To define the molecular basis of human chemoattractant receptor regulation, rat basophilic leukemia RBL-2H3 cells, which are thrombin-responsive, were transfected to stably express epitope-tagged receptors for C5a, interleukin-8 (IL-8), formylpeptides (e.g. N-formyl-methionyl-leucyl-phenylalanine (fMLP)), and platelet-activating factor (PAF). Here we demonstrate that both thrombin and a synthetic peptide ligand for the thrombin receptor (sequence SFLLRN) caused phosphorylation and heterologous desensitization of the receptors for C5a, IL-8, and PAF but not that for formylpeptides as measured by agonist-stimulated \([\text{GTP}_{S}]\)guanosine 5'-O-(thio)triphosphate binding to membranes. Consistent with the PAF receptor phosphorylation, both thrombin and thrombin receptor peptide inhibited phosphoinositide hydrolysis, Ca\(^{2+}\) mobilization, and degranulation stimulated by PAF. Unexpectedly, despite heterologous desensitization at the level of receptor/G protein activation, there was enhancement (“priming”) by thrombin of subsequent activities stimulated by C5a and IL-8 as well as fMLP. The priming effect of thrombin was blocked by its inhibitor, hirudin. However, two other activators of the thrombin receptor, the peptide SFLLRN and trypsin, stimulated Ca\(^{2+}\) mobilization in RBL-2H3 cells but did not cause priming. In addition, SFLLRN and the thrombin receptor antagonist peptide FLLRN both inhibited thrombin-induced Ca\(^{2+}\) mobilization but not priming. Furthermore, the proteolytically active \(\gamma\)-thrombin, which does not stimulate the tethered ligand thrombin receptor and caused little or no Ca\(^{2+}\) mobilization in RBL-2H3 cells, effectively primed the response to fMLP. These data demonstrate that heterologous receptor phosphorylation and attenuation of G protein activation are not, by themselves, sufficient for the inhibition of biological responses mediated by C5a and IL-8. Moreover, thrombin appears to utilize mechanism(s) independent of its tethered ligand receptor to selectively prime phospholipase C-mediated biological responses of the C5a, IL-8, and formylpeptide receptors but not PAF. Because C5a, IL-8, and formylpeptide activate phospholipase C\(_{1,2}\), whereas PAF stimulates a different phospholipase C, the striking selectivity of thrombin’s priming may be mediated via its ability to enhance receptor-mediated activation of phospholipase C\(_{1,2}\). Phagocytic leukocytes respond to multiple inflammatory signals and play a key role in immunological reactions. Among the well defined stimulants for leukocytes are chemoattractants: a cleavage product of the fifth component of complement (C5a), interleukin-8 (IL-8),\(^{3}\) platelet-activating factor (PAF), and N-formylated peptides (e.g. fMLP) (1-3). These chemoattractants mediate their biological responses via the activation of cell surface receptors that are coupled to phospholipase C via G proteins. Leukocyte responses to chemoattractants can be "primed" or desensitized by prior stimulation (1, 4, 5). Priming or enhanced responsiveness to subsequent stimuli may be caused by low concentrations of substance P, chemoattractants, phorbol esters, Ca\(^{2+}\) ionophores, and cytokines, including granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-\(\alpha\) (1, 6, 7). The molecular mechanism by which these agents prime responses in leukocytes have not yet been determined. Desensitization of cellular responses to a stimulus can either be homologous or heterologous (8). The former is specific for a given ligand/receptor, whereas the latter involves multiple ligands/receptors. There is convincing evidence that receptor phosphorylation is one mechanism by which many G protein-coupled receptors undergo both homologous and heterologous desensitization. Recent evidence indicates an additional mechanism for chemoattractant receptor desensitization, a process resulting in reduced phosphoinositide hydrolysis via a decreased activity of phospholipase C (9).

Thrombin, a serine protease generated at sites of vascular injury, is a key enzyme in the coagulation cascade but may also be important in regulating inflammatory and proliferative responses (10, 11). Thrombin activates a variety of cells including platelets, leukocytes, fibroblasts, and endothelial cells at least in part via its interaction with cell surface receptors (12-15). One such receptor is a member of the seven transmembrane domain receptor superfamily whose activation proceeds via a novel mechanism (16). Thrombin binds to and cleaves its receptor’s extracellular amino-terminal extension, thereby unmasking an amino-terminal receptor, whose binding site resides in the first six amino acids (sequence SFLLRN) (16, 17). This tethered ligand binds to the thrombin receptor and induces its activation. Thrombin also binds to the platelet membrane glycoprotein Ib (GP Ib) to activate Ca\(^{2+}\) mobilization and platelet aggregation (18, 19). However, thrombin-induced chemotaxis in monocytes and growth factor-like effect in fibroblasts appear to be mediated via the activation of as yet undefined thrombin receptors (14, 20, 21).

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1 The abbreviations used are: IL-8, interleukin-8; fMLP, N-formyl-methionyl-leucyl-phenylalanine; PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine; ET-4FR, ET-4CaR, ET-IL-8R, and ET-PAF, epitope-tagged receptors for fMLP, C5a, IL-8, and PAF, respectively; PMA, phorbol 12-myristate 13-acetate; TRP, thrombin receptor peptide; PIP2, phosphatidylinositol 4,5-bisphosphate; GTP\(_S\), guanosine 5'-3-O-(thio) triphosphate; GPlb, glycoprotein Ib; Ptx, pertussis toxin; PLC, phospholipase C.

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To define the molecular basis of human chemoattractant receptor regulation, rat basophilic leukemia RBL-2H3 cells, which are thrombin-responsive, were transfected to stably express epitope-tagged receptors for C5a, interleukin-8 (IL-8), formylpeptides (e.g. N-formyl-methionyl-leucyl-phenylalanine (fMLP)), and platelet-activating factor (PAF). Here we demonstrate that both thrombin and a synthetic peptide ligand for the thrombin receptor (sequence SFLLRN) caused phosphorylation and heterologous desensitization of the receptors for C5a, IL-8, and PAF but not that for formylpeptides as measured by agonist-stimulated \([\text{GTP}_{S}]\)guanosine 5'-O-(thio)triphosphate binding to membranes. Consistent with the PAF receptor phosphorylation, both thrombin and thrombin receptor peptide inhibited phosphoinositide hydrolysis, Ca\(^{2+}\) mobilization, and degranulation stimulated by PAF. Unexpectedly, despite heterologous desensitization at the level of receptor/G protein activation, there was enhancement (“priming”) by thrombin of subsequent activities stimulated by C5a and IL-8 as well as fMLP. The priming effect of thrombin was blocked by its inhibitor, hirudin. However, two other activators of the thrombin receptor, the peptide SFLLRN and trypsin, stimulated Ca\(^{2+}\) mobilization in RBL-2H3 cells but did not cause priming. In addition, SFLLRN and the thrombin receptor antagonist peptide FLLRN both inhibited thrombin-induced Ca\(^{2+}\) mobilization but not priming. Furthermore, the proteolytically active \(\gamma\)-thrombin, which does not stimulate the tethered ligand thrombin receptor and caused little or no Ca\(^{2+}\) mobilization in RBL-2H3 cells, effectively primed the response to fMLP. These data demonstrate that heterologous receptor phosphorylation and attenuation of G protein activation are not, by themselves, sufficient for the inhibition of biological responses mediated by C5a and IL-8. Moreover, thrombin appears to utilize mechanism(s) independent of its tethered ligand receptor to selectively prime phospholipase C-mediated biological responses of the C5a, IL-8, and formylpeptide receptors but not PAF. Because C5a, IL-8, and formylpeptide activate phospholipase C\(_{1,2}\), whereas PAF stimulates a different phospholipase C, the striking selectivity of thrombin’s priming may be mediated via its ability to enhance receptor-mediated activation of phospholipase C\(_{1,2}\).
Thrombin Primes Chemoattractant Receptor-mediated Responses

Figure 1. Phosphorylation of stably expressed chemoattractant receptors. 

**EXPERIMENTAL PROCEDURES**

Materials—[32P]Orthophosphate (8500–9120 Ci/mmol), myo-[2-3H(N)] -inositol (24.4 Ci/mmol), and [35S]GTPγS (1300Ci/mmol) were purchased from DuPont NEN. Monoclonal 12CA5 antibody was obtained from Berkeley Antibody Co. Earle's modified Eagle's medium and all tissue culture reagents were purchased from Life Technologies, Inc. Indo-1 acetoxyethyl ester and pluronic acid were purchased from Molecular Probes. C5a, fMLP, PAF, trypsin (Type I), α-chymotrypsin (Type VII: 1-chloro-3-tyosamido-7-amino-2-heptanone-treated, and hirudin were obtained from Sigma. Cephalin G and dextran from Athens Research and Technology Inc. IL-8 was purchased from Genzyme. Human α-thrombin (specific activity, 1052 NIH units/mg) was obtained from Calbiochem. Human α-thrombin (specific activity, 2680 units/mg), γ-thrombin (specific activity, 1.71 units/mg), and disopropylphosphofluoridate-inactivated α-thrombin (specific activity, 0.078 unit/mg) were generous gifts from Dr. J. W. Fenton II (New York Department of Health, Albany, NY). The thrombin receptor agonist peptide (SFLLRN) was purchased from Peninsula Laboratories, and the antagonist peptide FLLRN was synthesized by Quality Controlled Biochemicals Inc.

Phosphorylation of Epitope-Tagged Chemoattractant Receptors Stably Expressed in RBL-2H3 Cells—Phosphorylation of receptors was carried out exactly as described (4). Briefly, RBL-2H3 cells (2.0 × 10⁶) expressing chemoattractant receptors were seeded in 60-mm tissue culture dishes in normal growth medium and cultured overnight at 37 °C. The following day each dish was washed twice with 5 ml of phosphate-free Dulbecco's modified Eagle's medium and incubated with 150 μCi of [32P]orthophosphate for 90 min. Adherent cells were stimulated with the indicated stimulants, and the phosphorylated receptors were immunoprecipitated with 12CA5 antibody, analyzed by SDS-polyacrylamide gel electrophoresis, and visualized by autoradiography.

[35S]GTPγS Binding—RBL-2H3 cells expressing the appropriate chemoattractant receptors were treated with the indicated stimulants for 3 min at 37 °C, and membranes were prepared as described (4).[35S]GTPγS binding to 10 μg of membranes was performed as described previously (22).

**RESULTS**

Phosphorylation and Desensitization of Human Chemoattractant Receptors—RBL-2H3 cells stably expressing epitope-tagged receptors for fMLP (ET-FR), C5a (ET-C5aR), IL-8 (ET-IL-8R), and PAF (ET-PAFR) were incubated with [32P]orthophosphate and stimulated with their respective ligands, phorbol 12-myristate 13-acetate (PMA), thrombin (1 unit/ml), or TRP (sequence SFLLRN, 100 μM). All receptors underwent homologous phosphorylation (Fig. 1). PMA, thrombin, and TRP caused phosphorylation of ET-C5aR, ET-IL-8R, and ET-PAFR but not ET-FR. The extent of thrombin- and TRP-induced phosphorylation of the susceptible receptors was less than agonist-induced receptor phosphorylation. To determine whether receptor phosphorylation was correlated with desensitization at the level of G protein activation, cells expressing selected receptors (ET-C5aR and ET-PAFR) were treated with their respective ligands (C5a or PAF), PMA, thrombin, or TRP, and agonist-stimulated [35S]GTPγS binding to membranes was de-
Thrombin Primes Chemoattractant Receptor-mediated Responses

**Fig. 2. Effects of thrombin and TRP on chemoattractant receptor-stimulated [35S]GTP-γ-S binding to membranes.** RBL-2H3 cells expressing ET-C5aR and ET-PAFR were left untreated (CON) or treated with their respective ligands C5a or PAF, PMA, thrombin (THR), or TRP as in Fig. 1. Membranes were prepared and assayed for agonist-stimulated [35S]GTP-γ-S binding. The values are presented as percentages of net [35S]GTP-γ-S bound, which is defined as the maximum amount of agonist-stimulated response from untreated membranes. For ET-C5aR the basal and C5a-stimulated responses (untreated) were 7097 ± 216 and 15472 ± 47 cpm, respectively. For ET-PAFR, the basal and PAF-stimulated (untreated) responses were 6446 ± 350 and 12330 ± 46 cpm, respectively. The basal [35S]GTP-γ-S bound to membranes treated with ligand, PMA, thrombin, and TRP were similar to basal responses in the absence of pretreatment. The data are the means ± S.E. of three experiments performed in triplicate.

As shown in Fig. 2, thrombin, TRP, and PMA inhibited the responses to C5a and PAF. Similar to receptor phosphorylation, the extent of heterologous desensitization was less than homologous desensitization for both receptors (Fig. 2). Thrombin did not cause inhibition of fMLP-stimulated [35S]GTP-γ-S binding to membranes (4).

**Effects of Thrombin and TRP on Ca2+ Mobilization.** In Response to Chemoattractants, the peak [Ca2+]i associated with chemoattractant stimulation was determined by fMLP (10 nM), PMA, C5a (1 nM), IL-8 (3 nM), or PAF (0.2 M). The effects of thrombin and TRP on Ca2+ mobilization were also tested in cells expressing appropriate receptors. As shown in Fig. 3, thrombin but not TRP primed Ca2+ mobilization stimulated by the Ca2+ mobilization upon stimulation by agonist (data not shown). The effects of thrombin and TRP were also tested on Ca2+ mobilization stimulated by C5a, IL-8, and PAF in cells expressing appropriate receptors. 

**Fig. 3. Effects of thrombin and TRP on Ca2+ mobilization stimulated by fMLP, C5a, IL-8, and PAF.** RBL-2H3 cells expressing ET-FR, ET-C5aR, ET-IL-8R, and ET-PAFR were loaded with indo-1 and were left untreated (Control) or treated with thrombin (1 unit/ml) or TRP (100 μM) and 3 min later challenged with submaximal concentrations of fMLP, C5a (1 nM), IL-8 (3 nM), or PAF (0.2 M), and chemoattractant-stimulated Ca2+ mobilization was determined. The values presented are the peak Ca2+ mobilization stimulated by chemoattractants after the basal (1–150 nM) has been subtracted. The data are the means ± S.E. of three experiments.

**Effects of Thrombin and TRP on Chemoattractant Receptor-stimulated Phosphoinositide Hydrolysis and Secretion of β-Hexosaminidase.** In the absence of thrombin or TRP, fMLP caused a dose-dependent increase in the generation of [3H]inositol phosphates and release of β-hexosaminidase (Fig. 4). Thrombin enhanced the maximum responses stimulated by fMLP by 2–3-fold. TRP, however, had no effect on fMLP-induced responses. The dose-response effect of thrombin on fMLP-stimulated phosphoinositide hydrolysis demonstrated that the EC50 and EC100 values for priming by thrombin were approximately 0.1 and 1.0 unit/ml, respectively (data not shown).

**Fig. 4. Effects of thrombin and TRP on phosphoinositide hydrolysis and secretion of β-hexosaminidase.** Thrombin increased the maximum responses (measured as [3H]inositol phosphate release) by about 2–3-fold at [3H]inositol phosphate concentrations of 1 nM and 10 nM, which was determined to be the peak value for thrombin at each concentration. TRP, however, had no effect on fMLP-induced responses. The dose-response effect of thrombin on fMLP-stimulated phosphoinositide hydrolysis demonstrated that the EC50 and EC100 values for priming by thrombin were approximately 0.1 and 1.0 unit/ml, respectively (data not shown).

**The effects of thrombin and TRP on the generation of [3H]inositol phosphates and secretion of β-hexosaminidase.** In the absence of thrombin or TRP, fMLP caused a dose-dependent increase in the generation of [3H]inositol phosphates and release of β-hexosaminidase (Fig. 4). Thrombin enhanced the maximum response stimulated by fMLP by 2–3-fold. TRP, however, had no effect on fMLP-induced responses. The dose-response effect of thrombin on fMLP-stimulated phosphoinositide hydrolysis demonstrated that the EC50 and EC100 values for priming by thrombin were approximately 0.1 and 1.0 unit/ml, respectively (data not shown).

The effects of thrombin and TRP on the generation of [3H]inositol phosphates and secretion of β-hexosaminidase were determined in the absence of thrombin or TRP. fMLP caused a dose-dependent increase in the generation of [3H]inositol phosphates and release of about 20–30% of total cellular β-hexosaminidase. As shown in Fig. 5, thrombin but not TRP primed both phosphoinositide hydrolysis and secretion stimulated by C5a and IL-8. In contrast, both thrombin and TRP inhibited these responses to PAF.

**The fMLP-induced generation of [3H]inositol phosphates was used to further characterize the priming phenomenon.** The priming effect of thrombin was not dependent on its continuous presence because replacement of thrombin-containing buffer 3 min after its addition with fresh buffer without thrombin resulted in the same magnitude of priming as when thrombin
was not removed by washing (Fig. 6A). The priming effect of thrombin reached a maximum by 3–5 min, remained elevated for about 15 min, and returned to basal by 60 min (Fig. 6B).

When cells were preincubated for 5 min with thrombin in the presence of Ca\(^{2+}\) (1 mM) or EGTA (100 \(\mu M\); no added Ca\(^{2+}\)), washed, and stimulated with fMLP in the presence of Ca\(^{2+}\) (1 mM), the extent of priming was the same (data not shown).

To test whether the effect of thrombin correlated with an increase in substrate availability for phospholipase C, RBL-H3 cells were labeled with \(^{3}H\)inositol and exposed to thrombin (1 unit/ml) or TRP (100 \(\mu M\)) for 3 min and then the amount of \(^{[3]H}\)PIP\(_{2}\) was determined. The levels of incorporation of \(^{[3]H}\)inositol in to PIP\(_{2}\) in response to buffer, thrombin, and TRP were 4309 ± 695, 4329 ± 6210, and 4286 ± 105, respectively.

Specificity of Thrombin-mediated Responses in RBL-2H3 Cells—Thrombin receptor agonist and antagonist peptides SFLLRN and FLLRN (16) were utilized to test the role of the tethered ligand thrombin receptor on priming. As shown in Table I, the peptide SFLLRN stimulated a substantial Ca\(^{2+}\) mobilization and desensitized the thrombin-stimulated Ca\(^{2+}\) response by >75% but did not inhibit the priming effect of thrombin. The thrombin receptor antagonist peptide FLLRN caused no Ca\(^{2+}\) mobilization and inhibited the response to thrombin by >75% but did not block priming by thrombin (Table I).

Hirudin binds to the anion binding exosite and the enzymatic cleavage pocket of thrombin and prevents thrombin from binding and cleaving its receptor (25). Hirudin completely blocked both Ca\(^{2+}\) mobilization and priming of the fMLP response by thrombin but had no effect on the fMLP response itself (Table II). Thrombin inactivated by diisopropylphosphofluoridate has no catalytic activity but still binds to the seven transmembrane domain thrombin receptor via the anion binding exosite (26). Diisopropylphosphofluoridate-inactivated \(\alpha\)-thrombin did not stimulate Ca\(^{2+}\) mobilization in RBL-2H3 cells and had no effect on the fMLP response (Table II).

\(\gamma\)-Thrombin retains its catalytic activity, but its binding site for
**FIG. 6. Characteristics of thrombin-induced priming.** A. [3H]inositol-labeled RBL-2H3 cells expressing ET-FR were treated with buffer or thrombin (1 unit/ml for 3 min) containing LiCl (20 mM) and stimulated with fMLP (1 μM) in the continuous presence of thrombin (+). Alternatively, cells were preincubated with thrombin, washed, and exposed to a fresh medium without thrombin but containing LiCl (20 mM) and stimulated with fMLP (-). B, cells were exposed to thrombin for different time periods in the absence of LiCl, washed, and exposed to fMLP in a buffer containing LiCl. The reactions were quenched 10 min after the addition of fMLP and generation of total [3H]inositol phosphates were determined. The data shown are the means ± S.E. of one of four experiments performed in triplicate.

**TABLE I**

| Treatment | Intracellular Ca²⁺ concentration | Peptides | Thrombin | fMLP |
|-----------|---------------------------------|----------|----------|------|
| Control   | 173 ± 19                        | 454 ± 50 | 447 ± 47 |
| Thrombin  | 680 ± 60                        | 128 ± 5.7| 465 ± 35 |
| SFLLRN, thrombin | 0 ± 0                      | 120 ± 10.9| 483 ± 14 |
| FLLRN, thrombin | 0 ± 0                      | 120 ± 10.9| 483 ± 14 |

**TABLE II**

| Treatment | Intracellular Ca²⁺ concentration | First dose | Second dose (fMLP) | Fold priming |
|-----------|---------------------------------|------------|--------------------|--------------|
| Control   | 161 ± 5.2                       | 1074 ± 60  | 643 ± 17**         | 4.0          |
| Thrombin  | 167 ± 5.5                       | 0 ± 0      | 167 ± 5.5          | 0            |
| Hirudin + thrombin | 0 ± 0                      | 158 ± 13  | 0                  |
| DIP α-thrombin | 0 ± 0                      | 47 ± 14   | 440 ± 27**         | 2.5          |
| γ-Thrombin | 669 ± 18                        | 0 ± 0      | 159 ± 2            | 0            |
| Trypsin   | 272 ± 26*                      | 0 ± 0      | 272 ± 26*          | 1.7          |
| Cathepsin G | 23 ± 1.4                      | 264 ± 34* | 264 ± 34*         | 1.6          |

**DISCUSSION**

Regulation of receptor action has been the subject of intense investigation with one focus being the mechanisms of desensitization. There is abundant evidence for a role of receptor phosphorylation in homologous and heterologous desensitization (8). Indeed, the inability of the formylpeptide receptor to undergo heterologous desensitization was attributed to the absence of a phosphorylation site for protein kinase C on any of its predicted intracellular loops (4, 27). The data presented herein demonstrate that both thrombin and TRP stimulated phosphorylation of the chemoattractant receptors for C5a, IL-8, and PAF but not for fMLP. Furthermore, phosphorylation of susceptible receptors was correlated with heterologous desensitization at the level of receptor/G protein coupling. Unexpectedly, although both thrombin and TRP decreased G protein activation by C5a, thrombin primed subsequent phosphoinositide hydrolysis, Ca²⁺ mobilization, and degranulation stimulated by all the peptide chemoattractants (fMLP, C5a, and IL-8). The specificity of thrombin for the priming of peptide chemoattractant-mediated responses is demonstrated by the finding that thrombin inhibited these responses to PAF and had no effect on Ca²⁺ mobilization stimulated by the IgE receptors. These data suggest a surprising complexity in the regulation of receptor actions. Whereas signals can lead to...
receptor phosphorylation and desensitization at an early stage in the peptide chemoattractant signal transduction pathway, the same or other signals may result in markedly enhanced downstream activities.

The data herein indicate that the priming effect of thrombin requires its proteolytic activity but is unlikely to be mediated via its tethered ligand receptor. This contention is based on the following observations. The peptide agonist of the thrombin receptor, SFLLRN, stimulated Ca\textsuperscript{2+} mobilization in RBL-2H3 cells but did not prime the response to fMLP. Desensitization of the thrombin receptor by SFLLRN and treatment with the thrombin receptor antagonist peptide FLLRN both inhibited thrombin-induced Ca\textsuperscript{2+} mobilization by >70%, but neither blocked thrombin's ability to prime Ca\textsuperscript{2+} mobilization to fMLP. γ-Thrombin, which does not bind to the tethered ligand thrombin receptor but has proteolytic activity (26), stimulated little or no Ca\textsuperscript{2+} mobilization but effectively primed the response to fMLP. The priming signal is also unlikely to be derived from peptide chemoattractant receptors, because they desensitize each other's inositol 1,4,5-triphosphate formation and Ca\textsuperscript{2+} mobilization (9). The effect of thrombin is not solely due to its serine protease activity because trypsin and elastase, which are serine proteases (28), did not cause priming. However, other proteases such as cathepsin G and chymotrypsin primed fMLP-stimulated Ca\textsuperscript{2+} mobilization, albeit to a lesser extent than thrombin. Whether cathepsin G and chymotrypsin utilize the same or different mechanisms to cause priming remains to be determined.

In fibroblasts, both thrombin and TRP stimulate a transient Ca\textsuperscript{2+} mobilization of similar magnitude, but only thrombin is mitogenic (14). The mitogenic signal of thrombin persist long after (30 min) the transient Ca\textsuperscript{2+} mobilization has returned to basal (29). Based on these observations, the existence of an additional receptor for thrombin's mitogenic activity has been postulated (14, 29). The identity of this putative receptor or mechanism is yet to be determined. The data herein showing that both thrombin and TRP stimulate transient Ca\textsuperscript{2+} mobilization but only thrombin provides a sustained priming signal suggest that the priming and mitogenic responses of thrombin may be mediated via a similar pathway. In platelets, two glycoproteins (GPIb and GPV) interact with thrombin, but the biological significance of these interactions is not known (18, 19, 30–32). GPIb is a high affinity thrombin receptor in human platelets (18, 19). The demonstration that γ-thrombin, which does not bind to GPIb in human platelets (33), causes priming in RBL-2H3 cells suggests that GPIb is unlikely to be involved in priming. GPV (molecular mass, 82 kDa) is the only detectable membrane surface protein to be hydrolyzed by both α- and γ-thrombin as well as by chymotrypsin (30–32). Its proteolysis results in the generation and extracellular release of a 69.5-kDa soluble fragment. If GPV is expressed in RBL-2H3 cells, it is a candidate for involvement in priming.

The phenomenology of priming of chemoattractant receptor-mediated responses have been well delineated, but the molecular mechanisms are not known. For example, granulocyte-macrophage colony-stimulating factor enhances Ca\textsuperscript{2+} mobilization and superoxide generation stimulated by both peptide (fMLP and C5a) and lipid (PAF) chemoattractants as well as by direct activation of G proteins (6). It has been proposed that the priming effect of granulocyte-macrophage colony-stimulating factor may reside in its ability to cause de novo synthesis of cellular G proteins or activation of phosphoinositide kinase, which increases the availability of substrates for phospholipase C (7, 34). In the studies presented here, the effect reached a maximum 3–5 min after thrombin addition, and thus de novo protein synthesis is unlikely to be involved. In addition, thrombin actually decreased G protein activation by the C5a receptor yet primed its subsequent responses. Furthermore, under optimal conditions of priming, thrombin had no significant effect on PIP\textsubscript{2} levels in RBL-2H3 cells. The striking selectivity of thrombin for priming responses for peptide chemoattractants (fMLP, C5a, and IL-8) indicates that the target is distal to the receptor but is a component of the signal transduction pathway shared by fMLP, C5a, and IL-8 but not PAF.

The peptide chemoattractant receptors couple to a Ptx-sensitive G protein, presumably G\textsubscript{i2}, whereas PAF receptors utilize a predominantly Ptx-insensitive G protein (3, 35). Cotransfection studies in COS cells demonstrated that fMLP, C5a, and IL-8 utilize β\textsubscript{2} subunit (G\textsubscript{i2}) of G\textsubscript{i2} to activate PLC\textsubscript{β2}, whereas PAF utilizes the α subunit of a G\textsubscript{i2}-like G protein to activate a different PLC (2, 36, 37). The finding that Ptx blocks fMLP-induced Ca\textsuperscript{2+} mobilization in both thrombin-treated and untreated cells indicates that priming is not simply a switch in the coupling of the fMLP receptor from a Ptx-sensitive to a Ptx-insensitive G protein.

The activity of PLC is a likely locus for the priming event because phosphoinositide hydrolysis stimulated by peptide chemoattractants is enhanced, although the enzyme's substrate level is not. How thrombin primes chemoattractant receptor-mediated phospholipase C activation can only be speculated at present. Thrombin primes responses to chemoattractant receptors that utilize G\textsubscript{βγ} to activate PLC, so modification of either G\textsubscript{βγ} or PLC by thrombin may lead to enhanced generation of inositol phosphates. The γ subunits of heterotrimeric G proteins (G\textsubscript{γ}) are members of a family of proteins that are posttranslationally modified at their carboxyl termini by isoprenylation, proteolytic cleavage, and carboxymethylation (38). This modification of G\textsubscript{γ} is essential for membrane association of G\textsubscript{βγ} and activation of effector molecules such as PLC\textsubscript{β2} (39). Indeed, demethylation of G\textsubscript{βγ}, which does not affect receptor-mediated GTP\textsubscript{γS} binding to G proteins, is at least 10-fold less effective in stimulating PLC\textsubscript{β2} than its methylated counterpart (40). Moreover, fMLP-stimulated carboxymethylation of G\textsubscript{γ2} is associated with neutrophil activation (41, 42). It is therefore possible that thrombin enhances the carboxymethylation levels of G\textsubscript{γ2}, resulting in more efficient stimulation of PLC\textsubscript{β2}. This would explain the observation that thrombin enhances PLC-mediated responses to peptide chemoattractant receptors despite the fact that thrombin partially inhibits their G protein activation, as measured by GTP\textsubscript{γS} binding to membranes.

In human platelets, thrombin causes proteolytic cleavage of the 155-kDa PLC\textsubscript{β3} in a calpain-dependent manner to generate a 100-kDa fragment (43). The demonstration that this and a 110-kDa truncated form of the enzyme display markedly enhanced stimulation by G\textsubscript{βγ} when compared with the native PLC\textsubscript{β3} (43, 44) suggests that priming by thrombin could involve proteolytic cleavage of PLC\textsubscript{β}. In addition, a synthetic peptide corresponding to amino acids 448–464 of PLC\textsubscript{β2} stimulates the activity of this enzyme (45). It has been suggested that the peptide does not stimulate PLC\textsubscript{β2} activity per se but enhances the ability of an already active enzyme to hydrolyze additional substrates. It has not been determined whether thrombin leads to the formation of such a peptide in RBL-2H3 cells. There is evidence that PLC\textsubscript{γ1} possesses a domain within its structure that inhibits the catalytic activity of the enzyme (46). Thus, processes that enhance the ability of PLC to be activated may do so via the release of an inhibitory constraint. Therefore, the ability of thrombin to modify the G\textsubscript{βγ}, cause proteolytic cleavage of PLC\textsubscript{β2}, remove an inhibitory constraint from the enzyme or enhance its activation by other mechanisms could explain the observed specificity for thrombin-in-
duced priming. These possibilities are amenable to future investigations.

The data herein demonstrate that thrombin modifies RBL-2H3 cells to selectively enhance the phospholipase C-mediated responses to peptide chemoattractants (fMLP, C5a, and IL-8). We speculate that the striking selectivity of thrombin for priming peptide chemoattractant receptor-mediated responses is due to its ability to enhance the ligand-stimulated activation of PLCβ2. Of note, we have recently characterized a new form of cross-desensitization among receptors for peptide chemoattractants and suggested that the mechanism of this type of regulation involves inhibition of PLCβ2 activity (9, 47). Thus, modulation of the activity of phospholipases could have a major regulatory role in cross-receptor signaling. Understanding the molecular details of how differential regulation of PLCβ2 activity results in the priming and cross-desensitization of peptide chemoattractant receptors should provide new and important information on the mechanisms controlling receptor regulation.

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