Constriction of Retinal Arterioles to Endothelin-1: Requisite Role of Rho Kinase Independent of Protein Kinase C and L-Type Calcium Channels

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Purpose. Although endothelin-1 (ET-1) is a potent vasoconstrictor peptide implicated in several retinal pathologies, the underlying mechanism of vasoconstriction is understood incompletely. We addressed this issue by assessing the contributions of extracellular calcium (Ca^{2+}), L-type voltage-operated calcium channels (L-VOCCs), Rho kinase (ROCK), and protein kinase C (PKC) to ET-1-induced constriction of porcine retinal arterioles, all of which have been implicated commonly in vascular smooth muscle contraction.

Methods. Porcine retinal arterioles (~50–100 μm) were isolated for vasomotor study and molecular assessment of ROCK isoforms.

Results. Isolated arterioles developed stable basal tone at 55 cmH_2O luminal pressure and constricted to ET-1 (0.1 nM) with a 40 ± 6% reduction in resting diameter in 20 minutes. In the absence of extraluminal Ca^{2+}, arterioles lost basal tone and failed to constrict to ET-1. Although L-VOCC inhibitor nifedipine reduced basal tone and blocked vasoconstriction to PKC activator PDBu, vasoconstriction to ET-1 was unaffected. The broad-spectrum PKC inhibitor Gö-6983 abolished vasoconstriction to PDBu, but did not alter ET-1-induced vasoconstriction or basal tone. Incubation of arterioles with ROCK inhibitor H-1152 abolished basal tone and vasoconstrictions to ET-1 and PDBu. Both ROCK1 and ROCK2 isoforms were expressed in the retinal arteriolar wall.

Conclusions. Extracellular Ca^{2+} entry via L-VOCCs and basal ROCK activity play important roles in the maintenance of basal tones of porcine retinal arterioles. ET-1-induced constriction is mediated by extracellular Ca^{2+} entry independent of L-VOCCs and by ROCK activation without the involvement of PKC. However, direct PKC activation can cause vasoconstriction via L-VOCC and ROCK signaling. (*Invest Ophthalmol Vis Sci.* 2012;53:2904–2912) DOI:10.1167/iovs.12-9542

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Endothelin-1 (ET-1) is a 21 amino acid peptide synthesized by vascular endothelial cells.[1] It is a potent vasoconstrictor with roles in both physiologic and pathophysiologic contexts in the cardiovascular system.[2] In the eye, increased ET-1 has been implicated in the pathogenesis of retinal vein occlusion,[3] open angle glaucoma,[4] and diabetic retinopathy.[5,6] Since retinal arterioles are capable of synthesizing and releasing ET-1,[7] elevated levels of ET-1 may contribute to retinal hypoxia or ischemia and subsequently manifest as each of these diseases. However, the mechanistic action of ET-1 in retinal arterioles remains understood incompletely.

It has been well characterized that vascular smooth muscle contraction is calcium (Ca^{2+})-dependent, with Ca^{2+} entry occurring through several types of channels, including L-type voltage-operated calcium channels (L-VOCCs).[8,9] It also is known that the process of contraction is coupled to the level of myosin light chain (MLC) phosphorylation,[10–12] which is regulated by the Ca^{2+}-dependent activity of MLC kinase (MLCK)[13–15] and by the MLC phosphatase (MLCP).[16] Activation of Rho kinase (ROCK) has been implicated in vascular smooth muscle contraction, with its predominant role being enhancement of MLC phosphorylation via inhibition of MLCP.[17,18] However, the role of ROCK activation in mediating vasoconstriction to ET-1 and the expression/distribution of ROCK isoforms in retinal arterioles remain unknown.

We demonstrated previously the presence of the requisite components for ET-1 synthesis as well as this peptide's vasoconstrictor action in porcine retinal arterioles mediated by activation of the ET_A receptor subtype on vascular smooth muscle cells.[7] The ET_A receptor is a G-protein-coupled receptor, which upon stimulation leads to activation of several different downstream pathways, including activation of phospholipase C to produce inositol 1,4,5-triphosphate (IP_3) and diacylglycerol.[2] These molecules generally lead to increased intracellular Ca^{2+},[19] and activation of protein kinase C (PKC),[20] respectively, to elicit vasoconstriction.[21,22] Although this classic explanation conceptually is legitimate, there is a paucity of experimental studies regarding the signaling molecules and pathways involved in vasoconstriction to ET-1 in the retinal circulation at the level of small resistance arterioles.

In the present study, we investigated the contribution of extracellular Ca^{2+} entry through L-VOCCs, the role of ROCK, and the possible involvement of PKC in the maintenance of basal tone and mediation of ET-1-induced constriction of retinal arterioles. To address these issues directly, we used an isolated vessel approach, thereby eliminating confounding influences from surrounding neuroglial tissue and hemodynamic changes that are inherent commonly in in vivo preparations.

METHODS

Animal Preparation

All animal procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Scott and White Institutional Animal Care and Use Committee. Pigs of either sex (age range 8–12 weeks, weight 8–21 kg) purchased from Real Farms (San Antonio, TX) were sedated with Telazol (4–8 mg/kg, intramuscularly) and intubated. The procedure used for harvesting eyes has been described previously.[7]

Isolation and Cannulation of Microvessels

The techniques used for identification, isolation, cannulation, pressurization and visualization of the retinal vasculature have been described previously.[7,23] In brief, the isolated retinal arterioles (~80 μm in situ) were cannulated with a pair of glass micropipettes and pressurized to 55 cmH_2O intraluminal pressure without flow by two independent pressure reservoir systems.[24] Vasomotor activity of isolated vessels was recorded continuously using videomicroscopic techniques[25] throughout the experiments.

Study of Vasomotor Function

Cannulated, pressurized arterioles were bathed in physiological saline solution with albumin (PSS-albumin, 0.1%) at 36°C to 37°C to allow the development of basal tone (stable within 90 minutes). In the first series of studies, the involvement of extracellular Ca^{2+}, the role of Ca^{2+} entry via L-VOCCs, and the activation of PKC and ROCK as signaling molecules in the maintenance of resting tone and in the initiation of vasoconstriction to ET-1 were assessed. The arterioles with tone were incubated in Ca^{2+}-free PSS-albumin containing 1 mM EDTA, or in normal PSS-albumin containing the dihydropyridine L-VOCC blocker nifedipine (1 μM), the broad-spectrum PKC inhibitor Gö-6983[26] (3 μM), or the non-selective ROCK inhibitor H-1152 (10 μM). The resulting diameter changes over a period of 20 minutes were recorded. After establishing a new stable level of vascular tone, these vessels subsequently were exposed to ET-1 (0.1 nM) and the changes in vascular diameter were monitored for an additional 20 minutes. Because some of the aforementioned inhibitors caused a reduction in vascular tone, in another set of experiments the vessels were incubated with the endothelium-independent vasodilator sodium nitroprusside (SNP, 30 μM) for 20 minutes before administration of ET-1 (0.1 nM, 20 minutes) for comparison.

In the second series of studies, the contributions of extracellular Ca^{2+}, L-VOCCs, PKC and ROCK to the maintenance of vasoconstriction to ET-1 were examined. Arterioles with tone were exposed first to ET-1 (0.1 nM) for 20 minutes to establish stable vasoconstriction and then incubated with Ca^{2+}-free PSS-albumin or with nifedipine (1 μM), Gö-6983 (3 μM), or H-1152 (3 and 10 μM) for an additional 20 minutes.

In the final series of studies, the signaling pathway for vasoconstriction in response to direct PKC activation was investigated. Following development of basal tone, arterioles were incubated with the PKC activator phorbol-12,13-dibutyrate (PDBu, 0.1 μM) for 20 minutes before or after a 20-minute exposure to Gö-6983 (3 μM), nifedipine (1 μM) or H-1152 (3 μM). At the end of each experiment above, the maximum diameter of the vessels was obtained by incubating with a Ca^{2+}-free PSS-albumin solution containing 0.3 mM SNP.

Chemicals

ET-1 was obtained from BaChem (Bubendorf, Switzerland), PDBu from Tocris Bioscience (Ellisville, MO), albumin from USB (Cleveland, OH), H-1152 and Gö-6983 from EMD Chemicals (Gibbstown, NJ), and nifedipine and SNP from Sigma (St. Louis, MO). ET-1, SNP, and H-1152 were dissolved initially in water. PDBu and Gö-6983 were dissolved in dimethyl sulfoxide, and nifedipine in ethanol. All subsequent dilutions of drugs for use in experiments were performed using PSS.[7] The final concentrations of dimethyl sulfoxide in the vessel bath with PDBu and Gö-6983 were 0.001% and 0.03% by volume, respectively. The final concentration of ethanol in the vessel bath with nifedipine was 0.01% by volume. These solvent concentrations had no significant effect on vessel viability or maintenance of tone (data not shown).

Western Blot Analysis

Retinal arterioles of similar size to those used for functional studies were isolated and homogenized in lysis buffer. The protein content of each sample was quantified and separated by electrophoresis as described previously.[7] For electrophoresis, 20 μg of protein were loaded in each lane. Blotting and detection of proteins was carried out as described previously using the following primary antibodies: rabbit anti-ROCK1 or anti-ROCK2 (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-smooth muscle actin (anti-SMA, 1:20,000; Sigma).[7] After incubation with an appropriate secondary antibody (anti-mouse or anti-rabbit, 1:2000; Sigma), the proteins were visualized via enhanced chemiluminescence (Pierce, Rockford, IL).

Immunohistochemical Analysis

The immunohistochemical detection of ROCK1 and ROCK2 isoforms in the vascular wall was performed after the preparation of cryomicrotome sections of retinal arterioles. Techniques for immunohistochemical staining of the isolated retinal vasculature were described in our previous work.[7] Herein, we used four primary antibodies: goat anti-ROCK1 (1:100; Santa Cruz Biotechnology), rabbit anti-ROCK2 (1:100; Santa Cruz Biotechnology), mouse anti-endothelial nitric oxide synthase (eNOS, 1:100; BD Biosciences, San Diego, CA), or mouse anti-SMA (1:100; Sigma). Secondary antibodies that were used included FITC-conjugated anti-goat IgG (1:150; Jackson ImmunoResearch Laboratories, West Grove, PA) and Cy3-conjugated anti-rabbit IgG (1:150; Jackson ImmunoResearch Laboratories). Slides were observed using a fluorescence microscope (Axiovert 200, Zeiss, Thornwood, NY) for red (Cy3) and green (FITC) images. Merged images were created with ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

Data Analysis

Vessel diameters observed during experiments were normalized to resting vessel diameter following development of basal tone and are reported as percentages. A D'Agostino-Pearson test for normality was used to confirm normal distribution of the data. As appropriate, Student's *t*-test or repeated measures ANOVA with Tukey's multiple comparisons test were used to determine the level of significance of diameter changes in response to pharmacological interventions. Statistical analyses were carried out using Prism software (GraphPad, San Diego, CA). Data are reported as mean ± SEM. $P < 0.05$ was considered significant and n represents number of vessels (1 per pig per treatment group) used in functional studies.

RESULTS

Roles of L-VOCC, PKC, and ROCK in Maintenance of Basal Tone

All vessels ($n = 112$) developed a similar level of stable basal tone (42 ± 1% of maximum diameter) in PSS-albumin. The average resting and maximum diameters of the vessels were 36 ± 1 μm (range 18–68 μm) and 84 ± 1 μm (range 51–109 μm), respectively. Changing the vessel bath from PSS-albumin to a

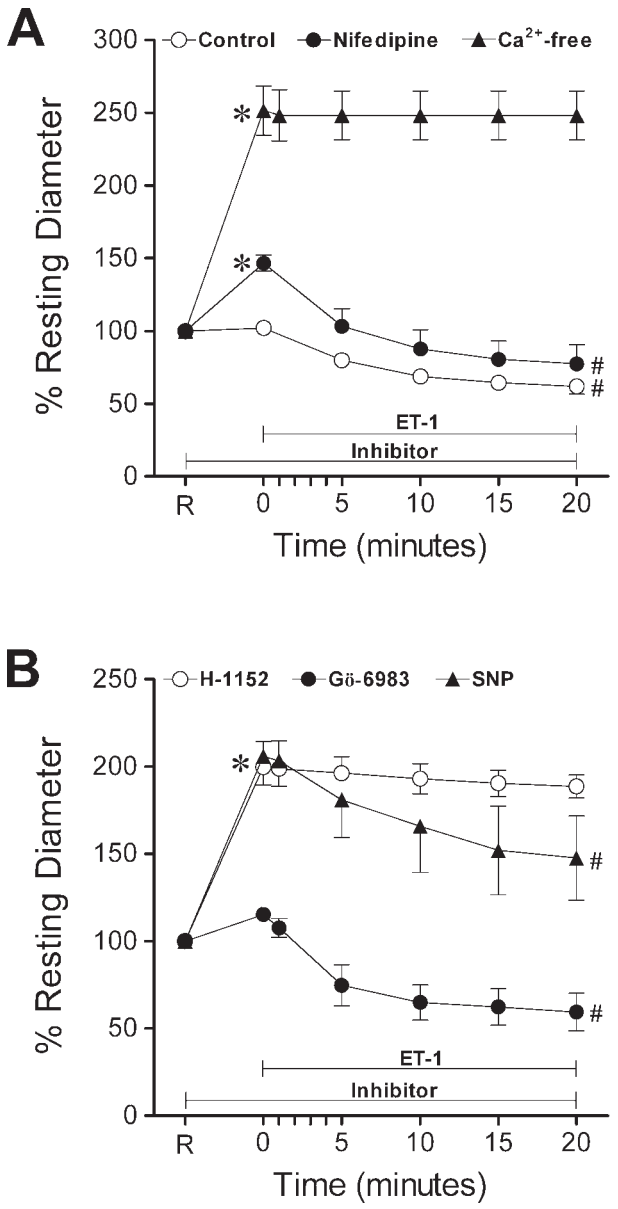

Figure 1. Prevention of ET-1-induced vasoconstriction. (**A**) Temporal course of vasoconstriction induced by ET-1 (0.1 nM, $n = 7$) in the presence of basal tone shown as the control response. Pre-treatment of vessels with basal tone with nifedipine (1 μM, $n = 4$) or pre-incubation in a Ca^{2+}-free solution ($n = 3$) for 20 minutes was followed by ET-1 (0.1 nM) treatment for 20 minutes. (**B**) Vessels with basal tone were pre-treated with SNP (30 μM, $n = 3$), H-1152 (10 μM, $n = 6$) or Gö-6983 (3 μM, $n = 5$) for 20 minutes before incubation with ET-1 (0.1 nM) for 20 minutes. R, resting diameter of vessels. $^*P < 0.05$ versus percent resting diameter at R. $\#P < 0.05$ versus percent resting diameter observed at time of addition of 0.1 nM ET-1 (0 minutes).

Ca^{2+}-free solution elicited a significant vasodilation, increasing resting diameter about 2.5-fold (i.e., reaching 97 ± 1% of maximum diameter, Fig. 1A). Incubation of vessels with the dihydropyridine L-VOCC blocker nifedipine (1 μM) led to a 41 ± 3% increase in resting diameter (Fig. 1A). The broad-spectrum PKC inhibitor Gö-6983[26] (3 μM) did not cause a significant change in resting diameter (Fig. 1B). In contrast, the ROCK inhibitor H-1152 (10 μM) and the endothelium-independent vasodilator SNP (30 μM) both caused approximately 2-fold increases in diameter, reaching near maximum dilation of the vessels (i.e., 98 ± 1% and 92 ± 2% of maximum diameter, respectively, Fig. 1B).

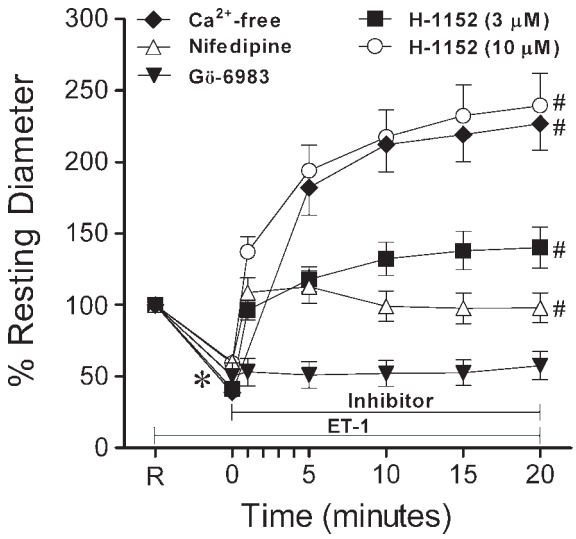

Figure 2. Reversal of ET-1-induced vasoconstriction. Vessels treated with ET-1 (0.1 nM) for 20 minutes constricted to a stable diameter, then were exposed to Ca^{2+}-free solution ($n = 6$) or treated with 10 μM ($n = 8$) or 3 μM ($n = 14$) H-1152, nifedipine (1 μM, $n = 6$), or Gö-6983 (3 μM, $n = 8$) for 20 minutes. R, resting diameter of vessels. $^*P < 0.05$ versus percent resting diameter at R. $\#P < 0.05$ for percent resting diameter values at 20 minutes versus percent resting diameter observed at time of addition of 0.1 nM ET-1 (0 minutes).

Roles of L-VOCC, PKC, and ROCK in Vasoconstriction to ET-1

As shown in Figure 1A, ET-1 (0.1 nM) caused a gradual vasoconstriction, stabilizing within 15–20 minutes and yielding an average of 40 ± 6% reduction in resting diameter. In the absence of extraluminal Ca^{2+}, ET-1-induced constriction was not observed. In contrast, retinal arteriolar constriction to ET-1 remained in the presence of nifedipine (1 μM) and vasoconstriction reached the same magnitude as that of control vessels (Fig. 1A). Pre-treatment of vessels with the PKC inhibitor Gö-6983 had no effect on the ability of vessels to constrict to ET-1, but the ROCK inhibitor H-1152 (10 μM) blocked ET-1-induced vasoconstriction (Fig. 1B). In contrast, while pre-treatment of vessels with SNP (30 μM) caused a reduction in tone similar to that observed with H-1152, no inhibition of ET-1-induced constriction was observed (Fig. 1B).

As shown in Figure 2, ET-1 (0.1 nM) caused a similar reduction in diameter in all groups of vessels tested. This vasoconstriction was reversed by a 20-minute exposure to Ca^{2+}-free solution, with vessel diameters increased to 227 ± 18% of the original resting level, corresponding to 95 ± 1% of maximum diameter. Treatment of ET-1-constricted vessels with nifedipine (1 μM) yielded vasodilation within one minute of drug administration, followed by a gradual constriction, whereas Gö-6983 did not cause any significant change in diameter (Fig. 2). Dose-dependent vasodilations to H-1152 (3 μM and 10 μM) were observed in vessels constricted with ET-1 (Fig. 2).

Roles of L-VOCC, PKC, and ROCK in Vasoconstriction to PDBu

Administration of PDBu (0.1 μM) caused significant constriction of retinal arterioles with an average of 59 ± 5% reduction

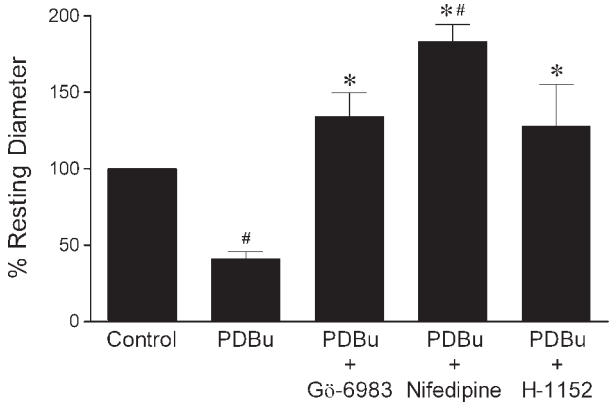

Figure 3. Reversal of PDBu-induced vasoconstriction. Vessels with basal tone were treated with PDBu (0.1 μM) for 20 minutes to produce vasoconstriction, followed by treatment with Gö-6983 (3 μM, $n = 5$), nifedipine (1 μM, $n = 6$), or H-1152 (3 μM, $n = 8$) for 20 minutes. #$P <$ 0.05 versus control. *$P < 0.05$ versus PDBu treatment.

in diameter, and this vasoconstriction was reversed by Gö-6983 (3 μM), nifedipine (1 μM), or H-1152 (3 μM) (Fig. 3). In the presence of Gö-6983 (3 μM), basal vascular tone was unaltered, but the vessels failed to constrict to PDBu (Fig. 4A). Nifedipine (1 μM) pre-treatment led to a decrease in tone of retinal arterioles, and subsequent addition of PDBu caused further dilation (Fig. 4B). As shown in Figure 4C, treatment of the vessels with H-1152 (3 μM) caused vasodilation and prevented PDBu-induced constriction.

ROCK Isoform Expression in Retinal Arterioles

Retinal arterioles express both ROCK1 and ROCK2 isoforms (Fig. 5A), and as shown by tissue immunofluorescence analysis, ROCK1 and ROCK2 staining in the arteriolar wall overlap with SMA (Fig. 6A) and eNOS (Fig. 6B) staining.

Discussion

Elevated vitreous and/or plasma levels of ET-1 have been implicated in several retinal pathologies, including diabetic retinopathy,[6] glaucoma[4] and retinal vein occlusion.[3] Although increased levels of ET-1 may contribute to ischemia in various disease contexts due to its marked potency as a vasoconstrictor,[3,4,27,28] the signaling pathways underlying retinal vasoconstriction remain elusive.[7] Herein, we have demonstrated a central role for extracellular Ca^{2+} and ROCK activation, and a lack of either L-VOCC or PKC involvement in ET-1-induced constriction of the porcine retinal vasculature. Moreover, we found that L-VOCC activity is important in maintenance of basal vascular tone and can be induced by PKC activation, thereby contributing to PKC-mediated vasoconstriction. PKC activation also leads to ROCK activation as part of its signaling cascade, causing constriction of the retinal vessels. The PKC-independent vasoconstriction to ET-1 is a unique feature in retinal arterioles because it is distinct from several other vascular beds wherein PKC activation has been shown to be involved in the signaling pathway for vasoconstriction to ET-1.[29,30]

It has been shown that ocular tissues, including the retinal vasculature, highly express ET-1,[31] which has been shown to decrease ocular blood flow in vivo.[32] Moreover, ET-1 participates in retinal blood flow regulation in humans because administration of an ET-1 receptor antagonist specifically blunts retinal arteriolar constriction in response to systemic blood

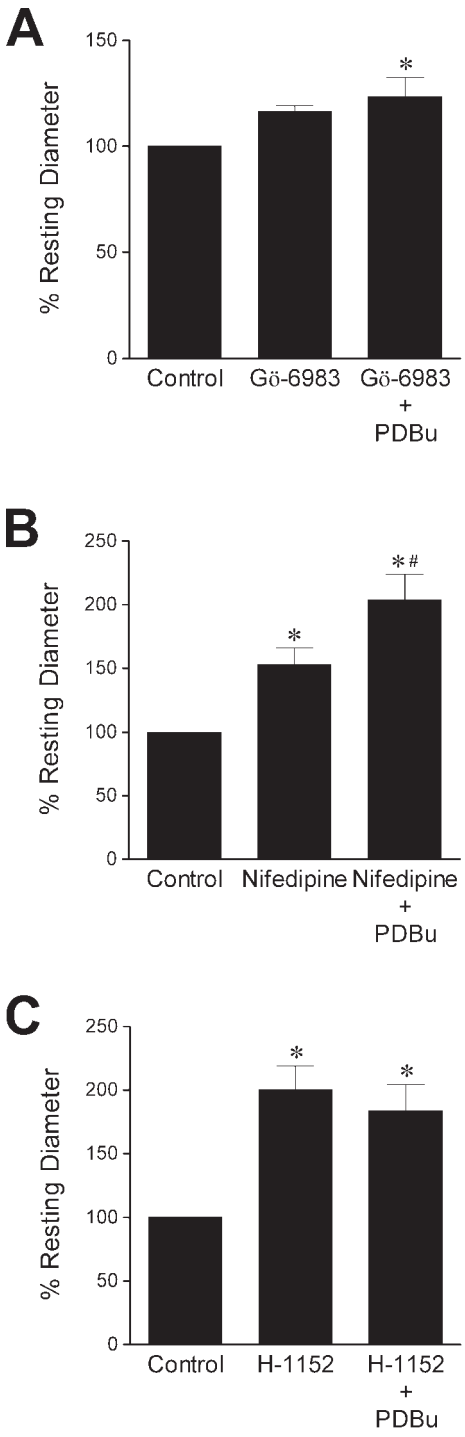

Figure 4. Prevention of PDBu-induced vasoconstriction. Vessels with basal tone were pre-treated for 20 minutes with (**A**) Gö-6983 (3 μM, $n =$ 6), (**B**) nifedipine (1 μM, $n = 5$), or (**C**) H-1152 (3 μM, $n = 7$), followed by treatment with PDBu (0.1 μM) for 20 minutes. *$P < 0.05$ versus control. #$P < 0.05$ versus nifedipine pre-treatment.

pressure elevation.[33] Physiologic plasma concentrations of ET-1 have been reported in the low picomolar range.[2,34] Normal vitreous concentrations of ET-1 are up to seven-fold higher than those in plasma,[35] and a range of elevated vitreous concentrations from as low as ~7 pM to as high as ~46 pM has been reported in patients with glaucoma,[36,37] branch retinal vein occlusion,[38] or diabetic retinopathy.[35,39] While the exact local

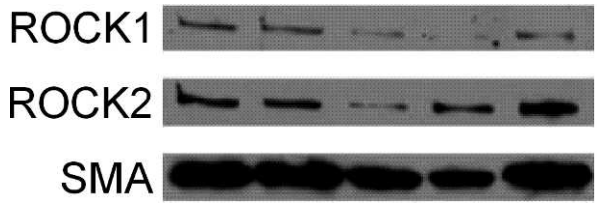

Figure 5. ROCK1 and ROCK2 protein expression in retinal arterioles. Immunoblot shows both ROCK isoforms expressed in arterioles with SMA as a loading control.

concentration of ET-1 at the level of the microvasculature is unknown, the concentration of ET-1 (0.1 nM) used in the present study is around the clinical and experimental range (nM) predicted.[40]

It has been suggested that the mechanisms involved with maintenance of basal tone are either distinct[41–43] or overlapping[44,45] with those for agonist-induced constriction. Hence, we characterized some of the signaling mechanisms involved with tone maintenance in an effort to obviate potential misinterpretation of our ET-1 constriction studies. We showed previously that extracellular Ca^{2+} is necessary for maintenance of basal tone at physiologic intraluminal pressure.[23] Administration of the L-VOCC blocker nifedipine caused a significant vasodilation, suggesting the importance of this channel for extracellular Ca^{2+} entry in maintenance of basal tone of retinal arterioles. It appears that initiation of basal tone also is dependent in part on activation of L-VOCC because nifedipine has been shown to diminish the development of pressure-induced tone in porcine[46] and bovine[47] retinal arterioles in vitro. However, the involvement of other Ca^{2+} entry pathways, including Na^+/Ca^{2+} exchangers,[48,49] and some members of the transient receptor potential channel family[50,51] have been reported for other vasculatures, and these may have a role in the retinal circulation as well.

As is the case for maintenance of basal tone, ET-1-induced vasoconstriction also is dependent upon extracellular Ca^{2+} because 1) ET-1 failed to elicit vasoconstriction in the absence of extraluminal Ca^{2+} (Fig. 1A), and 2) ET-1-induced vasoconstriction was abolished upon vessel exposure to Ca^{2+}-free solution (Fig. 2). However, entry of extracellular Ca^{2+} via L-VOCCs apparently has little role in mediating ET-1-induced constriction because nifedipine did not prevent this constriction. In fact, after 20 minutes of exposure of nifedipine-pretreated vessels to ET-1, the amount of constriction observed was indistinguishable from that seen under control conditions (i.e., without nifedipine, Fig. 1). Notably, constriction of retinal arterioles (i.e., reduction of diameter from a resting level of 40 ± 4% to 24 ± 4% of maximum diameter) to the L-VOCC activator Bay K 8644 (1 μM) was abolished by nifedipine (1 μM) in our pilot studies ($n = 8$, data not shown), supporting the specificity and efficacy of the nifedipine used in the present study. In light of nifedipine's inability to prevent vasoconstriction to ET-1, L-VOCCs appear not to contribute to the initiation of ET-1-induced vasoconstriction. Furthermore, nifedipine failed to reverse ET-1-induced vasoconstriction, as shown in Figure 2. In this series of experiments, the vessels were constricted to ~40% of their original diameter by ET-1 (Fig. 2). Based on the data shown in Figure 1, nifedipine is capable of reducing basal tone by ~40%. Taking into account the counteraction of these two vasomotor activities, it is predictable that the final vascular diameter observed after combined treatment with both compounds should be maintained at the original resting level if vasoconstriction to ET-1 is not affected by nifedipine. As shown in Figure 2, the steady state diameter of the vessels was returned to, and subsequently maintained at, the original resting level by offsetting constriction (due to ET-1) with dilation (due to loss of basal tone by nifedipine) of the vessels. It appears that the pathway of Ca^{2+} entry for vasoconstriction in response to ET-1 in retinal arterioles is distinct from that used for basal tone maintenance in that the former does not involve L-VOCC activation. In contrast to the present findings, a study addressing Ca^{2+} entry in ET-1-induced constriction of the bovine retinal vasculature showed that blockade of L-VOCCs with nitrendipine abolished tension development in response to ET-1.[53] This discrepancy may be related to the species difference, size of vessels (i.e., ~200 μm vs. ~80 μm in the present study) and/or the absence of basal tone in the previous bovine vessel preparations.[53] Nevertheless, our results are consistent with the minimal role of L-VOCCs in the sustained intracellular Ca^{2+} increase induced by ET-1 (0.1 nM) reported for freshly isolated smooth muscle cells from rabbit internal carotid artery.[52]

In addition to L-VOCC, store-operated calcium channels have been shown to contribute to Ca^{2+} entry in retinal arteriolar smooth muscle cells.[54] Depletion of IP_3-sensitive Ca^{2+} stores in the sarcoplasmic reticulum following agonist stimulation can lead to activation of store-operated calcium channels. Interestingly, recent evidence using confocal microscopic imaging demonstrates that ET-1 elicits an increase in intracellular Ca^{2+} via phospholipase C/IP_3 signaling in smooth muscle cells of rat retinal arterioles in situ.[55] Because the functional impact of this signaling pathway on vasoconstriction was not assessed, future studies investigating the Ca^{2+} entry mechanisms contributing to ET-1-induced constriction in retinal arterioles are warranted.

A role for PKC in myogenic tone has been suggested for vascular beds from various tissues in several different species.[44,56,57] However, in rat ophthalmic artery it was shown that PKC inhibition had no effect on vascular tone generated by a range of pressures between 75 mmHg and 160 mmHg, suggesting a relatively minimal role for PKC in tone maintenance.[58] The data presented in Figure 1B are in agreement with this finding because treatment with Gö-6983 had no effect on resting tone. This apparent lack of PKC involvement also is evident for ET-1-induced constriction because no prevention or reversal of constriction was observed with Gö-6983 treatment (Figs. 1B, 2). Importantly, we found that PKC activation by PDBu did cause retinal vasoconstriction, which was reversed (Fig. 3) and prevented (Fig. 4A) by treating vessels with Gö-6983. These data demonstrate the specificity of Gö-6983 for PKC inhibition and further support the conclusion that PKC is not involved in the retinal arteriolar constriction to ET-1, although its activation is capable of eliciting vasoconstriction. Contrastingly, a role for PKC in vascular contraction to ET-1 has been reported in several other vascular beds,[29,30,59,60] possibly due to the downstream production of diacylglycerol.[2,61] It appears that ET-1 elicits a distinct signaling pathway for vasoconstriction in the retinal microcirculation, at least in the porcine model, which recently has been shown to be similar to humans in vasomotor regulation.[23]

Given the comparable magnitude of vasoconstriction to PDBu with that to ET-1, we also tested the effects of nifedipine on PDBu-induced constriction to ascertain whether L-VOCC flux has a role in this context. As shown in Figure 3, nifedipine not only reversed PDBu-induced vasoconstriction but also caused vasodilation, that is, increased the vessel diameter beyond its resting level. Moreover, in the presence of nifedipine, PDBu failed to constrict retinal arterioles and instead, a vasodilation to PDBu was observed (Fig. 4B). Thus retinal vasoconstriction in response to PKC activation is dependent upon L-VOCC activation. Interestingly, some studies have implicated PKC isoforms in activation of eNOS.[62–64] Hence, it is possible that blocking L-VOCC-mediated contrac-

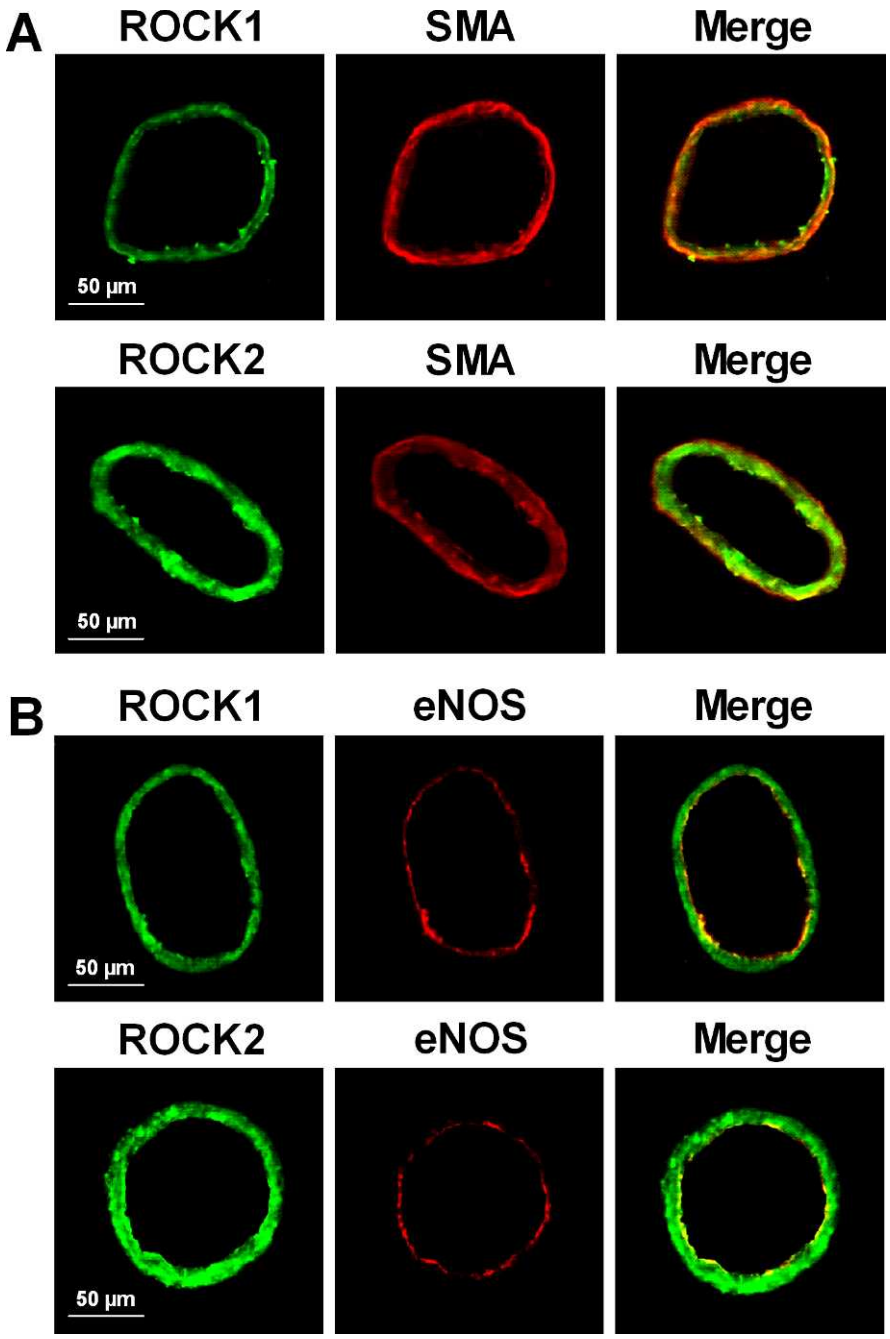

Figure 6. Immunohistochemical analysis of ROCK1 and ROCK2 isoforms in retinal arterioles. (**A**) Staining with anti-ROCK1 (*green*) or anti-ROCK2 (*green*) and anti-SMA (*red*) antibodies demonstrates expression of both ROCK isoforms and SMA. Merged images show overlap staining (*yellow*) of ROCK isoforms with SMA. (**B**) Staining with anti-ROCK1 (*green*) or anti-ROCK2 (*green*) and anti-eNOS (*red*) antibodies demonstrates expression of both ROCK isoforms and eNOS. Merged images show overlap staining (*yellow*) of ROCK isoforms with eNOS. Images are representative of three separate experiments.

tion of smooth muscle cells might unmask an endothelium-mediated response to PDBu, thus explaining the observed vasodilation. In light of a minimal role for L-VOCCs in ET-1-induced constriction, but a prominent role in PDBu-induced constriction, it seems that PKC activation linking to L-VOCC opening, although capable of evoking vasoconstriction, is not a part of the vasomotor signaling pathway induced by ET-1.

As shown in Figure 1B, ROCK inhibition led to a significant loss of basal tone, a finding in agreement with several studies from other vascular beds implicating ROCK in maintenance of vascular tone.[65–67] Pre-treatment with H-1152 prevented vasoconstriction to ET-1 (Fig. 1B), indicating that the development of ET-1-induced vasoconstriction depends on ROCK activity. The prevention of ET-1-induced vasoconstriction by ROCK inhibition was unlikely the result of vascular tone loss because vasoconstriction to ET-1 persisted in the presence of SNP, which produced a reduction of vascular tone comparable to that elicited by H-1152. As shown in Figure 2, vasoconstriction to ET-1 was reversed by H-1152 in a dose-dependent manner. Hence, these data show a prominent role of ROCK in maintenance of basal tone and ET-1-induced vasoconstriction. Interestingly, vasoconstriction to PDBu was reversed (Fig. 3)

and prevented (Fig. 4C) with H-1152, suggesting a pivotal role of ROCK in mediating vasoconstriction to PKC activation. This conclusion is in agreement with studies showing that PDBu-induced contraction of rat aortic rings involves activation of the RhoA/ROCK pathway[68] and that contraction of bovine coronary artery rings in response to PDBu is sensitive to ROCK inhibition.[69] At the concentrations used herein, H-1152 is capable of inhibiting Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII),[70] which has been suggested to have a role in vascular smooth muscle contraction.[71,72] However, in our pilot studies, pre-treatment of vessels with the CaMKII inhibitor KN-93 (3 μM) had no effect on either basal tone or ET-1-induced vasoconstriction ($n = 3$, data not shown). Hence, H-1152 is unlikely to be exerting the effects described here through inhibition of CaMKII.

Although the present functional studies suggest that ROCK is a convergent target for vasoconstriction to ET-1 and PKC activation, the expression of ROCK isoforms in the retinal microvasculature is unknown. The immunoblot data presented in Figure 5 show that ROCK1 and ROCK2 are expressed in retinal arterioles, and the immunofluorescence data in Figure 6 demonstrate expression of both isoforms in smooth muscle (Fig. 6A) and endothelium (Fig. 6B). Wang et al. demonstrated a direct interaction between the myosin binding subunit of MLCP and ROCK2, but not ROCK1 in A7r5 and primary rat aortic smooth muscle cells.[73] Moreover, silencing of either isoform led to reduced inhibitory phosphorylation of MLCP and reduced phospho-MLC levels, but there was significantly less contraction in ROCK2-silenced vascular smooth muscle cells relative to that in ROCK1-silenced or control cells,[73] suggesting a major role for ROCK2 in mediating vasomotor function. However, whether one isoform is responsible predominantly for ROCK-mediated tone maintenance or constriction to ET-1 and PDBu in the retinal vasculature is unknown and will be the subject of future investigation.

In summary, we demonstrated a central role for ROCK in maintenance of basal tone as well as in constriction of the retinal vasculature to ET-1 and PKC activation. We also showed that ET-1-induced constriction does not use the PKC/L-VOCC signaling pathway, although activation of this pathway does lead to vasoconstriction. To our knowledge, this is the first report of ET-1-induced vasoconstriction that does not lead to PKC activation. It is apparent that some of the classical mechanisms invoked for smooth muscle contraction may not be applicable necessarily to the constriction of retinal microvessels in response to ET-1. This study provides important insight into the ET-1-induced constriction mechanisms used by the retinal microcirculation, and lays a foundation for future, more detailed studies of this pathway. Understanding key components of this vasoconstriction mechanism and manipulating the ROCK signaling pathway may help in future development of more targeted therapeutics that could address the pathologic effects of ET-1 and PKC activation in the retina.

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