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Role of mPR α (PAQR7) in progesterone-induced Ca^{2+} decrease in human vascular smooth muscle cells

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Abstract

We have shown progesterone exerts a direct action on vascular smooth muscle cells (VSMCs) to induce relaxation through activation of membrane progesterone receptor alpha (mPR α)-dependent signaling pathways, but information on downstream events is lacking. Progesterone-induced changes in calcium concentrations in human umbilical artery VSMCs through mPR α -dependent signaling pathways and the involvement of Rho/ROCK signaling were investigated. Acute *in vitro* treatment with progesterone and the selective mPR α agonist 10-ethenyl-19-norprogesterone (Org OD 02-0, 02-0) blocked the rapid prostaglandin F $_{2\alpha}$ -induced calcium increase. This inhibitory progesterone action was prevented by knockdown of mPR α but not by knockdown of the nuclear progesterone receptor, confirming it is mediated through mPR α . The decrease in calcium levels and VSMC relaxation were abolished by treatment with FPL64176 (Ca^{2+} channel activator), supporting a role for decreased calcium channel activity in this progesterone action. The reduction in calcium was attenuated by pretreatment with pertussis toxin, 8-Bromo-cAMP and forskolin, indicating this progesterone action involves activation of an inhibitory G protein and downregulation of cAMP-dependent signaling. Inhibition of MAPK and Akt signaling with PD98059 and ML-9, respectively, prevented the progesterone-induced calcium concentration decrease and VSMC relaxation. Forskolin decreased progesterone-induced MAPK and Akt phosphorylation which suggests that the cAMP status influences calcium levels indirectly through altering these signaling pathways. Progesterone and 02-0 treatments decreased RhoA activity and ROCK phosphorylation, which suggests that reduced RhoA/ROCK signaling is a component of the mPR α -mediated progesterone actions on VSMCs. The results suggest that progesterone induces VSMC relaxation by reducing cellular calcium levels through mPR α -induced alterations in multiple signaling pathways.

Key Words

- ▶ mPR α
- ▶ calcium
- ▶ VSMC
- ▶ signaling

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Introduction

It is widely accepted that the sex steroids estradiol-17 β and progesterone have beneficial effects on cardiovascular functions in women. Premenopausal women have a significantly lower incidence of cardiovascular diseases

than men of the same age which has been attributed to their higher circulating levels of estradiol-17 β and progesterone (Orshal & Khalil 2004, Reckelhoff 2005). This conclusion is supported by the observation that the

decline in sex steroid levels in postmenopausal women is accompanied by an increased risk of cardiovascular disease (Gray *et al.* 2001, Wenner & Stachenfeld 2012). Studies in women and in animal models have shown that estradiol-17 β and progesterone exert beneficial effects on blood pressure by inducing rapid vasodilation of arteries and veins through increasing the synthesis of nitric oxide (NO) (Selles *et al.* 2001, 2002, Ross *et al.* 2008).

NO, synthesized in vascular endothelial cells by endothelial NO synthase (eNOS), is a principal regulator of vasodilation through its action on VSMCs, causing their relaxation (Freed & Gutterman 2017). Acute progesterone treatment increases eNOS activity and NO production in human umbilical vascular endothelial cells (HUVECs) (Simoncini *et al.* 2004). Recently, membrane progesterone receptor alpha (mPR α), which is a member of the progestin and adipoQ receptor (PAQR) family (Thomas *et al.* 2007), was identified as the receptor mediating this rapid progesterone action in HUVECs (Pang *et al.* 2015). However, until recently, it was not known if mPR α mediates other beneficial effects of progesterone on cardiovascular functions.

It has been shown that removal of vascular endothelial cells does not block the relaxation response to progesterone in rat and rabbit artery preparations, which suggests progesterone also acts directly on a progesterone receptor in VSMCs to induce their relaxation (Jiang *et al.* 1992, Li *et al.* 2001, Cairrao *et al.* 2012). Although the nuclear progesterone receptor (PR) has been detected in vascular smooth muscle cells (Ergun *et al.* 1997, Hodges *et al.* 1999, Hsu *et al.* 2011), its role in progesterone induction of VSMC relaxation remains uncertain.

We recently demonstrated that multiple mPRs are expressed on the cell membranes of human VSMCs and that both progesterone and 02-0, a selective mPR agonist, but not R5020, a selective PR agonist, rapidly activated MAP kinase and Akt signaling pathways and caused muscle relaxation (Pang & Thomas 2018). Knockdown of mPR α , but not PR, significantly blocked the muscle cell relaxant effect of progesterone. Collectively, these data demonstrate that progesterone exerts a direct action on VSMCs to cause their relaxation and that mPR α plays a key role in mediating this rapid progesterone effect. This previous study also determined that mPR α activates an inhibitory G protein (Gi) resulting in a decrease in cAMP levels in VSMCs (Pang & Thomas 2018). However, these cAMP findings are perplexing because it is well established that vascular smooth muscle relaxation is commonly mediated through increases in cAMP levels

(Morgado *et al.* 2012, Cuñas *et al.* 2013). In addition, several likely key components of the mechanism mediating this rapid progesterone action on VSMC relaxation through mPR α , particularly changes in intracellular calcium levels, their regulation by intracellular signaling pathways, and the involvement changes in RhoA/Rho kinase (ROCK) signaling, have not been investigated.

Therefore, in the present study, the roles of mPR α and PR in mediating progesterone-induced calcium level changes in human VSMCs were investigated using specific mPR and PR agonists and after knockdown of receptor expression with siRNAs. Specific pharmacological tools were used to examine the involvement of calcium channels, downstream signaling through G proteins, activation of PI3K/Akt and MAP kinase pathways, and decreased cAMP signaling in the progesterone-induced alteration in calcium levels. The potential role of decreased cAMP signaling in progestin-induced Akt and MAP kinase activation was also investigated to determine whether its action on calcium levels may be indirect through activation of these signaling pathways. In addition, the involvement of alterations of RhoA/ROCK signaling in progesterone actions on VSMCs was investigated. Taken together, the results provide evidence that progesterone regulation of intracellular calcium levels involves alterations of multiple signaling cascades.

Materials and methods

Reagents and chemicals

[2,4,6,7-³H]-progesterone ([³H]-P4, ~84 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). PI3K inhibitor Wortmannin, Akt inhibitor ML-9, and specific MEK1/2 inhibitor AZD6244 were purchased from Selleckchem (Houston, TX, USA). MEK inhibitor PD98059 and adenylyl cyclase inhibitor dd-Ado were purchased from Enzo Life Sciences (Farmingdale, NY, USA). Calcium ionophore A23187 was purchased from Cayman Chemicals (Ann Arbor, MI, USA) and 10-ethenyl-19-norprogesterone (Org OD 02-0, 02-0) was obtained from Organon (Oss, Netherlands), and the PR agonist promegestone (R5020) was purchased from Perkin Elmer. ROCK inhibitor RKI-1447 was obtained from APExBio (Houston, TX, USA) and Rho activator calpeptin was obtained from Cytoskeleton, Inc. (Denver, CO, USA), and FPL64176 was purchased from Tocris Bioscience (Minneapolis, MN, USA). All other chemicals were purchased from Sigma-Aldrich, unless noted otherwise.

Cell culture

Human placentas with attached umbilical cords were collected soon after birth from de-identified patients at CHRISTUS Spohn South Hospital, Corpus Christi, Texas. Ethical approval for the study was obtained from the Institutional Review Board (IRB) of CHRISTUS Health (IRB no. 2015-100). VSMCs were obtained by enzymatic digestion of umbilical arteries with 0.2% collagenase for 30 min at 37°C as described previously (Pang *et al.* 2015). The mixture of HUVECs and VSMCs perfused from the umbilical arteries was centrifuged at 1500g for 15 min, washed, centrifuged again, and seeded in flasks with smooth muscle culture medium (SMCM, ScienCell, Carlsbad, CA, USA) supplemented with 10% FBS. Cells were continuously sub-cultured and became an almost pure population of VSMCs after 3 weeks. Lack of endothelial cell contamination of VSMCs was confirmed by demonstrating negligible expression of eNOS mRNA by RT-PCR compared to HUVECs. VSMCs were used for experiments when they were 80–90% confluent.

Membrane [^3H]-progesterone receptor-binding assays

Plasma membranes were prepared from cultured VSMCs and specific [^3H]-progesterone ([^3H]-P4) binding was measured as described previously (Thomas *et al.* 2007, Pang & Thomas 2018). Saturation analysis of [^3H]-P4 binding was measured over the range of 0.5–24 nM in the presence (non-specific binding (NSB)) or absence (total binding (TB)) of 100-fold excess non-radiolabeled progesterone after 30-min incubation at 4°C. Two-point competitive binding assays were conducted with progesterone, 02-0 and R5020 competitors (1 and 10 μM) incubated with 1 nM [^3H]-P4 and the results expressed as a percent of maximum specific progesterone binding.

Western blot analyses

Western blot assays were performed on plasma membranes and lysates (~15–20 μg protein) of VSMCs following procedures published previously (Pang & Thomas 2018) using validated polyclonal mPR α , PR antibodies (Santa Cruz, 1:1000), and antibodies for ERK, phospho ERK, Akt and phospho Akt (Cell Signaling Technologies) and ROCK, phospho ROCK (Abcam, 1:1500). VSMCs, which had been serum-starved for 2 h, were treated with drugs or progestins. Membranes were then incubated with fluorophore-conjugated secondary antibodies (LI-COR)

for 1 h at room temperature, washed, and scanned and analyzed with an Odyssey® Infrared Imaging System (LI-COR). Relative densities of phosphorylated protein bands were normalized to those of total proteins using Image J software (<https://imagej.nih.gov/ij/>).

Immunocytochemical detection of mPR α and PR in VSMCs

Immunocytochemistry of mPR α and PR proteins in cultured VSMCs was performed with the same antibodies as those used in Western blot analyses following procedures published previously (Pang *et al.* 2015). Cells were fixed with formaldehyde, blocked with 2% BSA and incubated with the antibodies (1:2000) overnight at 4°C. Cells were washed and incubated with Alexa Fluor secondary antibodies for 1 h, washed, and then mounted on glass slides with ProLong Gold antifade reagent containing 4'6'-diamidino-2-phenylindole (DAPI, Invitrogen) to visualize nuclei. Images were recorded with a Nikon inverted fluorescent microscope and Nikon NIS elements Ar imaging system.

Quantitative PCR

Quantitative PCR (qPCR) was performed following procedures published previously (Pang *et al.* 2015). The mRNA levels of RhoA and ROCK were measured with 25 μL one-step Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies) containing 100 nM sense and antisense primers. The sequence of RhoA primers were sense, 5'-TAACAGCCCTCCTCTGCACT and antisense, 5'-TTCTGGTTGAGCCCATTTC; and human ROCK primers were sense, 5'-GAAGCTCGAGAGAAGGCTGA and antisense, 5'-TTGTCTGCCTCAAATGCTTG. A housekeeping gene, β -actin (sense, 5'-AAGAGAGGCATCCTCACCT and antisense, 5'-TACATGGCTGGGGTGTGAA), was used for loading control and normalization.

Muscle cell collagen gel contraction assay

Contractility/relaxation of VSMCs was measured in a collagen gel contraction assay as described previously (Pang & Thomas 2018). Collagen gel disks (0.5 mg/mL), prepared by mixing rat tail collagen with a suspension of cultured VSMCs to a final concentration of 1.5×10^5 cells/mL, were added (500 μL /well) to a 24-well plate. The solidified gel discs were incubated for 6 h at 37°C, 5% CO_2 in TCM-199 containing the various treatments. Diameters

of gel disks were measured using a stereo dissection microscope equipped with a micrometer.

Cell calcium assay

Calcium levels in VSMCs loaded with a fluorescent intracellular calcium indicator, Fura-2, were detected by fluorescence microscopy following a published method (Adams *et al.* 2017) with some modifications. Briefly, VSMCs were sub-cultured in glass-bottomed culture dishes (Biotechs, Butler, PA, USA) until 80–90% confluent, washed three times with modified HBSS supplemented with 1.3 mM CaCl_2 , 5.5 mM glucose and 4.4 mM NaHCO_3 (pH 7.4) and three times with the modified HBSS solution plus BSA (1 mg/mL). Cells were then incubated with 2 $\mu\text{g/mL}$ Fura-2 AM (Millipore-Sigma Life Science), in modified HBSS-BSA solution at 37°C for 40 min. Cells were then incubated for 20 min with either modified HBSS or HBSS containing inhibitors of signaling pathways followed by a further 15-min incubation with progestins, progesterone or 02-0. Dishes containing treated cells were mounted onto a temperature-control apparatus (Biotechs, Butler, PA, USA) maintained at 37°C on a Nikon inverted fluorescent microscope with fluorescent light (excitation 340 and 380 nm, emission 510 nm). Calcium increase was initiated with prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$, 1 μM) dissolved in modified HBSS and the fluorescent images were recorded for 6 min by time-lapse photography. Images of representative responsive cells (8–10 cells) were analyzed by Nikon NIS Ar imaging system.

cAMP assay

Cyclic AMP was measured in VSMC lysates after 20-min treatments with progestins and drugs using an EIA kit (Cayman Chemical) as described previously (Pang & Thomas 2018).

Knockdown of mPR α and PR in VSMCs

Human mPR α (GenBank access number: NM-178422), PR (NM-000926) and non-target control siRNA oligos (Silencer Select siRNA, ThermoFisher Scientific) were used for transient silencing of mPR α and PR expression in VSMCs as described previously (Pang *et al.* 2015). VSMCs, cultured in 25 cm culture flasks until 60% confluent, were transfected twice at 0 and 16 h with a transfection mix containing Opti-mem solution (Invitrogen), 3% Lipofectamine 2000 (Invitrogen) and mPR α or PR antisense and non-targeting control siRNA oligos

(10 nM). Cells were then incubated for 36–48 h prior to experimentation. Knockdown of mPR α and PR protein expression was confirmed by Western blot analysis.

RhoA activation assay

VSMCs were grown on six-well plates and serum-starved for 2–3 h prior to experimentation. Cells were treated with P4 and OD 02-0 (100 nM) and the Rho activator calpeptin (1 IU/mL) for 20 min prior to measurement of RhoA activity with a RhoA activation assay kit (Cytoskeleton Inc, Denver, CO, USA) following the manufacturer's instructions.

Statistics

All experimental data were calculated using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Results are expressed as means $\pm$ S.E.M. of at least three measurements. All experiments were repeated three or more times with separate batches of VSMCs from different donors. Experimental data were statistically analyzed with one-way or two-way ANOVA followed by Newman-Keuls' multiple comparison tests to determine differences between multiple experimental treatments or treatment groups.

Results

Expression of mPR α and PR in VSMCs and characterization of progesterone binding to cell membranes

A specific band of approximately 40 kDa was detected on Western blots of VSMC plasma membrane fractions with the mPR α antibody which corresponds to the molecular mass of the overexpressed mPR α -positive control (Fig. 1A). In addition, a second immunoreactive band was detected at the predicted molecular mass of the mPR α (~80 kDa), which could also be seen in the lysate sample and most likely represents dimers of mPR α as has been observed previously in HUVECs (Pang *et al.* 2015). The PR antibody detected an approximately 120 kDa band in the T47D positive control sample, but only a faint 120 kDa band and a 90 kDa band, which are the predicted sizes of PR-B and PR-A, respectively, could be seen in VSMC plasma membranes and in lysate samples (Fig. 1A). Immunocytochemistry (ICC) images also showed plasma membrane and perinuclear localization of mPR α , whereas no significant PR signal was detected (Fig. 1B).

Figure 1

Expression of mPR α and PR in human umbilical artery VSMCs and characteristics of $[^3\text{H}]\text{-P4}$ membrane receptor binding. (A) Western blot detection of mPR α and PR on cell membrane and lysate samples. Mk, protein size marker. α , mPR α . m, plasma membrane. lys, cell lysate. (B) Immunocytochemical detection of mPR α (left) and PR (right) expression in the incubated VSMCs. The blue staining shows the DAPI stained nuclei. (C) Saturation curve and Scatchard analysis of $[^3\text{H}]\text{-P4}$ specific binding to VSMC plasma membranes. (D) Displacement of $[^3\text{H}]\text{-P4}$ binding by progesterins in two-point competitive binding assays. Veh, vehicle control. P4, progesterone. 02, OD 02-0. R50, R5020. The binding assays were repeated three times and similar binding characteristics were obtained on each occasion.

The relatively small amount of PR protein detected in the VSMC lysate fraction in the Western blot (Fig. 1A) is consistent with the ICC results (Fig. 1B). Saturation analysis and Scatchard plots of $[^3\text{H}]\text{-P4}$ binding to plasma membranes of VSMCs showed the presence of a relative high affinity (K_d 6.685 nM), saturable, limited capacity (B_{max} 0.06 nM/mg membrane protein), single binding site on cell membranes, which are typical characteristics of mPRs (Fig. 1C). Both concentrations (100 nM and 1 μM) of the specific mPR agonist, 02-0, were as effective as progesterone in displacing $[^3\text{H}]\text{-P4}$ from VSMC plasma membranes, whereas the PR agonist, R5020, showed negligible displacement (Fig. 1D), indicating the

progesterone binding on VSMC membranes is primarily due to mPRs.

Role of mPR α in mediating progesterone-induced cellular calcium decreases in VSMCs

Treatments with 100 nM progesterone and 02-0 significantly attenuated the 1 μM PGF2 α -induced calcium increase in VSMCs (Fig. 2A and B), which suggests that this progestin effect is mediated through mPR α . The progesterone-induced decrease in cellular calcium concentrations was completely abolished in VSMCs in which mPR α expression was knocked down, whereas the

Figure 2

Effects of 100 nM progesterone and 02-0, a mPR-specific agonist, on 1 μM PGF2 α -induced calcium increase in VSMCs. (A) Representative trace of prostaglandin-induced attenuation of cellular calcium levels determined from Fura-2 fluorescence (340/380 nm) ratios measured with a Nikon inverted microscope and analyzed with Nis analysis software (Nikon). (B) Mean calcium levels calculated from the 340/380 ratios of 5–8 responsive VSMCs for each treatment. Refer to Fig. 1 for steroids abbreviations. (C) Effects of knockdown of mPR α and PR with siRNA oligos on progesterone attenuation of the PGF2 α -induced increase in calcium levels in VSMCs. NC, non-targeting control. si-mPR α , mPR α siRNA. si-PR, PR siRNA. $n = 3$. The imbedded images show Western blot analyses of mPR α and PR expressions in the non-targeting control (N) and siRNA (S) treated cells. Results were analyzed by one-way ANOVA, followed by Newman–Keul's multiple comparison test. Treatment groups that are significantly different from each other in the *post hoc* test ($P < 0.05$) are indicated by different letters. Experiments were repeated three or more times, and similar results and similar significant differences between treatment groups were obtained on each occasion.

calcium response to progesterone was not altered in the PR-silenced cells and was similar to that of no treatment controls (Fig. 2C). These results confirm that mPR α mediates the action of progesterone in reducing cellular calcium levels.

Involvement of calcium channels in the progesterone-induced decrease in cellular calcium levels

Treatments with nifedipine (L-type calcium channel blocker, 1 μM) and verapamil (voltage-gated calcium channel blocker, 1 μM) mimicked the inhibitory effect of progesterone on the 1 μM PGF2 α -induced calcium increase (Fig. 3A), displaying similar time-courses of inhibition to that of progesterone (Supplementary Fig. 1A and B, see section on [supplementary data](#) given at the end of this article). Pretreatment with FPL64176 (1 μM), a potent activator of L-type calcium channels (Liu *et al.* 2003) abolished the progesterone-induced decrease in cellular calcium levels (Fig. 3B). Similar results were obtained with the ionophore A23187 (2 μM), a selective calcium carrier (Jiron *et al.* 1983) (Supplementary Fig. 1C). FPL64176 also eliminated the increases in muscle cell relaxation caused by progesterone treatment (Fig. 3C). The results suggest that progesterone attenuation of the PGF2 α -induced calcium increase in VSMCs may involve decreased calcium channel activity.

Involvement of an inhibitory G protein and downregulation of adenylyl cyclase in the rapid action of progesterone on cellular calcium levels

Treatment with GTP γS (10 μM , inhibits continued G protein activation) significantly attenuated the progesterone-induced reduction in PGF2 α -stimulated calcium increase in VSMCs (Fig. 4A). Similarly, this progesterone effect on calcium levels was blocked by co-treatment of cells with activated pertussis toxin (50 μM PTX, an inhibitory G protein, G α_i , inhibitor), but not with inactivated PTX, suggesting that this progesterone action is mediated through an inhibitory G protein (Fig. 4B).

Treatment of VSMCs with an adenylyl cyclase inhibitor, 2',3'-dideoxyadenosine (dd-Ado, 50 μM), mimicked the action of progesterone to decrease cellular calcium levels (Fig. 4C), whereas addition of 100 μM 8-Br-cAMP, an analog of cAMP, to the culture medium together with progesterone significantly blunted the progesterone response (Fig. 4D). Similarly, the decrease in calcium levels was significantly attenuated when the

Figure 3

Involvement of calcium channels in progesterone attenuation of $\text{PGF}_2\alpha$ -induced calcium increase and cell relaxation of VSMCs. (A) Effects of progesterone (20 nM), nifedipine (Nife) and verapamil (Vera) (calcium channel blockers, 1 μM) on $\text{PGF}_2\alpha$ -induced calcium increase. (B) Effect of pretreatment with FPL64176 (FPL, 1 μM), an activator of L-type calcium channels, on progesterone (P4) reduction of $\text{PGF}_2\alpha$ -induced calcium increase. (C) Effects of pretreatment with FPL64176 (FPL, 1 μM) on progesterone-induced relaxation of VSMCs in the muscle cell contraction assay. Results were analyzed by one-way ANOVA, followed by Newman-Keul's multiple comparison test. Treatment groups that are significantly different from each other in the *post hoc* test ($P < 0.05$) are indicated by different letters. $n = 5$. Experiments were repeated three or more times, and similar results and similar significant differences between treatment groups were obtained on each occasion.

muscle cells were co-treated with forskolin, a potent adenylyl cyclase stimulator (Fig. 4E). Measurement of cAMP levels in VSMCs confirmed that these progestin and dd-Ado treatments significantly decreased cAMP levels, whereas forskolin treatment (1 μM) both in the absence and presence of the progestins increased cAMP levels (Fig. 4F). In contrast, a lower concentration of forskolin (0.5 μM) did not reverse the progestin-induced decrease in cAMP concentrations (Supplementary Fig. 1E). Collectively, these results suggest the effects of progesterone on calcium levels via $\text{mPR}\alpha$ are partially mediated through activation of an inhibitory G protein and downregulation of cAMP-dependent signaling.

Involvement of MAPK and PI3K/Akt signaling in the progesterone decrease of cellular calcium levels

Pre-treatment with two inhibitors of MAPK (AZD6244 (1 μM) and PD98059 (10 μM)) and PI3K/Akt (wortmannin, 1 μM , and ML-9, 25 μM) for 30 min before progesterone addition significantly attenuated the reduction of cellular calcium levels in response to progesterone (Fig. 5A, B, C and D). Both the MAPK and Akt inhibitors, PD98059 and ML-9, also blocked progesterone induction of muscle cell relaxation after 6 h of treatment (Fig. 5E and F). Western blot analysis confirmed that these PD98059 and ML-9 treatments blocked progestin-induced phosphorylation of ERK and Akt, respectively (Supplementary Fig. 1F). Similar attenuation of these responses to the progestins has been demonstrated previously with AZD and wortmannin (Pang & Thomas 2018). The results indicate that both MAPK and PI3K/Akt signaling pathways are involved in the progesterone reduction in calcium levels in VSMCs.

Involvement of adenylyl cyclase downregulation in progesterone activation of MAP kinase and PI3K/Akt signaling

Possible regulation of progesterone activation of MAP kinase and PI3K/Akt through downregulation of adenylyl cyclase was investigated by examining the effects of a stimulator of adenylyl cyclase, forskolin, on progesterone-induced ERK and Akt phosphorylation. Western blot analysis showed that pretreatment with 1 μM forskolin for 30 min significantly attenuated the phosphorylation of both ERK and Akt in response to 20 nM progesterone (Fig. 6A and B), which suggests alterations of cAMP signaling may influence calcium levels in VSMCs indirectly through influencing MAP kinase and PI3K/Akt signaling pathways.

Figure 4

Involvement of G proteins and alterations of cAMP signaling in progesterone attenuation of $\text{PGF}_2\alpha$ -induced calcium increase in VSMCs. (A and B) Effects of GTP γ S, a non-hydrolysable analogue of GTP (10 μM) and pertussis toxin (PTX, 50 μM), an inhibitor of inhibitory G protein, on progesterone reduction of the $\text{PGF}_2\alpha$ -induced calcium increase. aPTX, activated PTX. iPTX, inactivated PTX. (C and D) Effect of dd-Ado, an inhibitor of adenylyl cyclase, and 8-Br-cAMP (8-Br, 100 μM) on $\text{PGF}_2\alpha$ -induced calcium increase and the response to progesterone. (E) Effect of forskolin (FK, 1 μM), an adenylyl cyclase activator, on progesterone (P4) reduction of $\text{PGF}_2\alpha$ -induced calcium increase. (F) Effects of progesterone (P4), OD 02-0 (02-0), a specific $\text{mPR}\alpha$ agonist, dd-Ado, and forskolin (1 μM) alone and in the presence of P4 and 02-0 on cAMP levels after 20 min treatments in VSMCs. Results were analyzed by one-way ANOVA, followed by Newman-Keul's multiple comparison test. Treatment groups that are significantly different from each other in the *post hoc* test ($P < 0.05$) are indicated by different letters. $n = 6$. Experiments were repeated three or more times, and similar results and similar significant differences between treatment groups were obtained on each occasion.

Figure 5

Involvement of MAP kinase and PI3K/Akt signaling pathways in progesterone (P4) attenuation of $\text{PGF}_2\alpha$ -induced calcium increase in VSMCs. (A, B, C and D) Effects of MAP kinase inhibitors, AZD6244 (AZD, 1 μM) and PD98059 (PD, 10 μM) (A and C) and PI3K/Akt inhibitors, wortmannin (WM, 1 μM) and ML-9 (25 μM) (B and D) on the progesterone reduction of $\text{PGF}_2\alpha$ -induced calcium increase. (E and F) Effects of PD98059 and ML-9 on progesterone-induced relaxation of VSMCs in muscle cell relaxation assay at 6 h. Results were analyzed by one-way ANOVA, followed by Newman-Keul's multiple comparison test. Treatment groups that are significantly different from each other in the *post hoc* test ($P < 0.05$) are indicated by different letters. $n = 6$. Experiments were repeated three or more times, and similar results and similar significant differences between treatment groups were obtained on each occasion.

Progestin regulation of RhoA/ROCK signaling

RhoA activation was significantly decreased after 20-min treatment with 100nM progesterone and 02-0 (Fig. 7A).

Phosphorylation of ROCK at Ser1366 was also significantly decreased 1h after progestin treatment (20nM) which persisted for 2 and 4h (Fig. 7B). The mRNA levels of *RHOA*

Figure 6

Effects of forskolin pretreatment on progesterone-induced phosphorylation of ERK and Akt. (A and B) Representative Western blots (upper panels) showing the effects of treatments with progesterone and a combination of progesterone (20 nM) and forskolin (F, 1 μM) on the phosphorylation of ERK (A) and Akt (B) after 0.5 h. The bar graphs (lower panel) show relative densitometry changes of the bands in the Western blot images. Each bar represents the mean $\pm$ S.E.M. of three observations. Results were analyzed by two-way ANOVA (B), followed by Newman-Keul's multiple comparison test. Treatment groups that are significantly different from each other in the *post hoc* test ($P < 0.05$) are indicated by different letters. Experiments were repeated three or more times, and similar results and similar significant differences between treatment groups were obtained on each occasion.

and ROCK in the muscle cells were also downregulated by 20nM progesterone during the first 1–2h of treatment (Fig. 7C and D). Treatment with a Rho activator, calpeptin (1 IU/mL), abolished the progesterone-induced reduction of calcium levels in VSMCs (Fig. 7E), whereas treatment with 1 μM RKI-1447, a potent ROCK inhibitor, caused a similar decrease in calcium levels to that observed with progesterone (Fig. 7F). RKI-1447 also caused a marked relaxation of VSMCs in the muscle cell contraction assay which was not augmented by co-treatment with progesterone and O2-0 (Supplementary Fig. 1D). These results suggest that alterations of RhoA/ROCK signaling are also involved in mPR-dependent progesterone actions on VSMCs.

Discussion

A major finding of this study is that progesterone blocks the rapid $\text{PGF2}\alpha$ -induced calcium increase in human artery VSMCs and this effect is mediated through membrane progesterone receptor alpha ($\text{mPR}\alpha$). Although there are no reports, to our knowledge, describing a rapid attenuation of prostaglandin- or potassium-induced increase of calcium in human VSMCs by progesterone, it has been demonstrated to rapidly reduce the calcium current in human intestinal smooth muscles (Bielefeldt

et al. 1996) and decrease calcium influx in rodent, rabbit, pig, and monkey VSMCs (Murphy & Khalil 1999, Barbagallo *et al.* 2001, Li *et al.* 2001, Minshall *et al.* 2002). Both mPRs and PR have been detected in human VSMCs and are candidates for the receptor mediating this rapid, nongenomic action (Hodges *et al.* 1999, Nakamura *et al.* 2005, Thomas & Pang 2013, Pang & Thomas 2018). However, only minor expression of PR was detected in human artery VSMCs in the present study and the results clearly show using a variety of approaches that attenuation of the $\text{PGF2}\alpha$ -induced increase of calcium in these cells is mediated solely through $\text{mPR}\alpha$. Moreover, the observation that the $\text{mPR}\alpha$ agonist-induced decrease in cytosolic calcium levels is accompanied by smooth muscle relaxation is consistent with our previous finding that progesterone-induced human VSMC relaxation is mediated through $\text{mPR}\alpha$ (Pang & Thomas 2018). It was shown in this earlier study that the relaxation response through $\text{mPR}\alpha$ in these VSMCs involves activation of an inhibitory G protein and activation of ERK and PI3K pathways, resulting in MLC phosphorylation. The present results demonstrate that progesterone acts through these same pathways to regulate calcium levels in arterial VSMCs. Another calcium-dependent mechanism promoting VSMC contraction involves activation of RhoA/ROCK

Figure 7

Effects of progestin treatments on RhoA/ROCK expression and signaling and involvement of RhoA/ROCK in regulation of calcium levels in VSMCs. (A) Effects of P4 and O2-0 treatments (100 nM, 20 min) on Rho activator (calpeptin)-induced RhoA activity. (B) Representative Western blot (upper panel) showing the effects of progesterone and O2-0 treatments on the phosphorylation of ROCK at 1, 2 and 4 h. The bar graphs (lower panel) show relative densitometry changes of the bands in the Western blot images. Each bar represents the mean $\pm$ S.E.M. of three observations. V, vehicle; P, progesterone. O, O2-0. pROCK, phosphorylated ROCK. (C and D) QPCR detection of mRNA expression in the progesterone-treated VSMCs at 0, 1, 2 and 4 h. (E) Effect of Rho activator, calpeptin (RA, Rho-Acti, 1 IU/mL) on progesterone reduction of $\text{PGF2}\alpha$ -induced calcium increase in VSMCs. (F) Effects of progesterone and RKI-1447 (RKI, 1 μM), a ROCK kinase inhibitor, on $\text{PGF2}\alpha$ -induced calcium increase in VSMCs. Veh, vehicle control. Results were analyzed by one-way ANOVA (A, C, D, E and F) or two-way ANOVA, followed by Newman-Keul's multiple comparison test. Treatment groups that are significantly different from each other in the *post hoc* test ($P < 0.05$) are indicated by different letters. Experiments were repeated three or more times, and similar results and similar significant differences between treatment groups were obtained on each occasion.

which sensitizes the contractile apparatus to calcium resulting in MLC phosphorylation (Urena & Lopez-Barneo 2012). The results showing that the signaling of RhoA and ROCK is downregulated by a selective mPR α agonist suggests that mPR α signaling may also promote muscle relaxation via blocking this alternative mechanism. Taken together, these results indicate several plausible mechanisms by which progesterone through activation of mPR α and multiple signaling pathways induces a decrease in calcium levels or calcium sensitization resulting in relaxation of human VSMCs.

Calcium has a critical function in VSMC contraction and increases in intracellular calcium levels result in vasoconstriction and elevated blood pressure (Sonkusare *et al.* 2006, Nieves-Cintrón *et al.* 2008, Santana *et al.* 2008, Pulina *et al.* 2010). There is substantial evidence that estradiol-17 β - and progesterone-induced relaxation of VSMCs in a variety of animal models is mediated through decreases in intracellular calcium levels and that this decrease occurs through rapid inhibition of voltage-dependent L-type calcium channels (Nakajima *et al.* 1995, Murphy & Khalil 1999, Cairrao *et al.* 2012). For example, progesterone inhibits the KCl-induced elevation of cytosolic-free calcium in rat VSMCs and attenuates L-type calcium channel inward current in whole-cell patch-clamp experiments (Barbagallo *et al.* 2001). The finding that activation of the voltage-dependent calcium inward current in a rat VSMC line (A7r5 cells) by Bay K8644, a specific L-type calcium channel activator, is also decreased by progesterone treatment further suggests that progesterone inhibits L-type calcium channels in rat VSMCs (Cairrao *et al.* 2012). Several lines of evidence in the present study suggest progesterone attenuates prostaglandin-induced increases in intracellular calcium levels and induces smooth muscle relaxation by a similar mechanism in human VSMCs. The results of the present study suggest that the PGF2 α -induced increase of cellular calcium levels in human VSMCs involves activation of L-type calcium channels, because it is significantly decreased by treatment with nifedipine- and verapamil-specific L-type calcium channel blockers. Progesterone causes a similar attenuation of the calcium response to PGF2 α in these cells which was abolished by pretreatment with FPL64176, a potent L-type calcium channel activator. Furthermore, FPL64176 also significantly attenuated progesterone-induced relaxation of VSMCs. Taken together these results suggest that the progesterone-induced reduction of calcium levels in human VSMCs may involve inhibition of L-type calcium channel activity. However, progesterone has been shown to inhibit

calcium entry into HEK293 cells transfected with TRPM3 and TRPC5, two non-selective transient receptor potential cation channels that are permeable to calcium, (Majeed *et al.* 2011, 2012), which suggests that progesterone may act on multiple calcium channels in VSMCs.

Although these previous studies have clearly established that progesterone decreases calcium levels in mammalian VSMCs through inhibition of calcium channels, none of them have indicated the identity of the receptor mediating these progesterone actions. The finding that treatment with PR antagonists, J 867 and mifepristone, did not alter the relaxant effect of progesterone on VSMCs suggests this rapid progesterone action is not mediated through the PR (Glusa *et al.* 1997, Cairrao *et al.* 2012). The present results demonstrate that binding of [³H]-P4 to the plasma membranes of human artery VSMCs is characteristic of mPRs in that it is displaced by the specific mPR agonist, 02-0, but not by the PR agonist, R5020. Furthermore, the finding that this selective mPR agonist mimics the effects of progesterone and that this progesterone action is completely abolished by knock-down of mPR α expression with siRNA oligos provides compelling evidence that mPR α is the principal receptor mediating the inhibitory action of progesterone on cellular calcium levels. These results are consistent with our previous results showing that progesterone-induced relaxation of human vein VSMCs is mediated solely through mPR α and implicate alterations of calcium levels in this relaxation mechanism (Pang & Thomas 2018).

Currently, there is limited information on the signaling pathways mediating the progesterone-induced decrease in calcium levels in VSMCs. We recently obtained evidence that progesterone induction of human VSMC relaxation through mPR α is regulated through a Gi and activation of MAP kinase/ERK and PI3K/Akt pathways (Pang & Thomas 2018) and assumed that the same pathways mediate the progesterone-induced decrease in calcium levels in these cells. However, the results of several studies with different animal models and muscle cells are not consistent with our predictions with human cells. For example, MLC phosphorylation and contraction of rabbit endothelium-denuded aortic strips and intact swine carotid arteries appears to be dependent on PI3K activation, since they are blocked by PI3K inhibitors (Su *et al.* 2004, Wang *et al.* 2006). In contrast, estrogen-induced relaxation of human VSMCs involves activation of PI3K/Akt signaling (Han *et al.* 2007), similar to our findings with progesterone. In order to address these discordant findings and firmly establish the mPR α -dependent progesterone signaling pathways regulating relaxation of human VSMCs, the

present study examined the pathways which mediate decreases in calcium levels, a critical component of muscle relaxation. The observation that pretreatment with GTP γ S and pertussis toxin, blocked the action of progesterone on calcium levels confirms it acts through an inhibitory G protein (Gi). The demonstration that this response to progesterone was also blocked by inhibitors of MAP kinase (AZD6244 and PD98059), as well as by a PI3K inhibitor (wortmannin) and an Akt inhibitor (ML-9), indicates that the rapid action of progesterone on VSMC calcium levels, like its action on relaxation of these cells (Pang & Thomas 2018), involves activation of MAP kinase and PI3K/Akt signaling pathways. Interestingly, these pathways are also activated by progesterone through mPR α in human vascular endothelial cells (HUVECs) to increase production of nitric oxide, a major regulator of VSMC relaxation (Pang *et al.* 2015, Pang & Thomas 2017). Collectively, these studies suggest that mPR α exerts cardiovascular protective effects by two different mechanisms, but through the same signaling pathways.

Progesterone activation of mPR α was predicted to cause a decrease in cAMP levels in VSMCs in the present study because the receptor activates an inhibitory G protein and is coupled to it (Pang & Thomas 2018). However, the results showing that the progesterone-induced decrease in calcium levels was attenuated by pretreatment with agents that increase cAMP levels, 8-Br-cAMP and forskolin, and our earlier results demonstrating that 8-Br-cAMP also abolishes progesterone-induced VSMC relaxation (Pang & Thomas 2018), are not consistent with those of previous studies demonstrating that the decrease in calcium levels and smooth muscle relaxation are mediated by increases in cAMP levels (Wu & Shen 2010, Morgado *et al.* 2012, Cuñías *et al.* 2013). For example, progesterone inhibits L-type channel activity in guinea pig gallbladder smooth muscle cells through activation of a cAMP/PKA signaling pathway (Wu & Shen 2010). Moreover, estrogen and urocortin have been shown to exert their relaxant effects in several VSMC animal models through activation of Acy and increases in cAMP and inhibition of MLC phosphorylation (Keung *et al.* 2005, Wang *et al.* 2012, Lee & Choi 2013, Lindsey *et al.* 2014). The effects of altering cAMP levels on progesterone activation of MAP kinase and PI3K/Akt signaling were investigated to determine whether our disparate findings could be explained by an indirect action of cAMP through modulation of these signaling pathways. The results showing forskolin treatment attenuated progesterone-induced phosphorylation of both ERK and Akt are consistent this hypothesis, although additional studies will be required to confirm this indirect pathway.

The finding that progesterone significantly decreased *RHOA* and *ROCK* mRNA expression within 1 h of treatment and that both progesterone and the mPR agonist reduced RhoA activity and ROCK phosphorylation within a similar time period suggests that mPRs may also cause VSMC relaxation and exert cardiovascular protective effects by a third mechanism, through inhibition of RhoA-ROCK signaling. RhoA/ROCK regulates VSMC contraction through calcium-independent mechanisms by increasing the sensitivity of MLC to calcium (Uehata *et al.* 1997, Touyz *et al.* 2018). Progesterone may also increase degradation of RhoA in human VSMCs, since it has been shown to inactivate RhoA in rat VSMCs through cSrc-enhanced RhoA degradation (Hsu *et al.* 2011) and through phosphorylation of p27^{kip1} at Ser10, which forms a complex with RhoA and causes degradation of the complex through an ubiquitin-proteasome pathway (Wang & Lee 2014). An involvement of ROCK in calcium entry into VSMCs by various agonists has also been demonstrated (Ghisal *et al.* 2003, Martinsen *et al.* 2012). Interestingly, in the present study, treatment with RKI-1447, a potent ROCK inhibitor, caused a similar decrease in calcium levels to that observed with progesterone, whereas a RhoA activator, calpeptin, caused an increase in calcium levels and abolished the rapid progesterone-induced decrease in calcium levels. However, the implications of these findings remain unclear and warrant further study.

The results of this study demonstrate that progesterone reduces calcium levels in human VSMCs through multiple signaling pathways. The discovery that this nongenomic progesterone action is mediated through alterations in mPR α -dependent cAMP, PI3K/Akt, and MAPK signaling is consistent with our previous observations on the progesterone mechanism regulating relaxation of human VSMCs (Pang & Thomas 2018). In addition, evidence was obtained that progesterone regulates RhoA/ROCK activity in VSMCs through mPRs. Previously, we have shown that progesterone promotes smooth muscle relaxation by upregulating eNOS activity and NO production in HUVECs through the same mPR α -dependent signaling pathways (Pang *et al.* 2015, Pang & Thomas 2017). The fact that mPR α promotes human VSMC relaxation through multiple tissues and mechanisms suggests it has an important role in vascular protection and is a potential therapeutic target for treatment of hypertension.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-19-0019>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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