Pflugers Arch - Eur J Physiol
DOI 10.1007/s00424-014-1607-y

Kv1.3 channels modulate human vascular smooth muscle cells proliferation independently of mTOR signaling pathway

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Received: 23 June 2014 / Revised: 7 August 2014 / Accepted: 1 September 2014

Abstract Phenotypic modulation (PM) of vascular smooth muscle cells (VSMCs) is central to the process of intimal hyperplasia which constitutes a common pathological lesion in occlusive vascular diseases. Changes in the functional expression of Kv1.5 and Kv1.3 currents upon PM in mice VSMCs have been found to contribute to cell migration and proliferation. Using human VSMCs from vessels in which unwanted remodeling is a relevant clinical complication, we explored the contribution of the Kv1.5 to Kv1.3 switch to PM. Changes in the expression and the functional contribution of Kv1.3 and Kv1.5 channels were studied in contractile and proliferating VSMCs obtained from human donors. Both a Kv1.5 to Kv1.3 switch upon PM and an anti-proliferative effect of Kv1.3 blockers on PDGF-induced proliferation were observed in all vascular beds studied. When investigating the signaling pathways modulated by the blockade of Kv1.3 channels, we found that anti-proliferative effects of Kv1.3 blockers on human coronary artery VSMCs were occluded by selective inhibition of MEK/ERK and PLCγ signaling pathways, but were unaffected upon blockade of PI3K/mTOR pathway. The temporal course of the anti-proliferative effects of Kv1.3 blockers indicates that they have a role in the late signaling events essential for the mitogenic response to growth factors. These findings establish the involvement of Kv1.3 channels in the PM of human VSMCs. Moreover, as current therapies to prevent restenosis rely on mTOR blockers, our results provide the basis for the development of novel, more specific therapies.

Keywords Kv1.3 channels · Vascular smooth muscle cell proliferation · Phenotypic switch · Vascular remodeling · Cell signaling

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Electronic supplementary material The online version of this article (doi:10.1007/s00424-014-1607-y) contains supplementary material, which is available to authorized users.

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Introduction

The cellular responses to vascular injury lead to clinical events such as atherosclerosis, hypertension, and restenosis. One common feature of these lesions is the proliferation of vascular smooth muscle cells (VSMCs). While VSMC proliferation plays a key role in the development and homeostasis of blood vessels, it also contributes to the pathogenesis of vascular diseases such as hypertension and restenosis [31]. Aside from the complications related to acute rejection, heart allograft vascular disease is a major complication determining long-

Published online: 12 September 2014

term survival after heart transplantation. In spite of the improvements in prevention and treatment, up to 50 % of the patients undergoing heart transplantation are diagnosed of allograft vasculopathy within 10 years [38]. This condition represents also the most common complication after percutaneous vascular interventions and stent implantation. In all these pathologies, dedifferentiated VSMCs are the major cellular component of the thickened vessel [5].

Proliferation of VSMC occurs in response to mitogens produced by platelets, activated T cells, endothelial cells, macrophages, and VSMCs themselves, including vasoactive agents (angiotensin II, endothelin [15, 20]), cytokines such as interleukin I [19], and growth factors such as platelet-derived growth factor (PDGF) [31]. Mechanistically, mitogens can activate intrinsic protein tyrosine kinase receptors (such as PDGF and EGF) or G-protein-coupled receptors (endothelin I, angiotensin II). In both cases, subsequent signaling via mitogen-activated protein kinases (MAPKs) leads to the regulation of gene expression and cell cycle re-entry to stimulate cell proliferation [3, 22, 35].

PDGF-BB is the most potent known chemoattractant for VSMCs. Upon PDGF binding, the receptor tyrosine kinase autophosphorylates, creating docking sites for recruitment of SH2 domain-containing signaling molecules. Within minutes, many signaling modules are engaged, including several MAPKs, the phospholipase C gamma (PLCγ), and phosphoinositide 3 kinase (PI3K) [17, 29, 40].

K^+ channels have been implicated in the proliferation of a large number of cell types since the initial description of a voltage-dependent K^+-channel (Kv1.3) mediating proliferation in T cells [9]. Subsequently, a plethora of K^+ channels have been linked to migration and proliferation in numerous non-excitable tissues, including cancer cells, T lymphocytes, endothelial cells and VSMCs [1, 11, 32, 43]. Several K^+ channels, including KCa3.1, Kv3.4, and Kv1.3 have been shown to associate with VSMC proliferation [8, 24, 27, 41]. While in some cases this association depends on their ion-conducting properties, in others, it is unknown how their activity is linked to proliferation. In fact, there are some studies indicating that the effect of ion channels on cell proliferation relies on non-conducting properties of the channel proteins [7, 16, 28].

In our previous work, we postulate that Kv1.3/Kv1.5 ratio can be considered as a landmark of VSMC phenotype, because proliferation of VSMCs from several vascular beds in mice associates with a Kv1.5 to Kv1.3 channel switch [7]. Here, we explore if this role of Kv1.3 in the VSMC phenotypic modulation (PM) is also present in human vessels, and we investigate the signaling cascade linking Kv1.3 expression to increased VSMC proliferation. We confirm the anti-proliferative effect of Kv1.3 blockers in human VSMCs. The effects of Kv1.3 blockers can be occluded by selective inhibition of MEK/ERK and PLCγ pathways, but were additive to those of PI3K/mTOR blockers, opening interesting possibilities for the use of Kv1.3 blockers in the prevention and treatment of occlusive diseases.

Materials and methods

Sample collection

Human uterine (hUA), renal (hRA), and coronary arteries (hCA) and saphenous veins (hSV) belonging to the COLMAH collection of the HERACLES network (http://www.redheracles.net/plataformas/en_coleccion-muestras-arteriales-humanas.html) were obtained from donors at the Clinic Hospitals of Barcelona and Valladolid. Vessels were divided into two pieces, one was placed in RNAlater (Ambion) for RNA extractions and the other in a Dulbecco's modified Eagle's medium (DMEM) for cell isolation. Samples kept at 4 °C were received within 24 h after intervention. Cultured VSMCs were obtained from explants of the vessels as described elsewhere [27].

mRNA and protein determinations

RNA from tissue homogenates and from cultured VSMCs was isolated with TRIzol Reagent and reverse transcribed. mRNA levels were determined by real-time qPCR with TaqMan® Gene Expression Assays (Applied Biosystems) on a Rotor-Gene 3000 instrument (Corbett Research) using the $2^{-\Delta\Delta Ct}$ relative quantification method [26]. Western blot of protein lysates obtained from vascular tissues (contractile VSMCs) or primary cultures (proliferative VSMCs) were used for protein detection. Detection was carried out with the VersaDoc™ 4000 Image System (BioRad) with chemiluminescence reagents.

Electrophysiological methods and intracellular calcium measurements

Ionic currents were recorded at room temperature (20–25 °C) using the whole-cell configuration of the patch-clamp technique as previously described [27, 30]. Membrane potential (V_M) measurements were obtained at RT using the perforated patch technique [39]. For intracellular calcium measurements, hCA VSMCs were loaded with Fluo-4-AM (Molecular Probes, Invitrogen, OR, USA). Changes in fluorescence in response to the indicated stimuli were analyzed with Imaging Workbench 4.0 image software.

Proliferation assays

Proliferation was determined using a commercial kit (Click-iT® EdU Imaging Cell Proliferation Assay, Invitrogen). VSMCs at passages 3–8 were seeded onto 12-mm poly-L-

lysine-coated coverslips and synchronized in serum-free (SF) medium during 48 h before adding the proliferative stimulus (alone or in combination with specific inhibitors) during 24 h.

An expanded material and methods section with detailed protocols can be found in the Electronic supplementary material.

Results

The ratio of Kv1.3 to Kv1.5 mRNA can define the VSMC phenotype

In mice VSMCs, PM associates with a consistent change in the Kv1.3 to Kv1.5 ratio [7]. Here, we determined the relative abundance of Kv1.3 and Kv1.5 mRNA in VSMCs obtained from several human vascular beds, both in contractile (*Tissue*) and in proliferative phenotype (*Culture*). mRNA expression levels of another K^+ channel (the intermediate conductance Ca^{2+}-activated K^+ channel, KCa3.1) previously reported to associate with VSMC proliferation [24, 42] were also explored. Figure 1a shows mRNA levels in VSMCs from human saphenous veins (hSV), coronary (hCA), and renal arteries (hRA). Kv1.5 mRNA expression was predominant in all vascular beds in the contractile phenotype, decreasing dramatically upon PM. Otherwise, Kv1.3 and KCa3.1 mRNA levels were significantly lower and the changes upon PM smaller. Similar data has been found in human uterine artery (hUA) [27]. Nevertheless, when these data are represented as Kv1.3/Kv1.5 ratio ($2^{-\Delta\Delta Ct}$), it is evident that a clear switch from Kv1.5 to Kv1.3 upon PM is conserved in all vascular beds explored. This ratio is expressed in Fig. 1b in a logarithmic scale so that negative values reflect a higher expression of Kv1.5 mRNA while positive values reflect a higher expression of Kv1.3 mRNA. In all cases, Kv1.3 became the predominant Kv1 channel expressed in cultured VSMCs, mainly due to the dramatic decrease of Kv1.5 mRNA upon PM (Fig. 1c).

Changes in mRNA Kv1.3/Kv1.5 ratio upon PM correlate with changes in functional channel protein expression

We next explored the protein expression of Kv1.3 and Kv1.5 channels. Protein extracts obtained from hRA VSMCs, both in contractile (Tissue) and proliferating (Cultured) phenotype, were used for immunoblots with anti-Kv1.3 and anti-Kv1.5 (Fig. 2a). The expression of Kv1.5 protein significantly decreased in cultured VSMCs, in agreement with the mRNA expression levels. However, changes in Kv1.3 protein (almost not detectable in contractile VSMCs and robustly expressed in cultured VSMCs) were not anticipated from the mRNA expression data.

Electrophysiological studies in VSMCs obtained from hRA allowed functional characterization of the channels (Fig. 2b–d). Whole-cell patch-clamp experiments were carried out in VSMCs freshly dispersed (contractile) or from VSMCs maintained in primary culture. Kv currents were elicited by depolarizing pulses to +40 mV, and Kv1.3 and Kv1.5 contribution was estimated as the fraction of current sensitive to the selective blockers 5-(4-phenoxybutoxy) psoralen (PAP-1) or diphenyl phosphine oxide (DPO), respectively [7, 36]. Representative experiments (Fig. 2b) and average data (part c) are depicted. Kv1.5 currents represented a large fraction of the Kv currents elicited in contractile VSMCs, being almost absent in cultured VSMCs. On the contrary, the fraction of PAP-1 sensitive currents (Kv1.3) increased from contractile to cultured VSMCs. This increase remains when Kv1.3 currents are normalized by cell capacitance (Fig. 2d), in spite of the bigger size of proliferating VSMCs (43.4±4.0 pF vs. 29.27±2.34 pF of the freshly dissociated cells), indicating an increased expression of Kv1.3 channels in the plasma membrane of proliferative VSMCs. In addition, we explored whether Kv1.3 currents contribute to set the resting membrane potential (E_M) in cultured VSMCs with current clamp experiments (Fig. 2e). We found that 100 nM PAP-1 induced small, consistent depolarization, averaging 2.48±0.3 mV (n=6). Similar depolarizations were obtained in hCA cultured VSMCs (2.62±0.35 mV, n=12).

Comparable results were obtained in hCA and hSV (Online supplemental resource, Figure I). In all cases, the almost absence of DPO-sensitive currents and the large contribution of Kv1.3 channels to total Kv currents in cultured VSMCs were evident.

Exploring the contribution of changes in Kv1.3/Kv1.5 ratio to PM

We hypothesized that the Kv1.5 to Kv1.3 switch could be a relevant event needed to facilitate the acquisition of the proliferative and/or migratory capabilities of the PM. In explants from hRA, the switch on mRNA expression could be observed as soon as after 3 days in culture, becoming more pronounced at 7 days (Fig. 3a). No evident proliferation and migration could be observed at these times, suggesting that the Kv1 switch is needed for the PM and that the changes in their expression levels are modulated by the mitogenic signals initiating PM.

KCa3.1 channels in coronary VSMCs are upregulated upon PM as a consequence of the proliferative stimulus [37, 41]. To explore if this was also the case for Kv1.3 channels, we analyzed the changes in mRNA expression levels upon treatment of VSMC cultures with 20 % FBS (Fig. 3b) or 100 ng/ml PDGF (Fig. 3c). KCa3.1 mRNA increased both in hRA and hSV VSMCs upon stimulation with FBS or PDGF. However, Kv1.3 mRNA expression did not change significantly. These data point to a fundamental difference in the regulation of both ion channels during PM. While KCa3.1 channels seem to be regulated transcriptionally, Kv1.3 increased protein expression in the proliferative phenotype (Fig. 2) requires an alternate explanation.

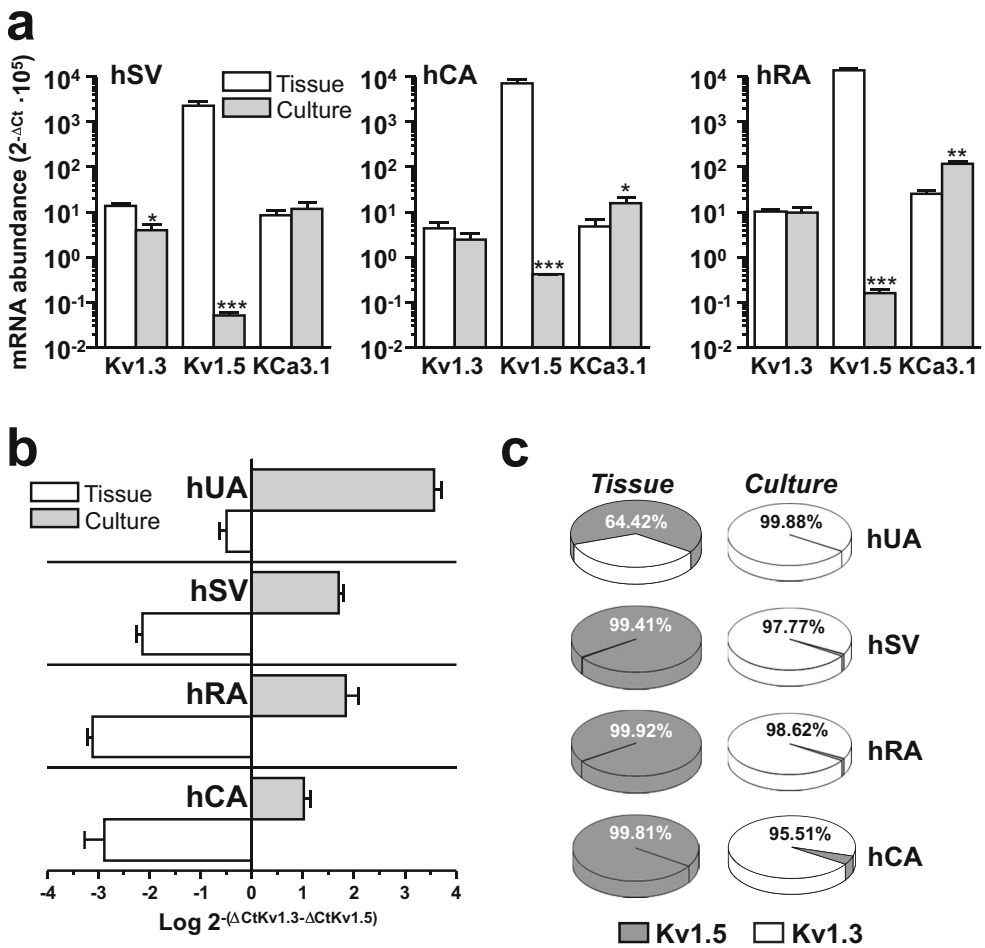

Fig. 1 mRNA expression levels of Kv1.3 and Kv1.5. **a** Relative abundance of Kv1.3, Kv1.5, and KCa3.1 mRNA was determined in human saphenous veins (hSV), coronary arteries (hCA), and renal arteries (hRA) both in contractile (*Tissue, open bars*) and in proliferative phenotype (*Culture*, *gray bars*). Expression levels were normalized to the housekeeping gene RPL18 and expressed as $2^{-\Delta Ct}$, where ΔCt=Ct*channel*-Ct*RPL18*. (see Electronic supplementary material). Each data is the mean ± SEM of 5–9 different preparations with triplicate determinations. *$p<0.05$, **$p<0.01$, *** $p<0.001$ (all through the text). **b** *Bar plots* show the Kv1.3:Kv1.5 ratio in four human vascular beds both in contractile (*white bars*) and proliferative (*gray bars*) phenotype. The ratio was expressed as log $2^{-(\Delta CtKv1.3-\Delta CtKv1.5)}$. In this scale, a value of 0 indicates equal expression (i.e., a Kv1.3:Kv1.5 ratio of 1), −2 denotes 100 times higher Kv1.5 expression than Kv1.3, and +2 Kv1.3 expression levels 100 times higher than Kv1.5. Each data point was obtained from at least six different vessels/cultures. **c**. The relative abundance of Kv1.3 (*white*) and Kv1.5 (*gray*) mRNA in the four preparations in both tissue and cultured VSMCs is illustrated by the pie charts

Selective blockade of Kv1.3 currents inhibits proliferation

The increased functional expression of Kv1.3 currents in cultured VSMCs suggests a link between the channel and the establishment and/or maintenance of the proliferative phenotype. To explore this possibility, we have tested the effect of 100 nM PAP-1 and 10 nM Margatoxin (MgTx) on the FBS-induced proliferation in VSMCs obtained from four different human vessels (Fig. 4a). In all cases, we found a significant decrease on the rate of FBS-induced proliferation in the presence of Kv1.3 blockers, suggesting a functional association of Kv1.3 expression with PM. To further investigate the signaling pathways linking the functional expression of Kv1.3 channels to VSMC proliferation, we explored the effects of Kv1.3 blockade on the proliferation induced by specific growth factors such as PDGF or angiotensin II (ATII) (Fig. 4b). ATII (1 μM) was a proliferative stimulus not as potent as PDGF (100 ng/ml). However, the inhibitory effect of 100 nM PAP-1 was the same in the two conditions, suggesting a common signaling pathway. Interestingly, when 100 ng/ml PDGF was used as the proliferative stimulus in VSMCs, proliferation rates were comparable to those obtained with 5–20 % FBS (see Online supplemental resource, Figure II), but the effect of 100 nM PAP-1 inhibiting proliferation was much stronger. Although this difference was particularly evident in hCA (~20 % inhibition with FBS vs. ~60 % inhibition with PDGF), similar results were obtained when VSMCs from other vascular beds were studied (Fig. 4c). Figure 4d shows the summary data obtained from hRA VSMCs when exploring the effect of different K^+ channel blockers on PDGF-induced

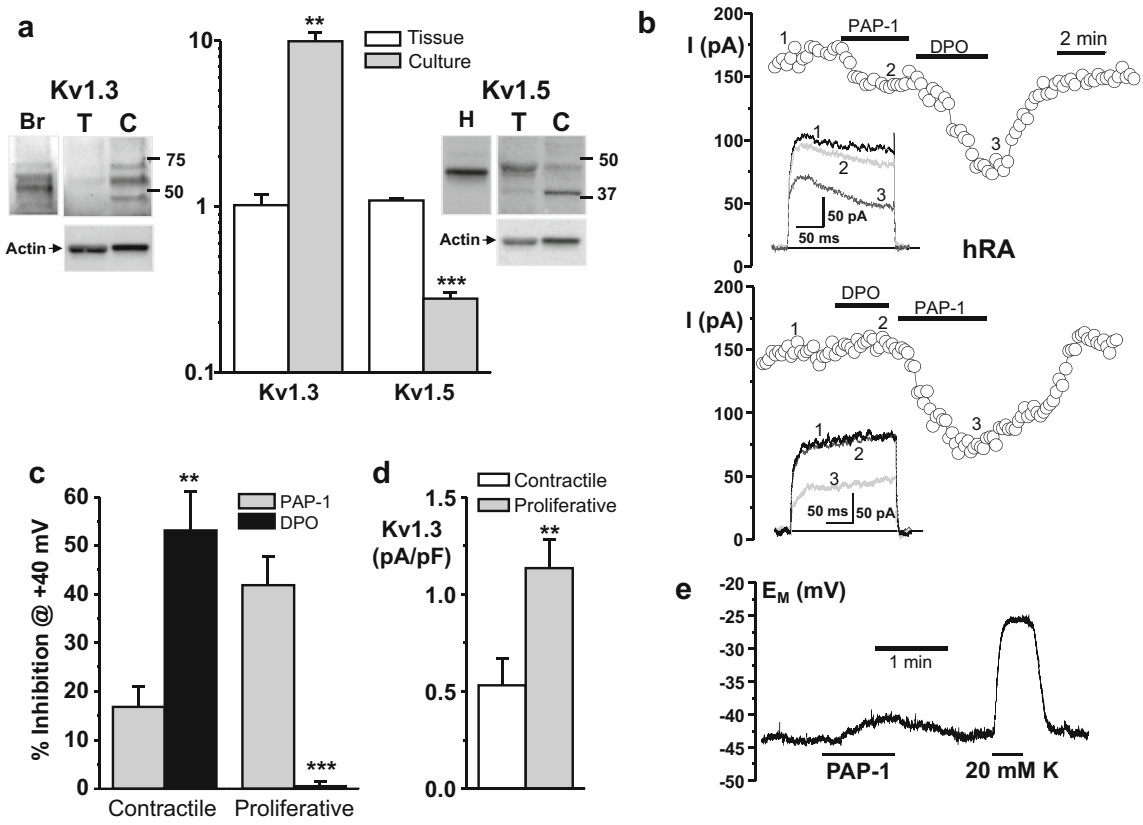

Fig. 2 Changes in the functional expression of Kv1.3 and Kv1.5 proteins during PM. **a** Representative immunoblots of VSMC lysates obtained from hRA homogenates (Tissue, *T*) or hRA primary cultures (*C*) with anti-Kv1.3 (*left*) or anti-Kv1.5 antibodies (*right*). Positive controls were brain (*Br*) and heart (*H*) lysates (Kv1.3 and Kv1.5, respectively), and β-actin was used as loading control. *Bar plots* show averaged data from 3 to 5 immunoblots. Kv1.3 or Kv1.5 protein expression was corrected for β-actin and normalized to the amount expressed in tissue. Note the logarithmic scale. **b** Time course of the peak current amplitude elicited by 200 ms pulses to +40 mV applied every 10 s in freshly dissociated (*upper* graph) or cultured (*lower* graph) hRA VSMCs. PAP-1 (100 nM) or DPO (100 nM) were applied to the bath solution as indicated. Representative traces at the time points labeled 1, 2, and 3 are depicted in the *insets*. **c** The effects of the blockers were expressed as percentage of inhibition of the current amplitude. Mean ± SEM values, n=8–12 cells in each group. **d** Absolute values of the Kv1.3 current density (pA/pF) obtained from contractile (n=9) and proliferative (n=12) VSMCs from renal arteries. Kv1.3 current density was defined as the 100 nM PAP-1 sensitive current. **e** Representative recording of membrane potential from a cultured renal VSMC obtained in current clamp with perforated patch. The indicated drugs/solutions were present in the bath solution as marked with the *lines*

proliferation. Both PAP-1 (100 nM) and MgTx (10 nM) show a similar inhibitory effect. A marked inhibition was also observed with the selective KCa3.1 blocker TRAM-34 (100 nM). The specific role of these two channels (Kv1.3 and KCa3.1) in the proliferative phenotype is supported by the lack of effect of selective blockers of BK_{Ca} channels, even though BK_{Ca} currents are present in cultured VSMCs (data not shown).

Kv1.3 effects on proliferation are mediated by ERK1/2 and PLCγ signaling pathway

We sought to identify the signaling pathway(s) contributing to PDGF-induced proliferation that can be affected by Kv1.3 blockade. We determined the effect of selective blockers of the different pathways activated by PDGF in hCA VSMCs on proliferation. No effect on proliferation was observed upon blockade of JNK or p38 kinases with 1 μM SP600125 or 20 μM SB203580, respectively (Fig. 5a). On the contrary, blockers of the ERK1/2, PI3K/mTOR, and PLCγ pathways were effective inhibiting VSMC proliferation. In these cases, we also studied if some additional effects could be observed upon selective blockade of Kv1.3 channels with PAP-1 (100 nM) or MgTx (10 nM). The effect of PAP-1 or MgTx was occluded in the presence of either ERK1/2 blockers or PLCγ blockers, suggesting that the pro-proliferative effects of Kv1.3 are mediated by these two pathways. In contrast, the effect of 100 nM PAP-1 is still present when either PI3K or mTOR was inhibited. In fact, the percent inhibition was not significantly changed when the proliferation obtained with the different PI3K/mTOR blockers were taken as control (51.5 % in control vs. 51.2 % in the presence of LY294002, 44 % with rapamicin 1 nM and 58 % with everolimus 0.1 nM) suggesting independent pathways and strictly additive effects.

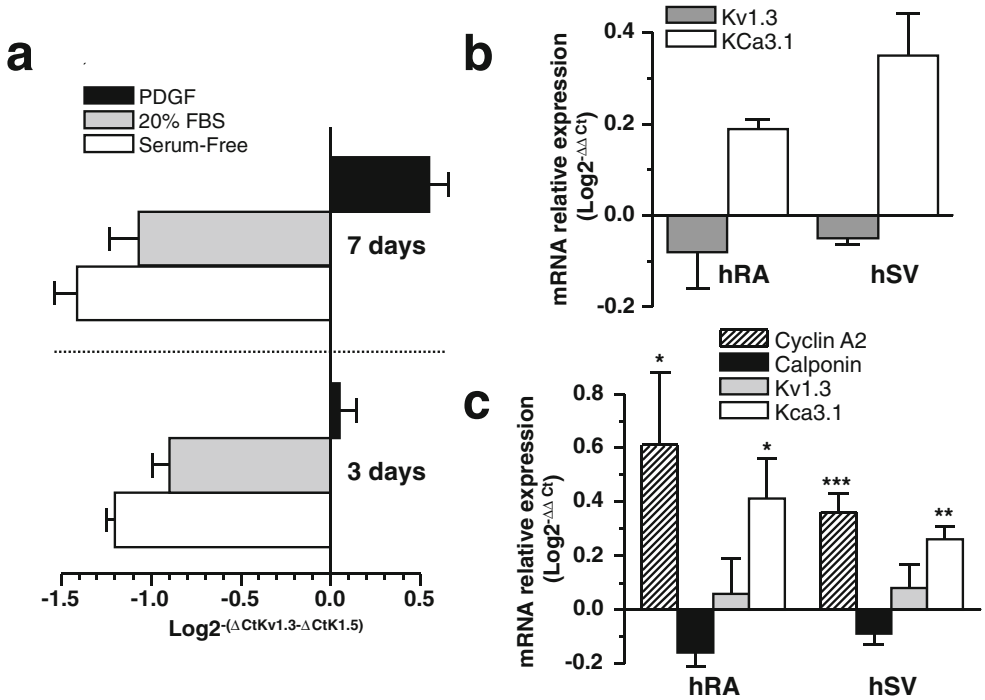

Fig. 3 **a** The Kv1.3:Kv1.5 ratio (as in Fig. 1) was obtained from renal explants incubated during 3 or 7 days in serum-free media or with 20 % FBS, or PDGF (20 ng/ml). Mean ± SEM, from three different vessels in triplicate determinations. **b**, **c** Changes in the expression of Kv1.3 and KCa3.1 mRNA in cultured hRA and hSV VSMCs after a 24-h incubation with 20 % FBS (**b**) or 100 ng/ml PDGF (**c**). The relative amount of mRNA ($2^{-\Delta\Delta Ct}$) was calculated using RPL18 mRNA as the housekeeping gene and the mRNA expression in serum-free VSMCs as the calibrator. In **c**, changes in the expression of cyclin A2 and calponin mRNA upon PDGF treatment were explored as internal controls for proliferation and differentiation, respectively. Mean ± SEM, n=4–6 experiments with triplicate determinations

Finally, the inhibitory effects of PD98059 (ERK1/2) and U73122 (PLCγ) were not additive, suggesting that these two signaling pathways converge in a common effector that could be the target of Kv1.3. However, the PI3K/mTOR pathway was clearly independent from ERK1/2 or PLCγ, since 0.1 nM everolimus clearly potentiated the effect of ERK1/2 and PLCγ blockers.

The additive effect of Kv1.3 blockers and mTOR blockers was explored in more detail by analyzing the dose-response curve for everolimus inhibition of PDGF-induced proliferation, either alone or in the presence of 100 nM PAP-1 (Fig. 5c). PAP-1 increased the inhibitory effect of everolimus at all the concentrations tested, with a similar effect at all the concentrations of everolimus, suggesting again independent mechanisms. PAP-1 (100 nM) had submaximal effects on proliferation, as illustrated in the PAP-1 dose-response curve obtained in hCA VSMCs (Online supplemental resource, Figure III).

When exploring the contribution of KCa3.1 channels in a series of similar experiments, we found that, as in hRA (Fig. 4d), 100 nM TRAM-34 inhibited PDGF-induced proliferation in hCA VSMCs (Fig. 6). However, this inhibitory effect of TRAM-34 was not additive to the effect of 100 nM PAP-1 (or 10 nM MgTx, not shown), suggesting a common effector. Finally, in a similar fashion to the effects of Kv1.3 blockers, the effect of TRAM-34 seems to be mediated by signaling through ERK1/2 (as previously described in A7r5 VSMCs, [37]) but not through PI3K/mTOR pathways. We conclude that both Kv1.3 and KCa3.1 channels contribute to VSMCs through some common effectors, indicating some redundancy on the mechanisms controlling VSMC proliferation.

Exploring the mechanisms involved in the anti-proliferative effect of Kv1.3 blockers

Data obtained in hCA VSMCs suggested that the effects of Kv1.3 channels on PDGF-induced proliferation were mediated through some common effectors of the ERK1/2 and the PLCγ signaling pathways. In order to identify this effector, we explored the effects of PAP-1 treatment on some well-known early events taking place upon activation of these signaling pathways, namely the phosphorylation of ERK1/2 or the increase in $[Ca^{2+}]_i$ upon PDGF activation of PLCγ (Fig. 7). ERK1/2 phosphorylation was evaluated by immunoblot analysis. pERK levels peaked around 10 min after PDGF stimulation, decreasing to levels close to basal ones for PDGF incubations up to 24 h (Fig. 7a). No significant differences in the levels of pERK production or in the temporal pattern were observed in VSMCs pretreated with 100 nM PAP-1.

The possible effect of Kv1.3 blockers on the initial steps of PDGF activation of PLCγ was studied by determining the

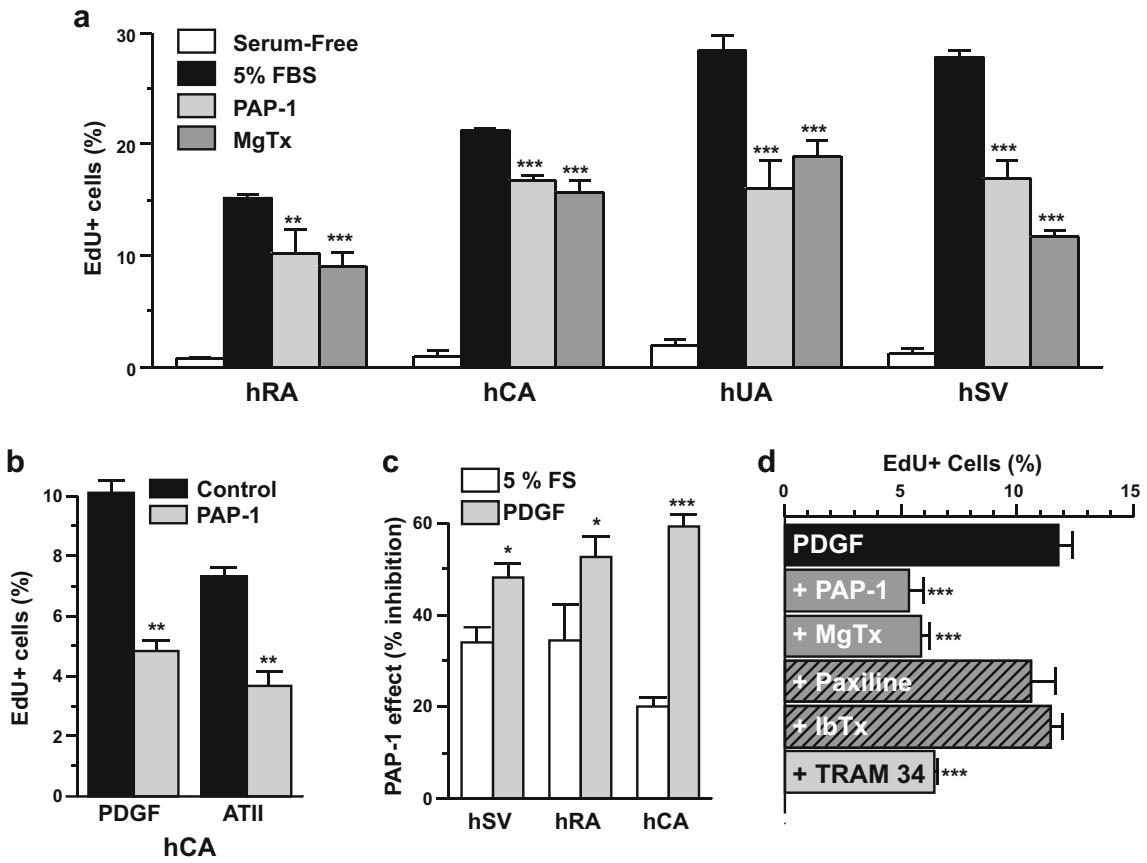

Fig. 4 **a** Proliferation rate in the four vascular beds studied was calculated as the percentage of cells incorporating EdU. After a 48-h incubation in serum-free media cells were kept during 30 h in serum-free or in the presence of 5 % FBS alone or in combination with 10 nM MgTx (MgTx) or 100 nM PAP-1 (PAP-1). EdU reagent was added to the media during the last 6 h of incubation. Each *bar* is mean ± SEM of at least six independent experiments from at least four different cultures. **b** Proliferation rate was also explored upon a 30-h treatment with two different mitogens, PDGF (100 ng/ml), and angiotensin II (ATII, 1 μM). In both cases, cells were incubated with the mitogens alone or in the presence of 100 nM PAP-1. Mean ± SEM, n=3. **c** The effect of PAP-1 (100 nM) on 5 % FBS or 100 ng/ml PDGF-induced proliferation is represented as the percent inhibition of EdU incorporation rate in three different vascular beds. Mean ± SEM, n=3–6. **d** Effects of the selective Kv1.3 blockers PAP-1 (100 nM) and MgTx (10 nM) and the selective BK_{Ca} blockers Paxilline (500nM) or Iberiotoxin (IbTx, 100 nM) and the selective KCa3.1 blocker TRAM-34 (100 nM) on PDGF-induced proliferation of hRA VSMCs. Cells were incubated during 30 h in the presence of 20 ng/ml of PDGF alone (*black bars*) or in the presence of the different blockers. Mean ± SEM, n=5–8 data from at least three different experiments

changes in $[Ca^{2+}]_i$ in response to acute application of PDGF. A short pulse of PDGF elicited a transient increase of $[Ca^{2+}]_i$ that was completely abolished by preincubation with the PLCγ blocker U73122, but unaffected by the presence of either MgTx or PAP-1 (Fig. 7b). Also consistent with these observations, the upregulation of cyclin D, one of the main growth factor-induced events in early G1, is not affected by treatment with 100 nM PAP, but is blunted with incubation with everolimus as previously described [3] (Fig. 7c). In fact, short (30 min) incubations with PDGF had a minimal proliferative effect when compared with long (24 h) incubations (Fig. 8a), suggesting that ERK1/2 phosphorylation and $[Ca^{2+}]_i$ increase may not suffice to promote PDGF-induced VSMC proliferation. In agreement with this observation, the inhibitory effect of PAP-1 on PDGF-induced hCA proliferation is not reduced when PAP-1 is added 30 min or 1 h after PDGF application, being only significantly different when its application is delayed several hours (Fig. 8b). Altogether, these data exclude a role of Kv1.3 channel at the initial steps of PDGF signaling cascade.

Discussion

The characterization of the mechanisms involved in the PM of VSMCs is a relevant issue with important clinical implications, as the cellular responses to vascular injury are important events in the formation of neointima in pathological states such as hypertension, atherosclerosis, and allograft vasculopathy. The knowledge of the signal transduction pathways controlling VSMC activation and PM may provide additional points of control that can represent novel therapeutical opportunities. Kv1.3

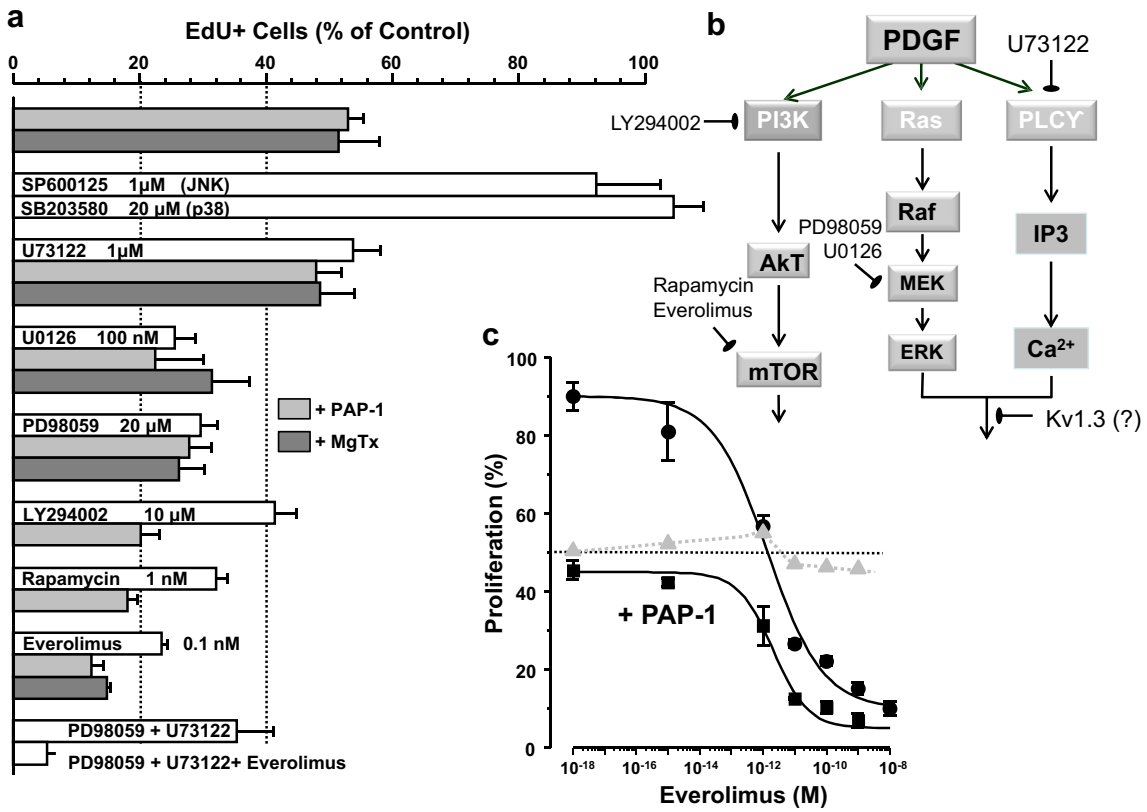

Fig. 5 **a** Effects of different treatments on the PDGF-induced proliferation of hCA VSMCs. VSMCs were serum starved for 48 h and then incubated 30 h with 100 ng/ml PDGF (control) alone or with blockers of several PDGF-activated signaling cascades at the indicated concentrations. The combined effect of these blockers with 10 nM MgTx (*dark gray*) or 100 nM PAP-1 (*light gray*) was also explored. Data were normalized to EdU incorporation in control conditions. Mean ± SEM of 5–20 determinations from at least three different cultures. Proliferation induced by PDGF treatment alone and in combination with PAP-1 were internal controls for each experiment. **b** Diagram showing the pathways explored and the targets of the blockers used. The putative location of Kv1.3 in the proliferative response is also indicated. **c** Dose-response curves for the effect of everolimus alone (*filled circle*) or in the presence of 100 nM PAP-1 (*filled square*) on PDGF-induced proliferation of hCA VSMCs. Data are normalized to the proliferation rate in 100 ng/ml PDGF, and the *solid lines* indicated the Boltzmann fit of the data. The effect of 100 nM PAP-1 at each everolimus concentration (*gray triangles*) was estimated by subtracting both curves. Each point is mean ± SEM of 3–6 determinations

channels could constitute one of those new therapeutical targets, as we show evidence indicating that the pro-proliferative role of Kv1.3 previously described in mice [8] can also be observed in human VSMCs obtained from different vascular beds. Moreover, the search for some mechanistic insights aimed to identify the signaling pathways involved in the effect of Kv1.3 in proliferation highlights nontrivial therapeutical opportunities.

The contribution of Kv1.3 channels to human VSMC proliferation seems to be a conserved, vascular bed-independent mechanism, as it could be observed in all vessels studied. Interestingly, unwanted remodeling is a relevant issue in most of the vascular beds studied.

PM in human VSMC associates with a change in mRNA Kv1.3/Kv1.5 ratio (Fig. 1). This change is mainly due to the large decrease of Kv1.5 transcripts (the most abundant Kv1 transcript in contractile VSMCs [6, 7, 30]) on proliferating cells. The switch in the ratio is an early event in the process of VSMC dedifferentiation, as it can be observed before VSMC

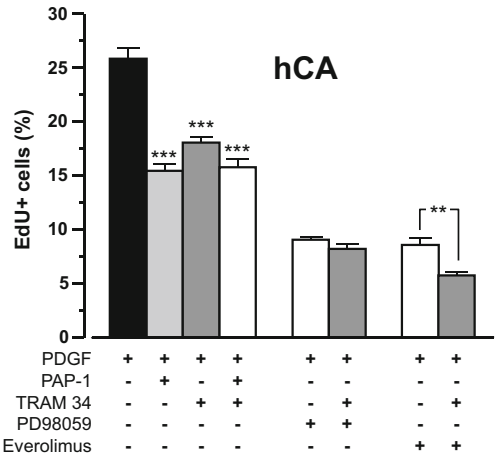

Fig. 6 Effects of TRAM-34 on hCA VSMC proliferation. VSMCs were serum starved for 48 h and then incubated 30 h with 100 ng/ml PDGF (control). The different blockers used were also present during 30 h. The effect of TRAM-34 (100 nM) was tested alone or in combination with 100 nM PAP-1, 20 μM PD98059, or 1 nM everolimus as indicated. Mean ± SEM of 4–7 determinations

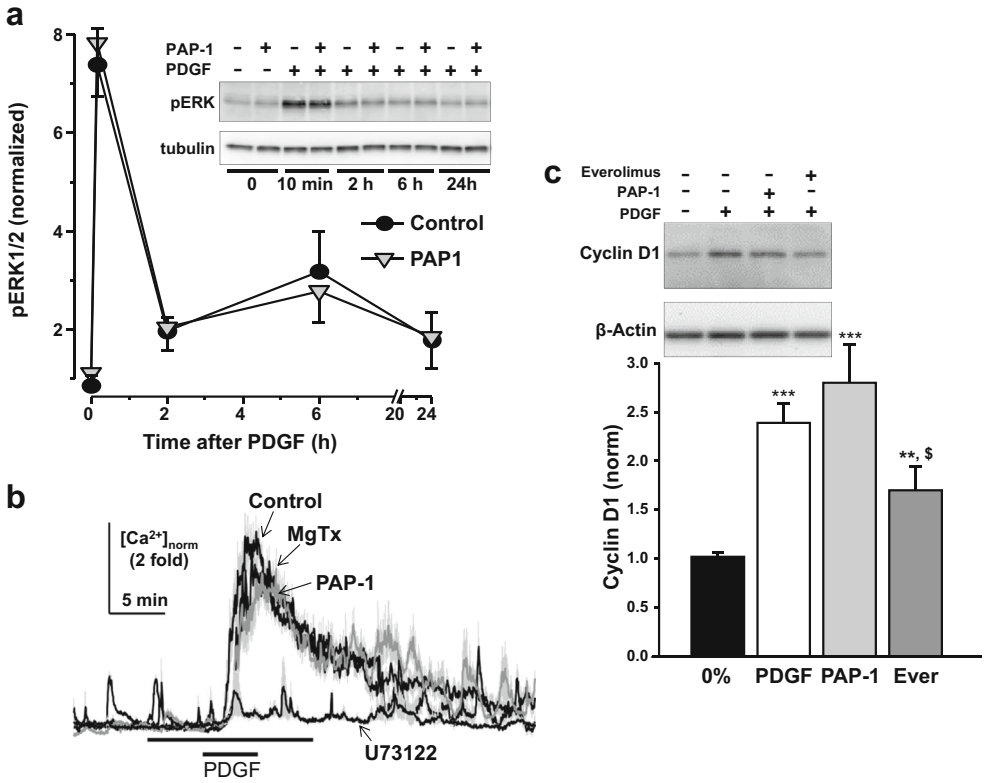

Fig. 7 **a** Time course of ERK1/2 activation. hCA VSMCs in culture were serum starved for 48 h and stimulated with PDGF (100 ng/ml) alone or with PAP-1(100 nM). ERK1/2 phosphorylation was assayed by immunoblotting with pERK antibody, with tubulin as loading control. The graph shows normalized data obtained from n=4 experiments. **b** Time course of the changes in $[Ca^{2+}]_i$ in response to acute application of PDGF. hCA VSMCs were serum starved for 24 h before loading with fluo-4-AM. Traces show changes in $[Ca^{2+}]_i$ in response to a short (5 min) application of 50 ng/ml PDGF alone or in the presence of 10 nM MgTx, 100 nM PAP-1, or 1 μM U73122 during 15 min as indicated by the *horizontal bar*. Each trace is mean ± SEM of 12–15 cells in the same plate. Data are representative of four similar experiments from different cultures. **c** Cyclin D1 expression in proliferating VSMCs. Representative immunoblots from hCA VSMC lysates obtained with anti-cyclin D1, using β-actin as the loading control. VSMC cultures were serum starved for 48 h before incubation for 24 h with PDGF alone or together with PAP-1 (100 nM) or everolimus (0.1 nM). The *bar plots* show average data from three similar experiments. * compared to serum-free (0 %); $ $p<0.05$ compared to PDGF

proliferation is evident. However, in spite of Kv1.3 increased functional expression in cultured VSMCs, Kv1.3 mRNA expression is not significantly different between contractile and proliferating VSMCs, suggesting that regulation of Kv1.3

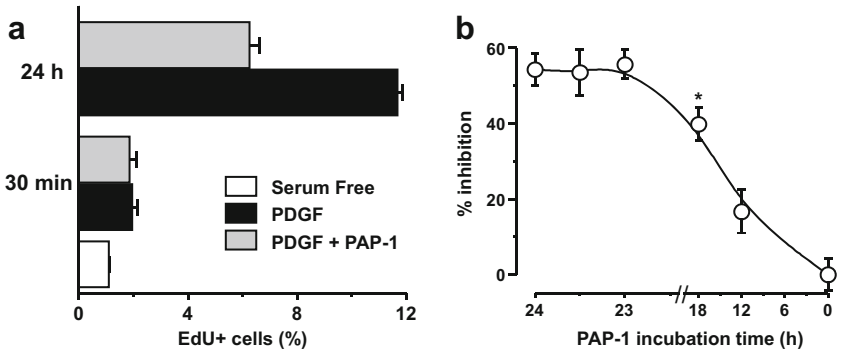

Fig. 8 **a** Time course of PDGF-induced proliferation of hCA VSMCs. Cells were serum starved for 48 h and then PDGF was added for 24 h or added for 30 min and then cells were maintained in serum-free media up to 24 h. In both cases, the effect of the simultaneous application of 100 nM PAP-1 was also explored. N=4. **b** Time course of the inhibitory effects of PAP-1 on VSMC proliferation. Inhibition of VSMC proliferation induced by 100 nM PAP-1 was plotted as a function of PAP-1 incubation time. hCA VSMCs were serum starved during 48 h and then incubated for 24 h with PDGF (100 ng/ml). PAP-1 was added at the same time than PDGF (24-h incubation time) or at the indicated intervals after PDGF application (0.5, 1, 6, and 12 h). The % inhibition was calculated as $(P_{PAP}\times 100)/P_C$ where P_{PAP} and P_C are the EdU incorporation rates in the presence and absence of PAP-1, respectively. Mean ± SEM, n=8–11 determinations from five independent experiments

protein expression is not mediated by transcriptional mechanisms. We also studied the changes in the expression of KCa3.1 channels, since they have been previously implicated in the control of VSMC proliferation both in vivo and in vitro [1, 24, 42]. We found variable PM-associated changes in KCa3.1 expression: increase in proliferating hRA and hCA VSMCs and no changes in hSV or hUA VSMCs [27]. However, in contrast to Kv1.3, KCa3.1 expression was upregulated in the presence of proliferating stimuli such as FBS and PDGF (Fig. 3, [37]), revealing a fundamental difference in the modulation of the expression of those two channels.

The functional expression of Kv1.3 channels was explored electrophysiologically, and their contribution to proliferation was demonstrated by the anti-proliferative effects of the selective blockers PAP-1 and MgTx. Our data indicate that the upregulated functional expression of Kv1.3 channels contributes to VSMC proliferation. An alternative explanation could be that the relevant change for PM is Kv1.5 downregulation so that VSMC proliferation will not take place if Kv1.5 decrease is prevented. This idea is consistent with the observation that while Kv1.3 overexpression is able to increase HEK293 cells proliferation, Kv1.5 overexpression significantly decreases it [7]. However, more research will be needed to determine whether in native VSMCs the functional expression of Kv1.5 channels is linked to anti-proliferative signaling or if it is the formation of Kv1.3/Kv1.5 heteromultimers what occludes the pro-proliferative signaling pathways mediated by Kv1.3 channels.

Selective blockade of Kv1.3 channels was able to inhibit FBS-induced proliferation in all human VSMCs tested, albeit with different potency. The anti-proliferative effect of Kv1.3 blockers was more homogeneous when cells were stimulated with a specific mitogen such as PDGF. PDGF activates multiple signaling pathways in VSMCs including Src, PLCγ, Ras, PI3K/mTOR, and MAPKs, which associate to cellular responses such as migration, proliferation, and gene expression (reviewed in [18, 29]). Most of these signaling pathways are present in VSMCs and activated by PDGF. However, despite (and possibly because of) the diversity of this complex network of signals, the precise association of each pathway to a particular cellular effect is incompletely understood. The possibility of crosstalk and compensation between pathways as well as their different contribution in different cell types also complicates their characterization [29, 40]. We found a contribution to VSMC proliferation of PI3K/mTOR, PLCγ, and ERK1/2 signaling, in agreement with previous reports [14, 25, 44]. Also in agreement with our data, p38 and JNK kinases have been previously found to have a minor contribution to VSMC proliferation, being more involved in VSMC migration and remodeling-related gene expression [44].

The activity of Kv1.3 channels modulates proliferation acting on ERK1/2 and PLCγ signaling pathways, as inhibition of proliferation by Kv1.3 blockers was occluded in the presence of selective inhibitors of these pathways, suggesting competition for the same site of action. We confirm this observation using different blockers with unrelated mechanisms of action or even different molecular target. Similarly, the fact that the effects of PAP-1 on proliferation were always reproduced by another structurally unrelated blocker such as MgTx [13, 36] supports the interpretation that their anti-proliferative effect is due to Kv1.3 channel inhibition. We also found that blockade of KCa3.1 inhibits VSMCs in all human vascular beds, but does not potentiate the effect of Kv1.3 channel blockers. These findings suggest in the one hand the presence of several alternate signaling pathways to ensure the activation of VSMC proliferation upon PM and in the other that the control of either E_M or $[Ca^{2+}]_i$ or both, through the activation of any of these K^+channels, is an important element of the signaling pathway leading to VSMC proliferation.

Regarding the downstream signaling pathways involved in cell cycle progression, previous reports demonstrate that there are two waves of growth factor-dependent signaling events required for a proliferative response. One is an acute signaling that occurs immediately and subsides even in the continuous presence of the growth factor [22], but is insufficient for cell cycle progression [21, 34]. The second wave overlaps temporally with the cell cycle program and may be directly responsible for engaging it, as PI3K/mTOR, PKC, and Ras activity during this second wave are essential for the mitogenic response to growth factors [22]. The pathways activated by these two waves may not be mutually exclusive, as there is a common signaling cascade that involves the temporally coordinated input of several effectors [21, 22], being the cellular responses depending on the timing, the duration, and the intensity of these signals [23, 33, 34]. Within this scheme, our data suggest that Kv1.3 channels play a role in that second wave of signaling events, as the same inhibition was found when PAP-1 is applied together with PDGF or 30 min or 1 h later (when all early events had taken place) while no significant inhibition was found when application was delayed for 12 h (when the second wave is over). Moreover, short time (30 min) application of PDGF only elicited a weak proliferative response not affected by PAP-1 (Fig. 8b).

The role of E_M in VSMC proliferation is an interesting issue. It has been postulated that K^+ channel inhibition depolarizes the cells, decreasing the driving force for Ca^{2+} entry, and the subsequent decrease of $[Ca^{2+}]_i$ could inhibit proliferation. However, we found that Kv1.3 blockade depolarize the membrane (Fig. 2b), but does not affect Ca^{2+} transients (Fig. 7b and Online supplemental resource, Figure IV). In the light of these observations, an alternative hypothesis could be that Kv1.3 modulates proliferation acting as a voltage sensor through cell cycle progression, coupled to ERK1/2 or PLCγ pathways [7]. Further experiments, measuring E_M changes and/or $[Ca^{2+}]$ along G0/G1 to S phase progression in native VSMCs, will contribute to clarify this aspect.

Interestingly, the pro-proliferative effect of Kv1.3 does not require PI3K/mTOR activation. This is a clear-cut result in our study. Both rapamycin and everolimus exhibit potent inhibition of growth factor-induced proliferation of lymphocytes and VSMCs and have been extensively used for maintenance of immunosuppression after transplantation [12] and to prevent neointimal hyperplasia after balloon angioplasty and/or stenting [10, 14]. In a similar fashion, Kv1.3 channels constitute a promising new anti-inflammatory drug target due to their roles in lymphocyte activation [4]. Kv1.3 is predominantly expressed in T cells and macrophages and is upregulated in effector memory T cells, and Kv1.3 blockers have been proposed as novel therapies for the treatment of autoimmune diseases [2, 36, 43]. The present work provides a role for these channels in the modulation of human VSMC proliferation, as we have demonstrate that they can serve also as therapeutical targets for the prevention and treatment of allograft vasculopathy. The fact that the anti-proliferative mechanisms involving Kv1.3 channel blockers and mTOR antagonist are additive represents a very interesting therapeutical opportunity.

Acknowledgments We thank Rodrigo de Pedro for the excellent technical assistance.

Funding Supported by grants from the Ministerio de Economía y Competitividad, Instituto de Salud Carlos III (RIC RD12/0042/0006, Red Heracles), Ministerio de Ciencia e Innovación (BFU2010-15898 to MTPG), Junta de Castilla y León (VA094A11-2 to, JRLL), and Fondo de Investigaciones Sanitarias (FIS PI11/00225 to MR).

Ethical standard For human samples, informed consent was given prior to inclusion. Protocols conforming the Declaration of Helsinki were approved by the Human Investigation Ethics Committees of the respective Hospitals.

All the experimental work performed complies with the Spanish legislation.

Conflict of interest The authors declare that they have no conflict of interest.

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