Ca\(^{2+}\) Influx Induced by Protease-activated Receptor-1 Activates a Feed-forward Mechanism of TRPC1 Expression via Nuclear Factor-κB Activation in Endothelial Cells* 

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Thrombin activation of protease-activated receptor-1 induces Ca\(^{2+}\) influx through store-operated cation channel TRPC1 in endothelial cells. We examined the role of Ca\(^{2+}\) influx induced by the depletion of Ca\(^{2+}\) stores in signaling TRPC1 expression in endothelial cells. Both thrombin and a protease-activated receptor-1-specific agonist peptide induced TRPC1 expression in human umbilical vein endothelial cells, which was coupled to an augmented store-operated Ca\(^{2+}\) influx and increase in endothelial permeability. To delineate the mechanisms of thrombin-induced TRPC1 expression, we transfected in endothelial cells TRPC1-promoter-luciferase (TRPC1-Pro-Luc) construct containing multiple nuclear factor-κB (NF-κB) binding sites. Co-expression of dominant negative IkBα mutant prevented the thrombin-induced increase in TRPC1 expression, indicating the key role of NF-κB activation in mediating the response. Using TRPC1 promoter-deletion mutant constructs, we showed that NF-κB binding sites located between −1623 and −871 in the TRPC1 5′-regulatory region were required for thrombin-induced TRPC1 expression. Electrophoretic mobility shift assay utilizing TRPC1 promoter-specific oligonucleotides identified that the DNA binding activities of NF-κB to NF-κB consensus sites were located in this domain. Supershift assays using NF-κB protein-specific antibodies demonstrated the binding of p65 homodimer to the TRPC1 promoter. Inhibition of store Ca\(^{2+}\) depletion, buffering of intracellular Ca\(^{2+}\), or down-regulation of protein kinase Ca downstream of Ca\(^{2+}\) influx all blocked thrombin-induced NF-κB activation and the resultant TRPC1 expression in endothelial cells. Thus, Ca\(^{2+}\) influx via TRPC1 is a critical feed-forward pathway responsible for TRPC1 expression. The NF-κB-regulated TRPC1 expression may be an essential mechanism of vascular inflammation and, hence, a novel therapeutic target.

Ca\(^{2+}\) signaling regulates endothelial cell and matrix tethering interactions and cell actin-myosin cell contractile machinery and, thus, is important in the control of vascular endothelial barrier function (1–3). An increase in [Ca\(^{2+}\)], induces barrier dysfunction leading to leakiness of the endothelial monolayer to plasma proteins (3). Mediators such as thrombin, histamine, and reactive oxygen species increase vascular permeability by activating Ca\(^{2+}\)-sensitive signaling pathways (3). We showed that Ca\(^{2+}\) entry through plasma membrane cation channels activated by Ca\(^{2+}\) store depletion is a critical determinant of increased endothelial permeability (4–6). We have also shown that activation of endothelial cell surface protease-activated receptor-1 (PAR-1)3 by thrombin caused a rapid and transient increase in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)],) secondary to the release of stored Ca\(^{2+}\) and subsequent longer-lived Ca\(^{2+}\) entry triggered by store depletion (4, 6). In endothelial cells, the plasma membrane cation channels, known as store-operated cation channels (SOCs), mediate the entry of Ca\(^{2+}\) (7–9). Several studies have shown that influx of Ca\(^{2+}\) in vascular endothelial cells occurs via SOCs (6, 8, 10, 11). Studies have also identified that the mammalian homologues of the transient receptor potential (TRP) gene family of channels function as SOCs (6, 8, 12, 13). These TRP genes encode a superfamily of proteins with six transmembrane helices that are divided into seven subfamilies: TRPC (canonical or classical), TRPV (vaniloid), TRPM (melastatin), TRPA (ankyrin), TRPML (mucolipin), TRPP (polycystin), and the TRPN (no mechanoreceptor potential C (NOMPC) (10, 14). Members of the TRPC subfamily contain 700–1000 amino acids, and 7 isoforms (TRPC1–7) are expressed in mammalian cells. Primary endothelial cells express TRPC1–7 (6, 12, 15). TRPC4 is the predominant isof orm expressed in mouse endothelial cells (6, 12). Deletion of TRPC4 in mice caused impairment in SOC current in mouse aortic and lung vascular endothelial cells (6, 12). We showed that SOC-mediated Ca\(^{2+}\) influx induced by thrombin causing increased microvascular permeability was impaired in

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3The abbreviations used are: PAR-1, protease-activated receptor-1; SOC, store-operated cation channel; TRP, transient receptor potential; ICAM-1, intercellular adhesion molecule 1; VCAM, vascular cell adhesion molecule; DAG, diacylglycerol; FBS, fetal bovine serum; HBVEC, human umbilical vein endothelial cell(s); HMEC, human dermal microvascular endothelial cell(s); RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBSS, Hanks’ balanced salt solution; hTRPC1, human TRPC1; 2-APB, 2-aminoethoxydiphenyl borate; EMDA, electrophoretic mobility shift assay; PKC, protein kinase C; siRNA, small interfering RNA; ER, endoplasmic reticulum; TER, transendothelial monolayer resistance; DN, dominant negative; NBD, NEMO binding domain; BAPTA/AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetra(acetoxymethyl)ester; Ab, antibody; IKK, IκB Kinase; NEMO, NF-κB essential modifier.
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TRPC4 knock-out mice (6); thus, TRPC4 is an essential SOC in endothelial cells mediating increased vascular permeability. Moore et al. (11) showed that antisense oligonucleotide-induced inhibition of TRPC1 expression, a predominant isof orm expressed in human vascular endothelial cells (15), reduced the thapsigargin-induced Ca\textsuperscript{2+} entry (11). We showed that overexpression of TRPC1 augmented the thapsigargin-induced Ca\textsuperscript{2+} influx in human endothelial cells (15), and this response resulted in markedly increased endothelial permeability (15). The cloning of human TRPC1 5\textsuperscript{\prime}-regulatory region revealed that it contains multiple nuclear factor-κB (NF-κB) binding sites (16). Tumor necrosis factor-α-activated TRPC1 expression in human endothelial cells was shown to occur via NF-κB signaling (16). In addition, tumor necrosis factor-α stimulation of human endothelial cells resulted in increased TRPC1 expression (15, 16).

Thrombin mediates the expression of genes such as ICAM-1 and VCAM-1 by activating NF-κB signaling in endothelial cells (17–19). In the present study we examined the role of Ca\textsuperscript{2+} influx induced by the depletion of Ca\textsuperscript{2+} stores in signaling TRPC1 expression in endothelial cells and its consequences in augmenting Ca\textsuperscript{2+} signaling. Utilizing TRPC1 promoter deletion mutant construct expression studies, we identified that NF-κB binding sites upstream of TRPC1 5\textsuperscript{\prime}-regulatory region were critical for thrombin-induced TRPC1 expression. We showed that store-operated Ca\textsuperscript{2+} influx signaled NF-κB activation and induced TRPC1 expression and thereby augmented Ca\textsuperscript{2+} influx in response to Ca\textsuperscript{2+} store depletion. Thus, augmented Ca\textsuperscript{2+} influx signal may play an important role in the pathogenesis of vascular inflammation and injury and provides a novel therapeutic target against vascular inflammatory diseases.

EXPERIMENTAL PROCEDURES

Materials—Human α-thrombin was obtained from Enzyme Research Laboratories (South Bend, IN). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). PAR-1 agonist (TFLLRNPNDK) and control (TFLLRNPNPD) peptides were synthesized as the C-terminal amide in-house (Biotechnology Center, University of Illinois at Urbana-Champaign). Endothelial growth medium (EGM-2) was obtained from Cambrex Bio Science Inc. (Walkersville, MD). Anti-TRPC1 polyclonal antibody (anti-TRPC1 Ab), anti-PKC\textalpha Ab, anti-PKC\textbeta Ab, anti-p50 Ab, anti-p52 monoclonal Ab, anti-c-Rel Ab, and anti-RelB Ab were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Anti-p65 (Rel A) Ab was obtained from Chemicon International Inc (Temecula, CA). TRlZol reagent, TaqDNA polymerase, Lipofectamine, Lipofectamine Plus reagent, MCDB 131, Opti-MEM I, and restriction enzymes were from Invitrogen. Fura-2 AM was obtained from Molecular Probes, Inc. (Eugene, OR). PCR primers were custom-synthesized from IDT (Coralville, IA). Cell-permeable Ca\textsuperscript{2+} chelator (1,2-bis(o-aminophenoxo)ethane-N,N,N',N\textprime-tetra(ace-toxymethyl)ester (BAPTA/AM)) was obtained from EMD Biosciences, Inc. (La Jolla, CA). The reporter vectors pGL2 (firefly, Photinus pyralis) and pRL (sea pansy, Renilla reniformis) luciferase and the dual luciferase assay system were from Promega Corp., Madison, WI. Non-phosphorylatable IκB\alpha dominant negative mutant cDNA cloned into pUSEamp+ vector was from Upstate Biotechnology, Inc. (Lake Placid, NY). PKC\textalpha dominant negative mutant lacking the functional catalytic domain due to substitution of lysine 368 was a gift from Dr. J. Soh (Columbia University, New York). Cell-permeable NEMO-binding domain (NBD) synthetic peptides (wild type, dqrkifwqrrnkwwkTALDWSLQTE (WT-NBD); mutant, dqrkifwgqrnmkwwkTALDASALQTE (MT-NBD)) were obtained from Biomol International LP, Plymouth Meeting, PA. Protease inhibitor mixture was obtained from Roche Applied Science.

Cell Culture—Human umbilical vein endothelial cells (HUVEC) obtained from Cambrex Bio Science Inc. (Walkersville) were grown in EGM-2 medium supplemented with 10% FBS as described (4, 16). HUVEC used in the experiments were between 3 and 6 passages. The human dermal microvascular endothelial cell line (HMEC) was grown in endothelial basal medium MCDB131 supplemented with 10% FBS, epidermal growth factor (10 ng/ml), 2 mM L-glutamine, and hydrocortisone (1 μg/ml) (16, 21).

Reverse Transcription (RT)-PCR—Confluent HUVEC monolayers were washed with serum-free medium and incubated with 1% FBS-containing medium for 2 h. After this period, the endothelial cells were treated with 50 nM thrombin in the presence or absence of actinomycin D (0.5 μM) in 1% FBS-containing medium for different time intervals. After thrombin treatment, total RNA was isolated using TRlZol reagent. RT-PCR was performed as described (16). Human TRPC1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified using the following primer sets: TRPC1 (forward, 5\textprime-GATT-TGGAAAATTTCTTGGGATGT-3\textprime; reverse, 5\textprime-GATTTTGCTCATGATTTGCTATCA-3\textprime); GAPDH (forward, 5\textprime-TATCGTGAGGACTCATGACC-3\textprime; reverse, 5\textprime-TACATGGCAACTGTGAGGG-3\textprime). RT product (2 μl) was amplified in a 50-μl volume containing 100 pmol of primers and 2.5 units of TaqDNA polymerase. Reaction conditions are as follows: 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min for 35 cycles, and then 72 °C for 7 min. Amplification of GAPDH was also performed following the protocol described above. PCR products were separated on 1.2% agarose gel and identified by ethidium bromide staining. The band intensity of scanned gel photograph was determined by using Scion imaging software (from the National Institutes of Health). Normalization of TRPC1 expression was achieved by comparing the expression of GAPDH for the corresponding sample.

Immunoblotting—TRPC1 expression in response to PAR-1 activation in HUVEC was determined by immunoblotting cell lysate proteins with anti-TRPC1 antibody (16).

Cytosolic Ca\textsuperscript{2+} Measurement—The cytoplasmic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}\textsubscript{i}]) in single endothelial cells was measured by Fura-2 fluorescence imaging (15, 16). Cells grown on 25-mm diameter glass coverslips were washed twice in Hanks' balanced salt solution (HBSS) and loaded with 3 μM Fura-2 AM for 20 min at 37 °C. Cells were then washed twice in HBSS and imaged by using an Attofluor RatioVision digital fluorescence microscopy system (Atto Instruments, Rockville, MD) equipped with a Zeiss Axiosvert S100 inverted microscope and F-Flur 40×40, 1.3 NA oil immersion objective. Regions of interest in individual cells were marked and excited at 334 and 380 nm with emission
at 520 nm at 5-s intervals. The 334/380 nm fluorescence ratio has been used to represent changes in [Ca\(^{2+}\)].

**Transendothelial Electrical Resistance**—The real-time change in endothelial monolayer resistance was measured to assess endothelial barrier function as described by us (20). In brief, HUVEC were grown to confluence on a small gold electrode (4.9 \(\times\) 10\(^{-4}\) cm\(^2\)). The small electrode and the larger counter electrode were connected to a phase-sensitive lock-in amplifier. An approximate constant current of 1 \(\mu\)A was supplied by a 1-V, 4000-Hz AC signal connected serially to 1-megaohm resistor between the small electrode and the larger counter electrode. The voltage between small electrode and large electrode was monitored by lock-in amplifier, stored, and processed by a personal computer. The same computer controlled the output of the amplifier and switched the measurement to different electrodes in the course of an experiment. Before the experiment, confluent endothelial monolayer was kept in 1% FBS-containing medium for 2 h, and then cells were either stimulated with thrombin or not stimulated with thrombin for 20 h. After this treatment, thrombin-induced change in resistance of endothelial monolayer was measured. The data are presented in resistance normalized to its value at time zero as described (20, 22).

**Plasmids and Mutagenesis**—The complete procedure for cloning the human TRPC1 (hTRPC1) promoter was described by us before (16). We cloned an \(-2.1\)-kilobase size hTRPC1 5′-regulatory region into pGL2 basic vector to generate hTRPC1-Pro-Luc expression construct (wild type hTRPC1-Pro-Luc) (16). Deletion mutant constructs for NF-κB sites in the hTRPC1-Pro-Luc were prepared by the PCR method using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The following sense and antisense oligonucleotides indicated in parenthesis were used (ΔNF-κB-1623, sense, 5′-GATAAACATCTTGTAGACA-3′, antisense, 5′-TGTCTACAAAGATGTTATC-3′; ΔNF-κB-880, sense, 5′-TGTCTACAAAGATGTTATC-3′; ΔNF-κB-708, sense, 5′-CATTGTTAGAGAACTA-3′; antisense, 5′-TTATTTGCTCAACTG-3′) for PCR amplification utilizing the hTRPC1-Pro-Luc (\(-1685\) to +40 (wild type hTRPC1-Pro-Luc)) plasmid template. These mutant constructs sequences were verified by DNA sequencing before using for transfection experiments.

**Expression of Reporter Constructs**—HMEC grown to 50% confluency in 6-well culture plates were used for reporter constructs transfection (16, 21). Plasmid DNA mixtures containing 1 \(\mu\)g of hTRPC1 promoter-luciferase (hTRPC1-Pro-Luc) in pGL2 vector and 0.035 \(\mu\)g of pRL/TK (Promega) were transfected using Lipofectamine. Lipofectamine-DNA complexes were diluted with 0.8 ml of Opti-MEM I before being added to HMEC and prewashed two times with Opti-MEM I for 2–4 h. To end transfection, 2 ml of MCBD131 medium supplemented with 10% FBS was added to each well.

**Dual Luciferase Reporter Assay**—At 48 h after transfection, the cells were incubated in MCBD131 medium containing 1% FBS for 2 h and then stimulated with thrombin or PAR-1 agonist peptide. In some experiments cells were pretreated with either 2-aminothoxydiphenyl borate (2-APB) (75 \(\mu\)M) for 30 min before thrombin treatment. After stimulation, cells were lysed, and 20 \(\mu\)l of lysate was used to measure reporter gene expression (16, 21). Firefly (\(P. pyralis\)) and sea pansy (\(R. reniformis\)) luciferase activity were assayed by the dual luciferase reagent assay system (Promega). Protein concentrations were determined using Bio-Rad reagents.

**Nuclear Protein Extraction**—Nuclear extracts were prepared from HUVECs after thrombin treatment as described (18). Cells grown in 100-mm cell culture dishes were washed twice with ice-cold Tris-buffered saline, scraped, and resuspended in 400 \(\mu\)l of buffer A (10 mM KCl, 10 mM HEPES, pH 7.9, 0.1 mM EDTA, pH 8.0, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). The suspension was homogenized for 10 strokes using a Glass Dounce homogenizer and centrifuging at 3000 \(\times\) g for 30 s. Nuclear pellets were then resuspended in 100 \(\mu\)l of solution B (20 mM HEPES, 1 mM EDTA, 0.4 M NaCl, 1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) and incubated on ice for 20 min. The nuclei were then pelleted by centrifugation at...
25,000 × g for 1 min. Supernatants containing nuclear proteins were used for electrophoretic mobility shift assay.

**Electrophoretic Mobility Shift Assay (EMSA)**—EMSAs were performed as described (18, 23). The probes used for EMSAs were 21-bp double-stranded TRPC1 promoter-specific NF-κB binding sequences that were listed in Fig. 4A. End labeling was performed by T4 kinase in the presence of [α-32P]ATP. Labeled oligonucleotides were purified on a Sephadex G-50 column. An aliquot of 10 μg of nuclear protein extract was incubated with the labeled double-stranded probe (∼80,000 cpm) in the presence of 2.5 μl of binding buffer (Promega). The binding reactions were incubated at 25 °C for 20 min. After adding non-denaturing sample buffer, the DNA-protein complexes were resolved by 6% native PAGE in low ionic strength buffer (0.5× Tris borate-EDTA). To study the effect of antibodies on DNA-protein binding, nuclear extracts were first incubated with NF-κB protein-specific antibodies (2 μg/assay) for 30 min at 25 °C, and then labeled double-stranded probe was added, and the incubation was continued for additional 20 min. After this incubation, non-denaturing sample buffer was added, and the DNA-protein complexes were separated as described above.

**PKC-α-specific siRNA Transfection**—Validated human PKCα-specific siRNA targeted to exon 4 was obtained from Ambion (Austin, TX). HUVEC grown to ~70 confluence on gelatin-
coated culture dishes were transfected with either 100 nm PKCo-siRNA or scrambled sequence using GeneEraser siRNA transfection reagent (Stratagene) according to the manufacturer’s protocol. At 48 h after transfection, cells were used for experiments.

Statistical Analysis—Statistical comparisons were made using two-tailed Student’s t test. Experimental values were reported as the means ± S.E. Differences in mean values were considered significant at p < 0.05.

RESULTS

PAR-1 Activation Induces TRPC1 Expression in Endothelial Cells—We addressed whether thrombin induces TRPC1 expression in endothelial cells. We exposed HUVEC to thrombin and determined TRPC1 mRNA expression by RT-PCR. We observed that thrombin increased TRPC1 mRNA expression in a time-dependent manner (Fig. 1A) without affecting housekeeping gene GAPDH expression (Fig. 1A). At 2 h after thrombin treatment, TRPC1 transcript level increased ∼2-fold, at 4 h the expression level was increased 3-fold, and at 6 h it was increased ∼4-fold over basal (Fig. 1, B and D). To address whether the increase in TRPC1 mRNA was due to increased transcription, we incubated HUVEC with actinomycin D and exposed the cells to thrombin. Thrombin-induced TRPC1 expression was blocked by actinomycin D (Fig. 1A). Thrombin activates PAR-1 by proteolytic cleavage of PAR-1, which couples to multiple heterotrimeric G-proteins to elicit cellular responses including NF-kB activation (17, 18, 21, 24, 25, 27). We also used PAR-1-specific agonist peptide (TFLLRNPNSDK) (24, 26) to mimic the effects of thrombin in inducing TRPC1 expression. Results showed that PAR-1 agonist peptide, like thrombin, activated TRPC1 expression in HUVEC (Fig. 1, B and D).

To address the functional relevance of TRPC1 expression, we measured TRPC1 protein expression after exposing HUVEC to thrombin or PAR-1 agonist peptide. Cells were exposed to PAR-1 control peptide (scrambled sequence, FTLLRNPNSDK), PAR-1 agonist peptide (TFLLRNPNSDK), or thrombin for 20 h, and then TRPC1 protein expression was determined by immunoblotting cell lysates with anti-TRPC1 antibody. TRPC1 protein expression was increased ∼3-fold after PAR-1 activation compared with control (Fig. 2, A and B). We measured the thrombin-induced increase in intracellular Ca2+ in control and thrombin- or TFLLRNPNSDK-exposed cells. In the presence of extracellular Ca2+ (1.26 mM), thrombin produced an increase in intracellular Ca2+ ([Ca2+]i) followed by a gradual decline to baseline value after thrombin stimulation (Fig. 2C). In the thrombin or TFLLRNPNSDK prestimulated cells, the increase in [Ca2+]i elicited by thrombin was significantly greater compared with control (Fig. 2C). We also compared thrombin-induced Ca2+ influx in control and thrombin-stimulated cells. Cells were first challenged with thrombin for endoplasmic reticulum (ER)-store Ca2+ depletion, and then Ca2+ was re-applied to the extracellular medium to assess Ca2+ influx. In both control and experimental group cells (i.e. thrombin- or TFLLRNPNSDK-pretreated cells), the thrombin-induced increase in the initial peak was similar; however, Ca2+ re-application produced more than a 2-fold increase in Ca2+ influx in the thrombin- or TFLLRNPNSDK-pretreated cells compared with controls (Fig. 2D). These results suggest that the increased TRPC1 protein expression induced by PAR-1 activation contributes to augmented Ca2+ influx in response to ER store Ca2+ depletion.

To address the functional relevance of increased Ca2+ influx associated with TRPC1 expression, we measured thrombin-induced permeability increase in endothelial permeability in control cells and cells exposed to thrombin for 20 h. Transendothelial monolayer resistance (TER) was measured to assess endothelial permeability increase as previously described (20). HUVEC grown to confluence on gold electrodes were incubated with 1% FBS containing medium and exposed to thrombin for 20 h. At 20 h after thrombin, cells were re-challenged with thrombin to assess changes in TER. In control cells (not pretreated with thrombin for 20 h), thrombin produced an ∼50% decrease in TER and TER return to normal level within 5 h (Fig. 2E). In the thrombin prestimulated cells, thrombin produced an ∼55% decrease in TER, but the TER failed to return to normal level after thrombin challenge (Fig. 2E), suggesting that increased Ca2+ influx via TRPC1 may augment the permeability increase.

Thrombin Induces TRPC1 Expression via NF-kB Activation—PAR-1 activation in endothelial cells is known to increase the expression of inflammatory genes by activating NF-kB signaling pathways (17, 18). In recent studies we cloned the 5′-reg-

**FIGURE 2.** Thrombin exposure induces TRPC1 protein expression and augments store-operated Ca2+ influx and increase in endothelial permeability. A, HUVEC grown to confluence were incubated in 1% FBS containing medium for 2 h, and then cells were exposed to 40 μM TFLLRNPNSDK (PAR-1 agonist peptide), FTLLRNPNSDK (PAR-1 control peptide), or thrombin (50 nM) for 20 h. After this treatment cells were washed and lysed using lysis buffer. Total cell lysate proteins (50 μg) from each sample were separated on SDS-PAGE and immunoblotted with anti-TRPC1 antibody. The membrane was stripped and immunoblotted with tubulin antibody (bottom panel). Results were representative of four experiments. B, TRPC1 induction -fold was calculated by measuring the ratio of TRPC1 to tubulin, ∗, p < 0.05, significantly different from the control. C, thrombin-induced increase in [Ca2+]i was measured. HUVEC grown on glass coverslips were stimulated with thrombin (50 nM) or TFLLRNPNSDK (40 μM) for 20 h as described above. Cells were then loaded with 3 μM Fura-2 AM for 30 min at 37°C (15, 16). After Fura-2 AM loading, cells were used to measure the increase in cytosolic Ca2+ (Fura-2 fluorescence). As expected, the extracellular medium contained a nominal Ca2+ concentration (1.26 mM). The arrow indicates the time at which cells were stimulated with thrombin (50 nM). D, Ca2+ entry was measured after thrombin-induced ER-stored Ca2+ depletion. The experiment was carried out as described above. Cells exposed to either thrombin or TFLLRNPNSDK for 20 h as described above were washed 2 times, placed in Ca2+- and Mg2+-free HBSS (Ca2+ free medium), and then stimulated with thrombin (50 nM). The return of [Ca2+]i to baseline levels, 1.5 mM CaCl2, was re-applied to the extracellular medium to induce Ca2+ influx. Arrows, times at which thrombin (7) or Ca2+ (8) was added. In this figure bottom tracing shows the Ca2+ entry in response to extracellular Ca2+ addition in the absence of thrombin stimulation in control cells. The experiments were repeated four times, and the data obtained were similar. The results show in C and D are from representative experiments. E, thrombin-induced decrease in TER was measured to assess endothelial permeability. HUVEC were grown to confluence on gold electrode (20) and then treated with or without thrombin (50 nM) in 1% FBS containing medium for 20 h. After this treatment cells were washed, incubated with 1% FBS containing medium for 2 h, and then challenged with thrombin (50 nM) to measure changes in TER (top panel). The arrow indicates the time at which thrombin (50 nM) or medium was added. Data from this experiment presented as a maximal decrease in TER at 0.5, 2, and 4 h after thrombin stimulation (bottom panel). Values are the means ± S.E. from four experiments. *p < 0.05 compared with 0 h thrombin-pretreated cells with control.
Store-operated Ca\(^{2+}\) influx induces TRPC1 expression

A. NF-κB1 (-1623) and NF-κB2 (-880) / NF-κB3 (-708) promoters

B. TRPC1 expression (RLU over basal) with thrombin exposure time (h)

C. IKKβ: 87 kDa

D. TRPC1 expression (RLU over basal) with IKKβ-DN construct (μg/ml)

E. [-1685 to +40] TRPC1-Pro-Luc

ΔNF-κB (-1623) TRPC1-Pro-Luc

ΔNF-κB (-880) TRPC1-Pro-Luc

ΔNF-κB (-708) TRPC1-Pro-Luc

TRPC1 expression (RLU over basal)
ulotary region of hTRPC1 gene (16) and showed that the hTRPC1 regulatory region contains multiple binding sites for the transcription factors NF-κB (Fig. 3A). As thrombin activates NF-κB signaling in endothelial cells, we addressed whether thrombin-induced TRPC1 expression is dependent on NF-κB activation. In the classical NF-κB activation signaling pathway, IκB phosphorylation and subsequent degradation promotes NF-κB translocation to the nucleus and initiates gene transcription (28–30). To address the role of NF-κB signaling, first we transfected TRPC1-Pro-Luc construct with non-phosphorylatable IκBα dominant negative mutant (IκBα-DN) and measured thrombin-induced TRPC1 expression (16). At 48 h after transfection, cells were stimulated with thrombin. In control cells (transfected only wild type TRPC1-Pro-Luc), thrombin-induced reporter expression was time-dependent. The reporter activity was at maximal level (~6-fold over basal) at 4 h after thrombin stimulation (Fig. 3B). Thrombin-induced TRPC1 expression was prevented by co-expression of IκBα-DN with TRPC1-Pro-Luc in HMEC (Fig. 3B). To address the role of IKK activation, we co-transfected kinase-defective dominant negative IκKβ mutant (IκKβ-DN) construct with a TRPC1-promoter-luciferase (TRPC1-Pro-Luc) construct in HMEC and measured thrombin-induced TRPC1 expression. We observed that IκKβ-DN mutant expression in a dose-dependent manner inhibited thrombin-induced TRPC1 expression (Fig. 3C), indicating that NF-κB activation is important in mediating TRPC1 expression.

Previous studies have shown that interaction of IKK/ NEMO with the IKK complex is critical for the activation of the IKK complex and the subsequent activation of NF-κB (30). A cell-permeable synthetic peptide (NBD peptide) corresponding to the NEMO N-terminal α-helical region was shown to block tumor necrosis factor-α-induced NF-κB activation (30). To further validate the role of IKK activation in the signaling mechanism of thrombin-induced TRPC1 expression, we pretreated HUVEC with WT-NBD peptide or MT-NBD peptide, and we measured thrombin-induced TRPC1 expression. In MT-NBD peptide-treated cells, thrombin-induced TRPC1 mRNA expression was not significantly altered compared with controls (i.e. in the absence of peptide) (Fig. 3D); however, in WT-NBD peptide-treated cells, thrombin-induced TRPC1 mRNA expression was markedly reduced (Fig. 3D). These results collectively suggest that IKK activation is critical in the signaling mechanism of NF-κB-dependent TRPC1 expression in response to thrombin in endothelial cells.

Because NF-κB activation is necessary for thrombin-induced TRPC1 expression, we constructed TRPC1 promoter mutants to identify NF-κB binding sites in the TRPC1 promoter. The schematics of the expression constructs are shown in Fig. 3E. The promoter mutants in pGL2-basic vector were transiently expressed into HMEC. At 48 h cells were treated with thrombin for 4 h, and reporter activity was measured to assess TRPC1 expression as described above. Transfection of the wild type (~1685 to +40) TRPC1-pro-Luc in HMEC showed ~6-fold increase in reporter expression over basal (Fig. 3E). Expression of the construct in which distal NF-κB site at ~1623 (Δ1623NF-κB) is deleted showed basal Luc activity, indicating that this NF-κB site is important for TRPC1 expression. Expression of the construct in which the NF-κB site at ~880 (Δ880NF-κB) is deleted markedly reduced thrombin-induced Luc expression (Fig. 3E). Deletion of the most proximal NF-κB site at ~708 (ΔNF-κB708) showed an ~25% inhibition on thrombin-induced reporter gene expression (Fig. 3E). These results demonstrate that two NF-κB sites located at position ~1623 and ~880 are essential in the mechanism of thrombin-induced TRPC1 expression.

To address further the mechanism of TRPC1 expression, we designed oligonucleotide probes for the three NF-κB consensus sites in hTRPC1 promoter to examine by EMSA the in vitro binding of NF-κB to the oligonucleotides. The nuclear extracts obtained from thrombin-stimulated HUVEC showed markedly increased DNA binding activity with NF-κB (~1623 oligos compared with control (not stimulated with thrombin) (Fig. 4, A and B). DNA binding activity increased within 30 min of thrombin treatment and remained elevated for up to 2 h with the ~1623 NF-κB oligos (see details under “Experimental Procedures”). The DNA binding of the NF-κB oligos at position ~880 was also elevated over basal (Fig. 4, A and B). We failed to detect DNA binding activity of NF-κB oligos at position ~708 in nuclear extracts obtained from control and thrombin-stimulated cells (Fig. 4, A and B). Thus, we observed utilizing both promoter deletion analysis and electrophoretic mobility shift

![FIGURE 3. PAR-1 activation induces TRPC1 gene expression. A. schematic of hTRPC1 5' -regulatory region reveals the binding sites for the transcription factors NF-κB, B, co-expression of IκBα (IκBα-DN) mutant inhibits thrombin-induced TRPC1 expression. HMEC were transfected into hTRPC1-Pro-Luc (wild type promoter, hTRPC1-Pro-Luc (~1686 to +40)) construct together with pRL/Tk plasmid as described under “Experimental Procedures.” In addition, the wild type hTRPC1 promoter (1 μg/ml), pRL/Tk (0.035 μg/ml) plasmid, was co-transfected with IκBα-DN (1 μg/ml). At 48 h after transfection cells were stimulated with thrombin (50 nM) for 4 h. After this treatment cells were lysed, and reporter activity was measured as described (16, 21). Luciferase reporter activity (relative light unit ratio (RLU)/mg of protein) was measured, and relative activity was expressed after subtracting the basal activity at each time point. The mean ± S.E. from four experiments is repeated in triplicate is shown. The asterisks indicate the difference from thrombin-stimulated control (*, p < 0.005). C, co-expression of dominant negative IκKβ (IκKβ-DN) inhibits thrombin-induced TRPC1 expression. The wild type hTRPC1 promoter (1 μg/ml), pRL/Tk (0.035 μg/ml) plasmid, was co-transfected with varying concentrations of IκKβ-DN expression constructs. At 48 h after transfection cells were stimulated with thrombin (50 nM) for 4 h. After this treatment cells were lysed, and the reporter activity was measured as described above. The mean ± S.E. from four experiments repeated in triplicate is shown. The asterisks indicate the difference from thrombin-stimulated control (*, p < 0.005). Cell lysates (25 μg of protein) were immunoblotted with anti-IκKβ antibody to assess the expression of IκKβ-DN (top panel). D, NEMO-binding domain peptide inhibits thrombin-induced TRPC1 expression in endothelial cells. HUVEC grown to confluence were incubated with cell-permeable wild type NB (WT-NBD) or mutant NB (Mt-NBD) peptides (150 μM) for 2 h in serum-free medium. Cells were then challenged with thrombin (50 nM) for the indicated times. Total RNA was isolated, and RT-PCR was performed to determine the expression of TRPC1 and GAPDH (see details under “Experimental Procedures”). The experiment was repeated three times with similar results. E, localization of thrombin-responsive NF-κB sites in the hTRPC1 promoter. Deletion mutant analysis was utilized to identify the NF-κB binding sites in the 5' -regulatory region of the hTRPC1 gene. PCR-based method was used to prepare the NF-κB site-deleted constructs from the wild type hTRPC1-Pro-Luc described under “Experimental Procedures.” TRPC1 wild type and the NF-κB site-deleted (ΔNF-κB –1623; ΔNF-κB –880; and ΔNF-κB –708) constructs were transfected into HMEC to assess TRPC1 promoter-driven reporter expression. At 48 h after transfection, cells were stimulated with thrombin (50 nM) for 4 h. After this treatment cells were lysed, and the reporter expression was measured (16, 21). The mean ± S.E. from four experiments repeated in triplicate is shown. The asterisk indicates the difference from the (~1685 to +40) TRPC1-Pro-Luc construct (*, p < 0.001).]
Store-operated Ca\textsuperscript{2+} influx Induces TRPC1 Expression

**FIGURE 4. Identification of thrombin-responsive DNA binding activity of NF-\kappa B in hTRPC1 promoter.** In A, the hTRPC1 promoter-specific NF-\kappa B oligonucleotide probes used for EMSA were shown. The underlined sequence represents the consensus NF-\kappa B sites in hTRPC1 promoter. In B, HUVEC grown to confluence were stimulated with 50 nM thrombin for 0.5, 1, and 2 h in serum-free medium. After this treatment nuclear extracts were prepared, and EMSA was performed using \textsuperscript{32}P-labeled double-stranded oligonucleotide containing the hTRPC1 promoter-specific three NF-\kappa B sequences and assayed for NF-\kappa B-DNA binding. The two upstream NF-\kappa B binding sites showed NF-\kappa B-DNA complex formation in response to thrombin. The experiment was repeated four times. The results from representative experiments are shown in this figure. In C, nuclear extracts prepared from HUVEC stimulated for 1 h with thrombin were incubated with antibodies specific to NF-\kappa B proteins for 30 min at room temperature before the addition of radiolabeled NF-\kappa B1 probe. The experiment was repeated four times. The results from representative experiments are shown in this figure.

For 60 min were incubated with the NF-\kappa B protein-specific antibodies, and then labeled NF-\kappa B1 double-stranded probe was added to determine the supershift of NF-\kappa B complex (see details under “Experimental Procedures”). The incubation of anti-p65 antibody resulted in a supershift of the DNA-protein complexes (Fig. 4C), whereas the addition of antibodies to p52, anti-p50, RelB, and c-Rel had no effect (Fig. 4C). We also performed supershift assay using NF-\kappa B2 double-stranded probe. We observed a supershift of the DNA-protein complex only with anti-p65 antibody (data not shown). These results suggest that p65 homodimer binds to the TRPC1 promoter in response to thrombin to initiate TRPC1 transcription in endothelial cells.

Ca\textsuperscript{2+} Signaling Induces TRPC1 Expression—Because thrombin-induced TRPC1 expression was dependent on NF-\kappa B activation, we addressed the role of Ca\textsuperscript{2+} signaling in the mechanism of thrombin-induced TRPC1 expression. In this experiment we first measured the effect of cell-permeable Ca\textsuperscript{2+} chelator BAPTA/AM on the thrombin-induced increase in TRPC1 mRNA expression. HUVEC were incubated for 30 min with 20 \mu M BAPTA/AM and challenged with thrombin for the indicated periods. BAPTA/AM prevented a thrombin-induced rise in cytosolic Ca\textsuperscript{2+} in HUVEC (data not shown). In control cells, PAR-1 signaling increased TRPC1 expression without altering GAPDH expression (Fig. 5A); however, pretreatment of cells with BAPTA/AM failed to increase TRPC1 mRNA expression in response to PAR-1 activation (Fig. 5B). Next we determined the effect of other G protein-coupled receptor agonists such as bradykinin and histamine (known to increase cytosolic Ca\textsuperscript{2+} level in endothelial cells (34–36)) and measured TRPC1 transcript expression. We observed that these agonists challenge increased TRPC1 mRNA expression in a time-dependent manner (Fig. 5, C and D). We treated HUVEC with thapsigargin (Ca\textsuperscript{2+}-ATPase inhibitor) to increase intracellular Ca\textsuperscript{2+} levels and measured TRPC1 expression. Thapsigargin also increased TRPC1 transcript expression (Fig. 5E). To further address the role of Ca\textsuperscript{2+} signaling in the mechanism of TRPC1 expression, we treated HUVEC with the inositol 1,4,5-trisphosphate receptor antagonist 2-APB to prevent an increase in intracellular Ca\textsuperscript{2+} and measured PAR-1 activation-mediated TRPC1 mRNA expression. 2-APB (75 \mu M) inhibited the thrombin-induced rise in intracellular Ca\textsuperscript{2+} in HUVEC (data not shown). We also observed that 2-APB treatment prevented thrombin-induced TRPC1 transcript expression in HUVEC (Fig. 5F), whereas 2-APB treatment had no effect on GAPDH expression (Fig. 5F). In addition, PAR-1 signaling-induced reporter expression in the presence and absence of 2-APB was measured in HMEC-transfected wild-type hTRPC1-Pro-Luc plasmid. We observed that either thrombin- or PAR-1-specific agonist peptide (TFFLRNPNDK) stimulation increased reporter expression ~6-fold over basal (Fig. 5G); however, in 2-APB-treated cells PAR-1 activation-induced reporter expression was markedly reduced (Fig. 5G), indicating that Ca\textsuperscript{2+} signaling is critical in the mechanism of thrombin-induced TRPC1 expression in endothelial cells.

Because 2-APB prevented the thrombin-induced TRPC1 expression, we addressed whether store-operated Ca\textsuperscript{2+} influx (i.e. Ca\textsuperscript{2+} influx through TRPC1) signaling is necessary for...
Store-operated Ca\(^{2+}\) Influx Induces TRPC1 Expression

TRPC1 expression in endothelial cells. To study whether Ca\(^{2+}\) influx signaling is necessary for thrombin-induced NF-κB activation-dependent TRPC1 expression, we performed EMSAs to determine the DNA binding activities of NF-κB. In this experiment, HUVEC were incubated in the presence and absence of Ca\(^{2+}\) in the extracellular medium, and cells were stimulated with thrombin. Nuclear extracts prepared from these cells were used for NF-κB-DNA binding activities. We used TRPC1 promoter NF-κB sites (NF-κB1 (−1623 to −1614)) and NF-κB2 (−880 to −871))-specific oligos as probes for EMSAs (Fig. 4A). Thrombin increased NF-κB-DNA binding activities in the presence of Ca\(^{2+}\) influx in a time-dependent manner (Fig. 6, A and B), whereas thrombin failed to increase NF-κB-DNA activities in the absence of Ca\(^{2+}\) influx (Fig. 6, A and B). To address the role of Ca\(^{2+}\) influx in this response, we measured thrombin-induced TRPC1 mRNA expression in the presence and absence of store-operated Ca\(^{2+}\) influx. We observed thrombin-induced TRPC1 mRNA expression in the presence of Ca\(^{2+}\) influx (Fig. 6C), whereas this response was abrogated in the absence of Ca\(^{2+}\) influx (Fig. 6C). These results collectively show that Ca\(^{2+}\) influx plays a critical role in the signaling mechanism of thrombin-induced TRPC1 expression.

Ca\(^{2+}\) Influx-Induced PKCα Activation Induces TRPC1 Expression—We next addressed the possible role of PKC activation in the signaling mechanism of thrombin-induced TRPC1 expression. In this experiment, HUVEC were incubated with PKC inhibitor calphostin C (a pan-PKC inhibitor) and measured thrombin-induced NF-κB activation by EMSA. We used NF-κB1 (Fig. 4A) site-specific oligos as probes. In control cells thrombin increased NF-κB-DNA complex formation in a time-dependent manner, whereas thrombin-induced increased NF-κB-DNA complex formation was markedly reduced in calphostin C-treated cells (data not shown). Because PKC signaling was required for NF-κB activation in response to thrombin, we addressed the possible role of the Ca\(^{2+}\)-dependent PKCα isoform in the signaling of TRPC1 expression. In this experiment HUVEC were exposed to PKCα-specific inhibitor Go6976, and DNA-NF-κB binding using EMSA was determined. The thrombin-induced DNA-NF-κB binding activity was markedly reduced in Go6976-treated cells (Fig. 7A) compared with control (not treated with Go6976). Also the thrombin-induced TRPC1 transcript expression was determined in

![Figure 5](https://example.com/image.png)

FIGURE 5. PAR-1-activated Ca\(^{2+}\) signaling is required for TRPC1 expression in endothelial cells. HUVEC grown to confluence on culture dishes were washed and incubated in serum-free medium (Ca\(^{2+}\) containing HBSS) for 2 h at 37 °C. After this treatment cells were incubated in the absence (A) or presence (B) of 20 μM BAPTA/AM for 30 min and washed, and then cells were challenged with thrombin (50 nM) for 0, 2, 4, and 6 h. After thrombin challenge, total RNA was isolated using Trizol reagent, and RT-PCR was performed to determine TRPC1 mRNA expression. PCR amplification of GAPDH was also performed for each sample. HUVEC incubated with serum-free medium for 2 h were challenged with 1 μM bradykinin (C), 10 μM histamine (D), or 1 μM thapsigargin (E) for 0, 2, and 4 h. After this, total RNA was isolated, and RT-PCR was performed to determine TRPC1 and GAPDH expression. In F, HUVEC were incubated with 75 μM 2-APB for 30 min, and then cells were challenged with thrombin (50 nM) for 0, 2, 4, and 6 h. The experiments in A, B, C, D, E, and F were repeated three times, and the results from representative experiment are shown. In G, wild type hTRPC1-Pro-Luc construct was transfected into HMEC as described under “Experimental Procedures.” At 48 h after transfection cells were first incubated in the presence and absence of 75 μM 2-APB for 30 min and then stimulated with thrombin (50 nM) or TFLLRNPN (40 μM) for 4 h. After this treatment cells were lysed, and reporter activity was measured (16, 21). The results are the mean ± S.E. from four separate experiments shown. *, p < 0.005, significantly different from thrombin- or TFLLRNPN/challenged control. RLU, relative light units.
Influx Induces TRPC1 Expression

We performed additional experiments to address the role of PKC\(_{alpha}\) signaling in the mechanism of TRPC1 expression. First, dominant negative PKC\(_{alpha}\) (PKC\(_{alpha}-DN\)) mutant expression construct with TRPC1-Pro-Luc were co-transfected in HMEC, and thrombin-induced reporter expression was recorded. The thrombin-induced TRPC1 expression was prevented by co-expression of PKC\(_{alpha}-DN\) mutant with TRPC1-Pro-Luc (Fig. 7C). Second, PKC\(_{alpha}\)-specific siRNA was transfected to down-regulate endogenous PKC\(_{alpha}\) expression. In PKC\(_{alpha}\)-siRNA transfected cells, PKC\(_{alpha}\) protein expression was inhibited <80% compared with control (scrambled sequence-transfected cells) (Fig. 7D). PKC\(_{delta}\) is known to be involved in the signaling mechanism of NF-\(kappa\)B activation-dependent ICAM-1, and VCAM-1 expression in endothelial cells (17, 37) was not altered by transfecting PKC\(_{alpha}\)-siRNA (Fig. 7D). We measured TRPC1 transcript expression in control and the PKC\(_{alpha}\)-siRNA transfected cells. Thrombin-induced TRPC1 expression was prevented in after PKC\(_{alpha}\)-siRNA transfection as compared with cells transfected with scrambled siRNA (Fig. 7E). We also measured ICAM-1 transcript expression in this experiment. The thrombin-induced ICAM-1 transcript expression was not significantly altered in PKC\(_{alpha}\)-siRNA-transfected cells compared with control (data not shown). These results collectively show that Ca\(^{2+}\) influx induced by ER store Ca\(^{2+}\) depletion activates the PKC\(_{alpha}\) to increase TRPC1 expression signaling via NF-\(kappa\)B in endothelial cells.

**DISCUSSION**

Thrombin, a serine protease, catalyzes the conversion of fibrinogen to fibrin (38, 39). Thrombin also activates a variety of cell types, including endothelial cells, smooth muscle cells, and leukocytes (40, 41); for example, thrombin induces leukocyte adhesion to endothelial cell surface by increasing the cell surface expression of adhesion molecules ICAM-1, VCAM-1, P-selectin, and E-selectin (18, 42, 43). Thrombin promotes the expression of ICAM-1 and VCAM-1 via the activation of the transcription factor NF-\(kappa\)B in endothelial cells (18, 37, 42). We and others have shown that thrombin also causes endothelial cell shape change and thereby increases endothelial permeability via opening of interendothelial junctions (3). Thrombin mediates these cellular responses by activating the G protein-coupled PAR-1 on the endothelial cell surface (24, 25, 40). We showed that ligation of PAR-1 increased endothelial permeability by depletion of ER Ca\(^{2+}\) stores and the subsequent activation of Ca\(^{2+}\) influx via SOC (6, 15, 16). The TRPC isoform TRPC1 is a prominent SOC present in human vascular endothelial cells (15, 16). In the present study we observed that thrombin induces TRPC1 mRNA and protein expression via NF-\(kappa\)B activation such that the resultant TRPC1 expression augmented the store-operated Ca\(^{2+}\) influx and increased endothelial permeability.

NF-\(kappa\)B is composed of homodimers and heterodimers of five different proteins (p50, p52, p65 (RelA), RelB, cRel) (44). These dimers exist in the cytoplasm in an inactive form bound to the inhibitory protein I-\(kappa\)B (I\(kappa\)B) (28–31). Agonist-induced signals activate I\(kappa\)B kinases \(alpha\) and \(beta\) (28–30, 44), which in turn phosphorylate serine residues 32 and 36 of I\(kappa\)B\(_{alpha}\) and serine residues 19 and 23 of I\(kappa\)B\(_{beta}\), respectively (28–31). These phosphorylation events lead to proteolytic degradation of I\(kappa\)B and dissociation of NF-\(kappa\)B, and the released NF-\(kappa\)B via its nuclear localization signal translocates to the nucleus to induce gene transcription (28–31). Because the hTRPC1 promoter contains multiple binding sites for NF-\(kappa\)B (16), we examined the possibility that the thrombin-induced TRPC1 expression was the result of activation of NF-\(kappa\)B. We first addressed the role of NF-\(kappa\)B in the mechanism of TRPC1 expression. We showed that co-express-
markedly reduced by treating endothelial cells with NEMO-binding domain peptide. These findings suggest that NF-κB signaling is a key requirement for thrombin-induced TRPC1 expression.

To determine the promoter NF-κB binding sites responsible for TRPC1 transcription, we made NF-κB deletion mutant constructs utilizing the wild type hTRPC1-Pro-Luc construct. We transfected these constructs in endothelial cells to identify the thrombin-induced TRPC1 promoter-driven reporter expression. Deletion of the upstream NF-κB site at position −1623 or at −880 from the transcription initiation site prevented the thrombin-induced reporter expression, whereas deletion of downstream site at −708 had only a minimal effect. Thus, thrombin appears to induce TRPC1 expression via the formation NF-κB-DNA protein complex upstream of these two TRPC1 promoter NF-κB consensus sites. To address further the specificity of NF-κB binding to TRPC1 5′-regulatory region, we performed EMSAs utilizing the NF-κB consensus sequence present in TRPC1 5′-regulatory region. Thrombin increased DNA-NF-κB complex formation with the TRPC1 promoter consensus NF-κB sites at position −1623 (NF-κB1) and −880 (NF-κB2); however, thrombin failed to induce DNA-NF-κB complex with consensus NF-κB site at −708 (NF-κB3). These results, in agreement with the mutant TRPC1-Pro-Luc expression studies described above, indicate the importance of NF-κB binding sites at position −1623 (NF-κB1) and −880 (NF-κB2) in regulating the transcription of TRPC1.

Previous studies have shown that NF-κB p65 homodimer binds to VCAM-1 and ICAM-1 promoters in thrombin-stimulated endothelial cells (17, 18). To identify the NF-κB proteins interacting with TRPC1 promoter, we carried out supershift assays utilizing antibodies specific to NF-κB proteins. We observed a supershift of NF-κB-DNA complex with anti-p65 antibody in thrombin-stimulated endothelial cells, suggesting that p65 homodimer
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![Diagram](https://via.placeholder.com/150)

FIGURE 8. Signaling pathways mediating thrombin-induced TRPC1 expression in endothelial cells. PAR-1 activation-induced ER store Ca\(^{2+}\) depletion via phospholipase C (PLC)–mediated inositol 1,4,5-trisphosphate (IP\(_3\)) generation activates Ca\(^{2+}\) influx via TRPC1 in endothelial cells. DAG generated from phosphoinositide hydrolysis by PLC and increase in cytosolic Ca\(^{2+}\) concentration activate the down-stream target the Ca\(^{2+}\)-dependent PKC\(\alpha\) isoform in endothelial cells. Thus, PKC\(\alpha\) signaling may be the upstream of the NF-κB activation to induce TRPC1 transcription in endothelial cells. IP\(_3\), inositol 1,4,5-trisphosphate receptor.

binds to TRPC1 promoter in response to thrombin to initiate TRPC1 transcription in endothelial cells.

Because TRPC1 promoter sequence has multiple NF-κB binding sites and thrombin-induced TRPC1 expression requires NF-κB activation, we addressed the possible role of thrombin-induced increase in cytosolic Ca\(^{2+}\) in signaling TRPC1 expression. These studies were based on the premise that a rise in cytosolic Ca\(^{2+}\) would be involved in signaling NF-κB-dependent gene expression (31–33). The activation of PAR-1 caused a rapid and transient increase cytosolic Ca\(^{2+}\) concentration (\([Ca^{2+}]_i\)) (phase I) secondary to the release of ER-stored Ca\(^{2+}\) and subsequent Ca\(^{2+}\) entry occurring via TRPC1 activated by store depletion (phase II) (6, 15, 16). The inositol 1,4,5-trisphosphate receptor antagonist 2-APB was shown to prevent both the phase I and phase II increases in \([Ca^{2+}]_i\) (45, 46). Strikingly, we also observed that exposure of endothelial cells to 2-APB prevented the PAR-1 activation-mediated TRPC1 expression. In other studies, we transfected TRPC1-Pro-Luc construct in endothelial cells and measured PAR-1-activated reporter expression. In this experiment, 2-APB treatment also significantly reduced thrombin-induced reporter expression, indicating that Ca\(^{2+}\) is a critical signal mediating TRPC1 expression.

We have shown previously that the thrombin-induced Ca\(^{2+}\) entry signal is a requirement for increased endothelial permeability (3, 6, 15). Thus, to address the role of Ca\(^{2+}\) entry in the mechanism of TRPC1 expression, Ca\(^{2+}\) was omitted from the extracellular medium such that there is no Ca\(^{2+}\) influx after ER-stored Ca\(^{2+}\) depletion. Both thrombin-induced TRPC1 mRNA expression and NF-κB interaction with the TRPC1 promoter were markedly reduced in the absence of Ca\(^{2+}\) influx, indicating that store-operated Ca\(^{2+}\) influx via TRPC1 plays an important role in the signaling TRPC1 expression in endothelial cells.

PKC-induced activation of NF-κB pathways can also signal the expression of inflammatory genes such as ICAM-1 and VCAM-1 in endothelial cells (17, 19, 37). Three different families of PKC isoforms (conventional, novel, and atypical) have been identified based on their domain structure and their ability to respond to Ca\(^{2+}\) and diacylglycerol (DAG) (47). The “conventional” PKC isoforms (α, βI, βII, and γ) require DAG and Ca\(^{2+}\) for activation (47). The “novel” PKC isoforms (δ, ε, η, and θ) require only DAG for activity (47). The “atypical” PKC isoforms (ζ, ι/λ, and μ) are activated independently of Ca\(^{2+}\) or DAG (47). Human vascular endothelial cells in culture express PKC\(\alpha\), PKC\(\delta\), PKCe, and PKCζ (4, 48). Rahman et al. (37) showed that PKCζ activation is required for thrombin-induced NF-κB activation and ICAM-1 gene expression in human vascular endothelial cells. We have shown that PAR-1 activation increases Ca\(^{2+}\)-dependent PKC\(\alpha\) activity and thereby contributes to signaling the increase endothelial permeability (4).

In this study we addressed the possibility that PKC signaling was involved in the mechanism of thrombin-induced TRPC1 expression. We showed that the pan-PKC inhibitor calphostin C prevented the thrombin-induced NF-κB binding to TRPC1 promoter, indicating that PKC signaling is crucial in activating TRPC1 transcription. Because the Ca\(^{2+}\) influx signal is required for NF-κB activation and TRPC1 gene expression, we addressed the possible role of Ca\(^{2+}\)-dependent PKC\(\alpha\) activation in the mechanism of thrombin-induced TRPC1 expression. The PKC\(\alpha\)-specific inhibitor G66976 prevented the thrombin-induced TRPC1 transcript expression. Moreover, co-transfection of PKC\()\alpha\)-DN expression construct with wild type TRPC1-Pro-Luc plasmid in endothelial cells prevented the thrombin-induced TRPC1 promoter-driven reporter expression. Also down-regulation of endogenous PKC\(\alpha\) expression by transfecting PKC\(\alpha\)-siRNA prevented the thrombin-induced TRPC1 expression. Taken together, these results demonstrate the essential role of Ca\(^{2+}\)-dependent PKC\(\alpha\) activation in signaling thrombin-induced TRPC1 expression in endothelial cells.

In summary, we show herein that PAR-1 activation of store-operated Ca\(^{2+}\) influx signals PKC\(\alpha\)-dependent NF-κB activation, which in turn induces TRPC1 gene transcription in endothelial cells (Fig. 8). This feed-forward mechanism of TRPC1 expression is important in augmenting Ca\(^{2+}\) influx in response to Ca\(^{2+}\) store depletion and, thus, in signaling increased endothelial permeability. The present results demonstrating the role of Ca\(^{2+}\) signaling in inducing TRPC1 expression may inform a novel therapeutic target in inflammatory diseases.

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