Thrombin modulates vectorial secretion of extracellular matrix proteins in cultured endothelial cells

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Thrombin, through as yet unidentified mechanisms, promotes in vivo angiogenesis and incisional wound healing (4, 39). Both of these processes involve EC activation and remodeling of the subendothelial extracellular matrix (ECM). ECM remodeling can occur either at the beginning of the angiogenic process by degradation of the basement membrane or at the end by de novo synthesis and deposition of basement membrane proteins (22). We hypothesize that thrombin’s angiogenic properties might, in part, be due to its capacity to initiate ECM remodeling. Indeed, previous studies have shown that long-term incubation of EC with thrombin causes a modification in the adhesive properties of the ECM (6), a decrease in its collagen content (13), and also an augmentation of the amount of some ECM proteins found in the culture medium, presumably due to a loss of these proteins from the subendothelial ECM (11). In addition, thrombin might
 affect the vectorial release of fibronectin and von Willebrand factor (1, 16, 24). A common characteristic of the above studies is the prolonged exposure of the cells to thrombin (hours to days), a condition that is unlikely to occur in vivo in which circulating thrombin is rapidly inactivated and/or removed from the circulation (8). Because thrombin activates both early (phospholipase C activation, permeability, and so forth) and late (protein synthesis, proliferation) events, we rationalized that early effects of thrombin stimulation on ECM protein secretion might differ from some of its previously described long-term effects on ECM remodeling.

To test our hypothesis, we assessed the effects of thrombin on ECM protein secretion from microvascular EC isolated from the rat adrenal medulla (RAMEC). We previously reported the time course for the constitutive secretion of ECM proteins from these cells (30). In this study, we show that a brief, 5-min exposure of confluent RAMEC to thrombin or a peptide agonist of the thrombin receptor (TRAP) significantly enhanced, within 3 h, basolateral ECM protein deposition in a bell-shaped, dose-dependent manner, while concomitantly reducing the apical release of these proteins. Prolonged (>1 h) stimulation with thrombin had the opposite effect. Furthermore, we found that two established early signaling events triggered by thrombin in EC, namely activation of PKC and elevation of intracellular Ca\textsuperscript{2+}, are not involved in this rapid modulation of ECM protein secretion by thrombin.

MATERIALS AND METHODS

Materials. Bovine α-thrombin, cycloheximide, actinomycin D forskolin, isoproterenol 1-[5-isquinolinsulfonyl]-2-methylpiperazine (II-7), 4-phorbol 12-myristate 13-acetate (PMA), A-23187, trypsin, type IV collagenase, plasmin, and EDTA were from Sigma Chemical (St. Louis, MO); Humana u- and γ-thrombin, as well as the inactive thrombin analogs, phenylalanine-propyl-arginine chloromethylketone (PPACK)-α-thrombin and diisopropyl (DIP)-α-thrombin, were prepared as previously described (10). The proteolytic activity of α- and γ-thrombin was verified for each batch by measuring the cleavage of S-2238, an established chromogenic substrate for thrombin (from KABI Diagnostics, Franklin, OH). Conversely, the functional inhibition of the thrombin analogs was inferred from their inability to cleave S-2238.

The human TRAP (SP(LRKNPDYKYPF) was from Peninsula Laboratories (Belmont, CA). The biologically inactive cyclohexyl-TRAP analog [S(CHA)LRKNPDYKYPF], in which the phenylalanine in position 2 is replaced by a cyclohexylalanine, was a generous gift from Dr. Thomas T. Andersen (Albany Medical College, Albany, NY). Cell-impermeant 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) and cell-permeant BAPTA-acetoxy-methyl ester (AM) were from Molecular Probes (Eugene, OR). Dulbecco’s modified Eagle’s cell culture medium (DMEM) was from Mediatech (Herndon, VA). The protein content of the wells was determined with a commercially available protein assay kit (Pierce, Rockford, IL) according to the manufacturer’s instructions.

Cell culture. RAMEC were isolated and characterized as previously described (30). The cells were routinely subcultured in DMEM supplemented with 7% fetal calf serum and 7% horse serum, 2.5 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin. All experiments were performed with postconfluent monolayers of RAMEC from passages 11 through 22. No passage-dependent differences were found in any of the results obtained. Unless stated otherwise, stimulation with thrombin and the subsequent incubations (up to 3 h) were carried out in serum-free medium.

Enzyme-linked immunosorbent assay. Ten thousand RAMEC were plated in each well of 96-well tissue culture plates. One day after reaching confluence, the cells were washed twice with serum-free medium and then stimulated with bovine α-thrombin or TRAP for 5 min in serum-free medium. Subsequently, they were washed twice and further incubated for 3 h in fresh serum-free medium. At the end of the incubation period, the medium was removed and stored at 80°C. To quantitate the ECM proteins deposited into the subendothelial ECM, the cells were removed from the plates by incubation (10 min) with 20 mM NiCl\textsubscript{2}OH at room temperature, as previously described (26). This method has been shown to completely remove the cells and leave an intact ECM attached to the wells (40). After cells were removed, the wells were washed three times with phosphate-buffered saline (PBS; pH 7.4) and incubated with blocking agent (1% bovine serum albumin in PBS, pH 7.4) for 2 h at room temperature. The levels of basolaterally deposited fibronectin, laminin, collagen IV, and collagen I were measured with an enzyme linked immunosorbent assay (ELISA) that was previously described in detail (30).

The following antibodies were used: 1) anti-human fibronectin (1:10,000 dilution, a generous gift from Dr. D. Amrani, University of Wisconsin Milwaukee, WI), 2) anti-laminin (1:5,000, raised in house against laminin purchased from Sigma), 3) anti-human collagen type IV (1:500, a generous gift from Dr. D. Grant, National Institute of Dental Research, Bethesda, MD), and 4) anti-rat collagen type I (1:200, Bio-Design International, Kennebunk, ME). The specificity of each antibody was ascertained by Western blotting with purified antigens, as described in Ref. 30. Also, using various ECM protein as antigens, we established by ELISA that the extent of cross-reactivity was minimal (<5%) for all antibodies (data not shown). Standard curves were generated by using human fibronectin (from Dr. D. Amrani), laminin (Sigma), human collagen type IV (Collaborative Biomedical Products, Bedford, MA), and rat collagen type I (Sigma).

Inhibition ELISA. In the same experiments in which we measured ECM protein deposition, we also quantitated the amounts of fibronectin, laminin, and collagen IV released apically into the culture medium by inhibition ELISA according to Ref. 33. In brief, microtiter plates were coated with standard amounts of antigens, diluted into 20 mM carbonate buffer at pH 8.6. The antibodies were allowed to adsorb to the microtiter wells overnight at 4°C. A standard amount of antibodies was added to the cell culture supernatant (100 μl) in a separate microtiter plate (same dilutions of the antibodies as used for the ELISA technique). After the antibody was allowed to bind to the antigen overnight at 4°C, each sample was transferred to the corresponding antigen-coated plate and incubated for 1 h at room temperature. The wells were then washed three times for 5 min with PBS (pH 7.4) and incubated for 1 h at room temperature with peroxidase-conjugated goat anti-rabbit immunoglobulin G (whole molecule) at a dilution of 1:1,000. After the plates were washed, the enzyme substrate was added and the reaction product was quantitated in a microplate reader as previously described (30).

Standard curves for fibronectin, laminin, and collagen type IV were generated by coating microtiter plates with the respective antigens (see Materials). Concomitantly, known amounts of the antigens in solution were incubated with a
constant amount of the corresponding antibody, as described above. We were unable to determine the amounts of collagen I released into the culture medium because the available antibody did not recognize the soluble antigen.

Quantitative reverse transcriptase-polymerase chain reaction. The quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was used with internal standard constructs, generated for each gene, to measure ECM mRNA levels (36). Isolation of RNA from RAMEC was performed with RNA-Stat (Tel-Test "B," Friendswood, TX) according to the instructions of the manufacturer. RNA was reverse transcribed into cDNA with oligo(dT) primer and Moloney murine leukemia virus RT (United States Biochemical, Cleveland, OH). Constant amounts of cDNA were amplified in the presence of gene- and species-specific primers with different dilutions of the appropriate internal standard DNA. Standard amplification conditions were 35 cycles of 30 s of denaturing at 95°C, 1 min of annealing at 57°C, and 1 min of extension at 72°C (10 min for the 35th cycle) in the presence of 1.2 mM MgCl₂. Internal standards were designed and generated with a PCR MIMIC kit (Clontech Laboratories, Palo Alto, CA). Because the rat α₁ (type IV) collagen sequence has not yet been reported, mouse α₁ (type IV) collagen-specific primers 5'-AGGTGGAGGAGACTGGACCAT-3' (sense) and 5'-TGGACAGGCCATGAGGATA-3' (antisense) were designed and synthesized (Operon Technologies, Alameda, CA) with the published sequence (27). Rat cDNA was amplified with these heterologous primers. The product was cloned with the use of a TA cloning kit (Invitrogen, San Diego, CA), and both strands were sequenced by the University of Wisconsin Milwaukee, Biological Sciences Department's automated sequencing service with DyeDeoxy terminator chemistry (Applied Biosciences, Foster City, CA; data not shown). Rat-specific primers 5'-AAGGAGAGACTGGACCAT-3' (sense) and 5'-TGGACAGGCCATGAGGATA-3' (antisense) were designed just internal to the mouse primers from the obtained rat sequence. Hybrid primers 5'-AAGGAGAGACTGGACCATT-3' (sense) and 5'-TGGACAGGCCATGAGGATA-3' (antisense) were then generated, containing rat collagen type IV primer sequences joined to ver B sequences (underlined) of the PCR MIMIC kit template. This template was amplified with the hybrid primers, and this product was cloned to generate the rat-specific collagen type IV internal standard.

Involvement of PKC and Ca²⁺. PKC was either directly inhibited by incubation of the cells with 10 μM H-7 for 30 min or downregulated by overnight incubation with a high concentration (10 μg/ml) of PMA (37). These treatments were, respectively, >95 and >99% effective in inhibiting and/or downregulating PKC activity, as determined by using a commercial, nonradioactive PKC assay (MESACUP from Medical and Biological Laboratories, Watertown, MA) according to the manufacturer's instruction (data not shown).

Extracellular Ca²⁺ was chelated by incubating the cells for 5 min with either 10 μM EDTA or 10 μM BAPTA in Ca²⁺-free PBS (pH 7.4). Because Ca²⁺-free PBS nominally contains <10 μM Ca²⁺, we established in preliminary studies that, under these conditions, chelation of extracellular Ca²⁺ did not cause detachment of the monolayer. Intracellular Ca²⁺ was chelated by incubating the cells for 1 h with 25 μM of permeant BAPTA-AM (7). Intracellular Ca²⁺ was elevated by incubation of the cells with the Ca²⁺ ionophore A-23187 (10 μM) for 5 min. After these treatments, the cells were stimulated for 5 min with 0.25 U/ml thrombin and further processed as described above. Cell viability was not affected by any of these treatments.

Statistical analysis. The significance of variability between the results from various groups was determined by one-way analysis of variance. Each experiment included triplicate wells for each condition tested. All results are expressed as means ± SE from at least three experiments with different batches of cells.

RESULTS

Thrombin stimulates basolateral ECM protein deposition.¹ Postconfluent monolayers of RAMEC were stimulated for 5 min with 0.025 U/ml thrombin in serum-free medium and subsequently incubated in fresh serum-free medium for 1–6 h. The amounts of basolaterally deposited ECM proteins (fibronectin and laminin) were significantly increased in thrombin-stimulated cells, whereas they remained virtually unchanged in the unstimulated controls (Fig. 1). Qualitatively similar results were obtained for collagen types I and IV (data not shown). For all ECM proteins studied, a significant increase in basolateral deposition could be reliably measured 1 h after the initial 5-min stimulation with thrombin, reaching a plateau at 3 h.

We also tested the effects of varying the length of thrombin stimulation on ECM protein deposition. As exemplified in Fig. 2 for fibronectin, maximal stimulation was obtained after a 5-min exposure to thrombin. Prolonged incubation of the cells with thrombin for >30 min resulted in a decline in the amount of basolaterally deposited fibronectin. If the cells were stimulated for 1

¹ Throughout this paper, for the sake of simplicity, the basolateral secretion of ECM proteins into the subendothelial cell space and incorporation into a nonextractable ECM has been termed "deposition." The apical secretion of ECM proteins into the luminal space (cell culture medium) has been termed "release." The term "secretion" refers to the phenomenon of (polarized) discharge of ECM proteins.
were stimulated for 5 min with thrombin, and ECM protein deposition and release were assessed 3 h later.

Exposure of RAMEC to increasing concentrations of thrombin induced a bell-shaped augmentation of the amounts of basolaterally deposited ECM proteins (Fig. 3). A significant dose-dependent increase was observed at concentrations of thrombin between 0.005 and 0.25 U/ml. The 50% effective concentration (EC₅₀) values for this stimulatory effect were 0.025, 0.032, 0.06, and 0.083 U/ml for fibronectin, laminin, collagen type IV, and collagen type I, respectively. Although the levels deposited under basal conditions varied significantly among the different ECM proteins (from 7.5 ng/well for collagen type I up to 120 ng/well for collagen type IV), maximal stimulation (at 0.25 U/ml thrombin) amounted to up to a three- to fourfold increase over basal values for all four ECM proteins. This stimulatory effect decreased gradually at higher thrombin concentrations. For example, at 2 U/ml of thrombin, the deposited levels of all ECM proteins were essentially identical to the unstimulated levels. At even higher thrombin concentrations, the “deposited” amounts of the ECM proteins decreased to below basal levels (not shown), presumably due to a partial dissolution of the subendothelial ECM (11). This finding confirms and extends previous studies in which prolonged (>1 h)
incubation of EC monolayers with high levels of thrombin resulted in the release of ECM proteins from the subendothelial matrix into the culture medium (6, 24). Inclusion of serum in the incubation medium during and after the 5-min stimulation with thrombin abrogated the increase in ECM protein deposition at thrombin concentrations up to 0.25 U/ml (data not shown).

**Thrombin attenuates apical ECM protein release.** In addition to quantitating basolateral deposition, we also measured, in the same experiments, the apical release of fibronectin, laminin, and collagen IV into the cell culture medium by using an inhibition ELISA. In contrast to the increase in basolateral ECM protein deposition described above, the same treatment resulted in a qualitatively opposite, bell-shaped decrease in apical ECM protein release (Fig. 3). At low concentrations of thrombin (<0.25 U/ml), apical ECM protein release was significantly reduced in a dose-dependent fashion (up to a three- to fourfold decline from basal levels). At higher concentrations, the inhibitory action of thrombin gradually decreased. It is noteworthy that the EC50 values and the maximally effective thrombin concentration for both the release and the deposition of each ECM protein were essentially identical.

The results presented so far suggest that 1) short-term stimulation of RAMEC with low concentrations of thrombin results in an enhanced basolateral deposition of ECM proteins and 2) ECM protein release and deposition appear to be reciprocally interrelated.

**Thrombin-induced ECM protein deposition does not require de novo mRNA or protein synthesis.** Two independent sets of experiments were carried out to determine whether, during the time course of our experiments, thrombin-stimulated ECM protein deposition requires de novo mRNA or protein synthesis.

First, as detailed in materials and methods, we designed a quantitative RT-PCR assay to assess the effects of thrombin stimulation on steady-state mRNA levels in RAMEC. As seen in Fig. 4, the steady-state mRNA levels for collagen IV were not detectably affected by treatment of RAMEC with 0.25 U/ml of thrombin for 5 min and subsequent incubation for another 3 h. Similar results were obtained with fibronectin (data not shown).

Second, inhibition of de novo mRNA or protein synthesis with, respectively, 10 μM actinomycin D or 10 μg/ml cycloheximide did not inhibit the stimulatory effect of thrombin on ECM protein deposition (Table 1). On the contrary, in the presence of these two ubiquitous inhibitors, thrombin-induced ECM protein deposition was significantly augmented presumably because these compounds indiscriminately inhibit de novo synthesis of all mRNAs and cellular proteins, including those that might affect ECM protein secretion. During the time course of this experiment (3 h), stimulation with thrombin did not cause an overall increase in the amount of total protein contents per well, as measured with the Bradford assay (not shown).

We conclude from these results that, under our experimental conditions, thrombin-stimulated ECM protein deposition is neither accompanied by an increase in ECM protein gene expression nor does it require global de novo synthesis of mRNA or proteins. Thus the enhancement of ECM protein deposition by thrombin does not reflect transcriptional or translational activation of the cells.

**Prolonged thrombin stimulation has opposite effects.** Previous studies have shown that prolonged (1 h) stimulation of human umbilical vein EC and bovine aortic EC with thrombin causes a time- and/or dose-dependent loss of ECM molecules from the subendothelial matrix (11, 24). We investigated whether a similar phenomenon occurs in RAMEC. In contrast to our standard (5-min stimulation) protocol, we stimulated the cells with increasing concentrations of thrombin for 1 h and measured the amounts of fibronectin, laminin, collagen IV, and collagen I in the ECM and in the culture medium 2 h later. As seen in Fig. 5, the amounts of these four proteins in the ECM were decreased in a dose-dependent fashion, whereas the amounts released into the culture medium were increased, correspond-
Table 1. Effects of actinomycin D and cycloheximide on basal and thrombin-stimulated deposition of extracellular matrix proteins

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Thrombin Stimulated</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>FN</td>
<td>LM</td>
</tr>
<tr>
<td>Control</td>
<td>20±4</td>
<td>23±4</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>6±1*</td>
<td>23±4</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>19±3</td>
<td>16±2</td>
</tr>
</tbody>
</table>

Values are means ± SE, expressed as ng extracellular matrix (ECM) protein/well. All experiments were performed in triplicate and repeated at least twice. Confluent monolayers were washed in serum-free medium and incubated (in serum-free medium) for 3 h with 10 μM actinomycin D or 10 μg/ml cycloheximide. In the case of thrombin stimulation, each of these compounds was included in the incubation medium during the 5-min exposure to 0.25 U/ml thrombin as well as during the subsequent 3-h incubation period. FN, fibronectin; LM, laminin; CIV, collagen type IV; CI, collagen type I. *Statistically significant (P < 0.05) from controls.

Thrombin receptor involvement. Treatment of RAMEC for 5 min with increasing amounts of TRAP increased the basolateral deposition while concomitantly decreasing the apical release of all ECM proteins tested in a bell-shaped, dose-dependent fashion (Fig. 6), which was similar to that seen for thrombin. At low concentrations (1.56-26 μM), TRAP induced a dose-dependent increase in ECM protein deposition, which was gradually abolished at higher concentrations. At 100 μM TRAP, the deposited amounts of the proteins had returned to basal levels. In the same experiments, low concentrations of TRAP decreased the amounts of ECM proteins released into the culture medium, whereas at higher concentrations this inhibitory effect was abrogated (Fig. 6). Thus TRAP could fully substitute for the effects of thrombin in this study. In contrast, the biologically inactive peptide cyclohexyl-TRAP analog, S(CHA)LLNPNDKYEPF, did not affect ECM protein deposition (Table 2). These results suggest that the action of thrombin on ECM protein secretion is mediated through activation of the thrombin receptor.

Requirement for proteolytically active thrombin. The above results suggest that the proteolytic activity of thrombin is required for the action of thrombin on ECM protein secretion. 
Fig. 6. Dose dependence of basolateral deposition and apical release of ECM proteins from RAMEC after brief stimulation with peptide agonist of the thrombin receptor (TRAP). Amounts of fibronectin (A), laminin (B), collagen IV (C), and collagen I (D), concomitantly deposited into the ECM (■) and released into the culture medium (▲), were measured 3 h after stimulating the cells for 5 min with increasing concentrations of TRAP (1.56–100 μM). Data points are means ± SE from 3 independent experiments, each performed in triplicate.

To confirm this notion, we stimulated RAMEC with γ-thrombin, which is proteolytically active but lacks the anion-binding site (10), as well as with two proteolytically inactive analogs of human α-thrombin, PPACK-α-thrombin and DIP-α-thrombin. As expected, γ-thrombin enhanced ECM deposition to a similar degree as α-thrombin, whereas both proteolytically inactive analogs were without effect. These results clearly establish that thrombin mediated ECM protein deposition requires proteolytic activation of the thrombin receptor.

Table 2. Effect of various thrombin analogs on ECM protein deposition

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Fibronectin</th>
<th>Laminin</th>
<th>Collagen Type IV</th>
<th>Collagen Type I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21.2 ± 3.7</td>
<td>26 ± 3.2</td>
<td>152 ± 21</td>
<td>19.2 ± 9.6</td>
</tr>
<tr>
<td>α-Thrombin, 0.25 U/ml</td>
<td>57.2 ± 7.1*</td>
<td>82.1 ± 6.3*</td>
<td>351 ± 46*</td>
<td>39.2 ± 4.1*</td>
</tr>
<tr>
<td>γ-Thrombin, 0.25 U/ml</td>
<td>53.1 ± 6.2*</td>
<td>79.3 ± 4.7*</td>
<td>321 ± 32*</td>
<td>40.1 ± 6.3*</td>
</tr>
<tr>
<td>PPACK-α-thrombin, 30 nM</td>
<td>22.7 ± 1.8</td>
<td>22.4 ± 2.6</td>
<td>141 ± 19</td>
<td>17.3 ± 3.2</td>
</tr>
<tr>
<td>DIP-α-thrombin, 30 nM</td>
<td>19.8 ± 3.1</td>
<td>24.5 ± 1.6</td>
<td>162 ± 27</td>
<td>20.2 ± 3.4</td>
</tr>
<tr>
<td>Active TRAP, 10 μM</td>
<td>63.2 ± 5.7*</td>
<td>87.5 ± 7.8*</td>
<td>369 ± 30*</td>
<td>44.2 ± 5.1*</td>
</tr>
<tr>
<td>Inactive TRAP, 10 μM</td>
<td>24.2 ± 3.2</td>
<td>22 ± 3.9</td>
<td>137 ± 26</td>
<td>15.9 ± 2.7</td>
</tr>
</tbody>
</table>

Values are means ± SE from 3 independent experiments, each performed in triplicate, and are expressed as ng protein/well. Cells were incubated with the various thrombin analogs and synthetic peptides at a nominal activity of 0.25 U/ml or at the respective activities and concentrations in the same fashion as thrombin. Cells were exposed to the various proteases for 5 min in serum-free medium, the medium was replaced, and the cells were then incubated for another 3 h. Deposition of different ECM proteins was determined by enzyme-linked immunosorbent assay (ELISA), as described in Materials and Methods. TRAP, thrombin receptor agonist peptide; PPACK, phenylalanyl-propyl-arginine chloromethylketone. DIP, diisopropyl. *P < 0.01, values are statistically significantly different from untreated controls.

Stimulation of ECM protein deposition by other serine proteases. Recent studies suggest that the thrombin receptor can be proteolytically activated also by other serine proteases, notably trypsin and plasmin (41). To test whether other proteases can mimic the effects of thrombin on ECM protein deposition, we exposed RAMEC under similar experimental conditions (e.g., similar proteolytic activities, same duration, and so forth) to the serine proteases trypsin and plasmin and to the metalloprotease collagenase type IV. Exposure for 5 min to 0.25 U/ml trypsin and plasmin stimulated protein secretion.
Table 3. Effects of various proteases on ECM protein deposition

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Fibronectin</th>
<th>Laminin</th>
<th>Collagen Type IV</th>
<th>Collagen Type I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.1 ± 3.2</td>
<td>23.9 ± 2.1</td>
<td>133 ± 39</td>
<td>21 ± 2.0</td>
</tr>
<tr>
<td>α-Thrombin, 0.25 U/ml</td>
<td>60.5 ± 6.8</td>
<td>91.3 ± 8.7</td>
<td>402 ± 62</td>
<td>43.5 ± 3.4</td>
</tr>
<tr>
<td>Trypsin, 0.25 U/ml</td>
<td>41.3 ± 3.3</td>
<td>62.8 ± 6.3</td>
<td>297 ± 28</td>
<td>34.1 ± 4.7</td>
</tr>
<tr>
<td>Plasmin, 0.05 U/ml</td>
<td>38.7 ± 4.1</td>
<td>59.3 ± 7.4</td>
<td>304 ± 42</td>
<td>31.9 ± 2.9</td>
</tr>
<tr>
<td>Type IV collagenase, 0.25 U/ml</td>
<td>24.5 ± 2.4</td>
<td>23.6 ± 3.2</td>
<td>141 ± 27</td>
<td>23.0 ± 2.7</td>
</tr>
</tbody>
</table>

Values are means ± SE from 3 experiments, each performed in triplicate, and are expressed as ng protein/well. All proteases were used at a nominal activity of 0.25 U/ml in the same fashion as thrombin. Cells were exposed to the various proteases for 5 min in serum-free medium, the medium was replaced, and the cells were then incubated for another 3 h. Deposition of different ECM proteins was determined by ELISA. *P < 0.001; †P < 0.01.

the deposition of the ECM proteins studied, albeit to a lesser extent than thrombin (Table 3). In contrast, type IV collagenase did not stimulate ECM protein secretion. Thus it appears that proteolytic activation of the thrombin receptor by other serine proteases is sufficient for stimulation of ECM protein deposition.

PKC and Ca2+ are not involved in thrombin-stimulated ECM protein deposition. Two of the best-characterized signal transduction pathways involved in thrombin-induced EC activation include elevation of intracellular Ca2+ and (activation of) PKC-mediated protein phosphorylation (12). We evaluated the possible involvement of these signaling pathways in the mechanism of both the basal and the thrombin-stimulated ECM protein deposition in cultured RAMEC with established stimulators and inhibitors.

Pretreatment of RAMEC with either H-7 or PMA under conditions that inhibit or downregulate PKC, respectively, resulted in an increase in the levels of deposited ECM proteins (Table 4). For example, for type I and type IV collagen, inhibition and/or downregulation of PKC resulted in an increase in the basolateral deposition approximately equivalent to that observed with thrombin. PKC inhibition and/or downregulation did not further augment the stimulatory effect of thrombin on collagen type I and collagen type IV deposition. Short-term PKC inhibition by H-7 significantly augmented the deposition of fibronectin but not laminin. In contrast, long-term PKC downregulation by PMA increased the deposition of laminin and, to a much lesser extent, of fibronectin. Moreover, both H-7 and PMA treatments augmented the thrombin-induced increase in the basolateral deposition of both of these ECM proteins in a fashion reminiscent of the inhibitors' effects on basal secretion.

As seen in Table 5, chelation of extracellular Ca2+ in either BAPTA- or EDTA-containing Ca2+-free PBS resulted in an increase in the deposited amounts of fibronectin, laminin, and collagen type I to levels similar to the maximum levels induced by thrombin stimulation. Subsequent stimulation with thrombin caused only a marginal, if any, further increase in the deposition of these ECM proteins. In contrast, chelation of extracellular Ca2+ resulted in stronger enhancement of collagen IV deposition, at approximately two to threefold above the level obtained with thrombin alone.

Depletion of intracellular Ca2+ by preincubation of the cells with cell-permeant BAPTA-AM resulted in an increased deposition of fibronectin, laminin, and collagen type I similar to that observed when extracellular Ca2+ was chelated. Subsequent thrombin stimulation yielded a statistically significant (P < 0.05) elevation in the basolateral deposition of fibronectin and collagen IV above that induced by BAPTA-AM (Table 5). Again,

Table 4. Effect of PKC inhibition on basal and thrombin-stimulated ECM protein deposition

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Fibronectin</th>
<th>Laminin</th>
<th>Collagen Type IV</th>
<th>Collagen Type I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.8 ± 1.1</td>
<td>23.2 ± 4.1</td>
<td>136 ± 27</td>
<td>17.1 ± 2.1</td>
</tr>
<tr>
<td>H-7, 1 µM</td>
<td>22.4 ± 4.1</td>
<td>64.2 ± 14</td>
<td>277 ± 46*</td>
<td>26.4 ± 1.9*</td>
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<tr>
<td>PMA, 10 µg/ml</td>
<td>22.4 ± 4.1</td>
<td>64.2 ± 14</td>
<td>277 ± 46*</td>
<td>26.4 ± 1.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE from 3 independent experiments, each performed in triplicate. Data were obtained as described in MATERIALS AND METHODS. For thrombin stimulation, 0.25 U/ml thrombin was used. *Basal protein deposition in the presence of inhibitor was slightly higher (P < 0.05) than in the absence of inhibitor. †Thrombin-stimulated protein deposition was significantly higher (P < 0.005) than the basal deposition under identical conditions.

Table 5. Effect of Ca2+ modulation on basal and thrombin-stimulated ECM protein deposition

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Fibronectin</th>
<th>Laminin</th>
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<tbody>
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<td>136 ± 27</td>
<td>17.1 ± 2.1</td>
</tr>
<tr>
<td>BAPTA, 10 µM</td>
<td>26.0 ± 5.0</td>
<td>58.6 ± 6.3</td>
<td>756 ± 156*</td>
<td>30.8 ± 2.3*</td>
</tr>
<tr>
<td>BAPTA-AM, 20 µM</td>
<td>24.5 ± 1.1*</td>
<td>44.9 ± 5.2*</td>
<td>227 ± 37</td>
<td>32.8 ± 2.5*</td>
</tr>
<tr>
<td>A-23187, 10 µM</td>
<td>15.8 ± 1.7</td>
<td>24.7 ± 1.7</td>
<td>122 ± 10</td>
<td>14.3 ± 1.5</td>
</tr>
</tbody>
</table>

Values are means ± SE from 3 experiments, each performed in triplicate. Data were obtained as described in MATERIALS AND METHODS. For thrombin stimulation, 0.25 U/ml thrombin was used. BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; AM, acetoxymethyl ester. *Basal protein deposition in the presence of inhibitor was slightly higher (P < 0.05) than in the absence of inhibitor. †Thrombin-stimulated protein deposition was significantly higher (P < 0.005) than the basal deposition under identical conditions.
collagen IV was unique in that chelation of intracellular Ca$^{2+}$ resulted in a smaller increase in the amount of deposited protein. In all cases, the increased deposition of ECM proteins was accompanied by a corresponding decrease in their release into the medium (data not shown). For all four ECM proteins tested, elevation of the intracellular Ca$^{2+}$ concentration by the Ca$^{2+}$ ionophore A-23187 did not affect either basal or thrombin-stimulated protein secretion either basolaterally (Table 5) or apically (data not shown).

From these data, we conclude that the effect of thrombin is mediated through neither PKC activation nor an increase in intracellular Ca$^{2+}$ concentration. Two other interesting observations also emerge from our results: 1) basal ECM protein deposition and release are negatively coupled to the activation of Ca$^{2+}$ and PKC, and 2) basal and thrombin-induced ECM protein deposition entail distinct signaling mechanisms for the various ECM proteins studied.

**DISCUSSION**

The main finding of this study is that a brief (5 min) exposure of confluent RAMEC cultures to low concentrations of thrombin within the physiological range significantly increased the deposition of fibronectin, laminin, and collagens I and IV into the subendothelial matrix. Concomitantly, the same treatment decreased the release of these proteins into the medium. Previous studies, which examined the effect of thrombin on ECM protein deposition or release by cultured EC, were based on the continuous presence of thrombin for up to 24 h in the culture medium (6, 11, 24). In those studies, thrombin increased the amounts of fibronectin and collagen released by the cells into the medium, presumably due to a loss of these proteins from the subendothelial matrix. These findings might be explained by the recent observation (43) that long-term stimulation of EC with thrombin induces the expression of matrix-degrading enzymes. In line with these findings, prolonged exposure of RAMEC to thrombin decreased collagen types I and IV, fibronectin, and laminin deposition into the matrix, accompanied by increased release into the medium (Fig. 5).

To the best of our knowledge, this is the first study to demonstrate the cellular regulation of ECM protein secretion triggered by a short-term (5 min) stimulation by thrombin. Studies on the effects of thrombin after short-term stimulation of cells are physiologically more meaningful than those after long-term stimulation. In vivo, α-thrombin is either actively incorporated into fibrin clots, rapidly inactivated by various proteases found in plasma (such as antithrombin III, heparin cofactor II, α-macroglobulin), or immobilized in the subendothelial basement membrane (8).

Because thrombin-stimulated ECM protein deposition and/or release does not require de novo mRNA or protein synthesis (Fig. 4, Table 1), the most likely explanation for our findings is that short-term exposure of RAMEC to thrombin might activate a polarized, vectorial secretion of these proteins at the basolateral surface of the cells. In the past, EC have been shown to polarize their secretion of certain ECM molecules, such as fibronectin and von Willebrand factor (16, 24). Alternatively, thrombin might act on cell surface molecules, such as integrins or other receptors responsible for ECM protein assembly, and thus modulate the amounts of molecules deposited into the ECM of the cells.

In addition to this strict time requirement for the observed stimulatory effects of thrombin on ECM protein deposition, our results also reveal a specific, biphasic dose requirement (Fig. 3). We suggest that thrombin has a dual role in ECM remodeling. In situations in which thrombin is present at low concentrations and for short periods of time (within a narrow “window”), it promotes the deposition of ECM proteins. When thrombin is present for long periods of time and at high concentrations, it causes enhanced loss of proteins from the ECM, presumably by degradation and/or proteolysis.

Two ubiquitous inhibitors of mRNA and protein synthesis, actinomycin D and cycloheximide, respectively, significantly augmented thrombin-stimulated deposition of three of the four ECM proteins studied, most prominently that of collagen type IV (Table 1). Interestingly, the basal deposition of the various ECM proteins was differentially affected by inhibiting the synthesis of proteins but was not sensitive to inhibition of mRNA transcription: cycloheximide significantly inhibited the basal deposition of fibronectin and collagen type IV without altering the deposited amounts of laminin and collagen type I. Together with our findings that 5-min stimulation of RAMEC with thrombin does not increase the total protein contents, these results provide further support for the notion that thrombin might differentially affect secretory processes rather than stimulating de novo expression of ECM protein genes and gene products.

Regulated secretion is a complex physiological process that involves numerous regulatory cellular and surface proteins (3). Global inhibition of mRNA or protein synthesis might disparately affect the expression of some of the proteins that regulate this process and thus might perturb the balance that normally restricts the secretory event. For example, the release of hormones or neurotransmitters is facilitated by removal of the perimembranal web of microfilaments, which are believed to constitute a physical barrier along the plasma membrane (2). Thus we hypothesize that the enhancement of thrombin-mediated deposition of ECM proteins by cycloheximide and actinomycin D might reflect a gradual removal or downregulation of barrier proteins. Indeed, incubation with 10 μM cytochalasin D, a selective disrupter of microfilaments, yielded an enhanced deposition of ECM proteins, comparable with the effects of thrombin in actinomycin- or cycloheximide-treated cells (unpublished observations). In conjunction with data discussed below, the disparate effects of these inhibitors on individual ECM proteins suggest the participation of distinct secretory and/or signaling pathways for their stimulated deposition.
The stimulatory effects of short-term incubation with thrombin were fully mimicked by a synthetic peptide, TRAP, which represents the new NH2 terminus of the cleaved thrombin receptor and serves as a full agonist of the thrombin receptor (5, 12). These data, together with our results with proteolytically active and inactive thrombin analogs (Table 2), clearly indicate that the effect of thrombin on ECM protein secretion is mediated through proteolytic activation of the thrombin receptor. Additional evidence is provided by the bell-shaped dose dependence of the effects of both thrombin and TRAP, which most likely reflects desensitization due to rapid proteolysis, phosphorylation, or internalization of the thrombin receptor at high concentrations of the agonist (15, 18).

Another interesting finding of the present study is that two other serine proteases, trypsin and plasmin, affect ECM protein secretion similarly to thrombin (Table 3). The ability of these two proteases to cleave and activate the thrombin receptor (albeit with lower efficiency than thrombin) has already been demonstrated (41, 42) and most likely accounts for their effects on ECM protein deposition. Alternatively, trypsin and plasmin may activate different receptors. Such a receptor (proteinase activated receptor 2), which is activated by trypsin but not by thrombin, has been cloned (29) and was recently also found in EC (25).

An early if not the initial event in EC activation by thrombin is the stimulation of phospholipase C, which results in IP3 formation and subsequent release of Ca2+ from intracellular stores concomitant with the stimulation of the influx of extracellular Ca2+. Phospholipase C activation results in the generation of diacylglycerol, which activates PKC and also leads to liberation of intracellular Ca2+ (12, 14). As in other EC, we found in RAMEC a transient elevation of intracellular Ca2+ after thrombin stimulation, confirming that this conventional pathway of thrombin receptor activation is operational also in this EC type (21a). However, our data argue against the involvement of the above "classical" pathway in thrombin-mediated ECM deposition. As detailed in Tables 4 and 5, agents that either eliminate extracellular Ca2+, chelate intracellular Ca2+, or inhibit and/or downregulate PKC do not inhibit thrombin-stimulated ECM protein deposition. On the other hand, our results suggest that basal ECM protein deposition is augmented by chelation of intracellular Ca2+ and/or inactivation of PKC. A similar partitioning between basal and thrombin-stimulated von Willebrand factor secretion has been reported in human EC (24).

Thrombin-mediated cellular events, such as platelet aggregation, involve both phosphorylation-dependent and -independent signaling pathways (21). Recent studies suggest that thrombin activation of protein tyrosine phosphatases constitutes a positive signaling pathway for diverse cellular functions (20). Thus, in view of the lack of the involvement of classical second messengers, we hypothesize that thrombin-mediated stimulation of ECM protein deposition might involve protein tyrosine phosphatase activation. This hypothesis is in line with current views on the role of protein (both serine/threonine and tyrosine) phosphatases in exocytotic secretion in numerous secretory cells (23, 32, 35). Although the mechanisms by which ECM proteins are secreted are not fully understood, there is sufficient evidence to suggest that this process can occur via regulated exocytosis (17, 34). Thus the above hypothesis is capable of explaining, at least in part, our finding that inhibition and/or downregulation of PKC strongly augments ECM protein deposition. By inhibiting PKC, the balance of phosphorylation-dephosphorylation is shifted toward the dephosphorylated state, which is equivalent to activating phosphatases. The continuing partially stimulatory effect of thrombin after PKC inhibition or downregulation might indicate either that the balance has not (yet) completely shifted toward the dephosphorylated state and/or that distinct phosphatases are activated by thrombin and play a role in ECM protein deposition. To test the involvement of phosphatases in thrombin-stimulated vectorial ECM release, we established that, in the presence of the tyrosine phosphatase inhibitor orthovanadate (1 mM), the stimulatory effect of thrombin on ECM deposition is inhibited (P. I. Lelkes and D. Woo, unpublished observations). This observation, in line with findings in other secretory cells (3a), suggests that thrombin-regulated ECM protein deposition may be controlled by tyrosine phosphatases and may be inhibited by hyperphosphorylation. Indeed, in platelets, thrombin activates a protein tyrosine phosphatase, SH-PTP1, in a Ca2+-independent manner via a pertussis-sensitive G protein (19), reminiscent of its action in RAMEC (21a). Detailed experiments are currently in progress in our laboratory that investigate the mechanism of basal and thrombin-stimulated ECM protein secretion in RAMEC.

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