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Mifepristone is a Vasodilator Due to the Inhibition of Smooth Muscle Cells L-Type Ca^{2+} Channels

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Abstract

Derived from the estrane progestins, mifepristone was the first synthetic steroid of this class employed as abortifacient in the first months of pregnancy. Mifepristone reduces high potassium-induced contraction and prevents calcium-induced contraction. At the vascular level, mifepristone induces direct relaxation in rat and human arteries, and this effect seems to be endothelium- and NO independent, suggesting that the vascular smooth muscle is its target. Moreover, mifepristone's effect could involve the modulation of different calcium channels. The aim of the present study is to analyze the involvement of calcium channels in the relaxation induced by mifepristone on vascular smooth muscle cells (VSMCs). Planar cell surface area (PCSA) technique was used to analyze the effect of mifepristone on the VSMC contractility, and the whole cell configuration of patch-clamp technique to measure the activity of L-type Ca^{2+} channels (LTCC) in A7r5 cells. Regarding the PCSA technique, mifepristone induced relaxation of the VSMC previously contracted by different agents. Also, a rapid inhibitory effect on basal and BAY K8644-stimulated calcium current was observed, which indicates that this drug has the ability to block LTCC. These results suggest that mifepristone induces relaxation on the VSMCs due to the inhibition of the calcium channels.

Keywords

mifepristone, calcium channels, relaxation, A7r5 cells

Introduction

The discovery of the first highly effective progesterone antagonist was made in the 80s and was designated as mifepristone or RU 486.[1-3] Structurally, mifepristone (RU 486) is a member of a class of compounds that is related to steroid hormones and is derived from the estrane progestins.[4] It was the first synthetic steroid of this class employed as an abortifacient, due to the blockage of progesterone action which increases the uterine contractility, sensitizes the myometrium to prostaglandins, and elicits cervical maturing.[4,5] As this is a drug used for early termination of pregnancy (TOP) and can affect vascular function, it is important to know the extent of its vascular effects and the mechanism(s) involved. In relation to the effect of mifepristone, there are 2 different mechanisms. In the genomic mechanism, this signaling drug modulates gene transcription due to the interaction with nuclear receptors. However, this drug can also induce rapid (seconds to minutes) nongenomic effects, which are reversible and insensible to transcription and protein synthesis inhibitors.[4,6,7] Regarding the genomic mechanism, mifepristone exhibits a strong affinity for the progesterone and glucocorticoid nuclear receptors and exerts a competitive antagonism in both receptors. Apart from these receptors, mifepristone has the ability to bind to a lesser extent to the androgen nuclear receptors. Thus, mifepristone is a potent antiprogesterone and antiglucocorticoid and a weak antiandrogen drug.[6,8]

Concerning the nongenomic effect of mifepristone, only a few studies described this effect. First, Haluska et al showed that during late pregnancy of the rhesus macaque, mifepristone does not induce delivery and may even cause a decrease in uterine contractile activity.[9] Moreover, Wolf et al showed that mifepristone has a nongenomic effect decreasing monkey uterine contraction.[10] More recently, Perusquia et al observed a relaxing effect induced by mifepristone in the rat myometrium that was slightly higher than the produced by progesterone.[11] Moreover, Perusquia and collaborators observed that the treatment with mifepristone reduces high potassium-induced contraction and prevents calcium-induced contraction in depolarized myometrium tissue and relaxes the oxytocin and

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prostaglandin-induced contractions.[4] These authors suggested that this effect is due to nongenomic mechanisms, since the relaxation produced by mifepristone may involve a reduction in calcium influx. Furthermore, they suggested that mifepristone may block different types of Ca^{2+} channels.[4,7] However, only Perusquia et al and Cairrao et al have demonstrated the vasorelaxant effect in vascular beds.[7,12]

However, it also induces nongenomic effects by mechanisms that were not elucidated yet. These actions induce smooth muscle relaxation and are responsible for side effects of mifepristone. Therefore, the purpose of this study was to analyze the mechanisms and the involved effectors implicated in the vasodilator effect of mifepristone. These effectors could be target of other drugs or endogenous substances, and this knowledge could disclose possible interactions. To elucidate this, the mifepristone effect on cell contractility and on calcium channels activity (I_{Ca}) was analyzed in A7r5 cells by planar cell surface area (PCSA) and the whole cell configuration of the patch-clamp technique, respectively.

Methods

Cell Culture of Vascular Smooth Muscle Cells

The A7r5 cells are a commercial cell line from aorta smooth muscle cells from rat (Rattus norvegicus) embryo that have the same typical properties of smooth muscle cells and are currently used as a model of vascular smooth muscle cells. This is a vascular smooth muscle cell line obtained from embryonic rat aorta (Promochem, Spain). Cells from A7r5 line from cultures performed in different flasks were used. The cells were grown in culture medium Dulbecco Modified Eagle Medium/Nutrient Mixture F-12 Hams (DMEF-F12; Sigma-Aldrich, Portugal) supplemented with $NaHCO_3$ (1.2 μg/L), L-ascorbic acid (20 μmg/L; Sigma-Aldrich), bovine serum albumin (0.5%; Sigma-Aldrich), heat-inactivated fetal 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 to 48 hours. 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 realization of the electrophysiological experiments or plated in specific Petri dishes for contractility experiments.

Contractility Experiments in Cell Culture of Vascular Smooth Muscle Cells

The studies on cell contractility with VSMCs were performed using the PCSA as described previously.[13,14] This technique allows the study of the changes in cell surface area by recording images of the cells and analyzing the decrease or the increase in the area showing cell contractility or relaxation, respectively.

Briefly, the A7r5 cell line was sparsely seeded onto 6-well culture plates in culture medium. After cell attachment, confluent cells were placed in culture medium without FBS (FBS-free culture medium) 24 hours before trypsinization, which was made using a solution of trypsin (0.3%) in a Ca^{2+}–Mg^{2+}-free, phosphate-buffered solution with EDTA (0.025%). After trypsinization, the cells were plated (500 μL) in specific Petri dishes coated with collagen (5 μg/cm^2) and placed at 37°C in an atmosphere of 95% air and 5% CO_2 during 4 hours. After this incubation period, the cells were washed 4 times with 500 μL of phosphate-buffered saline.

The cells were observed using an inverted fluorescence microscope (Zeiss Axio Observer Z1, Jena, Germany). Microphotographs of groups of 2 or more cells were taken along the length of each experiment. The microphotographs were taken before and after ($\sim$20 minutes) the addition of the different drugs and agents. This waiting time ($\sim$20 minutes) is the time needed to obtain a maximal response in which a plateau phase of the effect is achieved. The pictures of the cells were taken by a high-speed monochrome digital camera Axio Cam Hsm (Zeiss, Jena, Germany) and analyzed in a computer (HP Z800 Workstation) using the Axionvision software (Zeiss, Jena, Germany). This analysis consisted in the measurement of the cell area using the supplementary "Automatic Measurement program" (Zeiss). To assure variability in the data, the same experimental protocol concerning the drug addiction was performed in different cells.

Electrophysiology Experiments

The whole cell configuration of patch-clamp technique was used to analyze the L-type Ca^{2+} channels (LTCC) current ($I_{Ca,\ L}$). To analyze the $I_{Ca,\ L}$, the control external solution containing (mmol/L) NaCl 124.0, $CaCl_2$ 5.0, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (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 MΩ), 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 seconds to 0 mV test potential during 500 ms to measure $I_{Ca,\ L}$.

Basal $I_{Ca,\ L}$ was measured 3 to 5 minutes 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°C-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). Currents were sampled at a frequency of 10 kHz and filtered at 0.1 kHz using the analog–digital interface Digidata 1322A (Axon Instruments, Sunnyvale, California, USA) connected to a compatible computer with the Pclamp8 software (Axon Instruments). The external solution was applied to the cell proximity by placing the cell at the opening of a 250 μm

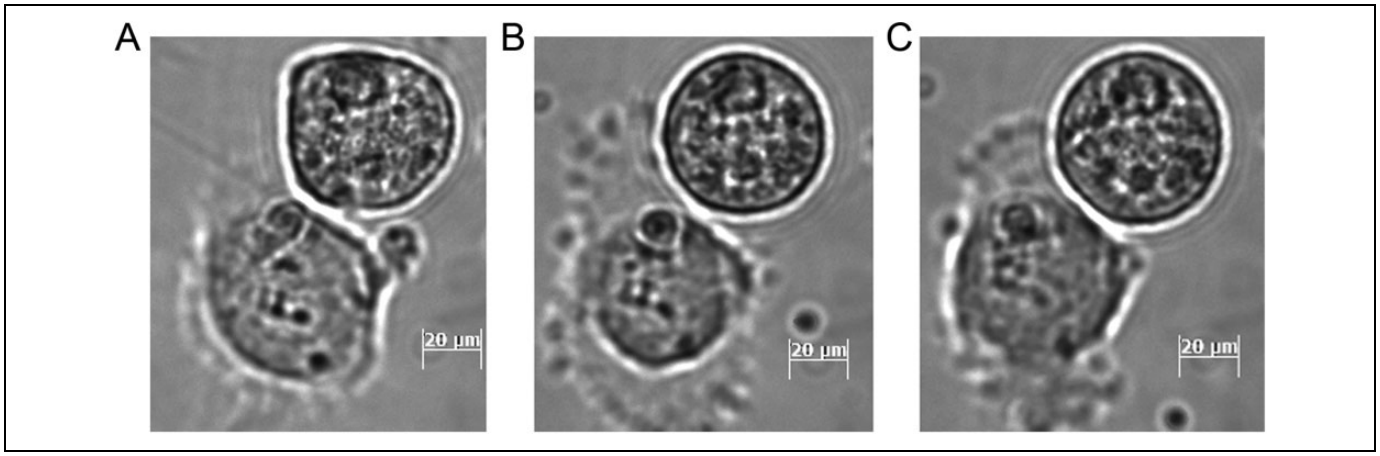

Figure 1. Pictures from a typical contractility experiment with A7r5 cells using the planar cell surface area (PCSA) technique. A cell in absence of drugs (A) has an area of 1559 02 μ^2 was contracted by noradrenaline 1 µmol/L (B) showing a decrease in the area to 1366 67 μ^2; after contraction, the application of mifepristone relaxed the cells (C) until an area of 1402 87 μ^2.

inner diameter capillary tube flowing at a rate of 20 µL/min. The basal and BAY K864-stimulated (10 nmol/L) $I_{Ca,\ L}$ were studied in the presence of different concentrations of mifepristone (0.1-100 µmol/L) dissolved in the external solution.

Drugs

Nifedipine, BAY K8644, serotonin (5-HT), noradrenaline (NA), NaCl, $CaCl_2$, HEPES, TEA, KCl, CsCl, $MgCl_2$, Na-ATP, Na-GTP, and EGTA were purchased from Sigma-Aldrich Química (Sintra, Portugal), except mifepristone, which was purchased from Biogen Cientifica (Madrid, Spain).

Mifepristone, BAY K8644, and nifedipine were initially dissolved in ethanol, and 5-HT and NA were made up in distilled water. Appropriate dilutions in the corresponding electrophysiology external solution were prepared every day before the experiment. Final concentration of ethanol never exceeded 0.02% 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 $\pm$ standard error of the mean of *n* experiments. Comparison among multiple groups was analyzed by using a 1-way analysis of variance followed by Tukey post hoc test to determine significant differences among the means. Comparison between 2 groups was analyzed using Students *t* test. Probability levels lower than 5% were considered significant ($P < .05$).

The $I_{Ca,\ L}$ amplitudes were automatically calculated between the maximum current peak and the stable current plateau near the final of the every 8-second pulse. The $I_{Ca,\ L}$ variations induced by the different drugs used are expressed as a percentage of the basal or BAY K8644-stimulated $I_{Ca,\ L}$.

Results

Effects of Mifepristone on Cellular Contractility

The changes in the cell area of A7r5 cells were quantified along the time and in presence or absence of different drugs (Figure 1). The maximal concentration of ethanol was 0.02%, the vehicle used to dissolve mifepristone did not affect basal (4.84% $\pm$ 0.92%, n = 8) or contracted by NA (9.63% $\pm$ 6.01%, n = 6) or by 5-HT (4.15% $\pm$ 2.95%, n = 8).

The effect of mifepristone (0.1-100 µmol/L) was studied after contraction of A7r5 cells by 5-HT and NA. These 2 contractile agents are physiological regulators of vascular contractility due to stimulation of their respective receptors.

Figure 2 summarizes the results of several experiments in which mifepristone induced a relaxant effect on cells previously contracted by NA (0.1 µmol/L) or by 5-HT (1 µmol/L). As shown in the figure, this effect is concentration dependent, since the percentage of relaxation increases with cumulative concentrations of the drug. However, in cells precontracted with 5-HT, the relaxant effect of mifepristone is lower from 2 concentrations, 10 and 100 µmol/L ($P < .05$, Student *t* test) than in cells contracted by NA, wherein the relaxant percentage varies between 32.99% and 57.06% in 5-HT contracted cells and between 40.93% to 82.49% when cells were contracted by NA. Thus, according to these results mifepristone induces relaxation of the A7r5 cells contracted either by NA or 5HT.

Effects of Mifepristone on LTCC

The whole-cell patch clamp technique was used to record LTCC currents in A7r5 cells.[15] The mean value of $I_{Ca,\ L}$ density was of 0.45 $\pm$ 0.06 pA/pF (n = 36). Basal current amplitude was measured 3 to 5 minutes after patch break to allow the equilibration between the pipette and the intracellular solutions.

Figure 3 summarizes these effects and shows that at concentrations of 10 and 100 µmol/L, mifepristone inhibits basal I_{Ca}, and this inhibition is rapid and reversible after washout. These

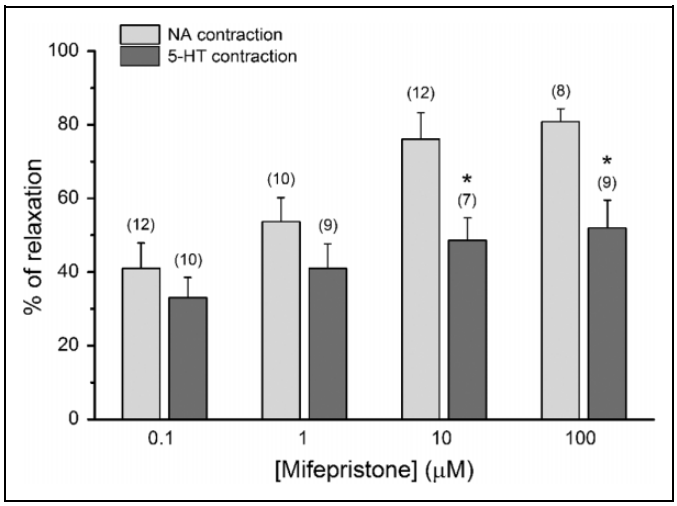

Figure 2. Relaxant effect of mifepristone (0.1-100 μmol/L) in A7r5 cells contracted with noradrenaline (0.1 μmol/L) and serotonin (1 μmol/L). Each column represents the mean value and the lines the standard error of the mean (SEM) of the number of experiments stated above the columns. *$P < .05$ noradrenaline contraction versus serotonin contraction, Student *t* test. The effects are expressed in percent relaxation over the initial area.

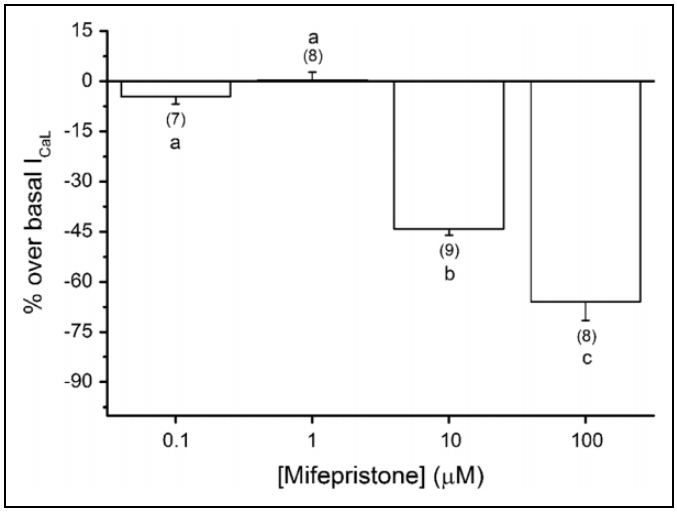

Figure 3. Effects of mifepristone on basal calcium channels activity (I_{Ca}). Summary of the inhibitory effects of mifepristone (0.1-100 μmol/L) on A7r5 basal L-type Ca^{2+} channel current ($I_{Ca,\ L}$). Each column represents the mean value and the lines the standard error of the mean (SEM) of the number of experiments stated above the columns. The effects are expressed in percent variation over the amplitude of basal I_{Ca}. Distinct letters (a, b, and c) indicate significant differences ($P < .05$, one-way ANOVA with Tukey post hoc test).

results indicate that mifepristone inhibits LTCC at the highest concentrations (10 and 100 μmol/L). Figure 4 also shows a typical experiment in which different concentrations (0.1-100 μmol/L) of mifepristone inhibited the basal $I_{Ca,\ L}$ in a reversible way.

Bay K8644, a direct activator of the LTCC, was used to analyze the mifepristone effect on stimulated I_{Ca}. The application

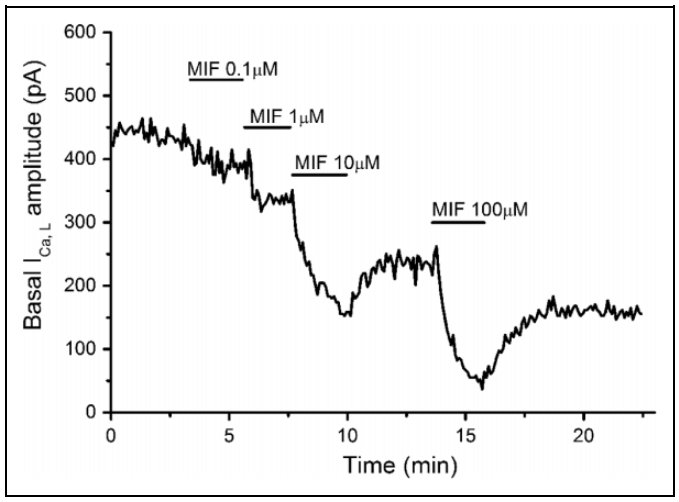

Figure 4. Original records showing the effect of increasing concentrations of mifepristone (0.1-100 μmol/L) on basal L-type Ca^{2+} channel current ($I_{Ca,\ L}$) amplitudes measured in Patch-clamp experiments performed with A7r5 cells.

of Bay K8644 (0.01 μmol/L) significantly stimulated the calcium current by 86.06% ± 14.08% (n = 6) above the basal level. On the other hand, the maximal concentration of nifedipine used (10 μmol/L; LTCC inhibitor) significantly reduced the current on 78.75% ± 3.76% (n = 7) of the basal current ($P < .05$). The effect obtained with concentrations of 0.1 and 1 μmol/L of nifedipine was not different from the maximal effect (Figure 5). Even so, the effects of BAY and/or nifedipine were completely reversible upon washout of the drug. These results indicate that the current analyzed is a $I_{Ca,\ L}$.

Figure 6 shows that mifepristone (0.1-100 μmol/L) inhibits the stimulated I_{Ca}. This effect is concentration dependent. These results indicate that mifepristone also inhibits stimulated I_{Ca}, and this inhibition is rapid and reversible after washout.

The maximal concentration of ethanol (0.01%), the vehicle used to dissolve mifepristone and nifedipine, did not affect basal nor stimulated $I_{Ca,\ L}$ (−1.93% ± 1.46%, n = 14 and 89.5793% ± 7.14%, n = 8, respectively).

Discussion

Mifepristone is used for early TOP, cervical dilatation prior to surgical TOP, preparation for prostaglandin-induced TOP during the second trimester, and expulsion of a dead fetus during the third trimester. Over the years, several studies have been conducted to study the mifepristone effects. This antiprogestin is implicated in some mechanisms, including genomic and nongenomic. Concerning the genomic mechanisms, this drug antagonizes mainly the progesterone and glucocorticoid receptors, being implicated in the modulation of gene transcription. The nongenomic effects of mifepristone don't require gene transcription or protein synthesis. According to other authors, the nongenomic effects may occur by different mechanisms: (1) direct effect in calcium channels; (2) binding to membrane receptors; and (3) binding to membrane nuclear receptors.

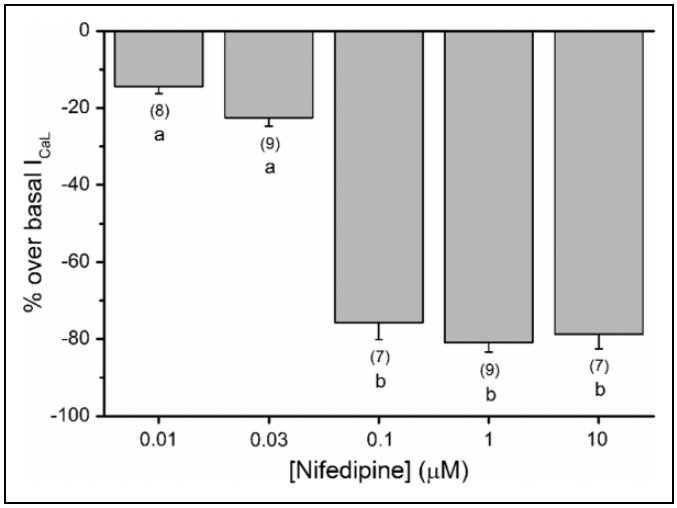

Figure 5. Effects of nifedipine on basal calcium channels activity (I_{Ca}). Summary of the inhibitory effects of nifedipine (0.01-10 μmol/L) on basal L-type Ca^{2+} channel current ($I_{Ca,\ L}$) in A7r5 cells. Each column represents the mean value and the lines the standard error of the mean (SEM) of the number of experiments stated above the columns. The effects are expressed in percent variation over the amplitude of basal I_{Ca}. Distinct letters (a and b) indicate significant differences ($P < .05$, one-way analysis of variance [ANOVA] with Tukey post hoc test).

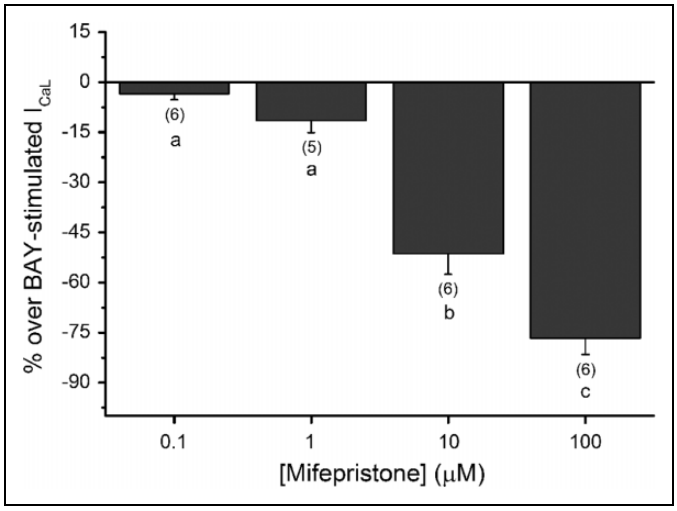

Figure 6. Effects of mifepristone on BAY-stimulated calcium channels activity (I_{Ca}). Summary of the inhibitory effects of mifepristone (0.1-100 μmol/L) on the I_{Ca} stimulated by BAY (0.01 μmol/L). Each column represents the mean value and the lines the standard error of the mean (SEM) of the number of experiments stated above the columns. The effects are expressed in percent variation over the amplitude of BAY-stimulated I_{Ca}. Distinct letters (a, b, and c) indicate significant differences ($P < .05$, one-way analysis of variance [ANOVA] with Tukey post hoc test).

The vascular effects were not extensively studied and it is crucial to analyze the extent of short-term (nongenomic) effects and the mechanisms involved. In this sense, Perusquia and collaborators suggested that the relaxation produced may involve a reduction in extracellular calcium influx, probably by blocking the voltage-operated Ca^{2+} channels.[4,7] In order to study the mechanism involved in the direct effects of mifepristone at the vascular level, we used the PCSA technique to measure cell contractility in A7r5 cells. These cells were precontracted with 2 different contractile agents, NA and 5-HT, and the effect of mifepristone was analyzed.

In previous studies using other techniques, several authors observed that mifepristone induces a rapid and reversible relaxation in vascular smooth muscle cells from rat aorta and relaxing effect on human and rat myometrium.[4,7,11] Our data also showed that the effect obtained with mifepristone is rapid and reversible, which suggested that mifepristone acts through a nongenomic mechanism. Other authors also observed similar results, with vascular smooth muscle relaxation being induced by testosterone in human umbilical artery and rat[16-22] and by progesterone and 17β-estradiol in rat aorta, rat uterus, and pig coronary arteries[12,19-21,23-25] because they are rapid in onset and not modified by inhibitors of protein synthesis.[26-29] Our results clearly show that mifepristone induces relaxation in the vascular smooth muscle cells, and this effect is dose dependent. Moreover, in cells contracted with NA, this relaxation is more pronounced than in cells contracted with 5-HT. This difference may be explained by the different vascular action mechanism of both contractile agents. Serotonin mediates the smooth muscle contractility through the activation of different receptors (5-HT_1, 5-HT_2, and 5-HT_7). The 5-HT_{1B} and 5-HT_{1D} receptors are coupled to Gi/G0 protein, which inhibits adenylate cyclase and induces contraction; 5-HT_{2A} is coupled to Gq protein and activates phospholipase C, resulting in contraction. On the other hand, the 5-HT_7 receptor is coupled to Gs protein, which stimulates adenylate cyclase inducing vasorelaxation.[30,31] The overall effect of 5-HT is the combination of the action of these receptors. It has been reported that multiple adrenoceptor subtypes, at least including α1A-, α1B-, α1D-, β1-, and β2-adrenoceptors, are expressed in vascular smooth muscle cells. Noradrenaline is involved in the contraction, by binding to α1-adrenergic receptors, which are associated with different Gq proteins, causing the increase in $[Ca^{2+}]_i$ which results in contraction.[32,33] On the other hand, the β1- and β2-adrenoceptors are coupled to Gs protein, which stimulates adenylate cyclase inducing vasorelaxation.[34,35] Moreover, since the stimulation of 5-HT and NA receptors can induce the influx of extracellular calcium via voltage- or receptor-operated calcium channels,[36] we can also hypothesize that this vasorelaxant effect induced by mifepristone in A7r5 could be due to a decrease in calcium influx by blocking the receptor-operated channel.

Knowing that the $[Ca^{2+}]_i$ is the main determinant of smooth muscle contraction/relaxation,[37] after analyzing the effect of mifepristone on the contractility, it became necessary to study the effect that this drug might have on Ca^{2+} currents. So, we analyzed the effect of mifepristone on the activity of LTCC in A7r5 cells. Like we previously showed, the calcium current measured in the A7r5 cells was only due to the LTCC channels.[12] These voltage-dependent slow inactivated inward currents were

obtained in cells by large depolarization of the plasma membrane. In our study, the electrophysiological and pharmacological characterization of the Ca^{2+} current in vascular smooth muscle cells from rat aorta was performed with the BAY (a dihydropyridine known as a specific agonist of LTCC) and previously with nifedipine (a specific blocker of LTCC) to insure the LTCC nature of this current.

Mifepristone relaxed contracted A7r5 cells in a concentration-dependent way and inhibited the basal I_{Ca}, this effect being bigger at the highest concentrations (10-100 μmol/L). These results show that this drug has the ability to inhibit LTCC activity in vascular smooth muscle cells from rat aorta. Furthermore, mifepristone also inhibits BAY-stimulated I_{Ca} at the higher concentrations (10 and 100 μmol/L) confirming the inhibitory effect on I_{Ca}. These data are according to the observed for several sex hormones, like Cairrao et al, Zhang et al, and Nakagima et al, which showed that sex hormones induce vasorelaxation, through the activation or inhibition of ionic channels.[38-42] This inhibition can be performed by different mechanisms, in a direct or an indirect manner. Concerning a direct effect, Montaño et al showed that the vasodilator effect induced by 5β-DHT selectively blocks voltage-dependent calcium channels by acting as a pure Ca^{2+} antagonist (from nmol/L to μmol/L), as well as testosterone at nmol/L concentrations.[43] The concentration range used in this study was lower than the plasma concentrations detected in patients after the administration of 200 to 600 mg mifepristone (2 mmol/L).[8] In this sense, the range of concentrations analyzed in this work is physiological.

Regarding the indirect effect of steroids, several investigators have demonstrated that the vasorelaxant effect of the sex hormones is due to increased levels of cyclic guanosine monophosphate (cGMP)[42,44] and cyclic adenosine monophosphate.[45] Cairrão et al showed that testosterone induces vasorelaxation because it increases the cGMP levels by activating particulate GC.[42] Mugge et al also showed that the increase of cyclic nucleotide levels is associated with the vasodilator effects of 17β-estradiol in human coronary artery,[46] indicating a participation of a second messenger which rules out the direct LTCC inhibition hypothesis. Concerning mifepristone, few studies tried to analyze the mechanism by which this drug induces relaxation (nongenomic effects). Perusquia et al suggested that mifepristone blocks the calcium channels in the same way as the other steroids and concluded that a nongenomic effect of mifepristone is produced before its journey into the cell for its genomic action.[4,7,47] Furthermore, Parra et al showed that cGMP is the intracellular mediator involved in the relaxant effect of mifepristone in the rat uterus, assuming that this effect may be cGMP dependent.[28] Our data seem to agree with the idea that the mechanism of mifepristone can be mediated by changes in the concentration of a cyclic nucleotide, once we obtained differences in the percentage of relaxation between NA and 5-HT.

In conclusion, our results demonstrate for the first time that there is an inhibition on I_{Ca} induced by mifepristone, which is consistent with the also observed vasodilator effect of this drug. Mifepristone inhibits basal Ca^{2+} current and also, and more powerfully, the LTCC agonist-stimulated I_{Ca} (BAY stimulated), by a rapid and nongenomic mechanism. As the blockage of LTCC reduces intracellular free calcium concentration, the vascular smooth muscle cells relax, as demonstrated by the results obtained by PCSA. Like hypothesized by Perusquia et al,[7] our data clearly shows that mifepristone induced a nongenomic relaxant effect due to the blockage of the LTCC. The mechanism of mifepristone can be direct or indirect and, in this sense, further studies must be done to clarify this subject.

Declaration of Conflicting Interests

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