Thrombin Receptor Peptide Inhibits Thrombin-induced Increase in Endothelial Permeability by Receptor Desensitization

Hazel Lum,* Thomas T. Andersen,* Alma Siflinger-Birnboim,* Chinnaswamy Tiruppathi,* Michael S. Goligorsky,† John W. Fenton II,*‡ and Asrar B. Malik*§

* Departments of Physiology and Cell Biology, and † Biochemistry and Molecular Biology, The Albany Medical College of Union University, Albany, New York 12208; and § Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201; and ‡ Department of Medicine, State University of New York, Stony Brook, New York 11794-8152

Abstract. Thrombin, a potent activator of cellular responses, proteolytically cleaves, and thereby activates its receptor. In the present study, we compared the effects of the thrombin receptor 14-amino acid peptide (TRP-14; SFLLRNPNFKYEPF), which comprises the NH2 terminus after cleavage of the thrombin receptor, and of the native α-thrombin on endothelial monolayer permeability. Addition of TRP-14 (1–200 μM) to bovine pulmonary artery endothelial cells increased [Ca2+]i in a dose-dependent manner. The peak increase in [Ca2+]i in response to 100 μM TRP-14 or 0.1 μM α-thrombin was similar (i.e., 931 ± 74 nM and 1032 ± 80 nM, respectively), which was followed by a slow decrease with t1/2 values of 0.73 and 0.61 min, respectively. Extracellular Ca2+ chelation with 5 mM EGTA abolished the sustained increases in [Ca2+]i induced by either TRP-14 or α-thrombin. α-thrombin (0.1 μM) increased transendothelial [125I]albumin permeability, whereas TRP-14 (1–100 μM) had no effect. Coincubation of 100 μM TRP-14 with 1 μM DIP-α-thrombin also did not increase permeability over control values. Stimulation of BPAEC with 0.1 μM α-thrombin induced translocation of protein kinase C (PKC) from the cytosol to the plasma membrane indicative of PKC activation, whereas TRP-14 had no effect at any concentration. TRP-14 at 100 μM desensitized BPAEC to thrombin-induced increases in [Ca2+]i and transendothelial permeability. The Ca2+ desensitization was reversed after ~60 min, and this recovery paralleled the recovery of the permeability response. These findings indicate that the TRP-14-induced Ca2+ mobilization in the absence of PKC activation is insufficient to increase endothelial permeability. In contrast, the increase in endothelial permeability after α-thrombin occurred in conjunction with Ca2+ mobilization as well as PKC activation. TRP-14 pretreatment prevented the α-thrombin-induced increase in endothelial permeability secondary to desensitization of the Ca2+ signal. The results suggest that combined cytosolic Ca2+ mobilization mediated by TRP-14 and PKC activation mediated by a TRP-14-independent pathway are dual signals responsible for the thrombin-induced increase in vascular endothelial permeability.

The procoagulant serine proteinase, α-thrombin, has a central bioregulatory role in hemostasis and functions as an agonist for diverse cellular activities including mitogenesis, prostaglandin synthesis, chemotaxis, and smooth muscle contraction (9). Additionally, α-thrombin can stimulate endothelial cells leading to increased vascular endothelial permeability and tissue edema (1, 12, 17, 21, 22). These responses require activation of thrombin receptor(s) on the endothelial cell and involve thrombin's proteolytic activity (1, 17).

The signaling pathways mediating the increase in endothelial permeability after thrombin receptor activation are poorly understood. Several lines of evidence suggest that the response is linked to a receptor-activated cascade of second messengers initiated by activation of phospholipase C (PLC). α-thrombin stimulates endothelial PLC, triggering the generation of inositol 1,4,5-trisphosphate [Ins(1,4,5)-P3] from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (5, 18, 20), which in turn increases [Ca2+]i (cytosolic Ca2+ concentration) (5, 13, 18, 21, 22, 28), as well as the

1. Abbreviations used in this paper: BPAEC, bovine pulmonary artery endothelial cells; ICAM-1, intercellular adhesion molecule-1; PKC, protein kinase C; PLC, phospholipase C.
Results

generation of diacylglycerol, which activates protein kinase C (PKC) (5, 23). We have reported that α-thrombin-mediated increase in endothelial permeability can be prevented by inhibition of PKC activation (23). An increase in [Ca2+]i, may also be involved in the response (21) because the thrombin-induced generation of Ins(1,4,5)P3 and the increase in [Ca2+]i, preceded the increase in transendothelial albumin permeability (22).

The deduced sequence determined from cloning of human (36) and hamster (27) thrombin receptors has indicated a novel mechanism of activation based on thrombin’s proteolytic activity. The thrombin receptor is coupled to GTP-binding proteins and has seven hydrophobic segments spanning the lipid bilayer (27, 36). The thrombin receptor is unique in that the long extracellular extension (∼75 amino acids) is the substrate target containing the proposed cleavage site (between R36 and S37) sensitive to thrombin’s proteolytic activity. The remaining shortened extracellular portion after cleavage contains a newly exposed NH2 terminus that binds to as yet an undefined region on the thrombin receptor, and functions as a “tethered ligand” to activate the receptor (27, 36). Synthetic peptides corresponding to the exposed NH2 terminus (i.e., SFLLRNPDKYEFP) (TRP-14) elicit cellular responses characteristic of native thrombin (e.g., platelet aggregation [15, 29, 36], increase in [Ca2+]i, in endothelial cells [26], activation of PLC [15, 35], and activation of neutrophil adhesion via P-selectin expression on endothelial cells [31]).

The issue has been raised whether all of thrombin’s responses are mediated by TRP-14, which corresponds to the α-thrombin-generated NH2 terminus of the receptor (29, 31, 35). The synthetic thrombin-receptor peptide induces PLC activation and serotonin release, but does not mediate proliferation of fibroblasts (35). In another study, the peptide desensitized the high affinity thrombin receptors activated by low concentrations of α-thrombin (<2 nM), but did not affect the responses mediated by higher thrombin concentrations (29). Furthermore, TRP-14 induced P-selectin expression and PMN adhesion to endothelial cells, whereas α-thrombin mediated the additional expression of Intercellular Adhesion Molecule-1 (ICAM-1) and ICAM-1-induced PMN adhesion (31). The present study demonstrates that TRP-14 increases [Ca2+]i in endothelial cells as is the case with α-thrombin. In contrast to α-thrombin, TRP-14 did not increase transendothelial permeability, and moreover, did not activate PKC. Prior exposure of cells with TRP-14 produced a transient desensitization of thrombin-induced [Ca2+]i, signal and prevented the increase in endothelial permeability. The results indicate that cytosolic Ca2+ mobilization is mediated by TRP-14, whereas PKC activation occurs by a TRP-14-independent mechanism. Both Ca2+ mobilization and PKC activation are, however, required to signal the thrombin-induced increase in endothelial permeability.

Materials and Methods

Materials

The following materials were used: Hapes, TCA, BSA (Fraction V), bradykinin, EGTA, iodonycin (Sigma Chemical Co., St. Louis, MO); DME, HBSS, PBS, (Gibco Laboratories, Grand Island, NY); polycarbonate micropore filters (Nucleopore, Pleasanton, CA); [125I]New England Nuclear, Boston, MA); furf2/AM (acetoxymethyl ester) (Molecular Probes, Inc., Eugene, OR).

Human α-thrombin and diisopropyl-fluorophosphate (DIP)-α-thrombin were prepared as described (10, 11). Human α-thrombin contained 98% α-thrombin (with residual amounts of β- and γ-thrombin), and a specific clotting activity of 3345 U/mg protein and ~100% enzymatic activity as determined by active-site titration with p-nitrophenyl-p’ guanidino-benzoate (NPGB). The DIP-α-thrombin had <1% clotting activity of the starting α-thrombin, and was 8% active with the NPGB assay.

Synthesis of Thrombin Receptor Peptide

The peptide TRP-14 (SFLLRNPDKYEFP) was synthesized on a Biosearch 9500 automated peptide synthesizer employing the tBOC strategy (25) and assembled on methylbenzhydrylamine resin, yielding the COOH-terminal amide after removal from the resin. Hydrofluoric acid cleavage utilized the “low-high” method of Tam et al. (32), and was followed by purification of Sep-Pak cartridges (Millipore, Bedford, MA) and HPLC (reverse phase on a CN column utilizing a gradient of 0–60% acetonitrile, 0.1% trifluoro-acetic acid). The purified peptide subjected to amino acid analysis (2) yielded the desired composition. All concentrations were obtained from amino acid analysis data. The correct sequence was confirmed by automated gas phase sequencing on a Proton 2090E sequencer. We also studied the effects of a control acetylated TRP-14. The TRP-14 dissolved in water was reacted with an equal volume of acetic anhydride for 1 hr at room temperature, and recovered by lyophilization. The acetylated TRP-14 was unreactive in a ninhydrin-based test for free amino groups indicative of acetylation of the NH2 terminus (19).

Endothelial Cell Culture

Bovine pulmonary artery endothelial cells (BPAEC) (cell line CCL-209) were obtained at the 16th passage from the American Type Culture Collection (Rockville, MD). These cells were characterized as endothelial cells by the presence of angiotensin-converting enzyme activity, factor VIII-related antigen, and uptake of acetylated low density lipoprotein (8).

Transendothelial [125I]Albumin Permeability

The transendothelial [125I]albumin clearance rates in cultured monolayers of BPAEC were measured using an in vitro system developed for assessment of endothelial permeability to proteins (7). This system measures the diffusive flux of the tracer molecule across cell monolayers in the absence of hydrostatic and oncotic pressure gradients. The system consisted of luminal and abluminal compartments separated by a polycarbonate microporous filter (0.8 μm pore diameter). BPAEC were seeded at 103 cells/filter and grown for 3 to 4 d to attain confluency. Both compartments contained the same medium (DME, 20 mM Hapes, 0.5 % BSA, pH 7.4) at volumes of 700 μl and 25 ml, respectively. The luminal compartment was fitted with a styrofoam outer ring, and floated in the abluminal medium so that fluid levels remained equal after repeated samplings from the abluminal compartment. The abluminal compartment was stirred continuously for rapid mixing and the entire system was kept at 37°C by a thermostatically-regulated water bath. Crystallized and lyophilized fatty acid-free BSA was purified by gel chromatography and labeled with [125I]using the chloramidine-T procedure (4). Noncovalently bound [125I] was removed by dialysis against 0.1 M NaI in PBS (pH 7.4). Transendothelial clearance of [125I]albumin was determined as the volume of luminal chamber radioactivity cleared into the abluminal chamber. The change in volume over time provided the clearance rate in μl/min as determined by weighted least-squares nonlinear regression (BMDP Statistical Software, Berkeley, CA).

At the beginning of the experiment, the luminal compartment was “floated” in the abluminal medium, and filled with medium containing ~6 μCi/ml [125I]albumin. Abluminal samples of 400 μl of tracer were obtained at 5 min intervals for 60 min, and counted for radioactivity in a gamma counter (Packard Instrument Co., Downers Grove, IL). At termination of the experiment, the percent free [125I] in luminal and abluminal compartments was determined using 12% TCA precipitation. The luminal medium contained 1–2% free [125I]. The transendothelial [125I]-albumin clearance rates were corrected for the free [125I].

Cytosolic Ca2+ Determination

Cytosolic [Ca2+]i was determined as reported (13, 21, 22). Briefly, BPAEC were seeded on 25-mm diam glass coverslips at 5 × 103 cells/coverslip, and grown to confluency (3–4 d). The cells were loaded with 5 μM furf2/AM for 60 min at room temperature, washed 3× with HBSS, and placed in a Sykes-Moore chamber (Bellco, Vineland, NJ). The chamber was placed onto the stage of an inverted Nikon Diaphot microscope which
was equipped with quartz optics, and coupled to a Delta scanning microspectrotometer (PTI, Inc., Princeton, NJ). An optically isolated group of 3-4 cells was illuminated by a 75W Xenon arc lamp at alternating excitation wavelengths of 340 and 380 nm. The emitted light was passed through an interference filter at 510 nm and collected via a photomultiplier. Fluorescence intensity was measured at 10 points/s. Background autofluorescence (in the absence of fura2) was determined at beginning of each day's experiments, and was subtracted automatically during data collection. At the end of each experiment, 10 μM ionomycin was added to obtain fluorescence of Ca2+-saturated fura2, and 0.1 M EGTA to obtain fluorescence of free fura2. The fluorescence ratio of excitation wavelengths 340 and 380 nm (340/380) and [Ca2+]i were computed using the PTI software. The [Ca2+]i was calculated based on a Kd of 224 nM (14).

**Immunocytochemical Protein Kinase C Localization**

Endothelial cell monolayers grown on coverslips (1 × 105 cells/coverlip) were preincubated with either buffer alone or challenged with TRP-14 or α-thrombin. The monolayers were then fixed with 1% paraformaldehyde and processed for immunocytochemistry of protein kinase C using monoclonal antibodies (Amersham Corp., Arlington Heights, IL) as described (16). Fluorescein isothiocyanate fluorescence was examined using a Zeiss universal microscope equipped with ×63 objective (numerical aperture 1.4, Carl Zeiss, Inc., Thornwood, NY). Images were obtained with a SIT camera (Hamamatsu Corp., Bridgewater, NJ), and processed using a Tracor Northern 8500 image processor (Tracor Inc., Austin, TX). Photographs were taken from the monitor screen using TMAX 400 ASA Kodak film.

**[125I]-α-Thrombin Binding**

Alpha-thrombin was labeled by the chloramine-T procedure (4), and the labeled reagent was separated from excess [125I] by chromatography on a Sephadex G-25 column. The [125I]-α-thrombin had specific activity of 2.4 μCi/μg. All binding experiments were made at 4°C with Hepes/Tris (pH 7.4). Binding of [125I]-α-thrombin was performed in a total buffer volume of 0.5 ml using 10 nM [125I]-α-thrombin and BSA (1 mg/ml). The BPAEC monolayers were washed 2× with buffer before incubation. The binding was terminated by washing the BPAEC rapidly 5× with buffer, and the cells were incubated with 0.6 ml of 0.3 N NaOH for 60 min. The solubilized contents were transferred to counting vials and the radioactivity was counted. Nonspecific binding was determined by adding 100-fold excess of unlabeled a-thrombin during the incubation; nonspecific binding for [125I]-a-thrombin varied between 25 and 30%.

**Data Analysis**

Single sample data were analyzed by the two-tail t test. A multiple range test (Scheffe's test) was used for comparisons of experimental groups with a single control group (30).

**Results**

**TRP-14 and α-Thrombin Mobilize Cytosolic Ca2+ and Differentially Activate Protein Kinase C**

TRP-14 (0.1, 1, 10, 100, and 200 μM) added to BPAEC caused concentration-dependent increases in the [Ca2+]i (Table I). At 10 and 100 μM TRP-14, [Ca2+]i increased 7- and 15-fold over basal levels, respectively; whereas <1 μM TRP-14 produced no effect (Table I). The 200-μM concentration of TRP-14 caused an increase in [Ca2+]i similar to that induced by 0.1 μM a-thrombin (a concentration shown to increase endothelial permeability by two to threefold). The t1/2 values (time at which the peak response decreased 50% of maximum value) were determined to assess the decay of the Ca2+ responses. Both TRP-14- and a-thrombin-induced increases in [Ca2+]i showed similar decay constants with t1/2 values of 0.73 min and 0.61 min, respectively (Table I). In medium containing 5 mM EGTA, the peak rise in [Ca2+]i after 100 μM TRP-14 (Fig. 1) or 0.1 μM a-thrombin stimulation (Fig. 1, inset) decreased to basal levels with the same time course. However, EGTA did not alter the initial rise in [Ca2+]i after either TRP-14 (Fig. 1) or a-thrombin challenge (Fig. 1, inset), indicating that the initial response in both cases was the result of intracellular Ca2+ mobilization. A control TRP-14 peptide with the NH2 terminus acetylated did not increase [Ca2+]i (Table I).

Fig. 2 shows the immunocytochemical localization of PKC. In quiescent (control) cells, PKC was distributed homogeneously within the cytosol (Fig. 2 c), α-thrombin caused the translocation of PKC from the cytosol to plasma membrane indicative of enzyme activation (Fig. 2 b). In contrast, TRP-14 (as high as 200 μM concentration) incubated with BPAEC did not alter the PKC distribution (Fig. 2 a).

**TRP-14 and α-Thrombin Mediate Different Effects on Endothelial Permeability**

Addition of TRP-14 (1, 10, and 100 μM) or acetylated TRP-14 (100 μM) did not increase the transendothelial albumin permeability (Fig. 3). In contrast, a-thrombin (0.1 μM) increased the permeability by ~200% over the control value (Fig. 3). We coincubated endothelial cells with 100 μM TRP-14 and 1 μM DIP-α-thrombin (which lacks enzymatic activity but retains high affinity binding capability [11]) to determine whether TRP-14 required the simultaneous binding of thrombin to its receptor to mediate the permeability response. The results indicated that the combination of TRP-14 and DIP-α-thrombin also did not increase transendothelial [125I]albumin permeability (Fig. 4).

**Receptor Desensitization Mediated by TRP-14 Prevents α-Thrombin-Induced Increase in Endothelial Permeability**

BPAEC preloaded with fura2 were stimulated with 100 μM TRP-14, and changes in [Ca2+]i were monitored to study the time course of desensitization. When the response had decayed to a new steady-state (i.e., ~<3 min after TRP-14 stimulation), the cells were restimulated with either 100 μM TRP-14 or 0.1 μM thrombin. The initial stimulation with 100 μM TRP-14 caused the characteristic increase in [Ca2+]i from 77 ± 10 to 984 ± 75 nM after which the response slowly decreased to a new steady-state value by ~<3 min (Fig. 5 a). The second challenge with 100 μM TRP-14 or 0.1 μM a-thrombin at this point failed to increase [Ca2+]i (Fig. 5 a).

### Table I. [Ca2+]i Responses in Bovine Pulmonary Artery Endothelial Cells

| Agonist     | Concentration | Basal [Ca2+]i | Peak [Ca2+]i | t1/2*  |
|-------------|---------------|---------------|--------------|-------|
| TRP-14      | 1             | 136 ± 40      | 147 ± 35     | 3     |
|             | 10            | 98 ± 9        | 677 ± 73     | 1.1   |
|             | 100           | 85 ± 10       | 931 ± 74     | 0.7   |
| AcTRP-14    | 100           | 74 ± 11       | 984 ± 142    | 0.8   |
| α-Thrombin  | 100           | 90 ± 4        | 118 ± 30     | 3     |

*Values are shown as mean ± SEM.

*Time at which the peak response decayed 50% from maximum value.

**Acetylated TRP-14 (a control peptide).**

- indicates not done.
However, when BPAEC were initially stimulated by 100 μM acetylated TRP-14, the second challenge with TRP-14 or α-thrombin, produced the typical increase in [Ca²⁺], indicating that acetylated TRP-14 did not desensitize the cells (Fig. 5 a, inset). The cells were also fully responsive to 0.1 μM bradykinin when BPAEC were initially stimulated with 100 μM TRP-14 (Fig. 5 b).

The effects of TRP-14-induced desensitization on the endothelial permeability response were studied using BPAEC grown on microporous filters. The cells were stimulated with TRP-14 for 5 min, washed, then restimulated with 0.1 μM α-thrombin, and endothelial permeability was determined. Preexposure of BPAEC with 100 μM TRP-14 (but not with 10 μM TRP-14) prevented the α-thrombin-induced increase in endothelial [³²P]albumin permeability, whereas preexposure with 100 μM TRP-14 did not affect the permeability increase in response to bradykinin (Fig. 6).

In a separate series of experiments, we determined the time required for BPAEC to recover from desensitization induced by TRP-14. Cells were initially stimulated with 100 μM TRP-14 for 5 min, washed, reincubated in fresh medium for 10, 30, or 60 min, at which time they were restimulated with 0.1 μM α-thrombin, and the increases in [Ca²⁺] and transendothelial [³²P]albumin permeability were monitored (Fig. 7 a and b). BPAEC were refractory to α-thrombin after incubation in fresh medium for 10 min and the response was of the level observed in control. However, at 30 min the rise in [Ca²⁺] in response to α-thrombin was ~43% of the response in control thrombin-exposed cells (Fig. 7 a). Alpha-thrombin did not cause increases in endothelial permeability at either 10 or 30 min after the initial stimulation with TRP-14 (Fig. 7 b). However, the cells recovered completely by 60 min after TRP-14 stimulation; that is, the magnitude of the α-thrombin-induced increase in [Ca²⁺], and endothelial permeability were >90% of the control cells at this time (Fig. 7, a and b).

We determined the effects of initial stimulation with TRP-14 on α-thrombin binding to BPAEC because the response to TRP-14-induced desensitization may be explained by decreased binding of α-thrombin to its receptor. BPAEC were pretreated with 100 μM TRP-14 for 5 min, washed, reincubated in fresh medium for 10, 30, or 60 min, and the binding of [³²I]-α-thrombin was determined. The results indicated that 100 μM TRP-14 did not decrease [³²I]-α-thrombin binding to BPAEC as compared to [³²I]-α-thrombin binding to control cells (Table II).

**Discussion**

The present study demonstrates that (a) TRP-14 is as competent an agonist as α-thrombin in increasing [Ca²⁺], in BPAEC, but in contrast to α-thrombin it does not activate PKC and it does not increase endothelial monolayer permeability to albumin; (b) TRP-14 desensitizes BPAEC to the increases in [Ca²⁺], and endothelial permeability mediated by α-thrombin; and (c) recovery from the TRP-14-induced desensitization of the Ca²⁺ response parallels the restoration of the endothelial permeability response.

We observed that TRP-14 at 100 μM elicited an increase in [Ca²⁺], of the same magnitude as observed with 0.1 μM α-thrombin. Vu et al. (36) have also observed that a 100-fold greater TRP-14 than α-thrombin concentration was required...
for maximal $\text{Ca}^{2+}$ release from oocytes expressing the thrombin receptor. The higher TRP-14 requirement is likely related to its lower binding affinity ($\sim 2 \mu M$) to the endothelial plasma membrane compared to $\alpha$-thrombin binding (33). Also, $\geq 100 \mu M$ concentrations of TRP-14 approach the saturating level because a similar $\text{Ca}^{2+}$ response was observed at 100 and 200 $\mu M$ TRP-14.

TRP-14 and $\alpha$-thrombin both produced rapid increases in $[\text{Ca}^{2+}]_i$ followed by slow decay to sustained values above baseline. Removal of extracellular $\text{Ca}^{2+}$ with EGTA in both cases abolished the sustained phase of the $\text{Ca}^{2+}$ response. Thus, the transient initial rise in cytosolic $\text{Ca}^{2+}$ is attributed to mobilization of $\text{Ca}^{2+}$ from intracellular stores, and the

---

**Figure 2.** Immunocytochemical localization of PKC in BPAEC monolayers (see Methods for details). (A) BPAEC stimulated with 200 $\mu M$ TRP-14 for 30 min; (B) BPAEC treated with $0.1 \mu M$ $\alpha$-thrombin for 30 min; and (C) control cells. Arrowheads indicate immunofluorescence of PKC translocated to the plasma membrane. Results are representative of three experiments. Magnification, 1,000.
Figure 5. Representative tracings showing the effects of sequential stimulation with agonists on the Ca$^{2+}\text{ response in BPAEC. Arrow indicates the time point of agonist addition. (a) BPAEC were initially challenged with 100 µM TRP-14 followed by a second challenge with either 100 µM TRP-14 (n = 4) or 0.1 µM α-thrombin (α-Thr) (n = 4) during the steady-state condition; BPAEC were stimulated with 100 µM acetylated TRP-14 (AcTRP-14), then followed by either 100 µM TRP-14 (n = 3) or 0.1 µM α-Thr (n = 3) (inset). The tracings are off-set for clarity. (b) BPAEC initially stimulated with 100 µM TRP-14 (arrow) followed by a second challenge with 0.1 µM bradykinin (BK) (n = 3).

The sustained phase can be attributed to influx of extracellular Ca$^{2+}. Prior exposure of BPAEC with TRP-14 prevented the increase in [Ca$^{2+}$], upon stimulation with either TRP-14 or α-thrombin. The desensitization of the Ca$^{2+}$ response suggests that α-thrombin and TRP-14 are both capable of activating the same receptor and signaling pathways that mediate the increase in [Ca$^{2+}$]. This would occur if the synthetic TRP-14 functions as a ligand in the same manner as the NH$_2$ terminus of the receptor after the cleavage of the thrombin receptor (36). The present results indicate that TRP-14 contains all of the essential structural information needed to activate the Ca$^{2+}$ signal in endothelial cells.

The control acetylated TRP-14 neither increased [Ca$^{2+}$], and endothelial permeability nor desensitized the BPAEC to subsequent α-thrombin challenge. Because the NH$_2$ terminus was acetylated, the results suggest that the first amino acid of TRP-14, S$^42$, is required to activate the endothelial thrombin receptor, and thereby to increase endothelial permeability. The importance of the terminal amino acid is also supported by the observation that the inverted TRP-14
Effects of combined TRP-14 and α-thrombin challenge on transendothelial \([^{125}I]\)albumin clearance rate. BPAEC monolayers pretreated with TRP-14 (10 or 100 μM) were stimulated with 0.1 μM α-thrombin (α-Thr), or 0.1 μM bradykinin (BK), and the transendothelial clearance rates of \([^{125}I]\)albumin were determined. The number of monolayers for the groups ranged from 12 to 46. Values are shown as mean ± SEM. (*) Indicate significant difference compared to the control group (p < 0.01).

(FSLLRNPDKYEPF) did not induce \(^{40}\text{Ca}^{2+}\) release in oocytes expressing the thrombin receptor (36).

Despite the ability of TRP-14 to increase \([\text{Ca}^{2+}]\), TRP-14 did not increase transendothelial albumin permeability at concentrations as high as 100 μM that did increase \([\text{Ca}^{2+}]\). At α-thrombin concentrations which raised \([\text{Ca}^{2+}]\) to the similar magnitude as 100 μM TRP-14, the transendothelial \([^{125}I]\)albumin permeability increased ~twofold. Although the desensitization studies indicated that TRP-14 and α-thrombin both activated the same receptor, it appears that the α-thrombin-induced increase in endothelial permeability is mediated by pathways in addition to those solely activated by TRP-14.

We have observed that γ-thrombin, an enzymatically active α-thrombin that does not compete for high-affinity binding sites (10, 11), was less potent than α-thrombin in increasing endothelial permeability (1). This suggests that binding of thrombin to high-affinity sites may play an obligatory role in mediating the thrombin-induced increase in permeability; therefore, it is possible that the inability of TRP-14 to increase endothelial permeability was related to a lack of occupancy of these sites. However, the addition of DIP-α-thrombin (which binds to high affinity thrombin receptors but is enzymatically inactive [11]) and TRP-14 in an attempt to reconstitute the signal did not increase permeability, suggesting that occupancy of the thrombin receptor is not essential for the permeability response.

We observed that α-thrombin shifted PKC activity from the cytosol to membrane indicative of PKC activation, which has also been noted previously (23). In contrast, TRP-14 did not translocate PKC to the plasma membrane. Because inhibition of PKC translocation was shown to prevent the increase in endothelial permeability in response to α-thrombin (23), we postulate that the lack of effect of TRP-14 in increasing endothelial permeability is somehow related to the inability of TRP-14 to activate PKC. The present study supports the critical role of PKC activation in signaling the increase in endothelial permeability.

Time course of recovery from TRP-14-induced desensitization of (a) \(\text{Ca}^{2+}\) response and (b) endothelial permeability. BPAEC were stimulated with 100 μM TRP-14 for 5 min, incubated in fresh medium for either 10, 30, or 60 min, and then stimulated with 0.1 μM α-thrombin. Changes in \([\text{Ca}^{2+}]\), and transendothelial \([^{125}I]\)albumin permeability rates were determined. The \(\text{Ca}^{2+}\) responses (in Fig. 7 a) are representative from three to four determinations per group. Short arrow indicates time point of the α-thrombin (α-Thr) addition; long arrow indicates experimental groups. The α-Thr group was not pretreated with 100 μM TRP-14. The permeability responses to α-thrombin at 10, 30, and 60 min after TRP-14 exposure are shown (in Fig. 7 b) along with the responses of control cells that were preexposed to DME for the same durations. The permeability data are from 8 to 36 monolayers per group. The results are reported as mean ± SEM. The control group (C) represents the basal permeability value of BPAEC monolayers. (*) Indicate significant difference from the DME group at the same time point (p < 0.01); (†) indicates significant difference from control (p < 0.01).

Accumulating evidence suggests that synthetic thrombin receptor peptides elicit some but not all of α-thrombin's actions. Vouret-Craviari et al. (35) found that peptides corresponding to the cloned hamster thrombin receptor's tethered ligand activated PLC and serotonin release in hamster fibroblasts, but did not induce mitogenesis as was the case with the native α-thrombin, indicating differences in α-thrombin and TRP-14-mediated responses. Synthetic peptides corresponding to the tethered ligand of the cloned human thrombin receptor induced the responses associated only with acti-
that α-thrombin and TRP-14 can activate different signal transduction pathways. The present results indicate that cellular responses mediated by TRP-14 and α-thrombin are distinct. A possible explanation of our data is that α-thrombin's proteolytic activity causes a conformational change of the thrombin receptor that may be different from that mediated by the nonproteolytic TRP-14 (34, 36) such that different signaling pathways are activated. Another possibility is that α-thrombin can activate additional pathways (e.g., phospholipase D) which can prolong the generation of diacylglycerol needed for the activation of PKC (3), and thereby increasing endothelial permeability (23).

Because TRP-14 resulted in the desensitization of the thrombin receptor and also did not independently increase endothelial permeability, we used the approach of desensitizing the thrombin receptor with TRP-14 to determine thrombin's effect on endothelial permeability. The results indicated that TRP-14 preexposure of BPAEC prevented the thrombin-induced increase in endothelial permeability. However, this effect was short lived in that inhibition was evident at 10–30 min after TRP-14 preexposure, and recovery was complete by 60 min. The inhibitory effect of TRP-14 on permeability was temporally related to the refractory period of the Ca²⁺ response, which supports an important facilitative role of Ca²⁺ in the permeability response. The results are consistent with the hypothesis that PKC activation signals the increase in endothelial permeability (23) because cells that are unable to mobilize intracellular Ca²⁺ may have an impaired PKC activation response (24).

Previous studies have suggested that the thrombin-induced increase in [Ca²⁺], is an important initial event mediating the increase in endothelial permeability (21, 22). The present results also point to the rise in [Ca²⁺] as a critical first step required for the thrombin-induced increase in permeability. The inhibition of the permeability response likely occurred at the receptor level since TRP-14 pretreatment did not interfere with bradykinin-induced increases in [Ca²⁺], and endothelial permeability. Our results suggest that the inhibitory effect of TRP-14 on the α-thrombin-induced increase in permeability is related to TRP-14-induced receptor activation, and to subsequent receptor desensitization, such that α-thrombin is now unable to activate its receptor.

TRP-14-induced desensitization is likely mediated by events downstream from the ligand-receptor level since TRP-14 pretreatment did not interfere with binding of ¹²⁵I-α-thrombin to BPAEC. Brass (6) recently showed in a leukemic cell line that the synthetic thrombin receptor peptide induces the desensitization of the Ca²⁺ response by receptor phosphorylation because treatment with phosphatase inhibitors prolonged the refractory period. This finding was in contrast to desensitization mediated by α-thrombin because thrombin modified its receptor by proteolytic cleavage, and thus the refractory period persisted for up to ~24 h (6). We found that the recovery time of BPAEC desensitized by TRP-14 pretreatment occurred within ~60 min, when the cells were fully responsive to α-thrombin. The time course of the recovery is consistent with the proposed receptor phosphorylation mechanism of desensitization (6).

In summary, TRP-14 and α-thrombin similarly increased [Ca²⁺] in BPAEC; however, TRP-14 did not increase transendothelial albumin permeability and also did not activate PKC in contrast to the actions of α-thrombin. In fact, TRP-14 pretreatment prevented the α-thrombin-induced increases in intracellular Ca²⁺ and endothelial permeability secondary to TRP-14-induced desensitization of the Ca²⁺ signal. The results suggest that Ca²⁺ mobilization and PKC activation are the dual signals required for the α-thrombin-induced increase in endothelial permeability.

Desensitization of the thrombin receptor by TRP-14 or related synthetic peptides may be a means of preventing α-thrombin-induced increase in vascular endothelial permeability and mitigating the pro-inflammatory properties of thrombin.

Table II. Effect of TRP-14-Exposure on ¹²⁵I-α-Thrombin Binding to Bovine Pulmonary Artery Endothelial Cells

| TRP-14-exposed (fmol/10⁶ cells) | Control | 10 min | 30 min | 60 min |
|---------------------------------|---------|--------|--------|--------|
|                                 | 540.0 ± 26.9 (7) | 518.2 ± 26.6 (7) | 518.3 ± 28.2 (7) | 506.8 ± 20.9 (7) |

Values are shown as the mean ± SEM.

Number in parenthesis indicate number of monolayers studied.

BPAEC were stimulated with 100 µM TRP-14 for 5 min, washed 2x, reincubated in fresh HBS for 10, 30, or 60 min, and binding of ¹²⁵I-α-thrombin was determined (see Methods for details). The control cells were exposed to DME.

References

1. Aschner, J. L., J. M. Lennon, J. W. Fenton, IIL M. Aschner, and A. B. Malik. 1990. Enzymatic activity is necessary for thrombin-mediated increase in endothelial permeability. Am. J. Physiol. 259:L770-L775.
2. Bidlingmayer, B. A., S. A. Cohen, and T. L. Tarvin. 1984. Rapid analysis of amino-acids using pre-column derivatization. J. Chromatogr. 336: 93-104.
3. Billah, M. M., and J. C. Antunes. 1990. The regulation and cellular functions of phosphatidylcholine hydrolysis. Biochim. J. 259:281-291.
4. Bocci, V. 1964. Efficient labeling of serum proteins with ¹¹¹I using chloramine T. Internat. J. Radiat. Isotopes. 15:445-456.
5. Brock, T. A., and E. A. Capasso. 1988. Thrombin and histamine activate phospholipase C in human endothelial cells via a phorbol ester-sensitive pathway. J. Cell Physiol. 136:54-62.
6. Brass, L. F. 1992. Homologous desensitization of HEL cell thrombin receptors. Distinguishable roles for proteolysis and phosphorylation. J. Biol. Chem. 267:6044-6050.
7. Cooper, J. A., P. J. Del Vecchio, F. L. Minnear, K. E. Barhop, J. G. N. Garcia, and A. B. Malik. 1987. Measurement of albumin permeability across endothelial monolayers in vitro. J. Appl. Physiol. 62:1076-1083.
8. Del Vecchio, P. J., J. W. Ryan, A. Chang, and U. S. Ryan. 1980. Capillaries of the adrenal cortex possess aminopeptidase A and angiotensin-converting enzyme activities. Biochem. J. 186:605-608.
9. Fenton II, J. W. 1988. Regulation of thrombin generation and functions. Semin. Thromb. Hemostasis. 14:234-240.
10. Fenton, J. W., II. 1988. Thrombin bioregulatory functions. Adv. Clin. Enzymol. 6:186-195.
11. Fenton, J. W., II, M. J. Fascio, A. B. Stackrow, D. L. Aronson, A. M. Young, and J. S. Finlayson. 1977. Human thrombins. Production, evaluation and properties of α-thrombin. J. Biol. Chem. 252:3587-3598.
12. Garcia, J. G. N., A. Sifflinger-Birnboim, R. Bizios, P. J. Del Vecchio, J. W. Fenton II, and A. B. Malik. 1986. Thrombin-induced increase in albumin permeability across the endothelium. J. Cell. Physiol. 128:96-104.
13. Goligorsky, M. S., D. N. Menton, A. Laslo, and H. Lum. 1989. Nature of thrombin-induced sustained increase in cytosolic calcium concentrations in cultured endothelial cells. *J. Biol. Chem.* 265:16771–16775.

14. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca²⁺ indicators with greatly improved fluorescent properties. *J. Biol. Chem.* 260:3440–3450.

15. Huang, R., A. Sorsby, W. R. Church, E. R. Simona, and E. Rittenhouse. 1991. Thrombin receptor-directed ligand accounts for activation by thrombin of platelet phospholipase C and accumulation of 3-phosphorylated phosphoinositides. *J. Biol. Chem.* 266:18435–18438.

16. Ho, T., T. Tanaka, T. Yoshiida, K. Osoda, H. Ohu, M. Hagiywara, Y. Itoh, M. Ogura, H. Saito, and H. Hidama. 1988. Immunocytocchemical evidence for translocation of protein kinase C in human megakaryoblastic leukemia cells: synergistic effects of Ca²⁺ and activators of protein kinase C on the plasma membrane association. *J. Cell Biol.* 107:929–937.

17. Horgan, M. J., J. W. Fenton II, and A. B. Malik. 1987. Alpha-thrombin-induced pulmonary vasconstriction. *J. Appl. Physiol.* 63:1993–2000.

18. Jaffe, E. A., J. Grulich, B. B. Weksler, G. Hampel, and K. Watanabe. 1987. Correlation between thrombin-induced prostacyclin production and inositol trisphosphate and cytosolic free calcium levels in cultured human endothelial cells. *J. Biol. Chem.* 262:8557–8565.

19. Kaiser, E., R. L. Colescott, C. D. Bossinger, and P. I. Cork. 1970. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* 54:593–598.

20. Lampugnani, M. G., M. Penedori, E. Dejana, D. Rotilio, M. B. Donati, F. Bussolino, G. Garbarino, D. Ghigo, and A. Bosia. 1989. Human α-thrombin induces phosphoinositide turnover and Ca²⁺ movements in cultured human umbilical vein endothelial cells. *Thromb. Res.* 54:75–87.

21. Lum, H., P. J. Del Vecchio, A. S. Schneider, M. S. Goligorsky, and A. B. Malik. 1989. Calcium dependence of the thrombin-induced increase in endothelial albumin permeability. *J. Appl. Physiol.* 66:1471–1476.

22. Lum, H., J. L. Aschner, P. G. Phillips, P. W. Fletcher, and A. B. Malik. 1992. Time-course of thrombin-induced increase in endothelial permeability: relationship to Ca²⁺ and inositol polyphosphates. *Am. J. Physiol.* 263:L1219–L1225.

23. Lynch, J. J., T. J. Ferro, F. A. Blumenstock, A. M. Brockenzauer, and A. B. Malik. 1990. Increased endothelial albumin permeability mediated by protein kinase C activation. *J. Clin. Invest.* 85:1991–1998.

24. May, W. S., Jr., N. Sahyoun, M. Wolf, and P. Cuatrecasas. 1985. Role of intracellular calcium mobilization in the regulation of protein kinase C-mediated membrane processes. *Nature (Lond.)* 317:549–551.

25. Merrifield, R. B. 1963. Solid phase peptide synthesis. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* 85:2149–2154.

26. Ngaiza, J. R., and E. A. Jaffe. 1991. A 14 amino acid peptide derived from the amino terminus of the cleaved thrombin receptor elevates intracellular calcium and stimulates prostacyclin production in human endothelial cells. *Biochem. Biophys. Res. Commun.* 179:1656–1661.

27. Rasmussen, U. B., V. Voursart-Craviari, S. Jallat, Y. Schlesinger, G. Pages, A. Pavirani, J. P. Lecoq, J. Pouysségur, and E. Van Obberghen-Schilling. 1991. DNA cloning and expression of a hamster α-thrombin receptor coupled to Ca²⁺ mobilization. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 288:123–128.

28. Ryan, U. S., P. V. Avdoinia, E. Y. Posin, E. G. Popov, S. M. Danilov, and V. A. Tkachuck. 1988. Influence of vasoactive agents on cytoplasmic free calcium in vascular endothelial cells. *J. Appl. Physiol.* 65:2221–2227.

29. Seiler, S. M., H. J. Goldenberg, I. M. Michel, J. T. Hunt, and G. B. Zavoico. 1991. Multiple pathways of thrombin-induced platelet activation differentiated by desensitization and a thrombin exosite inhibitor. *Biochem. Biophys. Res. Commun.* 181:636–643.

30. Sooder, G. W., and W. G. Cochran. 1967. Statistical Methods. The Iowa State University Press, Ames, Iowa.

31. Sugama, Y., C. Tiruppathi, K. Janasikedi, T. T. Andersen, J. W. Fenton, II, and A. B. Malik. 1992. Thrombin-induced expression of endothelial P-selectin and intercellular adhesion molecule-I: a mechanism for stabilizing neutrophil adhesion. *J. Cell Biol.* 119:935–944.

32. Tam, J. P., W. F. Heath, and R. B. Merrifield. 1983. Sn2 deprotection of synthetic peptides with a low concentration of HF in dimethyl sulfide: evidence and application in peptide synthesis. *J. Am. Chem. Soc.* 105:6442–6455.

33. Tiruppathi, C., H. Lum, T. T. Andersen, J. W. Fenton II, and A. B. Malik. 1992. Thrombin receptor 14-amino acid peptides binds to endothelial cells and stimulates calcium transients. *Am. J. Physiol.* 263:L595–L601.

34. Vassallo, Jr., R. B., T. Kieber-Emmons, K. Cichowski, and L. F. Brass. 1992. Structure-function relationships in the activation of platelet thrombin receptors by receptor-derived peptides. *J. Biol. Chem.* 267:6081–6085.

35. Vourret-Craviari, V., E. van Obberghen-Schilling, U. B. Rasmussen, A. Pavirani, J.-P. Lecoq, and J. Pouysségur. 1991. Synthetic chelating peptide that inhibits neutrophil adhesion and P-selectin and intercellular adhesion molecule-I: a mechanism for stabilizing neutrophil adhesion. *Cell.* 64:1–20.