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

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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 *RCAN1.4* 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]

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]

PMCAs are high-affinity calcium pumps that extrude 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 PMCAs 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 PMCAs and calcineurin interact in endothelial cells[15] although the functional significance of this interaction in endothelial pathophysiology has not been described thus far.
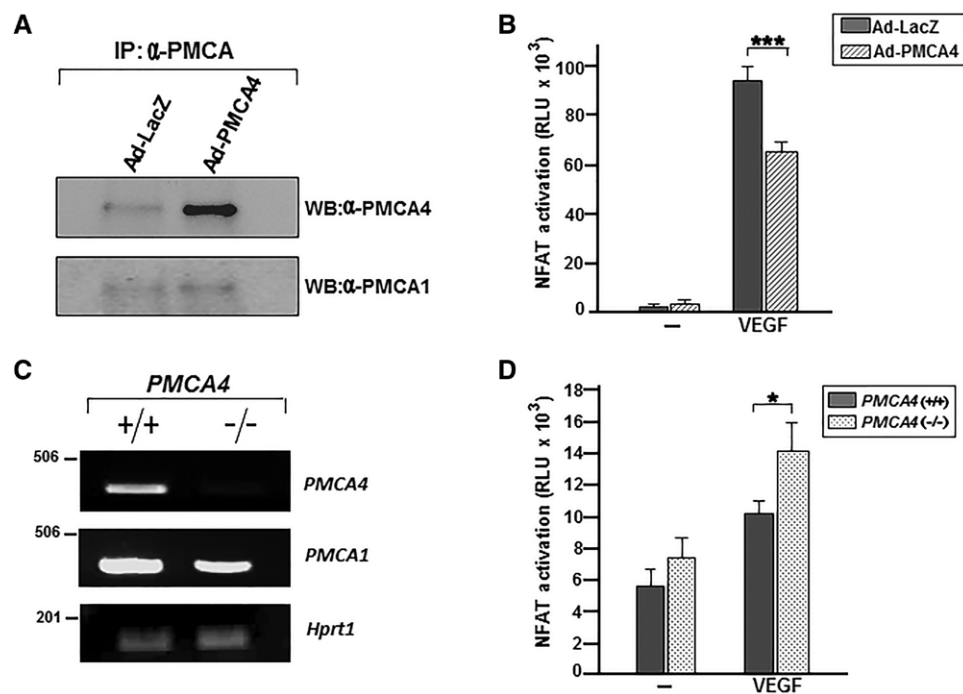
Figure 1. Plasma membrane calcium ATPase 4 (PMCA4) downregulates the activity of the calcineurin/nuclear factor of activated T cells (NFAT) pathway in endothelial cells. **A**, Ectopic expression of human PMCA4 in human umbilical vein endothelial cells (HUVECs) by infection with adenovirus Ad-PMCA4. 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 coinfected 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±SE of 5 independent experiments are shown. *** indicates statistically significant differences ($P\leq0.005$, 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 μg 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±SE of 5 independent experiments are shown. * indicates statistically significant differences ($P\leq0.05$, 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 cells[12] and cardiomyocytes[16] 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
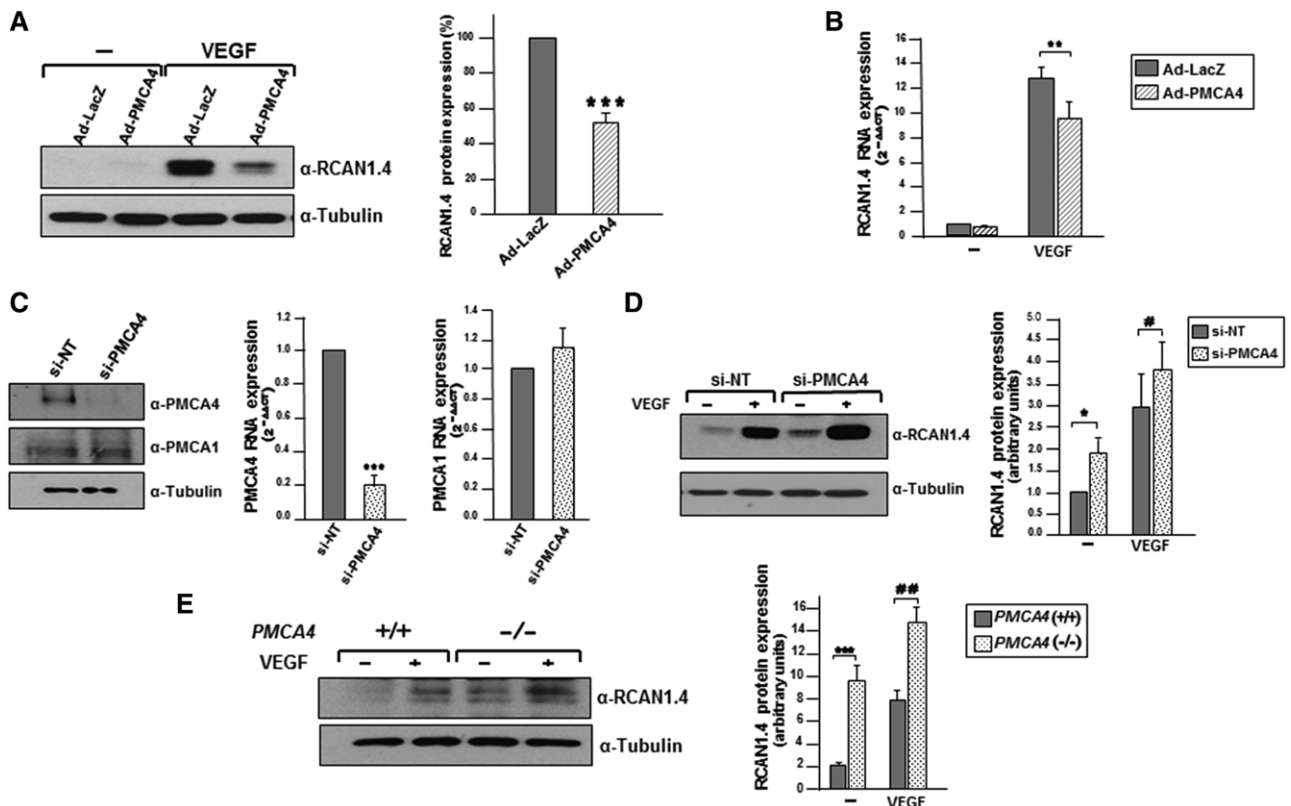
Figure 2. Plasma membrane calcium ATPase 4 (PMCA4) negatively regulates the vascular endothelial growth factor (VEGF)–mediated upregulation of the proangiogenic protein RCAN1.4 in endothelial cells. **A**, Overexpression of human PMCA4 attenuates the VEGF-induced upregulation of RCAN1.4 protein. Protein 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±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±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 PMCA1 and 4 proteins 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±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±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 HUVEC cells 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±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 IA 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 IB 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 IIA and IIB 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 IIA 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 IIIA in the online-only Data Supplement). Consistent with these results, analysis
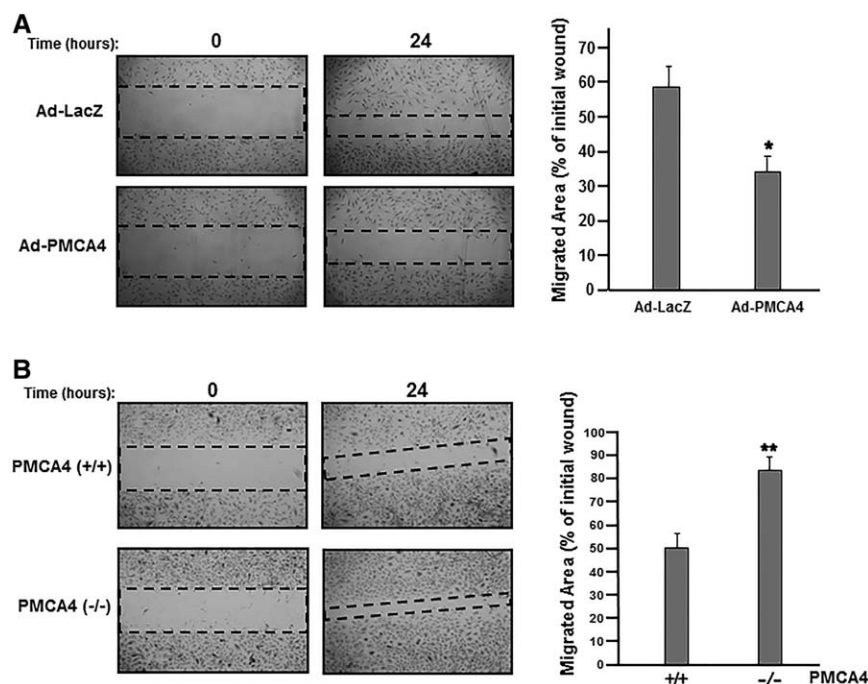
Figure 3. Plasma membrane calcium ATPase 4 (PMCA4) inhibits endothelial cell migration. **A**, Overexpression of PMCA4 significantly reduces endothelial cell migration. Representative images of wound-healing migration assays performed with human umbilical vein endothelial cells infected with adenovirus 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
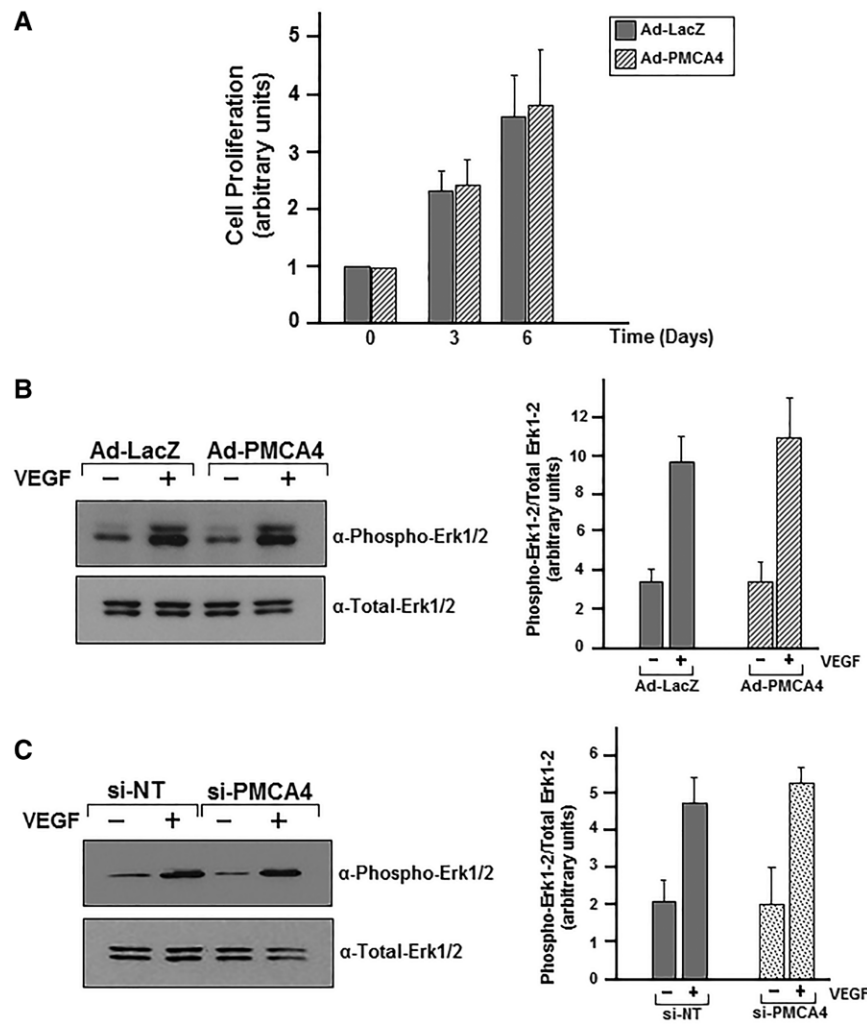
Figure 4. Plasma membrane calcium ATPase 4 (PMCA4) does not regulate endothelial cell proliferation. **A**, Human umbilical vein endothelial cells (HUVECs; $2×10^3$) infected with adenovirus Ad-PMCA4 or Ad-LacZ were plated on 96-well plates. The number of viable cells was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium 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±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-PhosphoErk1/2) 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±SE of ≥3 independent experiments.
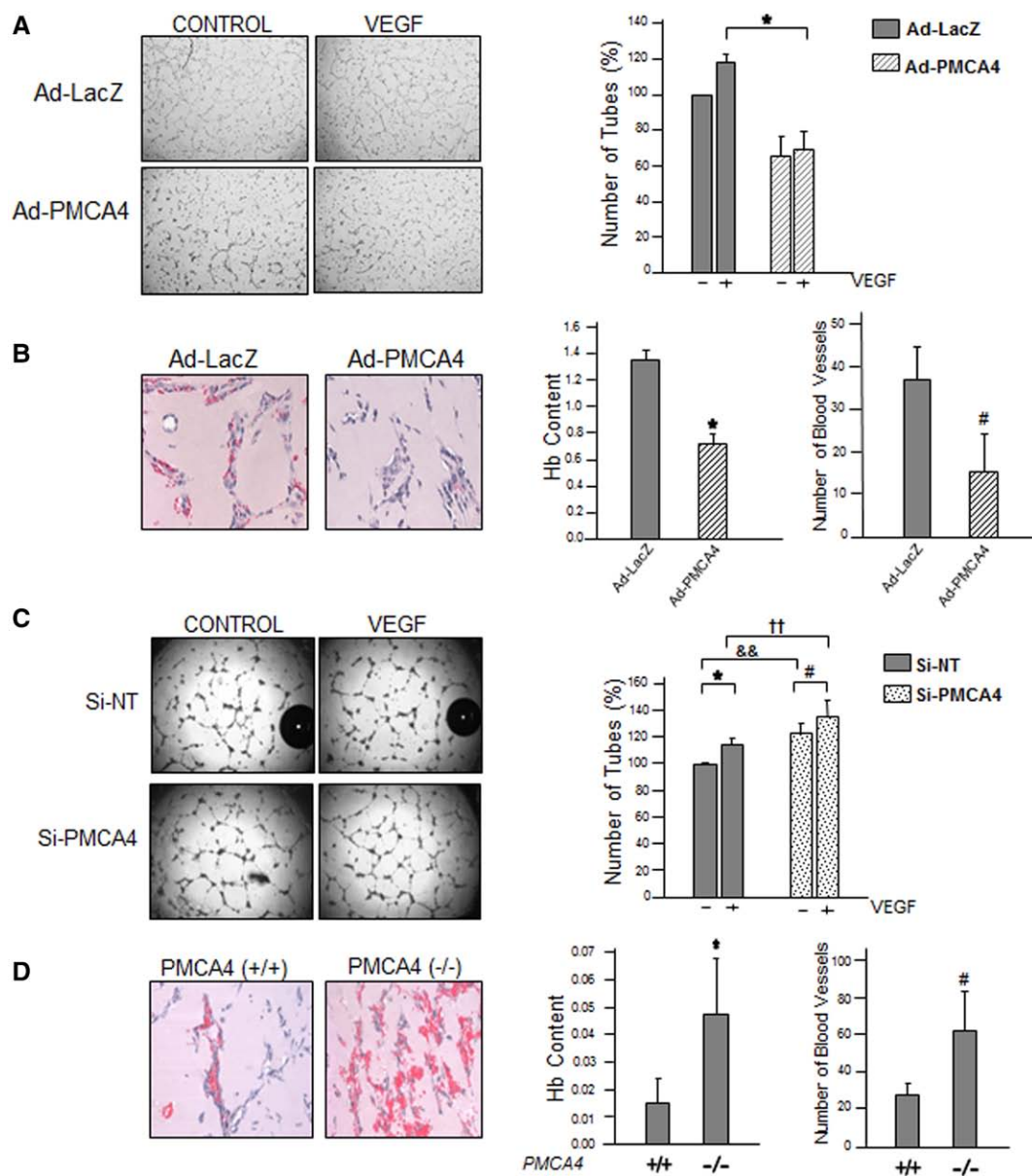
Figure 5. Plasma membrane calcium ATPase 4 (PMCA4) attenuates vascular endothelial growth factor (VEGF)–induced angiogenesis. **A**, PMCA4 overexpression in endothelial cells blocks in vitro tube formation in response to VEGF stimulation. Human umbilical vein endothelial cells (HUVECs) infected with 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±SE of 5 independent experiments. * indicates statistically significant differences (*P*≤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 µg/mL), and 2×10⁹ 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±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 ×20) was quantified in 10 random fields per sample. Histogram depicting number of blood vessels represents mean±SE of values calculated from 8 different 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 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±SE of 7 independent experiments. * and # indicate statistically significant differences in tube formation (*P*≤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 †† indicate statistically significant differences in tube formation (*P*≤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 calcineurin[11] (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

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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.
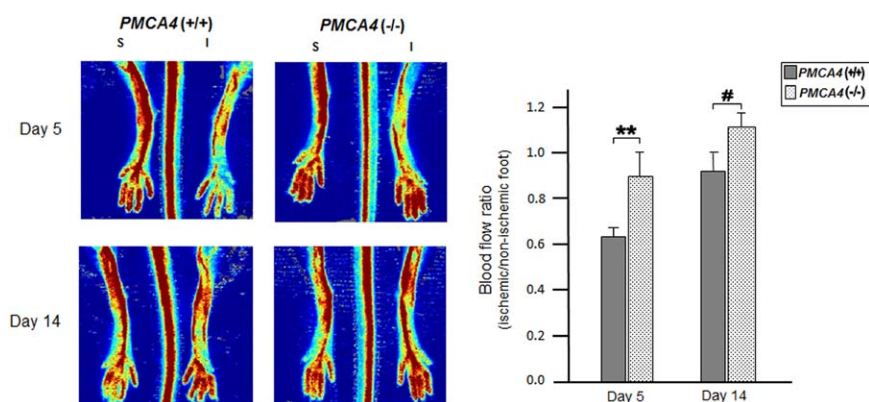
Figure 6. Plasma membrane calcium ATPase 4 (*PMCA4*) gene ablation enhances postischemic hindlimb perfusion. Representative laser Doppler images showing blood flow at 5 and 14 days after surgery, in sham-operated nonischemic legs (S) and femoral-ligated ischemic legs (I) of PMCA4 knockout (*PMCA4* (−/−)) or wild-type (*PMCA4* (+/+)) mice. Histogram represents mean±SE (n≥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*≤0.01 and *P*≤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 PMCA 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
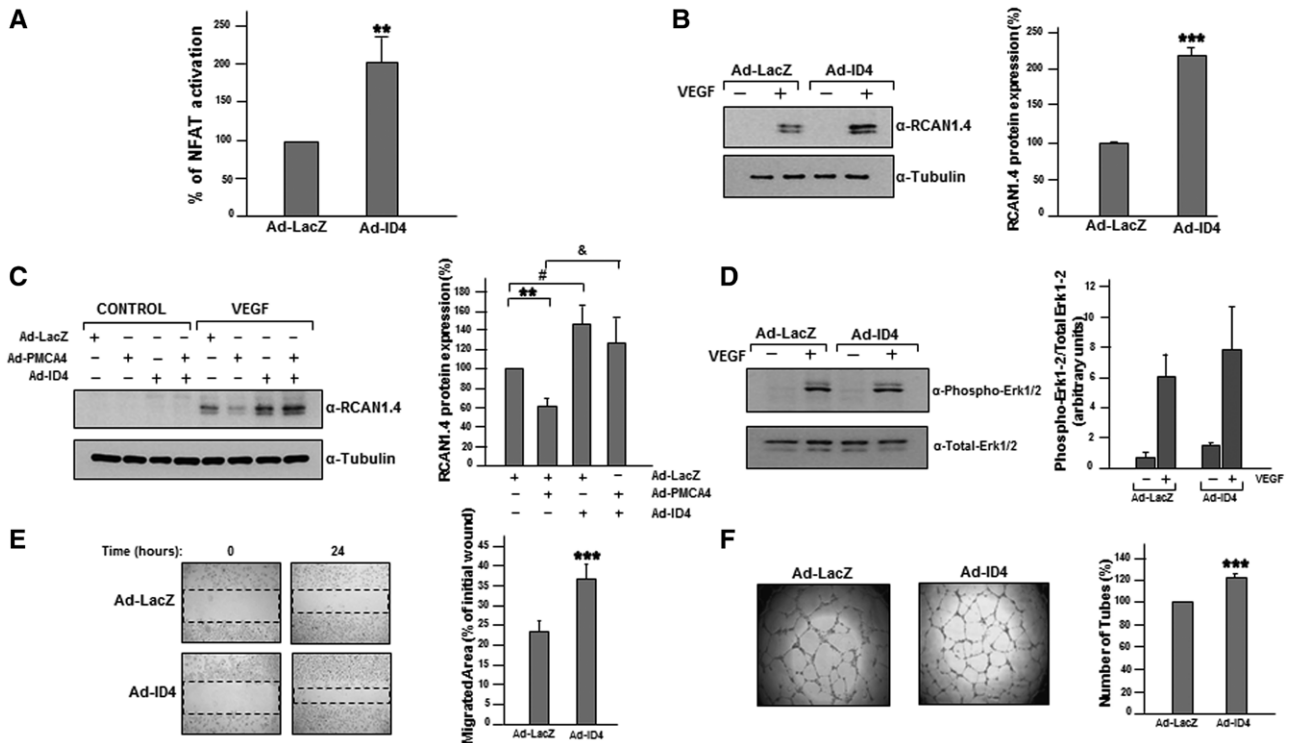
Figure 7. Plasma membrane calcium ATPase 4 PMCA4 downregulates nuclear factor of activated T cells (NFAT)–dependent proangiogenic events via interaction with calcineurin. **A**, Disruption of the PMCA4/calcineurin interaction increases the activity of the calcineurin/NFAT pathway in vascular endothelial 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±SE of 3 independent experiments. ** indicates statistically significant differences ($P \le 0.01$, 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±SE of 5 independent experiments. *** indicates statistically significant differences ($P \le 0.005$, 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 coinfected 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±SE of 4 independent experiments. ** indicates statistically significant differences ($P \le 0.01$, 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 ($P \le 0.05$, according to Student t test) when comparing VEGF-stimulated cells infected with Ad-LacZ vs Ad-ID4 (#) or with Ad-Laz+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±SE of 7 independent experiments. *** indicates statistically significant differences ($P \le 0.005$, 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±SE of 8 independent experiments. *** indicates statistically significant differences in tube formation ($P \le 0.005$, 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 cells[30] 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 al[33] 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 remodeling[34] 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.

Disclosures

None.

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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.

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 and Angel Luis Armesilla

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SUPPLEMENTAL MATERIAL

The Plasma Membrane Calcium ATPase isoform 4 inhibits VEGF-mediated angiogenesis through interaction with calcineurin

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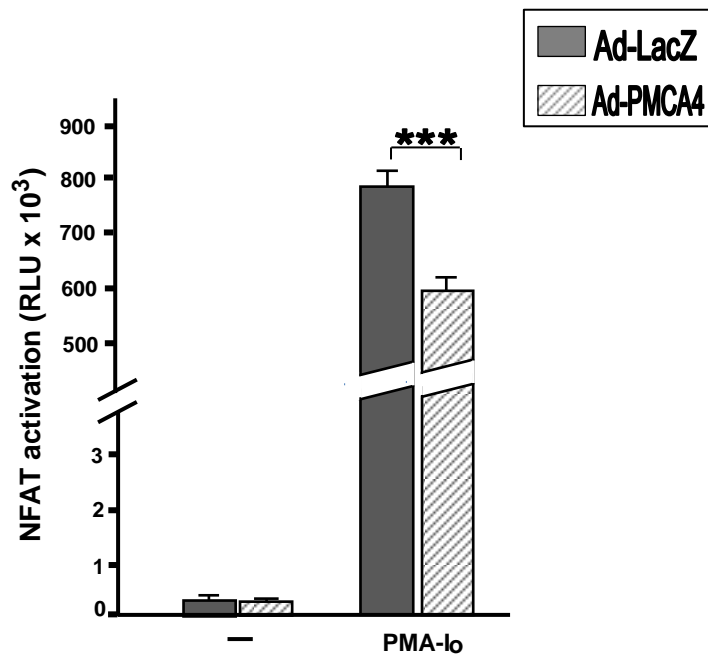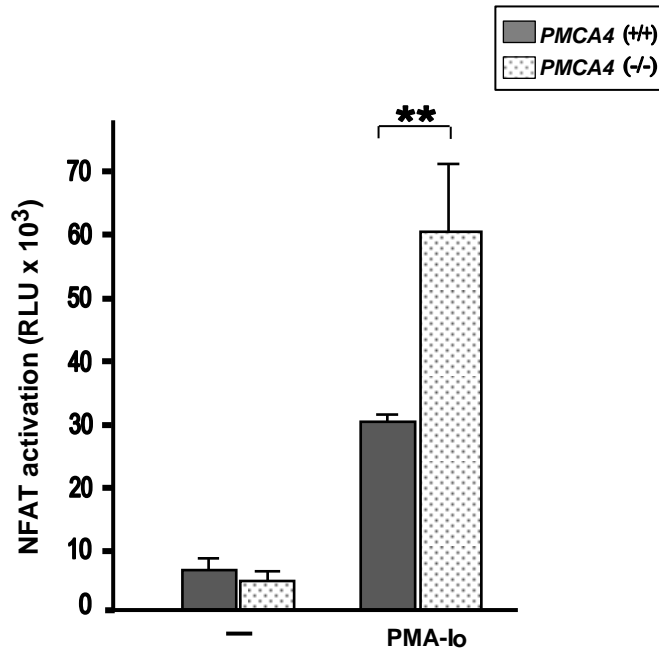
Figure I. PMCA4 significantly inhibits the activity of the calcineurin/NFAT pathway in response to PMA/Ionophore stimulation in endothelial cells. (A) HUVECs (3 x 10⁵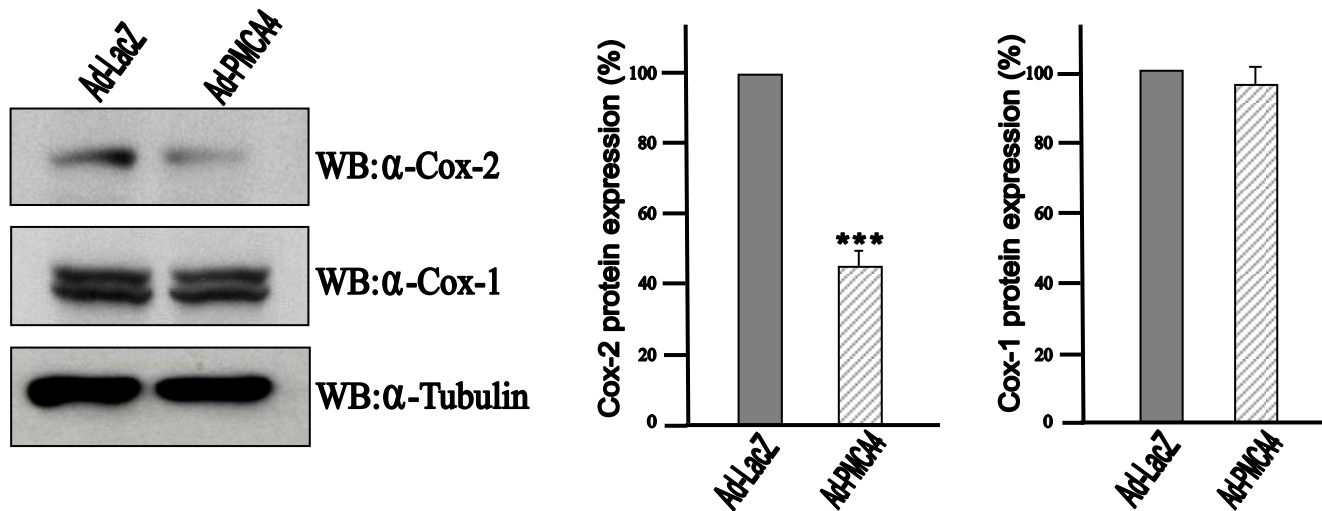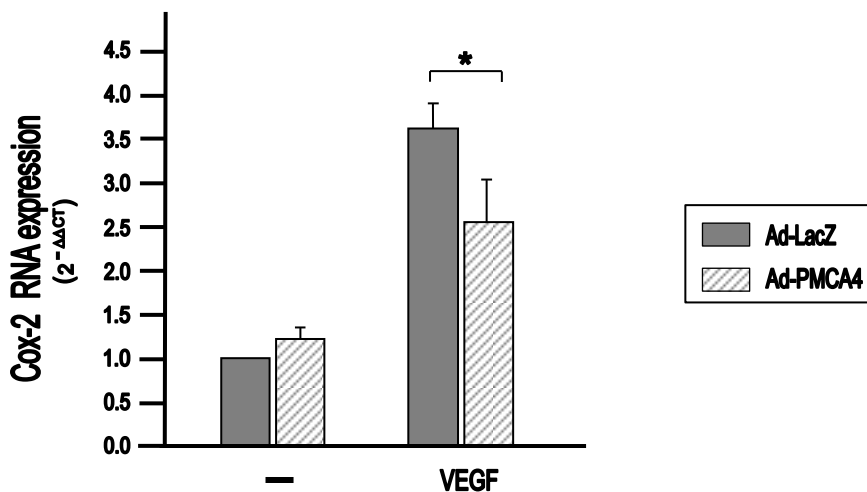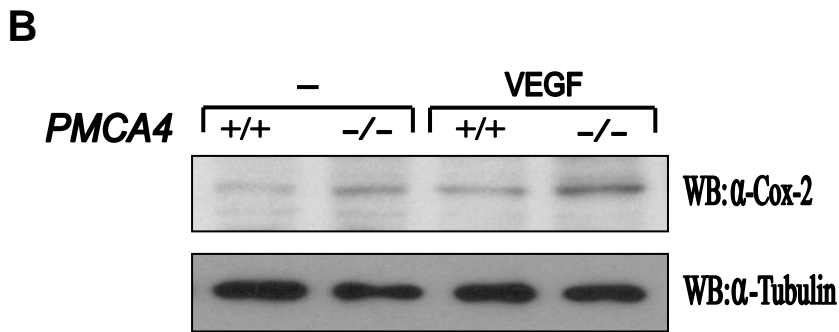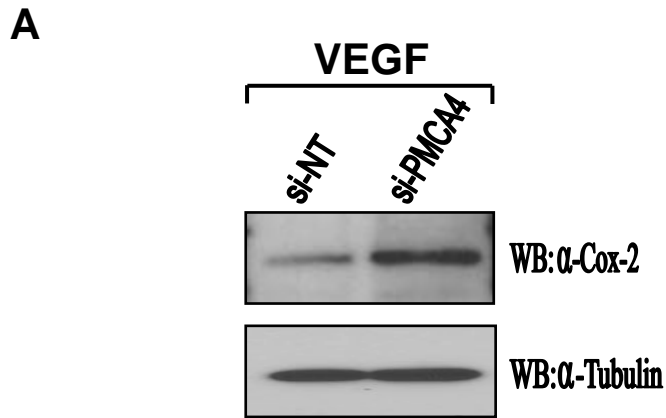) were co-infected with either Ad-LacZ or Ad-PMCA4 (MOI=50) for 24 hours, and then with Ad-NFAT-Luc (MOI=25) for an additional 24 hours. Infected cells were serum-starved in medium containing 0.5% fetal calf serum and left unstimulated (-) or treated with phorbol 12 myristate 13-acetate (PMA) (20 ng/ml) and the calcium ionophore A23187 (1 µM) for 6 hours (PMA-Io). Mean ± S.E. of five independent experiments are shown. *** indicates statistically significant differences ($P \leq 0.005$, according to Student's t-test) when comparing PMA/Io-stimulated HUVEC infected with Ad-LacZ vs those infected with Ad-PMCA4. (B) MLECs (3 x 10⁵) isolated from PMCA4 wild-type (+/+) or knock-out (-/-) animals were infected with Ad-NFAT-Luc (MOI=50) for 48 hours. After serum starvation in 0.5% fetal calf serum, cells were treated as described in (A) and luciferase activity was measured. Mean ± S.E. of five independent experiments are shown. ** indicates statistically significant differences ($P \leq 0.01$, according to Student's t-test) when comparing PMA/Io-stimulated PMCA4 wild-type vs knockout PMCA4 (-/-) MLECs.

Figure II. Ectopic expression of PMCA4 decreases the levels of Cox-2 in HUVECs stimulated with VEGF. HUVECs were infected for 72 hours with either Ad-PMCA4 or Ad-LacZ (MOI=50) and stimulated with VEGF (25 ng/ml) for 4 or 2 hours for protein and RNA analysis respectively. (A) Protein lysates were analysed by western blot with a 1:1000 dilution of a mouse anti-Cox-1 antibody (Alexis) (WB:α-Cox1) or a 1:1000 dilution of a mouse anti-Cox-2 antibody (Alexis) (WB:α-Cox-2). The expression of tubulin was analyzed using a mouse monoclonal anti-tubulin antibody (α-Tubulin) as loading control. Histograms show mean ± S.E. of five independent experiments. *** indicates statistically significant differences in Cox-2 protein expression ($P \leq 0.005$, according to Student t-test) when comparing VEGF-stimulated HUVEC infected with Ad-Lacz vs Ad-PMCA4. (B) RNA levels for Cox-2 were determined by qRT-PCR using the $2^{-\Delta\Delta CT}$ method. Mean ± S.E. of five independent experiments is shown. * indicates statistically significant differences in Cox-2 RNA levels ($P \leq 0.05$, according to Student t-test) when comparing VEGF-stimulated HUVEC infected with Ad-LacZ vs Ad-PMCA4.

A

VEGF

si-NT   si-PMCA4

WB:α-Cox-2

WB:α-Tubulin

B

PMCA4   –   VEGF

+/+   –/–   +/+   –/–

WB:α-Cox-2

WB:α-Tubulin

Figure III. Suppression of PMCA4 expression increases Cox-2 protein levels in endothelial cells stimulated with VEGF. (A) PMCA4 silencing enhances the expression of Cox-2 in VEGF-stimulated HUVECs. Western blot analyses of Cox-2 expression (α-Cox-2) in protein lysates of HUVECs transfected with a PMCA4-specific siRNA (si-PMCA4) or a non-targeting, control siRNA (si-NT). Cox-2 expression was triggered by stimulating transfected cells with VEGF (25 ng/ml) for 4 hours. An image representative of two independent experiments is shown. (B) Targeted ablation of PMCA4 increases Cox-2 protein expression in MLECs. Western blot analysis of Cox-2 expression in MLECs isolated from PMCA4 wild-type (+/+) or knock-out (-/-) mice. Where indicated, MLECs were stimulated with VEGF (50 ng/ml) for 4 hours. An image representative of two independent experiments is shown. (A-B) The expression of tubulin was analyzed using a mouse monoclonal anti-tubulin antibody (α-Tubulin) as loading control.
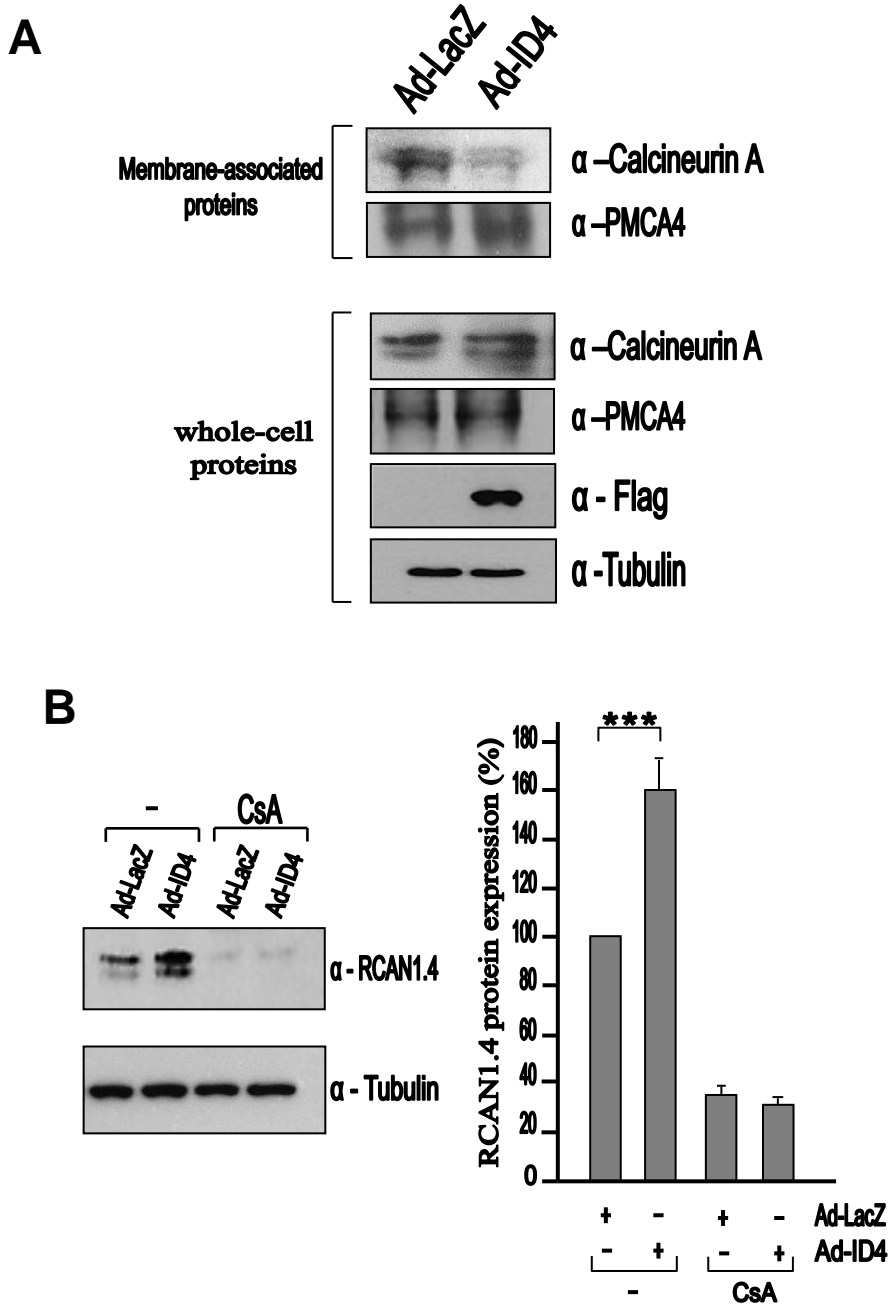
Figure IV. Disruption of the PMCA4/calcineurin interaction promotes upregulation of RCAN1.4 expression by a cyclosporine A-sensitive mechanism. (A) Ectopic expression of the region 428-651 of PMCA4 disrupts the interaction PMCA/calcineurin in the plasma membrane of endothelial cells. HUVECs were infected with Ad-LacZ (control) or Ad-ID4 at a MOI=150 for 72 hours. Membrane-associated and whole-cell total proteins were isolated from the infected cells and analysed by western blot with a 1:1000 dilution of an anti-calcineurin A antibody (α-Calcineurin A) or a 1:500 dilution of the JA3 anti-PMCA4 specific antibody (α-PMCA4) to determine the level of calcineurin or PMCA4 associated to the plasma membrane, and the total expression of calcineurin or PMCA4 respectively. Expression of 3xFlag-PMCA4(428-651) was examined by western blot in total extracts of infected cells using a 1:2000 dilution of the M2 anti-Flag monoclonal antibody (Sigma) (α-Flag). The expression of tubulin was analyzed using a mouse monoclonal anti-tubulin antibody (α-Tubulin) as loading control. (B) HUVECs were infected with Ad-LacZ or Ad-ID4 at MOI=150 for 72 hours and expression of RCAN1.4 was induced by stimulation with VEGF (25 ng/ml) for 4 hours. Where indicated, cells were pre-incubated with the calcineurin inhibitor cyclosporine A (CsA) (200 ng/ml) for two hours prior to VEGF stimulation. RCAN1.4 expression was examined by western blot using a rabbit anti-DSCR1 antibody (α-RCAN1.4)**.** The expression of tubulin was analyzed using a mouse monoclonal anti-tubulin antibody (α-Tubulin) as loading control. Histogram shows mean ± S.E of four independent experiments. *** indicates statistically significant differences in the levels of RCAN1.4 protein expression ($P \leq 0.005$, according to Student t-test) when comparing protein extracts isolated from VEGF-treated HUVEC cells infected with Ad-LacZ vs Ad-ID4.
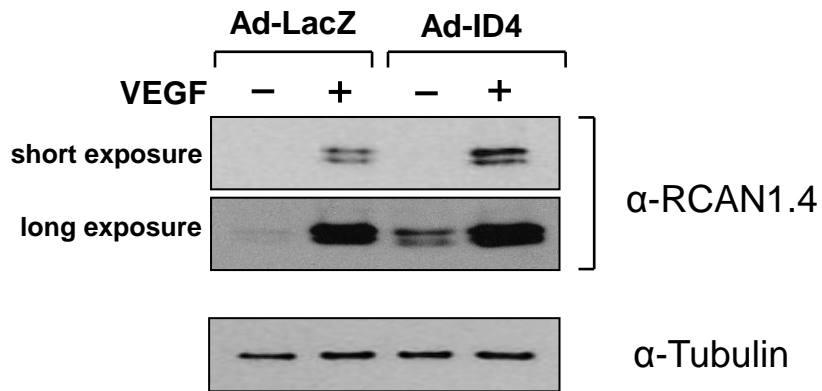
Figure V. Disruption of the interaction PMCA/calcineurin increases the basal expression of RCAN1.4 in endothelial cells. Western blot showing results of RCAN1.4 expression levels in Ad-LacZ or Ad-ID4 infected HUVECs, after short or long exposure of the autoradiography film to the western blot membrane during ECL detection. The image obtained after short exposure of the film (α-RCAN1.4 upper panel) shows the increase in RCAN1.4 expression observed in VEGF-stimulated HUVECs after disruption of the PMCA/calcineurin interaction by infection with Ad-ID4. Given the high level of RCAN1.4 expression triggered by VEGF stimulation, long exposure of the film (α-RCAN1.4 lower panel) masked the differences in RCAN1.4 expression as the system became saturated, however, long exposure of the film revealed that basal RCAN1.4 expression in unstimulated cells is augmented by disruption of the PMCA4/calcineurin interaction. This result is in agreement with the increase in RCAN1.4 basal expression observed in unstimulated PMCA4 knockout MLEC (Fig 2E).
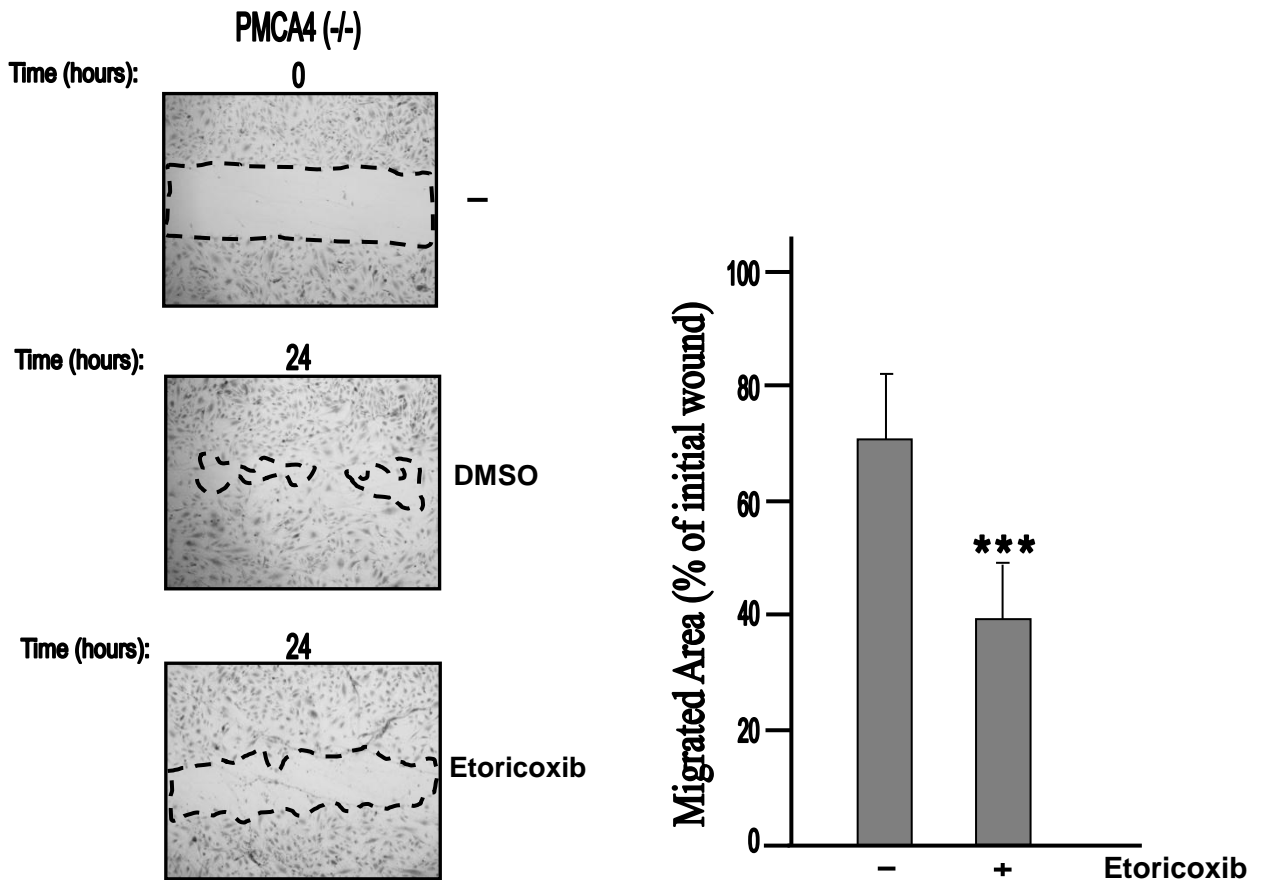
Figure VI. Enhanced endothelial cell migration in PMCA4 (-/-) MLECs is mediated by Cox-2. Cox-2 inhibition strongly abrogates the increase in migration observed in PMCA4 knockout cells. PMCA4 (-/-) MLEC cells were pre-treated with the Cox-2 specific inhibitor Etoricoxib (15 µM) (or DMSO vehicle as a control) for 1 hour before incubation for 24 hours in MLEC medium containing VEGF (50 ng/ml). Images were taken at time zero and after 24 hours incubation. The migrated area was calculated by subtracting the value of the non-migrated 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. Histogram represents mean ± S.E. of four independent experiments. *** indicates statistically significant differences ($P \leq 0.005$, according to Student's t-test) when comparing VEGF-induced migration of PMCA4 knockout MLECs after pre-incubation with Etoricoxib vs control vehicle DMSO.
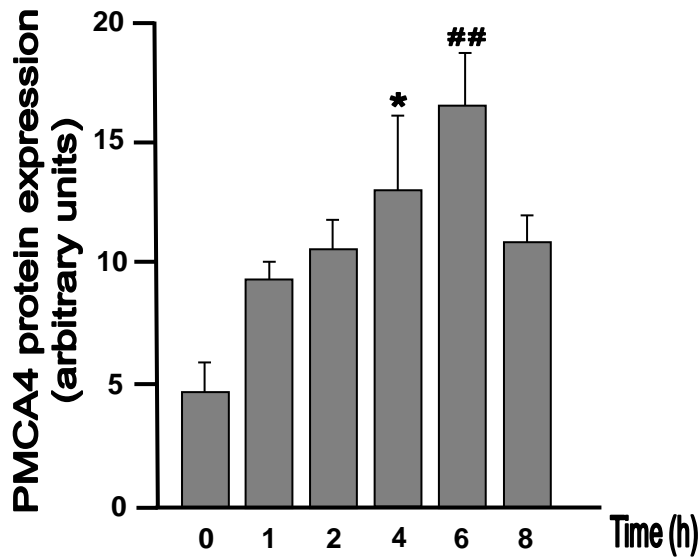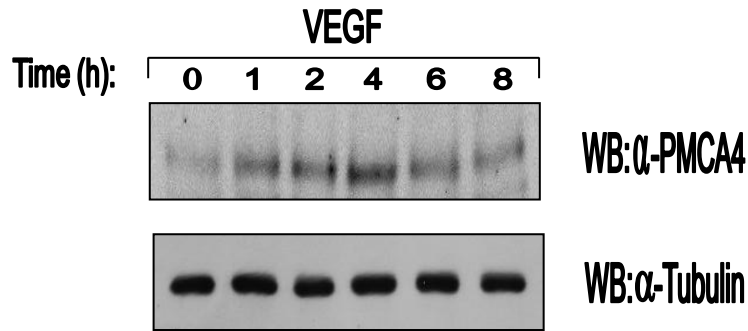
Figure VII. VEGF stimulation induces a transient increase of PMCA4 protein levels in HUVEC. Protein lysates from HUVECs stimulated with VEGF (25 ng/ml) for the indicated times were analysed by western blot with an anti-PMCA4 specific antibody (α-PMCA4) to determine the expression levels of PMCA4. Histogram shows data as mean ± S. E. of three independent experiments. Differences between results were evaluated by one-way ANOVA and Newman-Keuls post hoc test. * and ## indicate statistically significant differences $P \leq 0.05$ and $P \leq 0.01$, respectively when comparing PMCA4 expression levels at 4 or 6 hours vs expression in non-stimulated cells (0 h).

Materials and Methods

Cells and cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from PromoCell or TCS Cellworks, and were cultured in 0.1% gelatin-coated tissue culture flasks containing Endothelial Cell Growth Medium (ECGM; PromoCell) supplemented with ECGM-supplement mix (PromoCell) and 1% penicillin/streptomycin. HUVECs were used at passages 5-8.

Mouse lung endothelial cells (MLECs) were purified from wild-type or PMCA4 null mice. Purification and culture of MLECs was as described.[1] Mice deficient in PMCA4 have been previously characterized.[2]

When indicated HUVEC or MLEC were stimulated with VEGF at 25 or 50 ng/ml, respectively. RCAN1.4 and Cox-2 expression levels in endothelial cells were determined after 2 hours (for RNA) or 4 hours (for protein expression) of stimulation with VEGF, as these time points have been previously reported to show optimum expression levels of RNA and protein for these genes.[3,4] Assays of endothelial cell migration or tube formation in matrigel, which require activation of several cellular mechanisms, were performed using longer incubation times (24 h) as previously reported.[5]

Adenovirus

Generation of adenoviruses Ad-LacZ and Ad-PMCA4 have been previously described.[6] Ad-NFAT-Luc is a replication-deficient adenovirus harbouring an NFAT-dependent luciferase-based reporter system. To generate Ad-NFAT-Luc, the plasmid pNFAT-TA-Luc (Clontech) was digested with Kpn I and Sal I. The fragment containing the NFAT-dependent luciferase reporter system (three tandem copies of the NFAT consensus sequence, the TATA box of the herpes simplex virus thymidine kinase promoter and the firefly luciferase reporter gene) was purified and sub-cloned into the Kpn I and Sal I sites of the pENTR11 shuttle vector (Invitrogen). The resulting plasmid was subjected to recombination with the pAd-DEST vector (Invitrogen) using the LR Clonase II enzyme system following the protocol recommended by the manufacturer.

Adenovirus Ad-ID4 encodes a 3xFlag-tagged version of the region encompassing amino acids 428 to 651 of human PMCA4b (corresponding to the domain of PMCA4b implicated in the interaction with calcineurin A). To generate Ad-ID4, the region of pF-PMCA4b(428-651)[7] that encodes three copies of the Flag epitope fused to the domain 428-651 of PMCA4b was amplified by PCR using plasmid pF-PMCA4b(428-651) as a template and oligonucleotides BamHI-PMCA4b sense (5'-TCTTCgCggATCCTTAgTgAACCgTCAgAATTAACC-3') and XhoI-PMCA4b antisense (5'-TCTTCCgCTCgAgTCAAgAgggCTCTgTgTCATCgAA-3'). The amplified fragment was cloned into the BamHI and XhoI sites of the pENTR11 shuttle vector (Invitrogen). The resulting plasmid was subjected to recombination with the pAd-DEST vector (Invitrogen) using the LR Clonase II enzyme system following the protocol recommended by the manufacturer.

Adenovirus particles were generated in HEK293 cells as previously described.[6]

Luciferase reporter assay

HUVECs or MLECs infected with the corresponding adenovirus were serum-starved in basal medium containing 0.5% fetal calf serum for 16 hours and subsequently stimulated as indicated for 6 hours.

Luciferase activity was determined as described.[8]

Western blot

HUVECs or MLECs infected with the corresponding adenovirus for 72 hours were serum-starved in basal medium containing 0.5% fetal calf serum for 16 hours and stimulated as indicated. Western blot procedures were as described.[9]

Western blot membranes were incubated with a 1:500 dilution of the JA3 mouse monoclonal anti-PMCA4 antibody (Santa Cruz) or a 1:10000 dilution of a rabbit anti-PMCA4 antibody (Swant) (for PMCA4 detection), a 1:10000 dilution of a rabbit anti-PMCA1 antibody (Swant) (for PMCA1 detection), a 1:1000 dilution of a rabbit anti-DSCR1 antibody (Sigma) (for RCAN1.4 detection), a 1:2500 dilution of a mouse monoclonal anti-tubulin antibody (Sigma) (for tubulin detection), a 1:1000 dilution of a rabbit anti-phospho-Erk1/2 (Thr202/Tyr204) antibody (Cell Signaling) (for detection of phosphorylated (activated) Erk1/2) or a 1:1000 dilution of a rabbit anti-Erk1/2 antibody (Cell Signaling) (for detection of total Erk1/2). Primary antibodies were detected by incubation in either a 1:7500 dilution of peroxidase-conjugated sheep anti-mouse immunoglobuilin antibody (Sigma) or 1:7500 dilution of peroxidase-conjugated donkey anti-rabbit immunoglobulin antibody (GE Healthcare) depending of the origin of the primary antibody. All antibody dilutions were carried out in TBS-T.

Immunoprecipitation
Protein lysates from infected cells were immunoprecipitated using the anti-pan PMCA monoclonal antibody 5F10 as previously described.[10]
Immunoprecipitated proteins were analyzed by western blot.

Polymerase chain reaction
RNA from HUVECs or MLECs was extracted using the "Total RNA purification kit" (Norgen) and cDNA synthesis was performed with 0.5 µg of total RNA using the "High capacity cDNA reverse transcription kit" (Applied Biosystems).
Presence of RNA encoding for PMCA1 and PMCA4 in MLEC cells was determined by semi-quantitative PCR performed on retro-transcribed samples using oligonucleotides mousePMCA1sense (5'-TCTggCTACggAACCACCCA-3') and mouse PMCA1anti (5'-AAAggCTTCCCgCCAAACTg-3') or mousePMCA4sense (5'-CAAgCCTCTgATCTCCCgCA-3') and mousePMCA4anti (5'-CCACTgCTCCATggTCAggC-3'). Amplification of the housekeeping gene Hprt1 was performed with oligonucleotides mouseHprt1sense (5'- CCTAAgATgATCgCAAgTTg-3') and mouseHprt1anti (5'- CCACAgggACTAgAACACCTgCTAA-3') to verify equal RNA levels in both samples. PCR reactions were analyzed by agarose gel electrophoresis.
cDNA samples resulting from retro-transcription reactions with RNA isolated from HUVEC cells were subjected to SYBR green real-time PCR using oligonucleotides RCAN1.4forward (5'-CTCACTAggggCTTgACTgC-3') and RCAN1.4reverse (5'-CAggCAATCAgggAgCTAAA-3') for amplification of human RCAN1.4, or COX-2forward (5'-gAAgAAAgTTCATCCCTgATCCC-3') and COX-2reverse (5'-CTgggCAAAgAATgCAAACA-3') for amplification of human COX-2, in a Stratagene MX3000P thermal cycler using the following conditions, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The Ct value was normalized using the Ct values for the housekeeping genes Ubiquitin and YwHaz.
Analysis of RT-qPCR data was carried out using the comparative $2^{-\Delta\Delta CT}$ method.

siRNA gene knock-down
siRNA-mediated knock-down was performed using "ON-TARGET-*plus* SMART pool human ATP2B4" (Thermo Scientific) targeting PMCA4, or "ON-TARGET-*plus* non-targeting pool control duplexes" (Thermo Scientific). HUVECs (0.5×10^6) were transfected with 30 pmoles of siRNAs by electroporation using an AMAXA HUVEC nucleofector kit (AMAXA).

Wound healing migration assay
Cell migration of HUVECs (infected with the corresponding adenovirus) and MLECs was performed with the "Cytoselect™ 24-well wound healing assay kit" (Cell Biolabs). Images were recorded with a Nikon DSFi1 digital camera mounted on a Nikon

ECLIPSE TS100 microscope at 4x magnification. The cell-free area was calculated with ImageJ software.

Matrigel tube formation assay
HUVECs infected with adenovirus or transfected with siRNA, were seeded onto Geltrex Reduced Growth-Factor Matrix (Invitrogen) in Medium 200 without phenol red, supplemented with 2% fetal calf serum (containing 50 ng/ml VEGF where indicated) in 96-well tissue culture plates at a density of 3 x 10^4 cells/well. Tube formation was quantified after incubation at 37º for 24 hours. Tube formation was recorded with a Nikon DSFi1 digital camera mounted on a Nikon ECLIPSE TS100 microscope at 4x magnification.

***In vivo* Matrigel plug assay**
The use of adenovirus embedded in matrigel plugs is a well-established method for the delivery of exogenous proteins into endothelial cells that vascularize the plug.[11-13] Angiogenesis was quantified *in vivo* by Matrigel plug assays as described[4], with the following modifications: for analysis of the effect of PMCA4 overexpression, the Matrigel mixture contained Growth-Factor Reduced Matrigel (BD Bioscience) VEGF (500 ng/ml), heparin (376 µg/ml) and 2 x 10^9 p.f.u. of the corresponding adenovirus (Ad-LacZ or Ad-PMCA4). The Matrigel mixture was implanted subcutaneously in 4-week-old anesthetized C57BL/6 mice, and 7 days later implants were collected and analyzed. For angiogenesis measurements in the absence of PMCA4, no adenovirus was included in the implant mixture, and the experiments were performed in 4-week-old PMCA4 knock-out mice or their corresponding wild-type littermates.
After seven days, mice were euthanized and Matrigel plugs were collected and divided into two portions for measurement of hemoglobin content and blood vessel number as described.[4]
Experiments were performed in accordance with the guidelines of the EU on animal care, and approved by the Animal Care and Ethics committee at Centro Nacional de Investigaciones Cardiovasculares.

Murine hindlimb ischaemia
Unilateral hindlimb ischaemia was induced by double ligation, and subsequent removal, of the left femoral artery in 12-week-old PMCA4 wild type or knockout mice under 2% isoflurane anesthesia. Blood flow was measured in anesthetized animals at 5 and 14 days after surgery using a Laser Doppler Perfusion Imager System (Moor Instruments). The ischemic to non-ischemic foot blood flow ratio was used as an index of blood flow recovery.
Experiments were performed in accordance with the guidelines of the EU on animal care, and approved by the Animal Care and Ethics committee at Centro Nacional de Investigaciones Cardiovasculares.

MTT assay
HUVECs infected with Ad-LacZ or Ad-PMCA4 were seeded at a density of 2 x 10^4 cells/well in ECGM with supplement mixture in 96-well tissue culture plates (without gelatin pre-coating). The MTT assay was performed, as described[14], the morning after plating the cells (time=0) and then after 3 and 6 days of incubation in ECGM with supplement mixture containing VEGF (25 ng/ml).

Statistical analysis
Results are expressed as Mean ± S.E. The Student's *t*-test was used to determine the significance of differences between two groups. In supplementary figure Fig VII, comparison among groups was determined by one-way ANOVA with post hoc Newman-Keuls comparison test. Differences with $P \leq 0.05$ were considered statistically significant.

References Materials and Methods

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