

Kv1.3 Channels Can Modulate Cell Proliferation During Phenotypic Switch by an Ion-Flux Independent Mechanism

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Objective—Phenotypic modulation of vascular smooth muscle cells has been associated with a decreased expression of all voltage-dependent potassium channel (Kv)1 channel encoding genes but *Kcna3* (which encodes Kv1.3 channels). In fact, upregulation of Kv1.3 currents seems to be important to modulate proliferation of mice femoral vascular smooth muscle cells in culture. This study was designed to explore if these changes in Kv1 expression pattern constituted a landmark of phenotypic modulation across vascular beds and to investigate the mechanisms involved in the proproliferative function of Kv1.3 channels.

Methods and Results—Changes in Kv1.3 and Kv1.5 channel expression were reproduced in mesenteric and aortic vascular smooth muscle cells, and their correlate with protein expression was electrophysiologically confirmed using selective blockers. Heterologous expression of Kv1.3 and Kv1.5 channels in HEK cells has opposite effects on the proliferation rate. The proproliferative effect of Kv1.3 channels was reproduced by “poreless” mutants but disappeared when voltage-dependence of gating was suppressed.

Conclusion—These findings suggest that the signaling cascade linking Kv1.3 functional expression to cell proliferation is activated by the voltage-dependent conformational change of the channels without needing ion conduction. Additionally, the conserved upregulation of Kv1.3 on phenotypic modulation in several vascular beds makes this channel a good target to control unwanted vascular remodeling. (*Arterioscler Thromb Vasc Biol.* 2012;32:1299-1307.)

Key Words: gene expression ■ ion channels ■ vascular muscle ■ cell proliferation ■ phenotypic modulation

Vascular smooth muscle cells (VSMC) are essential components of the vessel wall that contribute to control blood flow through changes in their contractile activity. This function requires the expression of a set of proteins conducive for structural support and contraction. However, this contractile phenotype changes during several vascular disorders toward a proliferative phenotype, where the new set of expressed proteins provides the acquisition of a proliferative and migratory state. Because this phenotypic switch significantly contributes to the pathogenesis of vascular diseases, including atherosclerosis or hypertension, the study of its molecular determinants has a special significance.

One set of proteins particularly relevant in this context comprises ion channels required for controlling basic parameters, such as cell volume or membrane potential (V_M). Their contribution to VSMC function is well characterized in the contractile phenotype but is just beginning to be appreciated in the proliferative state.¹⁻⁵ The diversity of channels described as modulators of proliferation prompted our laboratory to carry out a global portrait of ion channel gene expression

changes associated with the phenotypic switch in 2 proliferative models of murine femoral arteries.⁶ We observed a general decrease of ion channels expression on proliferative switch, except for the voltage-dependent potassium channel (Kv) Kv1.3 and the $Kv\beta 2$ auxiliary subunit. Kv1.3 increase parallels an enormous decrease of Kv1.5, the dominant Kv1 channel in the contractile state. Moreover, Kv1.3 functional expression associates with proliferative phenotype because channel blocking induces a significant inhibition of cell proliferation. Interestingly, the relevant role of Kv1.3 channels in VSMC proliferation has recently also been shown in a human model of neointimal hyperplasia.⁷

Kv channels constitute a large and ubiquitous family of membrane proteins present in both excitable and nonexcitable cells. In nonexcitable cells, their function as feedback regulators of resting V_M has been proposed to participate in many cellular functions ranging from secretion to cell migration, proliferation, and apoptotic death. Kv channel genes can give rise to an even larger number of functional Kv currents, via heteromultimerization, association with accessory subunits,

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alternative splicing, and posttranslational modifications.⁸ Their contribution to these “nonexcitable” functions, particularly migration and proliferation, has been well documented for several Kv channels in different cellular systems, including Kv10.1, Kv11.1, and Kv1.3.^{9,10} Association between Kv1.3 expression and cell proliferation has been postulated in many cell types, including several cancer cell lines,^{9,10} endothelial cells,¹¹ microglia,¹² T lymphocytes,¹³ macrophages,¹⁴ and oligodendrocyte progenitors,¹⁵ but the underlying mechanisms linking Kv1.3 upregulation to proliferation remain controversial. In T lymphocytes, genetic or pharmacological Kv1.3 blockade has an antiproliferative effect due to membrane depolarization and consequent reduced calcium entry.¹³ However, this may not be the case in VSMC, because even though Kv1.3 inhibition leads to depolarization, proliferation was not affected by changes in V_M that were not mediated by Kv1.3.⁶ This intriguing observation suggests that the proproliferative effect of Kv1.3 upregulation may not be related to its role as a K^+ channel.

In this work, we sought to explore in more detail the functional meaning of the Kv1.5 to Kv1.3 channel switch associated with VSMC proliferation. First, we explored if this channel switch is a conserved feature linked to proliferation in different vascular beds. Next, we investigated if the association between Kv1.3 expression and increased cell proliferation could be reproduced in a heterologous system, which could be exploited for studying the mechanisms involved in this new function of Kv1.3 channels. We found that the Kv1.3 to Kv1.5 ratio is a landmark of the VSMC phenotype in all the preparations studied. Functional expression of Kv1.3 in HEK cells increased proliferation rate, and selective Kv1.3 blockers reverted this effect. Furthermore, the use of channel mutants with altered permeation and/or gating suggests that Kv1.3 could be a moonlighting protein that regulates intracellular signaling pathways leading to cell proliferation by a mechanism that is independent of potassium flux.

Materials and Methods

VSMC Isolation and Culture

BPN mice (Jackson Laboratories) were maintained with inbred crossing in the animal facilities of the University of Valladolid. Mice were killed by decapitation after isoflurane anesthesia, using protocols approved by the ethical committee of our University and in accordance with the European Community guiding principles. Femoral, aorta, or mesenteric arteries were dissected and cleaned of connective and endothelial tissues, and stored for RNA extraction or used directly to obtain fresh dispersed VSMC (contractile VSMC) or cultured VSMC (proliferative VSMC) as previously described.^{3,6}

Plasmids Construction and Transfection

Kv1.3 plasmids were obtained from 8860686 IMAGE mouse cDNA clone (Source Bioscience). Mutants were generated by site-directed mutagenesis using QuikChange (Stratagene). HEK293 cells were transiently transfected using Lipofectamine 2000 (Invitrogen).

RNA Expression Profile

mRNA levels were determined by qPCR with Taqman probes in a Rotor-Gene 3000 instrument (Corbett Research). Data were analyzed with the threshold cycle relative quantification method ($\Delta\Delta Ct$).⁶ Each sample derived from ≈ 500 ng of mRNA from contractile or proliferative VSMC.

Protein Expression and Function

Selective antibodies were used for protein detection by Western blot and immunocytochemistry.⁶ For functional studies, the whole-cell and perforated-patch configuration of patch-clamp technique were used in VSMC or transfected HEK cells, as previously described.⁶

Proliferation Assays

Proliferation was studied with Click-iT EdU incorporation assays (Molecular Probes) and counting cells with a hemocytometer.

For an expanded Methods section, please see the online-only Data Supplement.

Results

When characterizing the ion channels expression profile associated with the phenotypic switch of mice femoral VSMC, we found an upregulation of Kv1.3 channels, whose functional expression could be related to proliferation.⁶ However, looking at the expression levels of the Kv1 subfamily members (Figure 1A), it is noticeable that contractile VSMC have a predominant expression of Kv1.5 channels (whose functional contribution has been characterized^{16–18}); whereas Kv1.5 expression is almost absent in cultured VSMC, where Kv1.3 expression predominates. Data from 3 different vascular beds (Figure 1B) show that the Kv1.5 to Kv1.3 switch on proliferation is conserved in VSMC from conduit and resistance arteries, suggesting that the ratio Kv1.3:Kv1.5 is a representative parameter of the VSMC phenotype. We also confirmed that the relative functional expression of channel proteins parallels the mRNA expression levels of Kv1.3 and Kv1.5. Figure 1C shows representative experiments obtained from femoral artery VSMC, either freshly dissociated or cultured, when testing the effect of selective blockers of Kv1.5 channels (diphenyl phosphine oxide-1) or Kv1.3 channels (5-(4-phenoxybutoxy) psoralen [PAP-1]). Identical results were obtained from mesenteric VSMC (Figure 1D). Because the Kv1.5 to Kv1.3 switch is present in all the studied VSMC types, we hypothesized that it could be a relevant event needed to facilitate proliferation.

VSMC in primary culture are difficult to obtain in large amounts and hard to transfect efficiently as to study effects in cell populations. For these reasons, we explored if our hypothesis held true in a system more amenable to modify Kv channels expression in order to investigate the molecular mechanisms involved in their effects on cell proliferation. With this idea, we studied the effect of Kv1.3 and Kv1.5 overexpression on the proliferation rate of HEK cells, using 2 alternative methods: cell count and determination of the percentage of cells entering S-phase of the cell cycle using Click-iT EdU. Figure 2A represents the pooled data obtained from several experiments in which cell count was performed in HEK cells up to 4 days after transfection with vectors expressing Kv1.3 or Kv1.5. Because no differences in proliferation were detected among untransfected, mock transfected, and cells transfected with the empty vector, we have used this latter condition as control. Kv1.3 overexpression significantly increased proliferation rate up to 60 hours after transfection, with a loss of this effect at longer times probably due to plasmid dilution with cell divisions. On the contrary, the expression of Kv1.5 had an antiproliferative effect at all times explored. No evidence of increased apoptosis was

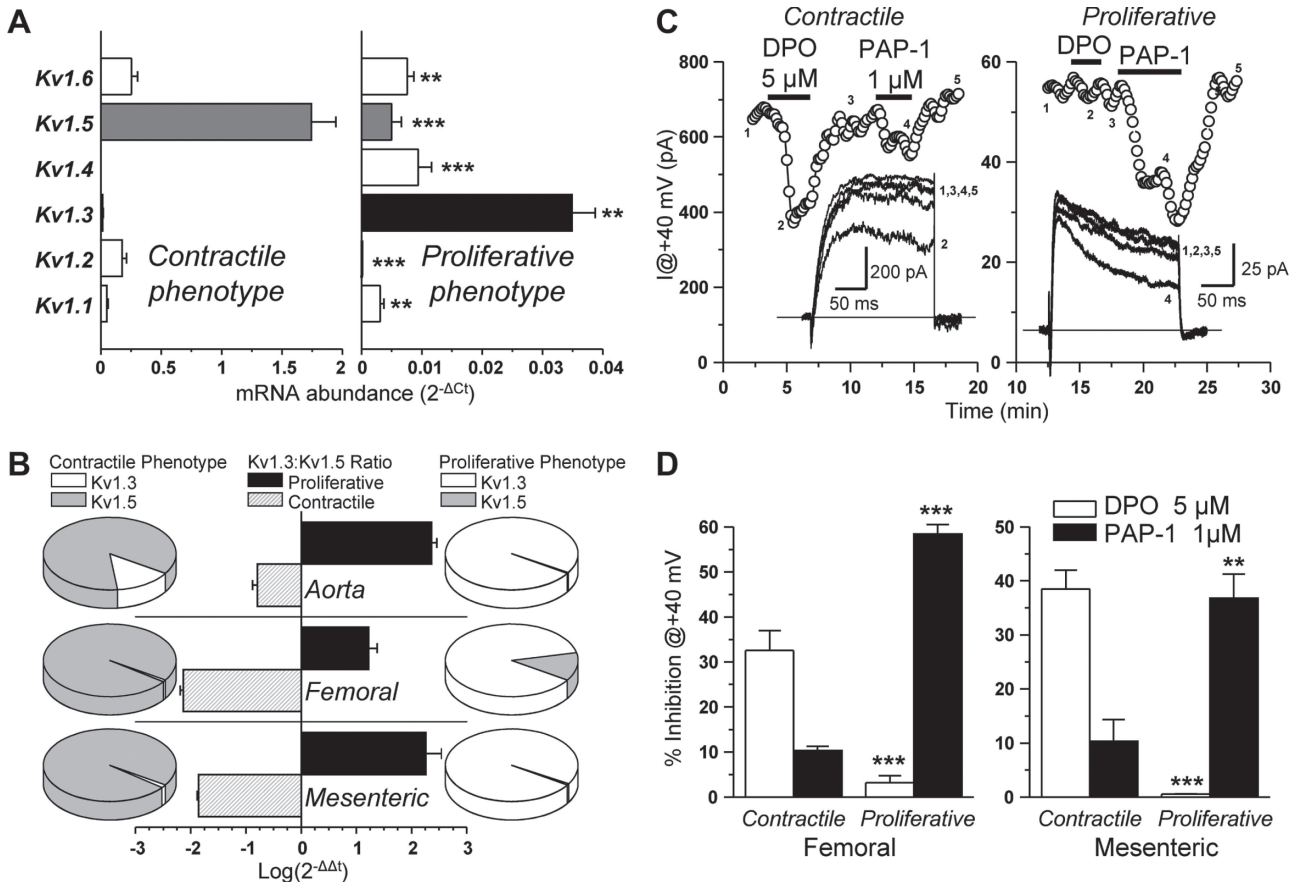


Figure 1. **A**, Relative mRNA abundance of the voltage-dependent potassium channel (Kv)1 family genes in contractile and proliferating vascular smooth muscle cells (VSMC) from mice femoral arteries. Expression levels were normalized with respect to RP18S and expressed as 2^{-ΔCt}, where ΔCt=Ctchannel-Ct18s. Each bar is the mean of 4 to 6 determinations obtained in 2 to 3 duplicate assays. All through the article, *P<0.05; **P<0.01, ***P<0.001. **B**, The relative abundance of Kv1.3 and Kv1.5 in 3 different vascular beds in both conditions is illustrated by the pie charts. The bars plot shows the Kv1.3:Kv1.5 ratio expressed as log (2^{-ΔΔCt}), where ΔΔCt was ΔCtKv1.3-ΔCtKv1.5. A value of 0 indicates a Kv1.3:Kv1.5 ratio of 1, a value of -2 denotes Kv1.5 expression levels 100 times higher than Kv1.3, and a value of +2 Kv1.3 expression levels 100 times higher than Kv1.5. Each data point was obtained from at least 4 independent determinations. **C**, Current amplitude at the end of depolarizing pulses to +40 mV from a holding potential of -80 mV, applied every 10 s, from a freshly dissociated (contractile) and a cultured (proliferative) femoral VSMC. Diphenyl phosphine oxide-1 (DPO) or 5-(4-phenoxybutoxy) psoralen (PAP-1) were present in the bath solution during the indicated times. The insets show sample current traces in each condition. **D**, Averaged data showing the fraction of the total Kv current represented by Kv1.5 currents (DPO sensitive) and Kv1.3 currents (PAP-1 sensitive) in contractile vs proliferative VSMC from femoral and mesenteric arteries. Mean±SEM, 4 to 7 cells/condition.

observed with TUNEL assays, in spite of previous reports suggesting a proapoptotic role of Kv1.5 channels in some VSMC preparations^{19,20} (Figure II in the online-only Data Supplement).

When transfection of HEK cells was carried out with decreased amounts of Kv1.3 plasmid (to tritate down the current density) we found a good correlation between the rate of proliferation and the amplitude of the Kv currents recorded in the transfected cells (Figure 2B, open symbols), that saturated when higher amounts of plasmid (and higher currents) were obtained. In femoral VSMC, phenotypic switch associates with the increased expression of both Kv1.3 and Kvβ2. Because Kvβ2 has a chaperone effect increasing the functional expression of Kv1.3 channels,²¹ we have explored if Kvβ2 coexpression could affect HEK proliferation. Using different amounts of Kv1.3 plasmid alone or in combination with excess Kvβ2 plasmid we have confirmed the chaperone effect of Kvβ2 in electrophysiological studies (Figure 2B, inset). However, Kvβ2 coexpression (Figure 2B, filled symbols) did not elicit a

proliferative effect different from the maximal effect obtained with Kv1.3 alone, suggesting that Kvβ2 effect on proliferation simply relates to its chaperone effect. Accordingly, Kvβ2 coexpression was able to significantly increase proliferation at low concentrations of Kv1.3 plasmid (Figure 2B, bars plot), but no additional effect was observed at saturating expression levels of Kv1.3 plasmid.

The specificity of the effect of Kv1.3 on proliferation rate was confirmed by Kv1.3 pharmacological blockade. Figure 2C plots the summary data of the proliferation rate of control or Kv1.3-transfected cells using Click-iT EdU assay. Serum-induced proliferation of HEK cells was significantly augmented in Kv1.3 transfected cells and could be abolished in the presence of MgTx (10 nmol/L) or PAP-1 (100 nmol/L). At these concentrations (within the range of complete blockade of Kv1.3 currents) the drugs had no effect on the proliferation rate of control cells. Similar results were obtained by cell counting (Figure 2D).

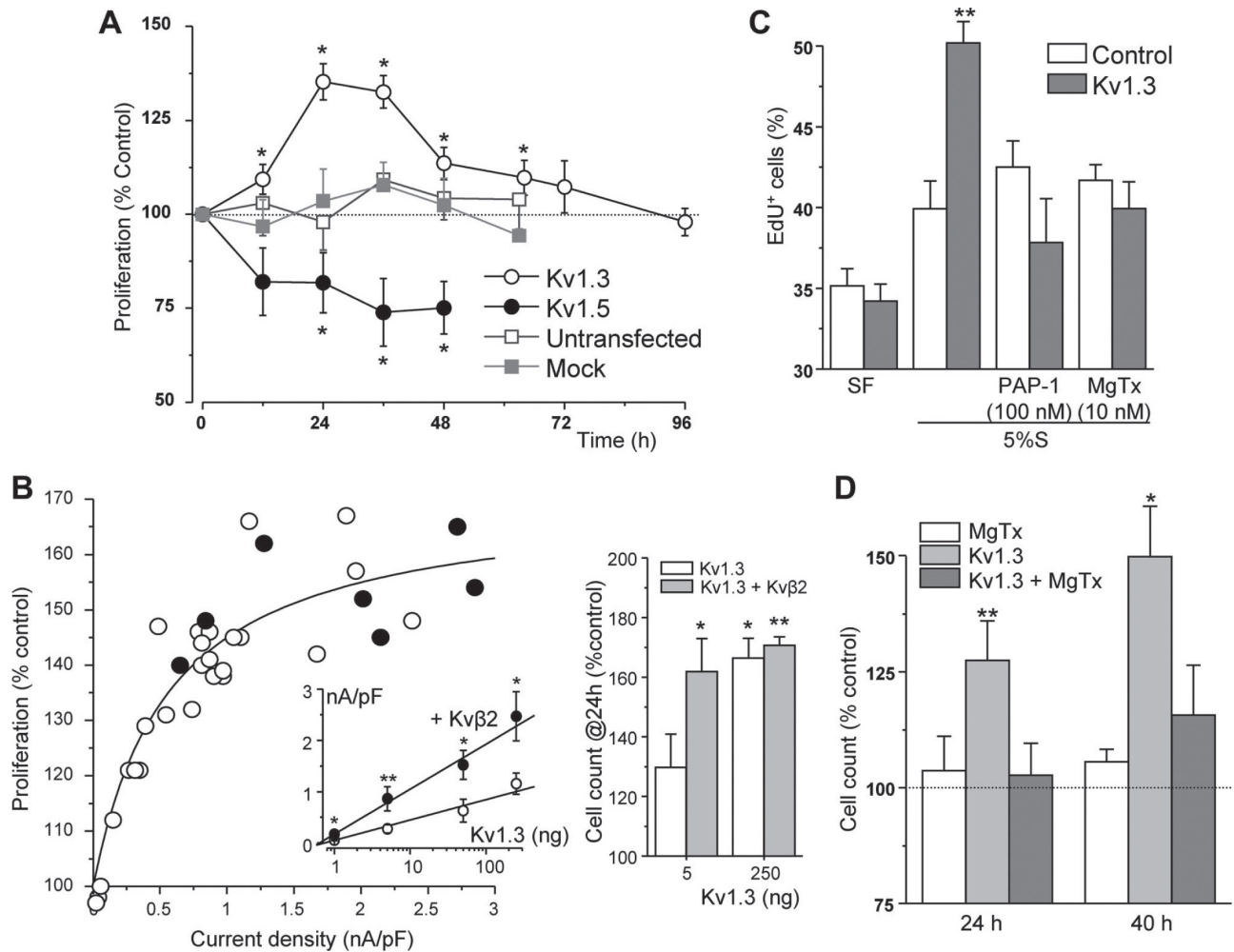


Figure 2. **A**, Proliferation time course of HEK cells explored in untransfected, mock transfected or cells transfected with plasmids expressing voltage-dependent potassium channel (Kv)1.3 (dsRED2ires-Kv1.3) or Kv1.5 (Kv1.5-EGFP). Data are normalized for the proliferation of dsRED2ires-transfected HEK cells (empty vector). Each point is the mean \pm SEM of 5 to 8 experiments, with 4 to 8 different data points each. **B**, HEK cells were transfected with increasing amounts (from 1–250 ng/35 mm dish) of Kv1.3 alone (open symbols) or together with Kv β 2 (filled symbols), and both proliferation rate (determined by cell counting at 24 hours after transfection) and current density of the transfected cells (obtained from the current amplitude elicited in depolarizing pulses to +40mV) were monitored. Each current density data point is the average of 3 to 6 cells/culture. The line shows the fit of the data to a hyperbolic function (P_{max} 170.6%, $P_{0.5}$ 0.57 nA/pF). The inset shows the current density obtained in HEK cells transfected with different amounts of Kv1.3 without (empty dots) or with (filled dots) Kv β 2 subunit. Both groups of data were fitted to linear functions. Each point is mean \pm SEM of 6 to 12 determinations. The proliferation rate of HEK cells transfected with low (5 ng) and high (250 ng) amounts of Kv1.3 alone or in combination with excess of Kv β 2 are represented in the bars plot. **C**, Proliferation rate of HEK cell transfected with dsRED2ires vector (control) or dsRED2ires-Kv1.3 was determined by measuring the fraction of cells incorporating EdU reagent after 24 h incubation in serum-free media (SF) or with 5% FBS (5%S), alone or in the presence of MgTx or 5-(4-phenoxybutoxy) psoralen (PAP-1) (mean \pm SEM, n=4–10). Statistical significance was determined against control cells incubated with 5% FBS. **D**, The effect of treatment with 10 nmol/L MgTx on the proliferation rate was also explored by cell counting at two different times after transfection (mean \pm SEM, n=4).

Voltage-dependent ion channels sense changes in membrane potential (V_M) and catalyze ion fluxes that modulate those changes. Theoretically, the channel proliferative effect could require the voltage-sensing, the V_M modulation, or both. To distinguish between these options, we designed some Kv1.3 mutant channels in which 1 or the 2 functions were lost. We created 2 pore mutants: Kv1.3A_{YA} (G399A/G401A), a dominant-negative pore mutant, and Kv1.3W_F (W389F), a nonconducting point mutant with intact gating properties. Finally, Kv1.3W_F3x (R320N/L321A/R326I), was devised as a nonconducting and voltage-insensitive channel, as the triple mutation in S4 region shifts channel activation to potentials below -170 mV, so that the channel remains

inactivated along the physiological range of voltages.²² All the constructs were created as GFP fusion proteins to allow their localization. Confocal microscopy using an extracellular Kv1.3 antibody in nonpermeabilized cells showed a clear membrane localization of all proteins but Kv1.3A_{YA} (Figure 3A). No differences in protein expression were observed for all Kv1.3 constructs, and the percentage of membrane expression was similar for Kv1.3, W_F, and W_F3x (Figure 3B, C). Moreover, immunoblot using anti-Kv1.3 revealed a band of the expected molecular size and an extra band of slightly higher molecular weight in all but Kv1.3A_{YA} (Figure 3B). This extra band reflects the N-glycosylation of the channel located at the plasma membrane, as it disappeared on treatment

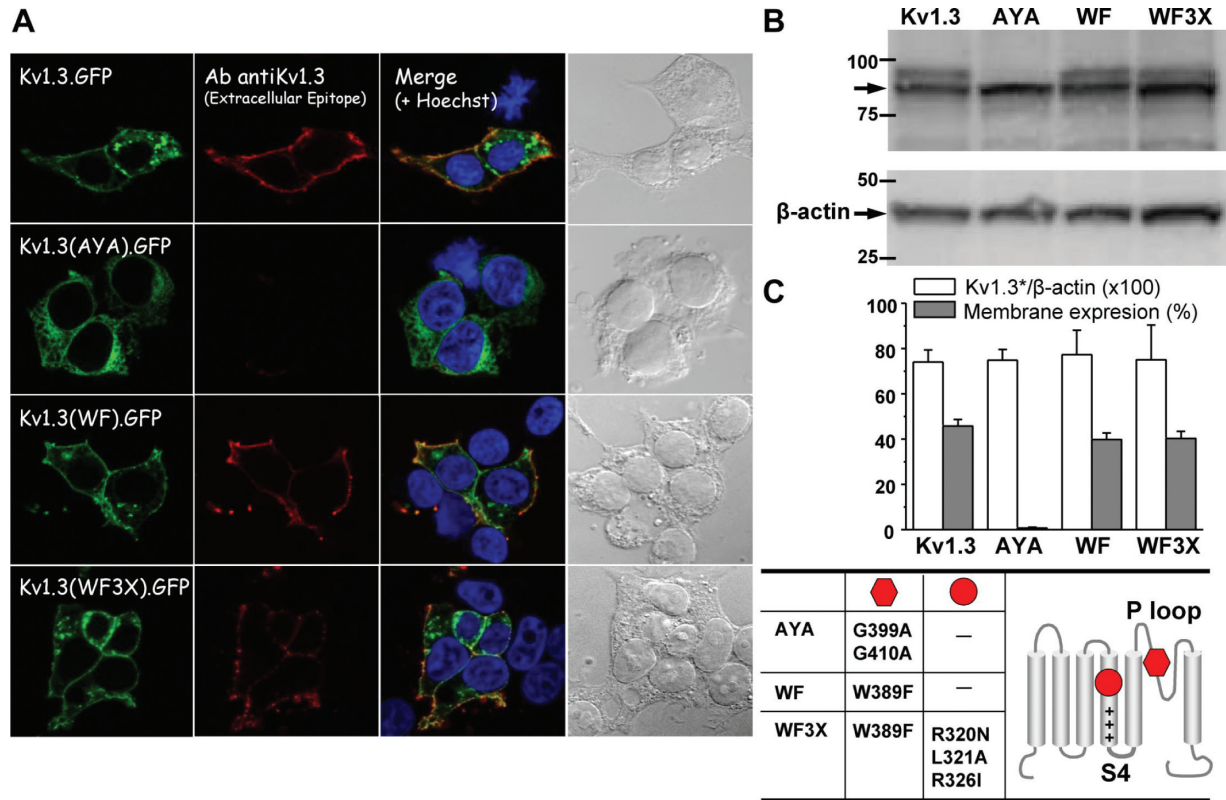


Figure 3. **A**, Confocal images obtained in HEK cells transfected with vectors expressing GFP fusion proteins of voltage-dependent potassium channel (Kv)1.3 or the different Kv1.3 mutants. (Mutations are depicted in the table). The panels show GFP fluorescence (green), labeling of nonpermeabilized cells with an extracellular anti-Kv1.3 channel (red), the nuclear staining with Hoechst (blue) and the bright field image. **B**, Immunoblot of cell lysates from these cultures with anti-Kv1.3 antibody shows a band (upper arrow) of the expected molecular weight (in kDa, for the Kv1.3.GFP fusion protein) and a band of higher molecular weight in all cases but in the Kv1.3AYA expressing cells. Loading control (β -actin) of the same membrane is also shown. **C**, White bars show average data of Kv1.3 protein expression normalized to β -actin protein from several immunoblots as the one shown in **B**. The gray bars represent averaged fraction of extracellular anti-Kv1.3 labeling obtained from confocal images of nonpermeabilized cells as in panel **A** (see also Figure I in the online-only Data Supplement).

of the samples with N-glycosidase (Figure III in the online-only Data Supplement).

Functional characterization of the channels was carried out with the patch-clamp technique (Figure 4). Although large voltage-dependent outward currents were recorded in Kv1.3 channels-expressing cells, only the gating currents could be recorded at the onset and the end of the pulse when cells expressed Kv1.3WF mutant. Gating currents were absent in the Kv1.3WF3X mutant, as there are not conformational changes along the range of voltages explored (Figure 4A). Gating currents similar to the Kv1.3WF mutant were disclosed in Kv1.3-transfected cells when K^+ fluxes were almost completely blocked by replacing intra- and extracellular K^+ with N-methyl-glucamide (Figure 4B). Additional confirmation of the behavior of the channels was obtained in current-clamp experiments, as only Kv1.3-transfected cells showed a significant hyperpolarization that was sensitive to Kv1.3 selective blockers (Figure 4C and 4D).

The effect of the Kv1.3 mutants on proliferation was assessed by cell counting and by Click-iT EdU assays with comparable results (Figure 5). Only Kv1.3WF was able to induce proliferation, being the magnitude and the time course of the effect indistinguishable from wild-type Kv1.3 channels. In fact, the proproliferative effect of Kv1.3WF was also

sensitive to 10 nmol/L MgTx. These results strongly suggest that the only requirement to induce the proproliferative effect is the presence of the voltage sensor in the membrane. However, they open the question of how MgTx (an open channel blocker that inhibits channel permeation²³) inhibits Kv1.3 induced proliferation. The characterization of the effects of MgTx and PAP-1 on Kv1.3WF gating currents (Figure 6) indicated that these 2 blockers are able to interfere with the conformational changes associated with the movement of the voltage sensor. Figure 6A shows an example of the reversible effect of PAP-1 on the gating currents of a Kv1.3WF transfected cell. MgTx and PAP-1 significantly reduced the maximal charge movement for the Q_{on} (Figure 6B) and the Q_{off} (Figure 6C), and PAP-1 produced a significant leftward shift in the voltage dependence of the Q_{on} movement. No significant changes in the slope of the Boltzmann fits were observed (data not shown). Therefore, MgTx and PAP-1 may inhibit Kv1.3 by producing some charge immobilization, in addition to their effects blocking K^+ fluxes.

Discussion

Many novel targets involved in VSMC phenotypic switch have been investigated in several in vitro and animal models

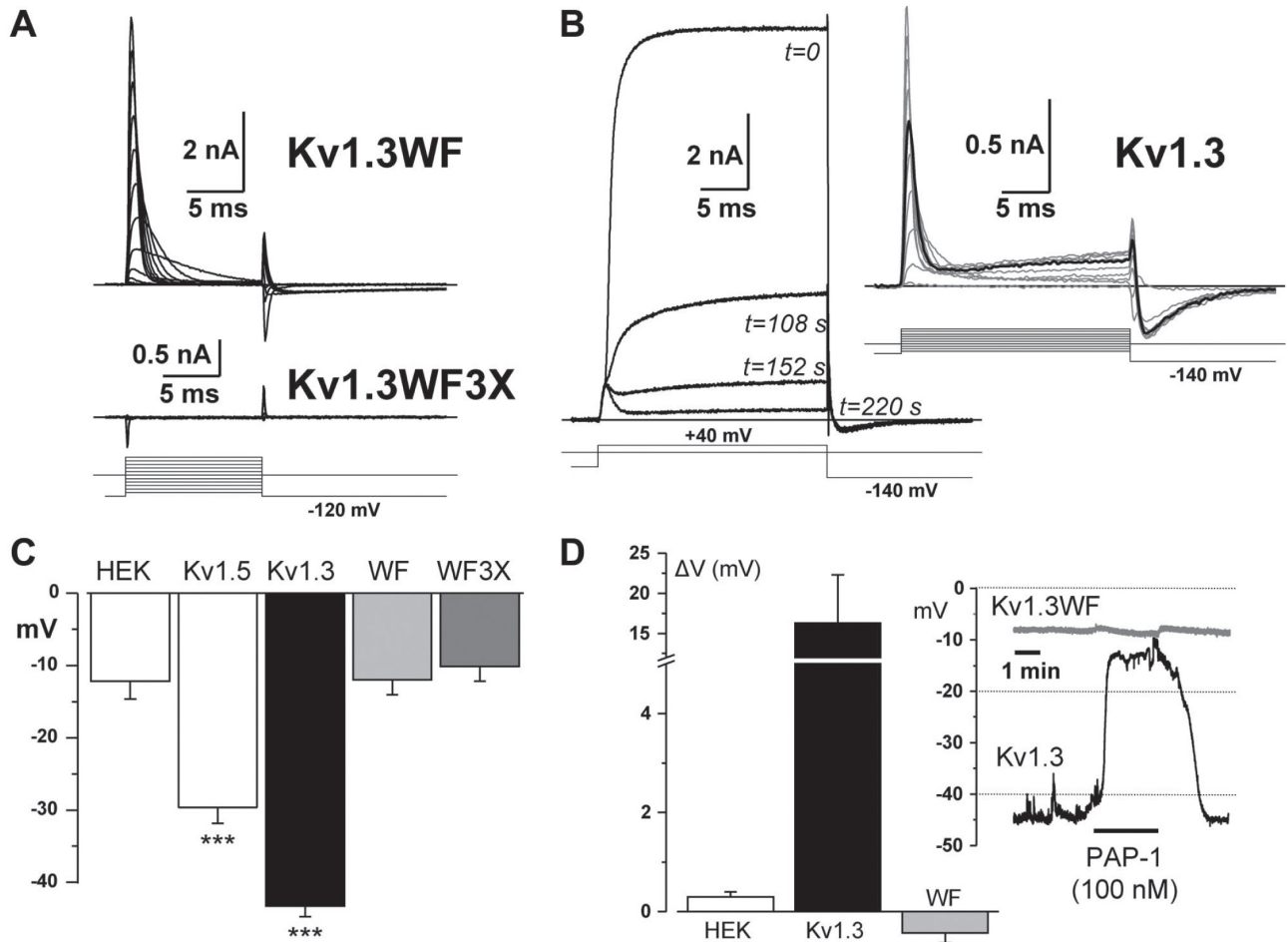


Figure 4. **A**, Representative current traces obtained from the indicated constructs with Standard_o and High-K_i solutions (bath and pipette, respectively) with the voltage protocol shown at the bottom. **B**, Whole-cell current traces from a Kv1.3-transfected cell illustrating K⁺ currents washout on intracellular dialysis with a solution free of K⁺ (replaced by N-methyl-D-glucamine). Trace labeled as t=0 was obtained immediately after breaking the patch. A family of current traces after complete dialysis is shown on the **right panel** (the thicker trace corresponds to the pulse to +40 mV). Recording solutions: N-methyl-D-glucamine (NMDG_o)/NMDG_i. **C**, Resting membrane potential (V_m) was measured with the perforated-patch configuration in all the conditions studied, including nontransfected HEK cells (HEK). Data are mean \pm SEM, n=8 to 15. **D**, Effect of voltage-dependent potassium channel (Kv)1.3 blockers on V_m . The bars plot shows the change in V_m (mean \pm SEM) on application of PAP-1, in control cells or cells transfected with Kv1.3 or Kv1.3WF channels (n=8–10). Sample traces are shown in the inset.

of VSMC proliferation. Among these, ion channels offer new venues for alternative treatments of restenosis and hypertension,^{2,5–7,24,25} but additional efforts are required to validate these targets and to develop optimized modulatory drugs that could be used as cardiovascular protective agents.¹⁰ Here, we endeavored to characterize the role of Kv1.3 channels upregulation in VSMC proliferation. We have explored if our initial findings, pointing to the upregulation of Kv1.3 currents in mouse femoral VSMC as an essential component of the phenotypic switch,⁶ could be reproduced in other vascular beds such as conduit (aorta) and resistance vessels (mesenteric). In both vessels we observed a consistent change in the Kv1.3 to Kv1.5 ratio, with a clear-cut functional correlate: Kv1.5 (diphenyl phosphine oxide-1-sensitive) currents represented the largest fraction of total Kv1 currents in contractile VSMC, being nearly absent in proliferating VSMC, where Kv1 currents were mediated by Kv1.3 channels (MgTx- or PAP-1-sensitive). The specificity and potency of diphenyl phosphine oxide-1 blocking Kv1.5 currents was determined in parallel

experiments using heterologously expressed Kv1.5 channels (data not shown), and the fraction of Kv1 current in both mesenteric and femoral VSMC was previously defined by using the correolide-sensitive fraction of the outward K⁺ current.^{6,18}

The fact that a proliferative role of Kv1.3 channels was described in VSMC from several human vessels^{6,7} could reflect an obligatory association of Kv1.3 channels with vascular remodeling, increasing its value as a new therapeutic target. Although there are several other ion channels (including TRPC1,² T-type calcium channels,¹ Kv3.4 channels,^{3,4} and IK1 channels^{5,25}) whose expression and/or function has been linked to VSMC proliferation, the information regarding conservation across different vascular beds and/or species is missed for most of these candidates. Besides, involvement of Kv1.3 channels in proliferation has been described in a substantial number of cell types (see introduction). In this work, we add another preparation to this long list, as this effect of Kv1.3 could be reproduced by its heterologous expression in HEK cells. The antagonistic effect

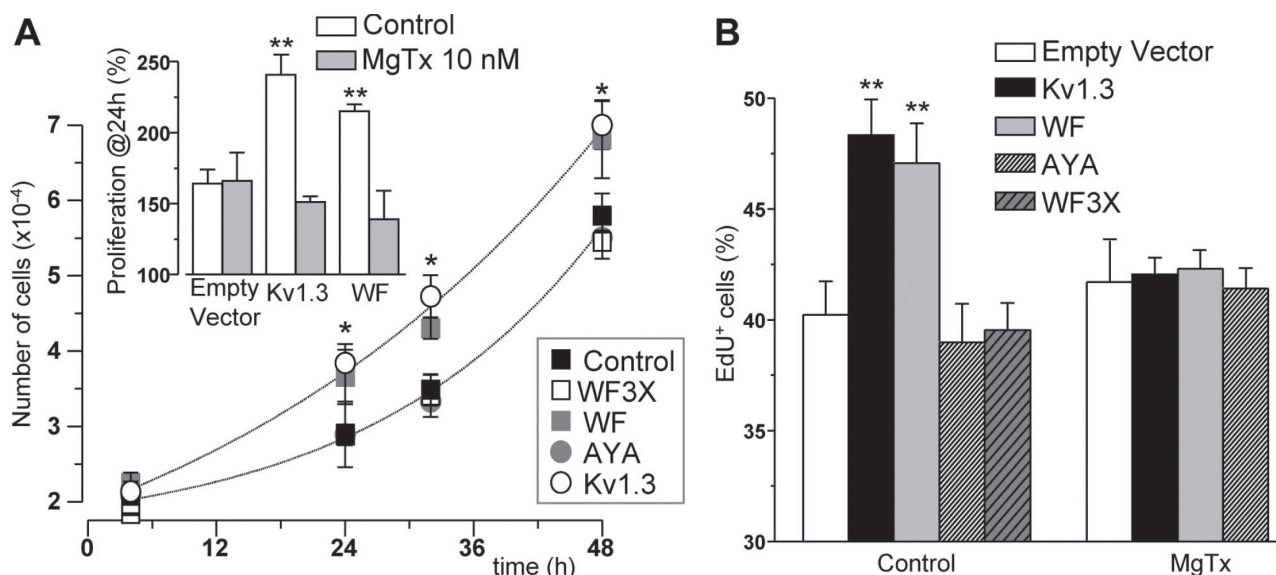


Figure 5. **A**, Cell counting was used to determine proliferation rate of the different voltage-dependent potassium channel (Kv)1.3 mutants in parallel with proliferation of HEK cells transfected with the empty vector (control) and with the wild-type Kv1.3 channel (positive control). Each data point is the mean±SEM of 3 to 5 independent experiments. The inset shows the effect of 10 nmol/L MgTx treatment on proliferation (measured also by cell counting) of control cells or cells expressing Kv1.3 or Kv1.3WF mutant at 24 hours post transfection. **B**, Percentage of cells incorporating EdU at 24 h posttransfection when transfected with the indicated constructs. Cells were maintained in 5% FBS (control) or in control media containing 10 nmol/L MgTx (MgTx). Data are mean±SEM of 4 independent experiments with internal controls in all cases (empty vector and Kv1.3 transfected cells).

of Kv1.5 and Kv1.3 channels in this system points to concurrent changes in Kv1.5 and Kv1.3 channels expression in native systems as a requirement for the functional changes associated with phenotypic switch. The effects on HEK cells were not very large but very consistent; moreover, considering our transfection efficiencies (42.58±1.75%, mean±SEM), they were probably underestimated. Finally, cotransfection of Kv1.3+Kvβ2, led to an increased HEK cell proliferation that could be simply explained by its role increasing functional expression of Kv1.3 channels (Figure 2B).

Kv channels may modulate proliferation by functioning either as V_M modulators, so that proliferation is affected by the change in V_M induced by ion fluxes through the channels or as V_M sensors, so that V_M induced conformational changes could affect associated proteins that participate in proliferation pathways. The former possibility underlies the current hypothesis for K⁺ channels-mediated proliferation, stating that cell hyperpolarization by K⁺ channels activation will increase the driving force for Ca²⁺ entry. However, our work suggests that membrane Kv1.3 channels only need their voltage-sensing capability to impact proliferation, being their effect on V_M irrelevant. This finding could accommodate the data obtained in native tissue, where we observed that although several Kv channel blockers depolarize VSMC, only the Kv1.3 channels blockade affected proliferation.⁶ Moreover, in femoral arteries, the phenotypic switch associates with a shift toward a more depolarized resting membrane potential (from -50.33±2.85 in freshly dissociated to -40.34±1.66 mV in cultured VSMC; mean±SEM of 12–20 cells), stressing the fact that the physiological relevance of the change in the Kv1.3:Kv1.5 ratio is not related to the hyperpolarizing role of K⁺ channels. Furthermore, the absolute value of V_M seems to be irrelevant for HEK cells,

because Kv1.3 and Kv1.3WF have similar effects on proliferation with a very different effect on V_M, whereas Kv1.3 and Kv1.5 have similar effects on V_M and opposite effects on proliferation (Figure 4B). Therefore, we propose that Kv1.3 behaves as a sensor of V_M changes occurring during the cell cycle, which translate into a proliferative signal through coupling to some yet unknown proliferative pathway (Figure IV in the online-only Data Supplement). This role of voltage-dependent ion channels as voltage sensors is reminiscent of other well characterized physiological processes such as the conformational coupling between the L-type calcium channel and the ryanodine receptor in the skeletal muscle.²⁶ In a more related scenario, similar mechanisms have been described for EAG-induced proliferation of fibroblasts and myoblasts, where the voltage-dependent conformational change of the channel seems to be the signaling switch that modulates cell proliferation.²⁷ Similarly, K⁺ efflux with the consequent hyperpolarization and enhanced Ca²⁺ entry are not necessary for IK1-induced HEK cell proliferation.²⁸ In this case IK1 does not even seem to behave as a voltage sensor because expression at the cell membrane is not required to potentiate proliferation.

The effect on proliferation of Kv1.3 and the nonpermeant Kv1.3WF is inhibited by selective Kv1.3 blockers (MgTx and PAP-1), that have been previously considered as open channel blockers with no effects on the kinetics of the channel.^{10,29,30} Their inhibitory effect on proliferation seems to be in open contradiction with the observation that ion flux through the channels is not required to induce proliferation. Characterization of the gating charge movements in the Kv1.3WF mutant shows that PAP-1 clearly affects the voltage-sensing mechanism. This effect could explain its inhibition of Kv1.3-induced proliferation. However, the

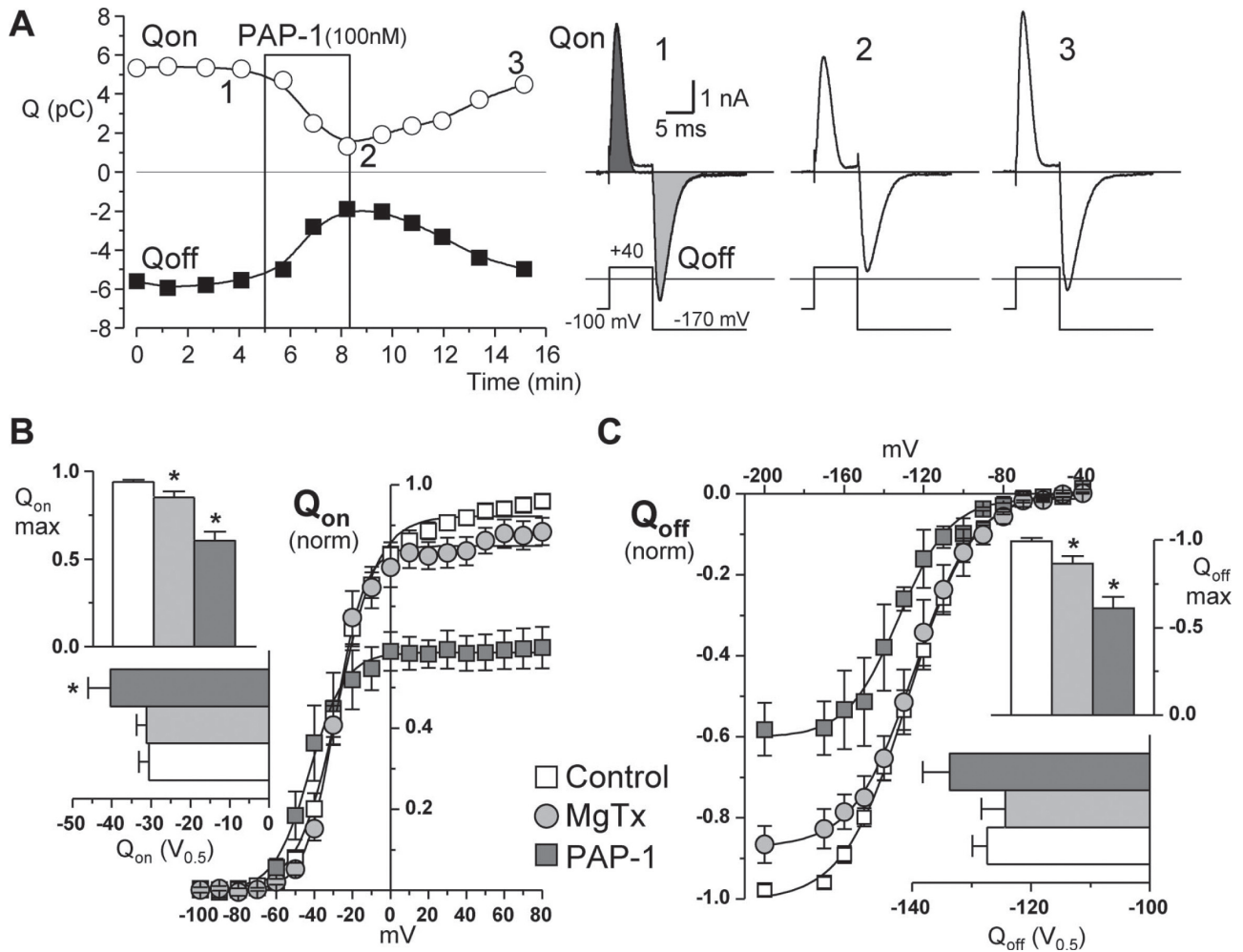


Figure 6. **A**, Time course of the effect of PAP-1 100 nmol/L on gating charge movement of voltage-dependent potassium channel (Kv)1.3WF transfected cells on depolarization to +40 mV (Q_{on} , **white dots**) and repolarization to -170 mV (Q_{off} , **black squares**). Representative gating current traces at the indicated times 1, 2, 3 are shown on the right, with the voltage protocol depicted below the current traces. Shaded areas in the control trace represent the gating charge movement (current integral) elicited on depolarization (Q_{on}) and repolarization (Q_{off}). **B**, Q_{on} voltage dependence of Kv1.3WF mutants obtained with the voltage protocol depicted in Figure 4A in N-methyl-D-glucamine (NMDG)_e solution alone or with 10 nmol/L MgTx or 100 nmol/L PAP-1. Data are mean \pm SEM of 4 to 6 different cells. **Solid lines** represent the data fit to a Boltzmann function. Insets represent the average \pm SEM of the fit parameters (Normalized $Q_{on(max)}$ and $V_{0.5}$) obtained in each individual cell. **C**, As in panel **B**, but exploring the Q_{off} voltage dependence. Q_{off} was obtained with a family of pulses like that shown in panel **A** with repolarization from -40 to -200 mV.

surprisingly small effect of MgTx does not seem to fit our hypothesis. Nevertheless, since the effect of MgTx appears to be voltage-independent (a decrease of Q_{max} without changes in the $V_{0.5}$ or the slope of the Boltzmann fits), this small fraction of channels could also be immobilized at resting V_M . Future studies will explore whether this effect suffices to explain proliferation inhibition, or if alternative mechanisms of action for MgTx are involved. In this regard, characterization of the effects of Kv1.3 blockers on the functional expression of the channels along the time scale used in proliferation studies (24–48 hours) may contribute to solve the puzzle.

In summary, the data presented here show that the change in the expression ratio of Kv1.3 and Kv1.5 channels could be a general requirement to facilitate VSMC phenotype modulation, due to differential effects of both channels on the proliferative response. Besides, we propose that conformational changes of Kv1.3 in response to V_M , but not changes in ion

fluxes, are the key element in the proliferation signaling pathway. Further studies in this direction will provide additional components of the cascade, offering new possibilities for the control and modulation of cell proliferation.

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Disclosures

None.

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