Thrombin Causes Pseudopod Detachment via a Pathway Involving Cytosolic Phospholipase A₂ and 12/15-Lipoxygenase Products

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Abstract
Thrombin causes rapid pseudopod detachment and shortening in Dunning rat prostatic carcinoma (MAT-Lu) cells. As seen by interference reflection microscopy and by immunofluorescence analysis with antibodies to paxillin and talin, the primary event is disassembly of adhesion sites. Biochemically, thrombin is a potent activator of cytosolic phospholipase A₂ and increases eicosanoid production in these cells. The pseudopod effects are blocked by lipoxygenase (but not cyclooxygenase) inhibitors. Arachidonic acid and 12(S)-hydroxyeicosatetraenoic acid or 15(S)-hydroxyeicosatetraenoic acid mimic the thrombin effect. We conclude that in certain cancer cells, thrombin is a pseudopod repellent that exerts its effect via a cascade involving cytosolic phospholipase A₂, 12/15-lipoxygenase, and 12(S)- and/or 15(S)-hydroxyeicosatetraenoic acid.

Introduction
Thrombin has gained renewed attention in recent years as a factor likely to be involved in the regulation of developmental phenomena and morphogenesis. Prothrombin and one of the thrombin receptors are expressed in diverse tissues, and thrombin is known to act as a mitogen on mesenchymal cells (1–4). In the developing nervous system, thrombin functions as an inhibitor of neurite growth and repellent of the nerve growth cone (5–8). This effect first was thought to be the direct consequence of the proteolytic activity of thrombin but later was shown to depend on thrombin receptor activation (8–10). These findings raise the possibility that thrombin acts as a pseudopod repellent on selected cells outside the nervous system, thus inhibiting their motility and, perhaps, helping to maintain tissue boundaries. The goal of the present study is to explore this possibility in a motile cancer cell line and to begin to analyze the mechanisms involved.

The thrombin receptor contains a “tethered ligand” that is unmasked by the proteolytic activity of thrombin (3, 11). The receptor has seven transmembrane domains and acts via heterotrimeric G proteins, but the signaling steps further downstream are less clear and seem to differ for different cell types (3). However, cell shape changes triggered by thrombin and lysophosphatic acid have been shown to involve tyrosine phosphorylation of focal adhesion proteins and the small GTP-binding proteins Rho and Rac (12–14).

A variety of amoeboid systems, such as macrophages, platelets, and nerve growth cones, exhibit high activity of different forms of cPLA₂ and generate high levels of AA (15–18). In platelets, cPLA₂ is activated via the thrombin receptor (19, 20). Also, ras-transfected cancer cells with increased motility exhibit increased cPLA₂ activity (21, 22). This may suggest a role of cPLA₂ and its product, AA, in the regulation of cell motility. The eicosanoid, 12(S)-HETE has long been known to affect leukocyte motility (23, 24) and has been implicated in cancer cell attachment (25, 26). 12-LO is the enzyme that converts AA into 12-hydroxyeicosatetraenoic acid, which is then reduced to 12(S)-HETE (27, 28). Leukocyte-type 12-LO actually generates both 12- and 15-HETE so that it has been renamed 12/15-LO (29). A correlation between metastatic potential and expression levels of 12-LO has been reported (30, 31). Again, these observations may implicate AA and HETEs in the regulation of cell attachment and/or motility, but the mechanisms involved have remained unknown.

We have begun a systematic investigation of a hypothetical mechanism that links receptor-mediated cPLA₂ activation and the generation of HETEs to the control of pseudopod attachment/detachment and, thus, motility in nonneuronal cells. As a model system, we selected a highly motile cancer cell line with long processes, the MAT-Lu subline of Dunning rat prostatic carcinoma (generous gift of Dr. J. T. Isaacs; Ref. 32). We found that thrombin acts as a pseudopod repellent in these cells, via disassembly of adhesion sites, and that it is a strong activator of cPLA₂. A variety of inhibitor experiments indicate that the generation of 12(S)-HETE and/or 15(S)-HETE is necessary for the repellent effect of thrombin.

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The abbreviations used are: cPLA₂, cytosolic phospholipase A₂; AA, arachidonic acid; HETE, hydroxyeicosatetraenoic acid; LO, lipoxygenase; IRM, interference reflection microscopy; TRAP, thrombin receptor activating peptide; NDGA, nordihydroguaiaretic acid; CDC, cinnamyl-3,4-dihydroxy-α-cyanoacetamide; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; DG, diacylglycerol; PLC, phospholipase C.
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Therefore, the present study begins to outline a signaling pathway that links adhesion site disassembly and pseudopod detachment to thrombin receptor activation and eicosanoid synthesis.

Results
MAT-Lu cells are highly motile (32) so that they immediately spread over the culture dish rather than forming clonal colonies. Under our standard culture conditions, they often form multiple, long processes (Fig. 1).

Thrombin Effects on Pseudopod Attachment. The exposure to thrombin of MAT-Lu cells causes process withdrawal within minutes, as shown in Fig. 1, A and B. The effect is reversible (Fig. 1A, 60 min); even without medium replacement, MAT-Lu cells regenerate their processes. The nonproteolytic TRAP mimics the thrombin effect (Fig. 1D) but at about 1000-fold higher concentration, in agreement with data in the literature (3, 33, 34). Fig. 1C shows that under identical conditions of extended microscopic observation, control cells continue to elaborate their processes rather than withdrawing them. Fig. 1B is a time sequence of a representative MAT-Lu cell treated with thrombin and examined at higher magnification. Process withdrawal is evident within 8 min. However, the processes retain their enlarged tips, and ruffling activity, an actin-based phenomenon, continues even as the processes become shortened. This is in contrast to cells treated with 1 μM cytochalasin D, which does not cause process withdrawal but blocks ruffling activity almost immediately (not shown). After longer exposure times (40 min) to thrombin, processes begin to regrow (Fig. 1B).

Process withdrawal may involve actin/myosin-based retraction and/or pseudopod detachment. To address this issue, we: (a) stained control and thrombin-treated MAT-Lu cells for filamentous actin; (b) performed IRM to analyze cell adhesions; and (c) examined the distribution of adhesion site proteins under these conditions. Fig. 2A shows phalloidin-labeled actin cables coursing through a control MAT-Lu cell and into the long processes. Accumulations of filamentous actin also are seen in ruffling edges. After thrombin treatment for 5 min (Fig. 2B) or 10 min (Fig. 2C), redistribution of filamentous actin is evident. Clumping of actin in distal (phase-dense) enlargements of withdrawing processes is commonly observed, whereas more proximal domains seem to be depleted of filamentous actin. The distal actin accumulations may later disappear or not always be evident, as shown in Fig. 2C. Yet, ruffling edges with their characteristic phalloidin staining pattern persist.

IRM has been used widely to image cellular adhesion sites, with the tightest adhesions appearing very dark and intermediate contacts being less dense than background (35–37). This technique was used to study the effect of thrombin on the attachment of MAT-Lu cell processes (Fig. 3). Extensive focal contacts (dark) are seen at the beginning of the experiment (0 min), but progressively these tight contacts give way to intermediate contacts or disappear altogether. Phase contrast images taken at the same time indicate that the cell process does not pull back prior to the adhesion changes. These data suggest that the primary effect of thrombin is a change in adhesion sites rather than retraction followed by passive detachment.

To investigate this issue further, the effects of thrombin and TRAP on adhesion site proteins were studied. In preparation for immunolocalization of the adhesion site proteins, talin and paxillin, we generated Western blots of MAT-Lu cells and of their Triton X-100-resistant adhesion plaques (Fig. 4). These blots reveal single, strongly immunoreactive bands at the appropriate molecular weight in whole cells and Triton X-100-resistant fractions, indicating the presence of both cytosolic and adhesion plaque-associated pools of talin and paxillin. These blots also ascertain specificity of the antibodies used for the immunolocalization experiments described below.

Control and TRAP-treated MAT-Lu cells were stained for paxillin and talin and examined by confocal microscopy. To reveal protein accumulations at adhesion sites, through-focus series are shown in Figs. 5 and 6. In a representative control process in Fig. 5, paxillin staining reveals bright spots of label (typically 0.5–1 μm in diameter), especially at the distal end of the process (middle panel, 2). One μm above and below this optical plane, paxillin spots are weaker. After 4 min of TRAP treatment of a similar cell, most paxillin spots have disappeared. Image planes 0.5 μm above and below exhibit weaker spots, indicating that the z plane does indeed go through the adhesion sites. Analogous experiments were performed with talin antibody (Fig. 6). In a representative control process, focused distal talin labeling is evident. At 4 min of TRAP treatment, however, labeled spots have disappeared. Instead, the level of diffuse background labeling in processes seems to have increased. These results indicate that thrombin and TRAP act on cellular adhesion sites.

Effects of Fatty Acids and Eicosanoids on Pseudopod Length. Because of the stimulation of AA release and eicosanoid synthesis by thrombin (see below), we studied the effects of these compounds on MAT-Lu processes. Fig. 7 shows that at 10 μM, stearic acid has little or no effect, whereas AA initiates pseudopod shortening in MAT-Lu cells within 5 min upon administration. At micromolar concentration, AA mimics the thrombin-induced length reduction within a similar time frame.

AA could itself act on a step downstream in the signaling mechanism that triggers pseudopod withdrawal. Alternately, AA might first be converted into an eicosanoid, a possibility that can be investigated with different inhibitors of AA metabolism. To obtain quantitative data, we performed morphometric assays of pseudopod length. A 15-min pretreatment with the cyclooxygenase inhibitor, indomethacin (10 μM), has no effect at all on AA-induced pseudopod shortening (data not shown). However, NDGA, which blocks LOs nonspecifically at 25 μM (38), interferes with the AA effect (39). By itself, NDGA (25 μM; 15-min pretreatment) has no effect on pseudopod lengths, but it inhibits the pseudopod shortening observed at 10−4 M AA so that control and AA + NDGA samples are not significantly different (P > 0.75; data not shown). Fig. 8 shows the results obtained with CDC, a more specific inhibitor selective for 12-LOs (39). At 63 nM (IC50) and at 126 nM, CDC pretreatment (15 min) reverses the AA effect so that controls and AA + CDC samples are indistinguishable sta-
tistically (whereas the values for AA alone are significantly lower, at \( P < 0.01 \)). These results strongly suggest the involvement of a 12-LO in process shortening. Therefore, subsequent experiments were focused on the 12-LO product of AA, 12(S)-HETE.

The result of 12(S)-HETE application to MAT-Lu cells in culture is shown in Fig. 7. When exogenous 12(S)-HETE (10^{-10} \text{ M}) is applied by medium replacement, pseudopod shortening is evident within \(<12\text{ min}\) and nearly complete for most cells within about 15 min. The observed morphologies

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Fig. 1. Morphological effects of thrombin and TRAP on MAT-Lu cells (phase contrast microscopy). A, group of cells exposed to thrombin for 1, 20, and 60 min. Process withdrawal is evident at 20 min, but regrowth of processes can be seen at 60 min in the presence of 135 nM thrombin (\(\ast\)). B, cell observed at higher power over a period of 40 min in the presence of 135 nM thrombin. While process shortening occurs, ruffling activity persists (arrowheads at 4, 8, and 12 min). New outgrowth is observed at 40 min in the presence of thrombin (\(\ast\)). C, controls observed at 18 and 32 min in the same experimental system. Process outgrowth continues. D, effects of 132 \(\mu\text{M}\) TRAP at \(<2\text{ min}\) and at 10 min. Numbers indicate time in minutes after the onset of the experiment.
are the same as those for thrombin (compare Figs. 1 and 7). Thirty min after withdrawal of 12(S)-HETE by medium replacement (done 30 min after onset of the experiment), pseudopods reappear, indicating reversibility of the eicosanoid effect (data not shown).

Dose responses of pseudopod lengths to different HETEs were measured after 15 min incubation. Raw data for 12(S)-HETE are shown in Fig. 9. As expected, pseudopod lengths do not form a Gaussian distribution, but the data can be fitted with a gamma regression model. The vertical lines in the histograms indicate the mode, the length of the majority of pseudopods. Control pseudopods are up to 140 μm in length, but the distribution’s mode is at about 30 μm only. At low concentrations of 12(S)-HETE (10^{-11} and 10^{-10} M), the mode shifts to a shorter pseudopod length of <20 μm. At ≥10^{-8} M 12(S)-HETE, the mode increases again to >20 μm. The whisker-box plot in Fig. 10A shows more clearly the change in pseudopod length distributions induced by 12(S)-HETE. At 10^{-11} and 10^{-10} M, values are significantly smaller than control (>45% length reduction for the median; \(P < 0.0001\)). The overall curvilinear depressed pattern, with pseudopod lengths returning to control levels at the higher concentrations (10^{-8} and 10^{-6} M), can be fitted with a quadratic polynomial across the log doses –12 through –8 (\(P < 0.0001\)). This curve displays a minimum at log –10.4 M. A time course study of process shortening for a population of 73 identified cells indicated a maximum population effect at 15–20 min upon application of 10^{-10} M exogenous 12(S)-HETE (data not shown). As a control for 12(S)-HETE, we used its regioisomers in the same experiments. 5(S)-HETE at 10^{-10} M has no effect on pseudopod length (data not shown). However, like 12(S)-HETE, 15(S)-HETE reduces process lengths of MAT-Lu cells in a biphasic manner (Fig. 10B). The effect is significant (\(P < 0.0001\)) and peaks at log –10.0 M. In summary, 12(S)- and 15(S)-HETE cause a rapid and dramatic biphasic reduction in pseudopod length, with the maximum effect observed between 10^{-11} and 10^{-10} M, whereas 5(S)-HETE is inactive.

Phospholipase Activity and Eicosanoid Generation in MAT-Lu Cells. The results of the behavioral and morphometric studies suggested that thrombin stimulates cPLA\(_2\) in MAT-Lu cells. Therefore, we measured AA release from exogenous 14C-AA-PE, 14C-AA-PC, and 14C-AA-PI. Although AA release from PI is higher than from the other substrates

![Fig. 2. Phalloidin-labeled filamentous actin in control (A) and experimental MAT-Lu cells treated with 135 nM thrombin for 5 (B) and 10 (C) minutes. Phase contrast images show dense distal processes characteristic of thrombin-induced shortening.](image)
under resting conditions, stimulation with thrombin causes a massive increase (for PC, >100-fold) in AA release, to about the same molar levels for all three substrates (Fig. 11, A and B). Under the same conditions of thrombin stimulation, we also looked for changes in \(^{14}\text{C}-\text{AA-DG}\) release from \(^{14}\text{C}-\text{AA-PI}\) (Fig. 11D). Under resting conditions, DG release from PI is much higher than that of AA, but thrombin actually reduces DG release rather than stimulating it. There is essentially no DG release from PE and PC, as expected. To test for the possible involvement of secreted PLA\(_2\), we performed PLA\(_2\) assays in the presence of reducing agent (5 mM DTT), which inhibits secreted PLA\(_2\) but leaves cytosolic PLA\(_2\) unaffected (40). As shown in Fig. 11B, DTT does not significantly inhibit the measured PLA\(_2\) activity. The dose response of cPLA\(_2\) activity to thrombin is shown in Fig. 11C for the PE substrate.

The inhibition of the thrombin effect on MAT-Lu pseudopods by NDGA and CDC suggests the involvement of a 12-LO. Therefore, we analyzed lipid extracts of MAT-Lu cells incubated with \(^{14}\text{C}-\text{AA-PC}\) for eicosanoid generation. The result is shown in Fig. 11E. AA-derived radioactivity that coextracts and comigrates in TLC with 12-HETE or 15-HETE is detectable in control cells. Radioactivity levels are nearly doubled upon stimulation of the cells with thrombin for 10 min. Consistent with this result, Western blots of MAT-Lu extracts probed with an antibody to leukocyte-type 12/15-LO (not shown) revealed strong immunoreactivity comigrating with the cognate enzyme in macrophages.

**Discussion**

**Thrombin Effects on MAT-Lu Cell Behavior.** Within <5 min, thrombin causes dramatic shortening of cellular processes. TRAP mimics the effect and, thus, confirms that this is a receptor-mediated phenomenon. Receptor identity is unclear, however. Thrombin dosages used in these experiments and necessary for full PLA\(_2\) activation are in the range of 100 nM, as opposed to ≤1 nM typically used for platelet activation. Yet, TRAP concentrations needed are similar to those used for platelets (in the 100 μM range). Several explanations are possible: (a) a thus far unidentified receptor
(but not PAR-2, which is thrombin-insensitive; Ref. 41) may be responsible (4, 42); (b) in MAT-Lu cells, the cloned thrombin receptor could be modified posttranslationally, e.g., by different glycosylation, which is known to affect its affinity (43); or (c) one or more of the many proteins known to interact with thrombin may affect its receptor binding in our experimental system (44).

Thrombin- or TRAP-induced shortening of cellular processes is accompanied by redistribution and, perhaps, partial depolymerization of filamentous actin, as shown by phalloidin labeling. IRM demonstrates loosening of tight adhesion sites by thrombin as an early event, prior to pseudopod withdrawal. The actin-depolymerizing drug, cytochalasin D, rapidly blocks ruffling activity. In thrombin-treated MAT-Lu cells, however, ruffling activity continues, indicating that the actin cytoskeleton is not the primary target of thrombin action. Rather, adhesion site changes seem to be involved. Our Western blots demonstrate substantial levels of talin and paxillin in Triton X-100-resistant fractions of attached MAT-Lu cells and confirm biochemically the presence of focal adhesions. Such adhesion sites also are evident by immunofluorescence in control cells. Within minutes of thrombin or TRAP application, however, talin and paxillin staining dissipates, and focal adhesions are greatly reduced in number. These observations demonstrate that thrombin and TRAP trigger the disassembly of adhesion sites. The overall effect is very similar to that observed with nerve growth cones (8). In other words, thrombin acts as a repellent for MAT-Lu cells.

In the nervous system, repellents are known to play a critical role in neurite pathfinding (45). Little is known, however, about pseudopod repellent action in nonneural cells. Repellents may be important to block cell migration into other tissues under normal conditions. It is of interest that levels of 12-LO, which seem to be involved in thrombin signaling (see below), are increased in cancer cells with increased metastatic potential (31). Therefore, behavioral changes induced by repellents are likely to be of major significance in normal biology and cancer.

Thrombin Activation of PLA₂. Our analysis of reaction products from radiolabeled PI substrate includes not only AA but also DG. In contrast to platelets where it stimulates PLC, thrombin decreases the level of radiolabeled DG compared with controls and, therefore, does not activate.
PLC in MAT-Lu cells. AA release from PI is stimulated about 18-fold by thrombin, and it is increased 36- or 113-fold, respectively, when PE or PC is used as substrate. However, the molar levels of AA released from these exogenous substrates at maximum thrombin stimulation are about the same. In principle, this effect could be: (a) attributable to the release of secreted PLA₂ (not very likely because the assays were performed on pelleted cells); or (b) it could be the result of cPLA₂ activation. Resistance to reducing agent and activity at low calcium levels (100 μM) indicate involvement of cPLA₂ rather than a secreted PLA₂ (40). Several forms of cPLA₂ have been characterized to date (40, 46). The substrate selectivity we observed is consistent with the activation of a PI-selective enzyme observed previously in nerve growth cones (8, 18), together with cPLA₂-85, the PE- and PC-selective M₄.

Fig. 6. Confocal microscopy of control and TRAP-treated (132 μM; 4 min) MAT-Lu cell processes, labeled with anti-talin. Control shows the focal adhesion plane with brightly labeled distal sites. The z-axis series (–0.8 μm, +0.8 μm) of the TRAP-treated process shows absence of focal adhesions in this process. Bar, 10 μm.

Fig. 7. Effects of exogenous stearic acid and AA and of 12(S)-HETE on MAT-Lu processes. AA and 12(S)-HETE mimic the effect of thrombin. Numbers indicate time in minutes after the onset of the experiment.
85,000 enzyme that requires low levels of calcium and has been cloned and sequenced (47, 48), but this remains to be demonstrated.

If cPLA₂ is causally involved in pseudopod withdrawal, then its inhibition ought to block the effect of thrombin. We cannot do this experiment because we have not yet identified a specific inhibitor that blocks selectively and completely the cPLA₂ activities in these cancer cells. However, if cPLA₂ is involved in the pathway leading to pseudopod shortening, then one of the products of the enzyme, AA or a lysophospholipid, should have the same effect. Indeed, micromolar AA mimics thrombin (but SA used as a control does not). Furthermore, inhibitors of 12-LOs, especially CDC, neutralize the process-shortening effect of thrombin. This supports strongly the causal involvement of cPLA₂ because eicosanoid synthesis depends on the cellular supply of AA (27, 28).

**Role of Eicosanoids in Thrombin Signaling.** The thrombin-like effect of AA on pseudopod length could be attributable to its direct interaction with a downstream effector, or it could occur indirectly, via a metabolite. The cyclooxygenase inhibitor, indomethacin, does not interfere with AA-induced pseudopod shortening. In contrast, the LO blockers NDGA and CDC inhibit the AA effect completely. At the concentrations used, CDC is quite selective for 12-LOs, suggesting that 12-HETE is necessary for pseudopod shortening. This is supported by the already mentioned fact that CDC blocks the effects of thrombin.

Complementary biochemical analyses did indeed demonstrate that thrombin stimulates the synthesis of an AA-derived compound(s) that seems identical to 12-HETE and/or 15-HETE. That the increase is not as great as that for AA may be explained as follows: (a) The HETE levels for controls are very low and difficult to dissociate from background radioactivity so that the actual increase may be greater; and (b) 12/15-LO activity appears to be rate-limiting so that only a small fraction of AA is converted into eicosanoid. Consistent with the finding of 12-HETE and/or 15-HETE generation, Western blots demonstrate the presence...
of significant amounts of leukocyte 12/15-LO in MAT-Lu cells (data not shown).

Although the 12-LO inhibitor, CDC, inhibits the repellent effect of thrombin, the products of 12/15-LO, 12(S)-HETE, and/or 15(S)-HETE replicate the thrombin effect at very low concentrations (10\(^{-11}\) to 10\(^{-10}\) M). Thus, the MAT-Lu response to these eicosanoids seems to be similar to the 12(S)-HETE-elicited retraction of endothelial cells (49). Another HETE isomer, 5(S)-HETE, causes no change in pseudopod length. These data indicate potent, regioisomer-specific action of 12(S)-HETE and 15(S)-HETE on the MAT-Lu pseudopods. The unusual biphasic response observed for both could be the result of a feedback loop downstream of HETE, or it could be the result of the direct, nonlinear interaction with the target molecule.

Honn et al. (31) have suggested that cellular 12(S)-HETE acts via a receptor, the activation of PLC, and the stimulation of PKC by the released DG. Our unpublished observations do indeed indicate the involvement of PKC in the effect of 12(S)-HETE on pseudopod length. However, the mechanism

Fig. 10. Whisker-box plots of pseudopod lengths of control cells and cells exposed for 15 min to different concentrations of various HETE isomers. For explanation of the plot, see Fig. 8. The statistical analysis of the data is described in the text.

Fig. 11. Thrombin activation of phospholipases and LO in MAT-Lu cells. A, the release, in the presence or absence of 200 nM thrombin, of AA (PLA\(_2\) activity) from \(^{14}\)C-AA-PI, \(^{14}\)C-AA-PE, or \(^{14}\)C-AA-PC used as substrates. B, thrombin-stimulated (200 nM) PLA\(_2\) activity as fold increase above control, in the presence or absence of the reducing agent DTT, which inactivates secreted but not cytosolic enzyme, for the three substrates. C, the dose response of PLA\(_2\) activation by thrombin, as AA release from PE. D, the release, in the presence or absence of 200 nM thrombin, of DG (PLC activity) from \(^{14}\)C-AA-PI, \(^{14}\)C-AA-PE, and \(^{14}\)C-AA-PC used as substrates. E, the formation of an AA-derived compound comigrating with 12-HETE and/or 15-HETE in the presence or absence of 100 nM thrombin. The substrate in these assays was \(^{14}\)C-AA-PC. All assays were run in triplicate. Bars, SD; where not present, bars were too small to be indicated.
must be different because: (a) rather than increasing DG release (which would be expected if that pathway were oper-ative), thrombin actually inhibits DG release in MAT-Lu cells; and (b) the maximum effect with 12(S)-HETE is ob-
served at 10−10.4 M, a concentration considerably below the K_D estimated for the putative 12(S)-HETE “receptor” (≥4.4 × 10−10, Refs. 50 and 51). Thus, the mechanism of PKC act-
viation by 12(S)-HETE remains to be elucidated.

Conclusions. Our observations indicate that thrombin may act on certain cell types, such as the MAT-Lu cells, or their pseudopods in a manner comparable with that of re-
pellents on nerve growth cones. The data indicate that cPLA2 may act on certain cell types, such as the MAT-Lu cells, or
HETE are sufficient for the repellent effect. Therefore, the repellent effect is mediated by cytosolic forms of PLA2 and
and the generation of 12- and/or 15-HETE, presumably by leuk-
ocyte 12/15-LO. Stimulation of this cascade triggers disas-
sembly of adhesion sites and pseudopod detachment. In
more general terms, our observations suggest the operation of repellent mechanisms in nonneural vertebrate tissues.
Hypothetically, thrombin and other repellents may be impor-
tant in all tissues to keep cells within their domains. If correct, this new concept is of significance for our understanding of the maintenance of normal tissue boundaries and their breakdown in invasive/metastatic disease.

Materials and Methods
Cell Culture. MAT-Lu cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml each of penicillin and streptomycin, and 250 μg dexamethasone, in 5% CO2 in air at 37°C, as described by Isaacs et al. (32). Cells were monitored morphologically to ascertain the constancy of the phenotype. For one day prior to experimentation, cells were transferred to medium containing 1% serum.

Microscopic Analyses. MAT-Lu cells were grown on laminin-coated and serum-quenched glass coverslips. Prior to experimentation, cells were first transferred to medium with 1% serum as stated above and then to serum-free medium overnight. For phase-contrast observations, the culture medium was changed to medium containing the reagent of interest (or control), the cultures were covered with a thin layer of paraffin oil to avoid evaporation and pH shift, and the dishes were transferred immedi-
ately to the heated stage of an inverted microscope (Zeiss IM35) and examined. These manipulations took from 40 to 80 s.

For IFM (35–37), MAT-Lu cells, grown on laminin-coated glass cover-
slips as described and mounted on a heated chamber (CoverWell; Grace Bio-Labs, Sumriver, OR), were analyzed with an appropriately equipped Zeiss Axioskop microscope. At different intervals after infusion of the appropriate reagent into the chamber, phase contrast and IRM pictures were recorded.

For immunofluorescence, cultures grown and treated as above were challenged with thrombin or a nonproteolytic TRAP, a hexapeptide con-
ducting the maintenance of normal tissue boundaries and their breakdown in invasive/metastatic disease.

Western Blots of Adhesion Plaque Proteins. Adhesion plaques were obtained from subconfluent MAT-Lu cells. Briefly, the cultures were rinsed twice with cold, modified Krebs buffer [220 mM sucrose, 50 mM NaCl, 5 mM KCl, 2 mM HEPES, 10 mM glucose, 1.2 mM NaH2PO4, and 1.2 mM MgCl2, pH 7.3], and then scraped off the plates and counted in a scintillation spectrometer (Beckman). In some experiments, especially those involving 12- and/or 15-LO, the DG-containing band (identified by comigration with AA standard) also was scored and counted to measure PLC activity.

To measure LO activity, cells were prepared as for PLA2 assays, preincubated with thrombin for 10 min on ice, and then incubated with radiolabeled phospholipid substrate as described above. Reaction products were extracted as described by Birke et al. (53), spotted on precoated silica gel 60 thin-layer plates, and the plates were develop-
ed in the upper phase of ethyl acetate/isooctane/acetic acid/water (100:60:20:100). The HETE product was identified by comigration with standard, scraped into scintillation fluid, and counted.

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To measure LO activity, cells were prepared as for PLA2 assays, preincubated with thrombin for 10 min on ice, and then incubated with radiolabeled phospholipid substrate as described above. Reaction products were extracted as described by Birke et al. (53), spotted on precoated silica gel 60 thin-layer plates, and the plates were develop-
ed in the upper phase of ethyl acetate/isooctane/acetic acid/water (100:60:20:100). The HETE product was identified by comigration with standard, scraped into scintillation fluid, and counted.

**Western Blots of Adhesion Plaque Proteins.** Adhesion plaques were obtained from subconfluent MAT-Lu cells. Briefly, the cultures were rinsed twice with cold, modified Krebs buffer [220 mM sucrose, 50 mM NaCl, 5 mM KCl, 22 mM HEPES (pH 7.3), 10 mM glucose, 1.2 mM NaH2PO4, 1.2 mM MgCl2, and 1 mM EGTA] and then extracted with lysis buffer [1% Triton X-100, 0.3 M sucrose, 3 mM MgCl2, 10 mM EGTA, 0.1 mM aprotinin, 0.1 mM leupeptin, 1 mM 4-(2-aminoxy)-benzenesulfonyl fluoride, and 20 mM HEPES, pH 7.3] for 10 min on ice to remove all proteins, except the adhesion complexes and associated cytoskeletal elements bound to the substratum (protease inhibitors were from Sigma). Adhesion plaques at-
tached to the dish were collected in 5% SDS, 2 μM DTT. Both fractions were precipitated with chloroform-methanol and redissolved in a small amount of SDS/DDTT. A modified Lowry assay was used to determine total protein [54].

Proteins from each sample (30 μg/lane) were resolved by SDS-PAGE (55) on 7.5% acrylamide gels, together with prestained standards to determine apparent molecular mass. Resolved proteins were transferred to nitrocellulose essentially as described by Towbin et al. (56), with a semidy blotting apparatus for 30 min at 300 mA. Ponceau S staining was used to monitor the efficiency of protein transfer. Blots were probed in the PBS and distilled water and dried. Prior to incubation with primary antibody, the blots were blocked with 5% nonfat milk powder and 0.2% Tween 20 in PBS for 2 h at room temperature. Blots were probed in the same blocking solution and conditions for all antibodies used. Antibody concentrations were 1:100 for anti-talin and 1:2000 for anti-panxillin. Blots were washed five times in blocking solution, followed by incubation with secondary antibody (horseradish peroxidase-conjugated goat antimouse; 1:2000) for 1 h in blocking solution. After extensive washing, bound antibody was detected by enhanced chemiluminescence according to the manufacturer’s directions (New England Nuclear, Boston, MA) by contact-exposing X-ray film (Kodak X-OMAT BLUE XG-1).

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**References**


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