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Ca_v3.1 (α_{1G}) controls von Willebrand factor secretion in rat pulmonary microvascular endothelial cells

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Abstract

The T-type Ca²⁺ channel Ca_v3.1 subunit is present in pulmonary microvascular endothelial cells (PMVECs), but not in pulmonary artery endothelial cells (PAECs). The present study sought to assess the role of Ca_v3.1 in thrombin-induced Weibel-Palade body exocytosis and consequent von Willebrand factor (VWF) release. In PMVECs and PAECs transduced with a green fluorescent protein (GFP)-tagged VWF chimera, we examined the real-time dynamics and secretory process of VWF-GFP-containing vesicles in response to thrombin and the cAMP-elevating agent isoproterenol. Whereas thrombin stimulated a progressive decrease in the number of VWF-GFP-containing vesicles in both cell types, isoproterenol only decreased the number of VWF-GFP-containing vesicles in PAECs. In PMVECs, thrombin-induced decrease in the number of VWF-GFP-containing vesicles was nearly abolished by the T-type Ca²⁺ channel blocker mibefradil as well as by Ca_v3.1 gene silencing with small hairpin RNA. Expression of recombinant Ca_v3.1 subunit in PAECs resulted in pronounced increase in thrombin-stimulated Ca²⁺ entry, which is sensitive to mibefradil. Together, these data indicate that VWF secretion from lung endothelial cells is regulated by two distinct pathways involving Ca²⁺ or cAMP, and support the hypothesis that activation of Ca_v3.1 T-type Ca²⁺ channels in PMVECs provides a unique cytosolic Ca²⁺ source important for G_q-linked agonist-induced VWF release.

Keywords

endothelial cells; thrombin

The endothelium plays an essential role in regulating blood flow through its dynamic interaction with circulating blood cells. Under physiological conditions, individual

endothelial cells produce an anticoagulant and antiadhesive surface that facilitates passage of plasma and cellular components through the vasculature. Changes in the local extracellular environment, as may take place at the site of inflammation, disrupt this homeostasis and stimulate endothelial cells to create a procoagulant and proadhesive microenvironment, thus initiating the adhesion process. Although the molecular mechanisms involved in circulating cell-endothelium adhesion are not yet fully identified, emerging evidence indicates that an essential step in endothelial cell membrane transformation from an anticoagulant, antiadhesive to a proadhesive, prothrombotic surface is the induction of Weibel-Palade body (WPB) exocytosis (8,47).

WPBs are endothelial cell-specific, regulated secretory organelles (13,54,56) that contain a number of components including the adhesive protein von Willebrand factor (VWF) (39,40,55), the leukocyte adhesion molecule P-selectin (4,28), and the chemotactic cytokine IL-8 (48,57). The exocytosis of WPBs delivers VWF, P-selectin, and IL-8 to the cell surface (4,18,19,28,48,57) where they contribute to hemostasis and inflammation. The most biologically active VWFs are high-molecular-weight multimers stored within the luminal space of WPBs (43,44). These multimers are important ligands for platelet receptor glycoprotein Ib α and integrin α Ib β 3, which are involved in platelet adhesion and aggregation during vascular injury (14,38). P-selectin is a WPB membrane protein whose regulated appearance at the apical plasma membrane of endothelial cells initiates the binding and rolling of leukocytes on the endothelium and the consequent recruitment into interstitial tissue at sites of inflammation (7,15,21,27). IL-8 is colocalized with VWF within the luminal space of WPBs (48,57). The presentation of IL-8 on the endothelial cell surface provides an effective means for controlling local leukocyte extravasation (33). Thus regulated exocytosis of WPBs furnishes endothelial cells with an active capacity to rapidly and selectively change the microenvironment of each individual vascular bed and modulate the interrelated processes of coagulation and inflammation. In spite of this importance, mechanisms underlying WPB exocytosis are still poorly understood.

Stimulation of WPB exocytosis can be triggered by a variety of naturally occurring inflammatory mediators that act by two distinct signaling pathways, elevating cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) or intracellular cAMP levels (12,16,19,22,37,41,52,53). The exocytosis stimulated by G_q -linked neurohumoral inflammatory agonists is a Ca^{2+} -dependent process that requires Ca^{2+} entry from the extracellular space (6,12,16,18,25). Although specific Ca^{2+} entry pathways that promote WPB exocytosis are not known, it has been established that in the inflamed circulation, G_q -linked agonists, e.g., thrombin, increased $[\text{Ca}^{2+}]_i$, and the resultant $[\text{Ca}^{2+}]_i$ transitions are sufficient to trigger WPB exocytosis, causing VWF secretion and P-selectin expression at the membrane surface (1,2,16,25,32,49). We previously reported that pulmonary microvascular endothelial cells (PMVECs) express a $\text{Ca}_v3.1$ (α_{1G}) voltage-gated T-type Ca^{2+} channel, whereas pulmonary macrovascular (i.e., pulmonary artery) endothelial cells (PAECs) do not express voltage-gated Ca^{2+} channels. Thrombin-induced transitions in membrane potential activate the $\text{Ca}_v3.1$ channel, resulting in a physiologically relevant rise in $[\text{Ca}^{2+}]_i$. Furthermore, activation of the $\text{Ca}_v3.1$ channel induces a procoagulant endothelial phenotype, i.e., channel inhibition attenuates increased retention of sickled erythrocytes in the inflamed pulmonary circulation (58). In the present study, we sought to resolve whether PMVECs and PAECs differ in mechanisms regulating WPB exocytosis, and, furthermore, to determine whether Ca^{2+} entry through $\text{Ca}_v3.1$ T-type Ca^{2+} channels is an important amplification step in promoting the exocytosis of WPBs from lung microvascular endothelium.

MATERIALS AND METHODS

Isolation and culture of rat lung endothelial cells

Rat PAECs and PMVECs were isolated, cultured, and characterized as described previously (11,23,45). The protocol was approved by the Institutional Animal Care and Use Committee of the University of South Alabama (Protocol 02010). Cells used in all experiments were below *passage 12*.

Generation of stably Ca_v3.1-transduced PAECs

PAECs were transfected with the expression vector pcDNA3 (Invitrogen, Carlsbad, CA) encoding the rat Ca_v3.1a (31) (GenBank accession no. AF027984), i.e., pcDNA3-Ca_v3.1a (24), kindly provided by Dr. Edward Perez-Reyes (Univ. of Virginia). Cells were transfected with FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN). Positively transfected cells were selected with neomycin (400 µg/ml; Sigma, St. Louis, MO) and further analyzed by RT-PCR, Western blotting, voltage clamp electrophysiology recordings, and fluorometric assessments.

Construction of small hairpin RNAs targeting on Ca_v3.1

Oligonucleotides coding for small hairpin RNAs (shRNAs) that target on rat Ca_v3.1 and an unrelated (scrambled) shRNA were designed. The complementary oligonucleotides were annealed and cloned into a retroviral shRNA expression vector, RNAi-Ready pSIREN-RetroQ-ZsGreen Vector (BD Biosciences Clontech, Mountain View, CA), or into an HRST lentiviral vector (29) derivative encoding the reporter mCherry (GenBank accession no. AY678264) (42). Individual transduction of each of the shRNAs into cultured PMVECs was achieved by retro- or lentiviral delivery. Five designed oligonucleotide sequences for Ca_v3.1 shRNA and scrambled shRNA used in this study are listed in Table 1.

Construction of VWF-GFP expression vector

The VWF-green fluorescent protein (GFP) expression vector was constructed by replacing the VWF A2 domain with a sequence encoding GFP as described previously (34,35). A recombinant retroviral vector for delivery of the VWF-GFP cDNA was constructed using the dicistronic retroviral LZRS vector as described previously (20).

Production of recombinant retro- and lentivirus and endothelial cell transduction

The helper-free recombinant retrovirus was produced after transfection of the retroviral vector DNA into Phoenix-A cells (20) using Lipofectamine 2000 (Invitrogen). Lentivirus was produced using a 293T producer cell line and ViraPower Lentiviral Expression System (Invitrogen) according to the manufacturer's instructions. Viral supernatant was collected at consecutive times following the 24 or 36 h transfection of retro- or lentiviral vector, respectively. The collected supernatant was filtered through a 0.45-µm filter. For endothelial cell transduction, PMVECs or PAECs were grown to 20–40% confluency and subsequently infected with the harvested virus supernatant of the packaging cells. The transduction efficiency was tested by determining the percentage of GFP or mCherry positive cells using flow cytometry analysis with a BD FACSVantage SE flow cytometry system (Becton Dickinson, San Jose, CA).

Real-time quantitative RT-PCR analysis of Ca_v3.1 mRNA expression

Quantitative RT-PCR (qRT-PCR) analysis was performed using total RNA isolated from cells with TRIzol reagent (Invitrogen) and digested with DNase I (Ambion, Austin, TX). The primers and probes for rat Ca_v3.1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed and synthesized by Applied Biosystems (Foster City, CA). qRT-

PCR was conducted with a 7500HT Real-Time PCR system (Applied Biosystems). The reaction was conducted in triplicate ($n = 3$). The gene expression ΔC_t value (the difference in threshold cycles for target and reference) of $Ca_v3.1$ from each sample was calculated by normalizing with internal housekeeping gene GAPDH, and relative quantitation values of gene expression were plotted.

Western blot analysis

Whole cell protein extracts were analyzed by SDS-PAGE and Western blotting. $Ca_v3.1$ was detected with an anti- $Ca_v3.1$ antibody (Alomone Labs, Jerusalem, Israel; diluted 1:200). Protein bands were visualized using SuperSignal West Pico Chemiluminescent System (Pierce Biotechnology, Rockford, IL). Actin was probed in the same membrane to ensure equal protein loading.

Electrophysiology, data acquisition, and analysis

Patch-clamp recordings were performed in whole cell configuration as previously described (58).

$[Ca^{2+}]_i$ measurement

$[Ca^{2+}]_i$ was estimated using fura 2-AM (Molecular Probes, Eugene, OR) fluorometric assessment according to the method described previously (30,58,59).

VWF secretion assay

Cultured endothelial cells were grown to confluence in 35-mm dishes. Cells were gently washed three times with PBS and were incubated at 37°C and 5% CO_2 either with serum-free medium (500 μ l/well) alone or accompanied by thrombin or isoproterenol, with or without mibefradil (10 μ mol/l, IC_{100}). The media were collected for VWF measurement after 10 and 60 min of incubation. The VWF-GFP levels present in the medium were measured by sandwich-style ELISA using paired capture and detecting anti-VWF antibodies (Cedarlane Laboratories, Hornby, Ontario, Canada) on triplicate medium samples (100 μ l each) according to the manufacturer's instructions. The standard curve was generated by using standard normal reference human plasma with verified VWF concentration (Precision Biologic, Dartmouth, Nova Scotia, Canada). The quantity of VWF secretion was normalized to the number of endothelial cells present in each of the original 35-mm dishes. Data are expressed in relative values as human VWF concentration equivalence (means $\pm$ SE). One-way ANOVA followed by Newman-Keuls tests were used, and $P < 0.05$ was considered significant.

Real-time imaging and confocal microscopy

Real-time imaging was performed on living VWF-GFP-expressing PAECs and PMVECs to measure the decrease of the number of VWF-GFP-containing vesicles in response to thrombin or isoproterenol using an Ultraview RS confocal microscope (PerkinElmer Life and Analytical Sciences, Boston, MA). Briefly, the VWF-GFP-transduced PAECs or PMVECs were grown on 25-mm glass coverslips for 3–4 days. Cells were then mounted in an Attotfluor cell chamber (Molecular Probes) in 500 μ l of HEPES-buffered physiological salt solution (HPSS) containing (in mmol/l) 107 NaCl, 6 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4 , 2 $CaCl_2$, 11.5 D-glucose, 25 HEPES, pH 7.40, with NaOH. A $\times 60$, 1.20 numerical aperture water-immersion objective along with 488-nm excitation and 525-nm emission filters and a 488-nm single wavelength dichroic mirror were used for GFP imaging. A set of serial optical sections (Z-stacks) was taken from apical to basal cell aspects at a 0.2- to 0.3- μ m interval. Time-lapse images of GFP were generated by taking Z-stacks every 3 min during the 60-min experiments. Selected cells were first imaged for 6 min in the initial medium of

HPSS. Subsequently, thrombin or isoproterenol (10 μ l in 500 μ l of media; EC₁₀₀ concentration, both from Sigma) was gently applied, without disturbing the cells or changing the focal plane, and the recordings were continued for the rest of the experiment. All of the experiments were performed at room temperature (22–25°C).

Time-lapse image processing and analysis

Changes in total GFP following the stimulations were determined from time-lapse image recordings using the image processing and analysis software ImageJ (Research Services Branch, National Institute of Mental Health Sciences; <http://rsb.info.nih.gov/ij/>) with voxel counter plug-in that counts the thresholded voxels, i.e., the volume equivalent of pixels, in each of the Z-stacks. Background voxels and the voxels of GFP fluorescent vesicles were discriminated by applying a threshold to each Z-stack. For each time-lapse recording, data were normalized to the voxel count at *time 0* and expressed as means $\pm$ SE. One-way ANOVA followed by Newman-Keuls tests were used, and $P < 0.05$ was considered significant.

RESULTS

Thrombin-induced Ca²⁺ entry via the T-type Ca²⁺ channel in PMVECs

Lung macro- and microvascular endothelial cells exhibit distinct Ca²⁺ entry pathways that govern [Ca²⁺]_i transitions in response to G_q-linked agonists (46,58). The typical global [Ca²⁺]_i response of endothelial cells to G_q-linked agonists, e.g., thrombin, comprises the initial immediate [Ca²⁺]_i rise (first phase) that reflects release of Ca²⁺ from inositol 1,4,5-trisphosphate-sensitive intracellular Ca²⁺ stores and the second phase of [Ca²⁺]_i elevation that reflects Ca²⁺ entry across the cell membrane after Ca²⁺ store depletion due to activation of store- and receptor-operated Ca²⁺ channels. Prior work revealed that the concentration of thrombin, which maximally activated both Ca²⁺ release and Ca²⁺ entry, was 1 U/ml in PAECs and 10 U/ml in PMVECs (9). To evaluate the role of Ca_v3.1 T-type Ca²⁺ channels in thrombin-stimulated [Ca²⁺]_i transitions, we performed fluorometric assay using fura 2-AM-loaded cells to assess changes in [Ca²⁺]_i following stimulation with thrombin in the presence and absence of the T-type Ca²⁺ channel blocker mibefradil [IC₁₀₀ concentration; 10 μ mol/l (58)]. In both cell types, thrombin stimulation triggered a typical biphasic [Ca²⁺]_i response, yet only the Ca²⁺ entry phase in PMVECs was attenuated by mibefradil pretreatment (Fig. 1A), suggesting a major portion of Ca²⁺ entry is sensitive to T-type Ca²⁺ channel blockade. Mibefradil at the same concentration had no effect on the thrombin-induced [Ca²⁺]_i transitions in PAECs (Fig. 1B), which lack expression of T-type Ca²⁺ channels. These data suggest that the reduction of thrombin-induced Ca²⁺ entry caused by mibefradil in PMVECs is due to the inhibitory effect on T-type Ca²⁺ channels. To confirm that expression of T-type Ca²⁺ channels contributes to thrombin-induced Ca²⁺ entry, similar studies were performed in PAECs overexpressing Ca_v3.1. The Ca_v3.1 stably transduced PAECs were generated by transient transfection of an expression vector encoding rat Ca_v3.1a (pcDNA3-Ca_v3.1a) (31) and selected with neomycin. Expression of Ca_v3.1 in transduced PAECs was confirmed by RT-PCR (data not shown) and Western blot analysis (Fig. 2A). Utilizing step depolarizations along with a two-step voltage protocol, where we could detect the maximally evoked T-type current (58), with the holding potential set to -90 mV, we demonstrated that expression of Ca_v3.1 resulted in a typical T-type current in PAECs. The current displayed low-threshold voltage activation, activated at -60 mV and above, rapid activation and inactivation kinetics that produce a criss-crossing pattern between successive traces of stepwise current-voltage (*I-V*) protocol (Fig. 2, B and C) (31a). In these Ca_v3.1 functionally overexpressing PAECs, we consistently observed that thrombin-induced Ca²⁺ entry was remarkably elevated (Fig. 2D), and the resultant elevation of Ca²⁺ entry was completely abolished by mibefradil (Fig.

2, *E* and *F*). These results further support that activation of the $\text{Ca}_v3.1$ T-type Ca^{2+} channel contributes to thrombin-induced physiological $[\text{Ca}^{2+}]_i$ transition in PMVECs.

Distinct regulation of Ca^{2+} - and cAMP-dependent exocytosis of WPBs from PMVECs and PAECs

In addition to G_q -linked agonists that trigger exocytosis of WPBs through elevating $[\text{Ca}^{2+}]_i$, the exocytosis of WPBs can be triggered by agonists that increase intracellular cAMP as well, e.g., β -adrenergic agonists (1,49). We next assessed the Ca^{2+} - and cAMP-dependent regulated exocytosis of WPBs in endothelial cells. A GFP-tagged VWF chimera (VWF-GFP) was stably introduced into cultured PMVECs and PAECs by retroviral infection. It has been previously demonstrated that this VWF-GFP chimera is targeted to WPBs together with endogenous VWF and colocalizes with P-selectin (35). Transduction of VWF-GFP resulted in a central cytosolic appearance of fluorescent vesicles in both PMVECs and PAECs (Fig. 3, *A* and *B*, Video 1. Supplemental data for this article is available online at the AJP-Lung web site). This is consistent with the distribution pattern observed in VWF-GFP-expressing endothelial cells in prior studies utilizing the same VWF-GFP chimeras (17,35). Accordingly, fluorescent vesicles revealed by confocal microscopy in VWF-GFP-expressing PMVECs and PAECs resembled WPBs. By exploiting the intrinsic GFP fluorescence, this approach enables direct visualization of the translocation and dynamics of WPBs in living endothelial cells.

We next examined the real-time secretory process of WPBs in living endothelial cells in response to thrombin or the β -adrenergic agonist isoproterenol. In each experiment, the EC_{100} concentration of thrombin (10 U/ml for PMVECs and 1 U/ml for PAECs) or isoproterenol (1 $\mu\text{mol/l}$ for PMVECs and 10 $\mu\text{mol/l}$ for PAECs) was applied to PMVECs and PAECs to induce maximal Ca^{2+} entry across the cell membrane (the 2nd phase of thrombin-induced elevation in $[\text{Ca}^{2+}]_i$) (9) or maximal elevation in intracellular cAMP levels (45). The GFP fluorescence of the cells was monitored in real time during 60 min of stimulation at intervals of 3 min using time-lapse confocal microscopy. Cells subjected to vehicle application served as control. In VWF-GFP-expressing PMVECs or PAECs with no stimulation, a slow, random movement of VWF-GFP-containing vesicles was observed throughout the cell body, suggesting the occurrence of arbitrary trafficking of WPBs. Additionally, a slight decrease in the number of VWF-GFP-containing vesicles was detected during the 60-min period, possibly due to spontaneous release of fluorescent vesicles or photobleaching of green fluorescence. It appears that some photobleaching is indeed occurring with 21 measurements over 60 min because no decrease in fluorescence was observed with just two measurements over 60 min, i.e., at the beginning and at the end of the experiment. Nonetheless, in PMVECs and PAECs, following thrombin stimulation, a dramatic movement of VWF-GFP-containing vesicles was observed, and a rapid, progressive decrease in the number of fluorescent vesicles was detected, i.e., 85% ($\pm 2\%$ SE) decrease in PMVECs and 60% ($\pm 5\%$ SE) decrease in PAECs (Fig. 4, *A* and *B*) during 60 min of stimulation. A similar pattern of movement and decrease in number of fluorescent vesicles were also observed following isoproterenol stimulation in VWF-GFP-transduced PAECs (Fig. 4*C*) but not in PMVECs (Fig. 4, *A* and *B*). These results imply that Ca^{2+} - and cAMP-dependent pathways regulating exocytosis of WPBs may differ in PMVECs and PAECs.

Thrombin-induced exocytosis of WPBs from PMVECs is sensitive to pharmacological blockade of T-type Ca^{2+} channels

To test whether Ca^{2+} entry through T-type Ca^{2+} channels is required for the exocytosis of WPBs, we first applied mibefradil to endothelial cells. The VWF-GFP-expressing PMVECs or PAECs were subsequently stimulated with thrombin or isoproterenol. Exposure of cells to

mibefradil alone did not evoke exocytosis of VWF-GFP-containing vesicles (data not shown). However, mibefradil nearly abolished the thrombin-induced decrease in the number of VWF-GFP-containing vesicles in PMVECs (Fig. 5, *A* and *B*) but not in PAECs (Fig. 5*C*). Additionally, the effect of isoproterenol on the decrease in number of fluorescent vesicles in PAECs was not affected by application of mibefradil (Fig. 5*D*).

The effect of T-type Ca^{2+} channel blockade on exocytosis of WPBs was further investigated by assessing the amount of VWF-GFP released in the medium following stimulation of PMVECs with thrombin (10 U/ml) by ELISA. As expected, we observed a rapid increase in VWF levels in the supernatant of thrombin-stimulated VWF-GFP-expressing PMVECs (Fig. 5*E*), which paralleled the thrombin-induced decrease in VWF-GFP-containing vesicles observed with confocal live cell imaging (Fig. 4*C*). While T-type Ca^{2+} channel blockade with mibefradil did not change the basal level of VWF in the supernatant of nonstimulated PMVECs, it indeed nearly abolished the increase in VWF in the supernatant of thrombin-stimulated PMVECs (Fig. 5*E*).

Effects of $\text{Ca}_v3.1$ RNA interference on thrombin-stimulated $[\text{Ca}^{2+}]_i$ transitions and WPB exocytosis

To confirm that the inhibition caused by mibefradil on thrombin-stimulated exocytosis of VWF-GFP-containing vesicles was indeed due to T-type Ca^{2+} channel blockade, the functional role of $\text{Ca}_v3.1$ in thrombin-stimulated WPB exocytosis was further investigated utilizing RNA interference approach to specifically silence the $\text{Ca}_v3.1$ gene in PMVECs. Five shRNA coding oligonucleotides were designed to target sequences within the coding region of the $\text{Ca}_v3.1$ gene. Each oligonucleotide was ligated into a retroviral lentiviral expression vector allowing for individual expression of the shRNA into cultured PMVECs through viral delivery. We initially employed GFP-expressing retrovirus for delivery of shRNAs to determine efficiency in $\text{Ca}_v3.1$ gene silencing. An mCherry-expressing lentiviral vector was used for delivery of a selected shRNA and the scrambled shRNA for fura 2 fluorometric $[\text{Ca}^{2+}]_i$ assessments and the studies requiring VWF-GFP coexpression. The shRNAs in transduced cells were processed into small interfering RNA-like molecules capable of carrying out $\text{Ca}_v3.1$ gene-specific silencing. Since the expression vector constitutively expresses GFP or mCherry, positively transduced PMVECs were easily identified, and the stable transfectants were selected to virtually 100% purity. This approach resulted in a remarkable reduction of $\text{Ca}_v3.1$ gene expression in virtually all cells, allowing for the use of a large number of cells for patch-clamp electrophysiology, Ca^{2+} epifluorescence studies, as well as real-time imaging and confocal microscopy studies on living VWF-GFP coexpressing PMVECs.

We first demonstrated the high efficacy of retro- and lentivirus-mediated delivery of shRNA to PMVECs (Fig. 6*A*). qRT-PCR analysis revealed that in shRNA retrovirally infected PMVECs, all five shRNAs markedly decreased the $\text{Ca}_v3.1$ mRNA level by a minimum of 86%, with shRNA #4 being the most efficient (92%) (Fig. 6*B*). The $\text{Ca}_v3.1$ gene silencing with shRNA #4 significantly attenuated the functional T-type Ca^{2+} channel in PMVECs as evidenced by the virtual disappearance of the deactivating tail current elicited at the peak of +80-mV pulse, where the maximally evoked T-type current could be measured, in transduced PMVECs (Fig. 6*C*). The T-type currents elicited in scrambled shRNA-transduced cells retained a similar current amplitude as well as current-voltage relationship (Fig. 6*D*) as we previously reported in normal PMVECs, i.e., currents were consistently activated at -60 mV, maximal current activation was observed at -10 mV (58). Elimination of the functional T-type Ca^{2+} channel by $\text{Ca}_v3.1$ gene silencing caused a significant attenuation of thrombin-stimulated Ca^{2+} entry as demonstrated by the decrease in the second phase of $[\text{Ca}^{2+}]_i$ elevation, but not Ca^{2+} release, in mCherry-labeled shRNA #4-transduced PMVECs (Fig. 7, *A* and *B*). This result is consistent with the data in Fig. 1*A* where

pharmacological T-type Ca^{2+} channel blockade with mibefradil reduced thrombin-stimulated Ca^{2+} entry.

Real-time imaging and confocal microscopy studies were finally performed on mCherry-labeled shRNA- and VWF-GFP-transduced living PMVECs to measure the decrease of the number of WPBs in response to thrombin stimulation. Notably, transduction of VWF-GFP with either shRNA #4 or scrambled shRNA exhibited the same distribution pattern and dynamics of VWF-GFP-containing vesicles in resting status as in PMVECs transduced with VWF-GFP alone. A rapid and progressive decrease in VWF-GFP-containing vesicles in response to thrombin was observed in scrambled shRNA-transduced PMVECs, virtually identical to that in nontransduced cells. However, the decrease in VWF-GFP-containing vesicles in response to thrombin was nearly abolished in shRNA #4-transduced PMVECs, similar to that of mibefradil-treated PMVECs (Fig. 7C).

DISCUSSION

Although Ca^{2+} entry has been recognized as the critical triggering mechanism in transducing thrombin- as well as other G_q -linked inflammatory agonist-stimulated exocytosis of WPBs (2,12,16,26), the molecular identities mediating this response remain to be elucidated. Indeed, thrombin activates multiple pathways including store-operated and receptor-operated Ca^{2+} entry pathways along with other reported signaling cascades in both macro- and microvascular endothelial cell types (for review, see Ref. 3). Our present study sought to specifically determine the relevance of the $\text{Ca}_v3.1$ T-type Ca^{2+} channel in thrombin-induced exocytosis of WPBs. The major findings in this study are that PMVECs differ from their macrovascular counterparts, PAECs, in mechanisms regulating the exocytosis of WPBs and that $\text{Ca}_v3.1$ T-type Ca^{2+} channels play a critical role in controlling WPB exocytosis from microvascular endothelial cells, i.e., T-type Ca^{2+} channel activation provides a unique $[\text{Ca}^{2+}]_i$ source important for thrombin-stimulated WPB exocytosis.

To address the role of endothelial cell $\text{Ca}_v3.1$ T-type Ca^{2+} channels in thrombin-stimulated exocytosis of WPBs, we initially introduced a fusion protein consisting of VWF, a prominent WPB constituent, and GFP (VWF-GFP) to directly visualize the secretory process of WPBs in living cells. Using live cell imaging analysis to assess changes in the number of VWF-GFP-containing vesicles, we observed a rapid and progressive decrease in fluorescent vesicles following stimulation of PMVECs or PAECs with thrombin or PAECs with isoproterenol. Notably, isoproterenol had no effect on the dynamics of VWF-GFP-containing vesicles in PMVECs. These results suggest that PMVECs are distinct from PAECs in regulated exocytosis of WPBs. Whereas the exocytosis of WPBs is predominantly Ca^{2+} dependent in PMVECs, it is both Ca^{2+} and cAMP dependent in PAECs.

Prior studies reported heterogeneity of lung macro- and microvascular endothelial cells in both Ca^{2+} and cAMP signaling pathways (45,46). Compared with PAECs, PMVECs exhibit a greater global $[\text{Ca}^{2+}]_i$ response to thrombin (9), but an attenuated intracellular cAMP response to forskolin or β -adrenergic receptor agonist isoproterenol (45,46), both activating transmembrane type 6 adenylyl cyclase. On the other hand, the intracellular cAMP response to the cAMP-specific type 4 phosphodiesterase inhibitor rolipram is more pronounced in PMVECs (45,46). Such heterogeneity in Ca^{2+} and cAMP signaling may be closely related to our observation of the diversity in regulated exocytosis of WPBs between Ca^{2+} - and cAMP-dependent pathways in the two endothelial cell types. Additionally, our results imply the possible existence of functionally distinct populations of WPBs whose exocytosis is differentially regulated. In support of this notion, a recent study revealed differential effects of thrombin, histamine, protease-activated receptor-activating peptides, and forskolin on secretion of VWF and cell surface expression of P-selectin in human umbilical vein

endothelial cells (HUVECs), which was proposed to be due to the existence of distinct populations of WPBs containing VWF with or without P-selectin (10). Moreover, previous real-time studies performed with the VWF-GFP fusion protein transduced into HUVECs revealed striking differences in the dynamics of WPBs between $[Ca^{2+}]_i$ and cAMP responses. For instance, stimulation with cAMP-elevating agonists, in addition to triggering fusion of WPBs with the plasma membrane, resulted in perinuclear clustering of a subset of WPBs that escaped exocytosis (35,36). Thus cAMP mediates release of VWF from peripheral WPBs. In contrast, $[Ca^{2+}]_i$ mediates release of both central and peripheral WPBs (51). These observations all suggest a selective coupling between $[Ca^{2+}]_i$ - or cAMP-dependent regulation of WPB exocytosis to specific populations of WPBs. Consistent with this idea, our findings indicate that WPB exocytosis in pulmonary macro- and microvascular endothelial cells is differentially regulated by two distinct pathways involving $[Ca^{2+}]_i$ and/or cAMP. Although not studied here in detail, the observation that the cAMP-mediated exocytosis of WPBs only occurred in PAECs, but not in PMVECs, is most likely caused by the existence of distinct populations of WPBs existing in the two cells types.

Based on our prior work, activation of $Ca_v3.1$ T-type Ca^{2+} channels by thrombin generates a physiologically relevant $[Ca^{2+}]_i$ rise in microvascular endothelial cells via depolarization of the plasma membrane (58). The present study provided further evidence that the $Ca_v3.1$ T-type Ca^{2+} channel is a critical downstream effector for thrombin signaling in endothelial cells. Although the T-type Ca^{2+} channel blocker mibefradil had no effect on thrombin-induced rise in $[Ca^{2+}]_i$ in PAECs, it did cause a remarkable decrease in the thrombin-induced rise in $[Ca^{2+}]_i$ in PMVECs. Consistently, $Ca_v3.1$ gene silencing in PMVECs nearly eliminated functional T-type Ca^{2+} channels and markedly reduced thrombin-induced Ca^{2+} entry. Furthermore, expression of recombinant $Ca_v3.1$ in PAECs resulted in the emergence of a T-type current and augmented thrombin-induced Ca^{2+} entry. The latter response was completely reversed by pharmacological blockade of the T-type Ca^{2+} channels.

Despite the fact that WPB exocytosis in both PMVECs and PAECs involves the activation of thrombin/ $[Ca^{2+}]_i$ and/or cAMP signaling pathways, only in PMVECs was the thrombin-induced release of VWF-GFP-containing vesicles diminished by pharmacological blockade of the $Ca_v3.1$ T-type Ca^{2+} channels. In addition, T-type Ca^{2+} channel blockade had no effect on the response of isoproterenol-stimulated release of VWF-GFP-containing vesicles in PAECs. These results are consistent with the observation that there are no T-type Ca^{2+} channels in PAECs (58) and support the specificity of mibefradil on T-type Ca^{2+} channel blockade. Furthermore, $Ca_v3.1$ gene silencing nearly prevented PMVECs from releasing VWF-GFP-containing vesicles in response to thrombin stimulation and significantly attenuated thrombin-stimulated Ca^{2+} entry to a similar extent as mibefradil. Together, these results indicate that the $Ca_v3.1$ T-type Ca^{2+} channel is a specific downstream target of thrombin, important for regulated exocytosis of WPBs in PMVECs.

In summary, we have utilized two distinct endothelial cell phenotypes, PMVECs and PAECs, to evaluate the dynamic activity and the role of T-type Ca^{2+} channels in thrombin-stimulated WPB exocytotic process. We have demonstrated that in microvascular endothelial cells, thrombin-induced Ca^{2+} entry via the $Ca_v3.1$ T-type Ca^{2+} channel plays a crucial role in promoting exocytosis of WPBs. These findings establish physiological relevance for the T-type Ca^{2+} channel in non-excitabile microvascular endothelial cells; namely, activation of the channel induces a procoagulant endothelial phenotype important for site-specific vascular endothelial activation. The significance of the findings can be exemplified by the observations in sickle cell anemia in which sickle erythrocytes adhere more readily to microvascular endothelium than to endothelium from conduit vessels (5). Indeed, our prior study also shows that inhibition of T-type Ca^{2+} channels attenuates increased retention of sickled erythrocytes in the inflamed pulmonary circulation (58). With

regard to the question raised by Varghese and Weir (50) as whether targeted delivery of T-type blockers can be used as therapy for vaso-occlusive crisis in sickle cell anemia, we propose that specific T-type Ca^{2+} channel antagonists or alteration of the $\text{Ca}_v3.1$ channel is a prospective therapeutic strategy for regulating endothelial activation in thrombotic disorders, especially in lung microcirculation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Mibefradil blockade of T-type Ca^{2+} channel inhibits the thrombin-induced rise in cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) in pulmonary microvascular endothelial cells (PMVECs). **A:** $[\text{Ca}^{2+}]_i$ response to thrombin (10 U/ml) in PMVECs with (red trace) or without (black trace) 10-min pretreatment of mibefradil (10 $\mu\text{mol/l}$) ($n = 7$ and 9). Mibefradil significantly reduced the 2nd phase, but not the 1st phase, of the thrombin-induced rise in $[\text{Ca}^{2+}]_i$ ($P < 0.05$). **B:** $[\text{Ca}^{2+}]_i$ response to thrombin (1 U/ml) in pulmonary artery endothelial cells (PAECs) with or without 10-min pretreatment of mibefradil (10 $\mu\text{mol/l}$) ($n = 4$ and 5). Mibefradil had no effect on $[\text{Ca}^{2+}]_i$ response to thrombin [$P = \text{not significant (ns)}$] in PAECs. Extracellular $[\text{Ca}^{2+}] = 2 \text{ mmol/l}$.

Fig. 2.

Functional expression of $\text{Ca}_v3.1$ T-type Ca^{2+} channel elevates thrombin-induced Ca^{2+} entry in PAECs. **A:** $\text{Ca}_v3.1$ protein expression appeared in $\text{Ca}_v3.1$ -transduced PAECs, PAECs (NT), PAECs transduced with $\text{Ca}_v3.1a$ (Trans), PMVECs (MV), rat brain. **B:** a representative set of macroscopic current traces in $\text{Ca}_v3.1$ -transduced PAECs. Currents were recorded by increasing depolarization from -70 to $+50$ mV with 10-mV increments from a holding potential of -90 mV. Ca^{2+} (10 mmol/l) was used as a charge carrier. **C:** averaged current-voltage (I - V) relationship of peak currents obtained from 5 $\text{Ca}_v3.1$ -transduced PAECs (■) and 12 nontransduced PAECs (□). V_m , membrane potential. **D:** $[\text{Ca}^{2+}]_i$ response to thrombin (1 U/ml) in $\text{Ca}_v3.1$ -transduced and nontransduced PAECs ($n = 4$ and 5, respectively). Functional expression of $\text{Ca}_v3.1$ considerably elevated the 2nd phase ($P < 0.05$), but not the 1st phase, of thrombin-induced rise in $[\text{Ca}^{2+}]_i$. **E:** $[\text{Ca}^{2+}]_i$ response to thrombin (1 U/ml) in $\text{Ca}_v3.1$ -transduced PAECs with or without 10-min pretreatment of mibefradil (10 $\mu\text{mol/l}$) ($n = 4$ each). Mibefradil reduced the 2nd phase of $[\text{Ca}^{2+}]_i$ responses to thrombin ($P < 0.05$) but had no effect on the 1st phase of $[\text{Ca}^{2+}]_i$ response to thrombin ($P = \text{not significant}$) in $\text{Ca}_v3.1$ -transduced PAECs. **F:** superimposition of the thrombin-induced $[\text{Ca}^{2+}]_i$ responses in $\text{Ca}_v3.1$ -transduced PAECs with pretreatment of mibefradil and nontransduced PAECs without pretreatment of mibefradil demonstrates that mibefradil completely abolished elevated Ca^{2+} entry resulting from functional expression of $\text{Ca}_v3.1$ in PAECs. Extracellular $[\text{Ca}^{2+}] = 2$ mmol/l in D–F.

Fig. 3.

Von Willebrand factor (VWF)-green fluorescent protein (GFP)-containing vesicles resemble Weibel-Palade bodies (WPBs) in VWF-GFP-expressing endothelial cells. *A*: PMVECs were transduced with the VWF-GFP chimera. VWF-GFP-containing vesicles were observed in the cytoplasm. *B*: images of serial apical-to-basal optical sections (Z-stacks) taken in a VWF-GFP-expressing PMVEC at 0.3- μ m intervals revealed that the vesicles were distributed throughout the cytoplasm, with the majority situated in the middle and lower sections of the cell.

Fig. 4.

Activated exocytosis of VWF-GFP-containing vesicles by thrombin and isoproterenol in PMVECs and PAECs. **A:** Representative sets of merged time-lapse GFP fluorescent images of all serial sections (Z-stacks) taken from apical-to-basal cell aspects at 0.25- μ m intervals every 3 min in live VWF-GFP-expressing PMVECs during 60-min experiments. A rapid, progressive decrease in total number of VWF-GFP-containing vesicles following thrombin (*top*, and Video 2) stimulation was observed. Arrow denotes when thrombin (10 U/ml) was applied. *A, bottom:* images taken in the same experimental setting in live VWF-GFP-expressing PMVECs following isoproterenol (1 μ mol/l) stimulation, illustrating virtually no decrease in total number of VWF-GFP-containing vesicles. **B** and **C:** the time-course summary of changes in total pixel volume of GFP fluorescence, normalized to the fluorescence value at the beginning of the recording (*time 0*) in VWF-GFP-expressing PMVECs (**B**) and PAECs (**C**) over 60 min. Arrows denote when thrombin or isoproterenol (at the concentrations depicted) was applied. Controls ($\square$) were cells without thrombin or isoproterenol exposure. Note thrombin induced a remarkable decrease in total number of vesicles in both PMVECs and PAECs ($\blacksquare$), whereas isoproterenol only induced a decrease in total number of VWF-GFP-containing vesicles in PAECs, not PMVECs (**B** and **C**, gray squares). *Time point where the data from 2 groups start to have significant difference ($P <$

0.01) through the end of observation. Data were acquired on a PerkinElmer UltraView RS confocal microscope with a $\times 60$ objective.

Fig. 5.

Mibefradil inhibits thrombin-activated exocytosis of VWF-GFP-containing vesicles from PMVECs but not from PAECs. *A*: a set of merged time-lapse GFP fluorescent images of all parts of Z-stacks taken from apical-to-basal cell aspects at 0.25-μm intervals in live VWF-GFP-expressing PMVECs during experiments in the presence of mibefradil (10 μmol/l). The decrease in total number of VWF-GFP-containing vesicles activated by thrombin was nearly abolished by mibefradil. Arrow denotes when thrombin (10 U/ml) was applied. *B–D*: the time-course summary for changes in total pixel volume of GFP fluorescence, normalized to the fluorescence value at *time 0* in VWF-GFP-expressing PMVECs (*B*) and PAECs (*C* and *D*) during the 60-min real-time image recordings with (gray squares) or without (■) mibefradil (10 μmol/l). Controls (dashed lines) were cells without thrombin or isoproterenol exposure. Note mibefradil markedly prevented thrombin-induced decrease in total number of VWF-GFP-containing vesicles in PMVECs (*B*). *Time point where the data from 2 groups (+mibefradil group and –mibefradil group) in PMVECs start to have significant difference ($P < 0.01$) to the end of the experiments. Mibefradil did not affect either thrombin or isoproterenol-stimulated changes in total number of VWF-GFP-containing vesicles in PAECs (*C* and *D*). Arrows denote when thrombin or isoproterenol (at the concentrations depicted) was applied. Data were acquired on a PerkinElmer UltraView RS confocal microscope with a $\times 60$ objective. *E*: the VWF release from PMVECs was determined by measuring the amount of VWF in extracellular media using ELISA following 10 and 60 min of thrombin (10 U/ml) stimulation of cells in the presence and absence of mibefradil (10 μmol/l). Mibefradil attenuated VWF release at each time point following thrombin stimulation but had no effect on basal VWF release. *Significantly different between the 2 sample sets depicted; $P < 0.05$.

Fig. 6.

Silencing $\text{Ca}_v3.1$ expression by RNA interference (RNAi). *A*: representative fluorescence image of mCherry-tagged small hairpin RNA (shRNA) #4-transduced PMVECs (*a*), phase-contrast image of the same cell monolayer (*b*), and merged image of the 2 (*c*) showing the high efficiency of virus-mediated delivery of shRNA to PMVECs. *B*: quantitative RT-PCR analysis of $\text{Ca}_v3.1$ gene expression by relative quantification. Rat GAPDH was used as internal standard for expression normalization. $\text{Ca}_v3.1$ gene expression was knocked down by 86–92% by 5 different shRNAs ($n = 3$ each). *Inset*: Western blotting analysis of $\text{Ca}_v3.1$ protein levels. $\text{Ca}_v3.1$ protein expression was markedly suppressed by shRNA #4. Scrambled shRNA had no effect on $\text{Ca}_v3.1$ protein expression. *C*: individual data points of the deactivating tail current ($I_{\text{Ca, tail}}$) elicited at the peak of +80 mV pulse in PMVECs transduced with shRNA #4 or scrambled shRNA. Lines in each data group are at median with interquartile range, i.e., for shRNA #4-transduced group (in pA/pF): median 0.0, 25th percentile –1.5, 75th percentile 0.0; for scrambled shRNA transduced group: median –6.9, 25th percentile –12.5, 75th percentile –3.8 ($P < 0.0001$, Mann-Whitney test). *D, left*:

representative traces of the current elicited at –10 mV from shRNA #4- (gray) and scrambled shRNA- (black) transduced PMVECs, respectively. *D, right*: *I*-*V* relationships of peak currents at each depolarizing pulse from shRNA #4- ($n = 8$, gray squares) and scrambled shRNA- ($n = 7$, ■) transduced PMVECs, showing shRNA #4-induced $\text{Ca}_v3.1$ gene silencing nearly eliminated T-type current in PMVECs. Ca^{2+} (10 mM) was used as a charge carrier.

Fig. 7.

Effects of shRNA-induced $\text{Ca}_v3.1$ gene silencing on thrombin-stimulated PMVEC $[\text{Ca}^{2+}]_i$ transitions and WPB exocytosis. **A:** $[\text{Ca}^{2+}]_i$ responses to thrombin (10 U/ml) in PMVECs transduced with shRNA #4 (red trace, $n = 4$) and PMVECs transduced with scrambled shRNA (black trace, $n = 4$) measured in buffer containing 2 mmol/l extracellular Ca^{2+} . $\text{Ca}_v3.1$ silencing with shRNA #4 considerably reduced the thrombin-stimulated Ca^{2+} entry, i.e., 2nd phase of $[\text{Ca}^{2+}]_i$ elevation ($*P < 0.05$ vs. scrambled shRNA) with no evidence of a decrease in Ca^{2+} release, i.e., no difference in the peak of thrombin-induced Ca^{2+} release phase between groups ($P > 0.05$). **B:** $[\text{Ca}^{2+}]_i$ responses to thrombin (10 U/ml) in PMVECs transduced with shRNA #4 (red trace, $n = 4$) and PMVECs transduced with scrambled shRNA (black trace, $n = 3$) measured in Ca^{2+} -free buffer. It was further confirmed that $\text{Ca}_v3.1$ silencing with shRNA #4 did not change the thrombin-stimulated Ca^{2+} release ($P > 0.05$ vs. scrambled shRNA). **C:** the time-course summary of changes in total pixel volume of GFP fluorescence, normalized to the fluorescence value at the beginning of the recordings (time 0), in PMVECs transduced with shRNA #4 (red squares, $n = 6$) and scrambled shRNA (■, $n = 5$) during the real-time image recordings. The rapid, progressive decrease in total number of VWF-GFP-containing vesicles activated by thrombin seen in PMVECs transduced with scrambled shRNA was nearly abolished in PMVECs transduced with shRNA #4. Arrow denotes when thrombin (10 U/ml) or isoproterenol (1 $\mu\text{mol/l}$) was applied. Top black dashed line shows the time control of GFP fluorescence changes in normal nonstimulated nontransduced PMVECs. Middle gray dashed line shows the time course of thrombin-stimulated decrease in the number of GFP-fluorescent vesicles when PMVECs were pretreated with mibefradil before thrombin stimulation (see Fig. 5C). Bottom red dashed line represents the time course of decrease in GFP-fluorescent vesicles during 60-min interval when normal PMVECs were stimulated with thrombin (see Fig. 5C). Note the shRNA #4 mimics the effect of mibefradil in Fig. 6B, and PMVECs transduced with scrambled shRNA retain the same response as normal nontransduced PMVECs to thrombin in release of VWF-GFP-containing vesicles. Data were acquired on a PerkinElmer UltraView RS confocal microscope with a $\times 60$ objective. $*P < 0.01$ between shRNA #4 and scrambled shRNA.

Table 1Oligonucleotide sequences for Ca_v3.1 small hairpin RNAs

Identification	Target Sequence (Bases)	Antisense	Sense
#1	877–894	TGGTGGTGAAGATGGTGG	CCACCATCTTCACCACCA
#2	1646–1664	CCTGTGCCTGGTGGTGATT	AATCACCACCAGGCACAGG
#3	1820–1837	AGCAGCCCGAAGGCTGGC	GCCAGCCTTCGGGCTGCT
#4	3263–3281	TAAAGTCCTCTACAACGGC	GCCGTTGTAGAGGACTTTA
#5	3498–3516	CACGCGGAACTACGAAAGA	TCTTTCGTAGTTCCGCGTG
Scrambled	Scrambled	CGTTGATTATGGTGCTAG	CTAGCACCATAATCAACG