Distinct Receptors and Signaling Pathways in α-Thrombin– and Thrombin Receptor Peptide–Induced Vascular Constrictions

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Abstract The vasoactive mechanisms of the serine protease α-thrombin were examined in isolated coronary arteries from dogs. In resting coronary arteries with endothelium, α-thrombin caused concentration-dependent contractions that were characterized by an initial transient relaxation followed by slowly developing sustained contractions. The vascular actions of α-thrombin were mimicked by the thrombin receptor-activating peptide (TRAP) SFLLRNFP, a synthetic peptide based on the cleaved amino terminus of the thrombin receptor domain. Treatment of the arteries with N'-nitro-L-arginine or removal of endothelium abolished the transient relaxations and enhanced the contractions, indicating that the transient relaxations were mediated by the concurrent release of endothelium-derived nitric oxide. α-Thrombin that had been catalytically inactivated with the irreversible inhibitor by use of D-Phe-Pro-Arg-chloromethyl ketone did not cause contractions, indicating the requirement of proteolytic cleavage by α-thrombin to induce contractions. In contrast to TRAP, α-thrombin–induced contractions were blocked by hirudin (a specific thrombin inhibitor), nifedipine and diltiazem (Ca2+ channel blockers), or staurosporine and calphostin C (protein kinase C inhibitors). Unlike α-thrombin, which undergoes homologous desensitization, TRAP failed to cause desensitization to subsequent stimulation by α-thrombin or TRAP. These observations support the hypothesis that vasoactive actions of α-thrombin are mediated by a mechanism that involves cleavage at the active site to expose a new NH2 terminus that activates the thrombin receptor. Further, the dissociation between α-thrombin and the synthetic receptor peptide in signal transduction and dissimilar desensitizing properties suggest the existence of distinct thrombin receptor subtypes and/or signaling events in vascular smooth muscle. (Circ Res. 1994;74:930-936.)

Key Words • active site • contraction • coronary arteries • hirudin • tethered-ligand receptor • signal transduction • α-thrombin

In addition to its role in the coagulation pathway, the serine protease α-thrombin causes contractions and relaxations of vascular smooth muscle depending on the loci of blood vessels and species. Some reports have shown that at higher concentrations, α-thrombin causes contractions that are inhibited by α-adrenergic receptor and prostaglandin synthesis inhibitors in canines basilar and rabbit femoral arteries.1-3 In contrast, others have shown that these inhibitors have no effect on α-thrombin–induced contractions in canine coronary and rabbit aorta, indicating the absence of a role involving α-adrenergic receptors and prostaglandins in mediating the contractions.2 At low concentrations, α-thrombin has been shown to induce endothelium-dependent relaxations in aorta, cerebral, femoral, and coronary arteries and in saphenous vein from various species including dogs and pigs.4-8 The vasorelaxant actions of α-thrombin are selectively blocked by thrombin active site inhibitors, suggesting that proteolytic cleavage of the thrombin receptor is crucial for the release of nitric oxide.6,8

The recent cloning and characterization of the thrombin receptor from human sources have elucidated a unique mechanism by which thrombin activates platelets and other cells.9 Thrombin binds to its receptor via its anionic binding exosite, which it cleaves after argi-nine-41 in the NH2-terminal portion of the receptor. The exposed new NH2 terminus has been proposed to function as a “tethered peptide ligand” that binds to an as-yet-undefined region of the thrombin receptor to cause receptor activation.9 Characteristically, the thrombin receptor is a member of the seven-transmembrane-spanning domain receptor family coupled to G protein and can be desensitized.9-11 Homologous synthetic peptides corresponding to the tethered-ligand sequence of the human platelet thrombin receptor have been reported to elicit various thrombin-signalenced cell responses in various cell types.9,12,13 It was found that the synthetic peptide that contains the sequence of the NH2 terminus of the thrombin receptor sequence possessed intrinsic thrombinlike activity, both in terms of platelet aggregation and activating receptors in xenopus oocyte receptor–transfected systems.9 Previously, we reported that the vasorelaxant actions of α-thrombin were mimicked by the heptamer amino acid thrombin receptor–activating peptide (TRAP) SFLLRNFP, which is consistent with involvement of the tethered-peptide-ligand thrombin receptor in the release of nitric oxide.4

The present experiments were designed to examine the direct smooth muscle actions of a wide range of concentrations of α-thrombin in isolated canine coronary arteries. The involvement of the proteolytic activity of thrombin was probed with catalytically inactivated α-thrombin by use of D-Phe-Pro-Arg-chloromethyl ketone and by hirudin, the inhibitor of the active site and the anion-binding exosite.4,15 In addition, we examined whether the vasoactive actions and receptor signaling of

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α-thrombin are mimicked by a synthetic analogue of the cleaved NH₂-terminal portion of the tethered-ligand receptor.

Materials and Methods
Canine Coronary Artery
Mongrel dogs (15 to 20 kg) were killed by exsanguination after anesthesia with dial urethane (0.4 mg/kg IV) composed of 40% urethane, 20% 5,5-diallybarbituric acid, and 40% ethyl urea administered in the forelimb. The left circumflex and left anterior descending coronary arteries were dissected from the heart, and the adhering perivascular tissue was carefully removed.

Rings of coronary arteries (≈5 mm long) were suspended between a fixed base and strain gauge for measurement of isometric circumferential force. The rings were placed in an organ bath filled with physiological salt solution containing (mmol/L) NaCl 118.3, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25.0, disodium EDTA 0.026, and glucose 5.5. The solutions were kept at 37°C and were continuously gassed with 95% O₂/5% CO₂ to maintain the pH at 7.4. The length of the smooth muscle was increased stepwise for 90 minutes to adjust basal tension to 10 to 11 g. This tension was found to be optimal for contractions of canine coronary arteries by testing the contractions to potassium (80 mmol/L). Once basal tension was established, the length of the rings was not altered thereafter. In some rings, the endothelium was removed by inserting forceps into the lumen and then gently rolling the rings over a wet filter paper.

Drugs
The pharmacologic agents used were the following: diltiazem, hirudin (from medicinal leeches; specific activity, 845 anti-thrombin units/mg protein), indomethacin, N⁷-nitro-L-arginine (L-NA), nifedipine, and staurosporine (Sigma Chemical Co, St Louis, Mo); calphostin C (Kamiya Biomedical Co, Thousand Oaks, Calif); α-thrombin (human, 2000 NIH U/mg and 1 NIH U/mL; ≈9.5 mmol/L) (Enzyme Research, South Bend, Ind); D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone (PPACK) (Calbiochem, La Jolla, Calif); phenolamine (CIBA-GEIGY, Summit, NJ); U-46619 (Cayman, Ann Arbor, Mich); and BMS-180742, BMS-180291, and BQ-123 (Bristol-Myers Squibb, Princeton, NJ). The specificity of BMS-180742, an anion-binding exosite inhibitor, has been shown by its ability to prevent thrombin cleavage of fibrinogen in clotting assays without affecting thrombin cleavage of S-2238, a synthetic substrate. BMS-180291 is a newly identified, potent, and selective prostaglandin (PG) H₂/thromboxane (TX) A₂ receptor antagonist. α-Thrombin was diluted using a physiological salt solution containing 0.5 mg/mL bovine albumin. PPACK-α-thrombin was prepared by adding excess PPACK (30-fold) to α-thrombin at room temperature for 15 minutes. The excess unbound PPACK was dialyzed by its lack of effect on thrombin cleavage of S-2238. BMS-180742 and BQ-123 were solubilized in 50% ethanol, and further dilutions were made in water. Calphostin C, nifedipine, and staurosporine were solubilized in dimethyl sulfoxide. Indomethacin was prepared in 2% Na₂CO₃ immediately before use. Potassium (120 mmol/L) was prepared by equimolar replacement of NaCl with KCl. Hirudin was added 3 to 5 minutes before obtaining the concentration response to α-thrombin. The concentration of hirudin (15 U/mL) used in the present study is estimated to be equivalent to that necessary to neutralize an equal amount of α-thrombin in the clotting assay. Other inhibitors were added 30 minutes before the addition of agonists.

Data Analysis
Contractions are expressed as a percentage of the maximal contractions caused by potassium (120 mmol/L). Data are expressed as mean ± SEM. A two-way ANOVA with repeated measures was used to compare the data. Paired comparison was then conducted using Student's t test for mean values. Values of P < .05 were regarded as significant. In all experiments, n equals the number dogs from which the rings are taken.

Results
α-Thrombin–Induced Contractions
In resting coronary arteries with intact endothelium, α-thrombin (1 to 100 nmol/L) caused concentration-dependent contractions (Figs 1 and 2). These contractions were characterized by transient relaxations at low concentrations followed by slowly developing contractions at higher concentrations of α-thrombin. Removal of the endothelium or treatment with L-NA (30 μmol/L) abolished the transient relaxations and significantly enhanced the contractions caused by α-thrombin (Figs 1
and 3, Table 1). α-Thrombin exhibited refractory desensitization to subsequent exposures of higher concentrations (≥30 nmol/L) in rings both with and without endothelium.

α-Thrombin–induced contractions were not affected by the treatment of arteries with indomethacin (3 μmol/L), a cyclooxygenase inhibitor (Table 1); BMS-180291 (3 μmol/L), a PGH₂/TXA₂ receptor antagonist; phentolamine (10 μmol/L), an α-adrenergic antagonist blocker; or BQ-123 (1 μmol/L), an endothelin receptor blocker (data not shown). BMS-180742 (3 μmol/L), an anion-binding exosite inhibitor, shifted the α-thrombin concentration-response relation to the right but had no effect on the maximal response (Fig 2).

Catalytically inactivated PPACK–α-thrombin did not induce contractions in rings with or without endothelium (Figs 1 through 3). α-Thrombin–induced contractions were markedly inhibited by hirudin, nifedipine (1 and 10 μmol/L), diltiazem (1 μmol/L), staurosporine (30 nmol/L), or calphostin C (30 and 300 nmol/L) in rings with and without endothelium (15 U/mL) (Figs 2 and 3).

In rings of coronary arteries with endothelium, the contractions caused by U-46619 (0.001 to 1 nmol/L) were not different in the absence or presence of hirudin (15 U/mL) (U-46619: EC₅₀, 0.8±0.008 versus 0.7±0.003 nmol/L, respectively; maximal contractions at 1 nmol/L, 71±6.1% versus 72±5.9%, respectively; n=5).

**TRAP-Induced Contractions**

TRAP (0.01 to 30 μmol/L) caused concentration-dependent contractions of canine coronary arteries that were markedly enhanced by treatment of the arteries with L-NA (30 μmol/L) or indomethacin (3 μmol/L) (Fig 4). Hirudin (15 U/mL), nifedipine (0.1 to 10 μmol/L), diltiazem (1 μmol/L), staurosporine (30 nmol/L), or calphostin C (30 and 300 nmol/L) had no significant effect on the contractions caused by TRAP (Fig 4, Table 2).

**Desensitization**

Exposure of deendothelialized rings of coronary arteries to submaximally (0.1 nmol/L) or maximally (10 and 100 nmol/L) effective concentrations of α-thrombin for 30 or 60 minutes caused complete refractory desensitization to subsequent stimulation by α-thrombin (0.1, 1, 10, and 100 nmol/L) but not to TRAP (1, 10, and 100 μmol/L) after washout of the first exposure (Figs 5 and 6). A complete desensitization was observed after 60 minutes of preincubation with α-thrombin compared with the desensitization after 30 minutes. Interestingly, the treatment of arteries with hirudin (15 U/mL) during the first exposure to α-thrombin blocked its action and prevented desensitization to the second stimulation by α-thrombin (Fig 5). Exposure of the arteries to TRAP (1, 10, and 100 μmol/L) for 30 or 60 minutes did not desensitize them to subsequent stimulation by TRAP (1, 10, and 100 μmol/L) and caused only a partial desensitization to subsequent stimulation by the lower (10 nmol/L), but not the higher (100 nmol/L), concentration of α-thrombin (Figs 5 and 6). As shown in Fig 5, the concentrations of α-thrombin (10 nmol/L) and TRAP (1 μmol/L) were selected on the basis of similar contractions caused by both agonists.

**Discussion**

In agreement with previous reports, the present studies demonstrate that α-thrombin causes contractions of canine coronary arteries. The lack of effect of indomethacin, BMS-180291, or phentolamine on α-thrombin–induced contractions indicates the absence of a role involving cyclooxygenase products, TXA₂, or adrenergic receptor activation in mediating the contractions. Similarly, previous reports have shown the lack of effect of blockade of α-adrenergic receptors or PG synthesis on α-thrombin–induced contractions in canine coronary
and rabbit aorta.\(^2\) It has been reported that α-thrombin enhances the release of endothelin from endothelial cells.\(^{18}\) In the present study, the absence of effect of BQ-123 indicates that α-thrombin does not mediate its contractions by the release of endothelin or the activation of endothelin receptors.

α-Thrombin–induced contractions were characterized by transient relaxations at lower concentrations, followed by slowly developing contractions in arteries with endothelium. Because the direct smooth muscle contractile action of α-thrombin overrides its ability to release nitric oxide, the transient relaxations were not observed at higher concentrations of α-thrombin. The enhancement of the contractions by inhibition of nitric oxide synthesis or by removal of the endothelium is likely due to the removal of the inhibitory influence of the endothelium. This is in agreement with previous reports that have shown endothelium-dependent relaxations caused by lower concentrations of α-thrombin in canine coronary arteries.\(^{5,6,8,19}\)

Similar to α-thrombin, TRAP caused concentration-dependent contractions in canine coronary arteries. The enhancement of the contractions by L-NA implies removal of the inhibitory influence of the endothelium by release of nitric oxide, which counteracts the contractions. These observations are consistent with previous reports showing that the synthetic peptide agonist causes endothelium-dependent relaxations in the canine saphenous vein and pig coronary artery, indicating involvement of tethered ligand in the release of nitric oxide.\(^{6}\) Others have reported that the rat aorta does not contract in response to human or rat α-thrombin but contracts to TRAP, implying species heterogeneity in the ability of α-thrombin to unmask tethered peptide ligand or that thrombin receptors are already cleaved.\(^{20}\) TRAP-induced contractions were enhanced by cyclooxygenase inhibition, suggesting simultaneous release of vasodilatory prostanooids by TRAP.

The synthetic chloromethyl ketone derivative of basic amino acids, PPACK, has been shown to alkylate the active center of thrombin, resulting in an irreversible inhibition of the enzymatic action of thrombin with the intact anion-binding exosite.\(^{14}\) The catalytically inactivated PPACK–α-thrombin gave no response in rings both with and without endothelium, suggesting that proteolytic cleavage at the active site is required for the vasoactive actions of α-thrombin. In this context, catalytic cleavage of α-thrombin led to exposure of the amino terminus, which presumably contains the tethered peptide ligand. The specificity of PPACK was ruled out by its lack of effect on the contractions caused by TRAP.

The inhibition by hirudin involves interaction at both the high-affinity noncatalytic site and another distinct site at or near the catalytic triad.\(^{11}\) In the present study, the inhibition of the contractions by hirudin may suggest that the active site and anion-binding exosite of the

![Graphs showing thrombin receptor–activating peptide (TRAP)-induced contractions induced by nifedipine and staurosporine (A) and N\(^{-}\)nitro-L-arginine (L-NA), indomethacin, and hirudin (B) in rings with endothelium (n=7 dogs). The contractions were not affected by nifedipine (0.1, 1, and 10 \(\mu\)mol/L), staurosporine (30 nmol/L), and hirudin (15 U/ml) but were markedly enhanced by L-NA (30 \(\mu\)mol/L) and indomethacin (3 \(\mu\)mol/L).](http://circres.ahajournals.org/)

Table 1. α-Thrombin–Induced Contractions in the Absence and Presence of Indomethacin, N\(^{-}\)Nitro-L-arginine, Diltiazem, or Calphostin C in Canine Coronary Arteries With Endothelium

<table>
<thead>
<tr>
<th>α-Thrombin, (-\log) mol/L</th>
<th>Control</th>
<th>Indo, 3 (\mu)mol/L</th>
<th>L-NA, 30 (\mu)mol/L</th>
<th>Diltiazem, 1 (\mu)mol/L</th>
<th>Calphostin C, nmol/L</th>
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<tbody>
<tr>
<td>10</td>
<td>1.2±0.4</td>
<td>2.0±0.4</td>
<td>1.2±1.0</td>
<td>0*</td>
<td>0*</td>
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<tr>
<td>9.5</td>
<td>2.1±0.8</td>
<td>4.6±0.7</td>
<td>2.2±0.9</td>
<td>0*</td>
<td>0*</td>
</tr>
<tr>
<td>9</td>
<td>6.8±2.2</td>
<td>4.5±4.4</td>
<td>4.1±1.2</td>
<td>0*</td>
<td>0*</td>
</tr>
<tr>
<td>8.5</td>
<td>13±3.3</td>
<td>8.7±8.6</td>
<td>10±2.5</td>
<td>1.9±1.4*</td>
<td>0.2±0.2*</td>
</tr>
<tr>
<td>8</td>
<td>20±2.5</td>
<td>26±11.6</td>
<td>24±3.5</td>
<td>2.7±1.4*</td>
<td>1.9±0.9*</td>
</tr>
<tr>
<td>7.5</td>
<td>22±1.4</td>
<td>23±5.7</td>
<td>33±1.7*</td>
<td>6.9±1.8*</td>
<td>9.8±3.6*</td>
</tr>
<tr>
<td>7</td>
<td>19±2.2</td>
<td>23±12.6</td>
<td>39±2.3*</td>
<td>9.7±2.1*</td>
<td>14±27*</td>
</tr>
</tbody>
</table>

*P<.05 vs control.

Indo indicates indomethacin; L-NA, N\(^{-}\)nitro-L-arginine. Contractions are expressed as a percentage of maximal contractions caused by 120 mmol/L K\(^+\). Values are mean±SEM; n=6 or 7.

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Table 2. Thrombin Receptor-Activating Peptide-Induced Constrictions in the Absence and Presence of Diltiazem or Calphostin C in Canine Coronary Arteries With Endothelium

<table>
<thead>
<tr>
<th>TRAP, –log mol/L</th>
<th>Control</th>
<th>Diltiazem, 1 μmol/L</th>
<th>Calphostin C, nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>0.6±0.4</td>
<td>0.3±0.2</td>
<td>0.2±0.1</td>
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<tr>
<td>7.5</td>
<td>1.4±0.8</td>
<td>0.8±0.4</td>
<td>1.2±0.3</td>
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<tr>
<td>7</td>
<td>3.2±1.2</td>
<td>2.6±0.6</td>
<td>3.3±0.7</td>
</tr>
<tr>
<td>6.5</td>
<td>6.0±1.2</td>
<td>8.8±8.6</td>
<td>12±1.6</td>
</tr>
<tr>
<td>6</td>
<td>18±2.3</td>
<td>23±3.9</td>
<td>24±3.1</td>
</tr>
<tr>
<td>5.5</td>
<td>33±3.5</td>
<td>40±3.5</td>
<td>41±2.6</td>
</tr>
<tr>
<td>5</td>
<td>50±3.5</td>
<td>56±3.6</td>
<td>60±2.1</td>
</tr>
<tr>
<td>4.5</td>
<td>57±3.7</td>
<td>58±3.6</td>
<td>63±2.5</td>
</tr>
<tr>
<td>4</td>
<td>58±4.3</td>
<td>60±3.1</td>
<td>63±2.5</td>
</tr>
</tbody>
</table>

TRAP indicates thrombin receptor-activating peptide. Constrictions are expressed as a percentage of maximal contractions caused by 120 nmol/L K+. Values are mean±SEM; n=6 or 7.

Thrombin domain may be required for optimal activity of thrombin to elicit smooth muscle contractions. The selectivity and specificity of hirudin on α-thrombin are demonstrated by its lack of effect on the constrictions caused by TRAP and U-46619. Blockade of the anion-binding exosite by use of BMS-180742 decreased the constrictions caused by lower concentrations of α-thrombin without altering the maximal response, indicating that the exosite of thrombin is required for recognition and affinity of the tethered-ligand thrombin receptor in the smooth muscle. Similarly, other reports have shown the lack of effect of anion-binding exosite inhibitors in platelet aggregation induced by higher, but not lower, concentrations of α-thrombin, suggesting multiple pathways of thrombin activation in platelets.16

Despite their overall similarity in causing contractions and endothelium-dependent relaxations, a close inspection of the data reveals interesting differences...
between α-thrombin and the receptor-activating peptide. α-Thrombin–induced contractions were markedly inhibited by structurally unrelated specific inhibitors of Ca\(^{2+}\) channels and the protein kinase C regulatory domain, indicating that the contractions were mediated by the opening of voltage-operated calcium channels and the activation of protein kinase C. In contrast, TRAP-induced contractions were not affected by the calcium channel blockers or by inhibitors of protein kinase C, implying that other distinct intracellular events and signaling mechanisms may be operative. Others have shown that the peptide stimulates phosphoinositide hydrolysis in rat aortic smooth muscle cells, suggesting that the contractions are primarily mediated by inositol triphosphate, which releases intracellular Ca\(^{2+}\). The reason for the apparent dissociation in signal transduction is unclear. This could hypothetically be due to alternative receptor and/or intracellular signaling events, because α-thrombin exists in the smooth muscle. Alternatively, the proteolytic activity of α-thrombin may cause a conformational change of the thrombin receptor that is different from that mediated by the nonproteolytic synthetic peptide, so that distinct signaling pathways are activated.

Another difference between α-thrombin and TRAP is the lack of heterologous thrombin receptor desensitization. One of the characteristics of cellular responses to thrombin is that the activation of receptors causes homologous desensitization in which the readadition of thrombin fails to evoke a second response. If the newly generated N-terminal sequence of the receptor would stimulate the same receptor site, then one might expect cross desensitization by TRAP and α-thrombin. By use of this strategy, the present study demonstrates homologous desensitization to the second stimulation by α-thrombin in smooth muscle. The nonspecific effects of prolonged incubation are ruled out by the fact that α-thrombin-mediated desensitization was prevented by hirudin. Nonetheless, a ring that had responded to the peptide was still responsive either to α-thrombin or to repeated exposures of the peptide. The lack of complete cross desensitization by α-thrombin and TRAP suggests that these agonists stimulate the smooth muscle at different receptor sites and use distinct intracellular signaling mechanisms. Another is that the tethered ligand may be binding in a manner different from the peptide, thus facilitating internalization or degradation of the N-terminal ligand. Alternatively, the differences in the desensitization responses between α-thrombin and TRAP suggest that the role of α-thrombin in smooth muscle activation may be more complex after scission of an extracellular portion of its receptor and creation of a new NH\(_2\) terminus. In this regard, α-thrombin causes the receptor to lose the NH\(_2\) terminus, which prevents further stimulation by α-thrombin but not by TRAP. Unlike TRAP, which is likely to activate cells solely by binding to the active site of the thrombin receptor, α-thrombin may also affect cellular responses by binding to other cell surface proteins (eg, thrombomodulin or glycoprotein Ib) or to the hirudin-like domain of the thrombin receptor itself after scission.

Our data support the notion that the receptor dynamics triggered by the proteolytically generated tethered receptor differ from the dynamics elicited by the synthetic peptide, likely by interacting at different receptor subtypes. In keeping with this notion, previous reports have proposed different receptor sites for the free synthetic peptide and α-thrombin, as demonstrated by the lack of desensitization to the subsequent exposure of guinea pig aorta to the 14-mer peptide ligand–induced contractions. Alternatively, using receptor-directed monoclonal antibodies and an epitope-tagged thrombin receptor, others have proposed that most of the internalized receptors are ultimately degraded and that some of the recycled receptors are in a state in which they respond to TRAP but not to thrombin, probably because of the additional proteolytic processing of the N-terminus receptors and/or the differences in rates of receptor activation. Alternatively, it may be possible that there is more rapid or complete internalization of the thrombin receptor when cleaved by α-thrombin as opposed to TRAP. In this respect, the complete recovery of the thrombin receptor is hypothesized to occur only after the expression on the cell surface of adequate numbers of newly synthesized receptors.

In summary, the present study indicates that vasoactive actions of α-thrombin are caused by a mechanism that involves cleavage at the active site to expose a new amino terminus, which presumably functions as a tethered ligand to activate thrombin receptors. Further, the dissociation in signal transduction and dissimilar desensitizing properties suggest the interaction of α-thrombin with more than one site in the receptor and/or different fates of the receptor after stimulation with α-thrombin or TRAP. It is of interest to identify the unique thrombin receptor subtypes so as to develop selective thrombin receptor antagonists.

Acknowledgments

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