Intracellular Targeting and Trafficking of Thrombin Receptors

A NOVEL MECHANISM FOR RESENSITIZATION OF A G PROTEIN-COUPLED RECEPTOR*

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The receptor for the protease thrombin is a member of the G protein-coupled receptor family, but is activated by a unique proteolytic mechanism. The irreversibility of this proteolytic mechanism and the fact that the ligand is tethered to its receptor raise special questions about inactivation of cleaved receptors and recovery of thrombin responsiveness. We compared the intracellular trafficking of the thrombin receptor to that of the β2-adrenergic receptor in transfected Rat1 fibroblasts. In unstimulated cells almost all β2 receptors were located on the plasma membrane; by contrast, part of a cell's thrombin receptors were found in an intracellular membrane compartment which co-localized with Golgi markers. Stimulation by agonist caused internalization and subsequent recycling of the β2-adrenergic receptor, but most activated thrombin receptors were internalized and targeted to lysosomes. The intracellular pool of thrombin receptors found in unstimulated cells was protected from activation by thrombin, but was translocated to the plasma membrane upon activation of cell surface thrombin receptors. Replenishment of plasma membrane thrombin receptors correlated with recovery of thrombin responsiveness. These observations reveal a novel trafficking mechanism for resensitizing the thrombin receptor as opposed to the internalization/recycling pathway of other G protein-coupled receptors.

G protein-coupled receptors constitute the largest family of plasma membrane receptors and are typically classified according to their pharmacological properties and the signal transduction systems they control. These receptors may also be differentiated by distinct mechanisms of desensitization, intracellular targeting, and trafficking. Previous studies have identified differences in subcellular distribution and agonist-mediated redistribution among adrenergic receptor subtypes (von Zastrow et al., 1993). These differences in intracellular trafficking may reflect special distribution of receptors in the membrane. These differences are especially marked in cells in vivo, such as at a synapse between neurons or between sympathetic nerves and smooth muscle cells. Receptor targeting to specific plasma membrane microdomains may also contribute to the specificity of receptor, G protein, and effector interactions (Neer and Clapham, 1988). Specific trafficking behavior may also be involved in the process of desensitization or of resensitization (Yu et al., 1993).

The thrombin receptor is a G protein-coupled receptor but is activated by a unique mechanism. Thrombin cleaves the receptor's amino-terminal exodomain to unmask a new amino terminus. This new amino terminus then serves as a tethered peptide ligand, binding to sites within the body of the receptor to effect receptor activation. This irreversible proteolytic activation mechanism stands in contrast to the reversible ligand-mediated activation mechanisms utilized by other members of the G protein-coupled receptor family and suggests profound differences in desensitization and resensitization pathways for thrombin receptor signaling. Recent studies indicate that thrombin receptors exhibit intracellular trafficking behavior that differs from that reported for β2- and α2-adrenergic receptors. Brass and colleagues (Hoxie et al., 1993) observed rapid internalization and targeting of thrombin receptors to lysosomes following stimulation. While β2-adrenergic receptors also undergo rapid internalization following stimulation, the process is reversible and receptors are reinserted in the plasma membrane following removal of agonist (von Zastrow and Koblika, 1992).

We have directly compared the cellular trafficking of thrombin receptors to that of β2-adrenergic receptors expressed in the same cell line. Our study revealed marked differences in subcellular distribution and trafficking behavior for the two receptor types and a novel mechanism for refreshing the cell surface with activable thrombin receptors.

EXPERIMENTAL PROCEDURES

Receptor Constructs and Expression—For immunofluorescence detection, the human thrombin receptor was epitope-tagged at its amino terminus with the Flag epitope (sequence DYKDDDD, Ishii et al., 1993), which is recognized by M1 and M2 monoclonal antibodies (Kodak Scientific Imaging Systems). For phosphorylation studies, a thrombin receptor with the 12CA5 epitope (sequence YPYDVPDYA) at its carboxyl terminus was used as described before (Ishii et al., 1994). Mutagenesis of the human thrombin receptor cDNA (Fig. 1) was performed by the method of Kunkel et al. (1987) or by insertion of double-stranded linkers or polymerase chain reaction products (Sambrook et al., 1989). Mutations were confirmed by dideoxy sequencing. For expression in cells, receptor cDNAs were subcloned into pBJ1 (provided by Dr. Mark Davis, Stanford University) and transfected into Rat1 fibroblasts (Hung et al., 1992). Stable transfectants were selected with G418 and screened for receptor expression by immunoblot and by surface antibody binding. Human β2-adrenergic receptor was epitope-tagged at the amino terminus with the 12CA5 epitope as described before (von Zastrow and Koblika, 1992).

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Tissue Culture—Human umbilical vein endothelial cells (HUVEC)\(^1\) and human erythroblasts (HEI) cells were obtained from American Type Culture Collection. Endothelial cells were maintained in M199 Earle's balanced salt solution medium containing 20% fetal calf serum (HyClone), 2 mM L-glutamine, acetic fibroblast growth factor (0.5 ng/ml), heparin (20 U/ml), L-glutamine (100 mM), sodium pyrophosphate (10 mM), and sodium fluoride (10 m). For the experiments, cells between generations 5 and 15 were used. The origin of endothelial cells was confirmed by positive immunofluorescence staining with a mouse monoclonal anti-human von Willebrand factor antibody (88/88; Dako Corp., Carpinteria, CA). HEI cells were grown in RPMI medium supplemented with 10% fetal bovine serum. Rat1 fibroblasts stably expressing wild-type and epitope-tagged human thrombin receptor were grown in high glucose DMEM supplemented with 10% fetal bovine serum and gentamicin containing 0.8 mg/ml Genetin (G418, Life Technologies, Inc.). Untransfected fibroblasts were kept in the same medium without addition of Genetin.

Immunofluorescence Microscopy—Two days before the experiments, cells were split on glass coverslips and 1 unit/ml hirudin (Calbiochem) was added to the medium. Following various treatments, cells were fixed in 3% formaldehyde or 4% paraformaldehyde and processed for immunocytochemistry as described before (Ishii et al., 1995). For experiments using permeabilized cells, fixed specimens were incubated 30 min in blocking buffer containing 0.2% Nonidet P-40, 5% non-fat dry milk, 50 mM Tris-HCl (pH 7.6). Subsequently, polyonal '1048' or '1809' antisera (final concentration 1 pg/ml) recognizing the hirudin domain epitope (Hung et al., 1992b) and monoclonal M1 anti-Flag antibody (final concentration 10 pg/ml; Eastman Kodak) were applied to the specimens in blocking buffer. Secondary antibodies (goat anti-rabbit F(ab')2 fragment of IgG conjugated to Texas Red, Jackson Immunoresearch; goat anti-mouse fluorescein isothiocyanate conjugate, Amersham Corp.) were diluted 1:500 and applied in the same buffer. For selective detection of receptor antigen localized on the cell surface, nonpermeabilized cells were labeled with primary antisera in DMEM medium containing 10% fetal bovine serum, 30 ng/ml HEPES, pH 7.6 at 4 °C. Immunofluorescence microscopy was performed using standard epifluorescence optics (Zeiss Axiophot, Zeiss x100/numerical aperture 1.3 objective).

Cell Markers—Monoclonal antibody 53F3C against mannosidase II (Burke et al., 1982) was purchased from Berkeley Antibody Co. (Berkeley, CA). For labeling of cellular Golgi membranes, the vital stain Bodipy-ceramide was used. Fluorescent C\(_2\)NBD-ceramide was covalently linked to defatted bovine serum albumin (Pagan and Martin, 1988). Cells on coverslips were fixed with 4% paraformaldehyde in PBS and incubated with 5 \(\mu\)g fluorescent C\(_2\)NBD-ceramide-defatted bovine serum albumin complex for 30 min at 4 °C. Cells were then washed and incubated for 60-90 min at room temperature with 3.4 mg/ml defatted bovine serum albumin in DMEM. The glass coverslips were mounted on depression slides and observed by fluorescence microscopy. Specimens were inspected by confocal laser scanning microscopy using a SARASTRO Bio Instruments 1000 instrument. Co-localization of the lysosomal marker and thrombin receptor (polyclonal 1909 antibody) was quantitated using the Molecular Dynamics image processing software.

Receptor Phosphorylation—Phosphorylation experiments were performed as described before (Ishii et al., 1994). Briefly, transfected Rat1 fibroblasts on six-well plates were labeled for 60 min at 37 °C with 0.2 mCi of \(^{32}P\)orthophosphate. After exposure to agonists, cells were solubilized in 150 mg NaCl, 50 mM Tris (pH 8.0), 5 mM EDTA, 1% (v/v) Nonidet P-40, 100 mg/ml sodium deoxycholate, 1% (v/v) SDS, 10 mg sodium fluoride, 10 mg sodium pyrophosphate, and protease inhibitors at 1 mg/ml phenylmethylsulfonyl fluoride, leupeptin, aprotonin, pepstatin A, and benzamidine. Solubilized protein was precleared with protein A-Sepharose beads (Pharmacia Biotech Inc.) and then immunoprecipitated with M2 or 12CA5 antibodies and protein A-Sepharose. Eluted protein was analyzed by 9% polyacrylamide-SDS gel electrophoresis and autoradiography.

Enzyme-linked Immunosorbent Assay of Receptor Antigen—To quantitate the amount of naive and transfected surface thrombin receptors in the medium pool, stably transfected Rat1 cells were split onto 24-well plates (Falcon) at 5 x 10\(^5\) cells/well. The next day, cells were washed with DMEM medium with 20 mg/ml HEPES (pH 7.4) and 1 mg/ml bovine serum albumin. Cells were incubated for various times with 10 ng thrombin at 37 °C and subsequently chilled down to 4 °C by rinsing with ice-cold PBS. Cell surface thrombin receptors were cleaved at 4 °C (15 min, 10 ng thrombin). After two rinsing steps with cold PBS, cells were permeabilized and fixed for 1 min with methanol at -20 °C. Plates were again washed twice with saline and reincubated in blocking solution (phosphate-buffered saline, 0.05% Tween 00, 1% bovine serum albumin (pH 7.4)). Primary antibody was applied in blocking solution (M1 antibody at 6 µg/ml) for 1 h at room temperature. Plates were washed with PBS and incubated with alkaline phosphatase-conjugated secondary antibody (Bio-Rad; 1:300 dilution in PBS, 0.05% Tween 00, 1% bovine serum albumin). Plates were developed with alkaline phosphatase chromogenic substrate p-nitrophenyl phosphate. OD\(_{405}\) was read after 20 min. Antibody binding data are expressed as specific binding (total minus nonspecific, with nonspecific being defined as the level of binding seen in untransfected Rat1 fibroblasts). In preliminary experiments (not shown), antibody binding was shown to be proportional to transfected cell number. Data shown are mean ± S.D. (n = 3) for a representative experiment out of four experiments.

Intracellular Calcium—To measure changes in intracellular calcium concentration, the long wavelength calcium indicator Fluo-3 was used (Molecular Probes, Eugene, OR) in combination with scanning microscopy as described before (Brooker et al., 1990; Niggli and Lederer, 1990). Fluo-3 acetoxymethyl ester (Fluo-3/AM) was dissolved in 50% pluronic F-127 in dimethyl sulfoxide and was diluted 1:100 in DMEM medium before use. Cells on coverslips were loaded with 5 µg Fluo-3/AM in DMEM for 30 min at 37 °C, rinsed twice with fresh medium and incubated for 15-30 min in DMEM medium to allow de-esterification. Subsequently, the coverslips with attached cells were mounted in PBS for inspection in a Sarastro Bio Instruments 1000 confocal laser scanning microscope. Images were recorded at 8-bit resolution using the fluorescein channel in time lapse mode in 5-s intervals (objective x 40; 256 x 256 pixels). Cells were stimulated with 10 ng thrombin, 250 µg agonist peptide (SFPLLR), or the calcium ionophore A23187. The images were stored on optical laser disk and processed using Image Space software (Molecular Dynamics, Mountain View, CA). Fluorescence intensity was measured by placing circular spots over the widest part of any given cell, and average intensity was determined for 5-15 individual cells per coverslip. For each experiment, three to four coverslips were measured under identical conditions and experiments were repeated at least three times. Data displayed indicate mean ± S.E.

RESULTS AND DISCUSSION

Subcellular Localization of Activated versus Naive Thrombin Receptors—To study the trafficking pathways of the thrombin receptor, we utilized a human thrombin receptor that was epitope-tagged (Flag epitope) at its amino terminus and stably transfected into Rat1 fibroblasts (Fig. 1, upper panel) (Ishii et al., 1993). With this modification, activated receptors could be differentiated from naive thrombin receptors by double antibody labeling (Fig. 1, lower panel). Prior to thrombin treatment, both Flag and hirudin domain epitopes were detected on the surface of nonpermeabilized cells (Fig. 1, a and b). As demonstrated previously (Ishii et al., 1993), brief exposure to thrombin resulted in a complete loss of cell surface binding of antibodies recognizing the Flag epitope (Fig. 1d) with persistent binding of antibodies which recognized the receptor's hirudin domain (Fig. 1c). The latter is carboxyl to the receptor's cleavage site and remains tethered to the receptor after cleavage. These and other data obtained with cleavage site mutant receptors show that loss of the Flag epitope binding distinguishes thrombin-activated from naive receptors (Ishii et al., 1993).

Unexpectedly, immunostaining of untreated permeabilized cells with both Flag and hirudin domain antibodies revealed

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\(^1\) The abbreviations used are: HUVEC, human umbilical vein endothelial cells; HEI, human erythroblasts; DMEM, Dulbecco's Modified Eagle's medium; NBD, 12-(N-methyl-N-[7-Nitro7-oxa-2-1,3 diazol-4-y]); PBS, phosphate-buffered saline.
thrombin receptors not only on the plasma membrane but also in a perinuclear distribution (Fig. 1, e and f). When cells were exposed to thrombin for 2 min before fixation and permeabilization, surface receptor was detected only by antibody to the hirudin domain, whereas the perinuclear pool was still recognized by the Flag antibody (Fig. 1, g and h). This perinuclear pool of thrombin receptor is therefore protected from proteolysis and activation by thrombin.

Phosphorylation studies support the notion of a protected intracellular pool of thrombin receptor (Fig. 2). Thrombin receptor cleavage and activation is rapidly followed by receptor phosphorylation, one of several mechanisms for terminating signaling (Ishii et al., 1994). Flag antibody immunoprecipitated phosphorylated thrombin receptors from cells activated by agonist peptide but not from cells activated by thrombin, whereas antibody to the carboxyl-terminal epitope (12CA5) immunoprecipitated phosphorylated receptor from cells activated by either agonist (Fig. 2). These data are consistent with the view that the perinuclear receptor pool recognized by the Flag antibody (Fig. 1 h) is protected from activation despite cleavage and activation of cell surface receptors by thrombin. As discussed below, this intracellular pool of naive receptors may be important for refreshing the cell surface with new thrombin-activatable receptors.

To determine the organellar location of the perinuclear pool of protected thrombin receptors, double immunofluorescence labeling studies were performed. The perinuclear thrombin receptor compartment co-localized with the resident Golgi protein mannosidase II (Fig. 3) and the fluorescent ceramide analog C6-NBD-ceramide (Pagano et al., 1989) (data not shown). This intracellular pool of thrombin receptors was noted over a range of receptor expression levels in different Rat1 stable transfectants as well as in human embryonal kidney 293 cells and COS 7 cells transiently transfected with thrombin receptor (data not shown). To determine whether the unusual perinuclear pool of the epitope-tagged thrombin receptor was somehow related to the epitope tag itself, we examined the distribution of wild-type thrombin receptor expressed in Rat1 cells. Again, both plasma membrane and perinuclear pools were found (data not shown).

Contrasting Trafficking Patterns of Thrombin Receptors and β2-Adrenergic Receptors—The unusual intracellular distribution of thrombin receptors described above differed from the known distribution pattern of β2-adrenergic receptors (von Zas-trow and Kobilka, 1992). We therefore compared the distribution of thrombin and β2-adrenergic receptors expressed in the same cell type. In unstimulated Rat1 cell transfectants, β2-adrenergic receptor was detected only on the surface (Fig. 4c) in contrast to both plasma membrane and Golgi localization of the thrombin receptor (Fig. 4, a and b). In response to stimulation by their respective agonists, both receptors were rapidly
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Fig. 3. Co-localization of thrombin receptors with the Golgi marker mannosidase II. a, thrombin receptor localized in Rat1 fibroblasts detected with the hirudin epitope antibody. b, the same cell stained for the Golgi apparatus with an antibody to anti-mannosidase II. Bar, 1 μm.

| Thrombin Receptor | β2 Receptor |
|-------------------|-------------|
| Hir Epitope       | Flag Epitope|
| 12CA5 Epitope     |              |

Fig. 4. A comparison of the trafficking of thrombin receptors and β2-adrenergic receptors in Rat1 fibroblasts before and after agonist stimulation. Thrombin receptors were localized in permeabilized cells by immunostaining with antibodies to the hirudin domain epitope (a, d, and g) or the Flag epitope (b, e, and h), β2-adrenergic receptors were detected with the 12CA5 antibody (c, f, and i). In unstimulated cells, thrombin receptors (a and b) and β2 receptors (c) are localized in the plasma membrane. In addition, thrombin receptors were detected in a perinuclear compartment (a and b). Upon stimulation with agonist, thrombin receptors (d and e) (10 min 10 nM thrombin) and β2 receptors (f) (10 min, 10 μM isoproterenol) are internalized into endosomes. 60 min after removal of the agonist, β2 receptors recycled to the plasma membrane (i), whereas activated thrombin receptors remained in intracellular vesicles (g and h). Bar, 10 μm.

Internalized into endosomal vesicles within 10 min (Fig. 4, d–f). Upon activation with thrombin, only cleaved thrombin receptors were internalized as the receptor in endosomes was stained with the hirudin domain antibody (Fig. 4d) but not with the Flag epitope antibody (Fig. 4e). In 293 cells co-transfected with both thrombin and β2 receptors and stimulated with both thrombin and isoproterenol, internalized thrombin receptors co-localized with internalized β2 receptors (data not shown). These data suggest that both receptors are initially internalized by a common activation-dependent mechanism despite their different G protein and effector coupling (β2-adrenergic receptors couple to α1, (Limbird et al., 1980), whereas thrombin receptors can couple to the α1 and α2 G protein families as well as to αq, α12, and G proteins (Brass et al., 1991; Hung et al., 1992a; LaMorte et al., 1993; Baffy et al., 1994; Offermanns et al., 1994)).

Following internalization, the intracellular trafficking of thrombin receptors was distinctly different from that of the β2 receptors. Whereas the β2 receptors recycled to the plasma membrane within 60 min of agonist removal (Fig. 4i), recycling of a significant population of activated thrombin receptors to the plasma membrane could not be detected after washout of thrombin or agonist peptide (Fig. 4, g and h). Although the distribution of immunostained thrombin receptors in Fig. 4g suggests that there is little plasma membrane receptor, the conditions used for photography emphasize the most intensely staining structures within the cell, such as receptor concentrations in vesicular structures. Receptors in the plasma membrane are more diffusely distributed and are therefore difficult to detect relative to the intensely stained vesicles in Fig. 4g. To best evaluate plasma membrane receptor, immunofluorescent labeling is done on nonpermeabilized cells (Fig. 1). Nevertheless, the comparison of Fig. 4i with Fig. 4f indicates that the majority of β2 receptors returned to the plasma membrane while the majority of thrombin receptors remained within vesicular structures.

Published studies of megakaryoblastic CHRF-288 cells demonstrated that following agonist activation internalized thrombin receptors were found in lysosomes (Hoxie et al., 1993). We also observe lysosomal targeting in transfected Rat1 cells (Fig. 5). In unstimulated cells, thrombin receptors were found in the plasma membrane and in the intracellular compartment. This intracellular compartment (which co-localized with the Golgi marker mannosidase II) was documented to be distinct from lysosomes in that it did not co-localize with staining for the lysosomal membrane glycoprotein lgp120 (Fig. 5, a–c). Five minutes after activation by 100 nM agonist peptide (SFLLRN), thrombin receptors were internalized into vesicles that did not stain with the lysosomal marker (Fig. 5, d–f). However, after 30 min of thrombin receptor stimulation, activated receptors co-localized partially with the lysosomal marker (Fig. 5, g–i). In the same cell line, no targeting of β2-adrenergic receptors to lysosomes was observed (not shown). Thus upon activation, thrombin and β2-adrenergic receptors are initially internalized into the same endosomal compartment, but subsequently follow distinct routes. Thrombin receptors are then sorted for delivery to lysosomes, but β2 receptors are recycled to the plasma membrane.

Recovery of Cell Surface Thrombin Receptors and Resensitization to Thrombin after Activation—Five minutes after stimulation with 10 nM thrombin, cell surface staining with the antibody to the Flag epitope was markedly diminished compared with staining by the antibody to the hirudin epitope (Fig. 6, c and d). Within 60 min of removing thrombin, nearly complete recovery of Flag epitope surface staining occurred (Fig. 6, e and f). Delivery of naive thrombin receptors to the cell surface was independent of protein synthesis as the same phenomenon was observed in the presence of 10 μM cycloheximide (Fig. 6, g and h). This concentration of cycloheximide blocked recovery of radioactivity in receptor immunoprecipitates from [35S]methionine-labeled cells by more than 95%, documenting blockade of
FIG. 5. Co-localization of thrombin receptors with the lysosomal membrane glycoprotein lgp120 at different times after stimulation by the agonist peptide SFLLRN. Immunofluorescence staining for thrombin receptor (Texas Red stain, primary antibody to the hirudin domain epitope) and lysosomal membrane glycoprotein lgp120 (fluorescein stain, primary antibody anti-lgp120). Co-localization of both antigens is indicated by the yellow color (overlapping of red and green fluorescence). Thrombin receptors are targeted to lysosomes by 30 min agonist-mediated endocytosis. Bar, 10 μm.

new thrombin receptor protein synthesis.

In order to examine the possibility that thrombin receptors newly delivered to the plasma membrane were mobilized from the protected intracellular pool upon stimulation of surface receptors, the time course of disappearance of this intracellular pool after blockade of protein synthesis was determined. Permeabilized cells were examined using both immunofluorescence and enzyme-linked immunosorbent assay techniques (Fig. 7). Even in the absence of new protein synthesis, unstimulated cells lost only a fraction of their intracellular thrombin receptor pool over 60 min (Fig. 7, a, b, and c). By contrast, thrombin stimulation resulted in nearly complete loss of the intracellular receptor pool over the same time frame (Fig. 7, c-e). Under these same conditions, mannosidase II staining remained unchanged, thus translocation of thrombin receptor does not reflect nonspecific movement or loss of all Golgi proteins.

The existence of the protected intracellular pool of thrombin receptors and its apparent mobilization to the plasma membrane upon activation of cell surface receptors suggested a trafficking mechanism that might play a role in restoring thrombin responsiveness. We therefore compared the recovery of cell surface thrombin receptors with recovery of thrombin responsiveness after initial exposure to thrombin (Fig. 8). Thrombin-induced elevation of cytosolic Ca²⁺ levels was determined at various times following initial thrombin exposure in the presence or absence of cycloheximide. In transfected Rat1 fibroblasts, stimulation of the thrombin receptor with thrombin resulted in a transient increase of the intracellular free Ca²⁺ concentration (Fig. 8). Cells were refractory to a second thrombin stimulation for 10–20 min. However, if the cells were allowed to recover for 40–90 min after removal of thrombin and in the presence of the thrombin inhibitor hirudin, they were again responsive to thrombin (Fig. 8, a and c). The recovery of thrombin responsiveness was observed in the presence of cycloheximide, indicating that it is independent of protein synthesis (Fig. 8b). The time course of recovery of thrombin responsiveness (Fig. 8c) correlated well with the loss of the intracellular pool of thrombin receptor (Fig. 7e) and the recovery of uncleaved cell-surface thrombin receptors.

Distribution of Native Thrombin Receptors in Naturally Expressing Cells—Experiments in Rat1 cells using epitope-tagged thrombin receptors demonstrated differences in the trafficking behavior between β-adrenergic receptors and thrombin receptors. To determine if these observations might be relevant to the function of thrombin receptors in vivo, thrombin receptor distribution and recovery was examined in cell lines which naturally express this receptor. Thrombin receptors mediate a variety of biological responses in vascular endothelium (for review see Coughlin (1993)). HUVEC were therefore examined to determine the distribution of thrombin receptors in a cell type which naturally expresses this protein. The HUVEC used in
these studies expressed von Willebrand factor, indicating that they retain the functional properties of endothelial cells. Thrombin receptors were detected with polyclonal antiserum to the hirudin-like domain of the receptor. In nonpermeabilized cells, thrombin receptors were detected in the plasma membrane (Fig. 9a). In permeabilized cells, additional staining was observed in an intracellular perinuclear compartment (Fig. 9b).

No staining was observed with preimmune serum in permeabilized or nonpermeabilized cells (data not shown). After stimulation with 10 nM thrombin for 15 min, many receptor-positive vesicles appeared in the periphery of the cell (Fig. 9c), suggesting endocytosis of activated thrombin receptors. With available antibodies we were unable to distinguish cleaved from non-cleaved naive thrombin receptors in HUVEC cells.

The potential functional role of the intracellular pool of thrombin receptors in resensitization of HUVEC to thrombin was examined. As with Rat1 fibroblasts, we observed protein synthesis-independent recovery of Ca²⁺ signaling within 60 min after a 10-min initial exposure to 10 nM thrombin (Fig. 9, d and e). In the continued presence of cycloheximide, a third exposure to thrombin produced a smaller Ca²⁺ response (Fig. 9e). This apparent inability to recover a second time is consistent with immunofluorescence studies which demonstrated depletion of the intracellular reserve of thrombin receptor following the first exposure to thrombin (data not shown).

These observations suggest that thrombin receptors in a naturally expressing cell line have targeting and trafficking properties similar to thrombin receptors transfected into Rat1 cells. However, the protein synthesis-independent re-sensitization that we observe for both Rat1 and HUVEC was unexpected in light of previous studies which demonstrated no recovery of responsiveness to thrombin within 1 h of stimulation of HEL
cells (Brass et al., 1991). In these studies recovery of responsiveness was observed after 22 h of culture and was dependent on protein synthesis. To address these contrasting results we examined resensitization in HEL cells and, in agreement with previous studies, observed little recovery of responsiveness to thrombin 60 min after a 10-min exposure to thrombin even in the absence of cycloheximide (Fig. Sf). Furthermore, using immunofluorescence staining we were unable to detect any significant intracellular pool of thrombin receptor in HEL cells (data not shown).

HEL cells have megakaryoblast-like properties, whereas HUVEC are endothelial cells. The biological consequences of thrombin receptor signaling in platelets and endothelial cells are markedly different. Stimulation of platelet thrombin receptors causes release of the platelets granule contents and platelet aggregation; these events presumably mark the end of a platelet’s need for thrombin responsiveness. Unlike platelets, stimulation of thrombin receptors in endothelial cells does not produce irreversible cellular events. Thus in endothelial cells and other cells that may need to respond to thrombin repeatedly or over time, mechanisms have evolved to permit recovery or maintenance of thrombin responsiveness; such mechanisms may not be needed in platelets.

Specialized subcellular targeting may be an important feature for the regulation of signal transduction for other G protein-coupled receptors as well. An intracellular pool of receptors is observed for one of the three closely related subtypes of α₁-adrenergic receptors (von Zastrow et al., 1993). Furthermore, cell fractionation studies have demonstrated an intracellular pool of N-formyl peptide receptor in neutrophils which is translocated to the plasma membrane upon agonist stimulation (Sengelov et al., 1994). Whereas the physiological relevance of the intracellular targeting of these receptors is unknown, intracellular retention of thrombin receptors may allow differentiated cells to quickly regain responsiveness to thrombin after activation of cell surface receptors.

Conclusion—The proteolytic activation of thrombin receptors by thrombin is a unique mechanism of activation among G protein-coupled receptors. Distinct intracellular trafficking pathways for thrombin receptors have evolved to dispose of cleaved receptors and to replenish the plasma membrane with uncleaved thrombin-responsive receptors (Fig. 10). Our results are consistent with previous reports that cleaved thrombin receptors are internalized and targeted to lysosomes (Hoxie et al., 1993). The contrasting reversible internalization of activated β₂-adrenergic receptors in the same cell line demonstrates that the lysosomal targeting of thrombin receptors represents a distinctive property of these receptors and not of the cell line. In some cell lines (HEL), resensitization of responsiveness to thrombin requires protein synthesis (Brass et al., 1991). However, in Rat1 fibroblasts and endothelial cells (HUVEC), resensitization of thrombin responsiveness is achieved by mobilizing a population of thrombin receptors retained in an intracellular

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**FIG. 8.** Calcium signaling in Rat1 fibroblasts transfected with human thrombin receptor (Ratl-TR). Cells were loaded with the calcium-sensitive dye Fluo-3/AM as described under "Experimental Procedures," and fluorescence intensity was measured by confocal laser scanning microscopy. Cells were incubated in the absence or presence of 10 nM thrombin as indicated. **c, time course of recovery of thrombin responsiveness after thrombin stimulation.**

**FIG. 9.** Detection of thrombin receptors in naturally expressing cells. Human umbilical vein endothelial cells were stained with a polyclonal antiserum to the hirudin-like domain of the thrombin receptor. **a** shows selective cell surface staining, whereas other panels (b and c) show staining of permeabilized cells. In nonpermeabilized endothelial cells, thrombin receptors were detected on the plasma membrane (a), however, receptor could be detected in small perinuclear vesicles in permeabilized cells (b). Upon stimulation with thrombin (10 nM for 5 min at 37°C), many receptor-positive endocytic vesicles appeared in the periphery of the cell (c). Bar, 20 μm. **d–f**, calcium signaling to repeated thrombin stimulation in human umbilical vein endothelial cells (HUVEC) and human erythroleukemia cells (HEL). Cells were attached on glass coverslips, loaded with Fluo-3/AM, and stimulated with thrombin as indicated. Fluorescence intensity was recorded by confocal laser scanning microscopy. In endothelial cells (d), thrombin was able to cause an increase of the intracellular calcium concentration repeatedly. Cells were incubated with 10 nM thrombin for 10 min. Between stimulations, cells were washed with regular medium containing hirudin for 90 min (w). In the presence of cycloheximide to block new receptor synthesis (e), HUVEC cells responded to a second thrombin stimulus, but the third response was significantly blunted. In HEL cells, thrombin was not able to elicit repeated calcium signals after the initial response (f).
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FIG. 10. Schematic representation of thrombin and β2-adrenergic receptor trafficking. Thrombin receptors and β2-adrenergic receptors undergo agonist-dependent endocytosis (arrows 1 and 4) and can be co-localized in the same endosomes (2, 5). At this point intracellular trafficking pathways diverge, with the β2 receptor recycling to the plasma membrane (arrow 3) and the activated thrombin receptors being delivered to lysosomes (arrow 6). Following thrombin stimulation, a pool of naive thrombin receptors in an intracellular compartment are translocated to the cell surface (7). This intracellular pool of thrombin receptor co-localizes with mannosidase II suggesting, although not proving, that these receptors are retained in the Golgi.

compartment and protected from thrombin cleavage. The intracellular pool of thrombin receptors co-localized with mannosidase II suggesting (although not proving) that these receptors may be retained in the Golgi. The process of resensitization is independent of protein synthesis. Mobilization is initiated by activation of the thrombin receptor; however, the nature of the signal for thrombin receptor mobilization remains to be determined. Stimulation of lysophosphatidic acid receptors present on Rat1 cells results in phosphatidylinositol turnover and an increase in intracellular calcium; however no mobilization of the intracellular thrombin receptor pool was observed in response to this agonist (data not shown). Future studies will attempt to determine the mechanism by which thrombin activates receptor mobilization, and to identify specific receptor domains and docking molecules which mediate the distinctive trafficking behavior of thrombin receptors.

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