Endothelial Cell Thrombin Receptors and PAR-2
TWO PROTEASE-ACTIVATED RECEPTORS LOCATED IN A SINGLE CELLULAR ENVIRONMENT

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Human endothelial cells express thrombin receptors and PAR-2, the two known members of the family of protease-activated G protein-coupled receptors. Because previous studies have shown that the biology of the human thrombin receptor varies according to the cell in which it is expressed, we have taken advantage of the presence of both receptors in endothelial cells to examine the enabling and disabling interactions with candidate proteases likely to be encountered in and around the vascular space to compare the responses elicited by the two receptors when they are present in the same cell and to compare the mechanisms of thrombin receptor and PAR-2 clearance and replacement in a common cellular environment. Of the proteases that were tested, only trypsin activated both receptors. Cathepsin G, which disables thrombin receptors, had no effect on PAR-2, while urokinase, kallikrein, and coagulation factors IX, X, XIa, and XIIa neither substantially activated nor noticeably disabled either receptor. Like thrombin receptors, activation of PAR-2 caused pertussis toxin-sensitive phospholipase C activation as well as activation of phospholipase A2, leading to the release of PGL2. Concurrent activation of both receptors caused a greater response than activation of either alone. It also abolished a subsequent response to the PAR-2 agonist peptide, SLIGRL, while only partially inhibiting the response to the agonist peptide, SFLLRN, which activates both receptors. After proteolytic or nonproteolytic activation, PAR-2, like thrombin receptors, was cleared from the endothelial cell surface and then rapidly replaced with new receptors by a process that does not require protein synthesis. Selective activation of either receptor had no effect on the clearance of the other. These results suggest that the expression of both thrombin receptors and PAR-2 on endothelial cells serves more to extend the range of proteases to which the cells can respond than it does to extend the range of potential responses. The results also show that proteases that can disable these receptors can distinguish between them, just as do most of the proteases that activate them. Finally, the residual response to SFLLRN after activation of thrombin receptors and PAR-2 raises the possibility that a third, as yet unidentified member of this family is expressed on endothelial cells, one that is activated by neither thrombin nor trypsin.

Endothelial cells occupy an environmental niche in which they are potentially exposed to a variety of extracellular proteases, particularly during vascular injury or inflammation. Thrombin is among these, but others exist as well, including the activated forms of other clotting factors, some components of the anticoagulant and fibrinolytic systems, and proteases such as cathepsin G and tryptase, which are released from neutrophils and mast cells. When added to endothelial cells, thrombin activates phospholipases A2, C, and D, elevates cytosolic Ca2+, increases permeability, and stimulates the release of von Willebrand factor, endothelin, NO, and PGL2 (e.g. Refs. 1–12). These effects are thought to be mediated by a G protein-coupled receptor that was originally cloned from a human megakaryoblastic cell line (13). The N terminus of this receptor is cleaved by thrombin between residues Arg-41 and Ser-42, exposing a new N terminus whose first five residues, SFLLRN, serve as a tethered ligand (13). Although other thrombin receptors may also exist (14, 15), the evidence that the cloned receptor is expressed on human endothelial cells includes the presence of mRNA encoding the receptor, several antibody binding studies (16–19), and the ability of agonist peptides, such as SFLLRN, to mimic the effects of thrombin on endothelial cells, although the interpretation of the latter observation has to be tempered by the recent observation that SFLLRN can activate at least one other receptor (20, 21).

In addition to thrombin receptors, endothelial cells express PAR-2, the only other member of the family of protease-activated receptors that has been identified to date. PAR-2 is structurally homologous to thrombin receptors and has an identical mechanism of activation. Activating proteases cleave the N terminus of PAR-2 between residues Arg-36 and Ser-37, exposing a tethered ligand domain whose sequence begins with SLIGRL in the human form of the receptor and SLIGRL in the mouse (20, 22, 23). The evidence that endothelial cells express PAR-2 is recent, but compelling. Nystedt et al. (24) and Mirza et al. (19) have shown that human umbilical vein endothelial cells (HUVECs) contain PAR-2 mRNA and bind a PAR-2 anti-

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The abbreviations used are: PGL2, prostacyclin; HUVEC, human umbilical vein endothelial cell.
body, observations that we have confirmed. Peptides based upon the sequence of the PAR-2 tethered ligand domain cause an increase in cytosolic Ca\(^{2+}\) and mitogenesis in endothelial cells (19) and stimulate endothelium-dependent relaxation of arterial rings (25–27) as well as the release of NO and endothelin (6). In addition to responding to peptide agonists based upon its own tethered ligand domain, human PAR-2 can also be activated by the thrombin receptor agonist peptide, SFLLRN (although the converse is not true) (20, 21). There is less information about the proteases that can activate PAR-2. The original description of the receptor showed that it could be cleaved and activated by trypsin but not thrombin (22). We have shown recently that it can also be activated by mast cell tryptase (28).

Protease-activated G protein-coupled receptors differ from other G protein-coupled receptors in several important respects: 1) because an intact N terminus is required for activation by proteases, they are inherently single-use receptors; 2) protease-activated receptors have the potential of being activated by more than one agonist. In addition to the peptide agonists, they can be activated by any protease that can bind to and cleave the N terminus at the unique site required for exposure of the tethered ligand domain. On the other hand, proteases that cleave the N terminus at the wrong site can disable the receptor and prevent its subsequent activation by an enabling protease (29, 30); and 3) studies on thrombin receptors show that its biology is dependent in part upon the cell in which it is expressed, most notably with respect to the key issue of how cleaved receptors are removed from the cell surface and replaced with intact ones (reviewed in Ref. 31).

With this as background, the goals of the present studies were: 1) to examine the enabling and disabling interactions of thrombin receptors and PAR-2 with proteases likely to be encountered in and around the vascular space, 2) to compare the responses elicited by the two receptors when they are present in the same cell, and 3) to compare the mechanisms of receptor clearance and replacement in a common cellular environment. Endothelial cells were chosen for these studies because they express both receptors endogenously and because of their exposure to proteases. The results show a high degree of similarity in the responses of endothelial cells to the activation of both receptors, suggesting that the expression of both serves more to extend the range of proteases to which endothelial cells can respond than it does to extend the list of potential responses.

The results also show that proteases that disable thrombin receptors and PAR-2 can distinguish between them, just as do most of the proteases that activate them. Finally, the results show that thrombin receptors and PAR-2 are cleared and replaced independently and provide preliminary evidence that a third member of this receptor family, one that is not a substrate for trypsin, may also be expressed on endothelial cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Highly purified α-thrombin was provided by Dr. J. Fen
to (New York State Department of Health, Albany, NY). Trypsin was obtained from Sigma. Cathepsin G was obtained from CalBiochem. Urokinase was a gift from Dr. J. Henkin (Abbot Laboratories). Plasmin was obtained from Boehringer Mannheim. Recombinant tissue kall
ton (New York State Department of Health, Albany, NY). Trypsin was obtained from Hematologic Technologies and ERL. Factor IXa was a gift from Dr. Peter Walsh (Temple Universi
ty School of Medicine, Philadelphia, PA). The DNA for human PAR-2 was generously provided by Dr. J. Sundelin (Lund University, Lund, Sweden).

**Antibodies**—Antibody PAR-2C is a rabbit polyclonal antibody ob
tained from ERL. Factor IXa was generously provided by Dr. J. Sundelin (Lund University, Sweden) and obtained from ERL. Factor XIIa was obtained from Hematologic Technologies and ERL. Factor XIa was ob
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RESULTS

Protease and Peptide Activation of PAR-2 and Thrombin Receptors in Endothelial Cells—Because endothelial cells express PAR-2 as well as thrombin receptors, a central issue is the identification of proteases that are capable of activating each of them. Thrombin has been shown to activate thrombin receptors but not PAR-2 when each is expressed in Xenopus oocytes (21, 22). Trypsin has been shown to activate PAR-2 (21, 22, 36), but there is some ambiguity about the ability of trypsin to activate human thrombin receptors; conflicting results have been reported in studies with fibroblasts, Chinese hamster ovary cells, and oocytes (13, 20, 21, 23, 36, 37). Because there is currently no completely effective way to selectively block thrombin receptors and PAR-2, we began by testing whether trypsin would activate human thrombin receptors expressed in COS-1 cells. 

This also seems to be the case in endothelial cells. When thrombin and trypsin were added to human umbilical vein endothelial cells loaded with Fura-2 so that changes in the cytosolic free Ca\(^{2+}\) concentration could be measured. Trypsin (100 nM), thrombin (4 units/ml), SLIGRL (100 μM), and SFLLRN (100 μM) were added at the times indicated. The results shown are from a single experiment. The aggregate results from several such experiments are included in Fig. 2. F, COS-1 cells were transfected with either human thrombin receptors or human PAR-2, loaded with \(^{3}\)Hinositol, and stimulated with either thrombin (4 units/ml) or trypsin (100 nM) for 45 min. Total \(^{3}\)Hinositol phosphate formation was measured. The results shown are the mean ± S.E. of five studies expressed as a fold-increase over the values obtained in each experiment with vector-transfected cells stimulated with buffer.

Other Proteases—Several other proteases that can come in contact with endothelial cells were also tested, beginning with the neutrophil protease cathepsin G. Cathepsin G has been shown previously to cleave the N terminus of the human thrombin receptor at two sites: 1) Arg-41–Ser-42 (the sole site of cleavage by thrombin), and 2) Phe-55–Trp-56 (29). Cleavage at the latter site disables the receptor by amputating the tethered ligand domain. As a result, cathepsin G was found to prevent thrombin receptor activation by thrombin, although it activated the receptor when the Phe-55–Trp-56 site was blocked with a monoclonal antibody or mutated. In the present

agonists were added sequentially, we found that exposure to trypsin inhibited a subsequent response to thrombin or to the PAR-2 agonist peptide SLIGRL by >90%, while exposure to thrombin inhibited the subsequent response to trypsin by only 60% (Figs. 1 and 2). Furthermore, activating thrombin receptors and PAR-2 simultaneously with either SFLLRN or the combination of thrombin plus SLIGRL completely inhibited the trypsin response (Fig. 2B).

Taken together, these results suggest that trypsin activates thrombin receptors as well as PAR-2 on endothelial cells, but given the pattern of desensitization that was observed, trypsin probably does not activate any other receptors unless those receptors are also sensitive to thrombin, SLIGRL, and SFLLRN. The results also suggest that the response of endothelial cells, at least in terms of the magnitude of the intracellular Ca\(^{2+}\) transient, can be greater when both receptors are activated than when either is activated alone.

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studies, cathepsin G was added first to endothelial cells that had been loaded with Fura-2. As previously reported (29), this had no positive effect on its own, but it completely abolished the response to thrombin (Figs. 3 and 4 A). In contrast, the response to trypsin was only modestly reduced in endothelial cells exposed to cathepsin G, as would be expected if cathepsin G disabled thrombin receptors but neither activated nor disabled PAR-2 (Fig. 4 A). To test the ability of cathepsin G to interact with PAR-2 in the absence of thrombin receptors, COS-1 cells expressing human PAR-2 were loaded with [3H]inositol and then stimulated with cathepsin G, trypsin, or both. Cathepsin G had no effect by itself and did not prevent a subsequent response to trypsin (Fig. 4 B), suggesting again that cathepsin G does not cleave human PAR-2.

Several other candidate proteases were tested in a similar manner. The results obtained with human factor Xa are shown in Fig. 3 C. When added to endothelial cells, factor Xa (33 nM) had no substantial effect on cytosolic Ca$^{2+}$ and little, if any, effect on subsequent responses to thrombin or trypsin. Factor IXa (29 nM), factor XIa (69 nM), factor XIIa (160 nM), and human kallikrein (200 nM) gave similar results, neither activating nor disabling thrombin receptors and PAR-2. Finally, human factor Xa (19 nM), urokinase (20 nM), plasmin (100 nM), tissue kallikrein (200 nM), and plasma kallikrein (20 nM) were also tested for their ability to stimulate phosphoinositide hydrolysis in COS-1 cells expressing PAR-2. None of them did so (data not shown).

Consequences of PAR-2 and Thrombin Receptor Activation—Thrombin causes phosphoinositide hydrolysis in endothelial cells that is relatively unaffected by pertussis toxin and is therefore likely to reflect phospholipase $C_\beta$ activation mediated by $C_{\alpha_\beta}$ or $C_{\alpha_\alpha}$ (3). The studies in Fig. 5 compare thrombin receptor activation (by thrombin) with PAR-2 activation (by SLIGKV) in [3H]inositol-loaded human umbilical vein endothelial cells. SLIGKV stimulated phosphoinositide hydrolysis at least as much as thrombin and was inhibited to a similar extent by preincubating the cells with pertussis toxin to inactivate $G_\alpha$ family members.

In addition to activating phospholipase C, PAR-2 agonists also activated phospholipase $A_2$. In the experiments shown in Fig. 6, endothelial cells were stimulated with either thrombin or SLIGKV, and PGI$_2$ formation was observed by measuring its stable metabolite, 6-keto-PGF$_{1\alpha}$. PGI$_2$ is formed in endothelial cells from the arachidonate released by phospholipase $A_2$ from membrane phospholipids. The responses to both agonists were maximal within 5 min and were greater with thrombin than with SLIGKV. Taken together with reports from other investigators (6, 19, 24), these results show that PAR-2 activation has many of the same effects on endothelial cells as thrombin.
receptor activation and seems to couple to at least some of the same G proteins.

**Receptor Desensitization**—Like other G protein-coupled receptors, thrombin receptors become quickly desensitized after activation and, as a result, are unable to respond a second time to either thrombin or SFLLRN (38–40). This is a distinct process from the desensitization that is due to cleavage of the N terminus. As for other G protein-coupled receptors, thrombin receptor desensitization is thought to be due to phosphorylation of cytoplasmic serine and threonine residues (39, 41). In addition, activated thrombin receptors are rapidly cleared from the surface of most cells that express the receptor, including endothelial cells, after which they are replaced by new receptors (31). In endothelial cells, replacement is a relatively rapid process because of an intracellular pool of preformed thrombin receptors that can repopulate the cell surface within 2 or 3 h (17, 18). We have previously shown that when endothelial cells are incubated with thrombin, all of the receptors are rapidly cleaved, but there is only a partial decrease in the response to a subsequent addition of SFLLRN, even when the SFLLRN is added too soon after the thrombin for new receptors to have emerged (18). This suggests that either some of the cleaved thrombin receptors never desensitize or that resensitization has occurred very quickly. Alternatively, of course, the residual response to SFLLRN that was observed in those studies could have been due (in hindsight) to the activation of PAR-2, which also responds to SFLLRN.

The studies in Figs. 1, 2, and 7 address some of these issues and compare the desensitization of PAR-2 and thrombin receptors in endothelial cells. As previously indicated, an initial exposure to trypsin completely prevented a subsequent response to either thrombin or trypsin, while thrombin only partially inhibited a subsequent response to trypsin, reflecting the ability of trypsin to activate both receptors, while thrombin activates only thrombin receptors (Figs. 2 and 7A). Trypsin also abolished the response to SLIGRL (Figs. 1A and 2B). Notably, however, trypsin (alone or in combination with thrombin) only
Loss and Recovery of Receptor Function—Exposure of endothelial cells to trypsin caused the cells to lose their ability to respond to either thrombin or trypsin within a few minutes (Fig. 7A). In the tracing shown in Fig. 7B, endothelial cells that had been exposed to trypsin were washed and then allowed to recover for 2 h before being stimulated in succession with thrombin and trypsin. In contrast to their inability to elicit responses when added immediately after trypsin, under these conditions, both agonists caused an immediate increase in cytosolic Ca\textsuperscript{2+}.

This experiment was repeated with varying times of recovery after the initial cleavage of thrombin receptors and PAR-2 by trypsin. The results are summarized in Fig. 8A, which compares the kinetics of the recovery of the Ca\textsuperscript{2+} response mediated by thrombin receptors and PAR-2. Recovery of PAR-2 was, if anything, faster than the recovery of thrombin receptors and, as has been previously shown for thrombin receptors, was unaffected by inhibiting protein synthesis with 2.5 mg/ml cycloheximide (data not shown). In some of the experiments, thrombin receptors were disabled with cathepsin G before adding trypsin to activate PAR-2. Early recovery of PAR-2 was seen under these conditions as well (Fig. 8B).

Loss and Recovery of Cell Surface Receptors—The similarity in the loss and recovery of responses mediated by thrombin receptors and PAR-2 suggests that the same processes of receptor clearance and replacement underlie both. To test this, we measured the amount of PAR-2 on the endothelial cell surface with a polyclonal antibody (PAR-2C) raised against the N terminus of human PAR-2 C-terminal to the trypsin cleavage site. This antibody would be expected to bind to intact as well as cleaved PAR-2. The results are shown in Fig. 5C. Within 5 min of the addition of trypsin, antibody binding decreased to approximately 30% of control. Recovery of antibody binding was detectable after 30 min and seemed to plateau at around 60% of baseline at 2 h, the longest time tested. As is the case for thrombin receptors, the loss of antibody binding sites was dependent on activation of the receptor and not proteolysis because it was also seen when the endothelial cells were activated with the PAR-2 peptide SLIGKV (Fig. 9A).

Finally, because of a recent report that PAR-2 activation can cause accelerated clearance of thrombin receptors in endothelial cells (19), we looked again at this issue as well as whether selectively activating thrombin receptors would affect PAR-2. Antibody PAR-2C was used to detect PAR-2, and monoclonal antibody WEDE15 (32, 33) was used to detect thrombin receptors. As we reported previously, thrombin caused a 70% decrease in WEDE15 binding (Fig. 9). Activation of PAR-2 with SLIGKV had no effect. Conversely, although SLIGKV caused a decrease in binding of the PAR-2 antibody (Fig. 9A), in 2 experiments thrombin caused PAR-2 binding to increase to 113 and 164% of control. Therefore, we found no evidence that activation of either class of receptor causes accelerated clearance of the other.
Thrombin Receptors, PAR-2, and Endothelial Cells

DISCUSSION

The human thrombin receptor and PAR-2 share a similar structure and a common mechanism of activation, but the two receptors are typically expressed in different cells. For example, human platelets and megakaryoblastic cell lines express thrombin receptors (13, 32, 38, 42, 43) but not PAR-2 (21, 27), while intestinal epithelial cells (40) express PAR-2 but not thrombin receptors. Since endothelial cells express both, they provide an opportunity to compare the biology of thrombin receptors and PAR-2 within the same cellular environment. The results of this study, together with those published recently by other investigators (6, 19, 24, 26, 27), show that there are many similarities between the two receptors in endothelial cells but also some differences. For example, both receptors activate phospholipase C in a largely pertussis toxin-resistant manner, and both activate phospholipase A₂, leading to PGI₂ formation. Both receptors also cause a transient increase in the cytosolic Ca²⁺ concentration, and, as noted by others (6), activators of PAR-2 cause NO formation and release from endothelial cells, just as thrombin does. Finally, a similar mechanism seems to underlie the clearance and replacement of thrombin receptors and PAR-2 on endothelial cells.

The differences that were noted between thrombin receptors and PAR-2 were most notable in their interactions with proteases and possibly in the process of receptor desensitization. The differences in the sequences of the two receptors in the N terminus around the activation site suggest that they do not respond to the same set of proteases. However, in contrast to an earlier report (21), we found that trypsin can activate thrombin receptors as well as PAR-2, at least in HUVECs and transfected COS-1 cells. One obvious possible explanation for the apparent discrepancy is that in the earlier study, trypsin was added to human thrombin receptors expressed in Xenopus oocytes, which may subject the receptor to different posttranslational modifications such as glycosylation.

Because endothelial cells in most anatomic locations are unlikely to encounter trypsin, it is likely that other proteases are normally responsible for PAR-2 activation in the vascular space. We have shown recently that trypsin can activate PAR-2 (28). Trypsin may have particular biological relevance for PAR-2 because it has been shown to be secreted by mast cells in perivascular areas, including sites of atherosclerotic plaque formation (44, 45). In the present studies we tested whether cathepsin G, plasmin, urokinase, kallikrein, and coagulation factors IXa, Xa, XIa, and XIIa would activate or disable PAR-2. None of them seemed to do so. Cathepsin G was of particular interest because it has previously been shown to disable thrombin receptors by cleaving the receptor N terminus downstream from the site of cleavage by thrombin, thus amputating the tethered ligand domain (29). The present studies show that PAR-2 is not a substrate for cathepsin G. The protease neither activates PAR-2 nor prevents trypsin from activating it. Theoretically, this would allow neutrophils, which are the source of cathepsin G, to modify endothelial cell responses mediated by thrombin receptors while leaving PAR-2 responses unaltered. We were able to take advantage of this difference to study PAR-2 in the absence of thrombin receptors. Finally, it should be noted that although none of the proteases tested seemed to cleave PAR-2 when added by themselves, it can not be ruled out that PAR-2 activation will occur if one of these proteases is bound to a cell surface (as is often the case) or is present in a complex with cell surface or soluble co-factors that alter the substrate specificity for a protease.

Clearance, Replacement, and Desensitization—Because thrombin receptors and PAR-2 appear to have to be intact to be activated by a protease, cleaved receptors have to be replaced to permit a second round of protease responses. The capacity of different cells to rapidly replace thrombin receptors varies enormously. Human platelets are essentially incapable of replacing activated thrombin receptors and remain unresponsive to thrombin once all of their receptors have been cleaved (46). In earlier studies, we and others have shown that activated thrombin receptors are rapidly cleared from the surface of human endothelial cells and then replaced from an intracellular pool of preformed receptors (16–19). Much less is known about PAR-2, but while the present studies were being completed, a report from Bunnett and co-workers demonstrated that activated PAR-2 in intestinal epithelial cells and transfected KNRK kidney epithelial cells is subject to rapid internalization and replacement (40, 47). As with thrombin receptors, PAR-2 internalization in those cells occurred via endosomes, and replacement seemed to occur initially from a pool of preformed receptors. Working with endothelial cells, Bahou and co-workers (19) have shown that cells exposed to the agonist peptide SLIGRL become desensitized but regain their ability to respond to the peptide over a period of approximately 45 min. However, lacking an antibody that recognizes PAR-2, they could not determine whether this was due to receptor resensitization or replacement. The present studies show that the activation of PAR-2 is followed by a 60–70% decrease in the number of binding sites for a PAR-2-directed antibody on the surface of HUVECs. This decrease occurred within 5 min when the receptors were activated with SLIGKV. It also occurred when the receptors were activated by trypsin. It may have been that the loss of receptors was related to receptor activation but was independent of receptor proteolysis. The recovery of PAR-2...
number paralleled the recovery of PAR-2 function and was unaffected by cycloheximide. It also occurred with strikingly similar kinetics to the loss and recovery of thrombin receptors on endothelial cells (Figs. 5 and 6 in Ref. 18).

Collectively, these observations suggest that in endothelial cells PAR-2 is subject to the same process of clearance and replacement that applies to thrombin receptors. Notably, however, we did not observe the phenomenon of cross-desensitization between thrombin receptors and PAR-2 that was recently reported by Mirza et al. (19), nor did we find that activation of PAR-2 caused the clearance of thrombin receptors. In the Ca²⁺ studies shown in Fig. 1, the initial response to trypsin, which activates thrombin receptors and PAR-2, was diminished by preincubating the endothelial cells with thrombin. However, the magnitude of the trypsin response after thrombin was similar to the magnitude of the response in untreated cells to SLIGRL, which activates only PAR-2. This suggests that activation of thrombin receptors has no indirect effect on PAR-2. In the antibody binding studies, the PAR-2 agonist, SLIGKV, caused a decrease in the binding of the PAR-2 antibody but had no effect on the binding of the thrombin receptor antibody, while thrombin caused a decrease only in the binding of the thrombin receptor antibody (Fig. 9). The reason for this discrepancy between these results and those reported earlier remains to be determined.

The desensitization that occurs when thrombin receptors are activated is thought to be due in part to the phosphorylation of serine and threonine residues in the cytosolic domain of the receptor by one or more members of the family of receptor kinases that includes the β-adrenergic receptor kinase (βARK) (39). This allows arrestin-like molecules to bind and may be part of the mechanism that leads to receptor clearance (48, 49). For other G protein-coupled receptors, phosphorylation can be reversed, and desensitization can occur, usually during passage of the receptor through the endosomal compartment. On HEL and CHRF-288 cells, where thrombin receptor recycling occurs, there is some evidence that passage through the endosomal compartment affects thrombin receptors in a similar manner (38). Phosphorylation of thrombin receptors has been demonstrated in transfected rat-1 fibroblasts and HEK 293 cells (50, 51) but not in endothelial cells. PAR-2 phosphorylation has not yet been demonstrated but is likely to occur.

One unexpected finding that stands out in the desensitization studies was the ability of SFLLRN to continue to elicit a response from the endothelial cells even when they had been exposed to trypsin. We had noted this residual response previously and speculated that it might be due to the activation of PAR-2 (18). This does not seem to be the case. In the present studies we found that there was a substantial residual response to SFLLRN even when activation of both receptor types eliminated the response to SLIGRL (Figs. 1 and 2). Among other possibilities, one explanation for the persistent response to SFLLRN is the presence of an as-yet-unidentified third member of the family of protease-activated G protein-coupled receptors. Based upon the present observations, this receptor would be predicted to be SFLLRN-responsive but unable to be activated by either thrombin or trypsin.

In conclusion, the results of this study suggest that on endothelial cells, the thrombin receptor and PAR-2 act independently of each other but elicit a similar range of cellular responses. The results also suggest that the constraints affecting thrombin receptor behavior, including the ability to respond to a protease only once and the mechanisms of receptor clearance and replacement, apply to PAR-2 as well and may prove to be generic to this subfamily of G protein-coupled receptors. Having (at least) two such receptors on endothelial cells extends the range of proteases to which these cells are able to respond, but it is likely that the full range of proteases to which these receptors respond, particularly PAR-2, is still to be determined. Recent knockout studies suggest that there is at least one more form of the thrombin receptor (14, 15), but whether there are additional members of this family that can respond to other proteases remains to be determined.
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