The intermediate conductance calcium-activated potassium channel KCa3.1 regulates vascular smooth muscle cell proliferation via controlling calcium-dependent signaling.*

Received for publication, October 10, 2012, and in revised form, April 12, 2013. Published, JBC Papers in Press, April 22, 2013, DOI 10.1074/jbc.M112.427187

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Background: The mechanism by which KCa3.1 regulates cell proliferation remains elusive.

Results: KCa3.1 regulates the expression of transcription factors and cyclins by controlling intracellular calcium levels in activated vascular smooth muscle cells (VSMCs).

Conclusion: KCa3.1 is an important regulator of the calcium-dependent proliferation machinery in VSMCs.

Significance: KCa3.1 modulation constitutes a therapeutic target for cell proliferative diseases such as atherosclerosis.

The intermediate conductance calcium-activated potassium channel KCa3.1 contributes to a variety of cell activation processes in pathologies such as inflammation, carcinogenesis, and vascular remodeling. We examined the electrophysiological and transcriptional mechanisms by which KCa3.1 regulates vascular smooth muscle cell (VSMC) proliferation. Platelet-derived growth factor-BB (PDGF)-induced proliferation of human coronary artery VSMCs was attenuated by lowering intracellular calcium. KCa3.1 blockade or knockdown inhibited proliferation by suppressing the rise in intracellular calcium, and attenuating the expression of phosphorylated CREB, c-Fos, and cyclin-dependent kinase inhibitors. The stimulation also attenuated the levels of phosphorylated CREB, c-Fos, and cyclin expression. After KCa3.1 blockage, the characteristic round shape of VSMCs expressing high l-caldesmon and low calponin-1 (dedifferentiation state) was maintained, whereas KCa3.1 stimulation induced a spindle-shaped cellular appearance, with low l-caldesmon and high calponin-1. In conclusion, KCa3.1 plays an important role in VSMC proliferation via controlling Ca2+-dependent signaling pathways, and its modulation may therefore constitute a new therapeutic target for cell proliferative diseases such as atherosclerosis.

The abbreviations used are: KCa, calcium-activated potassium channel; CREB, cAMP-response element-binding protein; CsA, cyclosporin A; EBIO, 1-ethyl-2-benzimidazolone; HCSMC, human coronary artery smooth muscle cell; NOR-1, neuron-derived orphan receptor-1; NS309, 6,7-dichloro-1H-indole-2,3-dione 3-oxime; PMA, phorbol-12-myristate-13-acetate; SCA-1, 2-amino-6-(methylsulfonyl)benzothiazole; SKA-31, naphthol[2,3-d]thiazol-2-yllamine; TRAM-34, 1-[[2-chlorophenyl]diphenylmethy]-1H-pyrazole; VSMC, vascular smooth muscle cell; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid; eGFP, enhanced green fluorescent protein; Luc, luciferase; tg, eGFP-tagged.

*This work was supported, in whole or in part, by National Institutes of Health Grants RO1 HL080173, HL080173-02S1, P20 RR018751, and P20 GM103513 (to H. M.); RO1 GM076063 (to H. W.); P01 HL68769 (to D. G.); and RO1 DK090123 (to F. P.). This work was also supported by an Advancing a Healthier Wisconsin grant from the Medical College of Wisconsin.

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KCa3.1 and Vascular Smooth Muscle Cell Proliferation

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ically change as they proliferate (13). In the contractile form of VSMCs, Ca^{2+} influx through voltage-dependent Ca^{2+} channels causes VSMC contraction and co-activation of large conductance KCa channels (BK), which in turn induces Ca^{2+} channel closure through repolarization. In contrast, in proliferating VSMCs, this mechanism is down-regulated, and Ca^{2+} influx through Ca^{2+} release-activated Ca^{2+} channels is maintained by membrane hyperpolarization induced by KCa3.1 activation. We recently found that vascular remodeling following myocardial infarction (14) and chronic inhibition of nitric oxide synthesis (15) is associated with increased KCa3.1 expression in rat hearts, suggesting a pathophysiological role for this channel. Consistently, KCa3.1 expression and activity are increased in VSMCs activated by mitogens, in intimal VSMCs of restenotic lesions following vascular injury, and in atherosclerotic plaques in humans, swine, rats, and mice (3, 11, 12, 16). Importantly, pharmacological blockade, siRNA knockdown, or genetic deficiency of KCa3.1 suppresses VSMC activation (including proliferation, migration, and excessive oxidant production) and attenuates the development of restenosis and atherosclerosis in swine, rats, and mice (3, 12, 17, 18). Activation of the transcription factor AP-1, which KCa3.1 regulates cell proliferative processes, remains unknown.

We hypothesized that KCa3.1 blockade or knockdown would suppress VSMC proliferation by inhibiting the mitogen-induced rise in [Ca^{2+}], and subsequent mitogenic signaling pathways, whereas KCa3.1 stimulation or overexpression would confer the opposite effects. To test this hypothesis, we examined the effect of pharmacological KCa3.1 blockade or activation, gene silencing, or overexpression on platelet-derived growth factor-BB (PDGF)-induced proliferation of VSMCs, focusing on changes in [Ca^{2+}], activation of mitogenic signaling pathways, cell cycle progression, morphology, and phenotypic characteristics in human coronary artery VSMCs (HCSMCs).

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HCSMCs (Cell Applications) were grown as reported previously (3). All experiments were performed between passages 5 and 7. Cells were seeded and cultured up to 70% confluence in smooth muscle growth medium (Cell Applications). Before each assay, cells were serum-starved and synchronized for 48 h in smooth muscle basal medium. HCSMCs in a quiescent state were stimulated for 1–48 h with PDGF (20 ng/ml; R&D Systems). None of the treatments performed in this study altered the viability of VSMCs, as judged by trypan blue exclusion (data not shown).

**Small Interfering RNA Transfection**—siRNA transfection was performed as reported previously (3). Optimization of siRNA concentration (12.5 nM; Ambion), transfection time, and cell density were determined by quantifying KCa3.1 mRNA and protein expression (3).

**RNA Extraction, Reverse Transcription, and Quantitative PCR**—Real-time PCR (iCycler, Bio-Rad or 7900 HT real-time PCR system, Applied Biosystems) was performed using iQ SYBR Green supermix to quantify transcript levels for KCa3.1 and GAPDH as described (3). Primers were designed using Beacon Designer software 3.0 (PREMIER Biosoft International).

**Western Blotting**—Total cell lysates or membrane fractions (10–50 μg) were analyzed by Western blotting as reported previously (3). Briefly, harvested cells or membrane fractions were lysed in a buffer containing CHAPS (1% w/v), protease inhibitor mixture (Sigma), 1 mM sodium orthovanadate, and 10 mM sodium fluoride. The protein content of each sample was determined using an RC DC protein assay (Bio-Rad). Proteins were resolved on a 10% SDS-polyacrylamide gel and subsequently transferred to a PVDF membrane (Bio-Rad). The following primary antibodies were used: a polyclonal antibody against human and mouse KCa3.1 (46 kDa) obtained from sera of rabbits immunized with the following oligopeptide: H-LNASYR-SIGALNQVRC-NH2 (S4-S5 of human and mouse KCa3.1) (3), anti-l-caldesmon, anti-calponin-1, anti-PDGF β-receptors, anti-cyclin A, anti-cyclin B1, anti-cyclin D1, anti-cyclin E, anti-β-actin (Santa Cruz Biotechnology), anti-cAMP-response element-binding protein (CREB), anti-phospho-CREB (Ser 133), anti-c-Fos (Cell Signaling), and anti-neuron-derived orphan receptor-1 (NOR-1) (R&D Systems). Secondary antibodies were from Santa Cruz Biotechnology. COLO 320DM cell lysate was used as a positive control for KCa3.1, and HeLa nuclear extract was used for c-Fos and NOR-1 (Santa Cruz Biotechnology). Representative immunoblots from three or more experiments are shown.

**Cell Proliferation and Migration Assays**—Cell proliferation (ELISA kit, Roche Applied Science) and migration were examined as we reported previously (3). VSMC migration was stimulated with PDGF for 8 h in Transwell plates (Corning).

**Cell Cycle Analysis**—As reported previously (3), HCSMCs were stimulated with 10% FBS for 24 h. After fixation, cells were treated with ribonuclease A (250 μg/10⁶ cells) for 30 min at 37 °C and stained with propidium iodide (50 μg/ml) for 30 min at 4 °C. The ratio of cells in each cell cycle phase was determined by flow cytometry (FACSCalibur: BD Biosciences) using CellQuest software (BD Biosciences). Intracellular Ca^{2+} Measurement—To measure [Ca^{2+}], in VSMCs after 48 h of treatment (24), cells were incubated with Fluo-4 AM (10 μM; Invitrogen) for 25 min at room temperature. Fluorescence images were captured and analyzed using an inverted epifluorescence microscope (Nikon TE200) with a 40× Plan Fluor objective, a high speed wavelength switcher (Lambda DG-4 from Sutter Instrument), a PC-controlled digital CCD camera (Hamamatsu C4742-95), and MetaMorph software (Universal Imaging). Fluorescence was measured at 488 nm, and recording emission was measured at 523 nm. Images were analyzed with MetaMorph software.
Patch Clamping—Serum-starved VSMCs or cells treated for 48 h with PDGF or with PDGF in the presence of TRAM-34 (a specific blocker of KCa3.1 (3, 25)), EBIO (an activator of KCa3.1 and small conductance KCa2 channels (26)), or SKA-31 or NS309 (more potent and specific KCa3.1 activators (27, 28)) were patch-clamped in the whole-cell mode of the patch clamp technique using an EPC-10 amplifier. KCa3.1 currents were elicited by dialysis with an aspartate-based pipette solution containing 3 μM free Ca2+ and voltage ramps from −120 to 40 mV of 200-ms duration applied every 10 s. Whole-cell KCa3.1 conductances were calculated from the slope at 80 mV where the KCa3.1 currents are not “contaminated” by inwardly rectifying potassium channel, voltage-gated potassium channel (Kv), or BK currents. The KCa3.1 whole-cell conductance was then divided by the KCa3.1 single channel conductance (11 picosiemens) to determine the KCa3.1 channel number per cell.

Determination of Cell Morphology—Cell morphology was analyzed as reported previously (29). After 48 h of treatment, phase-contrast images of ~20 randomly chosen fields per condition and per experiment were taken. The following morphological parameters were calculated from the cell boundary. 1) Cell length was determined along the principal axis of traction, which is a unique axis and presumably coincides with the principal actin bundles. 2) Cell width was measured in the direction perpendicular to the principal axis of traction. The maximum cell width was taken, ignoring thin cell protrusions. 3) The shape index was defined as the ratio of the cell length and width.

KCa3.1 Overexpression—For pharmacological induction, HCSMCs were treated with a combination of phorbol-12-myristate-13-acetate (PMA, 40 nM, a specific activator of protein kinase C; Calbiochem) and cyclosporin A (CsA, 100 nM, an inhibitor of calcineurin; Sigma-Aldrich) in smooth muscle basal medium for 48 h (7). For viral induction, replication-defective lentiviral vectors pseudotyped with vesicular stomatitis virus G-protein were produced as described previously (30). Positive colonies expressing eGFP were identified by fluorescence microscopy 3 days after the final transduction step. Using this approach, 50–75% of HCSMCs were eGFP-positive.

Statistics—All data are expressed as mean ± S.E. Student’s t test and analysis of variance (for one-way and nonparametric tests) were performed using SigmaStat version 3 (SPSS Inc.) (3). Computations were followed by a Bonferroni’s corrected t test when significant differences were noted. Statistical significance was defined as a value of p < 0.05.

RESULTS

KCa3.1 Regulates VSMC Proliferation via Controlling [Ca2+]i—We first examined whether KCa3.1 regulates VSMC proliferation via controlling [Ca2+]i. As we reported previously (3), a 48-h stimulation with PDGF caused a significant increase in de novo DNA synthesis in HCSMCs as demonstrated by a BrdU incorporation assay (Fig. 1A). Lowering [Ca2+]i, with BAPTA (30 μM, an intracellular Ca2+ chelator (31)) suppressed the PDGF-induced increase in DNA synthesis, whereas BAPTA alone had no effect (Fig. 1A). On the other hand, a forced rise in [Ca2+]i, with A23187 (0.01–1 nM, a Ca2+ ionophore (31)) increased DNA synthesis in the absence of PDGF and enhanced PDGF-induced responses in a dose-dependent manner (Fig. 1B). Fluorescence microscopy using Fluo-4 showed that a 48-h stimulation with PDGF increased fluorescence intensity in
HCSMCs (Fig. 1C), which was inhibited by co-treatment with a specific KCa3.1 blocker, TRAM-34 (100 nM (3, 25)), or by silencing KCa3.1 expression (Fig. 1C). NOR-1 and c-fos are immediate early genes that are activated in a [Ca\(^{2+}\)]\(_i\)-sensitive manner in proliferating VSMCs (32–34). As shown in Fig. 1D, PDGF-induced expression of c-Fos and NOR-1 was inhibited by TRAM-34 but not by TRAM-7 (100 nM, an inactive analog of TRAM-34 (3, 25)). In addition, KCa3.1 knockdown also reduced the expression of both pro-oncogenes. Inhibition of PDGF-induced VSMC proliferation by KCa3.1 blockade with TRAM-34 (100 nM) was suppressed by A23187 in a dose-dependent manner (Fig. 2A). Similarly, the inhibitory effect of KCa3.1 knockdown was also suppressed by co-treatment with A23187 (Fig. 2B). These data indicate that KCa3.1 regulates PDGF-induced VSMC proliferation via controlling the rise in [Ca\(^{2+}\)]\(_i\), and subsequent signaling pathways.

Paradoxical Inhibition of PDGF-induced HCSMC Proliferation by KCa3.1 Stimulation—To test the hypothesis that full activation of KCa3.1 with pharmacological activators would enhance VSMC proliferation during PDGF exposure by augmenting the rise in [Ca\(^{2+}\)]\(_i\), EBIO (an activator of KCa3.1 and small conductance KCa2 channels (26)) was applied at 100 or 300 μM during PDGF-induced proliferation of HCSMCs. These cells predominantly express KCa3.1 but not small conductance KCa2 channels (3). PDGF-induced increase in DNA synthesis was unexpectedly inhibited by co-treatment with EBIO in a dose-dependent manner, whereas EBIO alone had no significant effect (Fig. 3A). The antiproliferative effect of KCa3.1 stimulation with EBIO was also confirmed using a cell count assay (data not shown). PDGF-induced chemotaxis of HCSMCs was also inhibited by 300 μM EBIO (fold increase: PDGF + EBIO 7.5 ± 3.0, p < 0.05 versus PDGF 17.4 ± 4.0, n = 5), and EBIO alone had no effect (EBIO 1.2 ± 0.1). Fluorescence microscopy using Fluo-4 revealed that co-treatment with EBIO (300 μM) reduced the PDGF-induced rise in [Ca\(^{2+}\)]\(_i\), in HCSMCs (Fig. 3B). To confirm the antiproliferative effect of KCa3.1 stimulation, two more potent and specific KCa3.1 activators, SKA-31 (27) and NS309 (28), were also tested. SKA-31 inhibited PDGF-induced proliferation at 0.5 μM, whereas SKA-16, an inactive derivative of SKA-31, had no effect (Fig. 3C). NS309 also exhibited an antiproliferative effect at 10 nM. Both activators had no effect in the absence of PDGF (data not shown).

To clarify the mechanisms responsible for this paradoxical inhibition, we first analyzed the expression level of KCa3.1 in HCSMCs treated with PDGF in the presence of TRAM-34 or the KCa3.1 activators. As we reported previously (3), PDGF induced a remarkable increase in KCa3.1 mRNA expression in HCSMCs (Fig. 3D). TRAM-34 (100 nM) had little effect on PDGF-induced increase in KCa3.1 expression. Contrarily, EBIO (300 μM) markedly attenuated PDGF-induced KCa3.1 up-regulation. Western blotting corroborated the mRNA analysis, showing that EBIO abolished PDGF-induced membranous KCa3.1 expression in HCSMCs, whereas TRAM-34 modestly increased KCa3.1 expression. SKA-31 (0.5 μM) and NS309 (10 nM) also suppressed the PDGF-induced increase in KCa3.1 transcripts at 6 h of PDGF treatment, consistent with the inhibition of protein expression at 48 h of PDGF treatment. Whole-cell patch clamp experiments (Fig. 3E) further confirmed the mRNA and Western blotting observations. Although PDGF stimulation alone induced robust KCa3.1 currents, cells stimulated with PDGF in the presence of EBIO, SKA-31, and NS309 exhibited only barely detectable KCa3.1 currents, similar to serum-starved cells. TRAM-34-treated cells showed a trend toward reduced KCa3.1 expression, but did not differ significantly from PDGF-treated cells. These findings indicate the existence of a negative feedback mechanism whereby chronic pharmacological stimulation of KCa3.1 paradoxically inhibits the rise in [Ca\(^{2+}\)]\(_i\), by attenuating KCa3.1 expression and activity, leading to suppression of PDGF-induced VSMC proliferation.

KCa3.1 Regulates Cell Cycle Progression—We next studied the effect of TRAM-34 or EBIO treatment on the activation of CREB and on the expression of c-Fos, two factors playing an important role in PDGF-induced VSMC proliferation (32, 35). Western blot experiments showed that PDGF induced CREB phosphorylation at Ser-133 and c-Fos up-regulation in HCSMCs (Fig. 4A), which were inhibited by both TRAM-34 and EBIO (100 nM and 300 μM, respectively). Consistent with
the fact that the CREB/c-Fos pathway is associated with cell cycle progression via regulating cyclin expression (35), PDGF treatment increased the expression of cyclins A, B1, D1, and E in HCSMCs (Fig. 4B). TRAM-34 reduced the expression of cyclins A and B1, and EBIO suppressed all tested cyclins. Flow cytometry analysis of the cellular DNA content showed that 10% FBS evoked cell cycle progression with fewer G0/G1 and more S and G2/M cells (Fig. 4C), which was reduced by TRAM-34 (100 nM) at the transitions from G0/G1 to S and from StoG2/M phases, whereas the activators EBIO (16.7 ± 13.9 channels, n = 14, p = 0.007 versus PDGF), SKA-31 (12.2 ± 4.8, n = 15, p = 0.004 versus PDGF), and NS309 (16.1 ± 17.9, n = 12, p = 0.005 versus PDGF) drastically reduced functional KCa3.1 channel numbers. *, p < 0.05 versus control, and †, p < 0.05 versus PDGF alone.

VSMC Morphological and Phenotypical Changes after KCa3.1 Blockade or Stimulation—PDGF treatment changed the cell morphology, conferring a longer and thinner cell shape in HCSMCs (Fig. 5A). TRAM-34 (100 nm) reduced PDGF-induced cell elongation and thinning. In contrast, EBIO treatment (300 μM) remarkably enhanced PDGF-induced cell elongation and thinning, and this enhancement was inhibited by co-treatment with TRAM-34. HCSMCs treated with SKA-31 or NS309 also displayed similar morphological characteristics (ratio of cell length/width; PDGF/SKA-31 25 ± 2 and PDGF+NS309 23 ± 2, p < 0.05 versus PDGF alone 15 ± 2, n = 83 cells). Treatment of HCSMCs with PDGF increased the expression of \(\alpha\)-caldesmon, a dedifferentiation marker, and decreased the expression of calponin-1, a differentiation marker, accompanied by a decreased expression of membranous PDGF-β-receptors, a known negative feedback response (36) (Fig. 5B). TRAM-34 treatment did not affect these changes; however, the KCa3.1 activators abolished the membranous expression of PDGF-β-receptors, another dedifferentiation marker (37), and prevented PDGF-induced up-regulation of \(\alpha\)-caldesmon and down-regulation of calponin-1. Furthermore, A23187 failed to suppress the inhibitory effect of EBIO (300 μM) on PDGF-induced DNA synthesis (Fig. 5C). Therefore, these data indicate that KCa3.1 stimulation converted the...
VSMC phenotype to one that is resistant to PDGF-induced proliferation by disrupting the axis of PDGF β-receptors and its Ca²⁺ regulatory mechanism.

KCa3.1 Overexpression Initiates and Enhances VSMC Proliferation—Two methods of pharmacological and genetic manipulations were used to increase KCa3.1 expression in quiescent VSMCs. As reported in T cells (7), treatment with PMA and CsA for 48 h increased KCa3.1 mRNA and protein expression with little de novo DNA synthesis (PMA/CsA treatment 1.0 ± 0.2-fold of control, p = not significant versus control, n = 3) in HCSMCs, which can be compared with KCa3.1 expression 48 h after exposure to PDGF (Fig. 6A). Following removal of PMA/CsA, these cells displayed a modest but statistically significant proliferative activity in the absence of any mitogen as compared with quiescent cells (Fig. 6B). PDGF-induced proliferation was also enhanced in PMA/CsA-pretreated, KCa3.1-overexpressing cells 24 h after exposure to PDGF as compared with control cells.

A lentiviral vector system was subsequently utilized to genetically modify HCSMCs with either eGFP-tagged hKCa3.1 (KCa3.1-tg cells) or eGFP-tagged luciferase (Luc-tg cells) as a control. As confirmed by the expression of eGFP, KCa3.1-tg cells showed a high level of KCa3.1 mRNA and protein expression as compared with Luc-tg cells (Fig. 7A). BrdU incorporation assay performed in the absence of PDGF revealed that the activity of de novo DNA synthesis in KCa3.1-tg cells was higher than those in nontransduced and Luc-tg cells (Fig. 7B). PDGF-induced proliferation was also enhanced in KCa3.1-tg cells (Fig. 7B). The levels of phosphorylated CREB, c-Fos, and NOR-1 were higher in KCa3.1-tg cells than in Luc-tg cells before, and 1 h after, exposure to PDGF (Fig. 7C). These data confirm that KCa3.1 regulates PDGF-dependent signaling pathways of VSMC proliferation.

DISCUSSION

The major novel findings of this study are three-fold. First, both pharmacological blockade and gene knockdown of KCa3.1 inhibit HCSMC proliferation, whereas KCa3.1 overexpression has the opposite effect. Second, pharmacological KCa3.1 stimulation unexpectedly but markedly attenuates HCSMC proliferation by diminishing the expression of KCa3.1 and PDGF β-receptors and by inhibiting further dedifferentiation and cell cycle progression. Third, PDGF-induced HCSMC proliferation is associated with KCa3.1-dependent regulation of the rise in [Ca²⁺]i and the subsequent expression of transcription factors and cyclins that orchestrate cell cycle progression. Taken together, these findings suggest that KCa3.1 plays a key role in the regulation of VSMC proliferation.

KCa3.1 regulates the proliferation of a variety of cell types, including VSMCs. Previous studies have demonstrated that KCa3.1 regulates cell membrane potential and Ca²⁺ influx, and its blockade or knockdown inhibits the proliferation of B- and T-cells (6, 7), airway smooth muscle cells (4), fibroblasts (8), cancer cells (10), and VSMCs (3, 12, 13, 18, 21). Moreover, in stem cells, KCa3.1 regulates the cell cycle progression via cyclins D1 and E (9). However, the mechanism by which KCa3.1 regulates cell proliferation in many cell types is still not well understood.

Ca²⁺ is a major second messenger for a variety of cell activation processes. PDGF binds to and activates its specific tyrosine kinase receptors, leading to a sustained increase in [Ca²⁺], in VSMCs (31). Here, PDGF-induced HCSMC proliferation was suppressed by chelating [Ca²⁺], with BAPTA and augmented by a forced rise in [Ca²⁺], with A23187. These results are consistent with the effects of BAPTA and A23187 on PDGF-induced VSMC chemotaxis (31), indicating an essential role for [Ca²⁺], in the regulation of PDGF-induced VSMC activation. It
is widely accepted that in a variety of nonexcitable cells, KCa3.1 is activated by a small rise in [Ca^{2+}]_i (100 nM) following Ca^{2+} release from intracellular stores and subsequent Ca^{2+} influx. The resultant K^+ efflux causes membrane hyperpolarization that maintains Ca^{2+} entry by increasing an electrical gradient, thereby playing a role in the activation of Ca^{2+}-dependent signal pathways (38). In VSMCs, an increase in [Ca^{2+}]_i activates specific signaling pathways. CREB, a mitogen-induced transcriptional factor, is phosphorylated in response to a rise in [Ca^{2+}]_i and subsequently induces mitogenic immediate early genes such as c-fos and NOR-1 (32–34). In the present study, blockade or siRNA-mediated targeting of KCa3.1 suppressed PDGF-induced HCSMC proliferation, concomitantly with an inhibition of the rise in [Ca^{2+}]_i, CREB phosphorylation, and immediate early gene expression, and these Effects were abolished by a forced rise in [Ca^{2+}]_i with A23187. In contrast, KCa3.1 overexpression induced and enhanced VSMC proliferation by activating these signaling pathways. KCa3.1 is associated with VSMC proliferation to a variety of mitogens in different species (3, 12, 39). Taken together, these data indicate that VSMC proliferation is associated with KCa3.1-dependent regulation of Ca^{2+}-dependent signaling pathways.

EBIO at concentrations relatively specific to KCa3.1 (26) in HCSMCs (which predominantly express KCa3.1 but not small conductance KCa2 channels (3)) also suppressed PDGF-induced rise in [Ca^{2+}]_i, proliferation, and migration, accompanied by lower expression and activity of KCa3.1 and PDGF receptors on the membrane. Although TRAM34 did not change the expression pattern of these markers, KCa3.1 activators abolished the expression of PDGF β-receptors concomitantly with the suppression of PDGF-induced alteration in the expression of VSMC phenotypic markers. C. A23187 had no effect on EBIO-induced inhibition of VSMC proliferation. *, p < 0.05 versus control, †, p < 0.05 versus PDGF alone, and ‡, p < 0.05 versus PDGF + EBIO.
causes an excess of $\text{Ca}^{2+}$ entry that favors cell differentiation rather than proliferation (43). 3) Consistent with a study in HaCaT keratinocyte and C6 glioma cell lines where EBIO also inhibited proliferation (42), an incisive general mechanism of negative feedback abolishes KCa3.1 expression, leading to lowered $[\text{Ca}^{2+}]_i$, and thereby attenuated proliferation during the prolonged stimulation. It is unlikely that these activators nonspecifically evoke these effects, independently of KCa3.1 stimulation, because the morphological changes induced by EBIO were suppressed by TRAM-34; SKA-16, an inactive analog of SKA-31, had no effects; and KCa3.1-deficient VSMCs exhibit an almost complete loss of proliferative response to PDGF (3). As compared with TRAM-34, EBIO treatment resulted in a stronger inhibition of PDGF-induced signaling pathways and cell cycle progression, with a lesser induction of cyclins. This effect could be explained by the fact that KCa3.1 stimulation abolished the expression of PDGF $\beta$-receptors, whereas TRAM-34 had no additive effect to the PDGF-induced down-regulation of this receptor (36). Interestingly, the KCa3.1 activators were also more effective at reducing PDGF-induced increases in KCa3.1 expression. In addition, a forced rise in $[\text{Ca}^{2+}]_i$ with A23187 restored the proliferative response in KCa3.1 blocker-treated VSMCs (where $[\text{Ca}^{2+}]_i$ levels were lowered but the expression of PDGF $\beta$-receptors was unaltered) but not in KCa3.1 activator-treated cells (where both $[\text{Ca}^{2+}]_i$ levels and PDGF $\beta$-receptor expression were reduced). Therefore, the abolishment of PDGF $\beta$-receptor expression is an additional mechanism for diminished responsiveness to PDGF in KCa3.1 activator-treated VSMCs. Indeed, we show here that treatment with KCa3.1 activators prevents PDGF-induced up-regulation of $\gamma$-caldesmon and down-regulation of calponin-1. Because the mechanisms of PDGF-induced KCa3.1 gene activation in VSMCs remain to be determined, the precise mechanism by which KCa3.1 activators abolish KCa3.1 gene expression needs further investigation.

Cyclins bound to specific cyclin-dependent kinases control cell cycle progression; in particular, cyclin D1 mainly controls G1 phase, cyclins A and E control S phase, whereas cyclins A and B1 control mitotic phase (44). In addition, a rise in $[\text{Ca}^{2+}]_i$, phosphorylated CREB, and immediate early genes play important roles in the expression and activation of cyclins, in the induction of resting cell ($G_0$) reentry into the cell cycle, in DNA synthesis at $G_1$/S transition, and in mitosis at $G_2$/M transition in a cooperative fashion (45–47). In this study, TRAM-34 inhibited PDGF-induced expression of cyclins A and B1, but not D1 and E, along with a reduction in the rise in $[\text{Ca}^{2+}]_i$, CREB phosphorylation, and c-Fos and NOR-1 expression. This effect is consistent with the finding that TRAM-34 inhibited FBS-induced cell cycle progression at transitions from G1 to S and G2 to M phases. In contrast, EBIO more effectively inhibited FBS-induced cell cycle progression at the transition from G1 to S phase, concomitantly with a stronger inhibition in PDGF-induced expression of c-Fos and cyclins A, B1, D1, and E, indicating that KCa3.1 stimulation could prevent the reentry of resting cells ($G_0$) into the cell cycle.

PDGF induces VSMC elongation and thinning through the migratory machinery. Our previous observation that blockade of KCa3.1 inhibits PDGF-induced VSMC migration (3) is consistent with the present study showing that TRAM-34 inhibits PDGF-induced cell elongation and thinning, whereas KCa3.1 stimulation enhances these changes in a TRAM-34-sensitive manner. Thus, KCa3.1 may play a crucial role in the regulation of protrusive activity. Other possible mechanisms include the contribution of KCa3.1 to cell volume regulation that modulates morphological changes and the proliferative response (48, 49). Furthermore, in contrast to the effects of TRAM-34, a
forced rise in \([\text{Ca}^{2+}]_i\) with A23187 failed to suppress the inhibitory effect of EBI0 on proliferation. KCa3.1 stimulation decreased \(l\)-caldesmon and PDGF \(\beta\)-receptor expression, VSMC dedifferentiation markers, and increased calponin-1 expression, a differentiation marker. These effects were accompanied by a strong suppression of KCa3.1 expression, the appearance of a spindle-like shape, and the suppression of signaling pathways and cyclin expression. Thus, the effects of KCa3.1 stimulation cannot be explained only by their direct effects on channel function, implying a possible link between KCa3.1 and VSMC differentiation/dedifferentiation genes.

KCa3.1 overexpression induced VSMC proliferation in the absence of PDGF via signaling pathways similar to those activated by this growth factor. Moreover, KCa3.1 overexpression also enhanced the response to PDGF. Similar enhanced cell proliferation has been reported for the \textit{ether-a-go-go} \(K^+\) channel (Kv10.1), which also regulates \(\text{Ca}^{2+}\) influx and is expressed in up to 70% of human cancers (50). Possible mechanisms include: 1) increased channel expression may cause membrane hyperpolarization and \(\text{Ca}^{2+}\) influx through nonspecific cation channels, leading to CREB-dependent activation of mitogenic genes (33), and 2) KCa3.1 may be increased in intracellular organelles such as mitochondria, and may maintain the driving force for the store \(\text{Ca}^{2+}\) efflux with counter-influx of \(K^+\) (51).

In summary, KCa3.1 blockade or knockdown reduces PDGF-induced VSMC proliferation via inhibiting \(\text{Ca}^{2+}\)-dependent signaling pathways and cell cycle progression, whereas KCa3.1 overexpression has the opposite effects. Pharmacological KCa3.1 stimulation suppresses these responses more markedly via abolishing KCa3.1 and PDGF \(\beta\)-receptor expression. Therefore, targeting KCa3.1 with specific activators, in addition to blockers and gene therapy, may constitute a new therapeutic approach for the prevention of diseases with increased cell proliferative activity such as atherosclerosis.

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