Intercellular Gap Formation Induced by Thrombin in Confluent Cultured Bovine Retinal Pigment Epithelial Cells

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Purpose. Thrombin is formed at the site of intraocular hemorrhage and may be important in the development of progressive retinal damage. The authors observed that thrombin-treated bovine retinal pigment epithelial (RPE) cell cultures develop intercellular gaps and initiated this study to examine in detail the effects of thrombin on RPE cell morphology, adhesion, and cytoskeleton.

Methods. Confluent cultures of bovine RPE cells were incubated for various times (0 to 24 hours) with α-thrombin (0.1 to 100 U/ml) or enzymatically inactive thrombin. Intercellular gaps were quantitated by light microscopy in ten representative fields (magnification ×400) as number of gaps per field (gaps/f). RPE cytoskeleton was studied using immunofluorescent staining for vinculin and F-actin. The mechanism of thrombin-induced RPE cell gap formation was studied by preincubation with specific drugs, including a protein kinase inhibitor (staurosporine), protein kinase C inhibitors (H-7 and calphostin C), cyclic adenosine monophosphate (cAMP) inducer (forskolin), and cytoskeleton-disrupting agents (cytochalasin B or colchicine).

Results. Intercellular gaps (20 to 80 μm in diameter) were markedly increased in number in thrombin-treated cultures in a dose-dependent and time-dependent manner and were associated with an alteration in the distribution of F-actin and vinculin. Whereas control cultures showed 3.3 ± 2.4 gaps/f, incubation with 8 U/ml of α-thrombin for 3 hours resulted in 44.8 ± 15.3 gaps/f. These changes were most prominent shortly after the 3-hour coincubation, but the cultures did return to their original confluent state within 24 hours. Cultures treated with an enzymatically inactive thrombin showed fewer intercellular gaps than those treated with enzymatically active thrombin but had significantly more intercellular gaps than control cultures. Thrombin-induced intercellular gap formation was blocked by preincubation with forskolin (14.6 ± 7.1 gaps/f), staurosporine (10.2 ± 5.0 gaps/f), or H-7 (24.5 ± 9.8 gaps/f).

Conclusions. Exposure to an enzymatically active thrombin results in formation of intercellular gaps between cultured RPE cells. Inhibition of this phenomenon by protein kinase inhibitors and by a cAMP inducer suggests that this effect is mediated, at least in part, through protein kinase C- and cAMP-dependent pathways. Thrombin generation associated with intraocular hemorrhage may thus result in direct damage to the RPE monolayer, possibly via the same pathway(s). Invest Ophthalmol Vis Sci. 1994;35:720-729.
a product often found at the site of hemorrhage. In addition, thrombin exerts many other effects on diverse cell types, including bone marrow-derived cells, fibroblasts and endothelial cells; its effects include induction of cellular proliferation, release of growth factors, prostaglandin synthesis, chemotaxis and contractility. Furthermore, it has been reported recently that thrombin stimulates RPE cells to proliferate and activate inositol triphosphate. We observed that thrombin-treated RPE cells in culture develop intercellular gaps, and initiated this study to determine more precisely the effects of thrombin on cultured RPE cell morphology, intercellular adhesion, and cytoskeleton.

METHODS

Thrombin

Bovine a-thrombin was purified from commercially available thrombin (Sigma Chemical Co., St. Louis, MO), as described previously, using anion-exchange chromatography and gel filtration chromatography (Kabi-Pharmacia, Uppsala, Sweden). The specific activity of the a-thrombin was about 3000 U/mg. Enzymatically inactive thrombin, diisopropylfluorophosphate (DIP)-thrombin, was made from a-thrombin and DIP (Sigma), as previously described. The specific activity of DIP-thrombin was 0.1 U/mg. A specific thrombin blocker, hirudin, was purchased from Sigma. The enzymatic activity was evaluated by chromogenic assay using the S-2238 kit (Kabi-Pharmacia). To avoid an osmolarity effect, purified a-thrombin was extensively dialyzed with modified Eagle’s medium (MEM). Trypsin (type 1, bovine pancreas (Sigma), specific activity 10,600 U/mg) was also used as a positive control for serine protease activity.

Cell Culture

Bovine eyes were obtained from a local slaughterhouse and the RPE cells collected by a method described previously. Briefly, the eye was bisected and the anterior portion resected, leaving the intact optic cup. After resection of the retina, medium containing 0.1% trypsin and 0.02% ethylenediamine tetra-acetate (Sigma) was instilled into the eye cup and incubated at either 37°C or 4°C in MEM containing 0.35% bovine serum albumin and thrombin or DIP-thrombin at the indicated concentrations. The incubation time varied from 30 minutes to 24 hours. RPE cells incubated with MEM containing 0.35% bovine serum albumin only were used as controls. Hirudin (100 U/ml) was incubated simultaneously with 10 U/ml of thrombin. Trypsin (0.1 to 100 U/ml, 15 minutes to 4 hours) was tested also in place of thrombin.

Evaluation of Intercellular Gaps

 Cultures were observed and photographed were taken with a phase-contrast microscope (Zeiss, Ober Kochen, Germany). Intercellular gaps, each with a diameter of 20 to 80 μm, were quantitated in ten representative fields (as gaps per field [gaps/f]) at a magnification of ×400. Each experiment was performed in duplicate.

Stimulation of Confluent RPE Cells with Thrombin or DIP-Thrombin

To stimulate RPE cells, the confluent cultures were washed three times with MEM and incubated at either 37°C or 4°C in MEM containing 0.35% bovine serum albumin and thrombin or DIP-thrombin at the indicated concentrations. The incubation time varied from 30 minutes to 24 hours. RPE cells incubated with MEM containing 0.35% bovine serum albumin only were used as controls. Hirudin (100 U/ml) was incubated simultaneously with 10 U/ml of thrombin. Trypsin (0.1 to 100 U/ml, 15 minutes to 4 hours) was tested also in place of thrombin.

Pretreatment Before Thrombin Exposure

To elucidate the mechanism of thrombin-induced change, RPE cells were incubated with the following drugs just before thrombin exposure: cAMP inducer forskolin (1 or 10 μM for 40 minutes, Sigma); the analogue of forskolin, 1,9-dideoxyforskolin (1 or 10 μM for 40 minutes, Sigma), which does not bind to adenylate cyclase but which does mimic other functions of forskolin; protein kinase inhibitors staurosporine (10 or 100 nM for 40 minutes, Boehringer Mannheim, Indianapolis, IN), H-7 (5 or 50 nM for 40 minutes, LC Services Corp., Woburn, MA); and calphostin C (10 or 100 nM for 40 minutes under a light, LC Services); the microtubule-disrupting agent colchicine (0.1 or 0.5 μM for 5 hours, Sigma); the microfilament-disrupting agent cytochalasin B (0.005 or 0.05 ng/ml for 30 minutes, Sigma); and, as a control, the inactive analogue of a PKC inhibitor, HA-1004 (5 or 50 nM for 60 minutes, LC Services).
Distribution of Vinculin and Actin Filaments

RPE cells were fixed with 8% paraformaldehyde and washed three times with phosphate-buffered saline (PBS) containing 0.1% saponin (Sigma). Monoclonal antibody against vinculin (Biomakor, Rehovot, Israel; dilution 1:100) was added and the cells incubated for 1 hour at 37°C. After washing three times with PBS containing saponin, the cells were incubated with fluorescein-conjugated antimouse immunoglobulin antibodies for 1 hour at 37°C. After three more washes with PBS, the cells were reacted with rhodamine conjugated phalloidin (Sigma, dilution 1:200) for 1 hour at 37°C, washed three more times with PBS, mounted with aqueous mounting medium (Crystal Mount, Biomedica, Foster City, CA) and observed with a fluorescence microscope (Zeiss). As a negative control, normal mouse serum (diluted 1:1000 in PBS) was used instead of the primary antibody or rhodamine-phalloidin. All procedures, except for the incubation of antibodies, were performed at room temperature.

Ultrastructural Analysis

For transmission electron microscopy, RPE cells seeded on sterile glass cover slips (Nunc) were fixed with buffered 4% glutaraldehyde solution, post-fixed in 1% osmium tetroxide, dehydrated, and embedded in epoxy resin. Thin sections were stained with uranyl acetate–lead citrate. For scanning electron microscopy, specimens were critical point dried using evaporated liquid carbon dioxide and gold after dehydration. Observations were made using an Hitachi S-570 (Hitachi, Tokyo, Japan) or EM10 A/B (Zeiss) electron microscope.

RESULTS

Intercellular Gaps

Effect of Thrombin. In control cultures, the RPE cells proliferated in a monolayer with a cobblestone appearance; they were in contact with each other and displayed few intercellular gaps (Fig. 1A). After stimulation with α-thrombin the intercellular gaps were dramatically increased in number (Fig. 1B). Intercellular gap formation was most prominent between 3 and 6 hours of incubation; the RPE cells did, however, return to their original configuration within 24 hours (Fig. 2). The intercellular gap formation induced by thrombin was dose-dependent (Fig. 3). Significantly fewer intercellular gaps were formed when RPE cells were incubated with Dip-thrombin, thrombin + hirudin or thrombin at 4°C (Fig. 4). There was no apparent change in RPE cells treated with 0.1 U/ml trypsin. In cultures treated with 1 U/ml trypsin, however, intercellular gaps began to form after 15 minutes and then grew progressively larger; most of the cells started to detach and to float up in the medium after about 30 minutes. Higher concentrations of trypsin (4 U/ml) also resulted in detachment of cells from the culture dish within 30 minutes.

Effect of Preincubation with Various Drugs. Preincubation with forskolin or staurosporine reduced significantly the number of intercellular gaps formed by α-thrombin alone (α-thrombin, 44.8 ± 15.3 gaps/f; forskolin, 14.6 ± 7.1 gaps/f; staurosporine 10.2 ± 5.0 gaps/f, P < 0.001) (Fig. 5). Calphostin C and H-7 also reduced the number of gap formations (calphostin C, 26.5 ± 11.9 gaps/f; H-7, 24.5 ± 10.8 gaps/f, P < 0.01), but HA-1004 (51.0 ± 14.9 gaps/f) and 1,9-di-

FIGURE 1. Phase-contrast micrographs of RPE cells. (A) In control cultures, RPE cells showed a cobblestone appearance, and intercellular gaps were few in number. No gaps are present in the illustrated control culture field. (B) After α-thrombin stimulation (4 U/ml for 3 hours), intercellular gaps (arrow) were readily apparent. (Original magnification ×30.)
Thrombin-Induced RPE Intercellular Gaps

FIGURE 2. Time course of intercellular gap formation of RPE cells induced by thrombin stimulation. The intercellular gap formation reached its maximum after 3 hours of incubation, and RPE cells returned to their original configuration after 24 hours (α-thrombin, 4 U/ml). Values are expressed as mean ± standard error of ten representative fields.

deoxyforskolin (41.2 ± 16.0 gaps/f) had no blocking effect on the thrombin induced changes. The blocking effect was most prominent after preincubation with staurosporine. Preincubation with high doses of cytochalasin B (0.05 ng/ml) induced intercellular gap formation on its own, so it was impossible to evaluate its effect, if any, on thrombin-induced change. Interestingly, low doses of cytochalasin B (0.005 ng/ml) did block thrombin-induced intercellular gap formation (α-thrombin, 8 U/ml for 3 hours, 61.3 ± 10.4 gaps/f; cytochalasin B and α-thrombin, 19.2 ± 7.5 gaps/f).

FIGURE 3. Dose dependency of thrombin-induced intercellular gap formation by RPE cells. Each culture was incubated for 3 hours. The number of intercellular gaps increased with increasing doses of thrombin. Values are expressed as mean ± standard errors of ten representative fields.

FIGURE 4. Effects of various forms of thrombin. RPE cells were incubated under the indicated conditions. Thrombin (6 U/ml, for 3 hours at 37 °C). Thrombin (6 U/ml) + DIP-thrombin (DIP-Th) (9 × 10⁻⁷ M) were coincubated for 3 hours at 37 °C. DIP-Th (9 × 10⁻³ M, for 3 hours at 37 °C). Thrombin, cold (6 U/ml, for 3 hours at 4°C). Thrombin (6 U/ml) + hirudin (100 U/ml) were coincubated for 3 hours at 37 °C. The formation of intercellular gaps was not prominent with DIP-Th, thrombin under cold conditions, or coincubation with hirudin. Values are expressed as means ± standard errors of ten representative fields. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with thrombin alone. Thrombin + DIP-Th (P < 0.001), DIP-Th (P < 0.05), and thrombin, cold (P < 0.05) are statistically different from control; however, thrombin + hirudin is not.

FIGURE 5. Effect of pretreatment drugs on intercellular gap formation between RPE cells induced by thrombin. α-Thrombin, 4 U/ml, was added just after the incubation with the indicated drugs, and cells were incubated for another 3 hours. (Forskolin: top bar 10 nM, bottom bar 1 nM for 40 minutes; staurosporine: top bar 100 nM, bottom bar 10 nM for 40 minutes; calphostin C: top bar 100 nM, bottom bar 10 nM for 40 minutes; H-7: top bar 50 nM, bottom bar 5 nM for 40 minutes; HA-1004: top bar 50 nM, bottom bar 5 nM for 40 minutes.) Preincubation with forskolin, staurosporine, calphostin C, and H-7 inhibited the intercellular gap formation typically induced by thrombin; inhibition was greatest with staurosporine. HA-1004 did not block the thrombin-induced change. Values are expressed as means ± standard errors of ten representative fields. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with thrombin alone.
FIGURE 6. Double-stained micrographs of RPE cell cytoskeleton. Yellow green: vinculin (FITC-antivinculin antibody); orange: F-actin (rhodamine-phalloidin). (A) Control; (B) α-thrombin stimulation (1 U/ml for 1 hour); (C) α-thrombin stimulation (4 U/ml for 3 hours); (D) α-thrombin stimulation (4 U/ml for 24 hours). In control (A), vinculin plaques were distributed linearly at the cell borders (arrowhead), and F-actin was located mainly at the periphery of the cytoplasm, where it formed a dense peripheral band (arrow). After the stimulation (B, C), both vinculin plaques and F-actin assumed a diffuse pattern. Intercellular gaps were not apparent after 1 hour (B), but they did become apparent after 3 hours (C, double arrows). However, vinculin plaques returned to their original distribution after 24 hours, whereas F-actin staining remained diffuse (D). (Original magnification ×120.)

The microtubule-disrupting agent colchicine (0.5 μM for 5 hours) did not block the thrombin-induced intercellular gap formation (51.2 ± 12.9 gaps/f).

Actin Filament and Vinculin Plaques of RPE Cells

Effect of Thrombin. In control cultures, F-actin was distributed primarily at the periphery of the cytosol forming dense peripheral bands, and vinculin plaques were distributed linearly between the RPE cells (Fig. 6A). After stimulation by α-thrombin, the distribution of F-actin in the cytoplasm became more diffuse. Vinculin plaques assumed a dotlike or random pattern, and were located predominantly at the edge of F-actin filaments (Figs. 6B, 6C). The changes were not noticed after the 15-minute incubation with thrombin, but
were apparent after 30 minutes' incubation, and were maximal after 6 hours. They then returned to their original distribution within 24 hours, although some F-actin filaments remained diffusely scattered (Fig. 6D). The changes were more prominent with increasing concentrations of thrombin (0.1 to 100 U/ml for 3 hours). DIP-thrombin, coincubation with hirudin and α-thrombin, or incubation with α-thrombin under cold conditions did not change the distribution of F-actin or vinculin from that found in control.

Effect of Preincubation with Various Drugs. Incubation with forskolin, staurosporine, H-7, calphostin C, HA-1004 or colchicine, each used separately, had no effect on the distribution of F-actin and vinculin in RPE cells. Thrombin-induced distributional change of actin filament and vinculin was less prominent in cultures preincubated with staurosporine, calphostin C, H-7, or forskolin, than in cultures exposed to thrombin without preincubation. However, thrombin-induced changes occurred even after preincubation with high doses of staurosporine (Figs. 7A, 7B). Neither HA-1004 nor colchicine had any effect on thrombin-induced changes. It was not possible to study the effect of thrombin after preincubation with cytochalasin B because cytochalasin B alone altered the distribution of F-actin and vinculin into a diffuse pattern.

Ultrastructural Study

Scanning electron microscopic (SEM) examination of control cultures showed intimate contact between RPE cells at numerous points along their surfaces, with only very tiny intercellular gaps (<5 μm). After stimulation with α-thrombin (4 U/ml for 3 hours), large intercellular gaps were formed. The appearance of the gaps gave the impression that they were a result of cellular retraction (Figs. 8A, 8B). Transmission electron microscopic studies were performed to determine if intercellular gap formation was associated with degenerative changes in the cytosol. No ultrastructural difference between control and thrombin-treated cells were found in the cytosol (Figs. 8C, 8D).

DISCUSSION

In this study, intercellular gap formation and a change in distribution of F-actin and vinculin plaques was induced by thrombin in confluent cultured RPE cells. These gaps represent a focal enlargement of the extracellular space between confluent cultured RPE cells. Thrombin-induced intercellular gaps have been described in cultures of other cell types and have been reported to be due to a change in cell shape, rearrangement of the cell cytoskeletal elements, and alteration of tight junctions. 20-21 Thrombin is a multifunctional bioregulatory protein that is centrally involved in hemostasis, wound healing, cellular proliferation, growth factor secretion, prostaglandin synthesis, and intercellular gap formation in a variety of cells. 8,9,13,14 Thrombin-induced intercellular gap formation in other cell types has been shown to be mediated by thrombin receptors. 22-23 Although DIP binds to the catalytic active site of thrombin and abolishes the enzymatic activity, it does not block the binding of thrombin to surface receptors. 24 In contrast, hirudin does not inhibit enzymatic activity, but it does block the binding of thrombin to receptors. 25-26 This implies that enzymatic activity of thrombin and its binding are both necessary to this phenomenon. The characteristics of the current phenomenon are similar to those involving active-site-dependent thrombin receptors; it was a temperature-dependent slow reaction, which was spe-
FIGURE 8. Ultrastructure of RPE cells. (A) Scanning electron micrograph of untreated (control) RPE cells shows that the cells lay in contact with each other, and large intercellular gaps were not apparent between cell borders (arrow). (Original magnification X1200; bar 20 μm). (B) Scanning electron micrographs of RPE cells incubated with thrombin (4 U/ml for 3 hours). Large intercellular gaps were apparent between the cells (arrow). (Original magnification X1200, bar 20 μm). (C) Transmission electron micrograph of untreated (control) RPE cells shows that the cells were in contact with each other. Cytosolic organelles were well preserved. (Original magnification X16,000, bar 1 μm). (D) Transmission electron micrograph of RPE cells incubated with thrombin (4 U/ml for 3 hours). The cells were no longer in contact with neighboring cells. Cytosolic organelles were well preserved, however. (Original magnification X16,000, bar 1 μm.)

Specific for enzymatically active thrombin. However, even an enzymatically inactive thrombin (DIP-thrombin) had a minor effect, so the enzymatically inactive-thrombin receptor might play a minor role in this phenomenon. Assay of thrombin receptors in RPE cells will be necessary to elucidate this mechanism. In addition, these changes are reversible, implying that the response is not the result of RPE cell injury and that it may therefore be physiologically important. This contention is further supported by the fact that electron microscopy revealed no cytosolic degeneration in thrombin-stimulated RPE cells. It is known that fibroblasts can internalize or degrade thrombin by receptor-mediated endocytosis and secretion of protease nexin, and that the functional changes induced in fibroblasts by thrombin are mostly reversible. Although RPE cells might function similarly, it is also possible that thrombin acts directly on the cell surface of RPE cells or on some part of the extracellular matrix, because thrombin can cleave extracellular matrix glycoproteins such as laminin and fibronectin. In our study, one of the other serine proteases (trypsin) rapidly induced intercellular gaps, but most cells became detached after 30 minutes. Trypsin may therefore have a dissociating effect on the cell-substratum matrix that is stronger than the cytoskeletal rearrangement. Further studies are needed to determine whether or not RPE cells have thrombin-receptors and to examine the effects of other proteases.

It has also been reported that thrombin changes the distribution of microtubules and microfilaments in endothelial cells. In the current study, however, microtubules did not seem to play a major role in thrombin-induced gap formation in RPE cells, because the microtubule-disrupting agent colchicine could not block the thrombin-induced changes. A similar thrombin-induced rearrangement of actin filaments in vascular endothelial cells was reported by Galdal et al. Actin filaments form a cytoskeletal network that is involved in the preservation of cell shape, cell substratum adhesion, and cell motility, whereas vinculin is involved in the attachment of actin filament bundles to the plasma membrane. Therefore, the effect of thrombin on RPE cell shape may have re-
Thrombin-Induced RPE Intercellular Gaps

It cannot be definitely stated which signal transduction mechanisms are involved in the thrombin-stimulated changes seen in this study. However, some conclusions can be drawn from the current data and the findings of other studies. In the current study, pretreatment with forskolin inhibited the thrombin-induced gap formation, indicating that this phenomenon was transduced in part via a cAMP sensitive pathway. The regulation of cytosolic Ca^{2+} is important in maintaining the cytoskeleton and cell shape, and it has been established that thrombin stimulates the rapid hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP_{2}) and the accumulation of inositol 1,4,5-trisphosphate (IP_{3}) in endothelial cells, which acts as a second messenger within the cell to stimulate Ca^{2+} release from the endoplasmic reticulum.

Indeed, intracellular cAMP inducers, including forskolin, have been reported to decrease PIP_{2} hydrolysis and, consequently, to reduce the concentration of cytosolic Ca^{2+}. Recently, Crook et al.

The IP_{3}/DAG pathway is triggered by receptor mediated activation of phospholipase C. The subsequent formation of IP_{3} and DAG from PIP_{2} forms two separate transduction pathways, and DAG activates protein kinase C (PKC). In our study, the potent protein kinase blocker staurosporine, the relatively specific PKC blocker H-7, and specific PKC blocker calphostin C each blocked the thrombin-induced intercellular gap formation in RPE cells, which indicates that PKC is involved in the transduction system of this phenomenon. PKC plays an important role in the control or modulation of many metabolic processes, and it has been reported that intercellular gap formation in vascular endothelial cells is induced by thrombin via PKC pathways. What is more important is that staurosporine inhibited the thrombin-induced change more potently than did the other two drugs. Staurosporine acts at the ATP binding sites of protein kinases and inhibits the activity of a variety of protein kinases in a nonselective manner, unlike the action of calphos-
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