Contribution of Kv Channels to Phenotypic Remodeling of Human Uterine Artery Smooth Muscle Cells

Eduardo Miguel-Velado, Alejandro Moreno-Domínguez, Olaia Colinas, Pilar Cidad, Magda Heras, M. Teresa Pérez-García, José Ramón López-López

Abstract—Vascular smooth muscle cells (VSMCs) perform diverse functions that can be classified into contractile and synthetic (or proliferating). All of these functions can be fulfilled by the same cell because of its capacity of phenotypic modulation in response to environmental changes. The resting membrane potential is a key determinant for both contractile and proliferating functions. Here, we have explored the expression of voltage-dependent K+ (Kv) channels in contractile (freshly dissociated) and proliferating (cultured) VSMCs obtained from human uterine arteries to establish their contribution to the functional properties of the cells and their possible participation in the phenotypic switch. We have studied the expression pattern (both at the mRNA and at the protein level) of Kvα subunits in both preparations as well as their functional contribution to the K+ currents of VSMCs. Our results indicate that phenotypic remodeling associates with a change in the expression and distribution of Kv channels. Whereas Kv currents in contractile VSMCs are mainly performed by Kv1 channels, Kv3.4 is the principal contributor to K+ currents in cultured VSMCs. Furthermore, selective blockade of Kv3.4 channels resulted in a reduced proliferation rate, suggesting a link between Kv channels expression and phenotypic remodeling. (Circ Res. 2005;97:1280-1287.)

Key Words: Kv channel expression ■ vascular smooth muscle cells ■ cell proliferation ■ K+ currents

The vascular smooth muscle cells (VSMCs) of mature animals are highly specialized cells whose main function is contraction. Although during vasculogenesis, the principal role of VSMCs is proliferation and synthesis of the matrix components of the vessel wall, differentiated VSMCs proliferate at an extremely low rate and express a unique repertoire of contractile proteins, ion channels, and signaling molecules required for contraction. However, VSMCs can undergo relatively rapid and reversible phenotypic changes in response to local environmental conditions. Accelerated proliferation of VSMCs is known to play a key role in atherosclerosis, and raises [Ca2+]i, and cytosolic-free Ca2+ influx through voltage-activated Ca2+ (Cav) channels. The expression of these 4 types has been reported to vary among vascular beds as well as with vessel size.5,11 There is increasing evidence showing that K+ channels may have an important role in dedifferentiation and proliferation of VSMCs. Modulating Em,K may have an important role in dedifferentiation and proliferation of VSMCs. Modulating Em,K+ channels can affect not only Ca2+ influx, a well-established factor influencing cell proliferation, but also the driving force for Na+-dependent transport, the intracellular pH, and the regulation of cell volume, all factors that also participate in proliferation and apoptotic processes.9,10 The role of K+ channels on cell proliferation is a complex modulatory activity that only certain K+ channels at specific times and locations can perform. This fact, together with the broad diversity of functional K+ currents among different vascular beds, has unravelled the analysis of the participation of K+ channels on VSMCs proliferation. At least 4 different types of K+ channels have been identified in VSMCs: inward rectifier, voltage-gated (Kv), ATP-gated, and Ca2+-gated (BKCa) channels.2,4 The expression of these 4 types has been reported to vary among vascular beds as well as with vessel size.5,11 However, Kv and BKCa channels are present in virtually all vascular myocytes and strongly influence contractile respons-
K Cultured VSMCs

es. Moreover, several Kv channels have been described to participate in the proliferation process in pulmonary artery smooth muscles cells (SMCs). The goals of the present study were to characterize the expression profile of Kv channels in VSMCs from human uterine artery in contractile and proliferating phenotype and to evaluate their possible contribution to phenotypic modulation. Enzymatic dispersion of small pieces of uterine arteries provide VSMCs in contractile phenotype, which were studied within the same day, whereas proliferating VSMCs were obtained from explants of uterine arteries kept in culture for several passages. The molecular identification of the functional Kv subunits in both preparations shows that whereas Kv1 members are the main contributors to the Kv currents in contractile VSMCs, Kv3.4 expression is upregulated under proliferating conditions and represents the largest proportion of the Kv current in dedifferentiated VSMCs. Furthermore, selective blockade of Kv3.4 channels decreased proliferation rate, suggesting a direct relationship between channel function and uterine artery SMCs proliferation.

Materials and Methods

Uterine arteries were obtained from patients undergoing hysterectomy at the Clinic Hospital of Barcelona, with protocols approved by the Human Investigation Ethics Committee of the Hospital. Details of the materials and methods used in this study are in the online data supplement available at http://circres.ahajournals.org.

Results

Kv Currents in Freshly Dissociated and Cultured VSMCs

K+ currents were studied in acutely dissociated cells and in cultured cells obtained from human uterine arteries. After establishing the whole-cell configuration, current–voltage (I/V) relationships for K+ currents were obtained every 2 minutes. After several records of I/V curves in control conditions, BKCa currents were subtracted by using the selective blocker paxilline (500 nmol/L). Figure 1 shows average I/V curves and sample records (insets) obtained in acutely dispersed cells (Figure 1A) and in cultured VSMCs (Figure 1B) before and after paxilline application. As previously reported, cultured cells exhibited tetrodotoxin-sensitive inward Na+ currents, which were absent in acutely dispersed cells (Figure 1B, inset). Depolarization elicited outward currents with an apparent threshold for activation positive to −40 mV, and paxilline decreased current amplitudes at potentials positive to +30 mV. This effect was more evident in acutely dispersed cells. Cell capacitance was 40.21±2.45 pF in fresh VSMCs (n=28) and 44.58±6.17 pF in cultured cells (n=18). Current density was not significantly different between the 2 groups (19.04±2.76 versus 15.11±2.72 pA/pF at +80 mV), but BKCa current represents more than 70% of the outward K+ current in the freshly dissociated VSMCs and only 20% in the cultured cells. Furthermore, we consistently found a kinetic change with a larger proportion of inactivating K+ currents in cultured VSMCs (see traces in Figure 1). Resting E\text{m} measurements under perforated-patch conditions showed that contractile VSMCs had, on average, more hyperpolarized potentials than proliferating VSMCs (−45.0±1.9 mV versus −38.4±2.4 mV respectively, P<0.05; Figure 1C). Resting E\text{m} was not modified by paxilline in either of the 2 groups (data not shown). These observations indicate that the proliferation of human uterine artery VSMCs is associated with a decrease in the expression of BKCa currents and an increase in the contribution of Kv currents.

Pharmacological Characterization of Kv Currents

Whereas downregulation of BKCa currents with the phenotypic switch has been described previously, changes in Kv currents are not well characterized. Therefore, we characterized pharmacological Kv currents in both phenotypes after blocking BKCa currents with paxilline. Correolide was used to selectively block Kv1 currents, tetraethylammonium (TEA) sensitivity allowed the identification of Kv2 and Kv3 currents, and TEA- and correolide-insensitive current was attributed to Kv4 currents. The latter were also identified by
their sensitivity to block by toxins such as heteropodatoxin\textsuperscript{20} and phrixotoxin.\textsuperscript{21}

In acutely dispersed VSMCs (Figure 2), Kv1 currents were the predominant component of the K\(^+\) currents (Figure 2A). Correolide reduced Kv current amplitude by 21\% or 70\% at the predominant component of the K\(^+\) and phrixotoxin.\textsuperscript{21}

expression and subcellular location of Kv channels in acutely dispersed and proliferating VSMCs

Expression Profile of Kv\(\alpha\) mRNA

To study the molecular correlates of the Kv currents, real-time PCR was performed in both contractile and proliferating VSMCs. The results, normalized to RPL18 mRNA amount and corrected for the amplification efficiency of each reaction (see online data supplement), are plotted in Figure 4A. Unexpectedly, Kv4 channels represent the largest amount in both preparations, accounting for 67\% and 80\% of the total Kv\(\alpha\) mRNA (see online data supplement). We did not detect expression of Kv3.1, Kv3.2, or Kv1.1 in any preparation. With the exception of Kv4.2 and Kv3.4 subunits, which undergo an increased expression in cultured VSMCs, proliferation induced a downregulation of mRNA of all Kv\(\alpha\) subunits present in fresh tissue. Figure 4B shows the magnitude of the changes in the expression of Kv\(\alpha\) subunit mRNA from proliferating VSMC, taking the levels of mRNA in contractile VSMCs as the calibrator. In general, there is a correlation between changes in mRNA levels and changes in functional expression when comparing contractile and proliferating VSMCs (compare Figures 4B and 3E); whereas Kv1 channels are downregulated, Kv3.4 channels upregulate their expression and their functional contribution in cultured VSMCs.

Expression and Subcellular Location of Kv\(\alpha\) Proteins

The above data show, nevertheless, some discrepancies between the mRNA levels of a given Kv\(\alpha\) subunit and its contribution to the total Kv current, which could be attributable to the absence of correlation between mRNA and protein levels or, alternatively, to altered membrane trafficking of the protein. To distinguish between these 2 possibilities, we...
determined protein expression by immunoblot and cellular location with immunocytochemistry. Representative Western blots both in freshly isolated tissues and in protein extracts from cultured cells are shown in Figure 5. Kv1.1/H9251 subunit proteins were detected in fresh tissues, being absent or expressed at much lower levels in cultured VSMCs, with the only exception of Kv1.3, which appears to be more abundant in protein extracts from cultured VSMCs, contrary to what we will predict according to mRNA levels. A similar inconsistency was found in the case of Kv3.4 protein, which seems to be more abundant in fresh VSMCs than in cultured cells. Finally, with regard to Kv4/H9251 subunit proteins, bands for Kv4.2 and Kv4.3 were observed in both tissue and cell cultures, with substantially higher levels of expression in protein extracts from tissues, revealing the presence in both preparations of Kv4/H9251 subunit proteins, whose functional contribution has been found to be very small or almost absent.

Role of Kv3.4 in VSMCs Proliferation
The upregulation of Kv3.4α subunit mRNA during VSMC dedifferentiation and its transposition from a cytoplasmic to a membrane location suggest that the activity of Kv3.4 channels may be related to proliferation. To explore this hypothesis, we studied the proliferation rate of cultured VSMCs...
when the activity of the channels was blocked by low TEA concentrations (100 μmol/L or BDS-I toxin (Figure 7A). TEA application led to a decreased proliferation rate that was already significant after 24 hours of treatment. The difference increased with time and reached a plateau around 96 hours after TEA treatment. The same results were obtained when Kv3.4 channels were blocked with 2.5 μmol/L BDS-I, whereas blockade of Kv4 channels with 70 nmol/L phrixotoxin did not affect proliferation rate (inset in Figure 7A). These experiments demonstrate a specific link between Kv3.4 (but not Kv4) channels and VSMC proliferation, although the contribution of other Kv channels to this process (ie, some Kv1 family members, as previously found in other preparations22,23) cannot be excluded.

The decreased number of cells in the presence of Kv3.4 channel blockers could reflect a decreased proliferation rate or an increase in the number of cells undergoing apoptosis. Because among the hallmarks of late-stage apoptosis is the fragmentation of nuclear chromatin, we performed a terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling assay to detect apoptosis-induced DNA fragmentation both in control cells and in 100 μmol/L TEA–treated cells (Figure 7B). There was no significant cell death in either of the 2 samples (2.1±0.9% in control versus 2.0±0.7% in TEA treated, n=10 fields from 2 independent experiments), indicating that TEA block of Kv3.4 channels does not induce apoptosis.

**Discussion**

In this work, we have characterized the Kvα subunit profile underlying Kv currents of VSMCs from human uterine...
arteries and its changes when VSMCs switch from a contractile to a proliferating phenotype.

With the exception of pulmonary arteries, little is known regarding the expression profile of Kv channels in human resistance arteries. However, it is well established that Kv channels play an important role in regulating contraction through their effects on Em and by integrating a variety of vasoactive signals. Moreover, abnormal expression profiles of Kv channels have been involved in the pathogenesis of arterial hypertension and vascular proliferation, leading to new opportunities for developing drugs for targeting disease-specific changes in ion channel expression. Identification of the contributing Kv genes is crucial to pursue this approach.

The large diversity of Kv channel genes, together with their possibilities of heteromultimerization, association with accessory subunits, and alternative splicing, leads to an enormous diversity in the molecular composition and properties of Kv channel complexes. Often, the number of expressed Kv subunits appears to be much larger than the number of apparent Kv current components. The oligomeric composition of Kv channels and the factors regulating their folding and trafficking to the cell membrane could contribute to explain these discrepancies.

We have tried to correlate the electrophysiological and pharmacological properties of Kv currents with the expression of Kvα subunits, both at mRNA and protein levels. Although the role of BKCa currents in resistance arteries in vivo cannot be evaluated with this study, the apparent activation thresholds of BKCa and Kv currents (see Figure 1) suggests a small contribution of BKCa current to the resting Em in relaxed cells, which is supported by the measured resting Em values (Figure 1C) and its insensitivity to paxilline. Our observations echo the results obtained in other VSMC preparations, where Kv currents activated at potentials 20 to 30 mV more negative than BKCa currents.

Figure 6. Intracellular distribution of Kv proteins studied with double immunocytochemical labeling and confocal microscopy. A, One acutely dissociated VSMC labeled with antibodies against Kv1.3 (red) and calreticulin (green). The image was obtained with a Zeiss ApoTome microscope. Nucleus was labeled with 4',6-diamidino-2-phenylindole (blue). B, Kv3.4 (red) colocalizes with calreticulin (green) and is absent from the plasma membrane in contractile VSMC but not in cultured VSMC (C), where it appears restricted to the plasma membrane. D, Cultured VSMCs incubated with Dil (red) and Kv3.4 antibody (green). E. Kv4.3 (green) colocalize with Dil (red) staining in intracellular compartments. The same distribution pattern was found in acute dispersed cells (data not shown). B through D were obtained with a Bio-Rad confocal microscope. Each figure is representative of at least 3 experiments.

Figure 7. A, Absorbance at 490 nm (as an index of the number of viable cells) was taken at the indicated times in control VSMCs (●) and in cells incubated in the presence of 100 μmol/L TEA (●). TEA was added after 24 hours in culture. Each point is the mean±SEM of 4 determinations within the same experiment, and a total of 3 similar experiments where performed. Data were fitted to logistic functions. The inset shows the effect of 2.5 μmol/L BDS-1 and 70 nmol/L phrixotoxin (Phrix.) at 72 hours of culture (48 hours after drug application) obtained in a parallel experiment with n=4 data points in each condition. B, Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling assay performed in control cells and in cells after 2 days in the presence of TEA. Top panels show images of an assay performed in DNase-treated cells (C+). Left images are 4',6-diamidino-2-phenylindole–labeled nuclei. The data are representative of 3 experiments.
Our results showing that Kv1 members are the predominant components of the Kv currents are in good agreement with previous reports from other resistance vessels. The pharmacological data from acute VSMCs also indicate the contribution of Kv2, Kv3, and Kv4 channels. Kv2.1 currents have been described in rat mesenteric artery VSMCs and have been found to be the major contributor to the Kv current in VSMCs from conduit arteries such as rat aorta. mRNA and even protein for Kv3.4 and Kv4 channels have been previously described in rat mesenteric SMCs, but their functional correlates were not found.

Real-time PCR confirmed the presence of all the Kv studied but Kv1.1, Kv3.1 and Kv3.2. It also showed an inconsistency with the functional data, as the more represented Kvα subunit mRNAs (Kv4α subunits), represent only around 15% of the total Kv current. Western blot and immunocytochemistry data indicate that although Kv4 protein (mainly Kv4.3) is present in VSMCs, it is located mostly intracellularly and, therefore, cannot contribute to Kv currents.

On the other hand, the results from cultured VSMCs show remarkable differences, with relevant implications. First, our data demonstrate that VSMC proliferation leads to an important Kv channel remodeling; therefore, caution must be taken when interpreting data on ion channel expression or function obtained from cultured VSMCs, a common situation when studying human tissues. Also, the remodeling of Kv currents correlates well with the changes in mRNA, again with the notable exception of Kv4α subunits (see the online data supplement). Specific localization and trafficking mechanisms are relevant to Kv4 channel function in most cells where these channels have been studied. In addition, a surprisingly large number of ancillary subunits and scaffolding proteins that can interact with the primary subunits altering channel trafficking have been described (for a review, see Birnbaum et al). The study of the expression pattern of these auxiliary subunits may contribute to understanding the lack of functional expression of Kv4 channels in fresh and cultured VSMCs. For the other Kvα subunits, we observed an overall reduction in the expression of Kv1 and Kv2 mRNA and an increase in the amount of Kv3.4 mRNA. These changes are congruous with the electrophysiological and pharmacological profile of cultured VSMCs (Figure 3), where the predominant Kv current is an inactivating current, highly sensitive to micromolar doses of TEA.

Since the first description by DeCoursey et al of the possible role of K+ channels in T-cell mitogenesis, many other K+ channels have been implicated in normal and/or pathological cell proliferation in different preparations. Proliferation associates with either changes in the expression of channels already present (ie, the switch between Kv1.3 and intermediate-conductance Ca2+-activated K+ [IKCa] channels in T cells, between Kv1.5 and Kv1.3 in proliferating microglia, or between BKCa and IKCa in rat aortic SMCs) or with the concomitant appearance of channels absent in the native tissues, such as Kv10.1 channels in several tumor cell lines. We have also explored the expression of Kv10.1 and IKCa1 in acute and cultured VSMCs, finding a significant upregulation of Kv10.1 mRNA in cultured VSMCs and no changes in IKCa mRNA (see the online data supplement).

Regulation during VSMC proliferation has been described for L- and T-type Cav channels and for IKCa channels. For Kv channels, a role in VSMC proliferation through their effects on EK, is well established in the pulmonary circulation, where the decreased activity of Kv1.5 (and also Kv2.1) in hypoxic pulmonary hypertension has been shown to contribute to vascular hypertrophy by decreasing apoptosis.

Our results provide data in concordance with some of these previous reports and also some novel findings that increase the number of players contributing to VSMCs proliferation. (1) We demonstrate a marked decrease in the expression of all Kv1 channels. Downregulation of Kv1.5 is in agreement with the previous findings in pulmonary arteries, although its participation in the phenotypic change of human uterine artery SMCs was not further explored. (2) We found a decrease in Kv2 channel expression in proliferating VSMCs, an observation previously reported in immature rat aortic myocytes. Interestingly, some of the functional changes observed in cultured VSMCs, such as the decreased in BKCa currents and the increase in the inactivating component of the Kv current, have also been reported in neonatal myocytes, suggesting a relationship between the proliferating phenotype and the immature VSMCs. (3) We show, for the first time, the presence and functional upregulation of Kv3.4 channels with the phenotypic change, most likely associated with their translocation from an intracellular location to the plasma membrane and their contribution to cell proliferation. This translocation of Kv3.4 protein could be associated with the stimulation of mitogen-activated protein kinase pathways or growth factors that are probably involved in VSMC proliferation and that have been shown to contribute to the membrane trafficking of BK channels in neurons. Finally, the proliferation assays demonstrate that Kv3.4 channels are necessary for VSMCs to proliferate, as selective blockade of these channels decreases the number of viable cells without increasing apoptosis.

In summary, the present work provides a thorough functional and molecular characterization of the Kvα subunits expressed in VSMCs from human uterine artery and the modification by cell proliferation. We report Kv channel remodeling associated with the phenotypic change, and we found that Kv3.4 activity is related to cell proliferation; therefore, pharmacological modulation of this channel could become relevant for the regulation of VSMC proliferation associated with both physiological and pathological processes.

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MATERIAL AND METHODS

VSMCs isolation and culture
Uterine arteries were divided in two pieces, one of which was placed in RNAlater (Ambion) for RNA extractions and the other in Dulbecco’s modified Eagle’s medium (D-MEM), for cell isolation. Both samples were kept at 4 ºC and transported overnight to the lab. After cleaning of the surrounding connective tissue the endothelial layer was mechanically removed and the remaining tissue was cut into 1-3 mm pieces. Some of these pieces were used for obtaining acute dispersed cells for patch-clamp and immunocytochemical experiments (see bellow), and some were placed in 35 mm petri dishes and incubated for several hours in 2 % gelatine (Type B from bovine skin, Sigma) at 37 ºC in a 5% CO₂ humidified atmosphere. Then, the gelatine was removed and the pieces of artery explanted onto 6-well microplate. The arterial tissue was incubated in SMC-P-STIM medium (D-MEM medium supplemented with 20% SFB, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine). Migration and proliferation of VSMCs from the explants was apparent within 4-10 days. When cells reached confluence, they were mechanically detached with a rubber spatula and seed in a new flask at 1/10 density. VSMCs were subjected to several (5-10) passages without showing morphological changes. To obtaining fresh dispersed VSMCs, the small pieces of endothelium-free uterine artery were placed at 4 ºC for 30 min in PBS(-) (nominally Ca²⁺- and Mg²⁺-free PBS solution with 5 mM glucose) containing 0.2-0.1% collagenase IV and 0.1% BSA (Sigma) and then incubated at 37 ºC for 5-10 min in a shaking water bath. At the end of this first incubation, tissues were washed twice in PBS(-) solution and additionally incubated during 20 min in a 0.2-0.1% collagenase IV and 0.1-0.05% trypsin (Sigma) PBS (-) solution. After washing of this second incubation, single cells were obtained by gentle trituration with a wide-bore glass pipette. Dispersed cells were stored in PBS at 4ºC, to be used within the same day.
Electrophysiological methods

Ionic currents were recorded at room temperature (20-25 ºC, RT) using the whole-cell configuration of the patch-clamp technique. Freshly isolated cells were placed directly on the recording chamber and let to sit for a few minutes before starting superfusion with the external solution, and cultured cells were plated onto glass cover slips. The cover slips with the attached cells were placed at the bottom of a small recording chamber (0.2 ml) on the stage of an inverted microscope and perfused by gravity with the bath solution. This solution was connected to ground via a 3 M KCl agar bridge and an Ag-AgCl electrode. Patch pipettes were made from borosilicate glass (2.0 mm O.D., WPI), double pulled (Narishige PP-83) and heat-polished (Narishige MF-83) to resistances ranging from 5 to 7 MΩ when filled with the internal solution. The composition of the bath solution was (mM): 141 NaCl, 4.7 KCl, 1.2 MgCl₂, 1.8 CaCl₂, 10 glucose, 10 HEPES, (pH 7.4 with NaOH) and the pipette was filled with a solution containing (mM): 125 KCl, 4 MgCl₂, 10 HEPES, 10 EGTA, 5 MgATP; (pH 7.2 with KOH).

Whole-cell currents were recorded using an Axopatch 200 patch-clamp amplifier, filtered at 2 kHz (-3 dB, 4-pole Bessel filter), and sampled at 10 kHz. When leak-subtraction was performed, an online P/4 protocol was used. Recordings were digitized with a Digidata 1200 A/D interface, driven by CLAMPEX 8 software (Axon Instruments) in a Pentium clone computer.

Kv toxin blockers were acquired form Alomone (Alomone Labs.) and Correolide was a gift from María García (Merck).

Membrane potential measurements were performed at RT using the perforated-patch technique to avoid dialysis of intracellular medium. For these experiments, recordings were obtained with an Axopatch 4A patch-clamp amplifier. Pipette tips were briefly dipped into a solution containing (in mM): 40 KCl, 95 KGlutamate, 8 CaCl₂, 10 HEPES, pH 7.2, and backfilled with the same solution containing amphotericin B (250 µg/ml). After obtaining a high-resistance seal, electrical access to cell cytoplasm was assessed by monitoring the increase in cell capacitance. At this point, the amplifier was switched to current-clamp mode and membrane potential was continuously recorded. The high Ca²⁺ content of the pipette solution ensures the correct performance of the
perforate-patch technique, as accidental rupture of the patch (changing to whole-cell configuration) leads to a sudden Ca\textsuperscript{2+} load and cell death.

Electrophysiological data analyses were performed with the CLAMPFIT subroutine of the PCLAMP software and with ORIGIN 7.0 software (Microcal Inc.). Pooled data are expressed as mean± standard error of the mean (SEM). Statistical comparisons between groups of data were carried out with the two-tailed Student t test for paired or unpaired data, and values of p<0.05 were considered statistically different.

**Immunoblots of VSMC lysates**

The homogenized proteins from uterine arteries stored in RNAlater™ and from cultured VSMCs were recovered from organic phase created by addition of chloroform to TRIzol Reagent (Invitrogen, see manufacturer’s protocol). In parallel, tubes containing 60 \( \mu \)g of culture and tissue’s proteins, XT Reducing Agent (BIO-RAD) and XT Sample Buffer (BIO-RAD) were heated for 5 min. at 70 °C and sonicated in an ultrasonic bath, and the proteins were separated by SDS-PAGE and transferred to a PVDF membrane. After blockade of the membrane with 5 % non-fat dry milk in 1 X PBST (PBS with 0.1 % Tween 20), primary antibodies were diluted in blocking solution at a final concentration of 1:1000 and incubated for 1 h. Then the membranes were washed with 1 X PBST and incubated with horseradish peroxidase conjugated secondary antibodies (donkey anti-goat, goat anti-rabbit and goat anti-mouse, Santa Cruz) at final concentration 1:10000 for 1 h. The protein signals were detected with the VersaDoc™ 4000 Image System (BioRad) with chemiluminescence reagents (SuperSignal® West Pico Chemiluminescent Substrate, Pierce Biotechnology) and the relative amounts of each Kv\( \alpha \) subunit protein were calculated by densitometric analysis using the Quantity One software (BioRad).

The calculated molecular weight was obtained from http://bioinfo1.weizmann.ac.il/genecards/index.shtml

Primary antibodies were from Sigma (Kv3.4 and Kv1.5), Alomone (Kv4.2), Santa Cruz Biotechnologies (Kv4.3), Chemicon (Kv1.3) and Upstate Biotechnologies (Kv1.2). The anti-Kv antibodies were preincubated in control experiments with the corresponding Kv peptide (10 \( \mu \)g/ml), when this was
available (as it is the case for Kv1.3, Kv3.4, Kv4.2 and Kv4.3). Human or mouse brain protein (USBiological) were used as control.

**Immunofluorescence in VSMCs**

Freshly isolated VSMCs were plated onto glass cover slips and let to sit for 30 min at RT, and cultured VSMCs were directly seed in round cover slips in the bottom of petri dishes 2-3 days before the immunofluorescence assay. Cells were fixed with 4% paraformaldehyde (PF) in phosphate buffer, pH 7.5, for 15 min at 20 ºC, washed in PBTx (PBS, 0.1% Triton X-100), and blocked with PBTx-10 mg/ml BSA-2% normal goat serum for 10 min, Primary antibodies (anti-Kv subunits or anti-calreticulin -Stressgen-) were diluted in blocking solution and incubated with the cells for 40-60 min at RT at a final dilution of 1:100. After washes in PBTx, cells were incubated with secondary antibodies for 30 min. The fluorescently labelled secondary antibodies (Alexa 488/594-conjugated goat anti-rabbit/mouse secondary antibodies (Molecular Probes)) were used at a final dilution of 1:1000. After washes in PBS, the cover slips were mounted with Vectashield H-1000 (Vector Labs) with DAPI, and the cells were examined with the appropriate filters in a BioRad confocal microscope (Radiance 2100) or in an ApoTome Zeiss microscope. The anti-Kv antibodies were incubated in control experiments with the corresponding Kv peptide (10 µg/ml) for 2 hours before being added to the cells. Also, control labelling of secondary antibodies were included to discard non-specific labelling.

For DiI labelling, VSMCs were incubated for 40 min. at RT in PBS containing 20 µM DiI (Cell Tracker™ CM-DiI, C7000; Molecular Probes). After the incubation, the cells were fixed with 4% PF and treated as described above.

**RNA isolation and RT- PCR methods**

The pieces of uterine arteries stored in RNAlater™ were homogenized with a handheld homogenizer (Omni International Inc.). RNA from the tissue homogenates and from cultured VSMCs was isolated with TRIzol Reagent and the DNA was completely degraded with DNase I (Ambion) following manufacturer’s instructions. 0.35 µg total RNA was reverse transcribed with MuLvRT (5000 u/ml) in the presence of 20 u/µl of RNase inhibitor, 50 µM Random Hexamers, 10X PCR buffer, 25 mM MgCl₂ and 10 mM mixed dNTPs
at 42 °C for 60 min. All reagents were from Applied Biosystems. From the same samples, 0.35 µg total RNA was used as genomic control in reverse transcriptase reaction in the absence of MuLv and RNase Inhibitor at 42 °C for 60 min.

Quantitative real-time PCR (qPCR) reactions were performed on a Rotor-Gene 3000 instrument (Corbett Research). The mRNA levels for KCND2, KCNC4, KCNA5, CNN1 and KCNN4 genes were measured by quantitative real-time PCR using the TaqMan® Gene Expression Assays Hs00273378 m1, Hs00428198 m1, Hs00266898 s1, Hs00154543 m1 and Hs00158470 m1 (Applied Biosystems), and the mRNA levels for KCNH1 was measured by quantitative real-time PCR using a TaqMan® assay of Eurogentec (a gift of Dr. Luis Pardo). The amplification of cDNA was performed in 20 µl final volume and each reaction consisted of 10 µl Absolute QPCR Mix (ABgene), 1 µl TaqMan® Gene Expression Assays (or 1 µl TaqMan® assay of Eurogentec) and 1 µl cDNA, following manufacturer instructions (10 min at 95 ºC, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min).

The mRNA levels for KCNA1, KCNA2, KCNA3, KCNA4, KCNA6, KCNB1, KCNB2, KCNC1, KCNC2, KCNC3, KCND1, KCND3, NOS3 and RPL18 genes were measured by qPCR using SYBR Green I. The amplification of cDNA was performed in 20 µl final volume and each reaction consisted of 10 µl Absolute QPCR SYBR Mix (ABgene), 1 µl of 10 µM for each primer (MWG Biotech AG) and 1 µl cDNA. The PCR primers were designed using the Primer 3 website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and they are given in Table 1. The PCR conditions were: 15 min at 95 ºC; (15 s at 95 ºC, 30 s at Annealing temperature, 30 s at 72 ºC) X 40 cycles and 5 min at 72 ºC. Fluorescence was acquired at 72 ºC. A melting curve was carried out at the end of each experiment to ensure the specificity of the reaction and the absence of contaminating products (data no shown). Human brain total RNA was used as positive control (BD Biosciences).

The linearity of each qPCR assay was confirmed by serial dilutions of cDNA. Data were analyzed using the threshold cycle (Ct) relative quantification method², 2 ^(-∆∆Ct)\), where

\[
\Delta\Delta Ct = (Ct_{Kv} - Ct_{RPL18})_{Culture} - (Ct_{Kv} - Ct_{RPL18})_{Tissue}
\]
For comparison between the same gen in both conditions (tissue and culture), the mRNA amount for each transcript in the tissue samples, normalized to that of internal control, RPL18 (L18 ribosomal protein), was designated as the calibrator. Using the \(2^{-\Delta\Delta Ct}\) method, the data in cultured VSMCs are presented as the fold change in gene expression normalize to RPL18 and relative to tissue. For the tissue samples, \(\Delta\Delta Ct\) for each Kv subunit transcript equals 0, so that \(2^{-\Delta\Delta Ct}\) is 1. As the amplification efficiencies for the same gen did not vary between tissue and culture samples, the \(2^{-\Delta\Delta Ct}\) method could be used without correcting for the PCR efficiency for this normalization.

The relative abundance of RNA for Kv genes was calculated from

\[
\frac{(1 + E_{Kv})^C}{(1 + E_{L18})^C}
\]

where E was defined as PCR efficiency.

CNN1 (smooth muscle (h1) calponin) was used as a contractile phenotype marker in VSMC, and NOS3 (endothelial nitric-oxide synthase) was used as an endothelium marker (Figure 1). The mRNA amount for each transcript was normalized to that of internal control, RPL18 (L18 ribosomal protein).

**Detection of Apoptosis**

VSMC were plated onto glass cover slips in 4-well microplate. After incubation for 24 h in SMC-P-STIM medium, TEA 100 µM or BDS-I 2.5 µM were added to half of the wells and incubation proceeded for another 48 h. After this time, apoptosis was detected by TUNEL method (In Situ Cell Death Detection Kit, Roche Applied Science, Germany), that identifies DNA strand breaks by enzymatic labelling with terminal deoxynucleotidyl transferase (TdT), which catalyzes polymerization of labelled nucleotides (fluorescein –dUTP) to the free 3’-OH termini. he assay was performed following manufacturer instructions. Positive control were cells treated with DNAse I (Ambion) before TUNEL assay. As negative control the cells were incubated without TdT

**Proliferation assays**

VSMCs were plated in 96-well microtiter plates at a density of \(6 \times 10^3\) cells/well and cultured in 100 µl SMC-P-STIM medium at 37 °C in a 5% CO₂ humidified
atmosphere. Twenty four hours after cell harvest, half of the wells were treated with 10 µl TEA 1 mM (100 µM final [TEA]), 2.5 µM BDS-1 or 70 nM Phrixotoxin-I. The culture media of the wells (control media or drug-containing one) was replaced every 48h. The number of viable cells in proliferation was determined with CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA) colorimetric method, following manufacturer instructions. Briefly, 20 µl of CellTiter 96® AQueous One Solution Reagent were added to each well, and after 1-2 hours incubation at 37ºC the absorbance at 490 nm was determined using an ELISA plate reader. The quantity of coloured product is directly proportional to the number of living cells in each culture.

ADDITIONAL FIGURES AND SUPPORTING INFORMATION

Characterization of fresh and cultured uterine artery VSMCs preparations
In order to ensure that the endothelial cells were properly removed, and that VSMCs in culture undergo dedifferentiation, we analysed the presence of nitric oxide synthase (eNOS) and calponin mRNA by qPCR. The relative abundance of eNOS decreased by more than 75% with the mechanical removal of the endothelium layer (figure 1A), as it has been previously reported ³, and was undetectable in proliferating VSMCs in culture (data not shown). Phenotypic change was assessed by the reduction in the mRNA levels of calponin, a well characterized marker of mature contractile VSMCs⁴. As shown in figure 1B, the expression of calponin upon culture of VSMCs is reduced to less than 1% of the levels in the contractile cells.

Percentage distribution of Kv mRNAs in tissue and in culture.
In order to estimate the relative amount of each Kvα subunit mRNA, we use a normalized comparison between tissue and culture mRNA, in which the relative abundance of each Kvα subunit mRNA (calculated as described in the methods section) is expressed as percentage of the total Kv mRNA. The tart graph in figure 3 shows the results obtained. This representation clearly shows that Kv4 mRNAs account for a large majority of the Kv mRNAs in both preparations, representing the 68% of the Kv mRNA in tissue and almost an 80% in culture. It
is also evident that due to the general down-regulation of Kv1 and Kv2 subfamily members, the small up-regulation of Kv3.4 mRNA under cultured conditions results in a large increase in its representativeness, going from a 0.16% of the total Kv mRNA in the fresh tissues to a 7.03 in the cultured VSMCs.

**Quantification of mRNA levels from Kv10.1 and IKCa**

As many other K channels different from Kv channels have been implicated in proliferation processes in different preparations, we have also explored and quantified both in quiescent and proliferating VSMCs the presence of mRNA from some of these channels. We quantified the expression of eag channels because its presence and its association with cell proliferation has been documented in many different tumour cell lines. We have also look at the changes in the expression of IKCa1 channels (KCNN4), since an increased expression of this channels has been previously reported in rat aortic SMCs associated to VSMC proliferating phenotype. Our results using Taqman® assays for the detection of mRNA of these two channels are shown in figure 3. We did not find changes in the expression of IKCa when comparing acute dispersed (tissue) and cultured uterine artery VSMCs (figure 3A) suggesting that the reported up-regulation of this later gene upon proliferation may be species- and/or vascular bed- specific. However, there is a significant up-regulation of the Kv10.1 gene in proliferating VSMCs (Figure 3B). Interestingly, in addition to its potential role as an oncogen, Kv10.1 expression in normal mammalian tissues has only been described in central nervous system, placenta and myoblasts, where its expression occurs during a very narrow temporal window, contributing to the first hyperpolarization step linked to myoblasts differentiation.

**FIGURE LEGENDS**

**Online Figure 1. A.** Quantitation of mRNA levels for endothelial nitric oxide synthase (eNOS) in intact and endothelium-denuded arteries. mRNA levels were normalized to RPL18 mRNA and the fold change in expression \(2^{-\Delta\Delta Ct}\) was calculated from \(\Delta\Delta Ct\) as described in the methods section. Each bar is the
mean±SEM of four determinations. B. Down-regulation of calponin mRNA levels when comparing acute dispersed and cultured VSMCs preparations. Average data from 7 measurements (obtained with the same calculations than above) is shown in the bar plot. An example of the amplification plots for calponin mRNA in VSMCs from a tissue and from its corresponding culture is shown in the inset, where the fluorescence of the FAM-labelled Taqman® probe is plotted against the cycle number. NTC = non template control.

Online Figure 2. The levels of expression for each Kvα subunit mRNA obtained in the qRT-PCR are represented as a percentage of the total Kv mRNA both in fresh tissue and in cultured VSMCs. The Kvα subunits mRNAs whose percentage was less than 0.1 % of the total are not represented. Each determination was performed in 8-10 paired samples (the fresh tissue and its corresponding primary culture).

Online Figure 3. mRNA expression levels for IKCa1 (A) and Kv10.1 (B) in fresh dissociated (tissue, open bars) and cultured (grey bars) VSMCs. Data is expressed using the threshold cycle (Ct) relative quantification method, $2^{(-\Delta\Delta Ct)}$ (see methods), taken the normalized amount of each K channel mRNA in the fresh tissue as the calibrator. Each bar is the mean ± SEM of paired determinations (the tissue and its corresponding culture). n = 10 for IKCa1 and n = 7 for Kv10.1.
REFERENCES


Online Table 1. PCR primer sequences

<table>
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<tr>
<th>Gene</th>
<th>Primer sequence 5'-3'</th>
<th>Product length (bp)</th>
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<th>Annealing temp. (º C)</th>
<th>Efficiency</th>
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The obtained efficiency for KCNA5, KCNC4, KCND2, CNN1, KCNN4 and KCNH1 were: 1.99, 1.98, 2.01, 1.96, 1.99 and 1.77. N.C., not calculated. F, forward; R, reverse.
Online Figure 1

Tissue

- 62.83% (Overall)
- 4.27% (Kv4.3)
- 0.16% (Kv4.2)
- 0.16% (Kv4.1)
- 7.56% (Kv3.4)
- 0.58% (Kv2.1)
- 0.69% (Kv1.6)
- 1.25% (Kv1.5)
- 0.10% (Kv1.4)
- 17.26% (Kv1.3)

Online Figure 2

Cell culture

- 55.81% (Overall)
- 18.21% (Kv1.3)
- 7.03% (Kv1.2)
- 5.76% (Kv1.1)
- 3.86% (Kv1.4)
- 1.86% (Kv1.5)
- 1.10% (Kv1.6)
- 6.52% (Kv2.1)
- 1.10% (Kv3.4)

A

KCNN4 (IKCa1)

fold increase (2^\Delta C_t)

Tissue  Culture

B

Kv10.1 (eag)

Tissue  Culture

Online Figure 3