

Rapid inhibition of the contraction of rat tail artery by progesterone is mediated by inhibition of calcium currents

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Abstract

Progesterone induced rapid relaxation of KCl-contracted tail artery helical strips from rats. The effect was dose dependent, with an IC₅₀ (inhibitory concentration which produces 50% of the maximal response) of 8.9 μM progesterone. The actions of progesterone were not blocked by bicuculline, indicating that in this tissue the non-genomic actions of progesterone were not mediated via a γ -aminobutyric acid (GABA)-A receptor. Fura-2 was used to measure intracellular calcium levels ($[\text{Ca}^{2+}]_i$) in isolated vascular smooth muscle cells (VSMC). Incubation of cultured VSMC for 15 min with progesterone (10 μM) resulted in an inhibition of the KCl-induced $[\text{Ca}^{2+}]_i$ increase. The whole-cell patch-clamp technique was used to examine Ca^{2+} -channel currents in the membrane of isolated VSMC. Progesterone suppressed the L-type Ca^{2+} -channel currents in cells held at a potential of -40 mV. The effects of progesterone were quickly reversed by washout in all three experimental protocols suggesting that these effects on vascular tissues are non-genomic. The correlation of the effects on all these preparations, their time course and reversibility suggested that the rapid relaxation of the rat tail artery induced by progesterone is mediated at least in part by inhibition of L-type calcium channels, leading to inhibition of calcium responses in the VSMC of this tissue.

Introduction

The early observations that pre-menopausal women have a lower incidence of cardiovascular disease than post-menopausal women and men (Kannel et al 1976; Lerner & Kannel 1986), and that hormone replacement therapy reduces the cardiovascular morbidity and mortality in post-menopausal women (Henderson et al 1991; Ettinger et al 1996), has led to the hypothesis that estrogen and perhaps progesterone have cardioprotective effects. Many studies have focussed on the chronic actions of these two steroid hormones on the cardiovascular system. Clearly estrogen (Stampfer et al 1985) and progesterone (Rylance et al 1985), alone and in combination with each other (Regensteiner et al 1991; Punnonen et al 1995; McCrohon et al 1996), have been demonstrated to improve cardiovascular function. This improvement has been associated with the favourable expression of reduced vascular reactivity, and altered expression of elements of the calcium/contraction regulatory pathway such as protein kinase C translocation (Minshall et al 1998) and the GTP-binding Rho proteins (Loirand et al 1999).

In addition to the long-term effects of these steroid hormones, which likely involve altered gene expression mediated through cytosolic/nuclear receptors, estrogen and progesterone have both been reported to have acute actions, which occur at times too short to be explained by altered gene expression. The ovarian steroid hormones were found to acutely regulate the contraction of smooth muscle in the cardiovascular system. 17β -estradiol ($17\beta\text{-E}_2$) and progesterone caused vasorelaxation in isolated human omental arteries (Belfort et al 1996). In-vitro studies have indicated that progesterone caused relaxation in canine and rabbit coronary arteries (Miller & Vanhoutte 1991; Jiang et al 1992b), pig coronary arteries (Crews & Khalil 1999;

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Murphy & Khalil 1999) and rat aorta (Perusquia et al 1996; Glusa et al 1997). One possible mechanism of these effects is believed to be the modulation of Ca^{2+} -channel activity in the cell membrane. It has been reported that $17\beta\text{-E}_2$ decreased intracellular calcium levels ($[\text{Ca}^{2+}]_i$) in porcine coronary artery (Han et al 1995) and inhibited the activity of Ca^{2+} channels in vascular smooth muscle cells (VSMC) (Shan et al 1994; Zhang et al 1994) and cardiac myocytes (Jiang et al 1992a; Sheldon & Argentieri 1995). The effects of progesterone on calcium regulation have not yet been as clearly defined. Progesterone has been reported to increase $[\text{Ca}^{2+}]_i$ by opening Ca^{2+} channels in human (Mendoza et al 1995) and mouse sperm (Shi & Roldan 1995); this action in the mouse may be mediated via γ -aminobutyric acid (GABA)-A receptors. In rat myometrium (Rendt et al 1992), progesterone increased Ca^{2+} -channel current by increasing the density of channels. In human myocardium, progesterone had no effect on the positive inotropic action of BAYK8644, suggesting that there was no direct interaction with the dihydropyridine binding site (Sitzler et al 1996). Progesterone has been reported to relax coronary arteries (Jiang et al 1992b; Crews & Khalil 1999; Murphy & Khalil 1999) and aorta (Perusquia et al 1996; Glusa et al 1997). However, progesterone was recently reported to increase both Ca^{2+} -channel and K^+ -channel currents in coronary artery myocytes (Jacob & White 2000), an action mediated via GABA-A receptors. In this study, we examined the effects of progesterone on the rat tail artery, a model of a resistance vessel. Tension in isolated tissues, as well as intracellular Ca^{2+} levels and voltage-dependent Ca^{2+} channels in isolated VSMC were investigated. The onset and reversal by wash-out of the effects were closely observed.

Materials and Methods

Chemicals

Progesterone, collagenase/dispase, elastase, trypsin inhibitor, bovine serum albumin, collagenase, insulin, fetal bovine serum, dimethyl sulfoxide (DMSO), ionomycin, tetrodotoxin, bicuculline and salts for the bath solutions were purchased from Sigma Chemical Co. (St Louis, MO). Fura-2, penta-potassium salt, and Fura-2, acetomethoxy ester were purchased from Molecular Probes (Eugene, OR).

Tension measurement in rat tail artery

Isometric contraction was measured in tail artery helical strips following the method of Pang et al (1985). Male Sprague–Dawley rats, 250–350 g, were anaesthetized with pentobarbital and the tail arteries were removed. The helical strips were cut (1.5 cm) and then suspended in a Sawyer–Bartlestone tissue bath chamber. The bath solution used was Krebs–Henseleit solution with the following composition (in mM): NaCl 115, KCl 5, CaCl_2 2.1, MgSO_4 1.2, NaH_2PO_4 1.2, NaHCO_3 25 and glucose 11. The tissue inside the bath chamber was attached to an FT.03 Grass

force displacement transducer and the tension of the tissue was recorded on a Grass 79 D polygraph (Grass Instrument Co., MA) or a Gould polygraph (Gould Inc., Cleveland, OH). The bath was continuously aerated with a mixture of 95% O_2 and 5% CO_2 and maintained at 37°C . Before experimentation, the tissue was allowed to equilibrate for 60–120 min under a resting tension of 0.7 g. During the equilibration period, tissue in the bath chamber was washed with Krebs–Henseleit solution every 15 min. After equilibration, the tissue was tested for tension generation in response to the stimulation of 60 mM KCl. The stimulation procedure was repeated 3 times with half-hour intervals. Acetylcholine was found to have no effect in these tissues. Phasic tension was measured after an initial KCl (60 mM) stimulation, and the tension generated taken as control. After washing, a single dose of progesterone (0.1–100 μM) was added and incubated for 20 min before a second challenge with the same dose of KCl. The tension generated was measured and compared with the control.

Culture of vascular smooth muscle cells

VSMC were cultured following the method of Wang et al (1989). Male Sprague–Dawley rats, 150–350 g, were anaesthetized and the tail arteries were removed. Under the dissecting microscope, the arteries were cleaned of the surrounding connective tissues. The arteries were cut open longitudinally into 2–4-mm strips. The tissue was immersed in chilled Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY) and washed occasionally during the cleaning. After a 30-min rest at 4°C , the tissue was transferred to the first enzyme solution composed of: collagenase/dispase (1.5 mg mL^{-1}), elastase (0.5 mg mL^{-1}), trypsin inhibitor (1 mg mL^{-1}) and bovine serum albumin (BSA; 2 mg mL^{-1}) in HBSS with a low calcium concentration (0.2 mM). The tissue was incubated in the first enzyme solution for 60 min in a CO_2 incubator (5% CO_2 and 95% air) at 37°C before being washed with Ca^{2+} - and Mg^{2+} -free HBSS (HBSS-CMF; Gibco) 3 times. The second enzyme solution contained collagenase (1 mg mL^{-1}), trypsin inhibitor (0.3 mg mL^{-1}) and BSA (2 mg mL^{-1}) in HBSS-CMF. The tissue was incubated in the second enzyme solution for 50–60 min. After the enzyme incubation, the tissue was washed with HBSS-CMF and triturated for 3 min using a fire-polished Pasteur pipette. This was done to dissociate the smooth muscle cells from the tissue. The cells were placed at rest in HBSS-CMF for 30 min at 4°C . Ca^{2+} was then gradually added to the solution to a final concentration of 2 mM.

The cells were seeded in 3-cm Petri dishes in Dulbecco's modified Eagle medium (DMEM; Gibco) solution with insulin (31 $\mu\text{g mL}^{-1}$). Four to six hours later, the DMEM solution was replaced by the complete culture medium: 10% fetal bovine serum (FBS) in DMEM. The cells were placed in the CO_2 incubator overnight. For patch-clamp studies, the cells were used within 24 h. For intracellular calcium measurement, the cells were seeded on circular glass coverslips and cultured for 4–6 days before use. Cells with voltage-gated Ca^{2+} channels were presumed

to be VSMC, since endothelial cells do not express voltage gated Ca^{2+} channels (Wang et al 1999).

Patch-clamp study

Voltage-dependent inward calcium channel currents of the cell membrane were measured using the whole-cell version of the patch-clamp technique (Hamill et al 1981). The isolated cells grown in a 3-cm Petri dish were washed 2–3 times with the extracellular bath solution (see composition below). Only spherical cells with a clear border and firmly attached to the bottom of the dish were chosen for the electrophysiological experiments.

The patch microelectrode pipettes were pulled from borosilicate thin-wall glass capillary tubes (o.d. 1.2 mm, i.d. 0.9 mm; FHS, Brunswick, ME) with a two-stage microelectrode puller (Narishige PP-83, Tokyo, Japan). The tips of the pipettes were fire polished with a microforge (Narishige MF-83, Tokyo, Japan). The tip diameter was approximately 1 μm with a resistance of 2–8 $\text{m}\Omega$.

The intracellular solution used in the pipettes was composed of (in mM unless otherwise stated): Cs_2 -aspartate 70, EGTA 10, ATP- Na_2 2, MgCl_2 5, K-pyruvate 5, K-succinate 5, phosphocreatine- Na_2 5, creatine kinase 50 U mL^{-1} , HEPES 15 and glucose 5. The extracellular solution contained (in mM unless otherwise stated): BaCl_2 20, Tris 110, CsCl 5, KCl 5, glucose 20, HEPES 20 and tetrodotoxin (TTX) 0.5 μM . Ba^{2+} was the charge carrier.

In the experimental protocol, the patch pipette, controlled by a micromanipulator (Narishige Co. Ltd, Tokyo, Japan), was pressed onto the cell membrane. Once the gigaseal was established at the contact area by suction from the pipette, further suction was applied to rupture the patch and establish the whole-cell recording configuration.

The holding potential was set at -40 mV to measure the L-type inward Ca^{2+} -channel currents. A command voltage of $+10$ mV was applied for 250 ms at intervals of 5 s to monitor the current change. Test pulses from -30 to $+80$ mV (at 10-mV intervals) were used to depolarize the membrane and the data were recorded in order to plot the current–voltage relationship (I–V relationship). The membrane currents were monitored using a List EPC-7 patch clamp amplifier (List-Medical-Electronic, Darmstadt, Germany) and a digital oscilloscope (Nicolet Instrument Co., Madison, WI). The data were sampled using pClamp software (version 5.5) and an Axolab 1100 (Axon Instruments, Inc., Burlingame, CA) analog-to-digital converter and stored on the floppy disk of a personal computer (Zenith data system). Data were analysed and the I–V relationship curves were plotted using the pClamp and the PSI software. Peak currents were always used when analysing the data.

Intracellular calcium measurement

Fura-2 was used to measure the cytosolic free calcium ($[\text{Ca}^{2+}]_i$) in cultured VSMC. Primary cultured cells were plated on circular glass coverslips in Petri dishes for 4–6

days. When the cells were confluent on the cover slips, the culture medium was replaced, and Fura-2/AM (the aceto-methoxy ester of Fura-2) dissolved in DMSO was added to a final concentration of 3 μM . This loading process was carried out in the dark at room temperature for 50–60 min. The cells were then washed with, and kept in, recording buffer solution containing (in mM): NaCl 145, KCl 5, MgCl_2 2, D-glucose 10, NaH_2PO_4 0.5, HEPES 10 and CaCl_2 2. The coverslips with Fura-2 loaded cells were mounted in a 1-mL Sykes-Moore chamber on the stage of an inverted phase-contrast microscope (Diaphot-TMD; Nikon, Tokyo, Japan).

A Spex excitation fluorescence spectrophotometer and an IBM computer using CM3000DM program were used to measure the intensity of fluorescence of Fura-2 and to collect data during the experiments. The cells were illuminated alternately by excitation wavelengths of 340 nm and 380 nm. The intensity of fluorescence emitted from the Fura-2 loaded cells was recorded at 510 nm. $[\text{Ca}^{2+}]_i$ was calculated by using equation 1 (Grynkiewicz et al 1985):

$$[\text{Ca}^{2+}]_i = K_d(R - R_{\min}) / (R_{\max} - R) \times b \quad (1)$$

where K_d is the dissociation constant of Fura-2 for Ca^{2+} and is assumed to be 224 nM, and R is the ratio of the intensity of fluorescence measured at 340 nm and 380 nm. R_{\max} is the value of ratio R when the indicator is saturated with Ca^{2+} and R_{\min} is the value of ratio R when the indicator is in Ca^{2+} -free form; b is the ratio of fluorescence intensities measured at 380 nm under conditions of very low and saturating concentrations of Ca^{2+} . R_{\max} and R_{\min} were obtained by applying 2 μM ionomycin or 10 mM EGTA to the bath solution, respectively.

Statistics

The data were presented as mean \pm s.e. In the figures, n indicates the number of strips or number of cells used in the experiments. The paired or non-paired Student's t -test was used for comparisons between two groups. The Newman–Keul's test was applied when the comparison was among multiple groups. P values less than 0.05 were considered statistically significant.

Results

Progesterone inhibited the KCl-stimulated contraction of rat tail artery

Acute exposure of rat tail artery to progesterone inhibited the contraction induced by 60 mM KCl in a dose-dependent manner. The results are shown in Figure 1. Progesterone at a concentration of 100 μM completely inhibited the KCl-induced contraction. The solvent for progesterone used in these studies was a mixture of ethanol and DMSO (2:1) and itself had no significant effect on tension. The IC_{50} (concentration producing 50% of the maximal response)

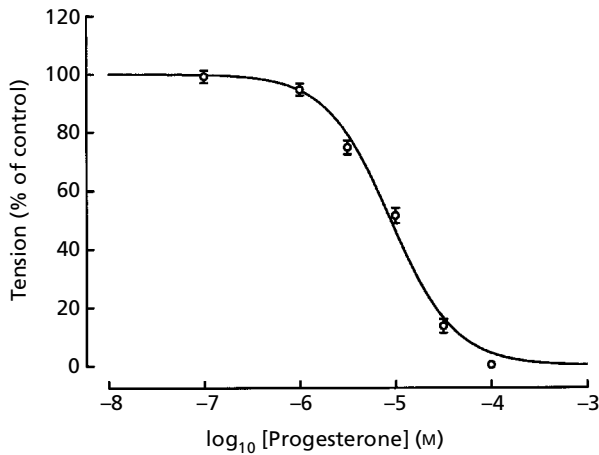


Figure 1 The effect of progesterone on contraction of the rat tail artery helical strip induced by 60 mM KCl. Each point is the mean \pm s.e.m. of 8 strips.

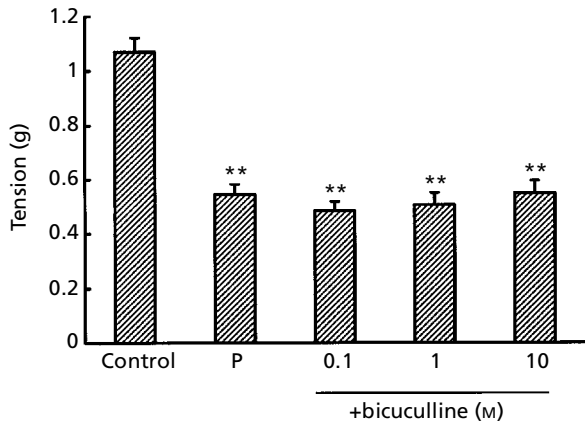


Figure 2 The effect of the GABA-A receptor antagonist bicuculline on the inhibition of contraction of the rat tail artery helical strip by 10 μ M progesterone. Bicuculline (0.1–10 μ M) was added to the bath at the same time as progesterone, and 15 min later the tissue was challenged with 60 mM KCl. P = progesterone alone. Each bar represents the mean \pm s.e.m., $n = 7$. ** $P < 0.01$ vs control (Newman–Keul’s test).

for inhibition of contraction by progesterone is $8.9 \pm 0.7 \mu$ M ($n = 8$). This method of stimulation depolarizes the tissue, and therefore activates voltage-gated Ca^{2+} channels in the tissue. Hence, under these conditions contraction will be due primarily to influx of extracellular Ca^{2+} . Others have reported that some actions on vascular smooth muscle Ca^{2+} channels may be mediated via GABA-A receptors (Jacob & White 2000). To examine this possibility, the acute vascular action of progesterone was measured in the presence of the GABA-A receptor antagonist, bicuculline. Results presented in Figure 2 show that the inhibition of KCl-induced contraction by 10 μ M progesterone of approximately 50% was not significantly altered in the presence of 0.1–10 μ M bicuculline ($n = 7$).

Progesterone inhibited the KCl-stimulated $[Ca^{2+}]_i$ increase in VSMC

Figure 3A presents the experimental recording traces showing the inhibitory effects of 10 μ M progesterone on $[Ca^{2+}]_i$ in primary cultured VSMC. The cells were sequentially stimulated with 30 mM KCl, followed by wash-out. This concentration of KCl transiently increased the $[Ca^{2+}]_i$ by approximately 4 fold from the baseline value. The measurement of the calcium response to the first KCl stimulation was taken as the control value. Cells were exposed to progesterone for 15 min between the first and second stimulation by KCl. The effect of progesterone was determined by comparing the maximal $[Ca^{2+}]_i$ of the two stimulations. In some experiments, after progesterone exposure, the cells were washed to remove progesterone from the bath, to determine whether the effect of progesterone was reversible. Figure 3 indicates that progesterone rapidly

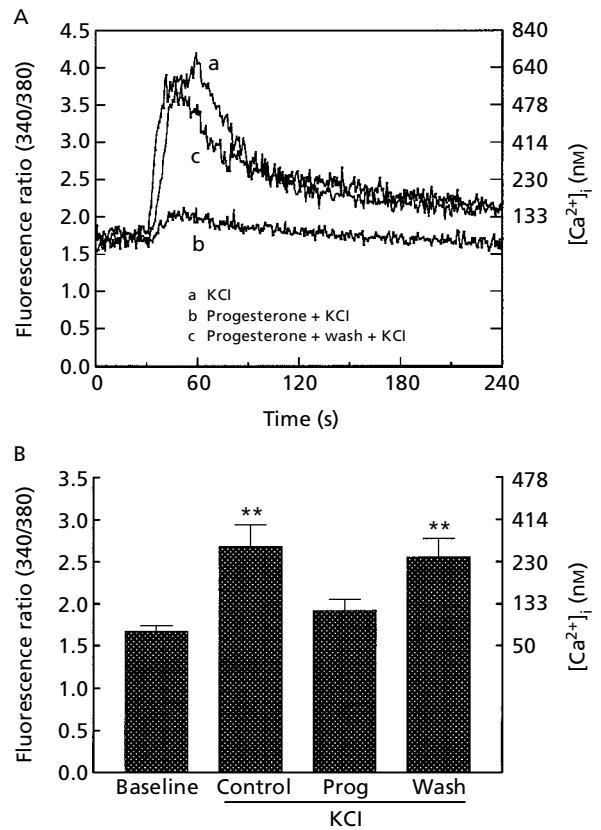


Figure 3 A. The effect of progesterone on $[Ca^{2+}]_i$ recorded in isolated vascular smooth muscle cells using Fura-2. Sample recording of: control response to exposure to 30 mM KCl (a); response to 30 mM KCl after pre-incubation with progesterone (10 μ M) for 15 min (b); and response to 30 mM KCl 15 min after washing progesterone out of the bath solution (c). B. The effect of progesterone (10 μ M) on $[Ca^{2+}]_i$ recorded in isolated vascular smooth muscle cells using Fura-2. Each bar represents the mean \pm s.e.m. $[Ca^{2+}]_i$ before (baseline) or after KCl (control, progesterone (prog) wash) stimulation with 30 mM KCl, for 6–8 recordings. The values for KCl, Prog and Wash are the peak $[Ca^{2+}]_i$ measured following addition of KCl to the bath. ** $P < 0.01$ vs control (Newman–Keul’s test).

inhibited the $[Ca^{2+}]_i$ increase induced by KCl in VSMC. The calcium response to KCl was reduced by $75 \pm 12.5\%$ from control in the presence of $10 \mu\text{M}$ progesterone. This inhibitory effect was completely reversed by washout for 15 min. Figure 3B gives a summary of a group of experiments ($n = 6-8$) indicating that progesterone incubation inhibited the KCl-induced $[Ca^{2+}]_i$ response, and that this inhibition could be reversed by washout of the progesterone-containing bath solution.

Effect of progesterone on L-type Ca^{2+} -channel currents in VSMC

Figure 4 illustrates three records of the inward L-type Ca^{2+} channel currents from the same VSMC before and after the application of progesterone ($10 \mu\text{M}$), and after washout. The currents were activated at potentials above $+10 \text{ mV}$ from the holding potential of -40 mV . The current-voltage (I-V) relationship curves plotted from this cell show the inhibitory effect of progesterone on the Ca^{2+} -channel currents at different membrane potentials. The voltage that elicited the peak current was not shifted, and the peak current was reduced by about 40% at this concentration of progesterone. Inhibition of the L-type Ca^{2+} -channel currents was first seen at 5 min after the application of progesterone. The inhibitory effect of progesterone on the Ca^{2+} currents reached a maximum at an average time of 15 min. After washout with extracellular solution for 10-15 min, the currents were returned to the control level. Figure 5 shows the concentration-dependent effects of progesterone on the Ca^{2+} -channel currents ($n = 4-8$). The effect of progesterone was significant above a concentration of $1 \mu\text{M}$. At a concentration of $1 \mu\text{M}$, progesterone suppressed the currents by $16 \pm 5.0\%$ from the control values and by $37 \pm 2.5\%$ at a concentration of $10 \mu\text{M}$. Con-

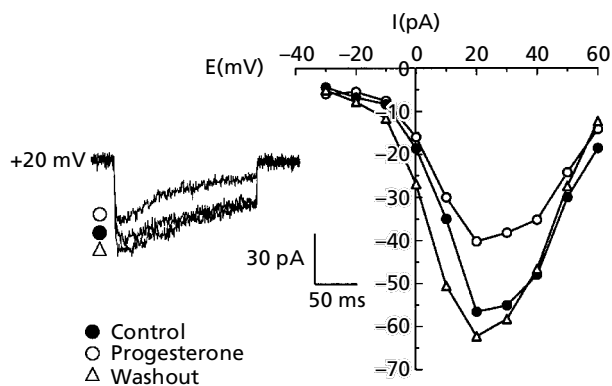


Figure 4 The effect of progesterone ($10 \mu\text{M}$) on Ca^{2+} currents recorded in isolated vascular smooth muscle cells. Ca^{2+} -channel currents were recorded using Ba^{2+} as the charge carrier. Cells were held at a potential of -40 mV , and test pulses from -30 to $+80 \text{ mV}$ (at 10-mV intervals) were applied to generate the I-V plot. The inset shows sample traces from one cell, and the I-V plot presents data from one representative cell. Currents were recorded before (●) or after (○) 15 min exposure to $10 \mu\text{M}$ progesterone, or 15 min following washout (△) of progesterone from the bath solution.

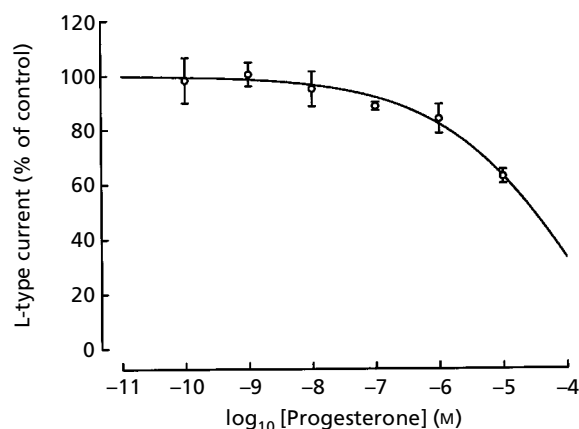


Figure 5 The dose dependence of the effect of progesterone on Ca^{2+} currents recorded in isolated vascular smooth muscle cells. Ca^{2+} -channel currents were recorded using Ba^{2+} as the charge carrier. Cells were held at a potential of -40 mV , and stepped to a test potential of 10 mV . Each point is the mean \pm s.e.m. of 4-8 recordings.

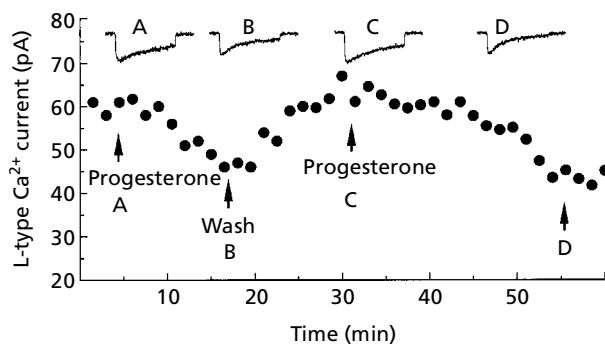


Figure 6 The time course of progesterone effects on the Ca^{2+} currents recorded from an isolated vascular smooth muscle cell. The data presented are typical for 5 cells recorded. Peak inward currents were recorded every 90 s. The cell was held at -40 mV , and stepped to a test potential of $+10 \text{ mV}$. Progesterone ($10 \mu\text{M}$) was initially applied (A). Once the maximal effect was obtained the bath solution was exchanged to remove progesterone (B). When the current returned to the control level, progesterone was applied again (C). The second application inhibited the current to the same extent as the first application (D).

centrations of progesterone above $10 \mu\text{M}$ were not investigated due to limited solubility in the bath solution. The solvent for progesterone, as described above, had no significant effect on currents.

To further investigate whether the inhibitory effect of progesterone on the Ca^{2+} channels occurred via the nuclear receptors and DNA transcription pathway, the washout procedure was employed. Figure 6 illustrates typical current recordings from a VSMC under above-mentioned holding and activating potential. The peak currents were recorded at regular intervals from a representative cell during the washout procedure. In this experiment, the cell was treated with progesterone ($10 \mu\text{M}$) after the currents were initially recorded to obtain a control value. When the

inhibitory effects of progesterone on the currents reached a maximum (approx. 15 min after progesterone application) the bath solution was washed out and replaced by normal extracellular solution. As the washout procedure continued, the inward Ca^{2+} current increased back to the control level. This recovery process took approximately 20 min. When the current remained stable, progesterone ($10 \mu\text{M}$) was applied once again. Over approximately 25 min, the current was suppressed to about the same level observed during initial progesterone exposure. For this particular cell, progesterone at $10 \mu\text{M}$ reduced Ca^{2+} currents by approximately 30% from the control value. Summary of a group of experiments ($n = 6-7$) indicates that progesterone ($10 \mu\text{M}$) caused reduction of the Ca^{2+} currents by $38 \pm 4.5\%$. This effect of progesterone was reversed after 15 min washout.

Discussion

The acute inhibitory action of progesterone seen in this study supports some previous observations made in other vascular tissues. This steroid hormone has been reported to relax coronary arteries (Jiang et al 1992b; Crews & Khalil 1999; Murphy & Khalil 1999), and aorta (Perusquia et al 1996; Glusa et al 1997). However, a recent report has also appeared which showed that progesterone increased Ca^{2+} -channel currents in coronary artery myocytes (Jacob & White 2000). The possible reasons for this difference are not certain, but may be related to difference in tissue. The rat tail artery has been reported previously to possess properties more consistent with a resistance vessel than a conduit vessel (Pang et al 1985). The difference in effects on Ca^{2+} -channel currents may also possibly be related to the observation that in the coronary artery the effect of progesterone involved a GABA-A receptor, whereas, in this study, no evidence was obtained for the involvement of this receptor. Furthermore, progesterone was reported to be vasorelaxant, suggesting the increased Ca^{2+} current did not contribute significantly to contraction of the smooth muscle. Progesterone also increased the Ca^{2+} -activated K^{+} -channel activity (perhaps via localized increase in Ca^{2+} concentration), which may have played a greater role mediating vasorelaxation.

The effective concentrations that were observed in this study are similar to the concentrations required for inhibition in other vascular tissues, and considerably higher than physiological concentrations. However, these concentrations may be reached in pregnancy or in women taking hormone replacement therapy, and therefore the vascular responses can be pharmacologically relevant. In addition to vascular smooth muscle, progesterone has been reported to directly relax airway smooth muscle (Perusquia et al 1997). In all cases this inhibitory action of progesterone appears not to be mediated by the classical steroid hormone receptor mechanisms involving genomic expression. Instead, these acute actions appear to be non-genomic, and may be mediated by plasma membrane receptors/acceptors. Several reports have demonstrated that actions of progesterone may involve activation of a GABA-A

receptor (Shi & Roldan 1995; Jacob & White 2000). This possibility was ruled out in this study, since the inhibition of KCl-induced contractions was not reversed by the GABA-A antagonist bicuculline. The non-genomic actions of progesterone were first studied in sperm, where this steroid plays a role in the induction of the acrosomal reaction (Baldi et al 1998). Other actions of progesterone on sperm have been reported more recently, as will be described below.

The role of $[\text{Ca}^{2+}]_i$ increase in smooth muscle contraction has long been widely accepted. In this study, it was shown that progesterone, at a concentration of $10 \mu\text{M}$, inhibited $[\text{Ca}^{2+}]_i$ increase stimulated by high K^{+} . The incubation time of VSMC with progesterone was 15 min. After washout of the progesterone-containing bath solution, $[\text{Ca}^{2+}]_i$ increased to the control levels within 15 min when the cells were again stimulated with K^{+} . These results were similar to those obtained with $17\beta\text{-E}_2$ and progesterone on VSMC (Shan et al 1994; Murphy & Khalil 1999), cardiac myocytes (Jiang et al 1992b) and hepatocytes (Sanchez-Bueno et al 1991). In these studies $17\beta\text{-E}_2$ showed a rapid inhibitory effect on $[\text{Ca}^{2+}]_i$ in VSMC stimulated by KCl and in cardiac myocytes activated by changing the membrane holding potential. Progesterone increased $[\text{Ca}^{2+}]_i$ in rat hepatocytes within a few minutes.

Voltage-dependent Ca^{2+} channels are a principal entry pathway for extracellular Ca^{2+} ions into smooth muscle cells. An increase in $[\text{Ca}^{2+}]_i$ in turn initiates contraction. Results presented in this study show that progesterone inhibited the L-type Ca^{2+} -channel currents activated by depolarisation of the cell membrane. At a concentration of $10 \mu\text{M}$, progesterone significantly suppressed inward currents by $37 \pm 2.5\%$. The result agrees with, and extends, results obtained by others (Crews & Khalil 1999), showing that progesterone inhibits ^{45}Ca influx into coronary artery smooth muscle. Progesterone has also been shown to inhibit calcium influx into airway smooth muscle (Perusquia et al 1997) and to inhibit calcium currents in intestinal smooth muscle cells (Bielefeldt et al 1996). Many reports indicate that different agents could regulate Ca^{2+} -channel currents in smooth muscle cells. Other steroids have also been found to exert their effects by regulating $[\text{Ca}^{2+}]_i$ and Ca^{2+} channels in VSMC. For example $17\beta\text{-estradiol}$ (Shan et al 1994) and Vitamin D_3 (Shan et al 1993) have both been shown to affect Ca^{2+} -channel conductance in VSMC.

The acute effects of progesterone on calcium channels have been investigated in other tissues. Progesterone was reported to affect human sperm maturation via the increase of $[\text{Ca}^{2+}]_i$ (Blackmore et al 1990). The increase in $[\text{Ca}^{2+}]_i$ was entirely due to the influx of Ca^{2+} from the extracellular compartment. This effect was very rapid (occurring within several seconds). The mechanism of progesterone action was proposed to be through the mediation of a progesterone receptor resident in the plasma membrane. Binding of progesterone to this receptor may activate a receptor-operated channel (ROC) or inhibit a store-operated channel (Blackmore 1999). Voltage-dependent Ca^{2+} -channel blockers such as verapamil and diltiazem do not block the Ca^{2+} influx efficiently (Blackmore 1993), and therefore it was suggested that voltage-dependent Ca^{2+} channels are

not involved in the response. Progesterone was also investigated for its ability to alter Ca^{2+} currents in CA1 hippocampal neurons (Spence et al 1991), but was found to have no effect in these cells.

The inhibitory effect of progesterone in this study was mediated by alteration of the L-type Ca^{2+} -channel current, although there is no data available from this study to show whether progesterone acts directly on the channel protein or through other intracellular messengers. Similarly, although KCl is known to elicit contraction of rat tail artery by stimulating influx of extracellular calcium, it is not possible to exclude the possibility that progesterone may have an additional effect on intracellular calcium stores.

In this study, the inhibitory effect of progesterone was observed as early as 5 min after addition, and reached its maximum point at 15 min. After washout of progesterone, the $[\text{Ca}^{2+}]_i$ and Ca^{2+} -channel currents returned to control values. These results suggested that progesterone exerted its inhibitory effect on L-type Ca^{2+} -channel current rapidly and that the effect lasted only in the presence of progesterone. In other words, the inhibitory effect of progesterone was both rapid in onset and reversible. Progesterone thus acted on VSMC possibly by a rapid membrane-associated mechanism other than the traditional gene expression and protein synthesis pathway of steroid hormones.

In this study, we have reported the inhibitory effect of progesterone on blood vessel contraction. That effect occurred immediately after the addition of progesterone and reached its maximum point within approximately 15 min. The time course for the effects of progesterone in tissue tension studies, cellular Ca^{2+} studies and Ca^{2+} -channel studies were very comparable and on all three levels the progesterone effect could be reversed by washout of the bath solution. This makes the inference reasonable that the inhibitory effects of progesterone on tension generation may be mediated at least in part by a $[\text{Ca}^{2+}]_i$ decrease resulting from the decrease of Ca^{2+} influx from Ca^{2+} channels in the cell membrane.

References

- Baldi, E., Luconi, M., Bonaccorsi, L., Forti, G. (1998) Nongenomic effects of progesterone on spermatozoa: mechanisms of signal transduction and clinical implications. *Frontiers Biosci.* **3**: D1051–D1059
- Belfort, M. A., Saade, G. R., Suresh, M., Vedernikov, Y. P. (1996) Effects of estradiol-17 beta and progesterone on isolated human omental artery from premenopausal nonpregnant women and from normotensive and preeclamptic pregnant women. *Am. J. Obstet. Gynecol.* **174**: 246–253
- Bielefeldt, K., Waite, L., Abboud, F. M., Conklin, J. L. (1996) Nongenomic effects of progesterone on human intestinal smooth muscle cells. *Am. J. Physiol.* **271**: G370–G376
- Blackmore, P. F. (1993) Rapid non-genomic actions of progesterone stimulate Ca^{2+} influx and the acrosome reaction in human sperm. *Cell Signal.* **5**: 531–538
- Blackmore, P. F. (1999) Extragenomic actions of progesterone in human sperm and progesterone metabolites in human platelets. *Steroids* **64**: 149–156
- Blackmore, P. F., Beebe, S. J., Danforth, D. R., Alexander, N. (1990) Progesterone and 17α -hydroxyprogesterone, novel stimulators of calcium influx in human sperm. *J. Biol. Chem.* **265**: 1376–1380
- Crews, J. K., Khalil, R. A. (1999) Antagonistic effects of 17 beta-estradiol, progesterone, and testosterone on Ca^{2+} entry mechanisms of coronary vasoconstriction. *Arterioscler. Thromb. Vasc. Biol.* **19**: 1034–1040
- Ettinger, B., Friedman, G. D., Bush, T., Queensberry, C. P. (1996) Reduced mortality associated with long-term postmenopausal estrogen therapy. *Obstet. Gynecol.* **87**: 6–12
- Glusa, E., Graser, T., Wagber, S., Oettel, M. (1997) Mechanisms of relaxation of rat aorta in response to progesterone and synthetic progestins. *Maturitas* **28**: 181–191
- Gryniewicz, G. M., Poenie, M., Tsien, R. Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Biol. Chem.* **260**: 3440–3450
- Hamill, O. P., Marty, A., Neher, E., Sakmann, B., Sigworth, F. J. (1981) Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* **39**: 85–100
- Han, S. Z., Karaki, H., Ouchi, Y., Akishita, M., Orimo, H. (1995) 17β -Estradiol inhibits Ca^{2+} influx and Ca^{2+} release induced by thromboxane A2 in porcine coronary artery. *Circulation* **91**: 2619–2626
- Henderson, B. E., Paganini-Hill, A., Ross, R. K. (1991) Decreased mortality in users of estrogen replacement therapy. *Arch. Intern. Med.* **151**: 75–78
- Jacob, M. K., White, R. E. (2000) Diazepam, γ -aminobutyric acid, and progesterone open K^{+} channels in myocytes from coronary arteries. *Eur. J. Pharmacol.* **403**: 209–219
- Jiang, C., Poole-Wilson, P. A., Sarrel, P. M., Mochizuki, S., Collins, P., Macleod, K. T. (1992a) Effect of 17β -oestradiol on contraction, Ca^{2+} current and intracellular free Ca^{2+} in guinea-pig isolated cardiac myocytes. *Br. J. Pharmacol.* **106**: 739–745
- Jiang, C., Sarrel, P. M., Lindsay, D. C., Poole-Wilson, P. A., Collins, P. (1992b) Progesterone induces endothelium-independent relaxation of rabbit coronary artery *in vitro*. *Eur. J. Pharmacol.* **211**: 16–17
- Kannel, W. B., Hjortland, M. C., McNamara, P. M., Gordon, T. (1976) Menopause and risk of cardiovascular disease. *Ann. Intern. Med.* **85**: 447–452
- Lerner, D. J., Kannel, W. B. (1986) Patterns of coronary heart disease morbidity and mortality in the sexes: a 26-year follow-up of the Framingham population. *Am. Heart J.* **11**: 383–390
- Loirand, G., Cario-Toumaniantz, C., Chardin, P., Pacaud, P. (1999) The Rho-related protein Rnd1 inhibits Ca^{2+} sensitisation of rat smooth muscle. *J. Physiol.* **516**: 825–834
- McCrohon, J. A., Adams, M. R., McCredie, R. J., Robinson, J., Pike, A., Abbey, M., Keech, A. C., Celermajer, D. S. (1996) Hormone replacement therapy is associated with improved arterial physiology in healthy post-menopausal women. *Clin. Endocrinol.* **45**: 435–441
- Mendoza, C., Soler, A., Tesarik, J. (1995) Nongenomic steroid action: independent targeting of a plasma membrane calcium channel and a tyrosine kinase. *Biochem. Biophys. Res. Comm.* **210**: 518–523
- Miller, V. M., Vanhoutte, P. M. (1991) Progesterone and modulation of endothelium-dependent responses in canine coronary arteries. *Am. J. Physiol.* **261**: R1022–R1027
- Minshall, R. D., Miyagawa, K., Chadwick, C. C., Novy, M. J., Hernsmeier, K. (1998) *In vitro* modulation of primate coronary vascular muscle cell reactivity by ovarian steroid hormones. *FASEB J.* **12**: 1419–1429
- Murphy, J. G., Khalil, R. A. (1999) Decreased $[\text{Ca}^{2+}]_i$ during inhibition of coronary smooth muscle contraction by 17β -estradiol, progesterone, and testosterone. *J. Pharmacol. Exp. Ther.* **291**: 44–52

- Pang, P. K. T., Yang, M. C. M., Shew, R., Tenner, T. E. (1985) The vasorelaxant action of parathyroid hormone fragments on isolated rat tail artery. *Blood Vessels* **22**: 57–64
- Perusquia, M., Hernandez, R., Morales, M. A., Campos, M. G., Villalon, C. M. (1996) Role of endothelium in the vasodilating effect of progestins and androgens on the rat thoracic aorta. *Gen. Pharmacol.* **27**: 181–185
- Perusquia, M., Hernandez, R., Montano, L. M., Villalon, C. M., Campos, M. G. (1997) Inhibitory effect of sex steroids on guinea-pig airway smooth muscle contraction. *Endocrinology* **118**: 5–10
- Punnonen, R. H., Jokela, H. A., Dastidar, P. S., Nevala, M., Laippala, P. J. (1995) Combined oestrogen-progestin replacement therapy prevents atherosclerosis in postmenopausal women. *Maturitas* **21**: 179–187
- Regensteiner, J. G., Hiatt, W. R., Byyny, R. L., Pickett, C. K., Woodward, W. D., Moore, L. G. (1991) Short-term effects of estrogen and progestin on blood pressure of normotensive postmenopausal women. *J. Clin. Pharmacol.* **31**: 543–548
- Rendt, J. M., Toro, L., Stefani, E., Erulkar, S. D. (1992) Progesterone increases Ca^{2+} currents in myometrial cells from immature and nonpregnant adult rats. *Am. J. Physiol.* **262**: C293–C301
- Rylance, P. B., Brincat, M., Lafferty, K., de Trafford, J. C., Brincat, S., Parsons, V., Studd, J. W. W. (1985) Natural progesterone and antihypertensive action. *Br. Med. J.* **290**: 13–14
- Sanchez-Bueno, A., Sancho, M. J., Bobbold, P. H. (1991) Progesterone and oestradiol increase cytosolic Ca^{2+} in single rat hepatocytes. *Biochem. J.* **280**: 273–276
- Shan, J., Resnick, L. M., Lewanczuk, R. Z., Karpinski, E., Li, B., Pang, P. K. T. (1993) 1,25-dihydroxyvitamin D as a cardiovascular hormone. *Am. J. Hypertens.* **6**: 983–988
- Shan, J., Resnick, L. M., Liu, Q. Y., Wu, X. C., Barbagallo, M., Pang, P. K. T. (1994) Vascular effects of 17β -estradiol in male Sprague-Dawley rats. *Am. J. Physiol.* **266**: H967–H973
- Sheldon, J. H., Argentieri, T. M. (1995) Acute administration of 17β -estradiol inhibits calcium currents in isolated guinea pig detrusor myocytes. *J. Pharmacol. Exp. Ther.* **274**: 723–729
- Shi, Q. X., Roldan, E. R. (1995) Evidence that a GABAA-like receptor is involved in progesterone-induced acrosomal exocytosis in mouse spermatozoa. *Biol. Reprod.* **52**: 373–381
- Sitzler, G., Lenz, O., Kilter, H., La Rosee, K., Bohm, M. (1996) Investigation of the negative inotropic effects of 17 beta-oestradiol in human isolated myocardial tissues. *Br. J. Pharmacol.* **119**: 43–48
- Spence, K. T., Plata-Salaman, C. R., Ffrench-Mullen, J. M. (1991) The neurosteroids pregnenolone and pregnenolone-sulfate but not progesterone, block Ca^{2+} currents in acutely isolated hippocampal CA1 neurons. *Life Sci.* **49**: PL235–PL239
- Stampfer, M. J., Willet, W. C., Colditz, G. A., Rosner, B., Speizer, F. E., Hennekens, C. H. (1985) A prospective study of postmenopausal estrogen therapy and coronary heart disease. *N. Engl. J. Med.* **313**: 1044–1049
- Wang, G. J., Wu, X. C., Chen, C. F., Lin, L. C., Huang, Y. T., Shan, J., Pang, P. K. (1999) Vasorelaxing action of rutaecarpine: effects of rutaecarpine on calcium channel activities in vascular endothelial and smooth muscle cells. *J. Pharmacol. Exp. Ther.* **289**: 1237–1244
- Wang, R., Karpinski, E., Pang, P. K. (1989) Two types of calcium channels in isolated smooth muscle cells from rat tail artery. *Am. J. Physiol.* **256**: H1361–H1368
- Zhang, F., Ram, L. J., Standley, P. R., Sowers, J. R. (1994) 17β -estradiol attenuates voltage-dependent Ca^{2+} currents in A7r5 vascular smooth muscle cell line. *Am. J. Physiol.* **266**: C975–C980