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Protein Kinase Cα Phosphorylates the TRPC1 Channel and Regulates Store-operated Ca^{2+} Entry in Endothelial Cells*

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The TRPC1 (transient receptor potential canonical-1) channel is a constituent of the nonselective cation channel that mediates Ca^{2+} entry through store-operated channels (SOCs) in human endothelial cells. We investigated the role of protein kinase Cα (PKCα) phosphorylation of TRPC1 in regulating the opening of SOCs. Thrombin or thapsigargin added to the external medium activated Ca^{2+} entry after Ca^{2+} store depletion, which we monitored by changes in cellular Fura 2 fluorescence. Internal application of the metabolism-resistant analog of inositol 1,4,5-trisphosphate (IP_3) activated an inward cationic current within 1 min, which we recorded using the whole cell patch clamp technique. La^{3+} or Gd^{3+} abolished the current, consistent with the known properties of SOCs. Pharmacological (Gö6976) or genetic (kinase-defective mutant) inhibition of PKCα markedly inhibited IP_3-induced activation of the current. Thrombin or thapsigargin also activated La^{3+}-sensitive Ca^{2+} entry in a PKCα-dependent manner. We determined the effects of a specific antibody directed against an extracellular epitope of TRPC1 to address the functional importance of TRPC1. External application of the antibody blocked thrombin- or IP_3-induced Ca^{2+} entry. In addition, we showed that thrombin or thapsigargin induced phosphorylation of TRPC1 within 1 min. Thrombin failed to induce TRPC1 phosphorylation in the absence of PKCα activation. Phosphorylation of TRPC1 and the resulting Ca^{2+} entry were essential for the increase in permeability induced by thrombin in confluent endothelial monolayers. These results demonstrate that PKCα phosphorylation of TRPC1 is an important determinant of Ca^{2+} entry in human endothelial cells.

In vascular endothelial cells, thrombin binding to protease-activated receptor-1 and cleavage of the receptor at the N terminus leads to activation of the heterotrimeric G-protein G_q and a subsequent increase in $[Ca^{2+}]_i$ (1, 2). Increased $[Ca^{2+}]_i$ results from the release of Ca^{2+} from intracellular stores (endoplasmic reticulum (ER)[1]) through inositol 1,4,5-trisphosphate (IP_3)-sensitive channels and activation of plasma membrane Ca^{2+} entry store-operated channels (SOCs) as a result of Ca^{2+} store depletion (3–7). Store-operated Ca^{2+} entry coupled with depletion of intracellular Ca^{2+} stores is essential for Ca^{2+} signaling by thrombin because it induces endothelial cell retraction and thus leads to increased endothelial permeability (8–11).

Endothelial SOCs (3, 10) are composed of transient receptor potential canonical (TRPC) channels (12–14) first identified in *Drosophila* and subsequently shown to mediate Ca^{2+} entry after store depletion in non-excitable cells (15–17). These channels are activated in response to stimulation of G-protein-coupled receptors (GPCRs) that induce Ca^{2+} store depletion. The likely constituents of endogenous SOCs are TRPC1, -2, -4, and -5 (alone or in combination) because these TRPC channels can be activated by Ca^{2+} store depletion (12, 18–21). Human endothelial cells predominantly express TRPC1 (11, 22–24). However, the regulatory signals mediating the store depletion activation of TRPC1 and the resulting Ca^{2+} entry via SOCs remain unclear.

Protein kinase Cα (PKCα) activation by GPCRs is known to induce increased endothelial permeability (9, 11, 25, 26). Although this effect of PKCα may be the result of activation of cellular Ca^{2+} entry, the role of PKCα in the regulation of SOCs is unclear, as the results of studies in several cell types have been contradictory (27–33). Thus, we addressed the role of PKCα in signaling TRPC1 activation and thereby triggering Ca^{2+} entry and endothelial barrier dysfunction. Our results demonstrate that PKCα-dependent phosphorylation of TRPC1 is an important signal contributing to SOC activation and triggering Ca^{2+} entry. We also show that PKCα-activated Ca^{2+} entry is critical in mediating the thrombin-induced increase in transendothelial permeability.

EXPERIMENTAL PROCEDURES

Materials—Human α-thrombin was obtained from Enzyme Research Laboratories (South Bend, IN). Human umbilical vein endothelial cells (HUVECs) and endothelial growth medium (EBM-2) were obtained from Clonetics Corp. (San Diego, CA). Human microvessel endothelial cells (HMECs), a human dermal microvascular endothelial cell line, were obtained from Dr. Edwin W. Ades (National Center for Infectious Diseases, Center for Disease Control, Atlanta, GA). Trypsin and Hanks' balanced salt solution and molecular cellular and developmental biology (MCDB) media 131 were obtained from Invitrogen. Superfect transfection reagent was obtained from QIAGEN Inc. (Valencia, CA). The "metabolism-resistant" form of IP_3, 3-deoxy-3-fluoro-D-myo-IP_3, was from Calbiochem, and anti-TRPC1 polyclonal antibody was purchased from Alomone Laboratory (Jerusalem, Israel) or Sigma. Green fluorescent protein was purchased from Clontech.

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[1] The abbreviations used are: ER, endoplasmic reticulum; IP_3, inositol 1,4,5-trisphosphate; SOC, store-operated channel; TRPC, transient receptor potential canonical; GPCR, G-protein-coupled receptor; PKC, protein kinase C; HUVECs, human umbilical vein endothelial cells; HMECs, human microvessel endothelial cells; Ad, adenovirus; dn, kinase-defective; Ab, antibody; TRP, transient receptor potential.

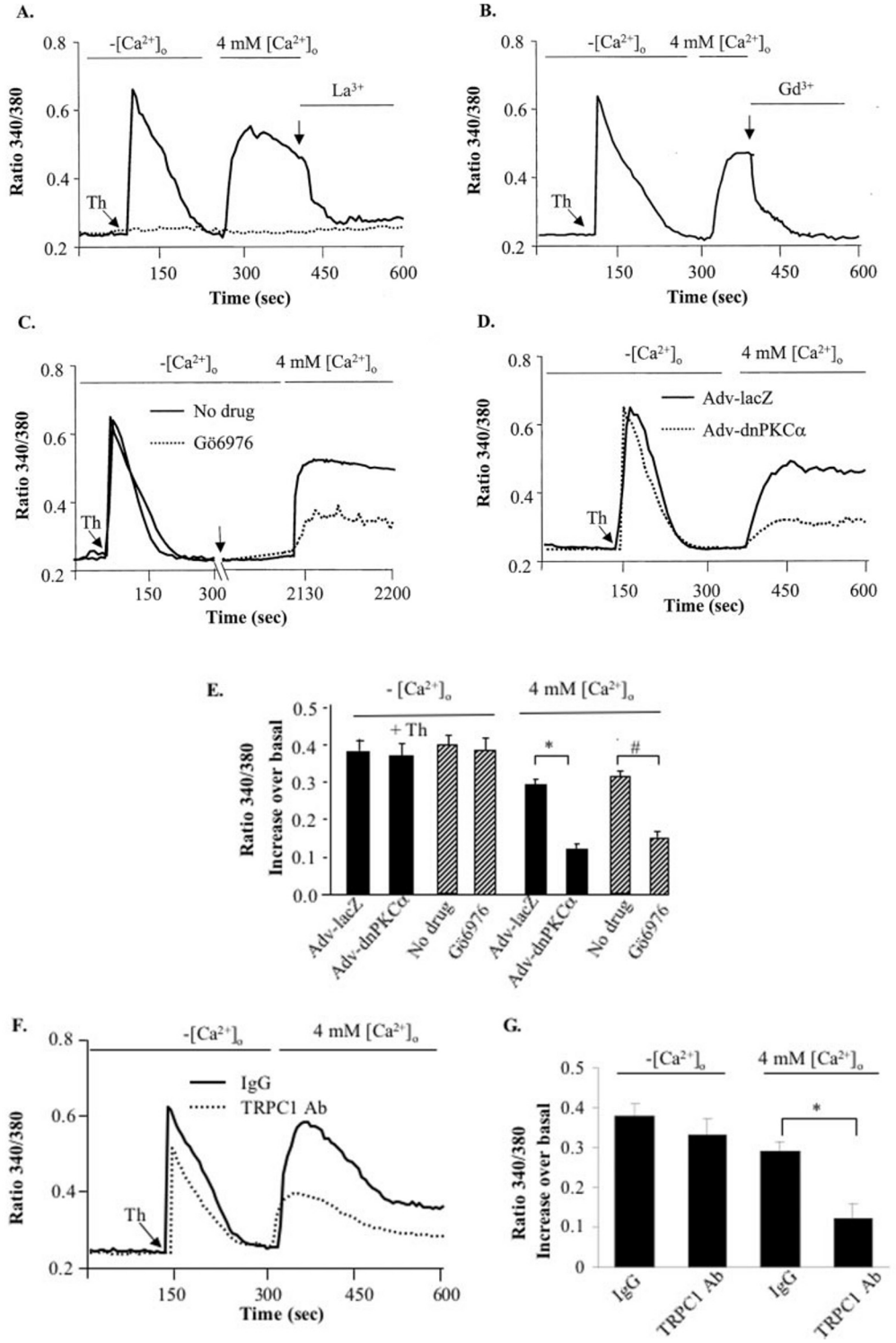

FIG. 1. **Effect of PKC*α* inhibition on thrombin-induced store-operated Ca^{2+} entry in HUVECs.** Cells were loaded with Fura 2/AM and stimulated with thrombin (*Th*) in the absence of extracellular Ca^{2+} to deplete ER Ca^{2+}. This was followed by repletion of $[Ca^{2+}]_o$ to 4 mM to determine Ca^{2+} entry. *A*, shown is La^{3+}-sensitive Ca^{2+} entry following depletion of ER stores with thrombin. *Arrows* indicate addition of 50 nM thrombin or 1 mM La^{3+} (*solid line*). Note that there was no Ca^{2+} entry upon calcium add-back in unstimulated cells that did not receive thrombin (*dotted line*). *B*, shown is Gd^{3+}-sensitive Ca^{2+} entry following depletion of ER stores with thrombin. *Arrows* indicate time of introduction of thrombin or Gd^{3+} (1 mM). *C*, after release of sequestered Ca^{2+} by thrombin (*first arrow*), cells were left untreated (*solid line*) or received Gö6976 (*dotted line*) for 30 min (note gap in recording ending at *second arrow*), after which 4 mM Ca^{2+} was added back to the medium to induce Ca^{2+} entry. *D*, shown is Ca^{2+} entry in cells infected with Ad (*Adv*)-dnPKC*α* or Ad-*lacZ* (control) following depletion of stores with thrombin. *E*, data show the mean ± S.E. of the thrombin-induced release of Ca^{2+} from stores ($-[Ca^{2+}]_o$) and Ca^{2+} entry due to calcium add-back ($4\ mM\ [Ca^{2+}]_o$) from multiple experiments calculated as the maximum increase over the basal value under various experimental conditions ($n = 4–7$). *, significant reduction ($p < 0.001$) in Ca^{2+} entry after transfection by Ad-dnPKC*α* *versus* the control (Ad-*lacZ*); #, statistically significant ($p < 0.001$) effect of Gö6976 treatment *versus* the control (*No drug*). *F*, shown are the Ca^{2+} transients in cells pretreated with 15 μg/ml IgG (*solid line*) or anti-TRPC1 antibody (*dotted line*) for 15 min; note that the reduction in calcium entry by anti-TRPC1 Ab was statistically significant. *G*, data show the mean ± S.E. of the release of Ca^{2+} from stores ($-[Ca^{2+}]_o$) by thrombin and Ca^{2+} entry ($4\ mM\ [Ca^{2+}]_o$) from multiple experiments calculated as the maximum increase over the basal value under each condition ($n = 4–5$). *, significant effect ($p < 0.001$) of anti-TRPC1 Ab *versus* isotype-matched IgG. The *traces* in *A–D* and *F* are averaged Ca^{2+} responses from at least 25 cells.

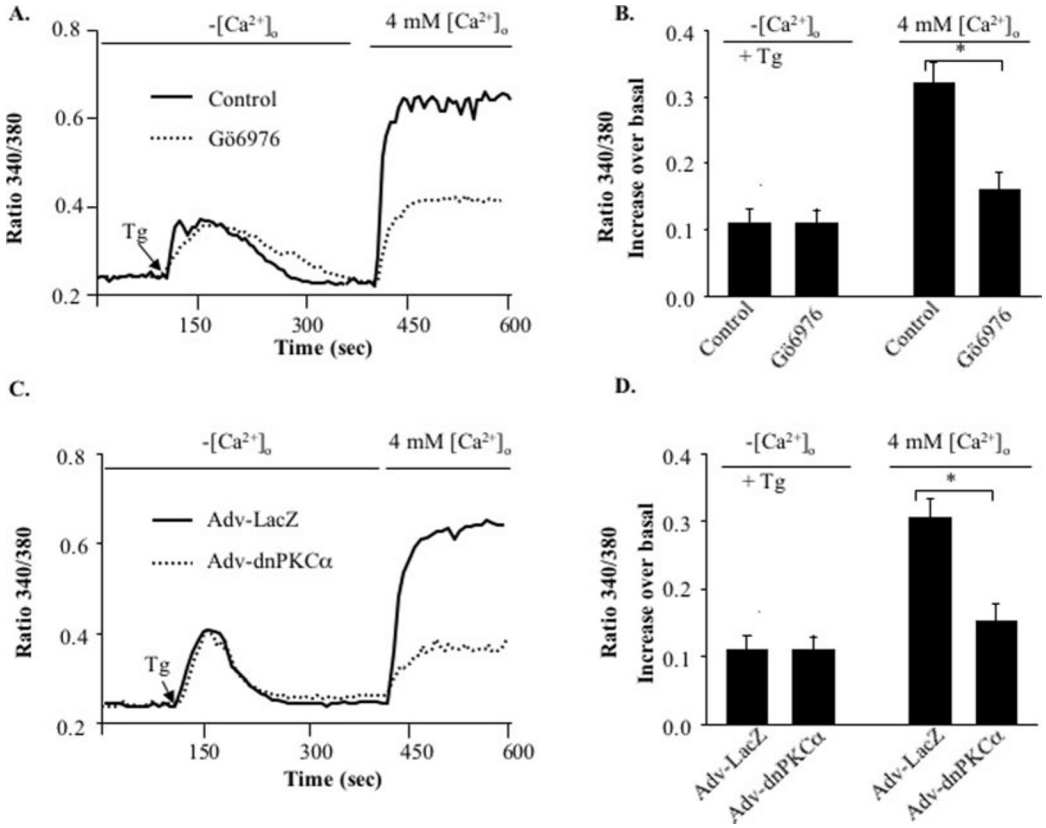

FIG. 2. **Effect of PKCα inhibition on thapsigargin-induced store-operated Ca^{2+} entry in HUVECs.** Fura 2-loaded cells were stimulated with thapsigargin (*Tg*) in the absence of extracellular Ca^{2+} to deplete ER Ca^{2+}. This was followed by repletion of 4 mM $[Ca^{2+}]_o$ to determine Ca^{2+} entry. *A*, Ca^{2+} entry in cells pretreated without (*solid line*) or with (*dotted line*) Gö6976. *B*, data shown as the mean ± S.E. of the release of Ca^{2+} from stores in calcium-free medium ($-[Ca^{2+}]_o$) and Ca^{2+} entry ($4\ mM\ [Ca^{2+}]_o$) from multiple experiments calculated as the maximum increase over the basal value under each condition ($n = 4$). *C*, Ca^{2+} entry in cells infected with Ad (*Adv*)-dnPKCα (*dotted line*) or Ad-*lacZ* (*solid line*) following depletion of stores with thapsigargin. *D*, data shown as the mean ± S.E. of the release of Ca^{2+} from stores and Ca^{2+} entry from multiple experiments calculated as the maximum increase over the basal value under each condition ($n = 4$). The *traces* in *A* and *C* are averaged Ca^{2+} responses from at least 25 cells. *, significant reduction ($p < 0.001$) in Ca^{2+} entry due to Gö6976 (*B*) or Ad-dnPKCα (*D*).

Endothelial Cell Cultures—Primary HUVECs were cultured in a T-75 flask coated with 0.1% gelatin in EBM-2 medium supplemented with 10% fetal bovine serum. HMECs were cultured in a T-75 flask in MCDB 131 medium supplemented with 10% fetal bovine serum. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air until they formed a confluent monolayer. Cells from each of the primary flasks were detached with 0.05% trypsin plus 0.02% EDTA, resuspended in fresh culture medium, and passaged as described below. In all experiments, unless otherwise indicated, a confluent monolayer of HUVECs was washed twice with phosphate-buffered saline and incubated in serum-free medium for 2 h before treatment with thrombin. HUVECs between passages 4 and 8 were used for all experiments as described (34, 35).

Cell Transfection—HMECs grown to 50–70% confluence on 8-mm coverslips (placed in 35-mm dishes) were transfected using the Superfect transfection reagent as described (34, 35). For transfection of the Myc-tagged *TRPC1* cDNA HMECs grown on 8-mm glass coverslips or COS-7 cells grown in 100-mm culture dishes were transfected with 1 μg/ml Myc-*TRPC1* cDNA or vector alone using LipofectAMINE Plus reagent (Invitrogen) as described (24).

Adenoviral Infection—The adenoviral construct containing a kinase-defective mutant of PKCα (referred to herein as Ad-dnPKCα) was a generous gift from Dr. Viswanathan Natarajan (Johns Hopkins University School of Medicine, Baltimore, MD). Cells grown to confluence in 100-mm dishes (for determination of phosphorylation) or on 25-mm coverslips (for $[Ca^{2+}]_i$ measurements) were infected with 1×10^7 plaque-forming units of the PKCα construct for 5 h in serum-containing medium, after which the medium was replaced with fresh medium supplemented with 10% fetal bovine serum. In parallel, monolayers were infected with adenovirus containing the β-galactosidase gene, *lacZ* (Ad-*lacZ*), as a control for nonspecific viral infection upon endothelial cell signaling (34). Adenovirus-infected cells were then used to determine TRPC1 phosphorylation or $[Ca^{2+}]_i$ as described below.

Whole Cell Patch Clamp Recording in Endothelial Cells—Patch clamp recording in the whole cell configuration was performed as described (20, 35, 36) on HMECs attached to a coverslip. Standard extracellular and pipette solutions (35) were provided. SOC currents were measured at a holding potential of −50 mV by depleting the cellular calcium store with 30 μmol/liter 3-deoxy-3-fluoro-D-myo-IP_3 (metabolism-resistant IP_3) and/or 2 μM thapsigargin included in the pipette solution. All experiments were performed at room temperature.

$[Ca^{2+}]_i$ Measurements—An increase in $[Ca^{2+}]_i$ was measured using the Ca^{2+}-sensitive fluorescent dye Fura 2/AM as described (8, 35). For loading of cells with Fura 2/AM, cells grown on 25-mm coverslips were incubated with 3 μM Fura 2 for 15 min at 37 °C. Cells were then washed twice with Hanks' balanced salt solution and imaged using an Attoflor Ratio Vision digital fluorescence microscopy system (Atto Instruments, Inc., Rockville, MD) equipped with a Zeiss Axiovert S100 inverted microscope and an F-Fluar ×40 1.3-numerical aperture oil immersion objective. Regions of interest in individual cells were marked and excited at 334 and 380 nm with emission at 520 nm. The 334/380 nm excitation ratio, which increased as a function of $[Ca^{2+}]_i$, was captured at 5-s intervals.

Immunoprecipitation and Phosphorylation of TRPC1—Cells grown to confluence in 100-mm dishes were serum-starved in phosphate-free medium for 2 h at 37 °C, after which cellular proteins were labeled by incubation with 150 μCi/ml ^{32}P for 4 h. Following incubation with ^{32}P, cells were stimulated with 50 nM thrombin or 2 μM thapsigargin at the indicated time intervals and lysed for 20 min on ice with 0.5 ml of radioimmune precipitation assay buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 1% Triton X, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml aprotinin) (34, 35). Cell lysates were immunoprecipitated with anti-IgG or anti-TRPC1 antibody (Ab) and Western-blotted with anti-TRPC1 antibody (2 μg/ml) to verify equal protein loading in each lane. To determine the specificity of anti-TRPC1 Ab, COS-7 cells transfected with Myc-tagged TRPC1 or vector alone were lysed in radioimmune precipitation assay buffer, followed by im-

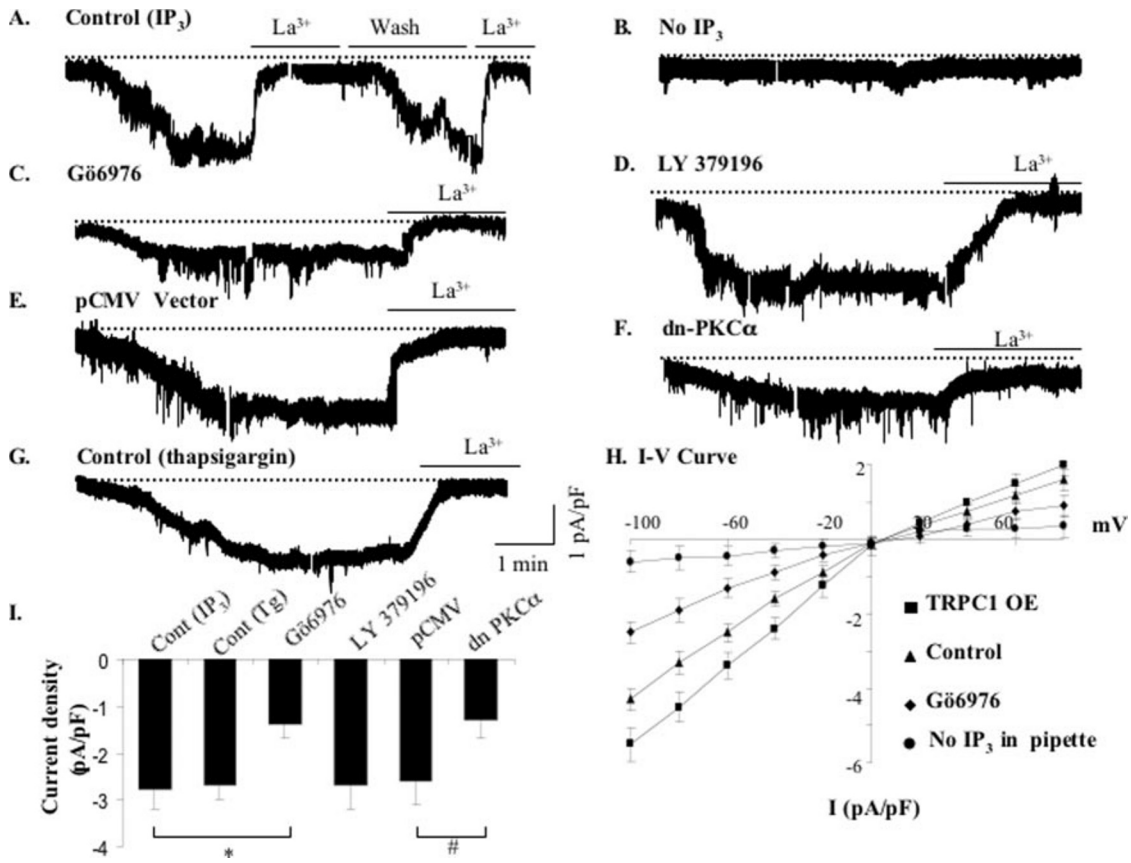

FIG. 3. **PKCα inhibition attenuates store-operated currents.** Shown are the effects of inhibitors of PKCα or PKCβ or transfection of dnPKCα on IP_3-induced activation of SOCs recorded continuously at a holding potential of −50 mV. *A*, reversible blocking of the IP_3-induced current by La^{3+} in non-transfected control cells; *B*, background current in untreated cells (no IP_3 or thapsigargin present); *C*, marked reduction in the IP_3-induced current in cells pretreated with 100 nM Gö6976; *D*, IP_3-induced current in cells pretreated with 50 nM LY379196 (PKCβ isoenzyme-specific inhibitor); *E*, IP_3-induced current in control cells transfected with the pCMV vector; *F*, inhibition of the IP_3-induced current in cells transfected with dnPKCα; *G*, La^{3+} sensitivity of the thapsigargin-induced current in control cells; *H*, current-voltage relationship (*I*-V curve) for the SOC current (IP_3-induced) in control cells (▲), TRPC1-overexpressing (*OE*) cells (■), and Gö6976-treated cells (♦), and for the background current in untreated cells (no IP_3 or any other additive; ●); *I*, data shown as the mean ± S.E. of maximum steady-state current density (pA/picofarad (*pF*)) above the basal level under the conditions indicated ($n = 4–7$). *, statistically significant reduction ($p < 0.001$) in the IP_3-induced current after treatment with Gö6976 *versus* the control (*Cont*; IP_3); #, significant reduction ($p < 0.001$) in the SOC current in dnPKCα-transfected cells *versus* the vector (pCMV) control. In *A–G*, the *dotted lines* mark the zero current level. In *A* and *C–G*, the La^{3+} concentration was 1 mM. The downward deflection in all current traces represents inward current.

munoprecipitation with anti-Myc Ab and Western blotting with anti-Myc and anti-TRPC1 antibodies. Both antibodies detected protein at ~90 kDa in TRPC1-transfected cells, confirming the specificity of anti-TRPC1 antibody as described (35, 37, 38).

Transendothelial Electrical Resistance Measurement—The time course of endothelial cell retraction, a measure of increased endothelial permeability, was measured using established protocols (35, 39). For transfection of the Myc-tagged TRPC1 construct, HMECs grown on gelatin-coated gold electrodes were transfected with 1 μg/ml Myc-*TRPC1* cDNA (TRPC1-overexpressing cells) or vector alone (control cells) using LipofectAMINE Plus reagent as described (24). The DNA/LipofectAMINE Plus mixture was incubated with cells for 4 h in serum-free Dulbecco's modified Eagle's medium, followed by addition of complete growth medium to the cells. After 48–72 h of transfection, the cells were used for experiments. Before measuring the transendothelial electrical resistance, cells were placed in reduced serum medium (1% fetal bovine serum) for 1 h. Cells were then incubated with or without Gö6976 for 1 h, after which they were stimulated with thrombin to determine the changes in electrical resistance of the endothelial monolayer. Data are presented as the change in the resistive (in-phase) component of the impedance normalized to its initial value at zero time.

Data Analysis—Statistical comparisons were made using unpaired Student's *t* test. Statistical differences of $p < 0.05$ were considered significant.

RESULTS

Regulation of Store-operated Ca^{2+} Entry by PKCα

Thrombin-induced Ca^{2+} Entry—In Fura 2-loaded endothelial cells, the thrombin response consists of an immediate but transient rise in intracellular Ca^{2+} due to release of sequestered Ca^{2+} from the ER, followed by a secondary rise in intracellular Ca^{2+} due to Ca^{2+} entry. We experimentally separated the two phases (Fig. 1*A*) using a Ca^{2+} add-back protocol. When administered under Ca^{2+}-free bath conditions, thrombin elicited only the Ca^{2+} transient due to release of sequestered Ca^{2+} into the cytoplasm. A more sustained Ca^{2+} signal appeared subsequently when extracellular Ca^{2+} was restored to the bath medium, signifying entry of extracellular Ca^{2+} into cells. To identify the Ca^{2+} entry pathway, we used the trivalent cation La^{3+} or Gd^{3+}, either of which is a known blocker of SOCs (11). Addition of La^{3+} or Gd^{3+} rapidly abolished the Ca^{2+} entry phase (Fig. 1, *A* and *B*), thus indicating that Ca^{2+} entry is mediated via SOCs. To exclude the possibility that the Ca^{2+} entry was not the result of background or tonic Ca^{2+} entry, we showed that there was no Ca^{2+} entry following repletion of Ca^{2+} in the medium in the absence of store depletion (Fig. 1*A*, *dotted line*). In another series, we observed the blocking effect of a specific inhibitor of PKCα on Ca^{2+} entry via SOCs (Fig. 1*C*). The marked inhibitory action of Gö6976 on Ca^{2+} entry was evident because the Ca^{2+} release from ER stores was equivalent in control and treated cells, *i.e.* Ca^{2+} store depletion was the same under both conditions. Expression of dnPKCα (a kinase-defective mutant of PKCα) also resulted in a

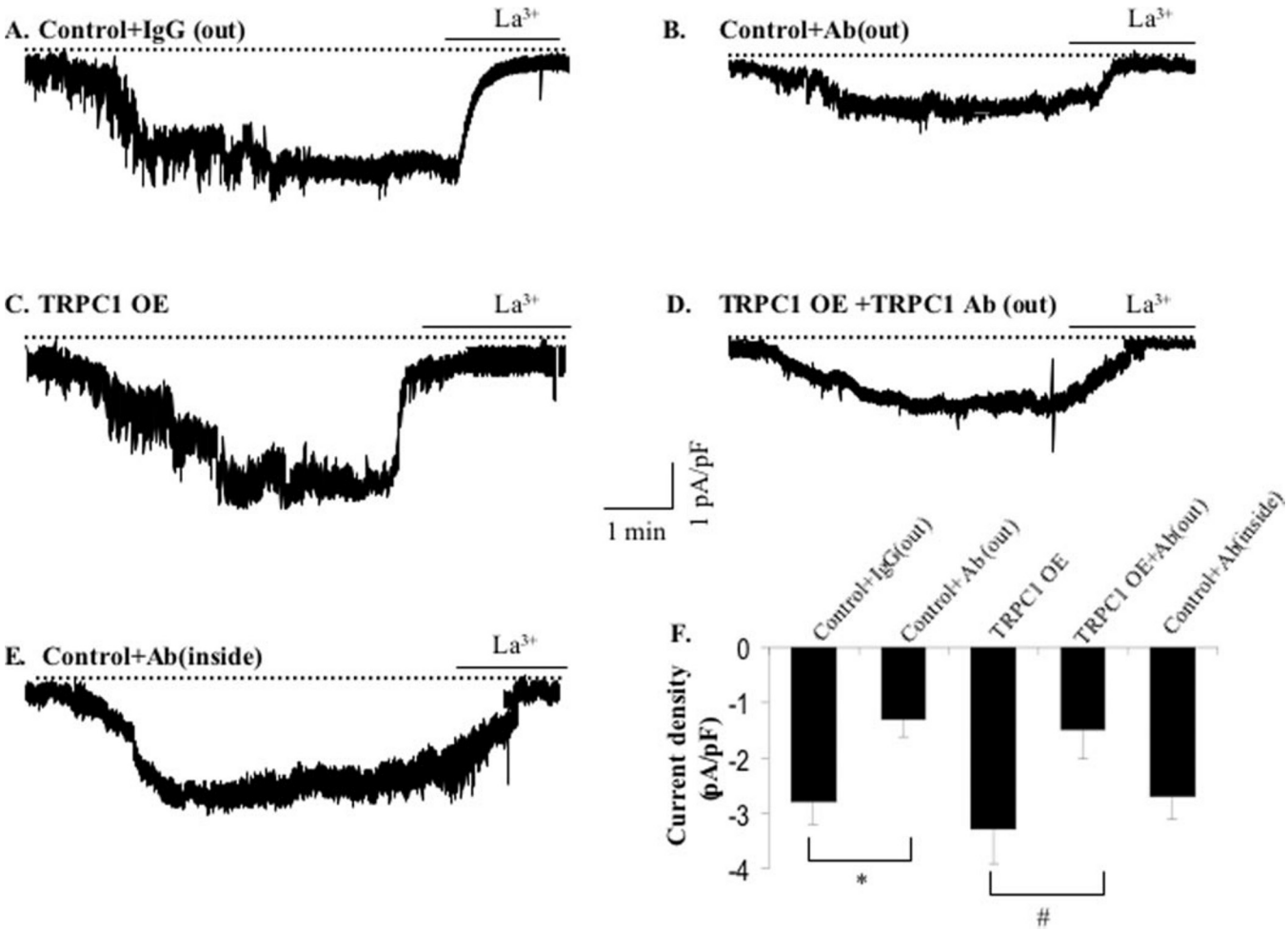

FIG. 4. **Effect of anti-TRPC1 antibody on store-operated currents.** IP_3-induced activation of SOCs is shown as whole cell continuous current recordings at a holding potential of −50 mV. The *traces* show the La^{3+}-sensitive current after intracellular application of IP_3. *A* and *B*, current from non-transfected control cells after 15 min of external application of 15 μg/ml IgG or anti-TRPC1 Ab, respectively; *C*, current from a cell overexpressing (*OE*) *TRPC1* cDNA; *D*, depressed current in a TRPC1-overexpressing cell pretreated with anti-TRPC1 Ab; *E*, essentially normal current recorded in a control cell internally dialyzed with anti-TRPC1 Ab; *F*, data shown as the mean ± S.E. of maximum steady-state current density over the basal value under the indicated conditions (n = 4–7). *, statistically significant reduction in current density in the presence of extracellular anti-TRPC1 Ab *versus* control IgG; #, significant reduction in current density in TRPC1-overexpressing cells due to external application of anti-TRPC1 Ab. In all current records, the downward deflection denotes inward current, and *dotted lines* mark the zero current level. In *A–E*, the La^{3+} concentration was 1 mM. *pF*, picofarad.

marked reduction in thrombin-induced Ca^{2+} entry via SOCs (Fig. 1*D*). As a procedural control, we showed that cells infected with a control construct (Ad-*lacZ*) gave normal responses to thrombin (Fig. 1*D*). The adenoviral vector used for these experiments guaranteed nearly 90% transfection of the endothelial monolayer (34). The summary graph in Fig. 1*E* demonstrates that 1) Ca^{2+} store depletion induced by thrombin was statistically indistinguishable in all groups and 2) Ca^{2+} entry caused by Ca^{2+} repletion was significantly reduced (>50% of the control) by genetic or pharmacological inhibition of PKCα. To demonstrate that TRPC1 is a functional component of SOCs in endothelial cells, we used anti-TRPC1 Ab against the extracellular epitope of TRPC1, which has been recently shown to block TRPC1 function in platelets (38). Ca^{2+} entry was measured following depletion of stores using thrombin. Anti-TRPC1 Ab pretreatment significantly inhibited the influx of Ca^{2+} following Ca^{2+} store depletion; however, control IgG had no effect (Fig. 1*F*). In these experiments, anti-TRPC1 Ab did not significantly reduce the Ca^{2+} release transient, but markedly inhibited Ca^{2+} entry, which fell to ~60% of the control. These findings indicate that normal Ca^{2+} entry via SOCs has a requirement for TRPC1 in endothelial cells.

Thapsigargin-induced Ca^{2+} Entry—Thapsigargin, an inhibitor of the Ca^{2+}-ATPase found in the ER (40), was used to activate SOCs by inducing Ca^{2+} store depletion independently of GPCRs (3, 4, 6, 10, 30). Ca^{2+} entry in response to thapsigargin was monitored in Fura 2-loaded endothelial cells with or without Gö6976 pretreatment (Fig. 2, *A* and *B*) or in endothelial cells expressing either dnPKCα or the *lacZ* control (Fig. 2, *C* and *D*). Inhibition of PKCα attenuated the store depletion-induced Ca^{2+} entry by 52 ± 5% (Gö6976) and 58 ± 6% (dnPKCα) (Fig. 2, *A–D*), indicating that PKCα contributes to SOC activation independently of GPCR pathways.

IP_3-induced Store-operated Current—IP_3 can induce Ca^{2+} store depletion and should thereby activate Ca^{2+} entry via SOCs. Poor selectivity for divalent cations and sensitivity to La^{3+} or Gd^{3+} are characteristic properties of store-operated channels in many tissues (4, 41, 42). Using the whole cell patch clamp technique (43), we internally applied IP_3 and measured the resulting SOC that we operationally defined as the La^{3+}-sensitive inward current at −50 mV. Continuous current recordings showed the rapid development of an La^{3+} (1 mM)-sensitive inward current upon internal dialysis with IP_3 or perfusion of thapsigargin. The action of La^{3+} was reversible as shown (Fig. 3*A*). Without IP_3/thapsigargin in

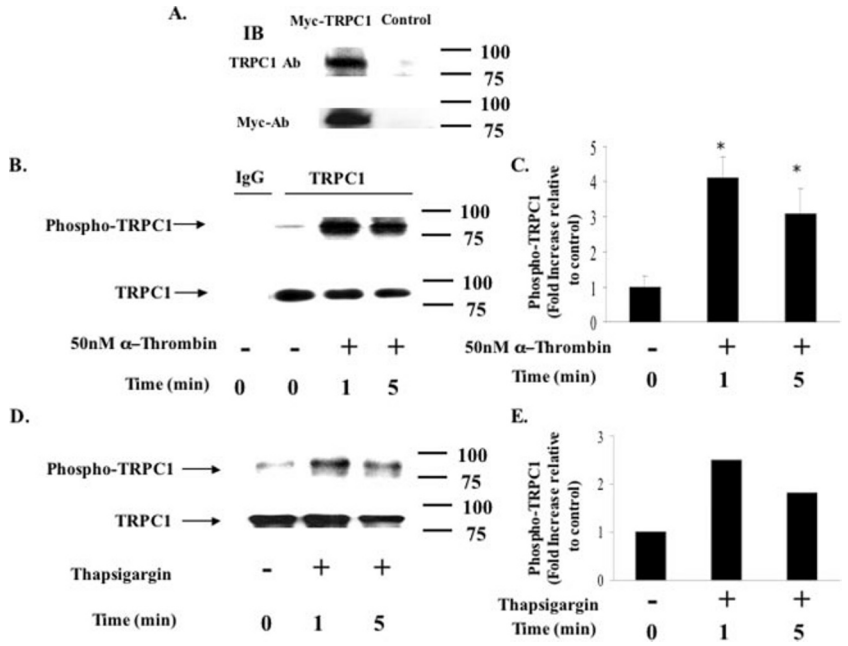

FIG. 5. **Thrombin- and thapsigargin-induced TRPC1 phosphorylation.** *A*, COS-7 cells overexpressing the Myc-tagged TRPC1 construct were lysed, and TRPC1 was immunoprecipitated with anti-Myc Ab, followed by Western blotting with anti-Myc or anti-TRPC1 Ab. Both antibodies recognized the TRPC1 band (~90 kDa). *B–E*, HUVECs grown to confluence were serum-starved and then labeled with ^{32}P for 4 h in phosphate-free medium. Cell lysates were immunoprecipitated with control IgG or rabbit anti-TRC1 Ab. Proteins were electrophoresed and transferred to nitrocellulose membranes, after which the membranes were exposed to radiography, followed by Western blotting with anti-TRPC1 Ab. *B* and *C*, thrombin-stimulated phosphorylation of TRPC1 for indicated time periods and quantitative results by densitometry, respectively. *, significant increase ($p < 0.05$) in TRPC1 phosphorylation compared with unstimulated cells. *D* and *E*, thapsigargin-stimulated phosphorylation of TRPC1 for the indicated time periods and quantitative results by densitometry, respectively. In *B* and *D*, the *upper panels* show autoradiograms of phosphorylated TRPC1, whereas the *lower panels* show Western blots of TRPC1. *C* and *E*, densitometric analysis of thrombin-stimulated ($n = 3$) and thapsigargin-stimulated ($n = 2$) TRPC1 phosphorylation, respectively. quantified as the increase in phosphorylation of TRPC1 above the level in unstimulated cells. *IB*, immunoblot.

the pipette, there was no current as shown in Fig. 3*B*, indicating that the current was not the result of a nonspecific leak. To determine whether the SOC current is regulated by PKCα, we pretreated cells with Gö6976 prior to membrane rupture with patch pipettes. Inhibition of PKCα by Gö6976 markedly reduced the SOC current amplitude from a mean value of 2.9 ± 0.3 to 1.3 ± 0.2 pA/picofarad (Fig. 3, *C* and *I*), indicating the role of endogenous PKCα in regulating SOC currents. Because Gö6976 has been shown to affect PKCβ in several cell types (30, 44), we also used LY379196, an inhibitor of PKCβ, to address the possible role of PKCβ in modulating SOC currents. As shown in Fig. 3*D*, inhibition of PKCβ had no effect on the IP_3-induced current. To demonstrate the specific role of PKCα, we also compared the magnitude of IP_3-induced currents in endothelial cells expressing the pCMV vector (control) and in cells expressing dnPKCα. As shown in Fig. 3 (*E* and *F*), expression of dnPKCα significantly reduced IP_3-induced currents; current amplitude was significantly decreased from 2.7 ± 0.3 to 1.2 ± 0.3 pA/picofarad (Fig. 3*I*). Perfusion of thapsigargin (2 μM) activated the SOC current to a similar amplitude as that induced by IP_3 (Fig. 3*G*). The current-voltage relationship in Fig. 3*H* shows a reversal potential close to 0 mV, indicating that the inward current was carried by cations as described previously for SOCs (4, 21, 45, 46). As TRPC1-overexpressing cells showed the same reversal potential as control cells, the results indicate that, in control cells, TRPC1 forms the main constituent of SOCs, as has been described by others (45, 47, 48). These results, which show that PKCα is critical in regulating SOC activity, are in agreement with the findings (see above) obtained using pharmacological inhibitors.

To further corroborate the role of TRPC1 in regulating Ca^{2+} entry observed above (Fig. 1*F*), we determined SOC currents in control endothelial cells as well as in TRPC1-overexpressing cells after pretreatment with anti-TRPC1 Ab (38, 49). Overexpression of TRPC1 increased the amplitude of IP_3-induced currents (Fig. 4, *C* and *F*). Pretreatment with anti-TRPC1 Ab significantly reduced the mean amplitude of IP_3-induced SOC currents in control and TRPC1-overexpressing cells (Fig. 4, *B*, *D*, and *F*). In contrast, preincubation of control cells with isotype-matched IgG (15 μg/ml) for 15 min had no effect on the current amplitude (Fig. 4*A*). In addition, anti-TRPC1 Ab had no detectable effect on IP_3-induced SOC currents when injected into the cell cytoplasm (Fig. 4*E*). Altogether, the data presented in Fig. 4 reinforce our observations, obtained in Fura 2-loaded cells, that PKCα activity controls Ca^{2+} entry via store-operated Ca^{2+} channels, most importantly, through the TRPC1 channel.

PKCα Phosphorylation of TRPC1

Thrombin- and Thapsigargin-induced Phosphorylation of TRPC1—As TRPC1 is a presumptive protein constituent of SOCs in human endothelial cells, and its sequence also contains several putative phosphorylation sites (50, 51), we hypothesized that thrombin activates SOCs by inducing the phosphorylation of TRPC1. We therefore determined TRPC1 phosphorylation in endothelial cells labeled with [^{32}P]orthophosphate upon stimulation with thrombin for various time periods. We first determined the specificity of anti-TRPC1 Ab using COS-7 cells transfected with Myc-tagged TRPC1 or vector alone. Cells were lysed in radioimmune precipitation assay buffer and immunoprecipitated with anti-Myc Ab, followed by Western blotting with anti-TRPC1 or anti-Myc Ab. Both antibodies detected protein at ~90 kDa in TRPC1-transfected cells (Fig. 5*A*). Thrombin induced rapid (within 1 min) and sustained (up to 5 min) phosphorylation of

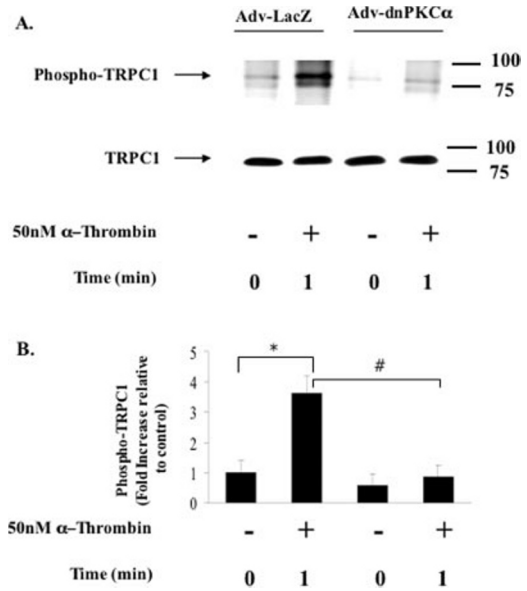

FIG. 6. **PKCα regulates thrombin-induced TRPC1 phosphorylation.** HUVECs expressing dnPKCα (Ad (*Adv*)-dnPKCα) or the control construct (Ad-*lacZ*) were serum-starved, labeled with ^{32}P for 4 h in phosphate-free medium, and stimulated with thrombin for 1 min. Cell lysates were immunoprecipitated with anti-TRPC1 antibody. Proteins were electrophoresed and transferred to nitrocellulose membranes, after which the membranes were exposed to radiography, followed by Western blotting with anti-TRPC1 Ab. In *A*, the *upper panel* shows an autoradiogram of phosphorylated TRPC1, whereas the *lower panel* shows a Western blot of TRPC1. In *B*, densitometric data summarize the thrombin-induced increase in phospho-TRPC1 at 1 min as a multiple of the unstimulated level. Note that thrombin produced a statistically significant increase in TRPC1 phosphorylation in the transfection control (Ad-*lacZ*; *, $p < 0.001$). In cells expressing the dnPCKα construct, the effect of thrombin was completely inhibited compared with the control construct (#, $p < 0.001$). Means ± S.E. are based on three independent experiments.

TRPC1 (Fig. 5, *B* and *C*). Thrombin-stimulated phosphorylation of TRPC1 was specific because it was not seen in lysates immunoprecipitated with control IgG (Fig. 5, *B* and *C*). In addition, we determined TRPC1 phosphorylation in response to depletion of stores with thapsigargin, which bypasses the GPCR pathway. Thapsigargin also induced the phosphorylation of TRPC1 within 1 min (Fig. 5, *D* and *E*), indicating that store depletion alone can trigger phosphorylation of TRPC1.

TRPC1 Phosphorylation Requires PKCα Activity—Because the results presented in Figs. 1–4 point to a role of PKCα in regulating SOC-mediated Ca^{2+} entry, we addressed the possibility that PKCα regulates SOCs by phosphorylating the TRPC1 channel protein. We therefore expressed dnPKCα in endothelial cells to determine its role in thrombin-induced TRPC1 phosphorylation. Endothelial cells infected with Ad-dnPKCα or Ad-*lacZ* (as a control) were metabolically labeled with [^{32}P]orthophosphate and stimulated with thrombin for 1 min. As shown in Fig. 6 (*A* and *B*), thrombin induced significant increases in TRPC1 phosphorylation in Ad-*lacZ*-transduced cells, but not in cells expressing dnPKCα. These findings indicate that the thrombin-induced phosphorylation of TRPC1 requires PKCα activity.

Role of PKCα in Induction of Increased Transendothelial Permeability

As the results implicated PKCα in regulating Ca^{2+} entry through SOCs, we determined the role of PKCα in regulating endothelial barrier function. We measured the changes in transendothelial electrical resistance in control HMEC monolayers and in cells overexpressing the TRPC1 construct with or without Gö6976 treatment. Thrombin caused a significant decrease in transendothelial electrical resistance in untreated control and TRPC1-overexpressing cells; pretreatment with Gö6976 reduced the effect of thrombin in both the control and TRPC1-overexpressing groups by 60 ± 6 and 58 ± 5%, respectively (Fig. 7, *A* and *B*), thus indicating the functional role of PKCα-induced Ca^{2+} entry in regulating endothelial barrier function.

DISCUSSION

The results demonstrate that PKCα activation in response to thrombin induces phosphorylation of the TRPC1 protein. The functional importance of TRPC1 phosphorylation in endothelial cells is evident by the following: 1) phosphorylation of TRPC1 in response to thrombin or thapsigargin occurred within minutes and preceded Ca^{2+} entry; 2) thrombin-induced phosphorylation of TRPC1 was blocked by overexpression of dnPKCα; and 3) the La^{3+}-sensitive IP_3-induced current (20, 21) through SOCs was markedly reduced when PKCα activity was inhibited. Ca^{2+} store depletion has thus far been considered the triggering event to open SOCs, resulting in store-operated Ca^{2+} entry (4, 5, 7, 52, 53). The present findings imply that phosphorylation of TRPC1 by PKCα can also increase Ca^{2+} entry through SOCs in endothelial cells. We found that such modulation of SOCs accounted for at least 50% of the current via SOCs in endothelial cells based on inhibitory effects of Gö6976 (100 nM) and expression of the catalytically defective PKCα mutant. In this study, we considered the whole cell currents through SOCs and did not address the detailed mechanisms of their modulation, which could theoretically include increases in channel open probability and amplitude of the single channel current. Either of these mechanisms could account for the partial inhibition of the macroscopic current through SOCs observed when PKCα was inhibited. Alternatively, it is arguable that the phosphorylated channel is preferentially opened by store depletion. Thus, the emerging picture of store-operated Ca^{2+} entry on the basis of the present findings includes channel opening by Ca^{2+} store depletion plus a functional modification of the channel by protein phosphorylation that enhances channel activity.

The precise relationship between Ca^{2+} store depletion and SOC activation remains controversial. However, despite intensive study, the ER-derived signal coupling store depletion and SOC activation is unknown. Evidence indicates that physical docking of the ER with the plasma membrane could be involved in SOC activation (6, 7, 52). Recently, we showed that, upon thrombin activation, RhoA signals interaction of the IP_3 receptor with TRPC1 at the plasma membrane and triggers SOC-induced Ca^{2+} entry in endothelial cells (35). We also showed that thrombin-induced Rho activation is dependent on PKCα, indicating that PKCα contributes significantly to Rho regulation of Ca^{2+} entry (26). There is also some evidence of involvement of both tyrosine kinase- and PKC-mediated protein phosphorylation/dephosphorylation in modulation of SOC activity (4, 12, 30, 54). It has been shown in photoreceptor cells that the TRPC is a substrate for PKC and that phosphorylation of TRPC by PKC regulates Ca^{2+} influx through the TRPC channel (55). This led us to investigate the potential function of PKCα phosphorylation of the TRPC1 channel in activation of SOCs (thus, Ca^{2+} entry) and in endothelial barrier function. In this regard, we showed that thrombin and thapsigargin stimulation led to an overall increase in TRPC1 phosphorylation in whole cell lysates as detected by Western blot analysis (Fig. 5, *B–E*). Our results further revealed that Ca^{2+} store depletion activated PKCα, which subsequently induced the phosphorylation of TRPC1. Our evidence for this concept is that inhibition of

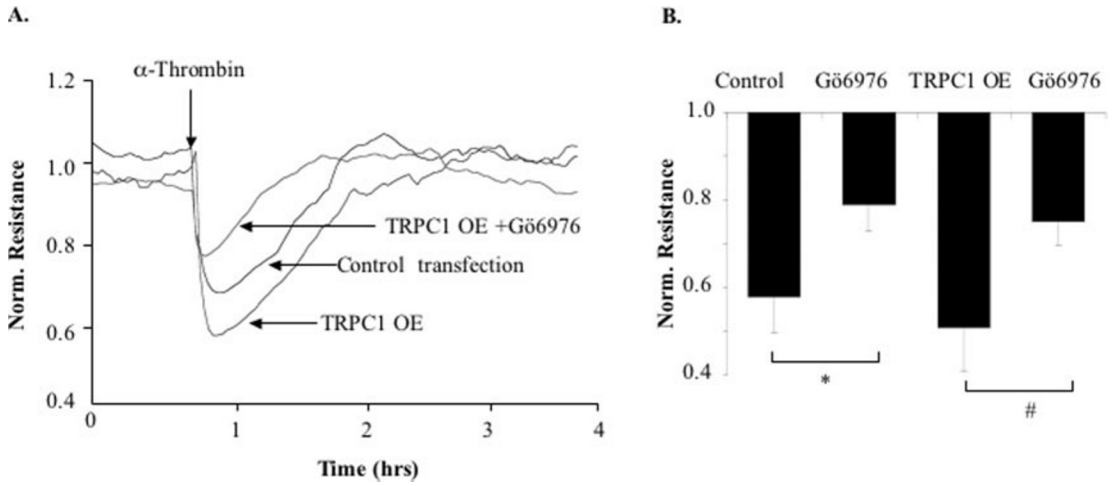

FIG. 7. **Effect of inhibition of PKCα on the thrombin-induced decrease in transendothelial monolayer electrical resistance in cells overexpressing TRPC1.** HMECs were transfected with either vector (control transfection) or the *TRPC1* cDNA (*TRPC1 OE*) construct. Transfected cells were grown to confluence on gold electrodes, and cells were placed in reduced serum medium (1% fetal bovine serum) for 2 h. Cells were then incubated with or without Gö6976 (100 nM) for 1 h, after which cells were stimulated with 50 nM thrombin to measure the changes in transendothelial electrical resistance. *A*, traces representative of a single experiment showing the inhibitory effect of Gö6976 in TRPC1-overexpressing (*OE*) cells; *B*, data shown as the mean ± S.E. of the peak reduction in electrical resistance produced by thrombin challenge (n = 5). * and #, values different from Gö6976-treated monolayers.

PKCα impaired Ca^{2+} entry through SOCs after store depletion induced by thapsigargin or internally applied IP_3.

TRPC1 is expressed in several cell types, including human endothelial cells (10, 14, 21–23), vascular smooth muscle cells (13), salivary gland cells (4, 56), human platelets (38), and airway smooth muscle cells (16). We have focused on TRPC1 in this study because human endothelial cell (HUVECs and HMECs) predominantly express this isoform; they also express TRPC3 and TRPC4, but to a considerably lesser extent (11, 22, 23, 35). These minor isoforms could contribute to the SOC, but may be regulated differently. A recent study by Venkatachalam *et al.* (29) using human embryonic kidney cells as a TRPC expression system showed that PKC inhibits TRPC4- and TRPC5-mediated Ca^{2+} entry, but that the kinase does not regulate Ca^{2+} entry through TRPC3 channels; the latter isoform is directly activated by diacylglycerol or its analogs. Because TRPC function varies with the cell type used, and its expression level *per se* determines channel activity, it is possible that these isoforms are regulated by distinct mechanisms. In our studies on endothelial cells, we would have observed an inhibition of SOC activity if the predominant effect of PKCα were to inhibit TRPC4. Because this was not the case, and expression level of TRPC4 was low in the endothelial cells used, the role of TRPC4 in the present experiments is inferred to be minor.

It has been shown that the SOC is inhibited by transfection with antisense TRPC1; extracellular addition of anti-TRPC1 antibody that specifically recognizes TRPC1-(557–571), which is present in TRPC1 and no other protein; or deletion of the *TRPC1* gene (12–14, 38, 49). We also found that pretreatment of endothelial cells in medium containing anti-TRPC1 Ab significantly inhibited SOC-induced Ca^{2+} entry in control cells as well as in cells overexpressing TRPC1, thus indicating that TRPC1 is a critical protein constituent of functional SOCs. Recently, it has been shown that the TRPC1 S5–6 region comprises the pore-forming unit of the channel and that mutation of TRPC1-(567–793) causes a reduction in the total number of functional channels without changing the channel properties (12). Although these studies provide convincing evidence that TRPC1 forms functional SOCs, they do not explain how TRPC1 becomes activated after store depletion. The TRPC1 protein has multiple serine/threonine phosphorylation sites in the putative pore-forming region and N and C termini (51). As PKCα is a serine/threonine kinase, we propose that phosphorylation of TRPC1 serine/threonine sites by PKCα regulates TRPC1 channel opening and hence store-operated Ca^{2+} entry.

PKC has been shown to modulate a variety of intracellular events by regulating the activity of ion channels. However, the results are extremely variable with respect to the regulation of SOCs by PKC (28–33) because there are multiple PKC isoforms with different functions. When activated, PKC normally translocates to its target site, which, in the case of SOCs, is the plasma membrane (17). It is probable that Ca^{2+} store depletion is involved in modifying the SOC via PKC activation, but it is unknown about how the kinase is activated.

Our results suggest that thrombin- or thapsigargin-induced phosphorylation of TRPC1 by PKCα strongly contributes to the regulation of SOC-induced Ca^{2+} influx. Phosphorylation of serine/threonine residues in the TRPC1 protein appears to maintain the channel activity. The precise phosphorylation site is unknown and requires further study. We observed the optimum phosphorylation by thrombin within 1 min, with continued activity for at least 5 min under our experimental conditions. Hence, PKCα-dependent channel phosphorylation may be required for full agonist activation of SOCs. The specific target for PKCα identified in this study is one or more phosphorylation sites in TRPC1.

Because Ca^{2+} signaling can activate PKCα, which, in turn, has an important role in SOC recruitment (9, 11), we also addressed the role of PKCα-induced Ca^{2+} entry in mediating the increase in endothelial permeability. We measured the transendothelial electrical resistance in endothelial cells to test the hypothesis that PKCα activation increases endothelial permeability. PKCα inhibition markedly decreased the effect of thrombin on endothelial permeability in both TRPC1-overexpressing and control cells. Therefore, SOCs arguably play an important role in the mechanism of endothelial barrier dysfunction.

In conclusion, our data show that 1) PKCα regulates the influx of Ca^{2+} through the SOC and 2) PKCα phosphorylation of TRPC1 is responsible for physiologically significant modulation of SOCs. The results indicate that PKCα regulates endothelial barrier function through the phosphorylation of TRPC1.

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