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# 17ß-Estradiol and Progesterone Inhibit L-Type $Ca^{2+}$ Current of Rat Aorta Smooth Muscle Cells

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**Abstract**

Sex hormones like 17ß-estradiol (ßES) and progesterone have shown rapid non-genomic vasodilator effects, which could be involved in the protection of cardiovascular system. However, the precise mechanism by which this effect occurs has not been elucidated yet, even if $Ca^{2+}$ influx inhibition seems to be implicated. The aim of this study was to study the influence of ßES and progesterone on the L-type $Ca^{2+}$ current measured by whole cell voltage-clamp in A7r5 cells. Voltage-operated $Ca^{2+}$ currents were elicited by square-step voltage pulses and pharmacologically characterized as L-type currents by (-)-Bay K8644 (BAY) and nifedipine. Both ßES and progesterone (1-100 µM), rapidly and reversibly inhibited, in a concentration dependent manner, either non-stimulated or BAY-stimulated $Ca^{2+}$ currents registered in A7r5 cells. These results suggest that ßES and progesterone inhibit L-type voltage-operated $Ca^{2+}$ channels through a non-genomic pathway. Consequently, these hormones inhibit the $Ca^{2+}$ entry into smooth muscle cells from rat aorta, an effect that can contribute for the protection of the cardiovascular system.

***Keywords:*** sex hormones, steroid non-genomic effects, L-type $Ca^{2+}$ currents, patch-clamp, A7r5 cells.

***Abbreviations***: BAY: (-)-Bay K8644; ßES: 17β-estradiol; $BK_{Ca}$: $Ca^{2+}$-activated $K^+$ channels; FBS: foetal bovine serum; $I_{Ca}$: L-type $Ca^{2+}$ current, TEA: tetraethylammonium sodium salt, LTCC: L-type $Ca^{2+}$ channels; VOCCs: voltage-operated $Ca^{2+}$ channels.

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## Introduction

Gender differences in morbidity and mortality by cardiovascular causes have been attributed to different hormonal levels between women and men, as the lower level of coronary heart disease observed in women before the onset of menopause [1, 2]. In general, these differences have been attributed to the feminine hormonal patterns related with the menstrual cycle [3].

According to the classic theory of steroid hormones, they modulate gene transcription by interaction with intracellular receptors, acting as ligand-dependent transcription factors. In contrast to this genomic action, non-genomic effects are rapid, reversible (seconds to minutes) and characterized by their insensitivity to inhibitors of transcription and protein synthesis [4-6].

Rapid effects of estrogens or progestins include vasodilatation, which has been shown in aorta [7-10], coronary arteries [11-13], cerebral arteries [14], and omental artery [15], from different species. This vasodilator effect does not seem to be mediated by the classic intracellular steroid receptors or by stimulation of protein synthesis [13, 16], indicating a non-genomic mechanism of action. Different pathways have been pointed to explain this effect, but a consensus between the investigators was not yet reached. Reduction of $Ca^{2+}$ entry through $Ca^{2+}$ channels has been implicated in estrogens-mediated vasodilatation [17, 18], but also the activation of some $K^+$ channel types has been suggested to participate in the vascular relaxant effect of sex hormones [7], [19].

Extracellular $Ca^{2+}$ can enter into the smooth muscle cells through different types of $Ca^{2+}$ channels placed in the plasma membrane: 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 [20-24].

The effects of 17ß-estradiol (ßES) and progesterone in VOCCs have been matter of previous studies. In A7r5 smooth muscle cells, Zhang *et al.* [25] have reported that ßES inhibited L type VOCCs. Nakajima *et al.* [17] observed that ßES has a $Ca^{2+}$ antagonist effect on L type $Ca^{2+}$ channels (LTCC), but failed to affect $Ca^{2+}$-permeable non-selective cation currents evoked by endothelin or vasopressin. The estrogen inhibition of $Ca^{2+}$ channels in a voltage-dependent manner has also been described in pregnant rat myometrium [26], although inhibition of $Ca^{2+}$ and $K^+$ channels by βES was also observed in smooth muscle cells from pregnant rat myometrium [18]. Other authors observed that environmental estrogenic pollutants and βES inhibit LTCC in vascular smooth muscle cells and also evoke a rapid and endothelium-independent relaxation of the coronary vessels [27]. Thus, the ionic mechanisms explaining the female sex hormones effects on vascular contractility were not yet clarify.

Activation of $K^+$ channels by sex hormones in vascular smooth muscle may induce repolarization of plasma membrane, which leads closing the VOCCs and contributes to vascular relaxation. The opening of $Ca^{2+}$-activated $K^+$ channels ($BK_{Ca}$) by ßES has been observed in human coronary myocytes [28], rat neurons [29], and A7r5 cells [27]. The functional implication of $K^+$ channels activation on the ßES-induced vasodilatation has been reported in rat cerebral arteries [19] and in aortas from hypertensive rats [7].

Therefore, the pathways implicated in the effects of female sex hormones on the regulation of vascular smooth muscle tone are still unknown. The purpose of this study was to analyse the mechanisms implicated in the sex steroids vasodilator effect in vascular smooth muscle cells. The whole cell configuration of the patch-clamp technique was used to analyse the effects of βES and progesterone on voltage-dependent L-type $Ca^{2+}$-channel current ($I_{Ca}$) in A7r5 cells.

## Methods

### *Cell culture and preparation*

The A7r5 cells used in this study is an established vascular smooth muscle cell line obtained from embryonic rat aorta. These cells were a generous gift from Drs. F. Orallo and M. Campos-Toimil (Santiago de Compostela, Spain).

The culture medium used was Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Hams (DMEF-F12; Sigma-Aldrich: D8900;) supplemented with $NaHCO_3$ (1.2 g/L), L-ascorbic acid (20 mg/L; Sigma-Aldrich: A5960), bovine serum albumin (BSA; 0.5%; Sigma-Aldrich: A6003), foetal bovine serum (FBS; 10%; Biochrom: F0113) and a mixture of penicillin (100 u/mL), streptomycin (100 μg/mL) and amphotericin B (250 ng/mL) (Sigma-Aldrich: P0781). Cell growth was perform at 37 ºC with culture medium in a fully humidified atmosphere of air with 5% $CO_2$. Confluent cells were placed in culture medium without FBS (FBS-free culture medium) 24-48 h before trypsinization, which 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 placed at 4 ºC in FBS-free medium until the realization of the electrophysiological experiments.

### *Electrophysiological experiments*

The whole cell configuration of patch clamp technique was used to record the L-type $Ca^{2+}$ current ($I_{Ca}$). The cells were maintained at –80 mV holding potential and routinely depolarised every 8 s to 0 mV test potential during 500 ms. Currents were not compensated for capacitance and leak currents. All experiments were done at room temperature (21-25 ºC) and the temperature did not vary by more than 1 ºC in a given experiment. The cells were voltage clamped using a patch-clamp amplifier (Axopatch 200B, Axon instruments). Currents were sampled at a frequency of 10 kHz and filtered at 0.1 kHz using an analog-digital interface (Digidata 1322A, Axon Instruments) connected to a PC compatible computer with the Pclamp8 software (Axon Instruments).

Control or drug-containing solutions were applied to the exterior by placing the cell at the opening of 250 μm inner diameter capillary flowing at a rate of 20 μL $min^{-1}$.

$I_{Ca}$ amplitudes were automatically calculated between the maximum current peak and the stable current plateau near the final of the pulse. $I_{Ca}$ variations were studied in control extracellular solution containing or not drugs. Results are expressed as mean ± s.e.m. When appropriate, the Student's *t*-test was used for statistical evaluation, and a $p < 0.05$ was considered as statistically significant.

***Solutions and drugs***

Control extracellular solution contained (mM): NaCl 107.1, CsCl 40.0, $CaCl_2$ 1.8, $MgCl_2$ 1.8, Na-piruvate 5.0, $NaHCO_3$ 4.0, $NaH_2PO_4$ 0.8, HEPES 10.0, glucose 5.0, pH 7.4 adjusted with NaOH. Patch electrodes (2-4 MΩ) were filled with intracellular solution (mM): 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, tetraethylammonium sodium salt (TEA) 10.0, pH 7.4 adjusted with CsOH. $K^+$ currents were blocked by replacing all $K^+$ ions with intracellular and extracellular $Cs^+$.

ßES, (-)-Bay K8644 (BAY), TEA and nifedipine were purchased from Sigma-Aldrich (E8875, B133, T2265, N7634). Progesterone was purchased from Calbiochem (5341). Stock solutions of ßES (100 mM), progesterone (100mM), BAY (0.1 mM) and nifedipine (10 mM) were prepared in absolute ethanol and stored at -20 ºC: appropriate dilutions in extracellular solution were prepared every day before the experiment was performed. Final concentration of ethanol never exceeded 0.1% in the experiments.

## Results

***Effects of BAY and nifedipine on the $Ca^{2+}$ current***

The whole-cell patch clamp technique was used to record $Ca^{2+}$ currents in A7r5 cells [30]. Basal current amplitude was measured 3-5 min after patch break to allow the equilibration between pipette and intracellular solutions. The depolarising protocol performed every 8 s induced a typical LTCC $I_{Ca}$, measured at 0 mV which slowly inactivates at the end of the depolarisation pulse (Fig. 1). The mean value of basal $I_{Ca}$ density was of 0.739 ± 0.071 pA/pF ($n$ = 70). The effect on basal or Bay-stimulated $I_{Ca}$ of the vehicle used to dissolve the steroids used in the study (0.001-0.1% of ethanol) was not significant ($p$ > 0.05, $n$ = 5).

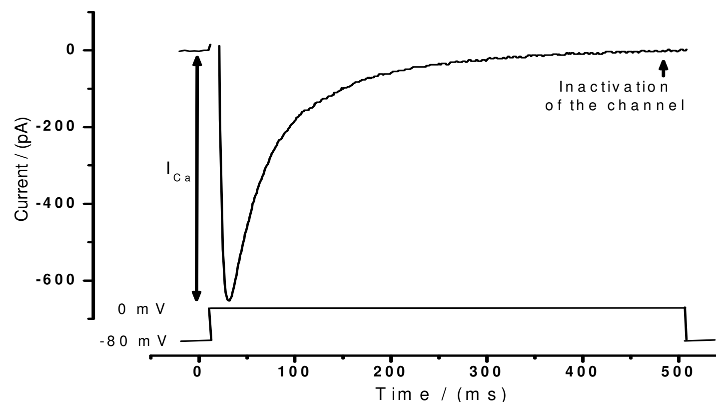


**Figure 1**. Typical individual calcium current trace obtained in our experiments. The graphic shows the current amplitude ($I_{Ca}$) and the time course inactivation of the channel.

The $I_{Ca}$ traces obtained shown a typical shape of L-type VOCC. To confirm this, and to exclude that the activity of the channels measured were not T-type $Ca^{2+}$ channel, the cells were challenged with BAY, a specific stimulator of L-type VOCCs, or with nifedipine, a known antagonist of L-type VOCCs. Fig. 2 illustrates a typical experiment showing the effect on the time course of the $I_{Ca}$ amplitude measured at 0 mV from a holding potential of –80 mV, where BAY (10 nM) stimulates and Nif (1 µM) inhibits the basal $I_{Ca}$ (A & B), and Nif (1 µM) inhibits the BAY-stimulated $I_{Ca}$ (B). As summarised in Fig. 3, two concentrations of BAY (10 and 100 nM) were applied to the cells and significantly stimulated $I_{Ca}$ by 186.8 ± 8.8% ($n$ = 5) and 249.9 ± 13.7% ($n$ = 5), respectively, above the control level ($p < 0.05$). On the contrary, nifedipine (1 µM) significantly reduces until a level of 17.7 ± 4.7% ($n$ = 5) the basal $I_{Ca}$ ($p < 0.05$) and also inhibits the BAY (10 nM) stimulated $I_{Ca}$ from ~187% to 71.2 ± 7.2% ($n$ = 5) of the basal $I_{Ca}$ (Fig. 3). Even so, the effects of BAY and/or nifedipine were completely irreversible upon washout of the drug. All together, these results indicate that the measured current is produced by the L-type VOCCs, confirming our initial hypothesis.

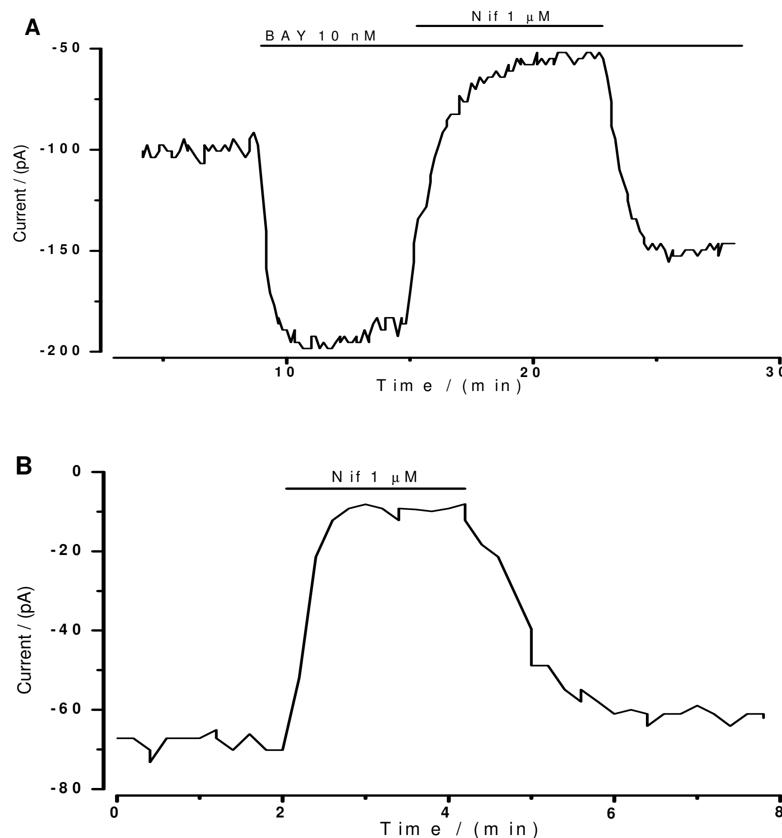


**Figure 2**. *Panel A*: time course of a typical experiment in which BAY (10 nM) stimulates $I_{Ca}$ and nifedipine (Nif; 1 µM) inhibits the BAY stimulation. *Panel B*: time course of another experiment in which nifedidipe (1 µM) inhibits the basal $I_{Ca}$. The inhibitory effects were completely irreversible upon washout of nifedipine.

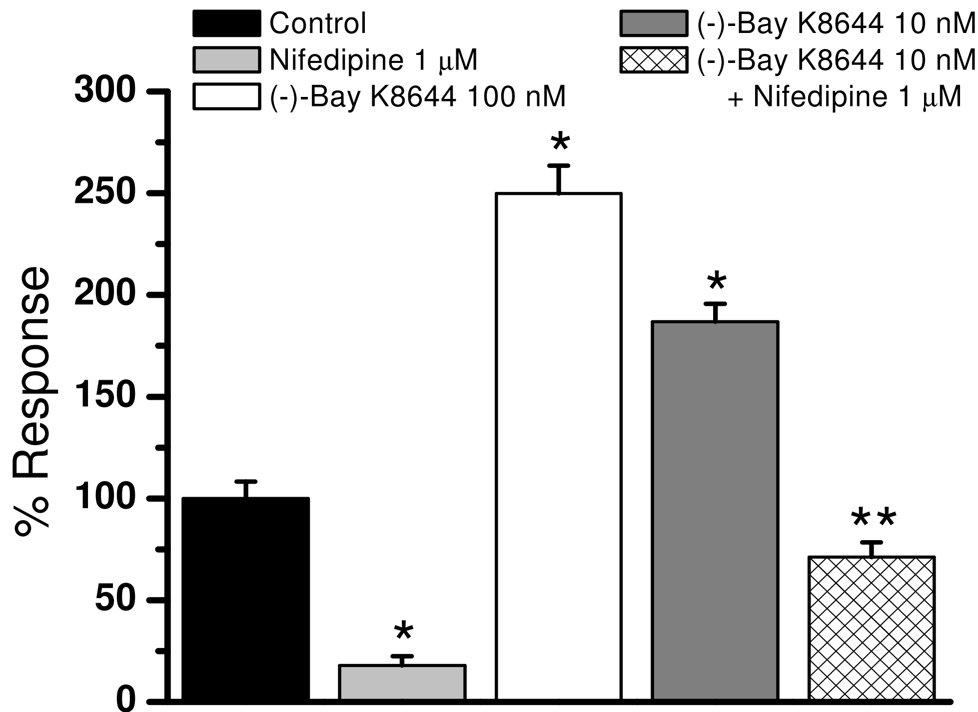


**Figure 3**. Effects of BAY and nifedipine on $I_{Ca}$. Summary of the effect of BAY (10 and 100 nM) on basal $I_{Ca}$, and the effects of nifedipine (1 μM) on basal and BAY-stimulated (10 nM) $I_{Ca}$. Each column represents the mean value ± S.E.M for at least 5 experiments. Basal $I_{Ca}$ was considered as 100%. * $p < 0.05$ with respect to basal $I_{Ca}$; ** $p < 0.05$ when compared with BAY-stimulated $I_{Ca}$ (10 nM).

### *Effects of ßES and progesterone on basal $I_{Ca}$*

As a proposed mechanism of ßES and progesterone to produce vasodilatation is the inhibition of L-type VOCCs, we tested the effect of these steroids on basal $I_{Ca}$ to analyse this possibility. Fig. 4A shows a typical experiment in which different concentrations of ßES (10, 30 and 100 μM) inhibited basal $I_{Ca}$, while Fig. 4B shows a similar effect of progesterone (10, 30 and 100 μM) in other experiment performed with an A7r5 cell. Fig. 5 summarises the results of several similar experiments in which ßES and progesterone at these three concentrations (10, 30 and 100 μM) inhibited basal $I_{Ca}$ in a concentration dependent way. As shown, both steroids inhibited significantly the basal $I_{Ca}$ only at concentrations up to 10 μM, and the effect of progesterone at 30 μM and 100 μM (48.3 ± 4.7% and 18.2 ± 4.5%, respectively; $n = 4$), was more powerful than the effect of ßES at the same concentrations (76.6 ± 5.5% and 65.9 ± 6.8%, respectively; $n = 4$; $p < 0.05$). These results indicate that either ßES or progesterone inhibits L-type VOCCs, and probably this is the cause of their vasodilator effect.

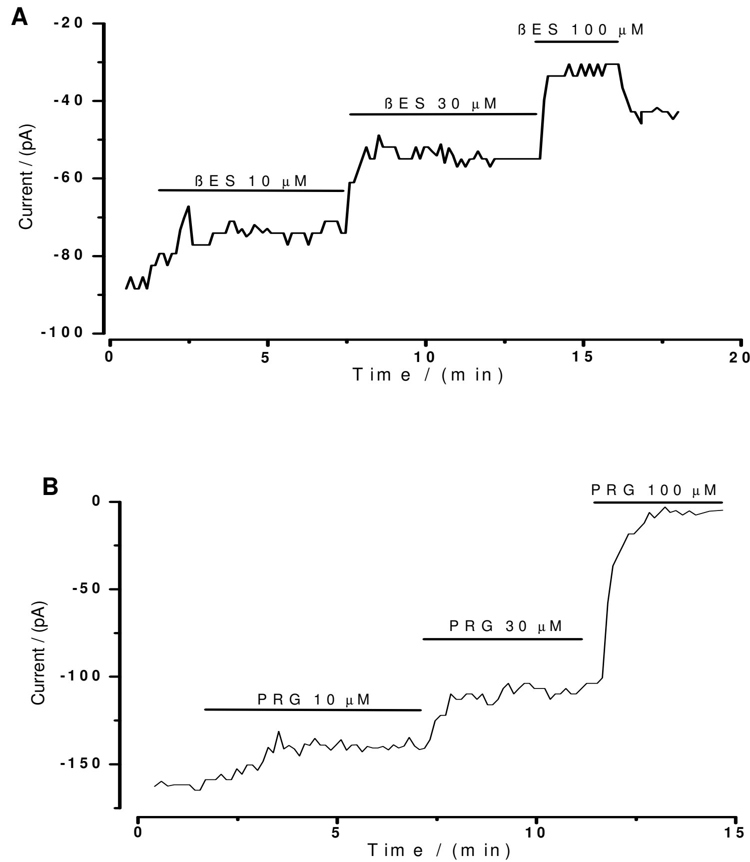


**Figure 4**. Time course of two typical experiments in which basal $I_{Ca}$ was inhibited by: *Panel A)* βES (10, 30 and 100 μM); *Panel B)* Progesterone (10, 30 and 100 μM).

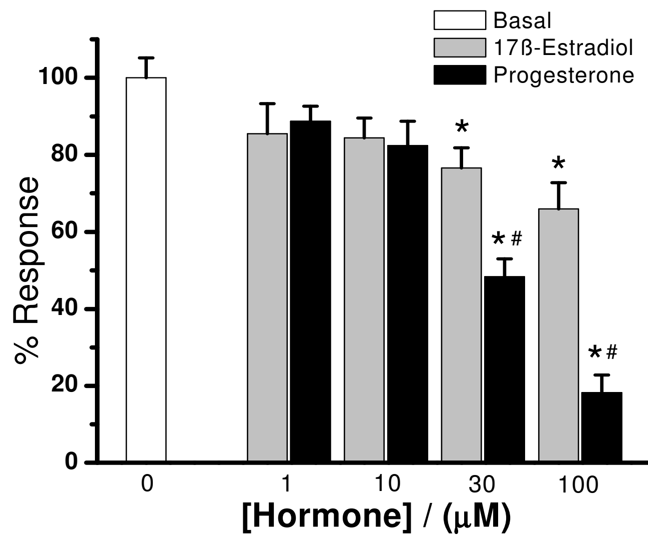


**Figure 5**. Effects of ßES and progesterone on basal $I_{Ca}$. Summary of the inhibitory effects on A7r5 basal $I_{Ca}$ of βES (1-100 μM) and progesterone (1-100 μM). Each point represents the mean value ± S.E.M for at least 3 experiments. *$p < 0.05$ *versus* basal $I_{Ca}$. # $p < 0.05$ when compared with βES effect at the same concentration.

***Effects of ßES and progesterone on BAY-stimulated $I_{Ca}$***

To further characterize the inhibitory effects of ßES and progesterone on vascular LTCC, we analyse their effect on the $I_{Ca}$ stimulated by the LTCC agonist BAY. Fig. 6A shows a typical experiment in which different concentrations of ßES (10, 30 and 100 µM) inhibited the $I_{Ca}$ stimulated by BAY (10 nM), while Fig. 6B shows a similar experiment in which progesterone (10, 30 and 100 µM ) also inhibited the $I_{Ca}$ stimulated by BAY (10 nM). As indicated before (Fig. 3), the stimulatory effect of BAY (10 nM) was 186.8 ± 8.8% (*n* = 5) of the basal $I_{Ca}$. ßES and progesterone at a concentration of 1 µM did not inhibit the $I_{Ca}$ stimulated by BAY (10 nM) (Fig. 7).

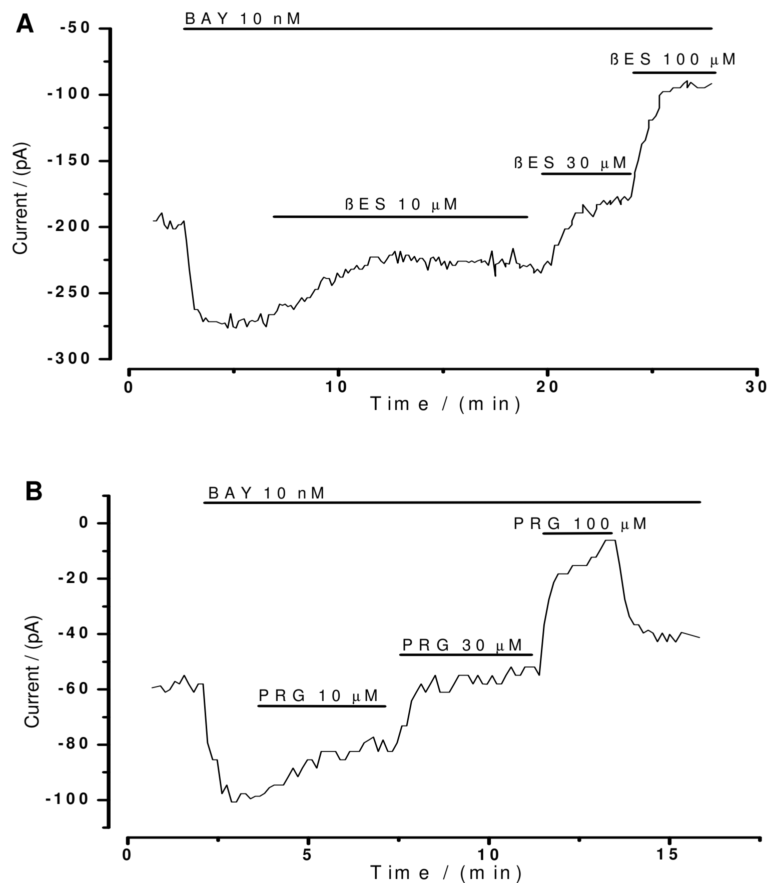


**Figure 6.** Time course of two typical experiments in which $I_{Ca}$ is stimulated by BAY (10 nM), and posterior this BAY-stimulated $I_{Ca}$ is inhibited by: *Panel A)* βES (10, 30 and 100 µM); *Panel B)* Progesterone (10, 30 and 100 µM).

However, a significant inhibition of BAY-stimulated $I_{Ca}$ was obtained with 10 µM of βES or progesterone (167.0 ± 8.0%; *n* = 5 and 160.8 ± 5.3%; *n* = 5, respectively; Fig. 7), a concentration that was inefficient to inhibit basal $I_{Ca}$ (Fig. 5). Maximal inhibition of BAY-stimulated $I_{Ca}$ by βES was obtained at 100 µM

where $I_{Ca}$ decreases until 131.1 ± 13.7% ($n$ = 5), an effect not statistically different to that induced by βES 30 μM (135.1 ± 12.4%, $n$ = 4; $p > 0.05$; Fig. 7). As illustrated in Fig. 7, the inhibition of progesterone 30 μM on BAY-stimulated $I_{Ca}$ is similar to that produced by βES at the same concentration (136.6 ± 15.7%, $n$ = 4; $p > 0.05$; Fig. 7). However, an upper concentration of progesterone (100 μM) not only inhibited completely the stimulation of BAY, but also reduced the $I_{Ca}$ until 25.1 ± 18.2% ($n$ = 5) of the basal level ($p < 0.05$). Even so, the inhibitory effect on both steroids on BAY-stimulated $I_{Ca}$ is dependent on the concentration.

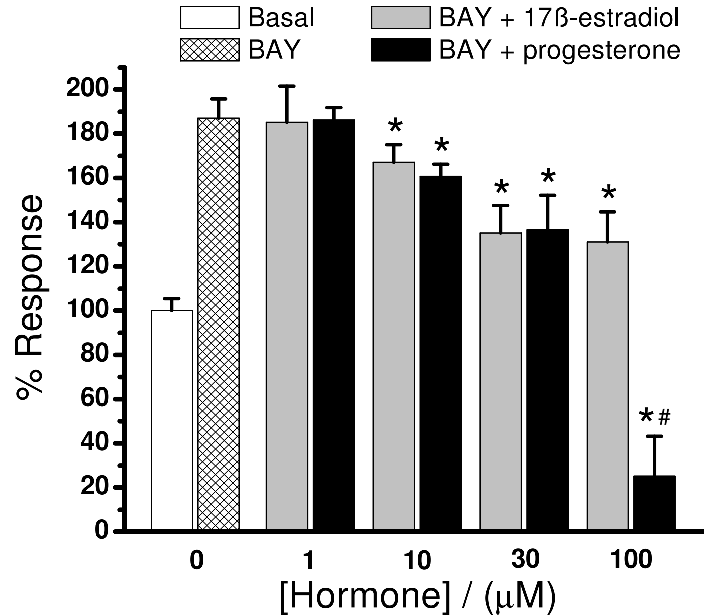


**Figure 7.** Effects of ßES and progesterone on BAY-stimulated $I_{Ca}$. Summary of the inhibitory effects of βES (1-100 μM) and progesterone (1-100 μM) on the $I_{Ca}$ stimulated by BAY (10 nM). Each point represents the mean value ± S.E.M for at least 3 experiments. *$p < 0.05$ *versus* BAY-stimulated $I_{Ca}$. # $p < 0.05$ when compared with βES effect at the same concentration.

## Discussion

In the present study, we analyzed the effect of βES and progesterone on the activity of LTCC in rat aorta cells. These voltage-dependent slow inactivated inward currents were obtained in A7r5 cells by large depolarisation of the plasma membrane. In our study we firstly characterised electrophysiologically and pharmacologically the $Ca^{2+}$ current in vascular smooth muscle cells from rat aorta (A7r5 cells) to insure the L-type nature of this current. Afterwards, we demonstrated a non-genomic inhibitory effect induced by the sex hormones studied (ßES and progesterone) on LTCC. Concerning this effect, progesterone was more efficient inhibiting vascular LTCC than βES.

In our experiments we avoid the interference of $K^+$ outward currents by the presence in the intracellular solution of TEA, an inhibitor of voltage-gated ($K_V$) and $BK_{Ca}$ potassium channels [31], and by the replacement of $K^+$ by $Cs^+$. The

characterisation of the $Ca^{2+}$ current was made by analysing the $Ca^{2+}$ traces shape, indicating a L-type current because its low inactivation time [32]. The A7r5 cells were also challenged with dihydropyridine modulators of LTCC. Thus, the perfusion of the cells with BAY, a known agonist of this type of channels [31], clearly stimulate the basal $Ca^{2+}$ current. On the other hand, nifedipine, a selective antagonist of LTCC [31], significantly blocked either basal or BAY-stimulated $Ca^{2+}$ current. All together, these results guarantee that the $Ca^{2+}$ currents measured in this study were L-type ($I_{Ca}$).

Concerning the nature of the ßES and progesterone effects, as indicated in the introduction, several authors described an inhibitory effect on LTCC on different animal blood vessels. Now, our results revealed a rapid concentration-dependent inhibitory effect on basal $Ca^{2+}$ current of both steroids, which indicates that these sex hormones have the ability to block LTCC in vascular smooth muscle cells from rat aorta. These results agree with previously reported by Zhang et al. [25], that showed an inhibitory effect of ßES on the basal L type $Ba^{2+}$ current in A7r5 cells. Also, inhibition of L-type $Ca^{2+}$ channel current by progesterone was previously observed in rat vascular smooth muscle cells from rat [33]. Nakagima *et al*. [17] determined that, while ßES 10 μM inhibited basal L type $Ba^{2+}$ current in A7r5 cells, progesterone 30 μM failed to affect these current. In contrast with our results, in that work the inhibitory effect of ßES on LTCC was significant at 10 μM and progesterone had no effect on LTCC, although these authors did not use sex hormones concentrations higher than 30 μM. Divergently, our results showed a more powerful effect of progesterone than βES at high concentrations (100 μM).

Instead the inhibitory effect of the sex hormones used on basal $I_{Ca}$, we observed inhibition of BAY-stimulated $I_{Ca}$ induced by ßES and progesterone. These measurements have not been referred before in the literature, and strongly confirm the inhibitory effect of LTCC by ßES and progesterone. Furthermore, these hormones more efficiently inhibited the BAY-stimulated than basal $I_{Ca}$, because their effect is patent at 10 μM, a concentration that does not inhibit basal $I_{Ca}$. In agreement with the data showed before about the effects on basal $I_{Ca}$, a concentration of 100 μM of progesterone inhibits more strongly than βES the BAY-stimulated $I_{Ca}$. This last inhibition completely annulated the BAY stimulatory effect, even more, the inhibitory effect achieved the level of ≈25% of basal $I_{Ca}$, indicating that, at this concentration, progesterone almost annulled the $Ca^{2+}$ current.

The genomic classical way of action of sexual hormones like ßES and progesterone is well known. They diffuse across the membrane of cells and bind to specific cytoplasmic receptors to form complexes that migrate to the nucleus, where they act as genetic transcription factors [34]. Therefore, this way needs some time to produce physiological effects. On the contrary, the inhibitory effects of ßES and progesterone observed in this study were rapid and reversible, because the effects disappeared after drug washing. Previously, some author already describes the existence of a non-genomic mechanism induced by sex steroids which regulates the vascular tone [35, 36]. Now, our results suggests that the βES and progesterone effects mismatch the classic genomic pathway of sex

hormones action, also indicating that this effect is due mediated by a non-genomic pathway. Whether the rapid effects of ßES or progesterone occur by the interaction of these hormones with their classic receptors, perhaps placed in the cell surface, or with a hypothetical new type of receptors is a matter that remains unsolved and need further investigations to be clarify [35, 37-39]. However, the antagonism of βES or progesterone classic receptors did not affect their vasodilator effect on rat aorta [40]. Even more, some works showed that ßES conjugated to bovine serum albumin, a steroid not membrane permeable analogue, still induced vasodilatation on rat arteries [35]. These last evidences, suggest that these hormones cause vasorelaxation acting through an unknown membrane receptor. In our case, it is also possibly to hypothesize that ßES and progesterone could block LTCC by direct binding to the channel protein, like other known $Ca^{2+}$ antagonists –i.e. nifedipine or verapamil. Mugge et al. showed that increase of cyclic nucleotide levels was associated with the vasodilator effects of ßES in human coronary artery [41], indicating a participation of a second messenger which, consequently, rule out the direct LTCC inhibition hypothesis. The implication of such an indirect mechanism has been recently proposed by White et al. [28] in human coronary arteries, where ßES would cause vascular smooth muscle cell repolarization and consequently VOCCs closing, by $BK_{Ca}$ activation through a cyclic guanosine 3',5'-cyclic monophosphate-dependent mechanism. In any case, further studies must be done to clarify the mechanism of non-genomic action of sexual hormones, and to justify the differential effects observed between progesterone and βES.
In summary, our results showed an inhibition on $I_{Ca}$ induced by ßES and progesterone, which confirms the previously observed vasodilator effects of these hormones in different animal vascular beds and humans [4, 6]. The steroids studied inhibit basal $Ca^{2+}$ current and also, and more powerfully, the LTCC agonist-stimulated $I_{Ca}$ (BAY-stimulated). As the blockage of LTCC, by the sex steroids studied, will reduce intracellular free $Ca^{2+}$ concentration, the vascular smooth muscle cells relax. Thus, the inhibition of $Ca^{2+}$ current in rat aorta cells by progesterone and βES is associated with the vasodilatation, a connection that was already reported in the case of progesterone in rat [42]. Our data, also correlate with the idea of a rapid vasorelaxation effect of progesterone and ßES through a mechanism independent of the endothelium, reported by other authors [40, 43], since $Ca^{2+}$ influx inhibition on smooth muscle cell directly facilitates muscle relaxation. Thus, independently of a possible role for endothelium [15, 44], vascular tissue relaxation by ßES and progesterone will be possible by their effects on LTCC.
In resume, the results of this study suggest that ßES and progesterone blocked LTCC in A7r5 cells by a non-genomic way of action and they help to understand the vasodilator mechanism of these hormones previously reported. In turn, these data contribute to confirm the evidence that the effect of hormone replacement therapy protects against the development of cardiovascular disease in postmenopausal women [45].

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