Cardiovascular Research (2010) **86**, 383–391
doi:10.1093/cvr/cvq011

Cell cycle-dependent expression of Kv3.4 channels modulates proliferation of human uterine artery smooth muscle cells

Eduardo Miguel-Velado[1], Francisco D. Pérez-Carretero[1], Olaia Colinas[1], Pilar Cidad[1], Magda Heras[2], José R. López-López[1], and M. Teresa Pérez-García[1]*

[1]Departamento de Bioquímica y Biología Molecular y Fisiología e Instituto de Biología y Genética Molecular (IBGM), Universidad de Valladolid y CSIC, Valladolid, Spain; and [2]Servicio de Cardiología, Institut Clinic del Tòrax, Hospital Clinic, IDIBAPS, Universidad de Barcelona, Barcelona, Spain

Received 30 July 2009; revised 8 January 2010; accepted 11 January 2010; online publish-ahead-of-print 21 January 2010

Time for primary review: 53 days

Aims Vascular smooth muscle cell (VSMC) proliferation is involved in cardiovascular pathologies associated with unwanted arterial wall remodelling. Coordinated changes in the expression of several K^+ channels have been found to be important elements in the phenotypic switch of VSMCs towards proliferation. We have previously demonstrated the association of functional expression of Kv3.4 channels with proliferation of human uterine VSMCs. Here, we sought to gain deeper insight on the relationship between Kv3.4 channels and cell cycle progression in this preparation.

Methods and results Expression and function of Kv3.4 channels along the cell cycle was explored in uterine VSMCs synchronized at different checkpoints, combining real-time PCR, western blotting, and electrophysiological techniques. Flow cytometry, Ki67 expression and BrdU incorporation techniques allowed us to explore the effects of Kv3.4 channels blockade on cell cycle distribution. We found cyclic changes in Kv3.4 and MiRP2 mRNA and protein expression along the cell cycle. Functional studies showed that Kv3.4 current amplitude and Kv3.4 channels contribution to cell excitability increased in proliferating cells. Finally, both Kv3.4 blockers and Kv3.4 knockdown with siRNA reduced the proportion of proliferating VSMCs.

Conclusion Our data indicate that Kv3.4 channels exert a permissive role in the cell cycle progression of proliferating uterine VSMCs, as their blockade induces cell cycle arrest after G2/M phase completion. The modulation of resting membrane potential (V_M) by Kv3.4 channels in proliferating VSMCs suggests that their role in cell cycle progression could be at least in part mediated by their contribution to the hyperpolarizing signal needed to progress through the G1 phase.

Keywords Vascular smooth muscle cells • Potassium channels • Cell proliferation • Cell cycle progression • Electrophysiology • Phenotypic switch

1. Introduction

The diversity of vascular smooth muscle cell (VSMC) functions requires different cell phenotypes. In contrast to terminally differentiated skeletal and cardiac muscle cells, VSMCs retain a high degree of plasticity and are able to switch from a contractile to a proliferative phenotype. This switch represents an essential element in the adaptation to physiological and pathological stimuli associated with injury, atherosclerosis, and hypertension.[1] Similar phenotypic modulation occurs when VSMCs are grown in primary culture in the presence of serum.[2,3] When VSMCs undergo the transition from contractile to synthetic phenotype and reenter the cell cycle, there are major changes in the expression of contractile proteins,[4] ion channels, transporters and receptors,[5,6] leading to changes in membrane potential and Ca^{2+} handling and to the loss of contractility.

We have previously described that phenotypic switch of human uterine artery (HUA) VSMCs in culture associates with increased functional expression of Kv3.4 channels, which mediate the largest

* Corresponding author: Departamento de Fisiología, Universidad de Valladolid, Edificio IBGM, c/ Sanz y Forés s/n, Valladolid 47003, Spain. Tel: +34 983 184590; fax: +34 983 184800, Email: tperez@ibgm.uva.es

proportion of the Kv current in dedifferentiated VSMCs. Furthermore, Kv3.4 channel blockade decreased proliferation rate, suggesting a link between channel function and VSMC proliferation in this preparation.[7] However, the molecular mechanisms of this process remain largely unexplored. It is not known whether Kv3.4 channels are important in HUA VSMC proliferation because of a general homeostatic role (maintaining membrane potential, intracellular [Ca^{2+}], pH, and cell volume) or because their activity is required at some special checkpoint during the cell cycle, which would imply that they have a precise role in proliferation. Besides, the molecular correlate of Kv3.4 currents in HUA VSMCs has not been studied. In other preparations, complexes of Kv3.4 and the accessory subunit MiRP2 (MinK-related peptide 2) recapitulate native currents,[8,9] as MiRP2 modifies the kinetics, unitary conductance, and drug sensitivity of Kv3.4 channels.

The cell cycle is a highly regulated process that ensures a homeostatic balance between cell proliferation and cell death in response to environmental signals. Dividing cells have internal timers which coordinate cell division, represented by cyclin-dependent kinases and oscillating cyclins.[10] Changes in the expression and activity of specific ion channels during cell cycle have been reported as part of the clock mechanism, together with other nucleus-independent cytoplasmic oscillators and the fluctuating activity of kinases.[11–13]

In the present work, we explored the existence of a link between the expression of Kv3.4 channels and cell cycle progression in cultured HUA VSMCs. We have studied: (i) the pattern of expression and activity of Kv3.4 as a function of the cell cycle stage and (ii) the changes in VSMC cell cycle distribution when Kv3.4 activity is suppressed. We found cell cycle-dependent variations in the expression of Kv3.4 and MiRP2 mRNA and protein, as well as in the channel contribution to total outward currents and to resting V_M. Moreover, Kv3.4 channels suppression rose the fraction of quiescent cells by increasing the number of cells that exit the cell cycle after M phase completion. These effects could be occluded by VSMC depolarization after raising extracellular K^+, suggesting that the role of Kv3.4 channels in HUA VSMCs proliferation is related to their contribution to resting V_M. Altogether, our data indicate that functional expression of Kv3.4 channels facilitates the entrance of uterine VSMCs to G1 phase, pointing to Kv3.4 channels as specific targets for new therapeutic approaches directed to modulate VSMC proliferation.

2. Methods

HUAs were obtained from patients subjected to hysterectomy at the Clinic Hospital of Barcelona, with protocols approved by the Human Investigation Ethics Committee of the Hospital and conforming the principles outlined in the Declaration of Helsinki. Cultured VSMCs were obtained from explants of HUAs as previously described.[7]

Synchronization of VSMC cultures was performed by pharmacological blockade or by serum deprivation, and evaluated by flow cytometry, Ki67 immunostaining and quantification of cyclins mRNA levels. Briefly, for G0 synchronization, VSMCs were placed for 48–72 h in serum-free (SF) medium. To arrest cells at the G1/S boundary, VSMCs initially synchronized at G0 were placed in control medium with 1 mM L-mimosine for 48 h. Finally, enrichment at G2/M stage was performed by two different strategies, incubation for 24–48 h with 0.1 μM Taxol or treatment with nocodazole after double thymidine block. In each case, Kv3.4 and MiRP2 mRNA and protein levels were determined by qPCR and western blot, respectively. qPCR data were analysed with the threshold cycle (Ct) relative quantification method (ΔΔCt),[14] using ribosomal protein L18 (RPL18) as housekeeper gene and control VSMC cultures as calibrator. In this way, data in experimental conditions were expressed as the fold change in gene expression compared with control.

The contribution of Kv3.4 channels to the outward currents and the resting V_M was evaluated by electrophysiological techniques, as previously described,[7,15] using the whole-cell and perforated-patch configurations of the patch-clamp technique. Briefly, VSMCs were placed in a bath solution containing (in mM): 141 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.8 $CaCl_2$, 10 glucose, 10 HEPES, 5×10^{-4} paxilline, 5×10^{-4} tetrodotoxin, 0.1 $BaCl_2$ (pH 7.4 with NaOH). Patch pipettes had resistances of 5–8 MΩ when filled with the following internal solution (mM): 125 KCl, 4 $MgCl_2$, 10 HEPES, 10 EGTA, 5 MgATP; (pH 7.2 with KOH). K^+ currents were recorded using a two pulse protocol, in which from a holding potential of −80 mV, 200 ms depolarizing prepulses from −80 to +80 mV in 10 mV increments were followed by a 200 ms depolarizing pulse to a fixed potential of +40 mV. From these records, current–voltage relationships and steady-state inactivation curves were constructed. For continuous V_M recordings, pipette tips were dipped into a solution containing (mM): 40 KCl, 95 K glutamate, 8 $CaCl_2$, 10 HEPES, pH 7.2 with KOH, and backfilled with the same solution containing amphotericin B (300 μg mL^{-1}).

Kv3.4 expression was suppressed either pharmacologically, with BDS-I[16] or tetraethilamonium chloride at micromolar concentrations,[7] or with gene knockdown with Kv3.4 siRNA transfections using predesigned siRNAs (Applied Biosystems) and Lipofectamine 2000 reagent (Invitrogen). Proliferation was evaluated by a BrdU incorporation assay (Roche Applied Science), following manufacturer instructions.

An Expanded Methods section with the detailed protocols and used solutions can be found in Supplementary material online.

3. Results

3.1 Cell cycle-dependent changes in Kv3.4 mRNA and protein expression

Kv3.4 and MiRP2 mRNA levels were determined in non-synchronized VSMCs, kept in control medium and in cell populations arrested in G0 (by 48–72 h serum starvation), at the late G1 phase (with 1 mM L-mimosine treatment) or in the G2/M boundary (by incubation with 0.1 μM Taxol). The effects of these manoeuvres were confirmed by flow-cytometry analysis and by quantification of the mRNA levels of cyclins D1, A2, and B1. *Figure 1A* shows representative flow-cytometry histograms obtained in the three conditions. Although serum starvation or L-mimosine did not fully synchronize cell cultures, both led to an increase of the percentage of cells in G0/G1 with respect to cells kept in control medium (from 56 ± 3.8 in control to 70.4 ± 1.4 and 74.4 ± 4.4 with SF or mimosine, respectively, *n* peak). On the contrary, collection of the cells detached by agitation after treatment with 0.1 μM Taxol provided a homogeneous population of cells in G2/M phase (2*n* peak).[17] Cyclins mRNA levels were also determined under these three treatments (*Figure 1B*). SF treatment decreased expression of all cyclins mRNA, L-mimosine application resulted in decrease A2 and B1 cyclins with no changes in D1 mRNA expression, and G2/M synchronization associated with augmented expression of A2 and B1 cyclins and decrease of D1 cyclin mRNA.

We also explored the expression levels of Kv3.4 and MiRP2 mRNA (*Figure 1B*) and protein (*Figure 1C*). G0-enriched cell cultures (SF) showed decreased Kv3.4 and increased MiRP2 mRNA expression, whereas G1-enriched cell cultures showed no significant changes and G2/M synchronization led to decreased expression of both Kv3.4 and MiRP2 mRNAs. Western blots from cell lysates in these

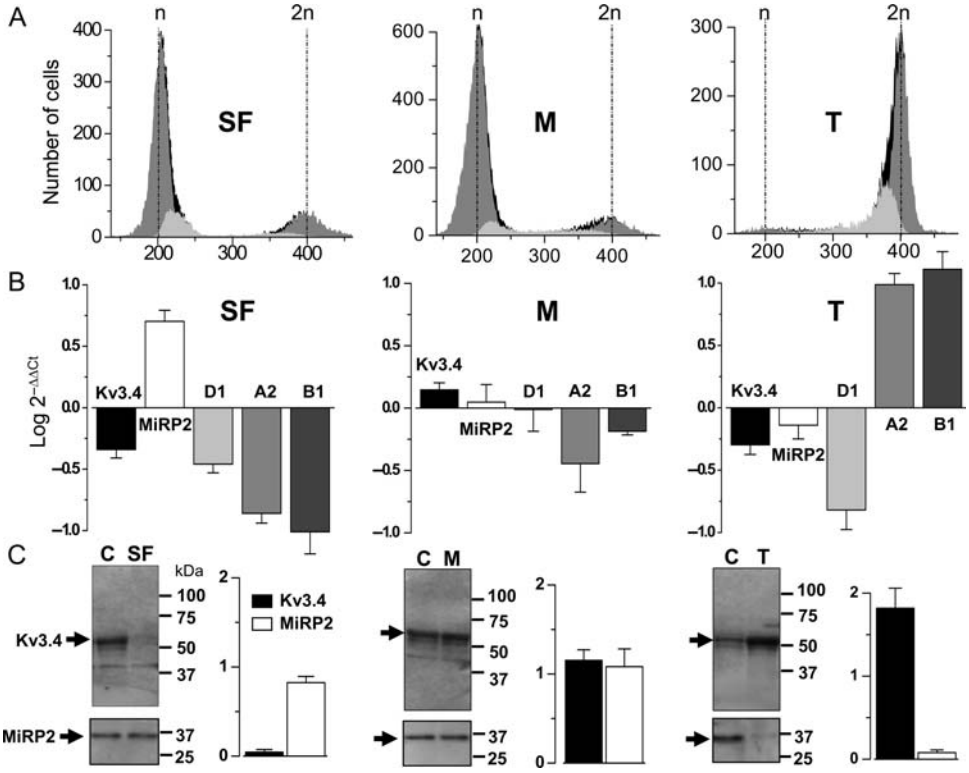

Figure 1 Molecular analysis of Kv3.4 expression along the cell cycle. (*A*) Representative flow-cytometry histograms obtained from cells arrested at G0 (serum free, SF), late G1 (mimosine 1 mM, M), or G2/M boundary (Taxol 0.1 μM, T). The plots show the distribution of VSMCs as a function of propidium iodide fluorescence intensity. The resulting histograms identify three populations: cells in G0/G1 (diploid cells, dark grey peak labelled as *n*), cells in G2/M (tetraploid cells, dark grey peak labelled as 2*n*), and cells in S phase (overlapping light grey histograms). (*B*) Fold-change in mRNA levels of Kv3.4, MiRP2, and cyclins D1, A2, and B1 were obtained by normalizing the data using RPL18 mRNA as internal control and non-synchronized VSMCs as calibrators. mRNA changes were expressed as $\log(2^{-\Delta\Delta Ct})$, so that positive values indicate increased expression and negative values decreased expression (see methods). (*C*) Representative immunoblots of Kv3.4 and MiRP2 protein (arrows) in control cultures and in the three experimental conditions. Bar plots show the protein quantification relative to control conditions in each case ($n = 3–4$).

three conditions are displayed in *Figure 1C*. While Kv3.4 protein levels increased as cells move along cell cycle (from G0 to G1 and G2/M) changes in MiRP2 protein showed opposite changes, being almost non-detectable in G2/M cells. Moreover, changes in mRNA expression along the cell cycle seemed to precede changes in protein, so that the expression levels of Kv3.4 and MiRP2 proteins correlated with mRNA expression only in L-mimosine treated cells.

3.2 Cell cycle-dependent changes in Kv3.4 currents

As Kv3.4 and MiRP2 expression changed along the cell cycle in HUA VSMCs, we explored their contribution to membrane currents with electrophysiological techniques, using unsynchronized (control) VSMCs and VSMCs synchronized with the same three strategies described in *Figure 1*. The average current–voltage (*I/V*) and steady-state inactivation curves (*Figure 2A*) showed that total outward K^+ current density decreased in SF VSMCs, and increased in Taxol or L-mimosine synchronized cells. Fit of the steady-state inactivation curves to a Boltzmann function revealed a progressive displacement of the $V_{0.5}$ of inactivation (left bars plot) when comparing control VSMCs with L-mimosine or Taxol treated cells, a change that has been previously reported when comparing Kv3.4 + MiRP2 complexes with Kv3.4 homotetramers.[8] The observed changes in current density are attributable to changes in the fraction of inactivating current (*Figure 2B*), without significant variations of the non-inactivating component. The absence of changes in the voltage dependence of activation (see conductance curves in *Figure 2C*), together with the increase of the inactivating current and the rightward shift of the steady-state inactivation led to a marked increase of the steady-state window current (inset of *Figure 2C*) at potentials around resting membrane potential (V_M). These kinetic properties make the transient current suitable for modulating cell excitability along the cell cycle.

Pharmacological experiments showed that the transient K^+ currents were mediated by Kv3.4 channels, as they were sensitive to 100 μM TEA and 2.5 μM BDS-1[7] (*Figure 3*). Although TEA sensitivity of the transient currents was similar in all experimental conditions, there was a small fraction of the sustained current sensitive to 100 μM TEA, that showed a significant increase in Taxol-treated cells (*Figure 3A*). This component could be mediated by another channel or by an increased contribution of Kv3.4 channels to the sustained current in this condition.

The contribution of Kv3.4 channels to resting V_M was explored in perforated-patch recordings. Resting V_M of SF cells was hyperpolarized when compared with control VSMCs, whereas cells entering cell cycle showed a progressive depolarization that was significant

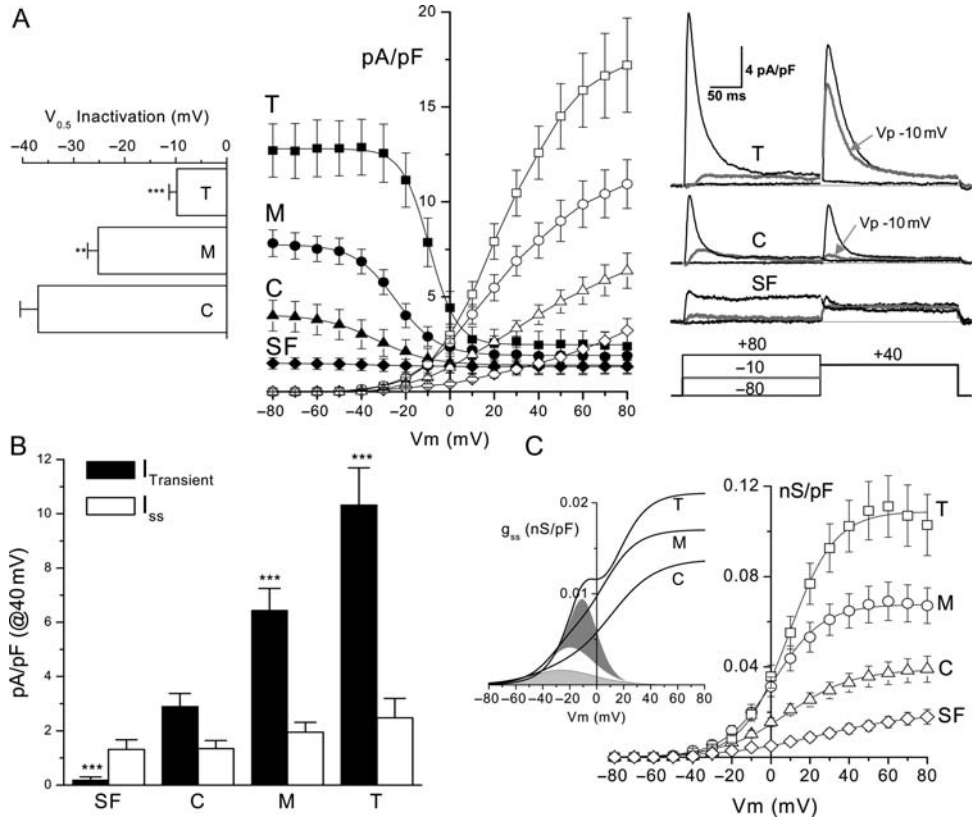

Figure 2 Functional expression of Kv3.4 channels along the cell cycle. (*A*) Mean current–voltage (open symbols) and steady-state inactivation relationships (filled symbols) obtained in VSMCs in control conditions (C), and synchronized with Taxol (T), Mimosine (M), or serum deprivation (SF) as in *Figure 1*. The average $V_{0.5}$ of inactivation obtained from the Boltzmann fit of the steady-state inactivation curves are shown in the left bar plot. The voltage protocol used and representative traces elicited at three different potentials in serum free, control and Taxol-treated cells are shown on the right. Data are mean ± SEM of 8–12 cells in each group. All through the figures: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ relative to control (non-synchronized) VSMCs. (*B*) Bar plots representing the current density (pA/pF) of the transient component (black bars) and the current remaining at the end of a 200 ms pulse to +40 mV (I_{ss}, white bars) of cells from the four experimental groups. (*C*) Conductance–voltage curves obtained from the current–voltage relationships and fitted to a Boltzmann function (see fit parameters in Supplementary material online, *Table SII*). The inset shows the steady-state conductance (g_{ss}) of control, mimosine- and Taxol-treated cells, computed as described in Supplementary material online. Shaded areas represent the fraction of this conductance mediated by the transient component in each experimental condition.

for the Taxol synchronized VSMCs (*Figure 3B*). Besides, 100 μM TEA induced resting V_M depolarization in all experimental groups but SF-treated cells. The amplitude of this depolarization was significantly larger in L-mimosine (6.1 ± 0.4 mV) and Taxol-treated (5.4 ± 0.2 mV) than in control cells (3.2 ± 0.5 mV, $P < 0.001$), in agreement with the increased expression of Kv3.4 channels along the cell cycle.

Transient K^+ currents were also sensitive to the selective Kv3.4 blocker BDS-I, but differences in the voltage dependence of BDS-I effect when comparing control and Taxol-treated cells were found (*Figure 3C*). In control cells, blockade was relieved by depolarization, whereas in Taxol-treated cells, it was voltage independent. Interestingly, the increased BDS sensitivity at depolarized potentials has been also described for Kv3.4 channels devoid of MiRP2 subunits.[8]

3.3 Effects of Kv3.4 blockade on cell cycle progression

The previous data indicated that cell cycle variations in Kv3.4 and MiRP2 mRNA and protein levels translate into functional changes, suggesting that the amplitude and kinetics of Kv3.4 currents could modulate the progression of HUA VSMCs along the cell cycle. To confirm this hypothesis, we explored whether Kv3.4 blockade interfered with cell cycle progression. After 48 h in control medium alone or in the presence of 100 μM TEA, cell cycle distribution was studied by flow cytometry. TEA increased in all cases the proportion of G0/G1 phase cells (*Figure 4A*). Additionally, immunocytochemical detection of the nuclear protein Ki67 (whose expression is restricted to proliferating cells[18]) showed a significant increase in the number of $Ki67^-$ VSMCs after 48 h treatment with 100 μM TEA or with 2.5 μM BDS-I (*Figure 4B*). Moreover, mRNA expression levels of D1 and A2 cyclins decreased in 100 μM TEA-treated cells (*Figure 4C*). There was also a decrease of the mRNA expression levels of Kv3.4, but no changes in the expression of calponin, a contractile protein used as a marker of VSMC differentiation.[19] In fact, when differentiation of cultured VSMCs is favoured (by serum starvation up to 96 h, *Figure 4C*), we observed more pronounced decreases of Kv3.4 and cyclins mRNA together with a five-fold increase of calponin mRNA levels. The mRNA profile observed upon TEA treatment indicated that Kv3.4 channels blockade increased the proportion of quiescent VSMCs.

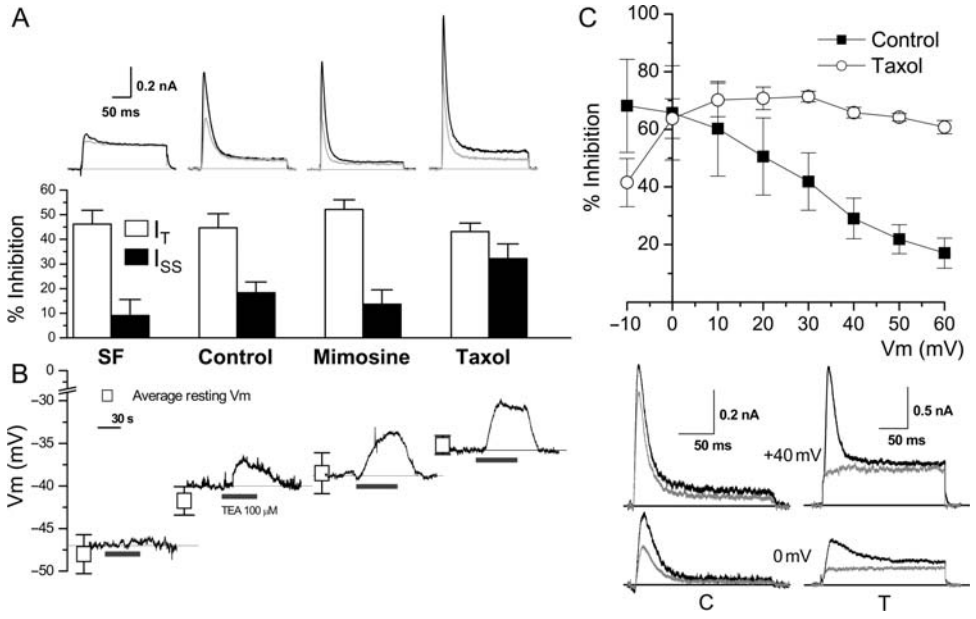

Figure 3 Pharmacological characterization of Kv3.4 channels along the cell cycle. (*A*) Representative current traces elicited by 200 ms pulses to +40 mV from a holding potential of −80 mV in cells under the indicated experimental conditions both in control solution (black traces) and in the presence of 100 μM TEA (grey traces). The bar plots show the mean effect of TEA (as % inhibition) on the transient (I_T, white bars), and the sustained components of the current (I_{ss}, black bars). Transient current was defined as the difference between peak current and current amplitude at the end of the depolarizing pulse; Mean ± SEM. (*B*) Average resting V_M (white squares) determined in perforated-patch recordings form the four experimental groups (mean ± SEM, $n = 7–12$ cells). The traces are representative current-clamp recordings showing the effect of 100 μM TEA application in a cell from each experimental group. (*C*) Voltage-dependent inhibition of K^+ currents elicited by application of 2.5 μM BDS-1 in unsynchronized (black squares) and in Taxol-treated VSMCs (open circles). Mean ± SEM, $n = 7$. Representative traces at two voltages in both conditions are also shown (black traces, control solution and grey traces, BDS-1).

3.4 Effects of Kv3.4 blockade on cell cycle duration

This effect of Kv3.4 blockade could reflect either a direct, specific effect, of Kv3.4 channels modulating cell cycle progression, or an indirect, non-specific, role of Kv3.4 channels in cell homeostasis, so that their blockade delayed the entire process. To elucidate between these two possibilities, we explored the time course of TEA effects on VSMC proliferation after synchronization of cultures at different checkpoints along cell cycle, determining the number of cells in S-phase with BrdU incorporation assays.

First, we estimated the duration of the S-phase (*Figure 5A*). After VSMCs synchronization on late G1 phase with L-mimosine, cells were kept in control media alone or with 100 μM TEA. BrdU incorporation was determined every 4 h. The time to reach the peak of BrdU incorporation set the S-phase duration, and was not different between control and TEA-treated VSMCs (6.56 ± 0.06 and 6.59 ± 0.10 h, respectively).

In another group of experiments, after VSMC synchronization at G0 with SF medium, control medium was reintroduced and half of the cells were also treated with 100 μM TEA up to 72 h. The time course of BrdU incorporation (*Figure 5B*) showed that TEA treatment led to a reduction in the cells entering S phase after 32 h. Together with the previous results (*Figure 5A*), these data suggested that Kv3.4 blockade had no effect on the progression of cycling cells up to S phase. Consequently, TEA effect could be interpreted as if Kv3.4 channels role were to facilitate reentrance in cell cycle after G2/M completion.

We tested this hypothesis with a double thymidine block (to synchronize cells at G1/S phase) followed by nocodazole treatment for 12 h, to obtain a large population of cells arrested in M phase.[20] After nocodazole release, VSMCs were placed for 12–14 h in control media alone or with 100 μM TEA and cell cycle distribution was explored. *Figure 5C* shows the flow-cytometry histograms obtained in a representative experiment after nocodazole treatment (0 h) and 12 h later in control conditions or in the presence of TEA. Summary data from three different experiments (*Figure 5D*) indicated that 100 μM TEA increased the proportion of cells in G0/G1 phase and decreased the proportion of cells in S phase, without changes in the G2/M fraction. Similar results were obtained with 2.5 μM BDS-I treatment (*Figure 5D*), when exploring the fraction of quiescent, $Ki67^-$ cells. Altogether, these results indicated a specific role of Kv3.4 channels modulating cell cycle progression, suggesting that Kv3.4 blockade arrest VSMCs at G0/G1 phase after completing the cell cycle.

3.5 Effects of Kv3.4 knockdown on cell cycle progression

To further confirm this hypothesis, we used siRNA-mediated knockdown of Kv3.4 channels in HUA VSMCs. The potency of the different siRNA commercially available reducing Kv3.4 mRNA and protein expression was assessed in a heterologous expression system. A set of these siRNAs showed a consistent reduction up to 80% of the Kv3.4 mRNA levels and correlated with a decreased amount of

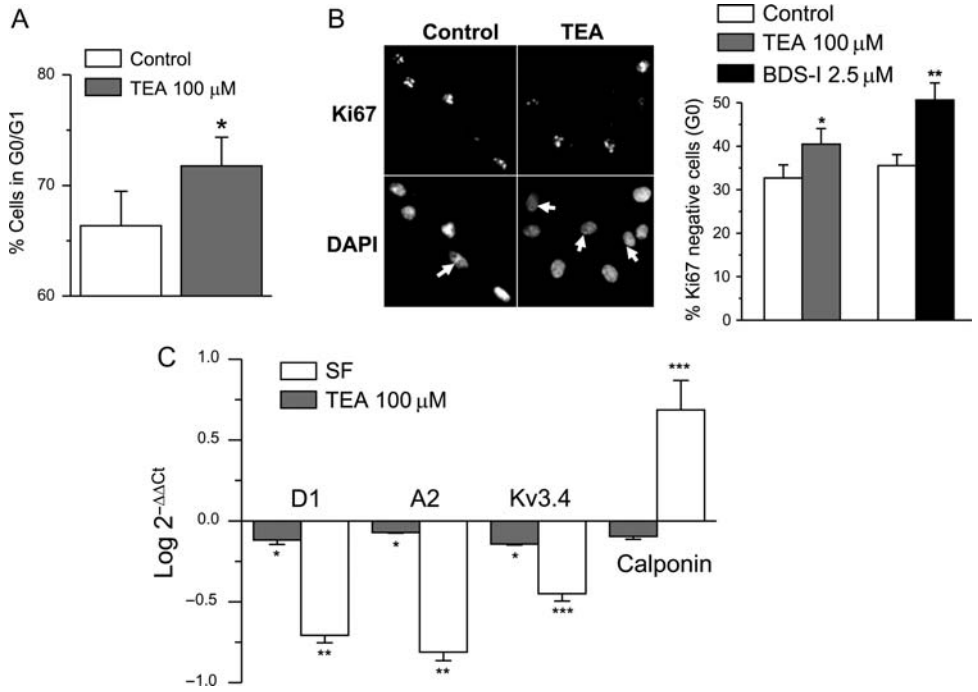

Figure 4 Effect of Kv3.4 channel blockade on cell cycle distribution. (*A*) Percentage of G0/G1 cells in VSMCs synchronized in SF during 48 h and cultured for 48 h in control media alone or with 100 μM TEA. Data obtained with flow cytometry ($n = 9$, mean ± SEM). (*B*) Percentage of Ki67$^-$ cells obtained in cultured cells kept in control media or in the presence of 100 μM TEA or 2.5 μM BDS-I ($n = 5$, 15–20 fields analysed in each experiment). Representative images of DAPI staining and Ki67 labelling in control and TEA-treated cultures are shown. (*C*) Changes in mRNA levels of D1-cyclin, A2-cyclin, Kv3.4, and calponin after treatment with 100 μM TEA (grey bars) or after serum withdrawal (SF) for 72 h (white bars). Non-synchronized cells were used as calibrator to compute the $\log(2^{-\Delta\Delta Ct})$ values. Each bar is the mean ± SEM of 3–4 triplicated assays.

Kv3.4 protein in HEK cell lysates (see Supplementary material online, *Figure S1*).

The two more efficient siRNAs (si303 and si304) were used to knockdown Kv3.4 expression in VSMCs. Transfection efficiency was routinely around 85–95%, as determined with a labelled non-silencing control siRNA. We explored the time course of the effects of Kv3.4 knockdown on mRNA levels and on proliferation rate in cells transfected after G0 synchronization (*Figure 6*). We found clear time dependent, cyclic variations of Kv3.4 mRNA levels that could reflect cell cycle associated changes in expression levels. Additionally, Kv3.4 siRNA led to a time-dependent decrease of Kv3.4 mRNA starting at 18 h after transfection and continuing up to the end of the assay at 72 h post-transfection (*Figure 6A*). Parallel characterization of proliferation rate demonstrated a decreased proliferation starting at 36 h after transfection (*Figure 6B*), together with an increase in the proportion of Ki67$^-$ cells (from 34.9 ± 3.9% in control to 41.2 ± 3.1% in siRNA-transfected cells, $P < 0.05$, data not shown). Moreover, decreased Kv3.4 mRNA in siRNA-transfected cells correlated with decreases in D1, A2, and B1 cyclins mRNA (*Figure 6C*). These results indicated that, similar to Kv3.4 blockade, Kv3.4 knockdown conduced to VSMCs to arrest in G0. Also resting V_M in siRNA transfected cells was significantly more depolarized than in mock-transfected cells (−28.6 ± 1.47 vs. −40.3 ± 2.5 mV, $P < 0.001$) and was not modified by 100 μM TEA (data not shown), confirming a role for Kv3.4 channels setting the resting membrane potential in cultured HUA VSMCs.

3.6 Effects of depolarization on cell cycle progression

Resting V_M changes could provide the link between functional expression of Kv3.4 and cell cycle progression. If this were the case, incubation of VSMCs in culture media with high K^+ concentrations should reproduce the effect of Kv3.4 channels blockade on proliferation rate. We explored this hypothesis by incubating VSMCs for 48 h in media containing 10 or 20 mM K^+. Under these conditions, proliferation rate decreased to values similar to those obtained with the blockade of Kv3.4 channels with 100 μM TEA or with siRNA (*Figure 6D*). Moreover, incubation of VSMCs with 100 μM TEA in the presence of 10 or 20 mM K^+ did not produce an additional inhibition. These results suggest that Kv3.4 channels regulation of cell cycle progression in uterine VSMCs is at least in part mediated by their effect on resting V_M.

4. Discussion

We describe here cell cycle-dependent variations in the mRNA and protein levels of Kv3.4 channels, as well as their functional contribution to the outward currents and to resting V_M in HUA VSMCs in culture. The blockade of Kv3.4 channels rose the fraction of quiescent cells by increasing the number of cells that exit the cell cycle after mitosis, an effect that was occluded by VSMC depolarization after raising extracellular K^+, suggesting that Kv3.4 channels contribution

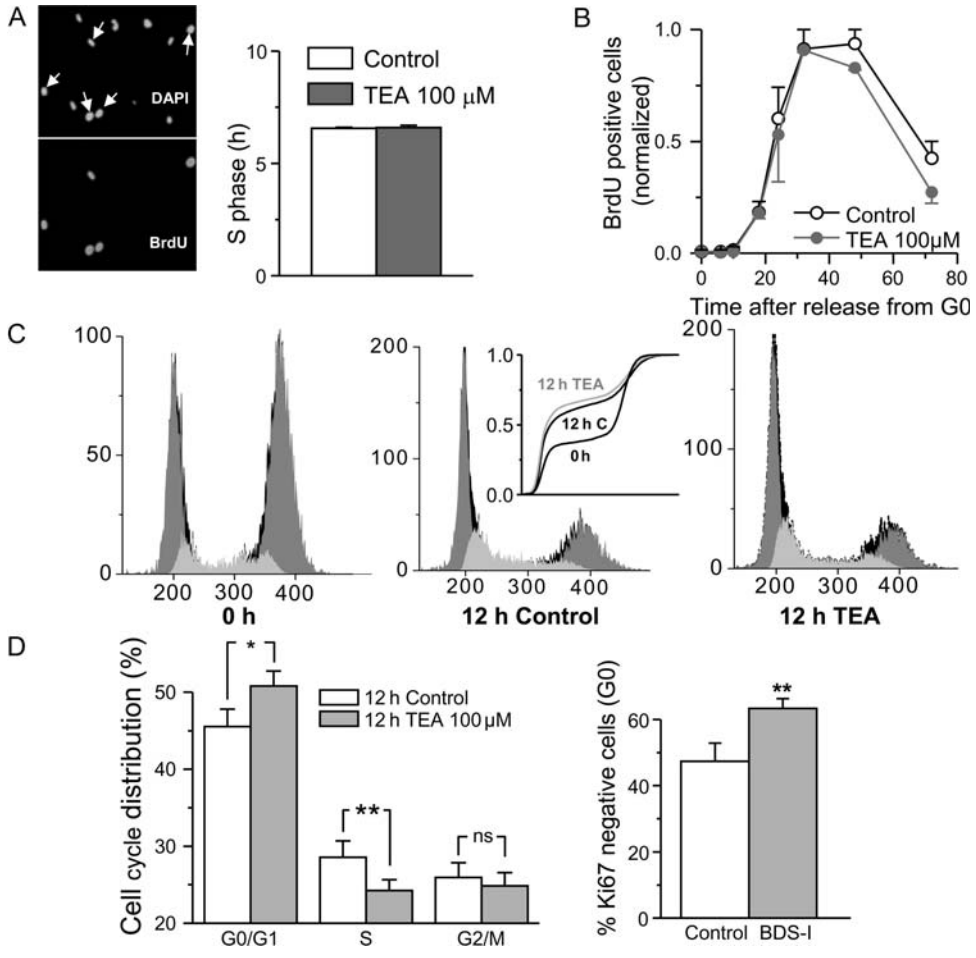

Figure 5 Effect of Kv3.4 blockade on cell cycle progression. (*A*) Effect of 100 μM TEA on S-phase duration. The length of the S phase was estimated by determining the time to reach the peak of BrdU incorporation in VSMCs previously synchronized in late G1 with L-mimosine. Data are mean ± SEM, $n = 3$. Representative immunofluorescence images of the same field labelled with DAPI and with BrdU are also shown. (*B*) Effect of TEA on cell cycle progression from G0/G1. The plot shows the time course of BrdU incorporation in cells previously synchronized with SF for 72 h, and maintained in control medium alone or with 100 μM TEA; $n = 3$. (*C*) Effect of TEA on cell cycle progression from G2/M. VSMCs were synchronized with a double thymidine block followed by nocodazole addition. Flow-cytometry histograms were obtained at nocodazole removal (0 h) and 12 h after culture in control media alone or with 100 μM TEA. The inset shows the normalized running integrals of the histograms in the three conditions. (*D*) Mean ± SEM results from three experiments as the one shown in (*C*). The right plot shows the percentage of $Ki67^-$ cells obtained in three experiments in which 2.5 μM BDS-I was applied for 12 h after double thymidine plus nocodazole treatment.

to resting V_M is relevant for their role modulating cell cycle progression.

Changes in cell cycle progression after Kv3.4 blockade or knockdown were always consistent and significant, although of small magnitude. These reduced effects are, at least in part, due to the lack of fully synchronized VSMC populations. In spite of the seeming slow proliferation rate, time-lapse studies for up to 48 h showed that cell cycle duration in HUA VSMC cultures was 23.4 ± 0.64 h ($n = 10$), suggesting that difficulties in synchronizing cultures could be due to the existence of a fraction of the cells entering a quiescent-like stage from which they require longer to re-enter S phase.[21] Our data indicate that functional expression of Kv3.4 channels facilitates the progression of HUA VSMCs through G1 phase, pointing to Kv3.4 channels as specific targets for modulating VSMC proliferation.

There is increasing evidence in the literature that ionic activity can influence cell cycle progression. Since K^+ channel blockade has been shown to be antiproliferative for numerous cell types, their currents have been proposed as determinants during cell cycle regulation. Cell cycle stage-dependent changes in K^+ currents have been described in many preparations, including immortalized cell lines, embryonic and tumoural cells.[12,22–24] However, there are no conclusive evidences that these changes suffice to regulate cell cycle, as the mechanisms by which ion channel activity translates into cell cycle signalling or vice versa are largely unknown. In principle, ion channels may affect proliferation in two different ways: any cell requires ion channel function to maintain basic homeostatic parameters ($[Ca^{2+}]$, pH, volume, and so on) and to allow uptake and release of metabolites, so that the inhibition of those channels will lower cell proliferation without interfering at a particular cell cycle step. On the other hand, ion channel activity, such as the hyperpolarizing activity of K^+ channels, is required at special checkpoints during cell cycle, and therefore it will have a specific role in proliferation.[12,13] Here, we

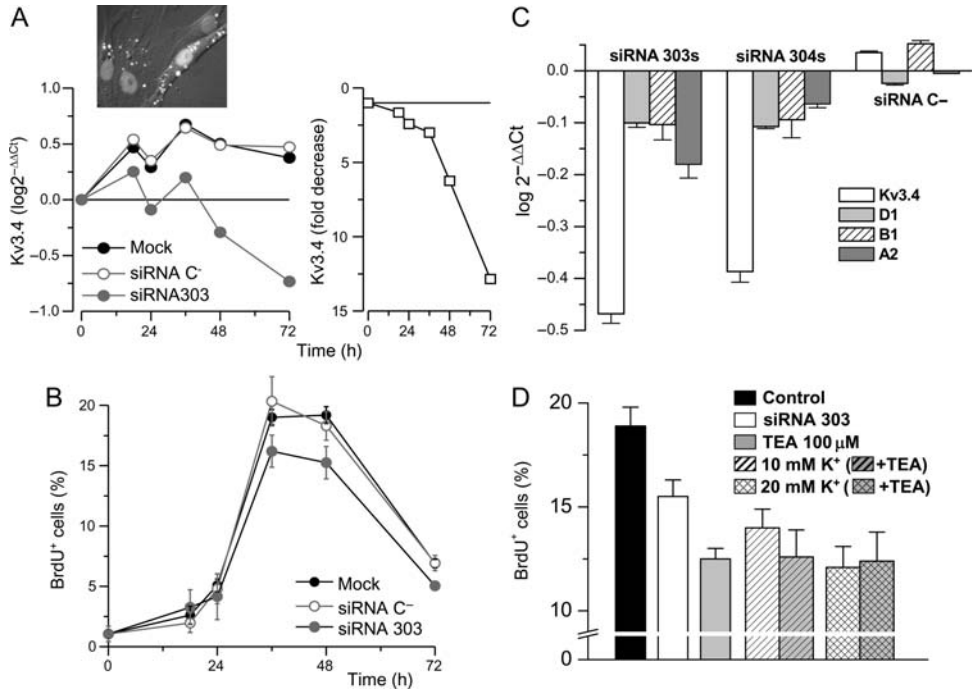

Figure 6 Effect of Kv3.4 knockdown on cell cycle progression. (*A*) Time course of changes in Kv3.4 mRNA levels obtained in VSMCs synchronized at G0 and transfected with Kv3.4 siRNA (siRNA303), negative control siRNA (C^-) or mock-transfected, relative to non-synchronized VSMCs. The Kv3.4 mRNA fold decrease obtained with siRNA303-transfected cells with respect to mock-transfected cells is shown in the right plot. Transfection efficiency was determined using the Block-it siRNA C^- (bright white spots in the image). (*B*) BrdU incorporation after siRNA transfection was studied with the same experimental protocol described in *Figure 5B*. $n = 3$. (*C*) Changes in cyclins and Kv3.4 mRNA levels are represented as in *Figure 1* and were determined 48 h post-transfection with the indicated siRNAs. In this case, mock-transfected cells were used as calibrator. (*D*) Average proliferation rate after 48 h incubation of VSMCs in the indicated conditions. $n = 3–5$ experiments.

provide evidence indicating that, in the case of Kv3.4 in HUA VSMCs, there is cell cycle-dependent oscillatory expression and activity of Kv3.4 channels. The cycling of the channel is not a simple epiphenomenon of the cell cycle, but rather a modulator, as specific pharmacological or genetic blockade of this current induces cell cycle arrest in G0, as demonstrated by flow cytometry, Ki67 expression and BrdU incorporation assays.

Moreover, there are also changes in MiRP2 expression leading to kinetic changes of Kv3.4 currents with relevant functional implications. The increased expression of Kv3.4 channels as the cells move from G0 to G1 and G2/M associates with a progressive decrease of MiRP2 subunits, which reduces the number of Kv3.4–MiRP2 complexes. The biophysical properties of the remaining homomeric Kv3.4 channels determine an increased functional contribution of Kv3.4 currents to resting V_M, as inferred from the magnitude of 100 μM TEA-induced depolarization (*Figure 3B*).

One mechanism by which K^+ channel activity is related to cell proliferation is through their role controlling resting V_M.[25,26] In agreement with previous data,[12,13] we observed cell cycle variations of resting V_M in proliferating HUA VSMCs, with a progressive depolarization as the cells move from quiescence (G0) towards G1, S, and G2/M phases. However, Kv3.4 contribution to resting V_M is minimal when cells are more hyperpolarized (G0) and increases in proliferating cells, implying that the fluctuations of resting V_M in cycling VSMCs depend on changes in the activity of other channels (such as inward-rectifier K^+ channels, as described in other preparations[23]), that oppose the hyperpolarizing influence of Kv3.4 channels. In spite of that the observed cyclic changes in Kv3.4 activity, via changes in V_M, can determine cell cycle progression. There are strong evidences indicating that transient hyperpolarization at a G1 control point is required for proliferation of both mitogen-stimulated and cycling cells,[12] and the expression and activation of Kv3.4 channels at this control point will allow the progression through G1 phase. The resulting hyperpolarization will be able to initiate cell signalling and must be synchronized with other cell cycle regulatory processes that tend to limit the duration of this change in V_M. This hypothesis is consistent with the pattern of activity of Kv3.4 channels along the cell cycle and can also explain that their blockade inhibits cell cycle progression at the passage through G1. The requirement for a transient hyperpolarization below a threshold V_M value could also explain the observation that high K^+-induced depolarization inhibits proliferation and mimics the effect of Kv3.4 blockade, as the two effects were non-additive.

The plasticity of VSMCs evolved as a mechanism of vascular repair after injury and/or vascular adaptation to changing demands. This phenotypic switch is under a tight control that reflects the interaction of genetic and environmental factors, which explains both the stable expression of vascular bed-specific transcriptional programs and the plasticity of VSMCs to respond with different genetic programs to pro-proliferative and pro-migratory signalling pathways.[1,5] Heterogeneity in ion channels expression pattern across different vascular beds has provided a large number of candidates implicated in the

phenotypic switch, some of them conserved in different VSMC preparations.[5,27] However, up-regulation of Kv3.4 channels has been associated with the proliferative phenotype only in HUA VSMCs[5,7] (and some non-vascular preparations such as tumoural cells[28] and developing neurons[29]). This specificity could be related to the peculiarities of this vascular bed regarding its potential for growth under physiological conditions.[30] Pregnancy-induced VSMC proliferation involves estrogen and peptide mitogens together with several local released factors; moreover, pregnancy-induced phenotypic changes in cultured aortic and uterine VSMCs show differences attributable to vascular cell heterogeneity.[31] In this regard, preliminary data indicate that Kv3.4 expression levels decreased in HUA VSMCs cultured in an estrogens-free media and can be recovered after the addition of 17β estradiol (data not shown), an observation that can contribute to our understanding about the physiological regulation of Kv3.4 channels in HUA. As a whole, the results gathered in this work open interesting new avenues to explore the molecular mechanisms involved in the VSMC phenotypic switch, which can be relevant for its regulation in pathophysiological processes.

Supplementary material

Supplementary Material is available at *Cardiovascular Research* online.

Acknowledgements

We thank Esperanza Alonso for excellent technical assistance and the reviewers for their shrewd comments.

Conflict of interest: none declared.

Funding

Work supported by Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III [R006/009-Heracles network, PI041044 to J.R.L.L.], Ministerio de Educación y Ciencia [BFU2004-05551 to M.T.P.G.; BFU2007-61524 to J.R.L.L.], predoctoral fellowship to OC], and Junta de Castilla y León [GR242].

References

1. Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev* 2004;**84**:767–801.
2. Thyberg J. Differentiated properties and proliferation of arterial smooth muscle cells in culture. *Int Rev Cytol* 1996;**169**:183–265.
3. Chamley-Campbell J, Campbell GR, Ross R. The smooth muscle cell in culture. *Physiol Rev* 1979;**59**:1–61.
4. Owens GK. Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev* 1995;**75**:487–517.
5. House SJ, Potier M, Bisaillon J, Singer HA, Trebak M. The non-excitable smooth muscle: Calcium signaling and phenotypic switching during vascular disease. *Pflugers Arch* 2008;**456**:769–785.
6. Burg ED, Remillard CV, Yuan JX. Potassium channels in the regulation of pulmonary artery smooth muscle cell proliferation and apoptosis: pharmacotherapeutic implications. *Br J Pharmacol* 2008;**153**:S99–S111.
7. Miguel-Velado E, Moreno-Dominguez A, Colinas O, Cidad P, Heras M, Perez-Garcia MT *et al.* Contribution of Kv channels to phenotypic remodeling of human uterine artery smooth muscle cells. *Circ Res* 2005;**97**:1280–1287.
8. Abbott GW, Butler MH, Bendahhou S, Dalakas MC, Ptacek LJ, Goldstein SA. MiRP2 forms potassium channels in skeletal muscle with Kv3.4 and is associated with periodic paralysis. *Cell* 2001;**104**:217–231.
9. Pannaccione A, Boscia F, Scorziello A, Adornetto A, Castaldo P, Sirabella R *et al.* Up-regulation and increased activity of Kv3.4 channels and their accessory subunit MinK-related peptide 2 induced by amyloid peptide are involved in apoptotic neuronal death. *Mol Pharmacol* 2007;**72**:665–673.
10. Harper JV, Brooks G. The mammalian cell cycle: an overview. *Methods Mol Biol* 2005;**296**:113–153.
11. Day ML, Pickering SJ, Johnson MH, Cook DI. Cell-cycle control of a large-conductance K^+ channel in mouse early embryos. *Nature* 1993;**365**:560–562.
12. Wonderlin WF, Strobl JS. Potassium channels, proliferation and G1 progression. *J Membr Biol* 1996;**154**:91–107.
13. Kunzelmann K. Ion channels and cancer. *J Membr Biol* 2005;**205**:159–173.
14. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. *Methods* 2001;**25**:402–408.
15. Moreno-Dominguez A, Cidad P, Miguel-Velado E, Lopez-Lopez JR, Perez-Garcia MT. *De novo* expression of Kv6.3 contributes to changes in vascular smooth muscle cell excitability in a hypertensive mice strain. *J Physiol* 2009;**587**:625–640.
16. Diochot S, Schweitz H, Beress L, Lazdunski M. Sea anemone peptides with a specific blocking activity against the fast inactivating potassium channel Kv3.4. *J Biol Chem* 1998;**273**:6744–6749.
17. Horwitz SB. Taxol (paclitaxel): mechanisms of action. *Ann Oncol* 1994;**5**:S3–S6.
18. Satoh K, Matoba T, Suzuki J, O'Dell MR, Nigro P, Cui Z *et al.* Cyclophilin A mediates vascular remodeling by promoting inflammation and vascular smooth muscle cell proliferation. *Circulation* 2008;**117**:3088–3098.
19. Yoshida T, Owens GK. Molecular determinants of vascular smooth muscle cell diversity. *Circ Res* 2005;**96**:280–291.
20. Harper JV. Synchronization of cell populations in G1/S and G2/M phases of the cell cycle. *Methods Mol Biol* 2005;**296**:157–166.
21. Coller HA. What's taking so long? S-phase entry from quiescence versus proliferation. *Nat Rev Mol Cell Biol* 2007;**8**:667–670.
22. Pardo LA, Bruggemann A, Camacho J, Stuhmer W. Cell cycle-related changes in the conducting properties of r-eag K^+ channels. *J Cell Biol* 1998;**143**:767–775.
23. MacFarlane SN, Sontheimer H. Changes in ion channel expression accompany cell cycle progression of spinal cord astrocytes. *Glia* 2000;**30**:39–48.
24. Ouadid-Ahidouch H, Ahidouch A. K^+ channel expression in human breast cancer cells: involvement in cell cycle regulation and carcinogenesis. *J Membr Biol* 2008;**221**:1–6.
25. Cone CD Jr. Variation of the transmembrane potential level as a basic mechanism of mitosis control. *Oncology* 1970;**24**:438–470.
26. Cone CD Jr. The role of the surface electrical transmembrane potential in normal and malignant mitogenesis. *Ann N Y Acad Sci* 1974;**238**:420–435.
27. Beech DJ. Ion channel switching and activation in smooth-muscle cells of occlusive vascular diseases. *Biochem Soc Trans* 2007;**035**:890–894.
28. Chang KW, Yuan TC, Fang KP, Yang FS, Liu CJ, Chang CS *et al.* The increase of voltage-gated potassium channel Kv3.4 mRNA expression in oral squamous cell carcinoma. *J Oral Pathol Med* 2003;**32**:606–611.
29. Pollock NS, Ferguson SC, McFarlane S. Expression of voltage-dependent potassium channels in the developing visual system of *Xenopus laevis*. *J Comp Neurol* 2002;**452**:381–391.
30. Osol G, Mandala M. Maternal uterine vascular remodeling during pregnancy. *Physiology* 2009;**24**:58–71.
31. Keyes LE, Moore LG, Walchak SJ, Dempsey EC. Pregnancy-stimulated growth of vascular smooth muscle cells: importance of protein kinase C-dependent synergy between estrogen and platelet-derived growth factor. *J Cell Physiol* 1996;**166**:22–32.