Characterization of Ion Channels Involved in the Proliferative Response of Femoral Artery Smooth Muscle Cells

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Objective—Vascular smooth muscle cells (VSMCs) contribute significantly to occlusive vascular diseases by virtue of their ability to switch to a noncontractile, migratory, and proliferating phenotype. Although the participation of ion channels in this phenotypic modulation (PM) has been described previously, changes in their expression are poorly defined because of their large molecular diversity. We obtained a global portrait of ion channel expression in contractile versus proliferating mouse femoral artery VSMCs, and explored the functional contribution to the PM of the most relevant changes that we observed.

Methods and Results—High-throughput real-time polymerase chain reaction of 87 ion channel genes was performed in 2 experimental paradigms: an in vivo model of endoluminal lesion and an in vitro model of cultured VSMCs obtained from explants. mRNA expression changes showed a good correlation between the 2 proliferative models, with only 2 genes, Kv1.3 and Kvβ2, increasing their expression on proliferation. The functional characterization demonstrates that Kv1.3 currents increased in proliferating VSMC and that their selective blockade inhibits migration and proliferation.

Conclusion—These findings establish the involvement of Kv1.3 channels in the PM of VSMCs, providing a new therapeutical target for the treatment of intimal hyperplasia. **(*Arterioscler Thromb Vasc Biol*. 2010;30:1203-1211.)**

Key Words: gene expression ■ ion channels ■ restenosis ■ vascular biology ■ vascular muscle ■ Kv1.3 channels ■ vascular remodeling

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Vascular smooth muscle cells (VSMCs) are differentiated cells that regulate vessel diameter and determine tissue perfusion. However, they can exhibit a variety of functionally dissimilar phenotypes. In response to local cues, VSMCs experience a phenotypic modulation (PM), with profound and reversible changes leading to proliferation, migration, and secretion of extracellular matrix components.[1] This plasticity is essential for injury repair, but it also contributes to the development and progression of vascular disease in response to abnormal environmental signals. It is becoming evident that contractile and proliferative phenotypes represent extreme cases of a spectrum of phenotypes that may coexist as the result of a developmentally regulated genetic program constantly modulated by environmental cues. This explains both a relatively stable expression of certain transcriptional programs in different VSMCs and a marked plasticity of these cells, including the ability to respond with different genetic programs to readjust cellular activity to mechanical and hormonal factors.[1–3]

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The switch in ion transport mechanisms associated with PM is getting increasing amounts of attention. Coordinate changes in ion channels are an integral component of VSMC plasticity, as they can redirect biochemical activity toward new functional responses.[4,5] Moreover, both contractile and proliferative signals require specific changes in intracellular [Ca^{2+}] and membrane potential that are determined by the ion channels expressed in VSMCs. Remodeling of several ion channels has shown to be functionally important for the PM of VSMCs in several preparations.[5–10] These data contribute to our understanding of VSMC modulation, but also, importantly, they can provide new targets for the treatment of vascular disorders. To date, studies of ion channel distribution within specific vascular beds and the modifications of ion channels on

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remodeling have been limited to a small number of candidate subunits believed to be of importance. In addition, the complexity of their characterization in the in vivo models has led most investigators to extrapolate from the data obtained from cultured VSMCs as a proliferative phenotype, because VSMCs in intimal hyperplasic lesions resemble dedifferentiated myofibroblasts or cultured VSMCs. However, the emerging picture regarding the phenotypic regulation of VSMCs shows a large degree of diversity, reflecting both intrinsic variability of VSMCs among vascular beds and the specific responses to the different proliferative signals present in cultured cells and in the in vivo lesions.

Here, we used a quantitative approach to obtain a global portrait of ion channel gene expression in contractile VSMCs from mouse femoral arteries and their changes on PM in 2 proliferative models: an in vivo model of neointimal hyperplasia induced by endoluminal lesion[11] and an in vitro model using cultured VSMCs from arterial explants. Two genes (Kv1.3 and Kvβ2) showed a concordant upregulation in both models, and the expression and function of Kv1.3 proteins was explored. Electrophysiological studies in cultured VSMCs and VSMCs from injured arteries show an increased functional expression of Kv1.3 currents. Pharmacological or genetic Kv1.3 blockade inhibits cultured VSMC migration and proliferation, and this inhibition cannot be mimicked by VSMC depolarization. Finally, the analysis of other vascular beds confirms that upregulation of Kv1.3 associates with PM, suggesting that they can represent a good therapeutical target by which to control VSMC proliferation.

Materials and Methods

An expanded Materials and Methods section is given in the supplemental material, available online at http://atvb.ahajournals.org.

Animals

Blood pressure normal (BPN) mice (The Jackson Laboratory) were maintained with inbred crossing in the animal facilities of the schools of medicine of the universities of Valladolid and Barcelona. Unilateral transluminal injury of femoral arteries was performed by passage of a 0.25-mm-diameter angioplasty guide wire, as previously described.[11]

VSMC Isolation

Mice were killed by decapitation after isofluorane anesthesia. Femoral arteries were dissected and cleaned of connective and endothelial tissues, and they were stored for RNA extraction or used directly to obtain fresh dispersed VSMCs or cultured VSMCs as previously described.[8,12]

RNA Expression Profile

TaqMan low-density arrays (Applied Biosystems) were used to simultaneously quantify the expression of 96 genes per sample.[12] Each sample derived from ≈500 ng of mRNA from contractile or proliferating VSMCs.

Protein Expression and Function

Selective antibodies were used for protein detection with immunologic methods.[8,11] The whole-cell and perforated-patch configurations of the patch-clamp technique were used for functional studies in isolated VSMCs[8,12]

Migration and Proliferation Studies

Migration of cultured VSMCs was studied by a scratch assay and proliferation was determined by 5-bromo-2′-deoxyuridine (BrdU) incorporation.

Results

Expression Profile of Ion Channel Genes in Femoral VSMCs

The transcriptional expression levels of 87 ion channel subunits in VSMCs from mouse femoral arteries were investigated by real-time polymerase chain reaction. These channel genes include α, β, and γ subunits of K^+ channels and α subunits of voltage-dependent Ca^{2+} channels, Cl^- channels, and Trp channels. The expression levels of markers of VSMCs (calponin, Cnn1) and endothelium (endothelial nitric oxide synthase, Nos3, and von Willebrand factor), were also explored, as well as other endogenous controls (Gapdh, B2m, Hprt-1, and Klf5). Expression of 26 channel genes was undetectable after 40 cycles of amplification under all conditions.

The relative abundance of the 54 channel genes found in the control preparation (mRNA from intact femoral arteries, with endothelium, C_{E+}) is shown in Figure 1. Genes are grouped by families, and their expression levels are normalized to the endogenous control ribosomal protein 18S. We detected expression of 1 Cl^- channel (Clcn3) and several inward rectifier and 2-pore domain K^+ channels. We found expression of all members of the Ca^{2+}-dependent K^+ channels (K_{Ca}) family, with the exception of the maxiK (BK_{Ca}) β subunit BKβ2; of them, the BKα and BKβ1 subunits were among the most abundantly expressed channel genes. Within the voltage-dependent K^+ channels (Kv channels), we found expression of several accessory subunits, as well as pore-forming α subunits of members of the Kv1 to Kv4 and Kv11 subfamilies. Voltage-dependent Ca^{2+} channels are represented by Cav1 and Cav3 subfamily members. Finally, we detected expression of a large number of Trp channels of the Trpc, Trpv, Trpm, and Trpp subfamilies, with Trpp2 being the most abundant transcript.

Changes in the expression profile of ion channels induced by the switch to a proliferative phenotype were studied in VSMCs obtained from explants of endothelium-denuded femoral arteries and kept in culture (in vitro model) and in endoluminal lesion-induced intimal hyperplasia (in vivo model), in which expression changes were studied at 3 different times after lesion (48 hours, 1 week, and 4 weeks). Each model had its own control: endothelium-free arteries (C_{E-}) for the in vitro model and endothelium intact arteries (C_{E+}) for the in vivo model. Differences between these 2 controls are summarized in the inset in Figure 1, where genes whose expression was modified in the C_{E-} samples (relative to C_{E+}) are shown.

Changes in proliferation were analyzed by 2 methods. First, we studied, for each individual gene, the differences

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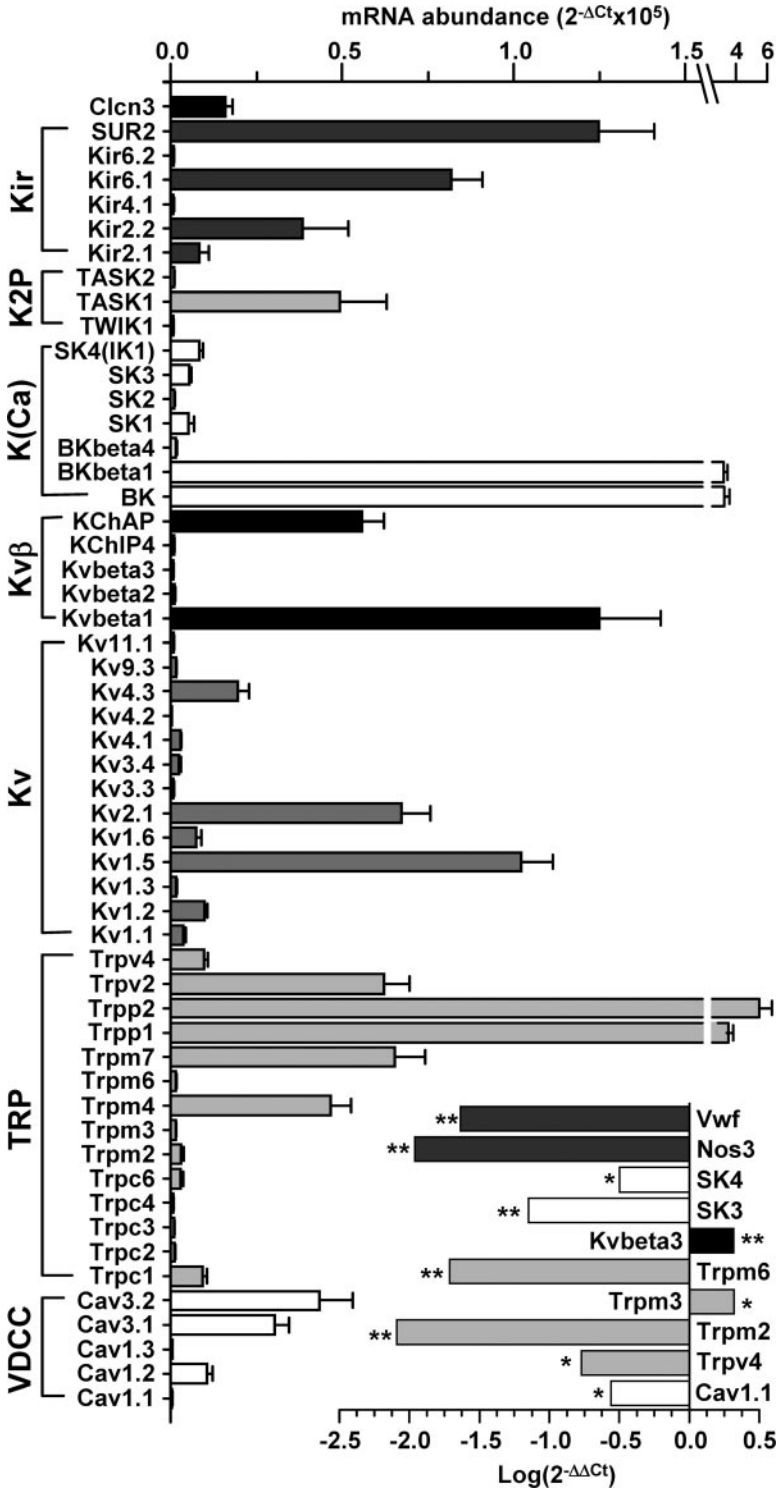

Figure 1. Relative abundance of the ion channel genes studied in complete femoral arteries (C_{E+}) expressed as $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{channel} - Ct_{18s}$. Each bar is the mean±SEM of 10 determinations obtained in 5 duplicate assays. Genes are grouped by families (see online supplemental data for the list of channel genes). The inset shows fold changes in expression in endothelium-free arteries (C_{E-}) expressed as log ($2^{-\Delta\Delta Ct}$), where $\Delta\Delta Ct = \Delta Ct(C_{E-}) - \Delta Ct(C_{E+})$. In all figures, $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$.

between control and proliferation in the 2 models by using the $2^{-\Delta\Delta Ct}$ relative quantification method[13] (Supplemental Figure III). Second, a 2-way hierarchical clustering analysis of genes and different experimental conditions[14] was performed and visualized with the Treeview software (Figure 2). This analysis categorized genes according to their responses to PM into upregulated and downregulated genes and also demonstrated time-course–related subclassifications in the model of endoluminal lesion (Figure 2A). Groups of genes with similarity in the pattern of expression (indicated by the correlation coefficient) are highlighted in Figure 2. Within downregulated genes, we identified clusters of genes with late decrease expression (Figure 2, box A), early decrease followed by a partial recovery (box B), or time-independent decrease (box C). Similarly, PM-upregulated genes can show a late increase

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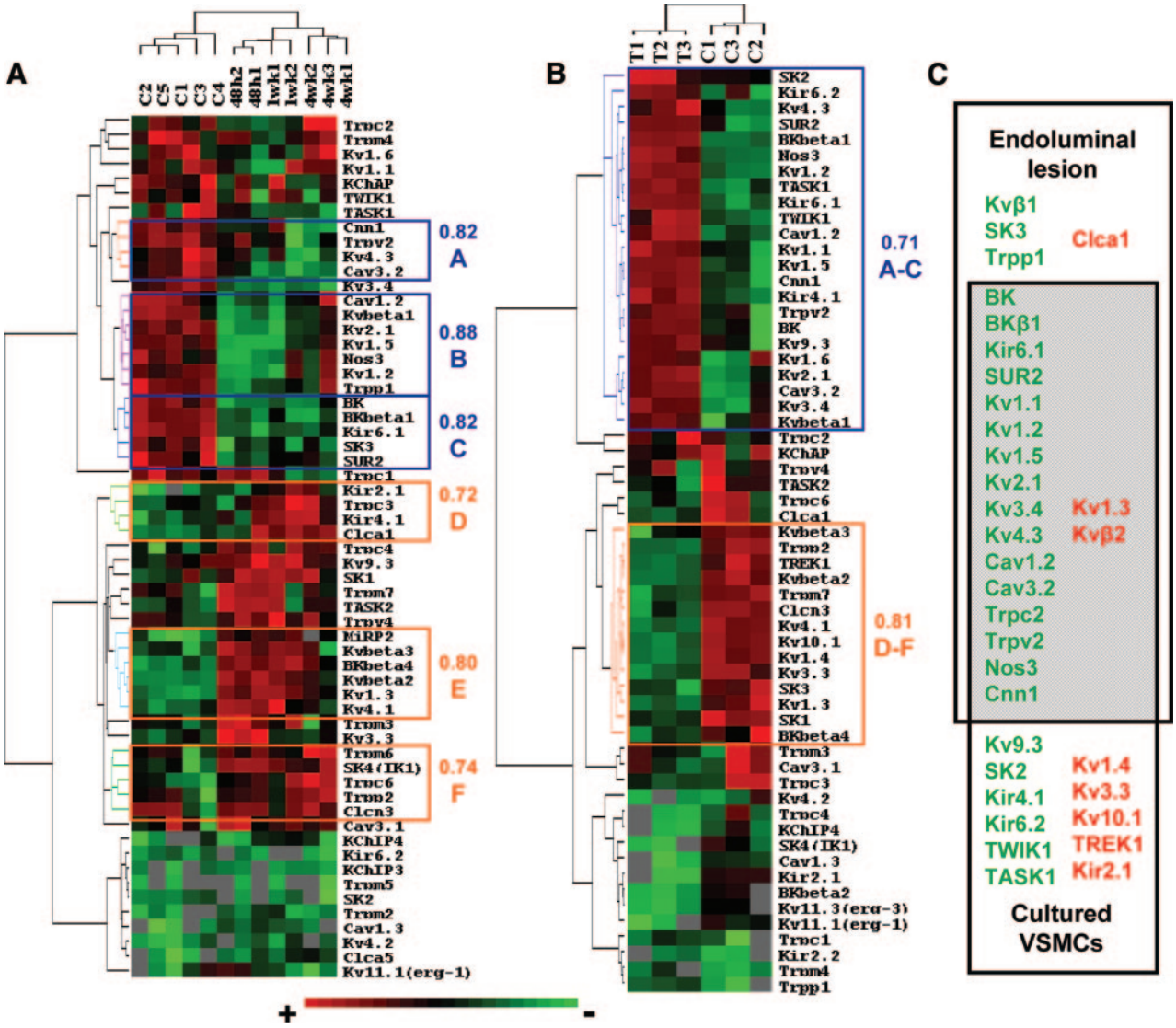

Figure 2. A, Two-way hierarchical agglomerative clustering applied to 59 genes (horizontally) and to 5 contractile VSMCs samples (C1 to C5), 2 samples at 48 hours after endoluminal lesion, 2 samples after 1 week, and 3 samples after 4 weeks (vertically). The input data were the ΔCt values for the genes. Color patch represents the expression level for each gene and sample. Expression levels were normalized within each sample, with a scale ranging from bright green (lowest) to bright red (highest). Missing values are shown as gray patches. The length of the tree branches is proportional to the correlation of the gene expression pattern, and some of these clusters and correlation coefficients are indicated. B, Same representation as in A, but comparing the contractile VSMCs (labeled here as tissues T1 to T3) with the cultured VSMCs (C1 to C3). C, Diagram showing genes exhibiting significant expression changes (upregulation, red font or downregulation, green font) in endoluminal lesion samples and in cultured VSMCs, when analyzed with the T-REX set of tools of the GEPAS suite (see Supplemental Methods). The light gray intersection contains the genes showing the same significant changes in both models.

(box D), an early increase with partial recovery (box E), or a sustained increase (box F). Following these criteria, the hierarchical clustering of the genes whose expression was modified in cultured VSMC (Figure 2B) also identified 2 groups of genes with decreased (boxes A through C) or increased (boxes D through F) expression with proliferation.

To discern whether these expression patterns were simply arbitrary structures or rather reflect biologically significant associations, we performed a statistical analysis of the data with the GEPAS suite (http://gepas.bioinfo.cipf.es/), with false discovery rate (FDR) correction.[15] We obtained a significant variation in the expression of 22 genes in the in vivo model and 29 genes in the in vitro model (Figure 2C). The endoluminal lesion induced a significant increase in 3 genes (ClCa1, Kv1.3, and Kvβ2), and a significant decrease in 19 genes. In cultured VSMCs, 7 genes were overexpressed and 22 exhibited significant decrease. The overlapping region in Figure 2C shows the genes with significant changes in their expression profile common to both proliferative models (18 genes). Of them, we focused on those with increased expression (Kv1.3 and Kvβ2), as they are more amenable to represent therapeutical targets. We studied the presence, distribution, and

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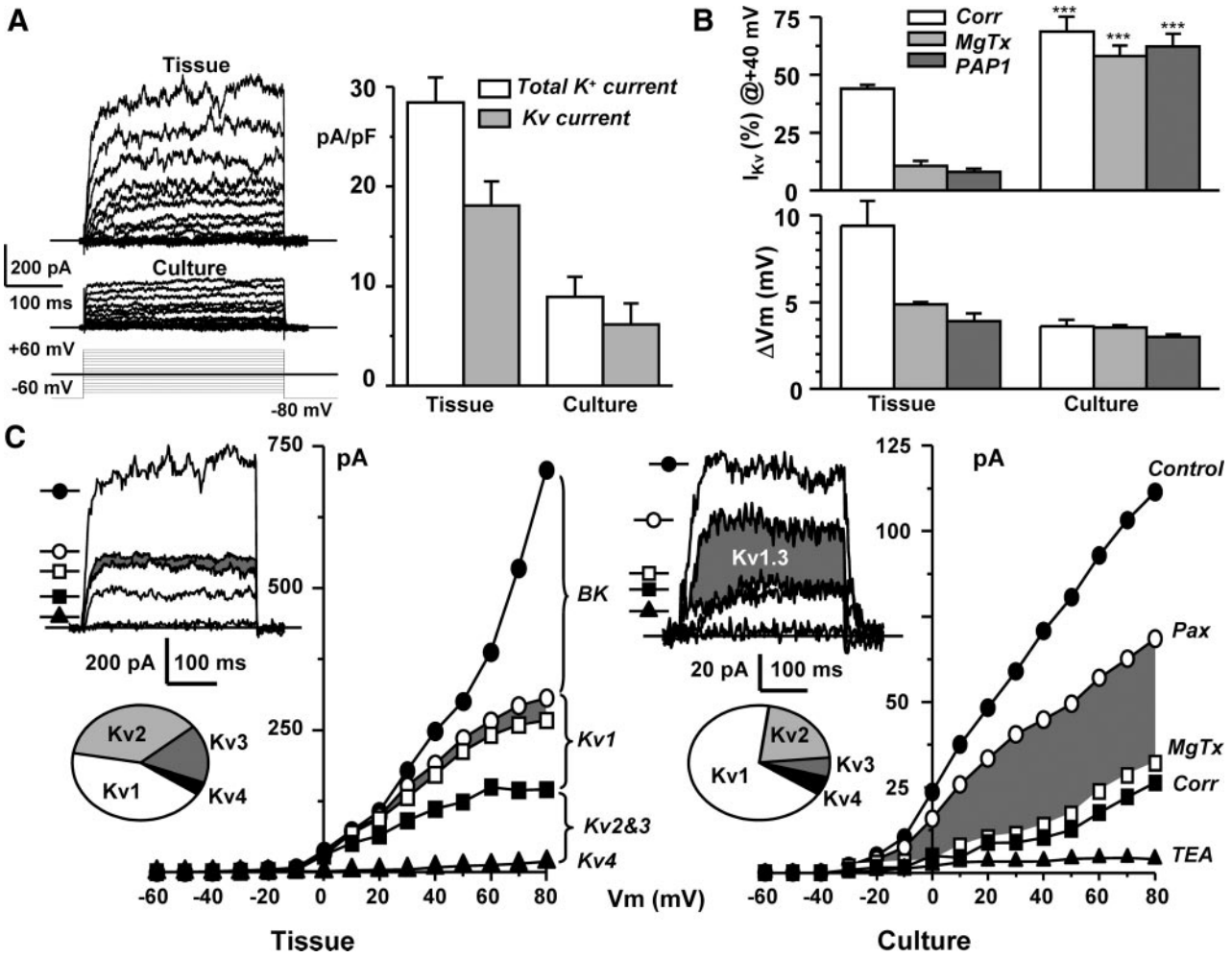

Figure 3. A, Family of current traces obtained in freshly dissociated and cultured VSMCs with the indicated pulse protocol. Current density of the total outward K^+ current was calculated in depolarizing pulses to +40 mV. Kv current density was obtained after BK_{Ca} block with 500 nmol/L paxilline. Mean±SEM of 14 to 18 cells. B, The upper plot shows the fraction of the total Kv current represented by Kv1.x currents (correolide-sensitive) and Kv1.3 currents (margatoxin- or PAP1-sensitive) in contractile (tissue) versus cultured VSMCs. The amplitude of the depolarization induced by these blockers in perforated-patch recordings is represented in the lower plot. Mean±SEM of 11 to 14 cells. C, Pharmacological characterization of outward K^+ currents in contractile and cultured VSMCs. Shown are current/voltage relationships obtained in 2 cells in control conditions and after sequential application of paxilline (500 nmol/L), margatoxin (10 nmol/L), correolide (5 μmol/L), and TEA (20 mmol/L). The Kv1.3 component is represented by the shaded areas. The traces show current at +80 mV in both cells with each blocker, and pie charts represent the proportion of Kv1-Kv4 currents in both preparations.

functional contribution of Kv1.3 in both proliferative models.

Functional Expression of Kv Channels in Femoral VSMCs

Outward K^+ currents were studied with the whole-cell patch clamp technique in freshly dissociated VSMCs and in cultured VSMCs from femoral arteries. Figure 3A shows representative traces obtained in each condition. Both total outward K^+ current density, and the Kv component of this current (ie, the 500 nmol/L paxilline-resistant current[16]) was significantly larger at all voltages in VSMCs in the contractile phenotype, but there were no significant differences in the proportion of the two components. However, the Kv1 fraction of the current (the 5 μmol/L correolide-sensitive current) was significantly larger in cultured VSMCs (from 44.12±1.6% in tissue to 68.69±6.5% in culture, Figure 3B). The increased functional contribution of Kv1.3 channels can account for this change, as revealed by selective Kv1.3 blockers (margatoxin and PAP-1[17,18]). Current sensitive to 10 nmol/L margatoxin represented 58.15±4.54% of the Kv current in cultured VSMCs versus 10.66±2.03% in freshly dispersed VSMCs. Similar results were obtained with 10 nmol/L PAP-1. The functional contribution of these channels to set resting V_M was explored with the same blockers, providing parallel results (Figure 3B, bottom). Figure 3C depicts examples of the pharmacological dissection of the outward K^+ currents. In the presence of paxilline (500 nmol/L), application of margatoxin or PAP-1 (10 nmol/L each) allowed quantification of the Kv1.3 component. After Kv1.3 blockade, correolide (5 μmol/L) was used to selec-

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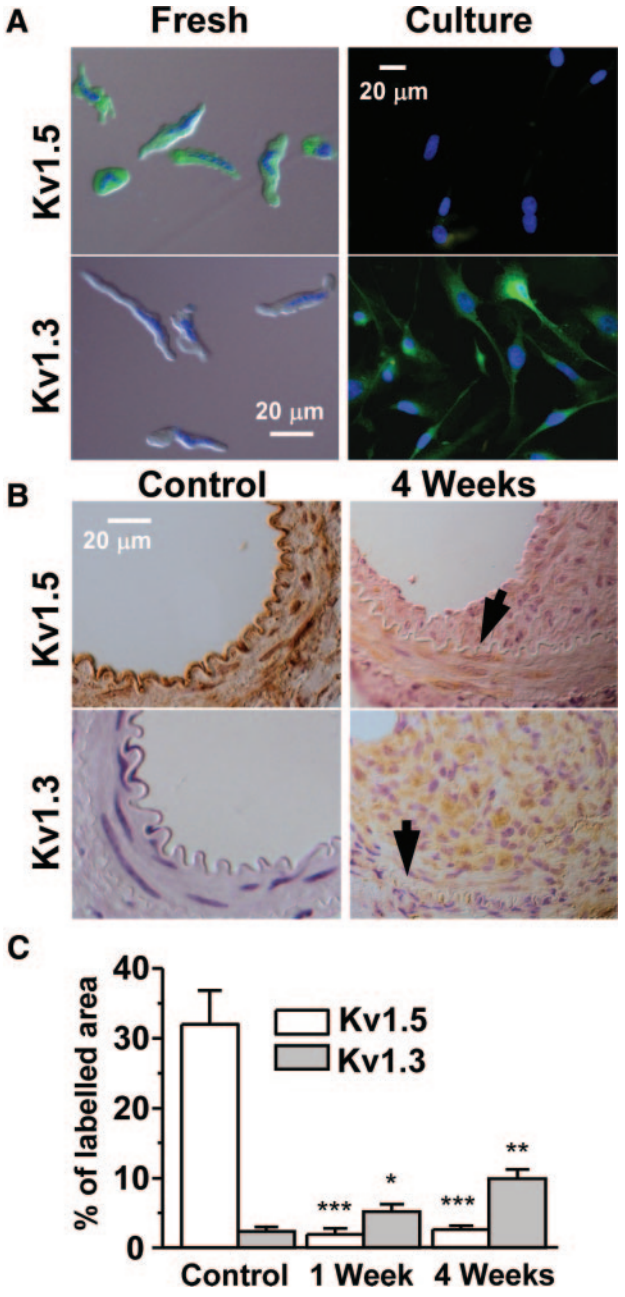

Figure 4. A, Immunocytochemical identification of Kv1.3 and Kv1.5 protein in freshly dispersed and cultured femoral VSMCs. Anti-Kv1.3 or anti-Kv1.5 labeling (green) is combined with 4′,6-diamidino-2-phenylindole staining (blue) B, Paraffin sections of control and injured femoral arteries were labeled with the indicated antibodies and counterstained with hematoxylin. Arrows indicate internal elastic lamina (IEL). C, Summary data represent the percentage of labeled area over the total vessel area. Mean±SEM of 10 to 25 sections from 6 to 13 animals.

tively block the remaining Kv1 currents. Subsequent application of tetraethylammonium chloride (TEA) (20 mmol/L) identified the amplitude of the Kv2+Kv3 component. The minimal residual current, which was insensitive to correolide and TEA, could reflect Kv4 current component. The molecular composition of Kv currents in each preparation is illustrated in the pie charts.

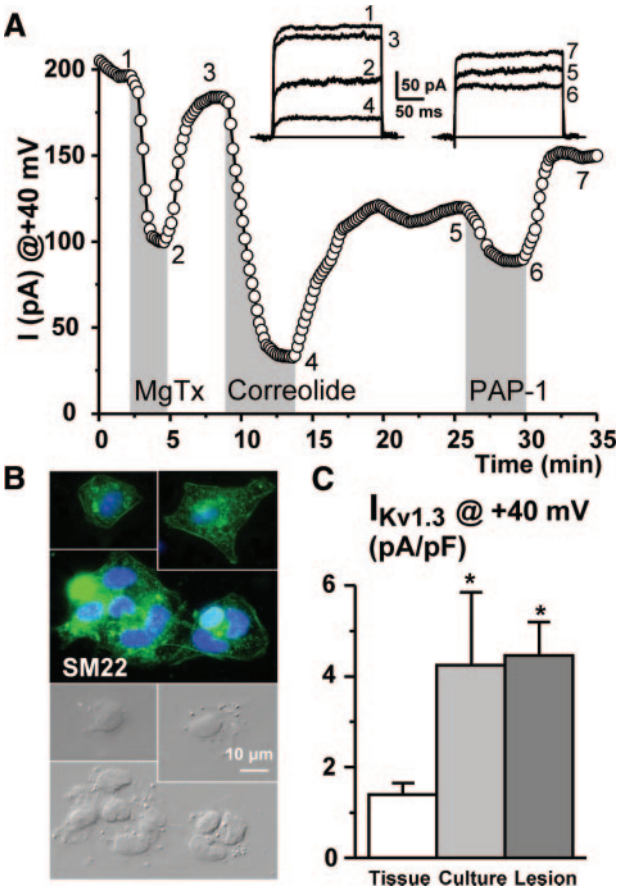

Figure 5. A, Representative example of the changes in peak current amplitude at +40 mV in a VSMC isolated from an injured artery with the indicated drugs. B, Labeling with anti-SM22 (green) demonstrates the vascular smooth muscle lineage of these cells. C, Absolute magnitude of the margatoxin-sensitive component ($I_{Kv1.3}$) in control (contractile) and in cultured and injured VSMCs. Mean±SEM of 11 to 15 cells.

In addition to the increased proportion of Kv1 currents, cultured VSMCs also showed a significant decrease in the Kv2 (from 36.52±2.97% to 21.34±3.71%) and Kv3 (from 16.4±2.74% to 5.56±0.97%) components. The Kv3 component was estimated as the fraction of the currents blocked by 100 μmol/L TEA after correolide, and the Kv2 component was calculated as the difference between 20 mmol/L and 100 μmol/L TEA-sensitive currents.

Kv1.3 Protein Expression in Femoral VSMCs

Changes in Kv1.3 protein expression were explored by immunocytochemistry in freshly dispersed and cultured VSMCs and by immunohistochemical labeling in sections of control and injured femoral arteries. We have also studied Kv1.5 protein expression as one example of channel downregulated on PM. Kv1.3 labeling increased in cultured VSMCs when compared with freshly dispersed cells, whereas Kv1.5 labeling disappeared (Figure 4A). Figure 4B shows similar results in sections of femoral arteries: Kv1.3 staining was weak in control sections, and increased after lesion (mainly in the intimal layer),

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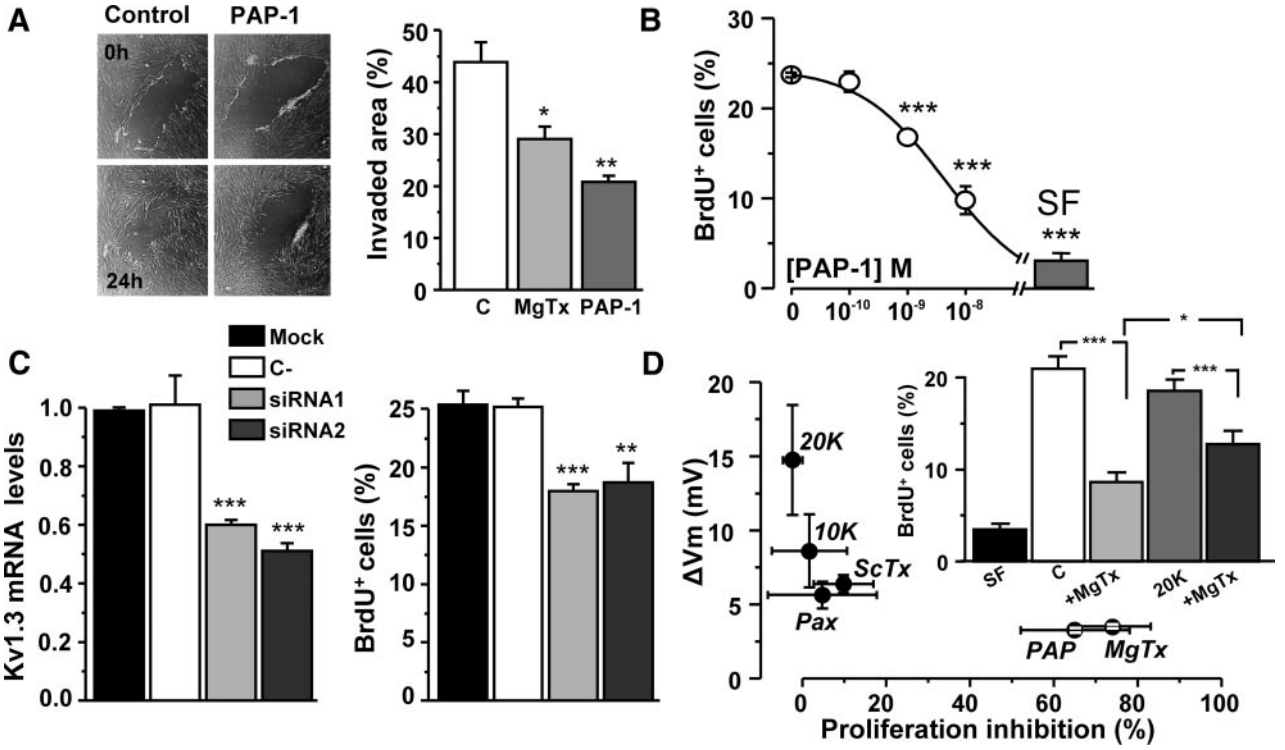

Figure 6. A, Effect of Kv1.3 blockers on femoral VSMC migration calculated as the percentage of invaded area in scratch assays. Images show a representative experiment with 10 nmol/L PAP-1. B, Dose-response effect of PAP-1 on the proliferation rate of cultured femoral VSMCs measured with a BrdU incorporation assay. n=3 to 8 experiments in each group; SF indicates serum-free medium. C, Two different Kv1.3 short interfering RNAs (siRNAs) were able to reduce mRNA expression (normalized to Gapdh) and BrdU incorporation in transfected VSMCs compared with mock-transfected or siRNA C – transfected VSMCs (n=4). D, Effects of K channel blockers or high $[K^+]_e$ on resting V_M (current-clamp experiments) and on proliferation inhibition (BrdUrd incorporation). Mean±SEM of 8 to 20 determinations. The inset shows the antiproliferative effect of MgTx (10 nmol/L) in control medium and in 20 mmol/L K^+-containing medium. n=7 experiments.

whereas labeling with anti-Kv1.5 antibody was detected in the muscular layer of almost half of the cells in control arteries, decreasing on injury. Functional characterization of Kv1.3 currents from VSMCs obtained from injured arteries (Figure 5A) showed an augmented expression of the Kv1 component (85.2±4.5% of the Kv current) due to an increase of the MgTx-sensitive component (61.5±5.2%), very similar to the case with cultured VSMCs (see Figure 3B). Moreover, increased Kv1.3 current was not simply reflecting downregulation of other Kv channels, as Kv1.3 current density increased in both proliferation models (Figure 5C).

Effects of Kv1.3 Blockade on VSMC Migration and Proliferation

The upregulated functional expression of Kv1.3 could reflect a link between the channel and the establishment and maintenance of the proliferative phenotype. To explore this, we studied the effect of Kv1.3 current blockade on the ability of cultured VSMCs to migrate and proliferate. Migration was determined in confluent femoral VSMCs by a scratch migration assay. After 24 hours in serum-free medium alone (control) or with 10 nmol/L PAP-1 or 10 nmol/L margatoxin, the invaded area was significantly larger in control cells than in cells treated with the Kv1.3 blockers (Figure 6A). Similarly, the proliferation rate, determined by the number of $BrdU^+$ cells, was significantly decreased by Kv1.3 channels blockade (Figure 6B through 6D). The effect of PAP-1 inhibiting proliferation was dose dependent (Figure 6B) and could be mimicked by selective knockdown of Kv1.3 currents with short interfering RNA (Figure 6C). PAP-1 and margatoxin depolarized VSMCs, but other maneuvers that also depolarized cultured VSMCs, such as increasing $[K^+]_e$ or blockade of Kv2 or BK_{Ca} channels, did not affect proliferation (Figure 6D). Moreover, proliferation inhibition by 10 nmol/L margatoxin was attenuated but still significant in the presence of 20 mmol/L K^+e (Figure 6D, inset).

Discussion

This work provides a comprehensive study of the expression pattern of ion channels in VSMCs, using a quantitative high-throughput technique, with the goal of elucidating PM-associated changes. We defined the relative expression of the ion channel genes studied and compared this profile among different vascular beds, observing a continuous pattern that correlates with the size of the arteries (see Supplemental Figure IV), in agreement with previous data focused on individual channels.[19–23] Characterization of the changes in expression pattern associated with proliferation in in vivo and in vitro models of PM

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showed a good correlation between both situations. Eighteen of 22 genes that changed in the in vivo model were conserved in cultured VSMCs. The larger number of genes that changed in cultured VSMCs can be attributed to their more homogeneous nature (a uniform population of synthetic VSMCs), whereas endoluminal lesion samples are partially contaminated with endothelial cells, contractile VSMCs, and connective tissue. These contaminants could also explain some of the changes present only in this model, as it is most likely the case of SK3 channels (small conductance K_{Ca} channels), whose expression is restricted to endothelial cells.[9,24] Although specific changes induced by the culture conditions cannot be excluded, our analysis indicates that ion channel expression profile in cultured VSMCs reproduces reasonably well the changes in proliferating lesions in vivo. This observation is important considering the technical limitations of the in vivo models for functional studies.

Only some of the channels highlighted here have been previously reported as relevant to VSMC proliferation. Downregulation of several Kv1 genes (Kv1.5, Kv1.2, and Kv2.1) has been reported in other preparations.[8,25] Also, decreased expression of Cav1.2 mRNA is consistent with the reported reduction in the functional expression of L-type Ca^{2+} channels in synthetic VSMCs,[5,26] although we did not detect the concomitant upregulation of genes of the Trpc and Cav3.x families described in these studies. Similarly, decreases of the BK channel genes (BKα and BKβ1 subunits) have been described,[5,8,9,27] but we did not find the associated increase in IK1 mRNA that was previously reported.[5,9,10] Nevertheless, a contribution to PM in our preparation of these channels (Trpc, Cav3.x, or IK1) via posttranscriptional modulation cannot be excluded. Overall, vascular bed variations may account for some of the recounted discrepancies, and it is tempting to speculate that not only the expression profile of ion channels but also the PM-induced changes could be vascular bed specific. In this context, the finding of conserved changes across different preparations is relevant, as it may reflect obligatory associations of certain channels with vascular remodeling, representing novel therapeutic opportunities.

With this idea, we explored the functional expression and the contribution to VSMC proliferation of the genes upregulated by the PM. We focused on Kv1.3 channels because the tools for determining the contribution of the modulatory subunit Kvβ2 are more limited. Furthermore, the concomitant increased expression of Kv1.3 and Kvβ2 may reflect the fact that they form heteromultimers, because Kvβ2 preferentially associates with Kv1.x channels,[28] and both association and upregulation of Kv1.3 and Kvβ2 have been described on mitogen-stimulated activation of T lymphocytes.[17,29] Functional expression of Kv1.3 proteins increased in both proliferative models, and selective blockade of Kv1.3 currents decreased cultured VSMC migration and proliferation, demonstrating a link between Kv1.3 channels functional expression and PM. Finally, although Kv1.3 inhibition leads to VSMC depolarization, proliferation was not affected if depolarization was induced with other channel blockers or with high $[K^+]_e$.

However, Kv1.3 inhibition could restrain hyperpolarizing signals required for proliferation. This hypothesis is consistent with our results showing an attenuated response to MgTx in high $[K^+]_e$.

We have previously reported a similar association between Kv3.4 channel upregulation and proliferation in human uterine artery VSMCs,[8] although in this preparation, high $[K^+]_e$ depolarization mimics proliferation inhibition by Kv3.4 blockade.[30] Interestingly, the Kv1.3 gene is also upregulated in these cells,[8] and Kv1.3 blockers inhibit proliferation (Supplemental Figure VIB and VIC), suggesting that the association between Kv1.3 upregulation and proliferation may be present in different vascular beds. In fact, the ion channel expression profile of cultured mesenteric VSMCs shows a remarkable similarity to that of femoral arteries (Supplemental Figure V), with Kv1.3 being the predominant Kv1 channel gene and Kv1.3 blockade inhibiting proliferation.

Kv1.3 channels have been reported to associate with proliferation in T cells,[17] endothelial cells,[31] microglia, macrophages, oligodendrocyte progenitors, and carcinoma cells,[32,33] but this is the first description of their role in VSMC proliferation and migration. In this scenario, Kv1.3 blockade could represent a new therapeutic approach to prevent unwanted remodeling.

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Disclosures

None.

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