Molecular Determinants of Kv1.3 Potassium Channels-induced Proliferation*

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Changes in voltage-dependent potassium channels (Kv channels) associate to proliferation in many cell types, including transfected HEK293 cells. In this system Kv1.5 overexpression decreases proliferation, whereas Kv1.3 expression increases it independently of K+ fluxes. To identify Kv1.3 domains involved in a proliferation-associated signaling mechanism(s), we constructed chimeric Kv1.3-Kv1.5 channels and point-mutant Kv1.3 channels, which were expressed as GFP- or cherry-fusion proteins. We studied their trafficking and functional expression, combining immunocytochemical and electrophysiological methods, and their impact on cell proliferation. We found that the C terminus is necessary for Kv1.3-induced proliferation. We distinguished two residues (Tyr-447 and Ser-459) whose mutation to alanine abolished proliferation. The insertion into Kv1.5 of a sequence comprising these two residues increased proliferation rate. Moreover, Kv1.3 voltage-dependent transitions from closed to open conformation induced MEK-ERK1/2-dependent Tyr-447 phosphorylation. We conclude that the mechanisms for Kv1.3-induced proliferation involve the accessibility of key docking sites at the C terminus. For one of these sites (Tyr-447) we demonstrated the contribution of MEK/ERK-dependent phosphorylation, which is regulated by voltage-induced conformational changes.

Voltage-gated K channels (Kv)7 comprise a large family of channels that are expressed in both excitable and non-excitable cells. In excitable cells they contribute to the control of resting membrane potential (resting EM) and action potentials frequency and duration. In non-excitable tissues they are involved in processes ranging from secretion to cell proliferation (1–3) by means of their ability to sense and modulate EM. Each Kv channel gene encodes a single protein, and functional Kv channels consist on homo- or heterotrimeric complexes within the same subfamily members (Kv1–Kv12). The large number of Kv channel genes combined with the possibility of heterotrimerization creates a large functional diversity of Kv currents. This diversity is increased by their association with accessory proteins capable of modulating gating properties and assist trafficking and multimerization (4). Finally, modulation of Kv currents may also rely on posttranslational modifications such as glycosylation and phosphorylation of the channel proteins, which can affect their folding, their trafficking, or their functional activity.

Among Kv channels, Kv1.3 was the first channel reported to modulate cell proliferation in T cells (5). Since then, many other Kv channels (such as Kv1.1, Kv1.5, Kv3.4, Kv10, and Kv11.1) have been linked to migration and proliferation in numerous non-excitable tissues, including cancer cells, T-lymphocytes, endothelial cells, macrophages, leukocytes, and vascular smooth muscle cells (VSMCs) (1, 2, 6–9).

Kv1.3 channels have been described as modulators of cell proliferation in many different tissues, most notably T-cells and VSMCs, but also in several cancer cell types, macrophages, microglia, and oligodendrocyte progenitors (10–12). In fact, the pro-proliferative effect of Kv1.3 could be also reproduced by its overexpression in a heterologous system in cells that do not normally express the protein (13). However, although the contribution of the Kv1.3 channels to activation, migration, and proliferation seems to be well established, the mechanisms by which Kv1.3 expression modulates these processes are mostly unknown to date. Several mechanisms, which may not be mutually exclusive, have been proposed, including the interaction of Kv1.3 with integrin receptors (14–16), the impact of K+ efflux through Kv1.3 channels on changes of resting EM needed for cell cycle progression, or the modulation of intracellular calcium levels that could influence the phosphorylation state of several proliferation-related signaling processes (17, 18).

In addition to this role of Kv1.3 channels in cell proliferation, there is evidence of a reciprocal modulation, as certain mitogens induce Kv1.3 channel up-regulation (5, 19, 20). In this regard it is interesting to highlight the observed up-regulation of Kv1.3 channels upon stimulation with PDGF, basic FGF, or FBS and the simultaneous down-regulation of Kv1.5, another
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Kv1 subfamily member (10, 11, 13, 19, 21, 22). This observation led us to hypothesize that the Kv1.3/Kv1.5 ratio could be a landmark to define VSMCs phenotype, as proliferation of VSMCs derived from different vascular beds in both mice and human associates with an expression switch from Kv1.5 to Kv1.3 (13, 22). Furthermore, in support of this hypothesis we have previously described that heterologous expression of Kv1.3 increased HEK cell proliferation, whereas Kv1.5 expression decreases basal HEK proliferation (13).

An important question regarding the mechanisms linking Kv1.3 expression to proliferation is whether this association depends on the ion-conducting properties of the channel (and hence of the feedback regulation of $E_M$) or not. There are previous studies demonstrating that the effects of several K channels on cell proliferation are not dependent on conducting properties of the channel proteins (13, 23, 24). In the first study we found that poreless Kv1.3 mutants can induce proliferation to the same extent that wild type channels, an effect that is lost in mutant voltage-insensitive channels or channels unable to localize to the plasma membrane. These results suggest that Kv1.3 proteins are docking sites that allow the activation of proliferative signaling cascades. In native VSMCs we found that PDGF-induced proliferation could be inhibited by either selective Kv1.3 blockers (Margatoxin or 5-(4-phenoxybutoxy)pso-ralen) or by blockers of MEK/ERK and PLCγ pathways, both effects being non-additive (22), which suggests a shared mechanism. However, the detailed description of the role of Kv1.3 channels modulating mitogen-induced signaling cascades awaits further characterization.

In this work we sought to explore in more detail the contribution of Kv1.3 channels to cell proliferation by addressing some of the questions raised above. First, on the basis of the opposite effects of Kv1.5 and Kv1.3 channels on HEK cell proliferation, we constructed chimeric Kv1.3/Kv1.5 channels to identify the molecular determinants involved in those effects. Our data pointed to the intracellular C-terminal domain of Kv1.3 as responsible for the increased proliferation rate. Next, we explored the contribution of individual residues within this C-terminal region by mutation to alanine of the eight predicted phosphorylation residues. This approach identified a short region in the C terminus (the YS segment) comprising two residues (Tyr-447 and Ser-459) whose mutation abolished Kv1.3-induced proliferation. The contribution of YS segment to Kv1.3-induced proliferation was confirmed by designing several mutant channels, including a C-terminal-truncated Kv1.3 channel retaining the YS segment and chimeric Kv1.5 channels harboring the YS segment at different positions within their C terminus. Second, we also identified one signaling pathway that could link Kv1.3 phosphorylation and proliferation, as genetic or pharmacological blockade of MEK/inhibited Kv1.3 induced proliferation and phosphoryrosine labeling of the channel. Finally, we observed that voltage-induced conformational changes of Kv1.3 channels are important for proliferation, regulating the accessibility of these tyrosine phosphorylation sites. Channel phosphorylation increased by maneuvers that facilitate the closed to open transition and was diminished in mutant channels that reside in the inactivated state.

Materials and Methods

HEK293 Cells Maintenance and Transfection—HEK293 cells were maintained in DMEM medium supplemented with 5% FBS, penicillin-streptomycin (100 units/ml each), 5 μg/ml Fungizone, and 2 mM l-glutamine at 37 °C in a 5% CO2 humidified atmosphere. HEK cells were transiently transfected with 1 μg of DNA by using Lipofectamine™ 2000 or TransIT-X2® reagent (Mirruss). For siRNA experiments, HEK cells were cotransfected with 1 μg of DNA together with one or a mixture of siRNA at a final concentration of 5 nM. Transfection efficiency was quantified in each experiment by fluorescence microscopy (35–60%). Treatments with specific inhibitors (PD98059 20 μM, Tocris Bioscience) or with modified media were applied during 4 h before determinations.

Plasmids Construction—Full-length hKv1.5 and hKv1.3 were obtained from either cDNA or genomic uterus artery VSMC DNA (MagNA Pure Systems, Roche Applied Science) to generate C-terminal fusion proteins when subcloned into pEGFP-N1 (addgene) or pmCherry-N1 (Clontech), respectively; pEGFP-N1-hKv1.5 and pmCherry-N1-hKv1.3. Chimeric K5N3 and K5C3 channels were generated by PCR using Phusion® Hot Start High Fidelity DNA Polymerase (Finnzymes). N or C termini from the Kv1.5 backbone were replaced by the corresponding domains of Kv1.3, creating the fusion proteins: pmCherry-N1-K5N3 and pEGFP-N1-K5C3. YS fragment is a 16-amino acid-residue fragment located at the proximal region of the C-terminal of Kv1.3 and containing residues Tyr-447 and Ser-459. The YS fragment was inserted within the hKv1.5 COOH terminus at two different positions, amino acid 532 (Kv1.5-YS$^{532}$) by overlap extension PCR or at the end of the C terminus (amino acid 613, Kv1.5-YS$^{613}$), by designing overlapping oligos. A truncated Kv1.3 containing the YS fragment and lacking C-terminal amino acids 461–523 (Kv1.3-YS) was also generated with overlapping oligos. Alanine substitutions were introduced at any of the potential phosphorylation residues Ser, Thr, and Tyr (predicted by NetPhos 2.0 Server) of Kv1.3 C terminus. With the exception of T439A that served as a control, all the selected residues were out of the Kv1.3-Kv1.5 consensus amino acid sequence. Mutagenesis was performed with the Stratagene QuikChange II site-directed mutagenesis kits using pmCherry-N1-hKv1.3 fusion protein as template. All constructs were sequence-verified.

Proliferation Assays—Once transfected, cells were counted with a hemocytometer and seeded at a density of 50,000 cells/well on 12-mm poly-lysine-coated coverslips. Proliferation was determined 24 h after seeding cells using a commercial kit (ClickIT® EdU Imaging Cell Proliferation Assay, Invitrogen) following previously described protocols (22). The percentage of cells at the S phase was quantified using 5-ethynyl-2′-deoxyuridine (EdU) incorporation for a 30-min period. Determinations were carried out in triplicate samples, and controls were included in all experiments.

qPCR Analysis of siRNA Efficiency—HEK cells were transfected with Ambion, Silencer® Select siRNAs. The siRNAs used were MAP2K1 (s11176 and s11168), MAP2K2 (s11170), and negative control (AM-4611). 48 h after transfection total RNA was isolated using TRIzol reagent (Invitrogen), and mRNA lev-
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eels were determined by qPCR with Taqman® probes in a RotorGene 3000 instrument (Corbett Research). Data were analyzed with the threshold cycle relative quantification method ($ΔΔCt$), normalized to an endogenous control (ribosomal protein L18). Taqman assays were used for MAP2K1 and MAP2K2. The corresponding siRNAs reduced MAP2K1 mRNA by 10-fold (from 0.96 ± 0.08 to 0.1 ± 0.003) and MAP2K2 by 3-fold (from 1.05 ± 0.1 to 0.36 ± 0.6). No cross-reactivity was observed.

Apopitosis Assays—Apopitosis was detected by TUNEL method (In Situ Cell Death Detection kit, Roche Applied Science) 24 h after seeding transfected HEK293 cells. Experimental positive and negative controls were also performed.

Electrophysiological Studies—Ionic currents were recorded at room temperature using the whole-cell or the cell-attached configuration of the patch clamp technique as previously described (13, 19). For the whole cell experiments we used an internal solution (High-K+) containing (125 mM KCl, 4 mM MgCl₂, 10 mM HEPES, 10 mM EGTA, 5 mM MgATP (pH 7.2 with KOH)). The composition of the bath solution (Standard-e) was 141 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl₂, 1.8 mM CaCl₂, 10 mM glucose, and 10 mM HEPES (pH 7.4 with NaOH). 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). Outward K+ currents were elicited by depolarizing pulses from a holding potential of −80 mV to +40 mV applied in 10-s intervals. In some cells full current/voltage curves were constructed from potentials ranging from −60 to +100 mV in 10-mV steps. Kv1.3 and Kv1.5 were defined by their sensitivity to the selective blockers 5-(4-phenoxybutoxy)psoralen (100 nM) and diphenyl phosphate oxide-1 (DPO; 1 μM), respectively.

Cell attached recordings were used for the kinetic characterization of the currents, with a pipette solution containing 120 mM NaCl, 30 mM KCl, 2 mM CaCl₂, and 10 mM HEPES (pH 7.4 with NaOH) and a bath solution (High-K+) with 150 mM KCl, 0.5 mM MgCl₂, 10 mM HEPES, 1 mM EGTA (pH 7.2 with KOH). Currents were elicited by 500 μs depolarizing pulses to +40 mV from a holding potential of −80 mV. IV curves were constructed with 800-ms-depolarizing pulses from −80 to +100 mV in 20-mV steps applied every 10 s. Conductance curves were obtained from the amplitude of the fit to a mono-exponential function of the tail currents upon repolarization to −80 mV. After normalization to the maximal conductance ($G_{max}$) the data were fitted to a Boltzmann function to obtain the $V_{0.5}$ of activation and the slope of the curve. The steady-state inactivation was studied with a two-pulse protocol, in which a 200-μs pulse to +40 mV was preceded by a family 1.8-s depolarizing pulses from −100 to +40 mV in 20-mV intervals applied every 20 s. The peak current amplitude of the +40-mV pulse was normalized to the maximal amplitude (obtained with the −100-mV prepulse), plotted as a function of the prepulse potential, and fitted to a Boltzmann function. To determine the membrane potential in the cell-attached patch, we assumed that in the High-K+ there was no voltage difference across the cell membrane. This was confirmed with the measurement of the K⁺, from an instantaneous IV curve. 100-ms depolarizing prepulses to +60 mV were followed by repolarization to potentials from −100 to +60 mV. The IV curve obtained allowed determination of the reversal potential of the current and calculation of the K⁺, so that we could correct the estimated transmembrane potentials.

Immunocytochemistry—Transfected HEK293 cells were fixed with 3.7% paraformaldehyde 24 h after seeding and blocked in 2% of normal goat serum with PBS (nonpermeabilized cells) or Triton X-100 0.1% (permeabilized cells). Nonpermeabilized cells were incubated with anti-Kv1.3 or anti Kv1.5 extracellular primary antibodies (APC101 or APC150, Alomone Labs), whereas permeabilized cells were incubated with anti-Kv1.3 COOH (75-009, NeuroMab) or anti-Kv1.5 COOH (APC004, Alomone Labs), all at a final concentration of 1:50. Goat anti-rabbit 488 (A-11008, Molecular Probes), goat anti-rabbit 532 (A-11009, Molecular Probes), or goat anti-mouse 532 (A-11002, Molecular Probes) secondary antibodies were used at 1:1000. Hoechst 33342 (1:2000)-labeled nuclei and coverslips were mounted with Vectorshield (Vector Laboratories). Photomicrographs were acquired with a LEICA SP5 confocal microscope using LAS software.

Image Analysis—Confocal images from non-permeabilized transfected HEK293 cells were used to assess the relative expression of Kv1.3 and Kv1.5 constructs at the plasma membrane. 63× (1.4NA) objectives were used to capture images and Z-stacks with a pinhole aperture of 1 airy unit (95.5 μm with our experimental settings) and a voxel size compatible with the Nyquist criterion. Images were binarized using a threshold that was automatically defined by Fiji software rsb.info.nih.gov). EGFP (488ex/507em) or mCherry fluorescence (587ex/610em) was taken as a measure of total expression of the fusion proteins ($A_{total}$), whereas secondary antibody images, 532 (531ex/554em) or 488 (495ex/519em) defined the membrane area ($A_{membrane}$). Both values were used to compute the % of membrane expression.

Immunoprecipitation—Transfected HEK293 cells incubated with the corresponding treatments were treated with 250 μM pervanadate during the last 5 min of incubation. Cell lysates collected in radioimmune precipitation assay buffer (150 mM NaCl, 50 mM Tris (pH 8), 1% Nonidet P-40, 0.2% sodium deoxycholate) with 1× protease inhibitor mixture (Roche Applied Science) and 1 mM phosphatase inhibitors NaF and Na₃VO₄ were incubated with RFP/GFP-Trap_A beads (ChromoTek) for 2–3 h and used for immunoblotting. They were separated by SDS-PAGE in 10% polyacrylamide gels and transferred to nitrocellulose membranes. Mouse monoclonal anti-phosphotyrosine antibody (clone 4G10, Millipore), rat monoclonal anti-RFP antibody (5F8, ChromoTek), rat monoclonal anti-GFP (3H9, ChromoTek), rabbit anti p-ERK1/2 (Cell Signaling 9101), and mouse monoclonal anti-β-actin (ab8226, Abcam) were used at a final concentration of 1:1,000 and incubated overnight at 4 °C. Secondary antibodies HRP-goat anti-rat (Abcam), or HRP-goat anti-rabbit (Santa Cruz, sc-2301) were used at final concentration 1:20,000 for 1 h and developed with VersaDoc 4000 Image System (Bio-Rad) using chemiluminescence reagents (SuperSignal West Femto
Chemiluminescent Substrate, Pierce). The relative amount of protein was calculated by densitometric analysis of the bands using Fiji (Image J) software.

Data Analysis—Electrophysiological analyses were performed with both the CLAMPFIT subroutine of the PCLAMP software (Axon) and ORIGIN 7.5 software. Pooled data are expressed as the means ± S.E. Statistical analysis was performed using paired or unpaired test as appropriate. For pairwise comparison of normal distribution, Student’s t test was used. For comparisons of several groups, one-way ANOVA was performed followed by a Shapiro-Wilk normality test of the fit of the residuals. In the case of normal distributions, comparisons among groups were performed with Tukey’s honest significant difference (HSD) test. In the case of un-normal distributions (i.e., p value in the Shapiro-Wilk test of residuals < 0.05), a Kruskal-Wallis one-way analysis of variance by ranks was used as a nonparametric method to test whether samples originate from the same distribution. When the null hypothesis was rejected, a pairwise Mann-Whitney-Wilcoxon (MWW) test with a Bonferroni correction was applied. In all cases, differences were considered to be significant when p < 0.05.

Results

The C-terminal Domain Contains the Molecular Determinants of Kv1.3-induced Proliferation—We constructed two chimeric channels (K5N3 and K5C3) using the backbone of Kv1.5 channels and substituting either the amino (K5N3) or the C-terminal (K5C3) intracellular domains with the corresponding regions of Kv1.3 channels. Both chimeras were constructed as fusion proteins with a fluorescent protein attached to the C terminus: EGFP for K5C3 and Cherry for K5N3. Upon expression, both chimeras formed functional channels with some mixed biophysical properties (Fig. 1A and Table 1): 1) current density was much larger for the K5C3 than for K5N3 channels; 2) inactivation time course and V_{0.5} of activation were similar to Kv1.5, K5C3, and K5N3-transfected cells. Data are represented as the mean ± S.E. of 7–11 cells. B, confocal images obtained in HEK293 cells transfected with vectors expressing K5C3-EGFP and K5N3-Cherry chimeric vectors. The left panels show the fluorescence of the fusion protein, the middle panels show the antibody labeling, and the right panels show the merged images with Hoechst to see nuclear staining (blue). Membrane expression was determined in nonpermeabilized cells with an extracellular anti-Kv1.5 antibody (shown in red for K5C3 and in green for K5N3). Subcellular expression was determined in permeabilized cells by using antibodies against the cytoplasmic C terminus (CT), an anti-Kv1.3-CT antibody (red) and an anti-Kv1.5-CT antibody (green), respectively. Constructs are depicted on the left schemes.
To test this possibility, HEK cells were co-transfected with Kv1.3 and Kv1.5 channels and the mutants and chimera constructs. Immunocytochemical labeling of non-permeabilized cells with extracellular Kv1.5 antibody (Fig. 1B and Table 2). The fluorescence overlap of the labeled antibody and the fusion proteins demonstrates that most K5C3 molecules localized to the cell surface, whereas a large amount of K5N3 was retained intracellularly and did not reach the plasma membrane.

The pharmacological profile of both chimeras resembles that of Kv1.5 channels (Fig. 2). Both mutants were sensitive to the Kv1.5-selective blocker DPO and did not respond to the selective Kv1.3 blocker 5-(4-phenoxybutoxy)psoralen (100 nM, data not shown). We tested proliferation after transient transfection of K5N3-, K5C3-, Kv1.3-, Kv1.5-, or EGFP-expressing plasmids. The data (Fig. 4) shows that five of the point mutants (Y447A, S459A, S473A, S475A, Y477A) altered the pro-proliferative effect of Kv1.3 channel expression. Interestingly, three of them (Y447A, S459A, and Y477A) completely abolish the effect, as the proliferation in the presence of any of them was no different from control, cherry-transfected cells. Evaluation of the current density obtained with these transfectants (Fig. 4) showed no difference with the wild type Kv1.3 channels in all the mutants explored, in agreement with the co-localization studies (Table 2).

**Confirmation of the Pivotal Role of Tyr-447 and Ser-459 Residues in Kv1.3-induced Proliferation—Modulation of Kv1.3 channel activity and kinetics in response to treatment with EGF or insulin has been shown to involve phosphorylation of tyrosine 477 but not of 447 (27). However, in our study point mutations to alanine of residues Tyr-447 and Ser-459 generated the two channel mutants that more efficiently abolished Kv1.3-induced proliferation. These residues are located at the proximal end of the Kv1.3 channel C terminus, in a region not previously identified as a conserved motif for interaction with growth fac-
tor-associated kinases. We constructed some additional chimeric channels to confirm the contribution of this region (that we named the YS segment; Fig. 4) to the effect of Kv1.3 channels on proliferation. We created a truncated Kv1.3 channel with a short C-terminal domain comprising only the YS segment (Kv1.3-YS). Two additional chimeric channels were constructed by inserting this YS segment at two different positions into the Kv1.5 channel C terminus: either proximal in the equivalent location in Kv1.3 channels (after residue 532, Kv1.5-YS532) or at the end of the protein (Kv1.5-YS613). Both Kv1.5-

FIGURE 2. Dose-response curves for the effect of DPO on K5N3 and K5C3 currents. A, the plots show the current amplitude at the end of depolarizing pulses to +40 mV from a holding potential of −80 mV, applied every 10 s, from a HEK cell transfected with K5N3 (upper graph) or with K5C3 (lower graph). DPO was applied to the bath solution at the concentrations indicated (in μM) during the time marked. The insets show sample current traces at the different DPO concentrations. B, normalized dose-response curves obtained for the inhibitory effect of DPO on the current amplitude in both chimeric channels as well as in Kv1.5 (open circles). Each data point is the mean ± S.E. of 9–18 cells. The lines show the fit of the data from K5N3 and K5C3 channels to a Hill function (IC50 = 0.66 μM for K5C3 and 0.26 μM for K5N3).

FIGURE 3. Effects of K5N3 and K5C3 channels on HEK cell proliferation. A, proliferation rate of HEK cell transfected with GFP-expressing vector (used as control) or with the indicated channels was determined by measuring the fraction of cells incorporating EdU reagent after 30 min of incubation in media with 5% FBS. Each bar is the mean ± S.E. n = 12–15. Data were from at least four different experiments. Comparisons were carried out with a one-way ANOVA and Tukey’s HSD test as post hoc analysis, and significant differences with control and GFP-transfected cells are shown. *, p < 0.05; **, p < 0.01. B, representative examples of family of currents elicited by 500-ms depolarizing pulses from −80 to +60 mV in 20 mV from a HEK cell expressing K5N3 alone or together with the chaperone subunit Kvβ2.1. In addition to the increased current amplitude, there was a leftward shift in the voltage dependence of activation upon Kvβ2.1 coexpression. The data are representative of seven cells in each group. C, the changes in the current density of K5N3 channels do not modify their effect on proliferation. The plot shows the inhibition of proliferation (left bars; n = 4) and the current density at +40 mV (right plot; n = 8–15) from whole cell experiments of HEK293 expressing Kv1.5, K5N3, and K5N3 + Kvβ2.1. Statistical differences from control (Kv1.5) were calculated with a Kruskal-Wallis test followed by a pairwise MWW with Bonferroni correction. pF, picofarads.
YS532 and Kv1.5-YS613 show kinetic and pharmacological properties similar to Kv1.5 channels. Moreover, their trafficking did not show significant differences compared with wild type channels (Fig. 5B, Table 2). Meanwhile, Kv1.3-YS showed biophysical properties similar to Kv1.3 channels (Fig. 5A), albeit we observed some differences in their activation kinetics (Table 1) as well as a decreased membrane expression (Table 1 and 2). The impact on proliferation of these constructs is summarized in Fig. 5C. The truncated Kv1.3 channel (Kv1.3-YS) was still able to induce proliferation, although to a lesser degree than wild type Kv1.3. Interestingly, the Kv1.5-YS chimera increased proliferation similar to Kv1.3, whereas Kv1.5-YS613 behaved as Kv1.5 channels. These data indicated that the YS segment suffices to convert Kv1.5 channels into Kv1.3 channels with regard to their proliferative properties, but the environment in which the YS segment is inserted is relevant for this effect. In fact, all the chimeras containing the Kv1.3 YS segment located in the homologous region of Kv1.5 channels (the proximal end of C termini), have a stimulating effect on HEK proliferation (Fig. 5D) that is independent of the amount of current they can carry (or their membrane expression).

Identification of the Proliferation-activated Signaling Pathway Modulated by Kv1.3 Expression—The pro-proliferative role of native Kv1.3 channels in VSMCs is mediated by ERK1/2 signaling pathway, as selective blockers of Kv1.3 or selective blockers of the ERK1/2 cascade inhibited proliferation in a non-additive fashion (22). We have explored if this pathway could also be involved in the Kv1.3 induced proliferation in HEK cells. Treatment of HEK cells with the ERK1/2 blocker PD98059 (20 μM) was able to suppress Kv1.3-induced proliferation and had no effect in control cells (Cherry-transfected) or in cells transfected with the mutant channels Y447A and S459A (Fig. 6A). Genetic blockade of MEK1/2 by co-transfection of cherry or Kv1.3 constructs with a mixture of three MEK1/2 siRNAs fully reproduced the effects of PD98059 on HEK cells proliferation (Fig. 6B). These experiments suggest that the Kv1.3 effect on proliferation requires ERK1/2, and we hypothesize that either Tyr-447 or Ser-459 needs to be phosphorylated for Kv1.3 channels to induce proliferation. Immunoblots with anti-phosphotyrosine antibodies of Kv1.3 or Y447A-transfected HEK lysates after immunoprecipitation with anti-Cherry antibody support this hypothesis. In Kv1.3-transfected cells we show a clear phosphorylation signal of Kv1.3 that decreased after PD98059 treatment. There was a decreased basal phosphotyrosine content of Y447A-transfected cells compared with Kv1.3, and the treatment with the inhibitor did not have any effect (Fig. 6C).

Besides, immunoprecipitation assays with anti-Cherry antibodies of Cherry- or Kv1.3-cherry-transfected cells demonstrated binding of pERK to the complexes containing Kv1.3 channels only upon treatment with phosphatase inhibitors, suggesting a brief, reversible interaction between them (Fig. 6D).

The correct location of Tyr-447 and Ser-459 within the channel protein seems to also be relevant for the effect on prolifer-
ation (as illustrated with the differences between Kv1.5-YS532 and Kv1.5-YS613). This could be attributed to conformational changes of the channel that expose these residues. Based on our previous data (13) we hypothesize that conformational changes in response to changes in EM may be necessary to expose the identified phosphorylation sites in the YS segment. We were able to explore this hypothesis for the tyrosine residues by studying the effect of high K\textsubscript{e}-induced depolarizations. Kv1.3-transfected cells were incubated with extracellular solutions containing 10, 20, and 60 mM K\textsubscript{e} during 20 min. High K\textsubscript{e} induced a significant increase of phosphotyrosine labeling of Kv1.3 channels at all the concentrations tested (Fig. 7A). The effect of depolarization on tyrosine phosphorylation was transient, returning to control values for incubations of 2–4 h in high K\textsubscript{e} (Fig. 7B). In addition we found that the phosphotyrosine content is higher in Kv1.3WF channels, a poreless channel mutant, and decreased in the pore and gate double mutant Kv1.3 WF3x, which does not have voltage-gating transitions at resting E\textsubscript{M} or in the Kv1.3 AYA mutant, a poreless channel that does not traffic to the plasma membrane (Fig. 7C). Strikingly, Kv1.3 WF3x and Kv1.3 AYA were not able to induce HEK cell proliferation, whereas Kv1.3WF had an effect similar to Kv1.3 channels (13).

**Discussion**

In this study we undertook the molecular dissection of the Kv1.3 channel and the parallel changes observed in HEK proliferation to conclude with an attractive hypothesis aimed to understand the role of ion channels in cell proliferation. The opposite effect on cell proliferation of two channels of the same subfamily (Kv1.3 and Kv1.5) has been of seminal importance for accomplishing our goals. This antithetical role has been described in different cell lineages, including VSMCs, oligodendrocyte progenitor cells, and microglia (10–13, 21). The fact that these antagonist effects on cell proliferation could still be observed upon heterologous expression of the channels represented an opportunity to explore the molecular mechanisms that associate the expression of these channels with the changes in the rate of proliferation. The data obtained from our analysis provided clear-cut results regarding the mechanisms by which Kv1.3 channel expression can facilitate cell proliferation.

All the chimeric and mutant channels were located at the plasma membrane. Two of them had a decreased functional expression (i.e. a decreased current density) compared with their corresponding wild type channels, namely Kv1.3-YS (compared with Kv1.3) and K5N3 (compared with Kv1.5). It has been reported that Kv1.3 channels harbor a di-acidic signal...
residues Glu-481/482 in human) essential for anterograde transport and surface expression (28) that is not conserved in Kv1.5 channels (see the schematic in Fig. 4). This could contribute to the decreased membrane expression of Kv1.3-YS and to the large increment both in current amplitude and membrane expression of K5C3 (see Tables 1 and 2). In this line, the reduced expression with K5N3 suggest that either the Kv1.3 N terminus has unique ER retention or retrograde trafficking signals or that the Kv1.5 N terminus has forward trafficking signals that are not present in Kv1.3.

Also, the changes in the biophysical properties of our mutant or chimeric constructs were in good agreement with the existing data regarding their molecular determinants. We confirm that the cytoplasmic N-terminal domains of Kv1.3 and Kv1.5 determine the voltage-dependent inactivation properties of the channels (29), and we reproduce the kinetic changes upon Kvβ2.1 coexpression (25, 26). Finally, the changes in the voltage dependence of activation observed in Kv1.3-YS and Kv1.5-YS (32) are consistent with previous observations indicating that the coupling of the voltage sensor movement to operation of the gate mainly involves physical interactions of the S4-S5 linker with the intracellular end of S6 and/or the cytoplasmic “C-linker” region (30).

The contribution of Kv channels to cell proliferation has been associated with several mechanisms that do not need to be mutually exclusive such as their ability to sense and regulate E_M, their role in the control of cell volume, or their contribution to the regulation of calcium fluxes and intracellular calcium concentrations by means of the hyperpolarization of the E_M. Most of these mechanisms rely on the potassium gradients and focus on the potassium fluxes that generate the changes in cell volume or in resting E_M and do not consider the contribution of the ion channel molecule itself. However, non-conducting signaling roles of ion channels can also accompany these effects and in some cases are the main signal to induce proliferation. In these cases the cell cycle-associated oscillations of resting E_M may be mere byproducts of the changes in the expression or the conformational states of the ion channel (31). There are very few studies dissecting the contribution to the cell cycle of both conducting and non-conducting properties of ion channels, as this requires the use of genetic or pharmacological tools to selectively abolish ion permeation, although these evidences are clear for a few Kv channel molecules in different systems (32). Even more scarce is the information regarding the link(s) between voltage-dependent gating of Kv channels and cell cycle progression, although some clues are provided by the charac-
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FIGURE 7. Phosphotyrosine labeling of Kv1.3 channels is modulated by voltage-dependent transitions. A, representative immunoblots and summary data from Kv1.3-transfected cells incubated with the indicated K⁺ solutions for 20 min before RFP-Trap immunoprecipitation (IP). p-Tyr labeling was corrected for anti-Cherry labeling and normalized to control data (cells incubated in 5.4 mM K⁺). Statistical differences from control condition were established with a Tukey’s HSD comparison after one-way ANOVA. B, time course of the effect of incubations with 60 mM K⁺. At each time point, p-Tyr labeling was shown as the ratio of the 60/5.4 mM K⁺ signal. Each data point is the mean ± S.E. of 5–12 determinations from at least three independent experiments. Statistics were obtained with a MWW test pairwise comparison with Bonferroni correction and compared with the p-Tyr ratio at t = 5 min. C, immunoblot and densitometric average values of GFP-Trap immunoprecipitated cells transfected with Kv1.3 or W389F-, WF3x-, and AYA Kv1.3-mutant channels. Data are normalized as above and are represented as the mean ± S.E. of 3–5 different experiments. Significant differences with Kv1.3 wild type channels were estimated with pairwise Student’s t tests. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

cellos can be fully abolished by three point mutations that eliminate three phosphorylation sites at the C-terminal domain. Moreover, we demonstrate that at least for one of these residues (Tyr-447) potentiation of proliferation is mediated by its phosphorylation and is dependent of an intact MEK/ERK signaling pathway (Fig. 6, A and C). Finally, our results with the chimeric channels containing the YS segment suggest that the location of this domain within the channel molecule also determines its ability to enhance proliferation. We speculate that the exposure of this region for being phosphorylated requires a conformational change of the molecule; differences in phosphotyrosine labeling of Kv1.3 channels in the presence of high K⁺ suggests that voltage-dependent transitions from closed to open state could represent this conformational change. Resting Eₘ of HEK cells changes from a mean value of −15 mV in mock-transfected (or Kv1.3WF-transfected) cells to −43 mV in Kv1.3-transfected cells (13). Interestingly, phosphorylation of Kv1.3WF is significantly higher than Kv1.3, as expected if we take into account that Kv1.3WF-transfected cells are more depolarized. Increasing K⁺ from 5.4 to 10, 20, or 60 mM depolarizes the cells to Eₘ values > Vₒ.5 of activation of the channel (−38, −30, and −14 mV, respectively), and in agreement with that we found increased Kv1.3 phosphorylation in the three conditions. Also, the requirement of a closed to open transition can explain the increased Tyr phosphorylation of Kv1.3WF (that shows normal gating currents) and the absence of Tyr phosphorylation of the pore and gating double mutant Kv1.3WF-3x (13). The contribution of phosphorylation of
Kv1.3 serine residues could not be determined with a similar approach. Unlike phosphotyroserines, which have a very specific epitope, there are no single antibodies that will recognize all or most phosphotyrosines independently of the surrounding amino acid sequence, and we failed to show phosphotyrosine labeling of Kv1.3 using several different experimental procedures. However, the physical association of pERK with the complexes containing Kv1.3 channels (Fig. 6D) could provide an indirect argument supporting a role for serine residues in proliferation signaling pathways, as ERKs substrates are phosphorylated in Ser/Thr residues.

Overall, our data suggest that the molecular nature of the voltage sensor that activates the signaling pathway facilitating proliferation is relevant. The effect on proliferation is lost when the channel is not able to sense the voltage and is increased when the voltage-dependent transitions are facilitated. However, the changes in E_A are not sufficient to promote proliferation, as illustrated by the overexpression of Kv1.5 channels, which promotes a similar hyperpolarization (13) but in the absence of the specific domains is not able to induce proliferation (in fact it has the opposite effect). These data open interesting questions to identify the signaling pathways by which Kv1.3 channels (and not Kv1.5 channels) potentiate cell proliferation in several native systems. The contribution of Kv1.3 channels to cell proliferation in these systems can be related to their role as effectors of the cell cycle machinery, participating in the regulation of signaling cascades via protein-protein interactions. The identification of these signaling cascades and of the molecules involved in this non-canonical function of Kv channels will provide a better understanding of these processes and will pave the way for the development of new, more specific therapies based on ion channels for controlling cell proliferation.

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