

Plasma Membrane Calcium ATPase Isoform 4 Inhibits Vascular Endothelial Growth Factor–Mediated Angiogenesis Through Interaction With Calcineurin

Rhiannon R. Baggott, Arantzazu Alfranca, Dolores López-Maderuelo, Tamer M.A. Mohamed, Amelia Escolano, Jorge Oller, Beatriz C. Ornes, Sathishkumar Kurusamy, Farjana B. Rowther, James E. Brown, Delvac Oceandy, Elizabeth J. Cartwright, Weiguang Wang, Pablo Gómez-del Arco, Sara Martínez-Martínez, Ludwig Neyses, Juan Miguel Redondo, Angel Luis Armesilla

Objective—Vascular endothelial growth factor (VEGF) has been identified as a crucial regulator of physiological and pathological angiogenesis. Among the intracellular signaling pathways triggered by VEGF, activation of the calcineurin/nuclear factor of activated T cells (NFAT) signaling axis has emerged as a critical mediator of angiogenic processes. We and others previously reported a novel role for the plasma membrane calcium ATPase (PMCA) as an endogenous inhibitor of the calcineurin/NFAT pathway, via interaction with calcineurin, in cardiomyocytes and breast cancer cells. However, the functional significance of the PMCA/calcineurin interaction in endothelial pathophysiology has not been addressed thus far.

Approach and Results—Using in vitro and in vivo assays, we here demonstrate that the interaction between PMCA4 and calcineurin in VEGF-stimulated endothelial cells leads to downregulation of the calcineurin/NFAT pathway and to a significant reduction in the subsequent expression of the NFAT-dependent, VEGF-activated, proangiogenic genes *RCANI.4* and *Cox-2*. PMCA4-dependent inhibition of calcineurin signaling translates into a reduction in endothelial cell motility and blood vessel formation that ultimately impairs in vivo angiogenesis by VEGF.

Conclusions—Given the importance of the calcineurin/NFAT pathway in the regulation of pathological angiogenesis, targeted modulation of PMCA4 functionality might open novel therapeutic avenues to promote or attenuate new vessel formation in diseases that occur with angiogenesis. (*Arterioscler Thromb Vasc Biol.* 2014;34:2310-2320.)

Key Words: angiogenesis effect ■ calcium ■ calcineurin ■ nuclear factors of activated T cells ■ plasma membrane calcium–transporting ATPase

Angiogenesis, the formation of new blood capillaries from pre-existing vessels, is a physiological process essential for proper embryonic development, organ growth, and tissue repair.¹ Blood vessels respond to a tightly regulated interplay of pro- and antiangiogenic factors to ensure proper vascular remodeling during development, wound healing, and pregnancy.¹ Abnormal growth of blood vessels, caused by deregulation of this process, plays a critical role in the pathophysiology of several human diseases, including tumor growth and metastasis, proliferative retinopathies, age-related

macular degeneration, neovascular glaucoma, ischemic disorders, and rheumatoid arthritis.² The proangiogenic factor vascular endothelial growth factor (VEGF) and its tyrosine-kinase receptor, VEGF-R, have been identified as crucial regulators of both physiological and pathological angiogenesis.³ VEGF activates several intracellular signal transduction pathways that trigger the angiogenic process. Among them, the calcineurin/nuclear factor of activated T cells (NFAT) signaling axis has emerged as a critical mediator of VEGF-stimulated angiogenesis.⁴⁻⁷

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From the Molecular Pharmacology Group, School of Pharmacy (R.R.B., S.K., A.L.A.), Brain Tumor UK Neuro-oncology Research Centre (F.B.R.), and Oncology Group (W.W.), Research Institute in Healthcare Science, Faculty of Science and Engineering, University of Wolverhampton, Wolverhampton, United Kingdom; Department of Vascular Biology and Inflammation, Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain (A.A., D.L.-M., A.E., J.O., B.C.O., P.G.-d.A., S.M.-M., J.M.R.); Human Genetics Department, Institute for Rare Diseases Research, Carlos III Health Institute, Madrid, Spain (A.A.); Institute of Cardiovascular Sciences, University of Manchester, Manchester Academic Health Sciences Centre, Manchester, United Kingdom (T.M.A.M., D.O., E.J.C., L.N.); Department of Biochemistry, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt (T.M.A.M.); Aston Research Centre for Healthy Ageing, School of Life and Health Sciences, Aston University, Birmingham, United Kingdom (J.E.B.); Department of Molecular Biology, Universidad Autónoma de Madrid, Madrid, Spain (P.G.-d.A.); and University of Luxembourg, Walferdange, Luxembourg (L.N.).

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Correspondence to Juan Miguel Redondo, PhD, Department of Vascular Biology and Inflammation, Centro Nacional de Investigaciones Cardiovasculares, Melchor Fernandez Almagro 3, E-28029 Madrid, Spain, E-mail jmredondo@cnic.es or Angel Luis Armesilla, PhD, Molecular Pharmacology Group, Research Institute in Healthcare Science, School of Pharmacy, Faculty of Science and Engineering, University of Wolverhampton, Wulfruna St, Wolverhampton WV1 1SB, United Kingdom, E-mail A.Armesilla@wlv.ac.uk

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Nonstandard Abbreviations and Acronyms	
CsA	cyclosporine A
Erk1/2	extracellular signal-regulated kinases 1 and 2
HUVEC	human umbilical vein endothelial cell
MLEC	mouse lung endothelial cell
NFAT	nuclear factor of activated T cells
PMA	phorbol 12-myristate 13-acetate
PMCA	plasma membrane calcium ATPase
VEGF	vascular endothelial growth factor

Calcineurin is a serine/threonine phosphatase activated in response to increments in the intracellular calcium concentration.⁸ The best characterized substrate of calcineurin is the NFAT family of transcription factors.⁹ NFATs are synthesized as constitutively phosphorylated proteins that reside in the cytoplasm of the cell. In response to VEGF stimulation, calcineurin mediates dephosphorylation of NFATs, promoting their translocation into the nucleus.^{4,6,7} Once in the nucleus, activated NFATs trigger the expression of endothelial target genes that participate in the formation of new blood vessels.⁴⁻⁷ The relevance of calcineurin/NFAT signaling to the progression of pathological angiogenesis highlights the clinical potential of this route for the design of therapeutic interventions aimed

at the treatment of neovascular-associated human diseases. Inhibition of calcineurin activity with the immunosuppressant drug cyclosporine A (CsA) has been shown to significantly decrease VEGF-mediated angiogenesis in animal models.⁵ However, long-term treatments with CsA are associated with undesirable side effects in patients precluding its use to treat chronic diseases including diabetic retinopathies and cancer.¹⁰ It is therefore essential to characterize endogenous cellular inhibitors of calcineurin that are less toxic and could be more suitable for chronic inhibition of this pathway. Toward this goal, we have identified recently a novel inhibitory interaction between endogenous plasma membrane calcium ATPase (PMCA) proteins and calcineurin.^{11,12}

PMCA is a high-affinity calcium pump that extrudes calcium from the cytosol to the extracellular medium.¹³ In humans, PMCA proteins are encoded by 4 independent genes termed PMCA1 to 4. PMCA1 and 4 are expressed ubiquitously, whereas the expression of PMCA2 and 3 is restricted to specific cells and tissues.¹³ It is thought that PMCA proteins inhibit the activity of calcium-dependent calcineurin by tethering it to small low-calcium microdomains created by the calcium extrusion activity of the pump.¹⁴ We previously reported that PMCA proteins and calcineurin interact in endothelial cells¹⁵ although the functional significance of this interaction in endothelial pathophysiology has not been described thus far.

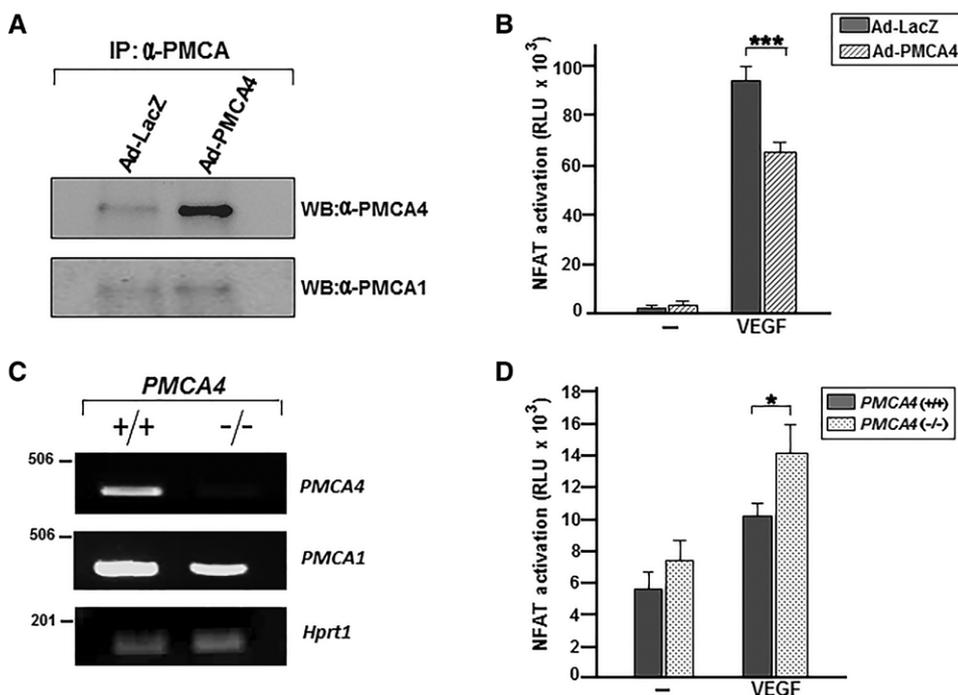


Figure 1. Plasma membrane calcium ATPase 4 (PMCA4) downregulates the activity of the calcineurin/nuclear factor of activated T cells (NFAT) pathway in endothelial cells.

A, Ectopic expression of human PMCA4 in human umbilical vein endothelial cells (HUVECs) by infection with adenovirus Ad-PMCA4. Protein lysates from 2.5×10^6 HUVECs infected with the indicated adenovirus (multiplicity of infection [MOI]=50) were immunoprecipitated with the anti-pan PMCA 5F10 monoclonal antibody. PMCA4 and PMCA1 proteins in the immunoprecipitated complexes were detected by Western blot using anti-PMCA4-specific (WB: α -PMCA4) or anti-PMCA1-specific (WB: α -PMCA1) antibodies, respectively. The figure shows a representative experiment of 3 performed. **B**, Ectopic expression of PMCA4 reduces the activity of the calcineurin/NFAT pathway. HUVECs (3×10^5) were coinfecting with either Ad-LacZ

or Ad-PMCA4 (MOI=50) for 24 hours, and then with Ad-NFAT-Luc (MOI=25) for an additional 24 hours. Infected cells were serum-starved in medium containing 0.5% fetal calf serum and left unstimulated (-) or treated with vascular endothelial growth factor (VEGF; 25 ng/mL) for 6 hours (VEGF). Mean \pm SE of 5 independent experiments are shown. *** indicates statistically significant differences ($P \leq 0.005$, according to Student *t* test) when comparing VEGF-stimulated HUVEC infected with Ad-LacZ vs those infected with Ad-PMCA4. **C**, Absence of PMCA4 expression in mouse lung endothelial cells (MLECs) isolated from PMCA4 knockout mice. A total of 0.5 μ g of total RNA, isolated from MLECs obtained from PMCA4 wild-type (+/+) or knockout (-/-) mice, was retro-transcribed and the expression of the mouse PMCA4 and PMCA1 genes analyzed by semiquantitative polymerase chain reaction. Amplification of the housekeeping gene *Hprt1* was used as a control. A figure representative of 3 independent experiments is shown. **D**, Targeted disruption of PMCA4 increases the activity of the calcineurin/NFAT pathway in MLECs. MLECs (3×10^5) isolated from PMCA4 wild-type (+/+) or knockout (-/-) animals were infected with Ad-NFAT-Luc (MOI=50) for 48 hours. After serum starvation in 0.5% fetal calf serum, cells were left unstimulated (-) or treated with VEGF (50 ng/mL) for 6 hours (VEGF) and luciferase activity was measured. Mean \pm SE of 5 independent experiments are shown. * indicates statistically significant differences ($P \leq 0.05$, according to Student *t* test) when comparing VEGF-stimulated PMCA4 (+/+) vs (-/-) MLECs.

In this work, we demonstrate that the interaction between PMCA4 and calcineurin impairs endothelial cell motility and blood vessel formation in response to VEGF stimulation. An inhibitory effect of PMCA4 on VEGF-mediated activation of the calcineurin/NFAT signaling pathway, and the subsequent downregulation of RCAN1.4 and Cox-2 gene expression, is likely to account for angiogenesis inhibition.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

PMCA4 Negatively Regulates the Calcineurin/NFAT Pathway in VEGF-Activated Endothelial Cells

The role of PMCA as an inhibitor of the calcineurin/NFAT pathway in breast cancer cells¹² and cardiomyocytes¹⁶ encouraged us to investigate whether this was also the case in VEGF-activated endothelial cells.

We ectopically expressed human PMCA4 in human umbilical vein endothelial cell (HUVEC) primary endothelial cells by

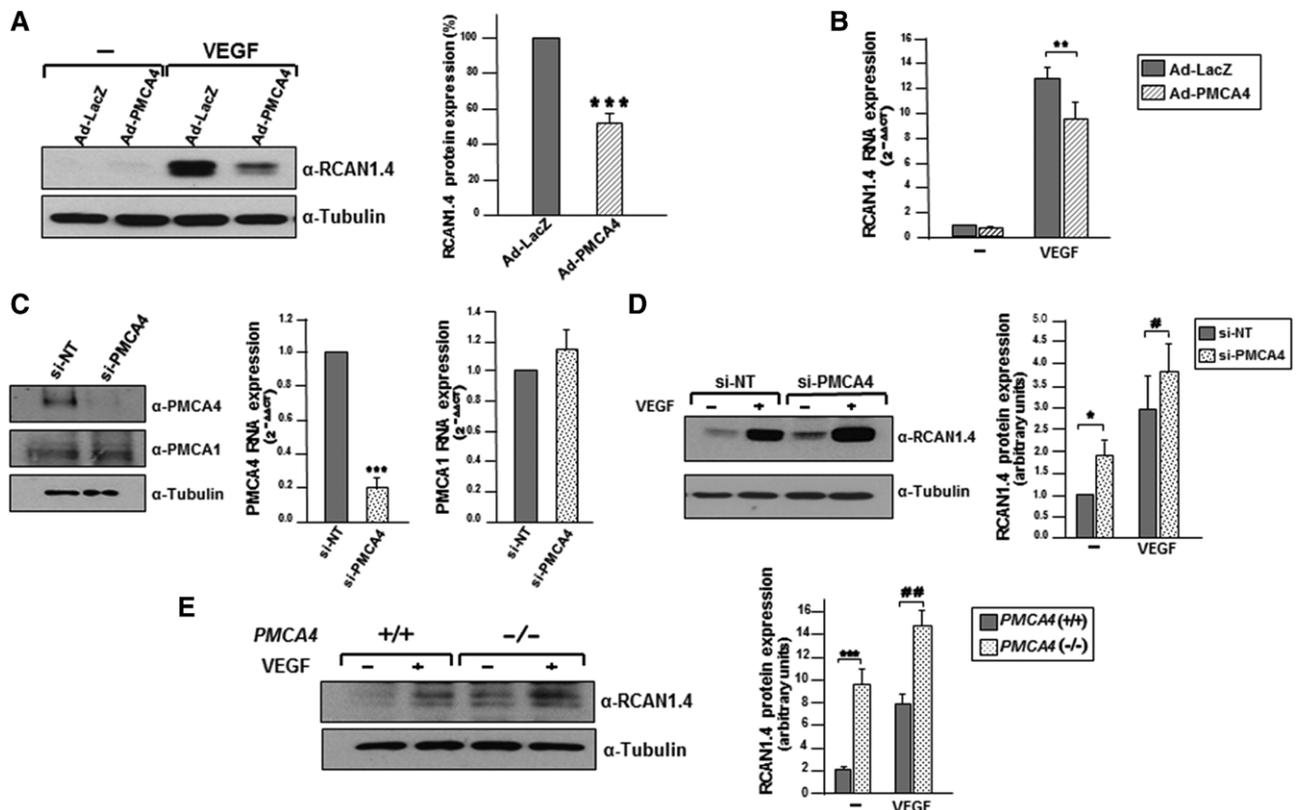


Figure 2. Plasma membrane calcium ATPase 4 (PMCA4) negatively regulates the vascular endothelial growth factor (VEGF)-mediated upregulation of the proangiogenic protein RCAN1.4 in endothelial cells. **A**, Overexpression of human PMCA4 attenuates the VEGF-induced upregulation of RCAN1.4 protein. Protein lysates obtained from human umbilical vein endothelial cell (HUVEC)-infected cells either unstimulated (-) or after stimulation with VEGF (25 ng/mL) for 4 hours (VEGF) were analyzed by Western blot with a rabbit anti-DSCR1 antibody (α -RCAN1.4) to determine RCAN1.4 expression. Histograms show data as mean \pm SE of 5 independent experiments. *** indicates statistically significant differences ($P\leq 0.005$, according to Student *t* test) when comparing VEGF-stimulated cells infected with adenovirus Ad-LacZ vs Ad-PMCA4. **B**, PMCA4 overexpression reduces RCAN1.4 gene expression. Infected HUVECs were left unstimulated (-) or stimulated with VEGF (25 ng/mL) for 2 hours. RNA levels for RCAN1.4 were determined by quantitative reverse transcription polymerase chain reaction (PCR) using the 2^{- $\Delta\Delta$ CT} method. Mean \pm SE of 6 independent experiments is shown. ** indicates statistically significant differences ($P\leq 0.01$, according to Student *t* test) when comparing VEGF-stimulated cells infected with Ad-LacZ vs Ad-PMCA4. **C**, Small interfering RNA (siRNA) mediated specific knockdown of PMCA4 in HUVECs. Protein expression of PMCA4 and PMCA1 in HUVECs transfected with a nontargeting control siRNA (si-NT) or a siRNA specific for human PMCA4 (si-PMCA4) was determined by Western blot. Images are representative of 3 independent experiments. Histograms represent RNA levels for PMCA4 and PMCA1 in the transfected cells determined by qRT-PCR. Mean \pm SE of 9 independent experiments is shown. *** indicates statistically significant differences ($P\leq 0.005$, according to Student *t* test) in PMCA4 RNA expression when comparing cells transfected with si-NT vs si-PMCA4. **D**, PMCA4 silencing upregulates the expression of RCAN1.4. Western blot analyses of RCAN1.4 expression (α -RCAN1.4) in protein lysates of HUVECs transfected with a PMCA4-specific siRNA (si-PMCA4) or a nontargeting, control siRNA (si-NT). Cells were stimulated with VEGF (25 ng/mL) for 4 hours where indicated. Histograms show mean \pm SE of RCAN1.4 protein expression calculated from 3 independent experiments. * indicates statistically significant differences ($P\leq 0.05$, according to Student *t* test) when comparing HUVEC cells transfected with si-NT vs si-PMCA4. # indicates statistically significant differences ($P\leq 0.05$, according to Student *t* test) when comparing VEGF-stimulated HUVECs transfected with si-NT vs si-PMCA4. **E**, Targeted disruption of PMCA4 increases RCAN1.4 protein expression. Western blot analysis of RCAN1.4 expression (α -RCAN1.4) in mouse lung endothelial cells (MLECs) isolated from PMCA4 wild-type (+/+) or knockout (-/-) mice. Where indicated, MLECs were stimulated with VEGF (50 ng/mL) for 4 hours. Histogram shows mean \pm SE of 3 independent experiments performed with 3 different batches of MLEC cells obtained from independent isolations. *** indicates statistically significant differences ($P\leq 0.005$, according to Student *t* test) when comparing RCAN1.4 protein expression in PMCA4 wild-type vs knockout MLEC. ## indicates statistically significant differences ($P\leq 0.01$, according to Student *t* test) when comparing VEGF-stimulated PMCA4 wild-type vs knockout MLEC. **A to E**, In Western blot assays the expression of tubulin was analyzed using a mouse monoclonal antitubulin antibody (α -Tubulin) as loading control.

infection with adenovirus Ad-PMCA4 (Figure 1A). To quantify the activity of the calcineurin/NFAT pathway, cells were also infected with a luciferase-based, NFAT-dependent adenoviral reporter vector (Ad-NFAT-Luc). Ectopic expression of PMCA4 significantly decreased (31.9% reduction, compared with cells infected with control Ad-LacZ) the VEGF-induced activation of the luciferase reporter (Figure 1B). Equivalent results were observed when the calcineurin/NFAT pathway was activated by stimulating the infected cells with phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore A23187 (Figure 1A in the online-only Data Supplement).

To substantiate this observation, we analyzed the activity of the calcineurin/NFAT pathway in mouse lung endothelial cells (MLECs) isolated from *PMCA4* knockout mice or from their corresponding wild-type littermates (Figure 1C). MLECs were infected with Ad-NFAT-Luc, and the calcineurin/NFAT pathway was activated by stimulation with VEGF (Figure 1D) or PMA+Io (Figure 1B in the online-only Data Supplement). In both cases, determination of luciferase activity in the infected cells revealed increased activation of the calcineurin/NFAT pathway in *PMCA4*-deficient cells compared with equivalent wild-type cells.

These results demonstrate that *PMCA4* plays a substantial role as an endogenous inhibitor of the calcineurin/NFAT signal transduction pathway in activated endothelial cells.

PMCA4 Inhibits the Expression of NFAT-Dependent, Proangiogenic Genes in VEGF-Stimulated Endothelial Cells

Stimulation of endothelial cells with VEGF has been reported to activate the expression of NFAT-target genes, including *RCAN1.4* and *Cox-2*, whose products actively participate in

the onset of angiogenesis.^{5-7,17-19} The inhibitory effect produced by *PMCA4* on the VEGF-induced activity of the calcineurin/NFAT pathway in endothelial cells prompted us to next examine the role of *PMCA4* as a negative regulator of angiogenesis gene expression.

Ectopic expression of *PMCA4* in HUVECs by infection with Ad-*PMCA4* markedly inhibited the VEGF-dependent upregulation of *RCAN1.4*, both at the level of protein (Figure 2A) and mRNA (Figure 2B) expression (48.4% and 24.8% reduction, respectively). Similarly, VEGF-mediated upregulation of *Cox-2* protein and mRNA was also diminished (52.1% and 29.4% reduction, respectively) by *PMCA4* overexpression (Figure 2A and 2B in the online-only Data Supplement). In contrast, expression of *Cox-1*, which is not regulated by the calcineurin/NFAT pathway, remained unchanged in *PMCA4*-overexpressing cells (Figure 2A in the online-only Data Supplement), highlighting the role of *PMCA4* as a regulator of NFAT-driven genes.

To corroborate these findings, we next measured angiogenesis gene expression in endothelial cells in the absence of *PMCA4*. Transfection of HUVEC cells with a small interfering RNA pool directed to *PMCA4* robustly silenced the expression of endogenous *PMCA4* at the protein and mRNA level, without affecting *PMCA1* expression (Figure 2C). *PMCA4* silencing increased the expression of *RCAN1.4* in both resting and VEGF-stimulated cells (1.9- and 3.86-fold increase, respectively, compared with a nontargeting small interfering RNA pool; Figure 2D). Similarly, small interfering RNA-mediated suppression of *PMCA4* in HUVEC cells significantly increased the expression of the proangiogenic protein *Cox-2* in VEGF-stimulated cells (Figure 2E in the online-only Data Supplement). Consistent with these results, analysis

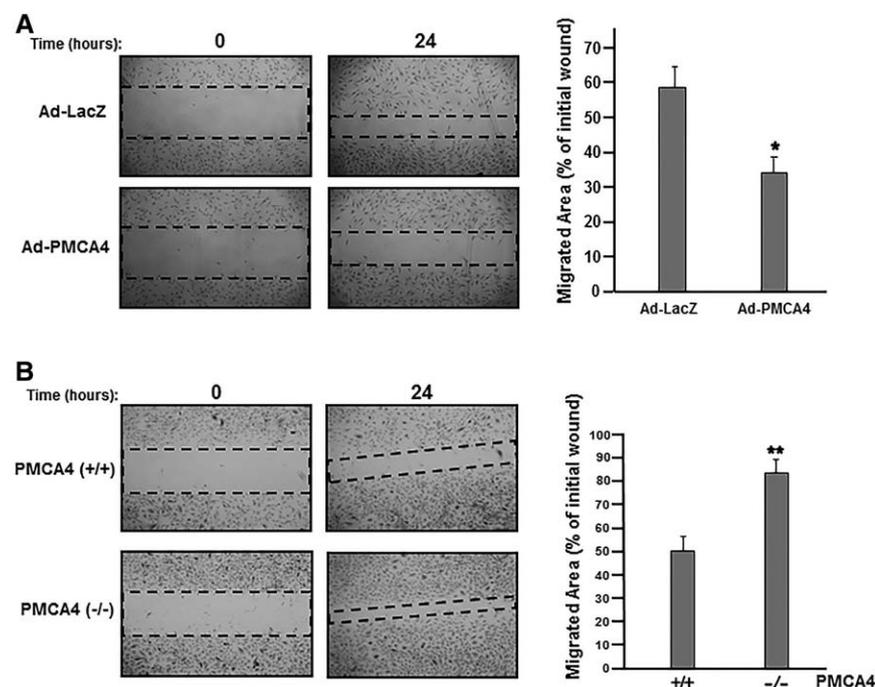


Figure 3. Plasma membrane calcium ATPase 4 (*PMCA4*) inhibits endothelial cell migration. **A**, Overexpression of *PMCA4* significantly reduces endothelial cell migration. Representative images of wound-healing migration assays performed with human umbilical vein endothelial cells infected with adenovirus Ad-*PMCA4* or Ad-LacZ at multiplicity of infection=50. Mean±SE of 6 independent experiments is shown. * indicates statistically significant differences ($P<0.05$, according to Student *t* test) when comparing the migration of cells infected with Ad-LacZ vs Ad-*PMCA4*. **B**, Targeted disruption of *PMCA4* increases endothelial cell migration. Representative images of wound-healing assays performed with MLECs isolated from wild-type (+/+) or *PMCA4* knockout animals (-/-). Histogram shows mean±SE of 4 independent experiments. ** indicates statistically significant differences ($P<0.01$, according to Student *t* test) when comparing the migration of *PMCA4* wild-type vs *PMCA4* knockout MLECs. In both cases (**A** and **B**) images were taken at time zero and after incubation for 24 hours in the corresponding complete medium supplemented with vascular endothelial growth factor. The

migrated area was calculated by subtracting the value of the nonmigrated area to the area of the wound at time zero. The migrated area is expressed as a percentage of the total area at time zero.

of *PMCA4*^{-/-} MLECs revealed higher levels of RCAN1.4 and Cox-2 protein, both in resting and VEGF-treated conditions, than those found in equivalent wild-type cells (Figure 2E; Figure IIIB in the online-only Data Supplement).

Taken together, these data indicate that inhibition of calcineurin/NFAT signaling by PMCA4 results in a downregulation of VEGF-induced RCAN1.4 and Cox-2 gene expression.

PMCA4 Reduces Endothelial Cell Migration and Blood Vessel Formation but Not Cell Proliferation

Blockage of the calcineurin/NFAT pathway by pharmacological doses of CsA has been shown to abrogate VEGF-driven endothelial cell migration, but not endothelial proliferation.⁵ We thus performed wound-healing migration assays with cells overexpressing or lacking PMCA4 to investigate whether PMCA4 might play a role in the regulation of endothelial cell migration. Notably, Ad-PMCA4-infected HUVECs had a reduced ability to migrate into the wound compared with control-infected cells (39.8% reduction; Figure 3A). Conversely, *PMCA4*^{-/-} MLECs displayed significantly increased cellular motility (66.6% increment over equivalent wild-type cells; Figure 3B). These results imply that PMCA4 plays a negative role in the regulation of endothelial cell migration.

As VEGF-induced endothelial cell proliferation is an additional essential step in the progression of angiogenesis, we next evaluated whether PMCA4 participates in the regulation of this process. No significant differences in proliferation were found between PMCA4-expressing cells and control Ad-LacZ-infected cells (Figure 4A). Because VEGF-dependent proliferation of endothelial cells requires activation of the mitogen-activated protein kinase kinase (MEK)-extracellular signal-regulated kinases 1 and 2 (Erk) signaling pathway,²⁰ we also tested whether PMCA4 modulates activation of Erk in HUVECs. Results showed that VEGF stimulation increased phosphorylation (activation) of Erk1/2 proteins to a similar extent in cells overexpressing either PMCA4b or β -galactosidase (Figure 4B). Consistent with this finding, HUVECs silenced for PMCA4 expression displayed similar levels of Erk1/2 phosphorylation to control (nontargeting small interfering RNA pool) cells after VEGF stimulation (Figure 4C). Taken together, these results indicate that PMCA4 does not participate in the regulation of endothelial cell proliferation.

Given that PMCA4 controls endothelial cell migration, we next addressed whether PMCA4 participates in the regulation of endothelial tube formation. Compared with control-infected cells, basal tubular morphogenesis in an in vitro Matrigel assay was reduced significantly (34%) in HUVECs

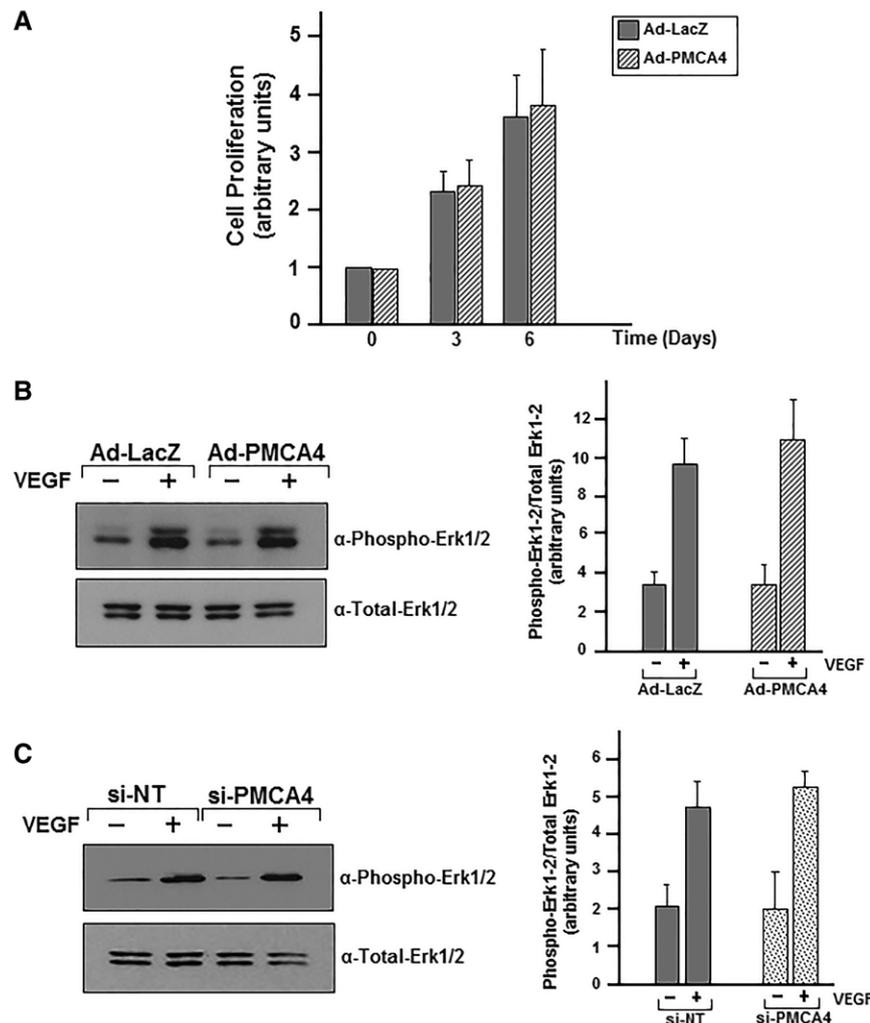


Figure 4. Plasma membrane calcium ATPase 4 (PMCA4) does not regulate endothelial cell proliferation. **A**, Human umbilical vein endothelial cells (HUVECs; 2×10^3) infected with adenovirus Ad-PMCA4 or Ad-LacZ were plated on 96-well plates. The number of viable cells was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) after overnight incubation ($t=0$) and after 3 and 6 days of incubation in complete endothelial cell growth medium supplemented with vascular endothelial growth factor (VEGF; 25 ng/mL). Histograms represent mean \pm SE of 6 independent experiments. **B** and **C**, Western blot analysis of the phosphorylation (activation) status of Erk1/2 proteins using an anti-phospho-extracellular signal-regulated kinases 1 and 2 (Erk1/2)-specific antibody (α -Phospho-Erk1/2) in HUVECs overexpressing (Ad-PMCA4) or deficient (si-PMCA4) in PMCA4 expression. Cells were stimulated with VEGF (25 ng/mL) for 5 minutes where indicated. Histograms represent quantification of Erk1/2 phosphorylation with respect to the levels of total Erk1/2 in PMCA4-overexpressing (**B**) or PMCA4-deficient (**C**) cells, compared with the values observed in the corresponding control cells infected with Ad-LacZ or transfected with nontargeting control small interfering RNA (si-NT), respectively. Histograms show mean \pm SE of ≥ 3 independent experiments.

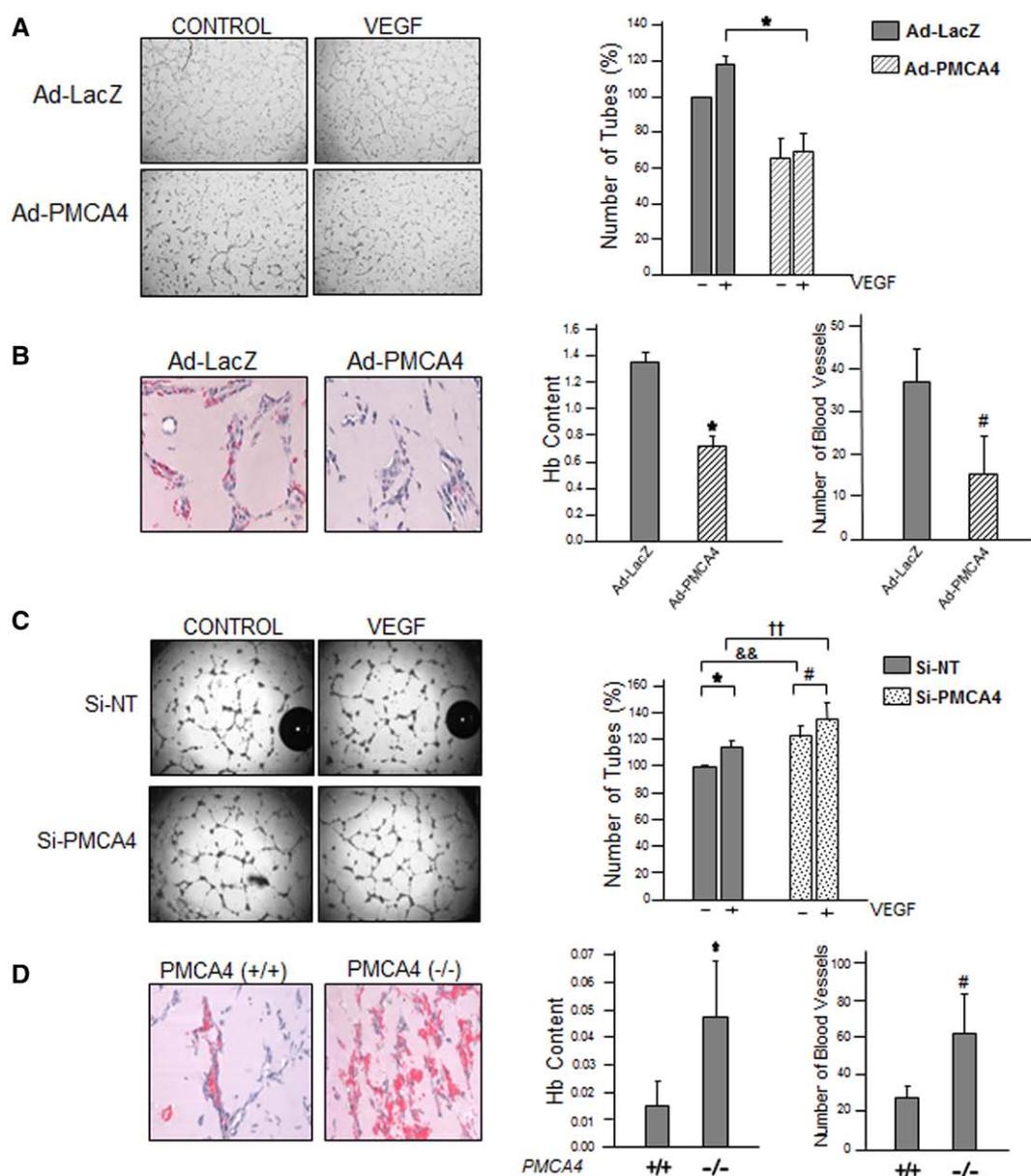


Figure 5. Plasma membrane calcium ATPase 4 (PMCA4) attenuates vascular endothelial growth factor (VEGF)-induced angiogenesis. **A**, PMCA4 overexpression in endothelial cells blocks in vitro tube formation in response to VEGF stimulation. Human umbilical vein endothelial cells (HUVECs) infected with adenovirus as indicated were plated on a layer of Growth-Factor Reduced Matrix (Geltrex) in Medium 200 containing 2% fetal calf serum, and where indicated VEGF (25 ng/mL), and incubated for 24 hours. Images show representative fields from experiments quantified in the histogram. Histogram represents mean \pm SE of 5 independent experiments. * indicates statistically significant differences ($P\leq 0.05$, according to Student *t* test) when comparing tube formation in VEGF-stimulated cells infected with Ad-LacZ vs those infected with Ad-PMCA4. **B**, PMCA4 overexpression attenuates in vivo tube formation. A mixture of Growth-Factor Reduced Matrigel (BD Bioscience) containing VEGF (500 ng/mL), heparin (376 μ g/mL), and 2×10^9 plaque-forming units of the corresponding adenovirus was implanted subcutaneously in mice, and 7 days later implants were collected and analyzed. Hemoglobin (Hb) content was measured spectrophotometrically. The number of blood vessels in each implant was determined in hematoxylin-eosin (H&E)-stained sections of the plug. Histogram of Hb content shows mean \pm SE of 3 independent experiments with ≥ 7 animals per group in each experiment. Pictures show representative fields of H&E-stained sections of plugs. The number of vessel containing blood cells per field (magnification $\times 20$) was quantified in 10 random fields per sample. Histogram depicting number of blood vessels represents mean \pm SE of values calculated from 8 different plugs per group, obtained from 3 independent experiments. * and # indicate statistically significant differences ($P\leq 0.05$, according to Student *t* test) in Hb content and number of blood vessels, respectively, when comparing Matrigel plugs containing Ad-LacZ vs those containing Ad-PMCA4. **C**, Knockdown of PMCA4 expression increases the angiogenic ability of endothelial cells. Matrigel in vitro tube formation assays of HUVECs transfected with a small interfering RNA (siRNA) specific for PMCA4 (si-PMCA4) or with control nontargeting siRNA (si-NT) were performed as described in **A**. Images show representative fields from experiments quantified in the histogram. Histogram represent mean \pm SE of 7 independent experiments. * and # indicate statistically significant differences in tube formation ($P\leq 0.05$, according to Student *t* test) in cells transfected with si-NT or si-PMCA4, respectively, when comparing nontreated vs VEGF-stimulated cells. && and $\dagger\dagger$ indicate statistically significant differences in tube formation ($P\leq 0.01$, according to Student *t* test) in resting or VEGF-stimulated HUVEC cells, respectively, when comparing cells transfected with si-NT vs those transfected with si-PMCA4. **D**, Targeted disruption of PMCA4 enhances endothelial tube formation in vivo. Matrigel assays were as in **B** only that no adenovirus was included in the Matrigel mixture and the plugs were implanted in 4-week-old PMCA4 wild-type (+/+) or knockout (-/-) mice. (Continued)

overexpressing PMCA4 (Figure 5A). Moreover, the additional VEGF-mediated tube formation was also clearly reduced by PMCA4 overexpression (Figure 5A), suggesting that PMCA4 negatively regulates endothelial tube formation. To validate this observation in a more physiological setting, we performed *in vivo* angiogenesis assays in mice by subcutaneous injection of a mixture of low growth-factor Matrigel, VEGF, and an adenoviral vector (either Ad-PMCA4 or control Ad-LacZ). Consistent with our *in vitro* findings, Matrigel plugs containing Ad-PMCA4 produced fewer vessels (58.2% reduction) and had lower hemoglobin content (47.1% decrease) than control implants (Figure 5B), indicating that ectopic expression of PMCA4 impairs the angiogenic capability of endothelial cells *in vivo*.

To verify that our observations were not merely an effect of PMCA4 overexpression, we next assessed the involvement of endogenous PMCA4 in the regulation of angiogenesis. Knockdown of PMCA4 expression significantly increased HUVEC tubular morphogenesis, in both basal and VEGF-stimulated conditions (23.07% and 22.05% increase, respectively, compared with tube formation of control cells; Figure 5C). Furthermore, vascularization and hemoglobin content of Matrigel plugs injected into *PMCA4*^{-/-} mice was increased 3.1- and 2.3-fold, respectively, compared with plugs implanted in wild-type littermates (Figure 5D). Thus, these data strongly support a negative role for endogenous PMCA4 in blood vessel formation. To further reassure the *in vivo* significance of these observations, we evaluated the role of PMCA4 in blood vessel formation using the mouse hindlimb ischemia model of pathological angiogenesis. We induced unilateral ischemia by femoral artery ligation in the lower limb of PMCA4 wild-type or knockout animals and examined blood flow perfusion of the ischemic limb by laser Doppler perfusion imaging, at 5 and 14 days after surgery. Postischemic perfusion at both times was significantly higher in the ischemic limbs of PMCA4 knockout animals than in those of their wild-type littermates, suggesting that lack of PMCA4 results in a stronger postischemic revascularization (Figure 6). No significant differences in blood flow were detected between the nonischemic contralateral limb of *PMCA4* (+/+) and (-/-) animals.

Altogether, these results demonstrate a novel role for PMCA4 as a negative regulator of angiogenesis *in vivo* and suggest the involvement of PMCA4 in the onset of vascular pathologies that occur with angiogenesis.

Interaction Between PMCA4 and Calcineurin Is Essential for PMCA-Mediated Inhibition of Angiogenesis

We previously reported that PMCA4 exerts its negative effects on the calcineurin/NFAT pathway via interaction with calcineurin A.¹¹ Indeed, disruption of the interaction between endogenous PMCA and calcineurin, by overexpression of

the PMCA-interacting domain, strongly increases the activity of the calcineurin/NFAT pathway in breast cancer cells.²¹ Therefore, we next assessed whether the PMCA4-mediated negative effect on angiogenesis required interaction between PMCA4 and calcineurin.

For this purpose, we disrupted the interaction between endogenous PMCA4 and calcineurin in HUVECs by infection with adenovirus Ad-ID4 that encodes a Flag-tagged region of PMCA4 encompassing amino acids 428 to 651, corresponding to the domain implicated in the interaction with calcineurin¹¹ (Figure IVA in the online-only Data Supplement). Plasma membrane-associated proteins were extracted from infected cells using a ProteoExtract Subcellular Proteome Extraction Kit, and the levels of calcineurin were analyzed by Western blot using a monoclonal anticalcineurin antibody. Overexpression of Flag-PMCA4(428–651) resulted in a significant decrease in the levels of calcineurin associated with the plasma membrane (Figure IVA in the online-only Data Supplement), suggesting that Flag-PMCA4(428–651) was competing with endogenous PMCA4 for binding to calcineurin and, therefore, disrupting the interaction between endogenous PMCA4 and calcineurin. PMCA4 expression in the membrane did not change significantly by infection with Ad-ID4, indicating that the decrease in membrane-associated calcineurin was not a consequence of a reduction in PMCA4 plasma membrane levels after cell infection with Ad-ID4 (Figure IVA in the online-only Data Supplement).

To discard the possibility that the changes observed in the levels of membrane-associated calcineurin were attributable to downregulation of calcineurin expression in Ad-ID4-infected cells, calcineurin total levels were determined by Western blot in proteins from whole-cell lysates. Total levels of calcineurin were equivalent in cells infected with Ad-LacZ or Ad-ID4 (Figure IVA in the online-only Data Supplement). Likewise, Western blot analysis of PMCA4 levels in whole-cell lysates did not show any significant changes in control or Ad-ID4-infected cells (Figure IVA in the online-only Data Supplement).

To quantify the activity of the calcineurin/NFAT pathway in Ad-ID4-transduced HUVECs, cells were further infected with Ad-NFAT-Luc. Measurement of luciferase activity after stimulation of transduced HUVEC with VEGF revealed that Flag-PMCA4(428–651) expression induced a 2.01-fold increase in reporter activity (Figure 7A).

Consistent with the results observed in PMCA4 knockout MLECs, disruption of the PMCA4/calcineurin interaction by expression of Flag-PMCA4(428–651) in HUVEC cells resulted in an increase in the basal (Figure V in the online-only Data Supplement) and VEGF-induced expression of RCAN1.4 (Figure 7B). To confirm that the increase in RCAN1.4 expression was a consequence of increased calcineurin activity, through disruption of its interaction with PMCA4, we repeated this assay after preincubation of

Figure 5. Continued Determination of hemoglobin content and number of blood vessels was as in **B**. Histogram of Hb content shows mean±SE of ≥9 different plugs per group, obtained from 3 independent experiments. Images show representative fields of H&E-stained sections of plugs. Histogram depicting number of blood vessels represents mean±SE of values calculated from ≥5 plugs per group obtained from 3 independent experiments. * and # indicate statistically significant differences (*P*≤0.05, according to Student *t* test) in Hb content and number of blood vessels respectively, when comparing assays performed in PMCA4 wild-type vs knockout mice.

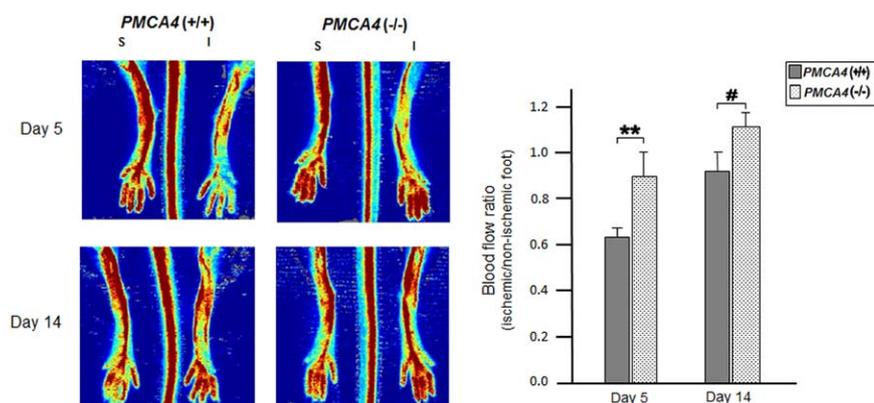


Figure 6. Plasma membrane calcium ATPase 4 (*PMCA4*) gene ablation enhances postischemic hindlimb perfusion. Representative laser Doppler images showing blood flow at 5 and 14 days after surgery, in sham-operated nonischemic legs (S) and femoral-ligated ischemic legs (I) of *PMCA4* knockout (*PMCA4* $-/-$) or wild-type (*PMCA4* $+/+$) mice. Histogram represents mean \pm SE ($n \geq 8$) of blood flow quantification at 5 and 14 days post surgery calculated as ratio of blood flow in ischemic to contralateral foot. ** and # indicate statistically significant differences $P \leq 0.01$ and $P \leq 0.05$, respectively (according to Student *t* test) in foot perfusion when comparing *PMCA4* wild-type vs knockout animals.

HUVECs with the calcineurin-inhibitor CsA before VEGF stimulation. As anticipated, CsA pretreatment abrogated not only the VEGF-mediated upregulation of RCAN1.4 expression but also the additional increase promoted by Flag-*PMCA4*(428–651) (Figure IVB in the online-only Data Supplement). Collectively, these data indicate that disruption of the *PMCA4*/calcineurin interaction in endothelial cells eliminates the inhibitory effect of *PMCA4*, leading to an increase in the activity of the calcineurin/NFAT pathway.

We have shown that overexpression of *PMCA4* in HUVECs significantly attenuates RCAN1.4 expression in response to VEGF stimulation (Figure 2A). To further consider the importance of an intact *PMCA4*/calcineurin interaction for inhibition of angiogenesis by *PMCA4*, we examined whether overexpression of *PMCA4* could downregulate RCAN1.4 expression under conditions that impaired this interaction. Thus, HUVECs were transduced with both Ad-*PMCA4* and Ad-ID4, and RCAN1.4 expression was assessed by Western blot. As expected, disruption of the *PMCA4*/calcineurin interaction, through overexpression of Flag-*PMCA4*(428–651), abolished the *PMCA4*-induced reduction in RCAN1.4 expression (Figure 7C), indicating that this interaction is essential for *PMCA4*-induced inhibition of NFAT-dependent, angiogenic gene expression. In agreement with our previous results illustrating that *PMCA4* is not involved in the regulation of the MEK-Erk1/2 pathway (Figure 4), disruption of the *PMCA4*/calcineurin interaction in HUVECs by infection with Ad-ID4 did not alter the activation (phosphorylation) of endothelial Erk1/2 proteins in response to VEGF stimulation (Figure 7D).

Finally, we evaluated the significance of the *PMCA4*/calcineurin interaction in endothelial cell migration and formation of capillary-like structures. In concurrence with our results in *PMCA4* knockout MLEC (Figure 3B), disruption of the *PMCA4*/calcineurin interaction by overexpression of Flag-*PMCA4*(428–651) augmented endothelial cell motility (Figure 7E). Interestingly, ectopic expression of Flag-*PMCA4*(428–651) significantly increased tube formation in Matrigel assays (even in the absence of VEGF stimulation; Figure 7F) to a degree similar to that previously observed in cells stimulated with VEGF (Figure 5A and 5C). This suggests that enhancement of angiogenesis by blockage of the interaction between *PMCA4* and calcineurin might have important therapeutic applications.

Altogether, these data demonstrate that disruption of the *PMCA4*/calcineurin interaction enhances significantly the activity of the calcineurin/NFAT pathway and consequently the expression of NFAT-dependent, proangiogenic genes, resulting in a significant increase in endothelial cell angiogenesis.

We have reported previously an inhibitory interaction between *PMCA4* and endothelial nitric oxide synthase that results in downregulation of nitric oxide production by endothelial cells.¹⁵ *PMCA4* also interacts with endothelial nitric oxide synthase through the region 428 to 651, so overexpression of Flag-*PMCA4*(428–651) might also disrupt the interaction between *PMCA4* and endothelial nitric oxide synthase, leading to endothelial nitric oxide synthase activation and an increase in nitric oxide production. It is well established that nitric oxide promotes angiogenesis.²² Therefore, although our experiments demonstrate the involvement of the calcineurin/NFAT pathway in the angiogenic increase elicited by Flag-*PMCA4*(428–651), we cannot rule out that overexpression of this region of *PMCA4* may be activating alternative proangiogenic signaling pathways that contribute as well to the observed increase in angiogenesis.

Discussion

In this work, we show that *PMCA4* inhibits the activation of the calcineurin/NFAT pathway on VEGF stimulation of endothelial cells, leading to a significant attenuation of VEGF-mediated angiogenesis. Consistent with these findings, *PMCA4* has also been shown to play a negative role in the progression of cardiac hypertrophy via downregulation of calcineurin signaling in cardiomyocytes.¹⁶

It is hypothesized that *PMCA4* negatively regulates the activity of calcineurin by recruiting the phosphatase to low-calcium microdomains created by the calcium extrusion ability of the pump.¹⁴ In support of this hypothesis we demonstrate here that the interaction between *PMCA4* and calcineurin is essential for the negative effect exerted by *PMCA4* on VEGF-induced signaling.

An interaction between calcineurin and calcium influx channels is thought to place the phosphatase into local high-calcium subcellular microdomains that promote activation of calcineurin/NFAT signaling.^{23–26} Here, we show that an interaction between calcineurin and the *PMCA4* calcium extrusion pump downregulates calcineurin/NFAT activity. It is

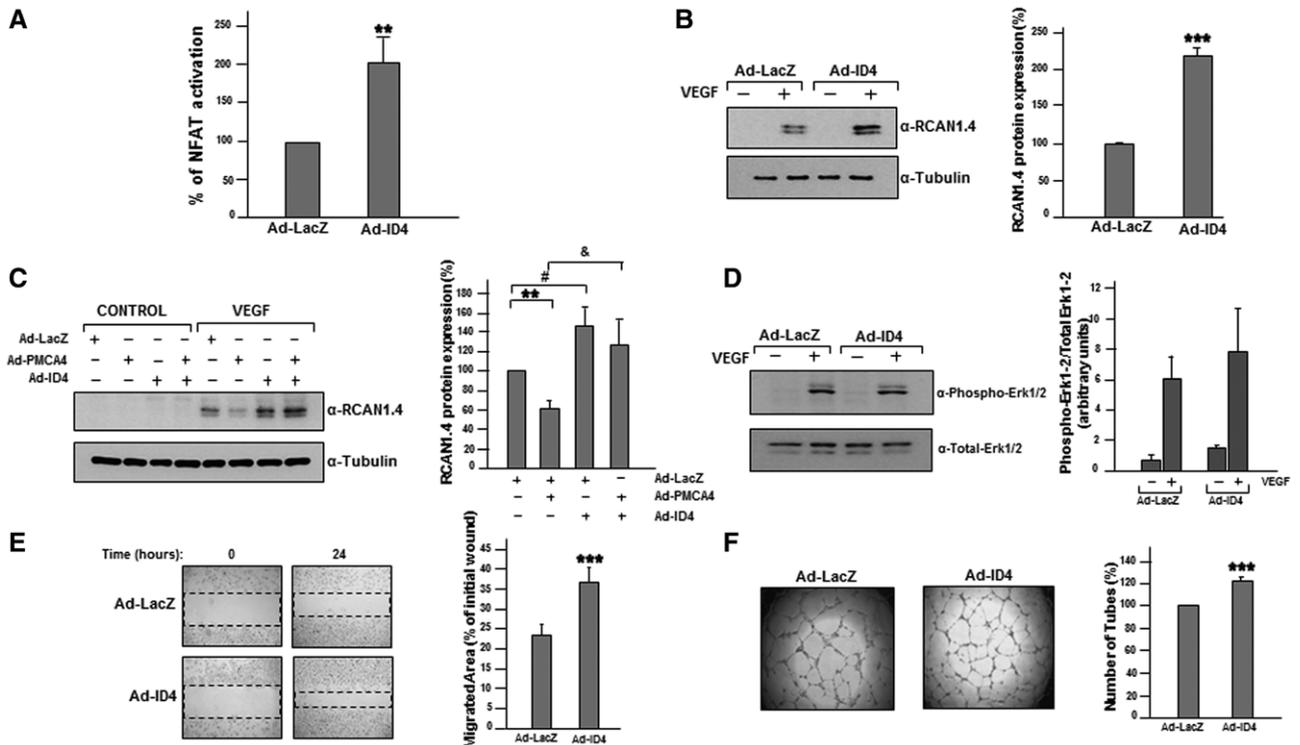


Figure 7. Plasma membrane calcium ATPase 4 (PMCA4) downregulates nuclear factor of activated T cells (NFAT)-dependent proangiogenic events via interaction with calcineurin. **A**, Disruption of the PMCA4/calcineurin interaction increases the activity of the calcineurin/NFAT pathway in vascular endothelial growth factor (VEGF)-stimulated endothelial cells. Human umbilical vein endothelial cells (HUVECs) were infected with adenovirus Ad-LacZ or Ad-ID4 (multiplicity of infection [MOI]=150) for 24 hours and subsequently with Ad-NFAT-Luc (MOI=25) for a further 24 hours. After serum starvation in endothelial cell growth medium (0.5% fetal calf serum), cells were stimulated with VEGF (25 ng/mL) for 6 hours. Induction of the luciferase activity of the reporter vector in cells infected with Ad-LacZ was taken as a reference (100%). Histograms show data as mean \pm SE of 3 independent experiments. ** indicates statistically significant differences ($P\leq 0.01$, according to Student *t* test) when comparing the percentage of NFAT activation in VEGF-stimulated cells infected with Ad-LacZ vs those infected with Ad-ID4. **B**, Disruption of the PMCA4/calcineurin interaction in endothelial cells enhances the expression of RCAN1.4 in response to VEGF. Western blot analysis of protein lysates obtained from 3×10^5 HUVECs infected with the indicated adenovirus (MOI=150) for 72 hours. Where indicated, cells were stimulated with VEGF (25 ng/mL) for 4 hours. Protein levels of RCAN1.4 were determined by Western blot with a rabbit anti-DSCR1 antibody (α -RCAN1.4). Histograms show data as mean \pm SE of 5 independent experiments. *** indicates statistically significant differences ($P\leq 0.005$, according to Student *t* test) when comparing RCAN1.4 protein expression in VEGF-stimulated cells infected with Ad-LacZ vs those infected with Ad-ID4. **C**, Impairment of the PMCA4/calcineurin interaction abolishes PMCA4-mediated downregulation of RCAN1.4 expression. HUVECs were coinfecting with Ad-PMCA4 (MOI=50) and either Ad-LacZ (MOI=150) or Ad-ID4 (MOI=150). Control cells were infected with Ad-LacZ (MOI=200). Cells were stimulated with VEGF (25 ng/mL) for 4 hours where indicated and protein expression of RCAN1.4 was analyzed by Western blot as described in **B**. Histograms show data as mean \pm SE of 4 independent experiments. ** indicates statistically significant differences ($P\leq 0.01$, according to Student *t* test) in RCAN1.4 protein expression in VEGF-stimulated cells when comparing cells infected with Ad-LacZ vs those infected with Ad-PMCA4. # and & indicate statistically significant differences ($P\leq 0.05$, according to Student *t* test) when comparing VEGF-stimulated cells infected with Ad-LacZ vs Ad-ID4 (#) or with Ad-LacZ+Ad-PMCA4 vs Ad-PMCA4+Ad-ID4 (&). **D**, VEGF-induced activation of extracellular signal-regulated kinase (Erk) signaling is not altered by disruption of the PMCA4/calcineurin interaction. Western blot analysis of protein lysates obtained from 3×10^5 HUVECs infected with the indicated adenovirus (MOI=150) for 72 hours. Where indicated, cells were stimulated with VEGF (25 ng/mL) for 5 minutes. Phosphorylation (activation) status of Erk1/2 proteins was determined using an anti-phospho-Erk1/2-specific antibody (α -PhosphoErk1/2). Histogram represents quantification of Erk1/2 phosphorylation with respect to the levels of total Erk1/2. **E**, Disruption of the PMCA4/calcineurin interaction enhances the motility of HUVEC cells. HUVEC cells were infected with Ad-LacZ or Ad-ID4 (MOI=150) for 72 hours. Migration analysis of infected cells was performed as described in Materials and Methods. Histograms show data as mean \pm SE of 7 independent experiments. *** indicates statistically significant differences ($P\leq 0.005$, according to Student *t* test) when comparing migration in cells infected with Ad-LacZ vs those infected with Ad-ID4. **F**, Disruption of the PMCA4/calcineurin interaction augments endothelial cell tube formation. HUVECs were infected with Ad-LacZ or Ad-ID4 (MOI=150) for 72 hours. Tubular morphogenesis was analyzed by plating infected cells onto a layer of Growth-Factor Reduced Matrix (Geltrex) in Medium 200 containing 2% fetal calf serum, for 24 hours. Images show representative fields from experiments quantified in the histogram. Histograms show data as mean \pm SE of 8 independent experiments. *** indicates statistically significant differences in tube formation ($P\leq 0.005$, according to Student *t* test) when comparing cells infected with Ad-LacZ vs those infected with Ad-ID4. **B** to **D**, In Western blot assays the expression of tubulin was analyzed using a mouse monoclonal antitubulin antibody (α -Tubulin) as loading control.

therefore tempting to speculate that regulation of calcineurin might involve shuttling between high- and low-calcium subcellular microdomains via interaction with calcium transporters responsible for influx or efflux of calcium. In accord with this notion, activation of the calcineurin/NFAT pathway in

cardiomyocytes has been shown to require a calcium signaling microdomain generated by calcium influx from a small fraction of L-type calcium channels specifically housed in caveolae.²⁷ PMCA4 expression has been localized to lipid rafts and caveolae in endothelial,²⁸ cardiac,²⁹ and vascular smooth

muscle cells³⁰ and, interestingly, the calcium-removal function of PMCA4 is impaired in smooth muscle cells of a caveolin-1 knockout mouse.³⁰ Whether targeting of PMCA4 to caveolae is necessary to downregulate VEGF-induced signaling of the calcineurin/NFAT pathway requires further investigation.

Our data also reveal that PMCA4-mediated inhibition of calcineurin signaling in endothelial cells leads to downregulation of *RCAN1.4* and *Cox-2* expression, together with an attenuation of VEGF-activated, proangiogenic cellular processes including migration and tubular morphogenesis. Although our results do not directly demonstrate that reduction of these cellular functions is linked to PMCA4-mediated downregulation of *RCAN1.4* and *Cox-2*, both proteins have been described to promote endothelial cell migration and tube formation,^{17,18,31} suggesting that they likely play a significant role in the PMCA4-directed inhibition of angiogenesis. In agreement with this idea, PMCA4-deficient MLECs presented higher expression of *Cox-2* (Figure IIIB in the online-only Data Supplement) and enhanced cellular migration (Figure 3B) than wild-type counterparts. However, specific inhibition of *Cox-2* functionality by incubation of *PMCA4(-/-)* MLECs with the *Cox-2* selective inhibitor Etoricoxib resulted in a strong reduction in cell motility (Figure VI in the online-only Data Supplement), indicating that elevated expression of *Cox-2* in PMCA4 knockout cells is linked to their enhanced motility.

The results of our gain- or loss-of-PMCA4 function experiments might be particularly relevant in the context of pathophysiological processes associated with changes in the expression of PMCA proteins. Initial investigations in our laboratory have revealed that stimulation of HUVEC cells with VEGF induces a transient upregulation of PMCA4 protein levels that peaks at 4 to 6 hours after VEGF treatment (Figure VII in the online-only Data Supplement). PMCA4 expression has been reported to increase in the late phase of osteoclast differentiation.³² Interestingly, PMCA4 upregulation in the differentiating osteoclast is dependent on the calcium-triggered activation of the calcineurin/NFAT pathway. In turn, increased osteoclast expression of PMCA4 leads to cytoplasmic calcium removal, creating a negative feedback regulatory loop.³² Our results suggest that VEGF-induced upregulation of PMCA4 might also be functioning as a negative feedback mechanism to regulate the activation of the calcineurin/NFAT pathway in endothelial cells. Whether VEGF-induced upregulation of PMCA4 in endothelial cells is mediated by NFAT-dependent transcriptional activation of the *PMCA4* gene promoter deserves additional investigation.

Further supporting the functional significance of changes in the expression of PMCA4 in cardiovascular pathophysiology, Ewart et al³³ recently reported that PMCA levels increase in mouse aortic tissue during atherosclerosis.

In this work, we demonstrate that PMCA4 modulates *RCAN1.4* expression in endothelial cells through regulation of the calcineurin/NFAT pathway. Interestingly, calcineurin-dependent expression of *RCAN1.4* in other cell types has been linked to pathological vascular wall remodeling³⁴ and atherosclerosis progression.³⁵ Hence, the potential role of PMCA4 in the modulation of vascular pathologies linked to *RCAN1.4* aberrant expression deserves further investigation.

Although in this study we have focused our attention on isoform PMCA4, we have shown previously that PMCA1 (the other major PMCA isoform expressed in endothelial cells)¹³ also interacts with calcineurin in endothelial cells.¹⁵ Thus, partial redundancy between the 2 isoforms might partly mask the total negative effect exerted by PMCA on endothelial calcineurin/NFAT signaling. Nonetheless, our data demonstrate that PMCA4 expression is essential for PMCA-mediated regulation of angiogenesis as suppression of PMCA4 led to a significant enhancement of angiogenesis in spite of background PMCA1 expression. Because targeted ablation of the *PMCA1* gene in mice is embryolethal,³⁶ generation of PMCA1 conditional knockout mice will be required to investigate its potential involvement in the regulation of calcineurin-dependent angiogenesis and to analyze the functional consequences of deleting both isoforms in PMCA1/4 double-knockout animals.

In conclusion, our results describe a novel role for PMCA4 as a negative regulator of VEGF-dependent angiogenesis via interaction with the signaling phosphatase calcineurin.

Given the relevance of the calcineurin/NFAT signaling axis in the regulation of pathological angiogenesis, targeted modulation of PMCA4 functionality might open new therapeutic avenues to promote or attenuate angiogenesis in patients experiencing angiogenic disorders.

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Disclosures

None.

References

1. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. *Nature*. 2011;473:298–307.
2. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature*. 2000;407:249–257.
3. Takahashi H, Shibuya M. The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions. *Clin Sci (Lond)*. 2005;109:227–241.
4. Armesilla AL, Lorenzo E, Gómez del Arco P, Martínez-Martínez S, Alfranca A, Redondo JM. Vascular endothelial growth factor activates nuclear factor of activated T cells in human endothelial cells: a role for tissue factor gene expression. *Mol Cell Biol*. 1999;19:2032–2043.
5. Hernández GL, Volpert OV, Iñiguez MA, Lorenzo E, Martínez-Martínez S, Grau R, Fresno M, Redondo JM. Selective inhibition of vascular endothelial growth factor-mediated angiogenesis by cyclosporin A: roles of

- the nuclear factor of activated T cells and cyclooxygenase 2. *J Exp Med*. 2001;193:607–620.
6. Zaichuk TA, Shroff EH, Emmanuel R, Filleur S, Nelius T, Volpert OV. Nuclear factor of activated T cells balances angiogenesis activation and inhibition. *J Exp Med*. 2004;199:1513–1522.
 7. Ryeom S, Baek KH, Rioth MJ, Lynch RC, Zaslavsky A, Birsner A, Yoon SS, McKeon F. Targeted deletion of the calcineurin inhibitor DSCR1 suppresses tumor growth. *Cancer Cell*. 2008;13:420–431.
 8. Klee CB, Ren H, Wang X. Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. *J Biol Chem*. 1998;273:13367–13370.
 9. Hogan PG, Chen L, Nardone J, Rao A. Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev*. 2003;17:2205–2232.
 10. Martínez-Martínez S, Redondo JM. Inhibitors of the calcineurin/NFAT pathway. *Curr Med Chem*. 2004;11:997–1007.
 11. Buch MH, Pickard A, Rodriguez A, Gillies S, Maass AH, Emerson M, Cartwright EJ, Williams JC, Oceandy D, Redondo JM, Neyses L, Armesilla AL. The sarcolemmal calcium pump inhibits the calcineurin/nuclear factor of activated T-cell pathway via interaction with the calcineurin A catalytic subunit. *J Biol Chem*. 2005;280:29479–29487.
 12. Holton M, Yang D, Wang W, Mohamed TM, Neyses L, Armesilla AL. The interaction between endogenous calcineurin and the plasma membrane calcium-dependent ATPase is isoform specific in breast cancer cells. *FEBS Lett*. 2007;581:4115–4119.
 13. Strehler EE, Zacharias DA. Role of alternative splicing in generating isoform diversity among plasma membrane calcium pumps. *Physiol Rev*. 2001;81:21–50.
 14. Holton ML, Wang W, Emerson M, Neyses L, Armesilla AL. Plasma membrane calcium ATPase proteins as novel regulators of signal transduction pathways. *World J Biol Chem*. 2010;1:201–208.
 15. Holton M, Mohamed TM, Oceandy D, Wang W, Lamas S, Emerson M, Neyses L, Armesilla AL. Endothelial nitric oxide synthase activity is inhibited by the plasma membrane calcium ATPase in human endothelial cells. *Cardiovasc Res*. 2010;87:440–448.
 16. Wu X, Chang B, Blair NS, Sargent M, York AJ, Robbins J, Shull GE, Molkentin JD. Plasma membrane Ca²⁺-ATPase isoform 4 antagonizes cardiac hypertrophy in association with calcineurin inhibition in rodents. *J Clin Invest*. 2009;119:976–985.
 17. Urso K, Alfranca A, Martínez-Martínez S, Escolano A, Ortega I, Rodríguez A, Redondo JM. NFATc3 regulates the transcription of genes involved in T-cell activation and angiogenesis. *Blood*. 2011;118:795–803.
 18. Holmes K, Chapman E, See V, Cross MJ. VEGF stimulates RCAN1.4 expression in endothelial cells via a pathway requiring Ca²⁺/calcineurin and protein kinase C-delta. *PLoS One*. 2010;5:e11435.
 19. Minami T, Yano K, Miura M, Kobayashi M, Suehiro J, Reid PC, Hamakubo T, Ryeom S, Aird WC, Kodama T. The Down syndrome critical region gene 1 short variant promoters direct vascular bed-specific gene expression during inflammation in mice. *J Clin Invest*. 2009;119:2257–2270.
 20. Yu Y, Sato JD. MAP kinases, phosphatidylinositol 3-kinase, and p70 S6 kinase mediate the mitogenic response of human endothelial cells to vascular endothelial growth factor. *J Cell Physiol*. 1999;178:235–246.
 21. Baggott RR, Mohamed TM, Oceandy D, Holton M, Blanc MC, Roux-Soro SC, Brown S, Brown JE, Cartwright EJ, Wang W, Neyses L, Armesilla AL. Disruption of the interaction between PMCA2 and calcineurin triggers apoptosis and enhances paclitaxel-induced cytotoxicity in breast cancer cells. *Carcinogenesis*. 2012;33:2362–2368.
 22. Bir SC, Xiong Y, Kevil CG, Luo J. Emerging role of PKA/eNOS pathway in therapeutic angiogenesis for ischaemic tissue diseases. *Cardiovasc Res*. 2012;95:7–18.
 23. Oliveria SF, Dell'Acqua ML, Sather WA. AKAP79/150 anchoring of calcineurin controls neuronal L-type Ca²⁺ channel activity and nuclear signaling. *Neuron*. 2007;55:261–275.
 24. Nieves-Cintrón M, Amberg GC, Navedo MF, Molkentin JD, Santana LF. The control of Ca²⁺ influx and NFATc3 signaling in arterial smooth muscle during hypertension. *Proc Natl Acad Sci USA*. 2008;105:15623–15628.
 25. Kim JY, Saffen D. Activation of M1 muscarinic acetylcholine receptors stimulates the formation of a multiprotein complex centered on TRPC6 channels. *J Biol Chem*. 2005;280:32035–32047.
 26. Poteser M, Schleifer H, Lichtenegger M, Scherthaner M, Stockner T, Kappe CO, Glasnov TN, Romanin C, Groschner K. PKC-dependent coupling of calcium permeation through transient receptor potential canonical 3 (TRPC3) to calcineurin signaling in HL-1 myocytes. *Proc Natl Acad Sci U S A*. 2011;108:10556–10561.
 27. Makarewich CA, Correll RN, Gao H, Zhang H, Yang B, Berretta RM, Rizzo V, Molkentin JD, Houser SR. A caveolae-targeted L-type Ca²⁺ channel antagonist inhibits hypertrophic signaling without reducing cardiac contractility. *Circ Res*. 2012;110:669–674.
 28. Fujimoto T. Calcium pump of the plasma membrane is localized in caveolae. *J Cell Biol*. 1993;120:1147–1157.
 29. Hammes A, Oberdorf-Maass S, Rother T, et al. Overexpression of the sarcolemmal calcium pump in the myocardium of transgenic rats. *Circ Res*. 1998;83:877–888.
 30. El-Yazbi AF, Cho WJ, Schulz R, Daniel EE. Calcium extrusion by plasma membrane calcium pump is impaired in caveolin-1 knockout mouse small intestine. *Eur J Pharmacol*. 2008;591:80–87.
 31. Iizuka M, Abe M, Shiiba K, Sasaki I, Sato Y. Down syndrome candidate region 1, a downstream target of VEGF, participates in endothelial cell migration and angiogenesis. *J Vasc Res*. 2004;41:334–344.
 32. Kim HJ, Prasad V, Hyung SW, Lee ZH, Lee SW, Bhargava A, Pearce D, Lee Y, Kim HH. Plasma membrane calcium ATPase regulates bone mass by fine-tuning osteoclast differentiation and survival. *J Cell Biol*. 2012;199:1145–1158.
 33. Ewart MA, Kennedy S, Macmillan D, Raja AL, Watt IM, Currie S. Altered vascular smooth muscle function in the ApoE knockout mouse during the progression of atherosclerosis. *Atherosclerosis*. 2014;234:154–161.
 34. Esteban V, Méndez-Barbero N, Jiménez-Borreguero LJ, Roqué M, Novensá L, García-Redondo AB, Salas M, Vila L, Arbonés ML, Campanero MR, Redondo JM. Regulator of calcineurin 1 mediates pathological vascular wall remodeling. *J Exp Med*. 2011;208:2125–2139.
 35. Méndez-Barbero N, Esteban V, Villahoz S, Escolano A, Urso K, Alfranca A, Rodríguez C, Sánchez SA, Osawa T, Andrés V, Martínez-González J, Minami T, Redondo JM, Campanero MR. A major role for RCAN1 in atherosclerosis progression. *EMBO Mol Med*. 2013;5:1901–1917.
 36. Prasad V, Okunade GW, Miller ML, Shull GE. Phenotypes of SERCA and PMCA knockout mice. *Biochem Biophys Res Commun*. 2004;322:1192–1203.

Significance

Vascular endothelial growth factor has been identified as a crucial regulator of physiological and pathological angiogenesis. Among the intracellular pathways triggered by vascular endothelial growth factor, activation of the calcineurin/nuclear factor of activated T cells signaling axis has emerged as a central mediator of angiogenic processes, highlighting the clinical potential of this route for the design of therapeutic interventions aimed at the treatment of neovascular-associated human diseases. Here, we have identified a critical role for the plasma membrane calcium ATPase 4 as a negative regulator of vascular endothelial growth factor–induced angiogenesis via downregulation of the calcineurin/nuclear factor of activated T cells pathway and the subsequent expression of nuclear factor of activated T cells–dependent, proangiogenic genes. Plasma membrane calcium ATPase 4–dependent inhibition of calcineurin signaling translates into a reduction in endothelial cell motility and blood vessel formation that ultimately impairs in vivo angiogenesis by vascular endothelial growth factor. Targeted modulation of plasma membrane calcium ATPase 4 functionality might open novel therapeutic avenues to promote or attenuate new vessel formation in human diseases that occur with angiogenesis.