminopyridine (2 mmol/L), BK_{Ca} channel blocker tetraethylammonium (1mmol/L) and K_{ATP} channel blocker glibenclamide (10 μ mol/L) did not significantly modify the relaxant effect of the hormones. On the other hand, the blockage of the intracellular β ES and PRG receptors with estradiol receptor antagonists ICI 182,780 (1 μ mol/L) and PRG receptor antagonist mifepristone (30 μ mol/L), respectively, did not significantly modify the relaxant action of the hormones. In A7r5 cells, both the hormones (1–100 μ mol/L) rapidly and reversibly inhibited the basal and BAY-stimulated LTCC. However, these hormones had no effect on the basal K⁺ current.

Conclusion: The vasorelaxant effects of β ES and PRG are due to the inhibition of LTCC. The K⁺ channels are not involved in the effects.

Keywords: female sex hormone; 17 β -estradiol; progesterone; non-genomic action; vasorelaxant effect; Ca²⁺ channel; K⁺ channel; rat aorta artery ring; A7r5 cell

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Introduction

Cardiovascular diseases are one of the most common causes of death in western countries and there are different degrees of incidence of these diseases between both sexes^[1]. Concerning the role of sex hormones in these diseases, it was reported that estrogen replacement therapy can reduce the risk of coronary and cerebrovascular diseases in postmenopausal women^[2, 3]. Also, the results of distinct observational and clinical studies suggested that female and male sex hormones can modulate the vascular function^[4, 5]. Nevertheless, the molecular mechanisms underlying the effects of these hormones remain undefined due to controversial data and to relatively small number of experimental studies undertaken.

According to the classic theory to explain the steroid hormones effects, these signalling molecules modulate gene tran-

scription due to the interaction with intracellular receptors. However, these hormones can also induce rapid (seconds to minutes) non-genomic effects which are reversible and insensitive to transcription and protein synthesis inhibitors^[1, 6, 7]. Non-genomic effects of sex hormones include vasodilatation, one of the most relevant and promising action of sex steroids. Different pathways have been proposed to explain this effect. Progesterone (PRG) and 17 β -estradiol (β ES) may activate endothelium-dependent vasorelaxant pathways, including pathways mediated by nitric oxide, prostacyclin and hyperpolarizing factors^[1, 8, 9]. However, these steroids can also relax arterial smooth muscle via endothelium-independent mechanisms, mainly involving modulation of membrane ionic flux^[10, 11]. This direct vasodilator effect of PRG and β ES has been observed in different arteries from different species such as aorta^[12, 13], coronary arteries^[8, 10, 14], cerebral arteries^[15], omental artery^[16], tail artery^[17] and mesenteric artery^[9, 18, 19].

The inhibition of Ca²⁺ channels and the activation of K⁺ channels was suggested as a leading cause of the sex hor-

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mones' effect. The extracellular Ca^{2+} can enter into the smooth muscle cells through different types of Ca^{2+} channels such as store-operated Ca^{2+} channels, voltage-operated Ca^{2+} channels (VOCCs), Ca^{2+} -permeable non-selective cation channels and the controversial receptor-operated Ca^{2+} channels^[20]. Some authors showed that the inhibition of Ca^{2+} channels is associated to the vasodilatation mediated by estrogens^[21–23] and PRG^[24, 25]. The effects of βES and PRG on VOCCs were the objective of some studies. Zhang *et al* have reported that in A7r5 cells estradiol inhibits two types of VOCCs: L- and T-type Ca^{2+} channels (LTCC and TTCC respectively)^[26]. Nakajima *et al*, using tight-seal whole cell clamp technique, observed that βES inhibits LTCC, but failed to affect Ca^{2+} -permeable non-selective cation currents evoked by endothelin or vasopressin^[21]. In addition, these authors also observed that PRG and testosterone fail to inhibit calcium channels^[21]. Other authors observed that environmental estrogenic pollutants and βES induced a rapid and endothelium-independent relaxation by inhibiting LTCC in vascular smooth muscle cells^[27]. Activation of K^+ channels by PRG and by βES in vascular smooth muscle may induce repolarization of plasma membrane, which will close the VOCCs and contribute to the vascular relaxation^[27–30]. This mechanism does not appear to be very important for PRG^[29], but the opening of Ca^{2+} -activated K^+ (BK_{Ca}) channels by βES has been observed in human coronary artery smooth muscle cells^[30] and rat aorta A7r5 cells^[27]. The activation of K^+ channels was suggested to be involved in the vasodilatation induced by βES in arteries from different species^[8, 31] and could also be important to prevent or reduce hypertension^[32, 33]. On the contrary, K^+ currents can be attenuated by 17 α -estradiol or βES , an effect that could be mediated by estrogen-induced proteasomal degradation of these channels. These sex hormones specific bind to K^+ channels and induce proteasome-dependent proteolytic degradation. This action can be elicited independently of the activation of the nuclear estradiol receptors or the accessory $\beta 1$ -subunit, but in the presence of $\beta 1$ -subunits, specific binding of estradiol to BK_{Ca} channels is significantly increased^[34].

In summary, PRG and βES have vascular non-genomic actions which include vasodilatation. Although the mechanistic pathways implicated in this effect are still unknown, some studies have reported, regardless of the specific site of action, the involvement of an ionic-related transduction mechanism. The purpose of this study was to analyse the mechanisms implicated in the vasodilator effect of these hormones in rat aortic smooth muscle. The relaxation induced by these hormones in endothelium-denuded rat aorta was analysed. Also, the whole cell configuration of the patch-clamp technique was used to analyse the effects of sex hormones on voltage-dependent Ca^{2+} current (I_{Ca}) and on the K^+ current (I_{K}) in A7r5 cells.

Materials and methods

Contractility experiments in isolated rat thoracic aorta rings

Male adult Wistar rats (Charles-River, Barcelona, Spain) weighing 400–500 g were housed and acclimatized with light

cycles of 12 h light: 12 h dark and food and water *ad libitum* for at least one week before performing the experiments. The rats were used in accordance with the European regulations about protection of animals (Directive 86/609) and the Guide for the Care and Use of Laboratory Animals promulgated by the US National Institutes of Health (NIH Publication No 85–23, revised 1996).

The rats were sacrificed by decapitation and, after thoracotomy, the thoracic aortas were removed, placed in a thermostated (37°C) Krebs' modified solution and the fat and connective tissue was cleaned. Also, the vascular endothelium was mechanically removed by gentle rubbing with a cotton bud introduced through the arterial lumen. The rat aorta artery rings were placed in an organ bath (LE01.004, Leticia) containing Krebs-bicarbonate solution at 37°C continuously gassed with carbogen. The composition of the Krebs' modified solution was (mmol/L): NaCl 119, KCl 5, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.5, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2, KH_2PO_4 1.2, NaHCO_3 25, EDTA- Na_2 0.03, L-(+)-ascorbic acid 0.6 and glucose 11 (pH 7.4). The rings were suspended by two parallel stainless steel wires and tension measurement was performed using isometric transducers (TRI201, Panlab SA, Spain), amplifier (ML118/D Quad Bridge, ADInstruments), interface PowerLab/4SP (ML750, ADInstruments), interface PowerLab/4SP (ML750, ADInstruments), and computerised system with Chart5 PowerLab software (ADInstruments). During the resting periods, the organ bath solution was changed every 15 min.

Initially, the rings were equilibrated for 60 minutes until a resting tension of 1.0 g. After the equilibration period, aortic rings were firstly contracted with high isosmotic KCl (60 mmol/L) and the absence of endothelium functionality was confirmed by the lack of relaxant response to acetylcholine (1 $\mu\text{mol/L}$). After that, the arteries were washed many times for at least 45 min before the next stimuli. The rings were contracted using KCl (60 mmol/L) or (-)-Bay K 8644 (BAY; 0.1 $\mu\text{mol/L}$) and vasorelaxation induced by βES and PRG (1–100 $\mu\text{mol/L}$) on these contractions was analysed.

The contraction induced by BAY (0.1 $\mu\text{mol/L}$; specific LTCC activator) was performed to observe the direct effect of these sex hormones on LTCC. The contractions induced by BAY were obtained in presence of KCl 10 mmol/L.

In some experiments, specific antagonists for the intracellular hormonal receptor were used in order to analyse the involvement of these receptors in the vasorelaxant effects of βES and PRG. In these cases, after a stable contraction with KCl (60 mmol/L), the arteries were incubated for 15 min with ICI 182,780 (1 $\mu\text{mol/L}$; a specific antagonist for the classical estradiol receptor) or with mifepristone (30 $\mu\text{mol/L}$; a specific antagonist for the classical progesterone receptor). After this incubation the vasorelaxation induced by βES or PRG in the presence of this specific antagonist was analysed.

To analyse the role of K^+ channels in the effects of these sex hormones, different inhibitors of these channels were used: tetraethylammonium (TEA; 1 mmol/L), that inhibits of large-conductance Ca^{2+} -activated potassium (BK_{Ca}) channel; glybenclamide (Gly; 10 $\mu\text{mol/L}$), that inhibits ATP-sensitive potas-

sium (K_{ATP}) channel; and 4-aminopyridine (4-AP; 1 mmol/L), that inhibits voltage-sensitive potassium (K_V) channels. In these cases, after a stable contraction with KCl (60 mmol/L), the arteries were incubated 15 min with the potassium channel inhibitors. After this incubation the effect of the sex hormones was analysed. Control experiments were performed using ethanol, the vehicle used to dissolve the channel inhibitors.

Cell culture of vascular smooth muscle cells

The A7r5 cell line, used in this study, is a commercial vascular smooth muscle cell line obtained from embryonic rat aorta (Promochem, Spain). The cells were grown in culture medium Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 Hams (DMEM-F12; Sigma-Aldrich, Portugal) supplemented with NaHCO_3 (1.2 $\mu\text{g/L}$), L-ascorbic acid (20 mg/L; Sigma-Aldrich), bovine serum albumin (0.5%; Sigma-Aldrich), heat-inactivated foetal bovine serum (FBS; 10%; Biochrom), and a mixture of penicillin (100 U/mL), streptomycin (100 g/mL), and amphotericin B (250 ng/mL) (Sigma-Aldrich). The cells were kept in culture at 37°C in a humidified atmosphere with 5% CO_2 in air. After confluence, the cells were placed in culture medium without FBS (FBS-free culture medium) for 24–48 h. Trypsinization was made using a solution of trypsin (0.3%) in Ca^{2+} - Mg^{2+} -free phosphate buffered solution with EDTA (0.025%). Subsequently, the cells were kept at 4°C in FBS-free medium until the realisation of the electrophysiological experiments.

Electrophysiology experiments

The whole cell configuration of patch clamp technique was used to analyse the L-type calcium current ($I_{\text{Ca,L}}$) and the K^+ current (I_K). To analyse the $I_{\text{Ca,L}}$, the control external solution contained (mmol/L): NaCl 124.0, CaCl_2 5.0, HEPES 5.0, tetraethylammonium sodium salt (TEA) 10.0, KCl 4.7 and glucose 6.0, pH 7.4 adjusted with NaOH. Patch electrodes (2–4 M Ω) were filled with internal solution (mmol/L): CsCl 119.8, CaCl_2 0.06, MgCl_2 4.0, Na-ATP 3.1, Na-GTP 0.4, EGTA 5.0, HEPES 10.0 and TEA 10.0, pH 7.3 adjusted with CsOH. The presence of Cs^+ instead of K^+ in the solutions blocked the potassium currents. The cells were maintained at a holding potential of -80 mV and routinely depolarized every 8 s to 0 mV test potential during 500 ms to measure $I_{\text{Ca,L}}$.

To analyse the I_K , the control external solution contained (mmol/L): NaCl 134.3, CaCl_2 1.0, HEPES 5.0, KCl 5.4 and glucose 6.0, pH 7.4 adjusted with NaOH. Patch electrodes (2–4 M Ω) were filled with internal solution (mmol/L): KCl 125.0, MgCl_2 1.0, Na-ATP 5.0, Na-GTP 0.5, EGTA 0.1, HEPES 20.0 and glucose 10.0, pH 7.3 adjusted with KOH. For I_K analysis we used the same holding potential and depolarizations to 60 mV for 300 ms were performed every 8 s.

Basal $I_{\text{Ca,L}}$ and I_K were measured 3–5 min after patch break to allow the equilibration between pipette and intracellular solutions. Currents were not compensated for capacitance and leak currents. All experiments were done at room temperature (21–25°C) and the temperature did not vary by more than

1°C in a given experiment. The cells were voltage clamped using the patch-clamp amplifier Axopatch 200B (Axon instruments, USA). Currents were sampled at a frequency of 10 kHz and filtered at 0.1 kHz using the analog-digital interface Digi-data 1322A (Axon Instruments, USA) connected to a compatible computer with the Pclamp8 software (Axon Instruments, USA). The external solution was applied to the cell proximity by placing the cell at the opening of a 250 μm inner diameter capillary tube flowing at a rate of 20 $\mu\text{L/min}$. The basal and BAY-stimulated (10 nmol/L) $I_{\text{Ca,L}}$ were studied in the presence of different concentrations of βES (1–100 $\mu\text{mol/L}$) and of PRG (1–100 $\mu\text{mol/L}$) dissolved in the external solution.

Drugs

All drugs were purchased from Sigma-Aldrich Química (Sintra, Portugal), except 4-aminopyridine and PRG that purchased from Biogen Científica (Madrid, Spain).

Mifepristone, ICI 182,780, BAY, nifedipine, βES and PRG were initially dissolved in ethanol. 4-Aminopyridine, glibenclamide, apamin and tetraethylammonium were initially dissolved in deionised water. Appropriate dilutions in Krebs' modified solution or in the corresponding electrophysiology external solution were prepared every day before the experiment. Final concentration of ethanol never exceeded 0.1% in the experiments.

Statistical analysis

Statistical treatment of data was performed using the SigmaStat Statistical Analysis System, version 1.00 (1992). Results are expressed as mean $\pm$ SEM of n experiments. Comparison among multiple groups was analysed by using a one-way ANOVA followed by Dunnett's or Tukey *post hoc* test to determine significant differences among the means. Comparison between two groups was analysed by using Students *t*-test. Probability levels lower than 5% were considered significant ($P < 0.05$).

In the contractility experiments, the relaxant responses induced by βES and PRG are expressed as a percent of the maximal contraction ($E_{\text{max}} = 100\%$) produced by each vasoconstrictor agent. In these experiments, sigmoidal concentration-response curves for the vasorelaxant effects were fitted and IC_{50} values (*ie* concentrations inducing 50% of relaxation) of βES and PRG were estimated for KCl- or BAY-induced contractions. The antagonist of classical progesterone receptors, mifepristone, relaxed by itself the arteries contracted by KCl, and in this case the maximal effect used to perform the concentration-response curves was the tension obtained in presence of mifepristone.

The $I_{\text{Ca,L}}$ amplitudes were automatically calculated between the maximum current peak and the stable current plateau near the final of the every 8 s pulse. The $I_{\text{Ca,L}}$ variations induced by the different drugs used are expressed as a percent of the basal or BAY-stimulated $I_{\text{Ca,L}}$. The I_K variations are expressed as a percent of the basal I_K obtained by depolarization in the absence of any drug.

Results

Vasorelaxant effects of female sex hormones on rat isolated aorta

The rat aortic rings without endothelium were contracted by depolarisation with isosmotic KCl (60 mmol/L) solution or by the calcium channel opener BAY (0.1 μ mol/L). The maximal contractions elicited by KCl and BAY, 1134.3 ± 39.9 mg ($n=52$) and 1222.8 ± 83.1 mg ($n=20$) respectively, were not significantly different ($P < 0.05$, Student's *t*-test). These contractile effects were reversible after washing out with Krebs' solution.

Afterwards, the effect of β ES or PRG (1–100 μ mol/L) on the contraction induced by KCl and Bay were analysed. Increasing concentrations of both hormones were administered and, at higher concentrations, almost completely relaxed the contractions induced by KCl and BAY (Figure 1A and 1B). The vasorelaxation induced by each dose of β ES or PRG was observed 10–15 min after the application. The effect of the hormones was reversible because, after washing out, a second administration of the contractile agents elicited similar contractile effects than the previous one ($P > 0.05$, data not shown). The maximal relaxation induced by β ES and by PRG was similar in arteries contracted by KCl or BAY. However, the vasorelaxant effect induced by PRG 10 μ mol/L in the arteries contracted with BAY is lower than the induced by β ES at the same concentration (Figure 1B). Also, in the arteries contracted with KCl the vasorelaxant effect induced by β ES 10 μ mol/L is lower than the induced by PRG at the same concentration, and similar to the control arteries (Figure 1A).

The IC_{50} values corresponding to the relaxant effects of β ES or PRG on KCl-induced contraction were almost similar, being β ES slightly more effective than PRG, although this difference was not significant ($P > 0.05$; Student's *t*-test; Table 1). Similarly, in BAY-contracted arteries, the vasorelaxant effect of β ES seems to be slightly more effective than that of PRG, as revealed the IC_{50} values obtained in both cases (Table 1). However, there are no significant differences between the IC_{50} values of β ES and PRG, independently of the contractile agent (KCl or BAY) ($P > 0.05$; Student's *t*-test; Table 1). Thus, in general, the vasorelaxant effect induced by β ES and by PRG was similar.

Influence of K^+ channels on the vasorelaxant effects of the female sex hormones

The effects of three K^+ channels inhibitors (Gli, 4-AP, and TEA) were investigated to analyse the involvement of these channels in the female sex hormones-associated relaxant mechanism. Initially, after contraction with KCl 60 mmol/L, rat aorta rings were exposed for 15 min to glibenclamide (Gli; 10 μ mol/L), 4-aminopyridine (4-AP; 1 mmol/L), and tetraethylammonium (TEA; 1 mmol/L), either together or separately, and these inhibitors did not have a significant effect on the contraction induced by KCl 60 mmol/L (data not shown). After that, the administration of cumulative concentrations of β ES or PRG induced the total relaxation of the contracted aortic rings. None of the K^+ channel inhibitors tested modified significantly the relaxant effect of these hormones (Figure 2)

Figure 1. Relaxant effect of different concentrations of β ES and PRG (1–100 μ mol/L) on contractions of endothelium-denuded rat aortic rings induced by KCl (60 mmol/L) (A) or by BAY (0.1 μ mol/L after KCl 10 mmol/L) (B). Each point represents the mean value and the vertical lines indicate SEM of at least 5 experiments. Control experiments were performed using with ethanol, the vehicle used to dissolve the hormones. ^b $P < 0.05$ versus control. ^f $P < 0.01$ β ES versus PRG. One-way ANOVA followed by with Tukey *post hoc* test.

($P > 0.05$, one-way ANOVA with Dunnett's *post hoc* test). The relaxant IC_{50} values for β ES and PRG in the presence of any one of the K^+ channels inhibitors did not differ significantly from the IC_{50} values calculated in the absence of them ($P > 0.05$; Student's *t*-test; Table 1). Thus, the inhibition of K_v , BK_{Ca} and K_{ATP} channels did not reduce the relaxing effect of β ES or PRG.

Implication of β ES and PRG intracellular receptor in the vasorelaxant effects of these hormones

To analyse if the vasorelaxant effects of β ES and PRG are mediated by the activation of the intracellular receptors, we used specific antagonists for these receptors. The arteries were contracted by KCl or BAY, after reaching a plateau of contraction the antagonists were administered and increasing concentrations of β ES or PRG were added to test the relaxant effect of these hormones in these conditions. The ICI 182,780 (1 μ mol/L) was used to block β ES receptors and mifepristone (30 μ mol/L) was employed for the receptors of PRG.

When administrated alone, mifepristone induced a significant relaxation of the contracted rat aortic rings ($53.9 \pm 3.1\%$).

Table 1. Values of IC_{50} ($\mu\text{mol/L}$) for the relaxant effect of 17β -estradiol and progesterone on rat aortic contractions induced by BAY (10 nmol/L) and with KCl (60 mmol/L). The influence of potassium channel inhibitors was analysed using the following drugs: the K_v channel blocker 4-aminopyridine (4-AP; 2 mmol/L); the BK_{Ca} channel blocker tetraethylammonium (TEA; 1 mmol/L); and the K_{ATP} channel blocker glibenclamide (GLI; 10 $\mu\text{mol/L}$). The influence of the blockage of intracellular hormone receptors was analysed using the estradiol receptor antagonists ICI 182,780 (ICI; 1 $\mu\text{mol/L}$) and the progesterone receptor antagonists mifepristone (30 $\mu\text{mol/L}$). Each value represents the mean $\pm$ SEM from the number of experiments shown in brackets.

AGENTS	17β -Estradiol	Progesterone
BAY	14.24 $\pm$ 0.05 (n=6)	22.33 $\pm$ 0.30 (n=5)
KCl	19.54 $\pm$ 1.15 (n=9)	23.04 $\pm$ 0.02 (n=9)
KCl+TEA	22.66 $\pm$ 2.76 (n=4)	20.25 $\pm$ 1.11 (n=6)
KCl+4-AP	17.14 $\pm$ 1.33 (n=7)	22.60 $\pm$ 1.06 (n=6)
KCl+GLI	30.44 $\pm$ 2.43 (n=3)	18.79 $\pm$ 2.01 (n=4)
KCl+ICI	16.97 $\pm$ 1.26 (n=6)	
KCl+Mifepristone		24.49 $\pm$ 1.35 (n=8)

4-AP, 4-aminopyridine; BAY, (-)-Bay K8644; GLI, glibenclamide; TEA, tetraethylammonium sodium salt; ICI, ICI 182,780.

Figure 2. Relaxant effect of different concentrations of 17β -estradiol (A) and progesterone (B) on KCl-contracted arteries in presence or absence of the following different potassium channel inhibitors: glibenclamide (GLI; 10 $\mu\text{mol/L}$); 4-aminopyridine (4-AP; 1 mmol/L); and tetraethylammonium (TEA; 1 mmol/L). Each point represents the mean value $\pm$ SEM (indicated in vertical bars) from the number of experiments shown in brackets.

However, mifepristone and ICI 182,780 did not alter the relaxant effects of PRG or β ES, respectively, because the IC_{50} values obtained in the absence and in the presence of these antagonist were similar ($P>0.05$, Student's *t*-test; Table 1). These results indicated that the vasorelaxant effects of β ES and PRG are not mediated by classical hormonal receptor activation.

Effects of β ES and PRG on $I_{Ca,L}$ in A7r5 cells

The whole-cell patch clamp technique was used to analyse calcium current through the LTCC ($I_{Ca,L}$) in A7r5 cells. The mean value of basal $I_{Ca,L}$ density was of 0.90 ± 0.05 pA/pF ($n=70$). The application of BAY (10 nmol/L; specific stimulator of LTCC) significantly stimulated the calcium current by $97.7\pm8.3\%$ ($n=17$) above the basal level. On the contrary, nifedipine (0.1 $\mu\text{mol/L}$; LTCC inhibitor) significantly reduced the basal $I_{Ca,L}$ until a level of $24.3\pm4.5\%$ ($n=7$) of the basal activity ($P<0.05$). The effects of BAY and/or nifedipine were completely reversible upon washout of the drug (data not shown). These results indicate that the current measured was due to the LTCC.

Like a proposed vasodilatation mechanism of the female sex hormones is the inhibition of LTCC, we tested the effect of β ES or PRG on the $I_{Ca,L}$. The Figure 3A shows a typical experiment in which different concentrations of β ES (1–100 $\mu\text{mol/L}$) inhibited the basal $I_{Ca,L}$ in a reversible manner. Concerning PRG, Figure 3B shows a typical experiment in which PRG (1–100 $\mu\text{mol/L}$) almost completely inhibits the basal $I_{Ca,L}$. The data obtained in this type of experiments demonstrate that PRG (1–100 $\mu\text{mol/L}$) inhibits the basal $I_{Ca,L}$. Thus, PRG seems to have similar effects with β ES on basal $I_{Ca,L}$ ($P>0.05$, Student's

Figure 3. Original records showing the effect of increasing concentrations of 17β -estradiol (A) and progesterone (B) on basal $I_{Ca,L}$ amplitudes measured in patch-clamp experiments performed with A7r5 cells.

t-test).

To further characterize the effect of β ES on these channels, we analyse their effect on the BAY-stimulated $I_{Ca,L}$. Figure 4A shows a typical experiment in which β ES reversibly inhibited the $I_{Ca,L}$ stimulated by BAY (10 nmol/L) and this inhibitory effect was dependent on the concentration. The maximal effect of β ES was an inhibition of $53.8\pm11.8\%$ on the BAY-stimulated $I_{Ca,L}$ (Figure 5B). Concerning PRG effect, we also analyse its effect on the $I_{Ca,L}$ stimulated by BAY (Figure 4B). PRG also inhibited BAY-stimulated $I_{Ca,L}$. At a concentration of 100 μ mol/L, PRG almost completely inhibited the stimulation of BAY and reduced the $I_{Ca,L}$ below the basal level (Figure 5B).

Figure 4. Original records showing the effect of increasing concentrations of 17 β -estradiol (A) and progesterone (B) on BAY-stimulated $I_{Ca,L}$ amplitudes measured in patch-clamp experiments performed with A7r5 cells.

A comparison between the effects of both hormones showed that the effects of β ES and PRG on BAY-stimulated $I_{Ca,L}$ are similar ($P>0.05$, Student's *t*-test). On the other hand, ethanol (0.001%–0.1%), the vehicle used to dissolve β ES and PRG did not affect basal or stimulated $I_{Ca,L}$ (data not shown).

Effects of β ES and PRG on I_K in A7r5 cells

The whole-cell patch clamp technique was used to analyse potassium current (I_K) in A7r5 cells. The mean value of basal I_K density was of 6.1 ± 0.7 pA/pF ($n=37$). In order to determine the types of K^+ channels that were responsible for the total potassium current measured, we used selective blockers of dif-

Figure 5. Inhibitory effect (% of reduction) of 17 β -estradiol and progesterone on basal $I_{Ca,L}$ (A) and BAY-stimulated (10 nmol/L) (B) $I_{Ca,L}$ in A7r5 cells. Each column represents the mean value $\pm$ SEM (indicated in vertical bars), in percent of the basal (A) or BAY-stimulated (B) $I_{Ca,L}$ from the number of experiments shown in brackets.

ferent channels. The K_V channel blocker 4-AP reduced basal I_K by $37.4\pm2.5\%$ at +60 mV. TEA (1 mmol/L), which is used as a BK_{Ca} channel blocker, reduced net current by $38.3\pm3.9\%$ at +60 mV (Figure 6). We also tested the presence of the low conductance K_{Ca} channels using the selective blocker apamin (10 μ mol/L), which induced a small reduction on the basal I_K ($11.7\pm3.8\%$, $n=9$). Glibenclamide, usually used as a K_{ATP} channel blocker also induced a small reduction on the I_K ($8.3\pm0.7\%$, $n=6$) (Figure 6). The effects of the K^+ channels blockers used were completely reversible upon washout of the drug. Thus, our data suggest that the potassium current measured is mainly constituted by potassium exit through K_V and BK_{Ca} channels.

In order to analyse the role of K^+ channels in the relaxant mechanism of β ES and PRG, the effects of these steroids on A7r5 I_K were analyzed. The results show that different concentrations (1–100 μ mol/L) of β ES and PRG did not inhibit the I_K (Table 2).

Discussion

In the present study, we analyzed the vascular responses

Figure 6. Inhibition of I_K (% of reduction) induced by different potassium channel inhibitors in A7r5 cells. The bars represent the effect on I_K of the following potassium channel blockers: the K_V channel blocker 4-aminopyridine (4-AP; 2 mmol/L); the BK_{Ca} channel blocker tetraethylammonium (TEA; 1 mmol/L); the low conductance K_{Ca} channels blocker apamin (APM, 10 µmol/L); and the K_{ATP} channel blocker glibenclamide (GLI; 10 µmol/L). Each column represents the mean value $\pm$ SEM (indicated in vertical bars), in per cent of the inhibition of I_K from the number of experiments shown in brackets.

Table 2. Inhibitory effect of the basal potassium current (% of basal I_K) induced by 17 β -estradiol and progesterone (1–100 µmol/L) on A7r5 cells. Each value represents the mean of the % of variation of basal I_K $\pm$ SEM from the number of experiments shown in the brackets.

Concentration	17 β -Estradiol	Progesterone
1 μ mol/L	2.1% $\pm$ 2.4% (n=5)	-1.4% $\pm$ 3.8% (n=8)
10 μ mol/L	-1.1% $\pm$ 5.7% (n=6)	1.8% $\pm$ 3.1% (n=7)
30 μ mol/L	-3.7% $\pm$ 2.8% (n=5)	-0.9% $\pm$ 4.3% (n=5)
100 μ mol/L	-2.1% $\pm$ 3.2% (n=5)	-3.8% $\pm$ 0.8% (n=5)

of β ES and PRG in endothelium-denuded aorta from male adult rats and their effects on the $I_{Ca,L}$ and I_K measured by whole cell voltage-clamp in A7r5 cells. The female hormones (1–100 µmol/L) equally relaxed, in a rapid and concentration-dependent manner, the aortic rings contracted with KCl or BAY, suggesting an inhibitory effect on voltage-dependent Ca^{2+} influx currents. These voltage-dependent slow inactivated inward currents were measured in A7r5 cells and were characterised electrophysiologically and pharmacologically as $I_{Ca,L}$. The sex hormones studied, β ES and PRG, inhibited the basal and BAY-stimulated $I_{Ca,L}$. On the other hand, the vasorelaxant effects of β ES and PRG on rat aorta were not mediated by classic hormone receptors or by K^+ channels opening. This data, obtained performing contractility experiments, were also confirmed using the whole cell configuration of the patch clamp, which show that β ES and PRG failed to stimulate I_K in A7r5 cells. Therefore, altogether, our results demonstrated a non-genomic inhibitory effect induced by β ES and PRG on LTCC that seems to be the responsible for the endothelium-independent vasorelaxant effect of these hormones.

The vasorelaxant effects of β ES and PRG on aortic rings contracted with KCl were dose-dependent (1 and 100 µmol/L) and the maximal relaxation achieved with the hormones was

100%. Because we studied the effect of these hormones at the smooth muscle level, the vascular endothelium was previously removed. Previous studies have shown the vasorelaxant effect of the female sex hormones in rat aorta, but there is no consensus about the mechanistic pathway involved in this action. Some authors defended a main role of the endothelium in the relaxant effects of these hormones^[1, 9]. Our results demonstrate that there is a relaxant effect independent of the endothelium. These data agree with the that obtained by Perusquia *et al* using PRG in rat thoracic aorta^[35]. Also, Unemoto *et al* described a vasorelaxant effect of β ES and PRG on agonist-induced contractions in the aorta of Wistar-Kyoto and spontaneously hypertensive rats^[12]. For these authors, the vasorelaxant effect of β ES seemed to be more gifted than PRG, however they did not find statistical differences between the IC_{50} values calculated for the inhibitory action of the female hormones. In opposition, Rodriguez *et al* showed that 17 α -estradiol, but not β ES, relaxes calcium-dependent contractions in rat aortic strip^[36]. On the other hand, we previously showed that testosterone and cholesterol also relax rat aorta by inhibiting LTCC^[37]. Thus, in the sense, the vasodilator effect of cholesterol, testosterone, β ES and PRG seems to be similar.

The relaxant effect induced by β ES and PRG on the contractions induced by KCl or by BAY is similar, which, attending to the mode of action of both drugs, suggests that these hormones inhibit Ca^{2+} influx into vascular smooth muscle cells. High extracellular KCl concentrations induce plasmatic membrane depolarization, which activates the Ca^{2+} entry by VOCCs (mainly LTCC) and this leads to muscle contraction. BAY directly and specifically opens LTCC and induces vascular smooth muscle contraction also due to intracellular Ca^{2+} elevation. Thus, β ES and PRG inhibited KCl and BAY-induced contractions and presumably inhibit Ca^{2+} influx through LTCC. This hypothesis was also supported by other investigators that studied the sex steroids effects in rat aorta^[12, 38] and in other arteries^[15, 23, 39].

Activation of K^+ channels in vascular smooth muscle may induce repolarization of the plasma membrane, which leads to close VOCCs and contributes to vascular relaxation. To test the possible implication of this pathway in the vascular effects of β ES and PRG we used inhibitors of these channels: TEA (BK_{Ca} channels inhibitor), glibenclamide (K_{ATP} channels inhibitor) and 4-aminopyridine (K_V channel inhibitor). None of them significantly modified the rat aorta relaxant effects of β ES or PRG, suggesting that potassium channel opening is not involved in the vascular action of these hormones. This conclusion may not surprise for PRG because its vascular effects were never associated to the potassium channel activation^[12, 29]. However, in rat cerebral arteries, β ES seems to enhance nitric oxide production from vascular endothelium and, by this way, activate BK_{Ca} ^[40]. According to Unemoto *et al*, the relaxation induced by β ES in aortas from spontaneously hypertensive rats is, at least partially, mediated via K_{ATP} and K_V channels stimulation in addition to Ca^{2+} influx blockage^[12]. Nevertheless, these authors suggest that the K^+ channels stimulation by β ES does not occur in aorta of Wistar rats. Other authors dem-

onstrate that β ES can activate BK_{Ca} channels and induce artery relaxation in human coronary artery smooth muscle cell^[31] and in rat coronary arteries^[8]. On the contrary, potassium currents can be attenuated in transfected HEK293 cells by 17 α -estradiol or by β ES, an effect that could be mediated by estrogen-induced proteasomal degradation of the channels^[34]. To further investigate the effect of β ES and PRG on the K^+ channels, we performed patch clamp studies in A7r5 cells. The potassium current measured was significantly inhibited by the K_V channel blocker (4-AP) and by the BK_{Ca} channel blocker (TEA), but the SK_{Ca} (low conductance) channel blocker (apamine) and the K_{ATP} channel blocker (glibenclamide) did not have a significant effect on the potassium current measured. In agreement with previous data^[37], our study show that the potassium current measured in A7r5 cells is mainly due to K_V and BK_{Ca} channels. On the other hand, for the first time our study also demonstrates that β ES and PRG fail to stimulate I_K in A7r5 cells. These data confirm the contractility data and demonstrate that K^+ channels are not implicated in the vasorelaxant effect of β ES and PRG in rat aorta.

The blockage of the intracellular receptors for β ES and PRG did not modify significantly the vasorelaxant action of these hormones, demonstrating that their intracellular receptors are not involved in the vascular effects induced by these hormones. This fact was previously observed by other authors^[23, 38]. On the contrary, it has been described that the antiprogesterin mifepristone reduced the PRG-induced relaxation on human placental arteries and veins, suggesting a classic receptor-activated mechanism for PRG in these vessels^[41]. Furthermore, Han *et al* showed that β ES activates K^+ channels is due to the activation of the intracellular α receptor for estradiol^[31]. On the other hand, the relaxation induced by mifepristone on KCl-contracted arteries was never referred by other authors. However, a similar effect in rat aorta was described for flutamide, a specific antagonist of the intracellular testosterone receptor^[42, 43]. To explain this effect of flutamide, Iliescu *et al* suggested the involvement of the NO-cGMP pathway activation^[42]. Also, Ba *et al* also observed this effect in rat arteries and a bigger relaxation in arteries from males than from females, suggesting a sex dependent mechanism for the flutamide effect^[43].

To further investigate the effects of the female sex hormones on calcium channels, we performed patch clamp studies in A7r5 cells. The characterisation of the I_{Ca} was made by analysing the effect of BAY, a known agonist of this type of channels, which clearly stimulate the basal I_{Ca} , and nifedipine, a selective antagonist of LTCC, that significantly blocked either basal or BAY-stimulated calcium current. These data confirm that calcium current measured is due to calcium entry through LTCC ($I_{Ca,L}$). Concerning the β ES and PRG effects in the calcium currents, our results revealed a rapid concentration-dependent inhibitory effect on basal $I_{Ca,L}$, which indicates that these sex hormones have the ability to block LTCC. These results agree with previously report by Zhang *et al*, that showed an inhibitory effect of β ES on the basal $I_{Ca,L}$ in A7r5 cells^[26]. Nakagima *et al* determined that, while β ES (10 μ mol/L) inhibited basal

$I_{Ca,L}$ in A7r5 cells, PRG (30 μ mol/L) failed to affect these current^[21]. In agreement with our results, these authors indicated that the inhibitory effect of β ES on LTCC was already significant at a concentration of 10 μ mol/L. Our results showed a more powerful effect of PRG than β ES at high concentrations (100 μ mol/L). The inhibition of LTCC current by PRG in A7r5 cells is here reported for the first time, although Zhang *et al* observed in rat tail vascular smooth muscle cells that PRG reduced the LTCC^[25]. We already studied the effect of PRG and β ES on the BAY-stimulated $I_{Ca,L}$. We show for the first time that PRG and β ES inhibit BAY-stimulated $I_{Ca,L}$, confirming the inhibitory properties of these hormones on rat aorta LTCC.

The action of sex steroids on the genetic protein expression is due to diffusion of these molecules across the cell membrane that, afterwards, bind to specific intracellular receptors that regulate this expression as genetic transcription factors^[44]. Therefore, this action needs some time to produce physiological effects. On the contrary, the inhibitory effects of β ES and PRG observed in this study were rapid and reversible, because the effects disappeared after drug washing. Some author already described the existence of a non-genomic mechanism for some sex steroids through which the hormones can regulate the vascular tone^[45, 46]. Our study also demonstrates that the effects of β ES and PRG are mediated by a non-genomic pathway. Furthermore, the vasodilator effects of these female hormones are not mediated by their intracellular receptors and further investigations are needed to identify the pathway involved in this effect. In these sense, recent data have suggested that the sex steroids receptors are distributed at the membrane surface, throughout the cytosol, in the mitochondria and in the nucleus of the cells. However there is controversy regarding the exact characterization of membrane estrogen receptors. Some studies suggested the activation of the membrane surface receptors, for example, the receptor GPR30, that may activate multiple effects, including adenylate cyclase, Scr and spingosine kinases^[47–49]. Other authors suggested a direct modulation of the ionic channels by steroids^[1]. In this sense, further studies must be done to clarify the mechanism of non-genomic action of sex hormones on vascular tissue.

In summary, our results showed for the first time an inhibition on $I_{Ca,L}$ induced by β ES and PRG in vascular smooth muscle cells, which supports and confirms the observed vasorelaxant effects of these hormones in rat aortic rings. These sex steroids inhibit basal and BAY-stimulated $I_{Ca,L}$. This blockage of LTCC will reduce intracellular free Ca^{2+} concentration and is responsible of the rat aorta relaxation. Our data also correlate with the idea of a rapid relaxant effect of β ES and PRG through a mechanism independent of the endothelium and not mediated by intracellular receptors or by potassium channel activation.

The results of this study help to further understand the non-genomic vasodilator mechanism of sex hormones. Moreover, the accumulating evidences about the cardiovascular effects of sex hormones provide promising data about their role in preventing and retaining the progression of cardiovascular diseases.

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Author contribution

Ezequiel ALVAREZ: designed research, performed research, analyzed data; João Miguel CARVAS: performed research; Antonio Jose SANTOS-SILVA: contributed; Ignacio VERDE: designed research, wrote the paper.

Abbreviations

BAY, (-)-Bay K8644; β ES, 17 β -estradiol; BK_{Ca}, large conductance Ca²⁺-activated K⁺ channels; FBS, foetal bovine serum; I_{Ca}, voltage-dependent Ca²⁺ current; LTCC, L-type Ca²⁺ channels; PRG, progesterone; VOCCs, voltage-operated Ca²⁺ channels.

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