

Thrombin induces Ca^{2+} -dependent glutamate release from RPE cells mediated by PLC/PKC and reverse $\text{Na}^+/\text{Ca}^{2+}$ exchange

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Purpose: We analyzed the molecular mechanisms leading to glutamate release from rat primary cultures of RPE cells, under isosmotic conditions. Thrombin has been shown to stimulate glutamate release from astrocytes and retinal glia; however, the effect of thrombin on glutamate release from RPE cells has not been examined. Our previous work showed that upon the alteration of the blood–retina barrier, the serine protease thrombin could contribute to the transformation, proliferation, and migration of RPE cells. In this condition, elevated extracellular glutamate causes neuronal loss in many retinal disorders, including glaucoma, ischemia, diabetic retinopathy, and inherited photoreceptor degeneration.

Methods: Primary cultures of rat RPE cells were preloaded with $1 \mu\text{Ci/ml}$ ^3H -glutamate in Krebs Ringer Bicarbonate (KRB) buffer for 30 min at 37°C . Cells were rinsed and super-perfused with 1 ml/min KRB for 15 min. Stable release was reached at the 7th minute, and on the 8th minute, fresh KRB containing stimuli was added.

Results: This study showed for the first time that thrombin promotes specific, dose-dependent glutamate release from RPE cells, induced by the activation of protease-activated receptor 1 (PAR-1). This effect was found to depend on the Ca^{2+} increase mediated by the phospholipase C- β (PLC- β) and protein kinase C (PKC) pathways, as well as by the reverse activity of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger.

Conclusions: Given the intimate contact of the RPE with the photoreceptor outer segments, diffusion of RPE-released glutamate could contribute to the excitotoxic death of retinal neurons, and the development of thrombin-induced eye pathologies.

The RPE is the predominant component of the outer blood–retina barrier (BRB), and plays an essential role in the maintenance of the functional and structural integrity of the neural retina required for visual function. The RPE is involved in the trans-epithelial transport of nutrients, the storage and metabolism of vitamin A derivatives, the renewal of photoreceptor outer segments, and the release of trophic compounds, cytokines, chemokines, and growth factors required for the proper function of the neural retina [1,2].

As a main component of the epithelium that separates the inner eye from the bloodstream, the RPE also contributes to establishing the immune privilege of the eye. Under pathological conditions, which alter the BRB, such as rhegmatogenous retinal detachment, contact with blood-contained thrombin may contribute to the induction of the epithelial-mesenchymal transition (EMT) of RPE cells [3-5], which, in turn, results in the death of retinal neurons and the loss of vision [6-8]. Within the eye, several pathological insults which disrupt the BRB may induce RPE cells to undergo EMT, which has been identified in several proliferative

ocular diseases [9]. Among these ailments, the pathogenesis of proliferative vitreoretinopathy (PVR), a major cause of failure in retinal surgery aimed to the correction of retinal detachment or severe ocular trauma, includes a fibrotic reaction leading to the formation of contractile membranes on both retinal surfaces formed mainly by transformed RPE and glial cells [9]. This process involves EMT through which RPE cells become fibroblastic, proliferate, and acquire the ability to migrate [10].

In addition to the well-known role in hemostasis, the proinflammatory serine protease thrombin, activated upon tissue injury, is involved in the regulation of cell proliferation, invasiveness, and tumor growth [11]. Through the activation of distinct signaling cascades, thrombin may provide cytoprotective effects or lead to cell degeneration [12,13]. Thrombin is present in the brain during primary cerebral hemorrhage, ischemia, or after trauma episodes which disrupt the blood–brain barrier [14,15]. Thrombin also seems to be involved in neurologic complications in HIV [16], and it is accumulated in Alzheimer senile plaques [17]. Despite this evidence, information concerning the signaling mechanisms mediating thrombin actions in the retina is scarce.

Cell responses to thrombin occur via the protease-activated receptors 1, 3, and 4 (PARs) [18], coupled to members of the G-protein families, particularly Gq, Gi,

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and $G_{12/13}$. Through this interaction, thrombin elicits various downstream signaling cascades [12,19]. Thrombin effects are direct, mediated by the activation of PAR signaling, or indirect, triggered by the release of chemokines, growth factors, neurotransmitters, and angiogenic factors [20]. In RPE cells, thrombin activation of PAR-1 induces the release of epidermal growth factor (EGF) [21] and the stimulation of monocyte chemoattractant protein 1 (MCP1) and GRO chemokine expression and release [22].

Glutamate (Glu) has been shown to regulate the proliferation, migration, and survival of several cell types within the nervous system [23,24], and to play a key role in synaptic plasticity and gene expression [25]. However, the excessive release of glutamate is an early and critical event in the Ca^{2+} -mediated death of neurons implicated in neurodegenerative processes associated with ischemia, epilepsy, and other neuropathological conditions [26,27].

Glutamate is the main excitatory neurotransmitter in the radial signaling pathway of the vertebrate retina from photoreceptors to ganglion cells, acting at ionotropic and metabotropic glutamate receptors (GluRs) [28]. Stimulation of GluRs has been shown to induce the release of glutamate from the retina [29], and to promote the excitotoxic death of retinal neurons [30]. In such a system, any additional factor enhancing glutamate efflux from retinal cells will exacerbate the excitotoxic damage.

Retinal photoreceptors lie between the inner retina and the RPE. Efficient photoreceptor function depends on short- and long-term support from the RPE [31]. The apical membrane of the RPE is separated from the plasma membrane of the photoreceptor outer segments by an extracellular space of only 10–20 nm [32]. Thus, glutamate released by RPE cells may influence synaptic transmission at the main signaling pathway of the retina. Although glutamate release from photoreceptors to inner retinal neurons has been extensively documented, the role of glutamate in communicating the RPE with the neural retina is largely unexplored. Thrombin has been shown to stimulate glutamate release from astrocytes and retinal glia in response to ATP and osmotic stress [33–35]. In turn, glutamate promotes ATP release and RPE cell proliferation acting through N-Methyl-D-aspartic acid (NMDA) and metabotropic receptors [31,36,37]. Thus, the autoactivation of glutamate receptors may contribute to the induction of RPE cell transformation and the development of PVR. However, the effect of thrombin on glutamate release from RPE cells is unexplored. We demonstrated that thrombin-induced activation of PAR-1 glutamate release from RPE is protein kinase C (PKC)-regulated and calcium-dependent.

METHODS

Reagents: All reagents used were cell culture grade. Dulbecco's modified Eagle's medium (DMEM), Opti-MEM, Hanks' balanced salt solution (HBSS), trypsin-EDTA, fetal bovine serum (FBS), penicillin, streptomycin, and dispase were obtained from Invitrogen (Carlsbad, CA). Bovine serum albumin (BSA) was from Equitech-Bio Inc. (Kerrville, TX). Thrombin, hirudin, KN-93, and Ro-32-0432 were from EMD Millipore (Burlington, MA). Ser-Phe-Leu-Leu-Arg-Asn-amide trifluoroacetate salt (SFLLRN), aminoxyacetic acid (AAOA), L-methionine sulfoximine, EGTA, ryanodine, dantrolene, and wortmannin were obtained from Merck KGaA (Darmstadt, Germany). L-trans-2, 4-L-trans-Pyrrolidine-2,4-dicarboxylic acid (PDC), U73122, U0126, and KB-R7943 were from Tocris Bioscience (Bristol, UK). 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA AM) was obtained from Molecular Probes (Eugene, OR). D-Phenylalanyl-prolyl-arginyl Chloromethyl Ketone (PPACK) was from Enzo Life Sciences (New York, NY).

Cell culture: RPE cells were isolated as previously described [36]. Briefly, 8- to 10-day-old Long Evans rats (RRID:RGD_2308852) were anesthetized with pentobarbital (100 mg/kg) and euthanized following the Animal Care and the Guidelines for Animal Care of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and Mexican NOM-062-ZOO-1999. Eyes were enucleated, rinsed in DMEM, containing 100 U/ml penicillin and streptomycin, and incubated in DMEM containing 1% v/v dispase for 30 min at 37 °C. The RPE was detached from the neural retina, the choroid, and the sclera in calcium- and magnesium-free HBSS. The tissue was then incubated in the presence of 0.1% trypsin-EDTA, for 5 min at 37 °C. The cells were mechanically dissociated using a Pasteur pipette. Trypsin digestion was stopped with the addition of 4% FBS. Cells were separated with centrifugation at 195 ×g for 5 min, suspended in Opti-MEM containing 4% FBS, and seeded onto six-well culture plates at 2×10^5 cells/cm² density. After 4 days in the presence of FBS, the medium was replaced with serum-free Opti-MEM for 24 h to synchronize the cells. All experiments were performed in confluent, passage 1 cultures 2 weeks after seeding.

Immunocytochemistry: RPE cells from the primary cultures were seeded on 22 mm glass coverslips and allowed to reach confluence for 2 weeks with the medium (Opti-MEM containing 4% FBS) changed every 4 days. Cells were fixed in 4% paraformaldehyde for 10 min, washed with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4), and permeabilized with 0.1% Triton X-100 for 5 min,

and then blocked for 1 h with either PBS-Tween-0.05%, FBS 10% (for zonula occludens 1 [ZO-1]), PBS-Tween-0.05%, FBS 10%, 1% BSA (for RPE), and PBS-casein 0.5%-saponin 0.1% (for CRALPB). Primary antibody was incubated overnight at 4 °C in blocking buffer. For ZO-1 (Santa Cruz, Santa Cruz, CA, Cat No sc33725), the antibody was diluted 1:50, RPE65 (Novus Biologicals, Centennial, CO, Cat No NB100355) 1:250, and CRALPB (Abcam, Cambridge, UK, Cat No 15051), 1:50. Fluorescein isothiocyanate (FITC)-labeled secondary antibodies were incubated in blocking buffer 1:100 for ZO-1 and cellular retinaldehyde-binding protein (CRALBP); 1:500 for RPE65. The cells were washed three times with PBS for 5 min. Samples were mounted with Dako Fluorescence Mounting Medium (Dako North America, Inc., Carpinteria, CA), and visualized using a microscope Eclipse TE 2000-U. Images were acquired with a DXM1200F camera and 40X objective (0.6 NA) using ACT-1 software (All from Nikon, Tokyo, Japan).

Release experiments: Cultures of rat RPE cells were incubated in the presence of the glutamate decarboxylase inhibitor amino-oxyacetic acid (1 mM), methionine sulfoximine (0.5 mM), and reuptake inhibitor 1-transpyrrolidine-2,-4-dicarboxylic acid (trans-PDC) 50 μ M for 30 min before loading with 1 μ Ci/ml 3 H-glutamate in Krebs Ringer Bicarbonate (KRB; 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.17 mM MgSO₄, 25 mM NaHCO₃, 5.6 mM glucose, pH 7.4) solution for 30 min at 37 °C. Cells were rinsed and superfused with KRB, and samples of 1 ml were collected every minute for 10–15 min. Once stable release was attained, stimulus was applied in fresh KRB. The values in the bar graphs represent the percentage of 3 H-Glu released on the first minute following stimulation, compared to the value in an unstimulated control. The results in the line graphs represent the percentage of 3 H-Glu released each minute compared to the total 3 H-Glu incorporated in the cells. When inhibitors were used, they were included 30 min before thrombin or SFLLRN stimulation.

Statistical analysis: The paired Student *t* test was applied for statistical analysis, using the Prism V7.0 program (GraphPad Prism, RRID:SCR_002798). Results are expressed as the mean \pm standard error of the mean (SEM) of at least three independent experiments.

RESULTS

RPE cell morphology and identity: RPE cells in culture are considered a suitable model in which dedifferentiation and proliferation of these cells can be studied [38]. Previous studies conducted by our group showed some of the biochemical features of cultured RPE cells in response to thrombin

and the glutamate receptors expressed by these cells [22,36]. To confirm the identity of the cultured cells, we tested the expression of RPE65. RPE-specific 65 kDa protein (RPE65) is a critical enzyme in the vertebrate visual cycle that catalyzes the conversion of all-trans-retinyl esters to 11-cis-retinol during phototransduction. RPE65 expression is considered a hallmark of RPE cells [39], and as shown in Figure 1A, it is widely expressed in these cultures. CRALBP also plays an important role in the visual cycle, and it is considered a marker for RPE cells [39]. As shown in Figure 1E, CRALBP was negative in these cells, suggesting that they were not mature RPE cells. However, the RPE cultures showed a typical epithelial morphology, with the presence of melanosomes (Figure 1B,D,E). The tight junction (TJ) protein ZO-1 is a membrane-associated TJ adaptor protein that plays a key role in cell-cell adhesion and epithelial function, particularly in BRB function. Figure 1C shows that ZO-1 expression is restricted to the cell membrane, and reveals a polygonal structure characteristic of differentiated RPE. However, it is noticeable that not all cells express ZO-1. These results suggested that although these cultures have many features of differentiated RPE cells, they are probably not fully mature cells, and could resemble RPE cells transitioning into PVR cells.

Thrombin induces 3 H-glutamate release from RPE cells: Our previous work showed that thrombin activation of PAR-1 signaling promotes RPE cell transformation, proliferation, and migration, characteristic of fibroproliferative eye pathologies [22]. In addition to these effects directly derived from PAR signaling, Glu release from RPE may induce RPE proliferation by stimulating Glu receptors on the same RPE cells [36], which may contribute to excitotoxicity through diffusion to the inner retina [29]. To assess this possibility, we tested the effect of thrombin (2 U/ml) on the release of 3 H-glutamate from rat RPE cells in primary culture, and showed that the inclusion of 2 U/ml thrombin stimulated Glu release by about 40% over control, which was increased to 60% upon preincubation with the glutamate metabolism inhibitor AAOA and the Glu reuptake inhibitor PDC (Figure 2A). Thrombin-induced Glu release was fast, attained 1 min after the addition of thrombin. Glu release was shown to be specifically prevented by the thrombin inhibitors hirudin (4 U/ml) and PPACK (10 μ M; Figure 2B).

Thrombin-induced Glu release is dose-dependent, mediated by the activation of PAR-1: The dose–response curve for thrombin stimulation of glutamate release from 1 pM to 20 nM concentration showed that glutamate release was evident from 100 pM, and reached a saturation value at 10 nM thrombin (Figure 3A). These results indicated that thrombin

concentration in plasma [40] suffices for the induction of glutamate release upon RPE exposure to blood due to disruption of the BRB.

To determine if the thrombin effect is mediated by the activation of PAR-1, RPE cells were stimulated with the highly specific PAR-1 agonist peptide SFLLRN. Figure 3B shows that PAR-1 activation induces the dose-dependent release of glutamate, with the maximum effect (100% stimulation over control) attained at 10 μ M SFLLRN concentration.

This result demonstrated that thrombin-induced Glu release is mediated by activation of PAR-1.

PAR-1-induced glutamate release requires calcium increase from intra- and extracellular stores: Thrombin has been shown to increase intracellular calcium through PAR-1 activation. To investigate if PAR-1-mediated Glu release is calcium-dependent, RPE cells were stimulated with 10 μ M SFLLRN in nominally calcium-free medium. As shown in Figure 4A, SFLLRN-induced release was decreased by

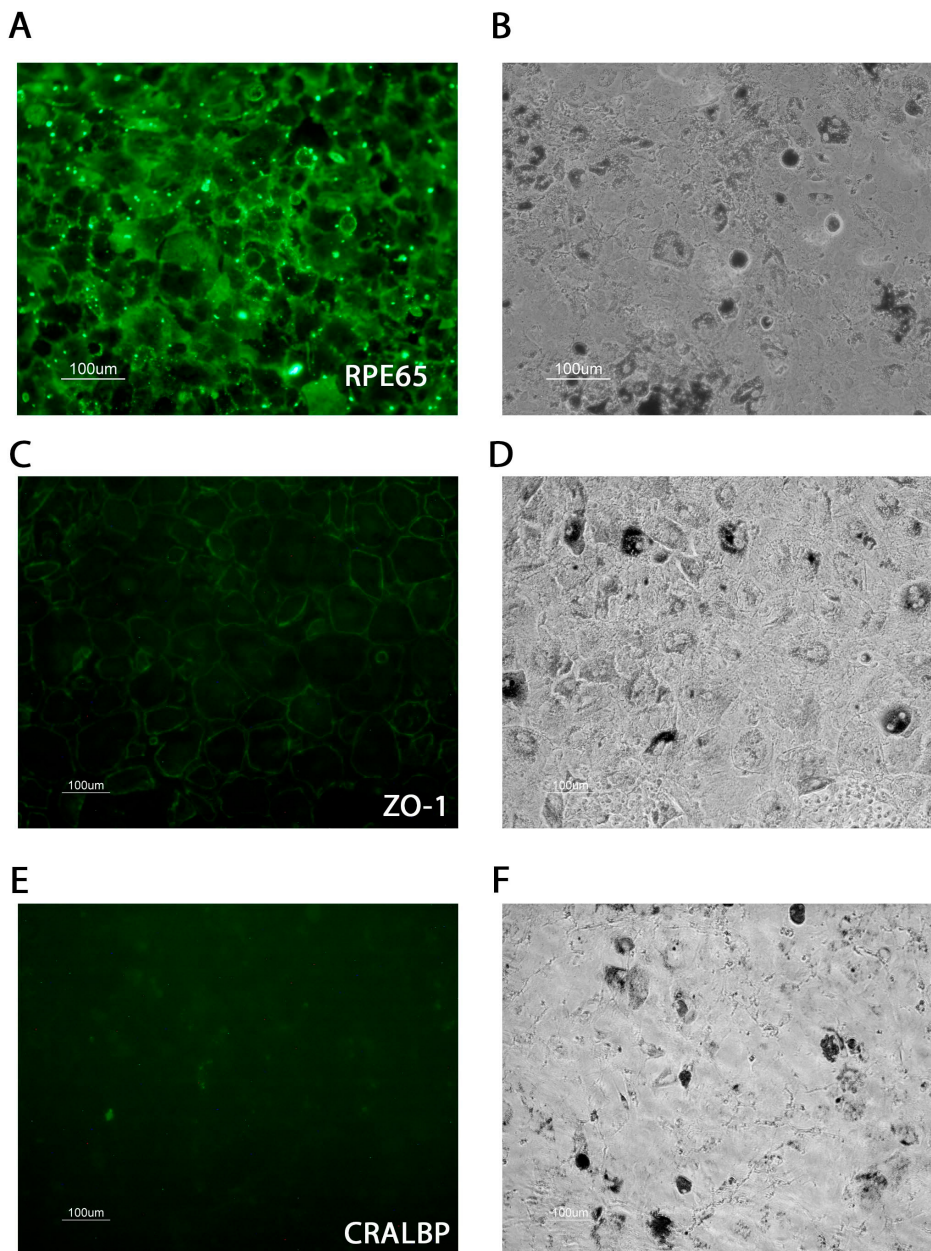


Figure 1. RPE cell morphology and identity. RPE cultures without any treatment were stained with antibodies against RPE65 (A), zonula occludens 1 (ZO-1) (C), and cellular retinaldehyde-binding protein (CRALBP) (E), as described in the experimental procedure section. The corresponding bright-field images are shown in the right column (B, D, F).

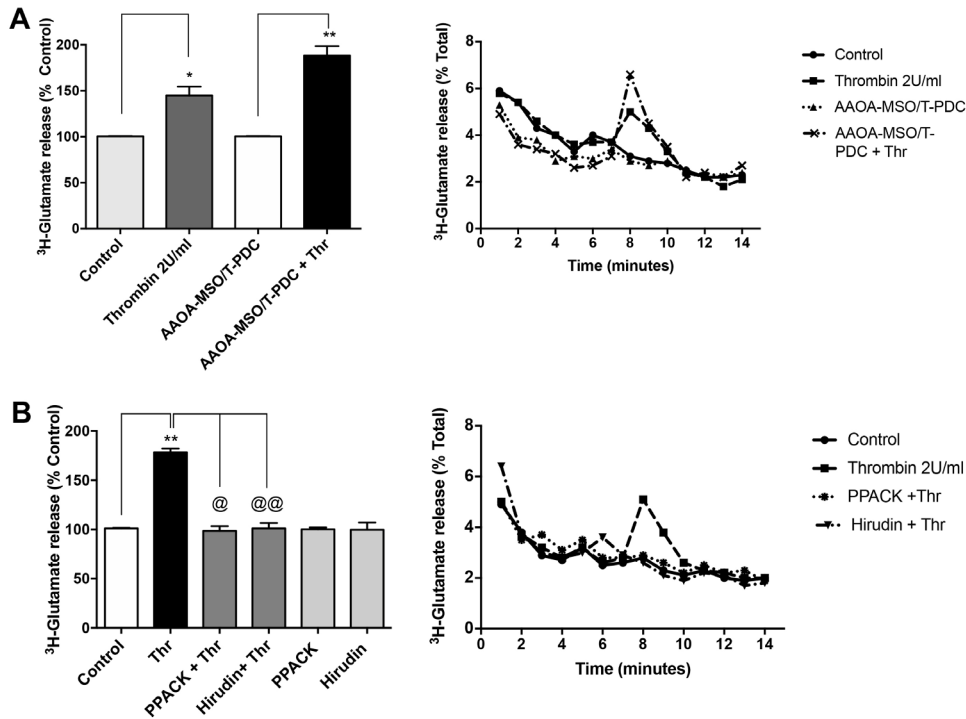


Figure 2. Thrombin induces glutamate release from rat RPE cells. Primary cultures of rat RPE cells were incubated for 30 min with 1 mM amino-oxycetic acid and 0.5 mM methionine sulfoximine (AAOA-MSO) before loading with ³H-glutamate in Krebs ringer bicarbonate (KRB; 1 μ Ci/ml 30 min). Cultures were superfused with KRB containing 50 μ M 1-trans-pyrrolidine-2,4-dicarboxylic acid (trans-PDC) and stimulated with 2 U/ml thrombin. One milliliter samples were collected at 1 min intervals. Results are expressed as % of total ³H-Glu incorporation or as a % of unstimulated control. **A:** Thrombin stimulates glutamate release. Thrombin-induced ³H-Glu release was increased by the inhibition of glutamate (Glu) metabolism and reuptake. Values are the mean

\pm standard error of the mean (SEM) of three independent experiments. The Student *t* test with respect to negative control was statistically significant (* $p < 0.05$; ** $p < 0.005$). **B:** Specificity of the thrombin effect. Thrombin-induced Glu release was prevented by the inclusion of thrombin inhibitors hirudin (4 U/ml) and D-Phenylalanyl-Prolyl-Arginyl Chloromethyl Ketone (PPACK; 10 μ M) during superfusion. Values are the mean \pm SEM of three independent experiments. The Student *t* test with respect to negative control was statistically significant (** $p < 0.005$); the Student *t* test with respect to thrombin stimulation was statistically significant (@ $p < 0.05$; @@ $p < 0.005$).

about 40% in this condition. Furthermore, the removal of extracellular calcium by the inclusion of 1 mM EGTA inhibited PAR-1 stimulation of Glu release to the same extent as calcium-free medium (about 40%). In contrast, preloading of the cells with the permeable calcium chelator BAPTA-AM completely prevented SFLLRN-induced ³H-glutamate release; this effect was not modified by the joint inclusion of BAPTA-AM plus EGTA. These results indicated that thrombin induction of Glu release from RPE cells depends primarily on intracellular calcium increase, although extracellular calcium is required to attain the maximum effect. To further confirm the requirement of calcium from intracellular stores for glutamate release, RPE cells were treated with 5 μ M ionomycin, known to promote the release of calcium from the endoplasmic reticulum (ER) to the cytosol, and showed a highly significant increase in thrombin-stimulated glutamate release in this condition (Figure 4B).

Additional mechanisms promoting Ca^{2+} entry into RPE cells, such as the reverse activity of the Na^+/Ca^{2+} exchanger (NCX) [41] and the L-type calcium channels, have been identified [42]. We tested the effect of the NCX inhibitors KB-R7943 (75 μ M) and SEA0400 (50 μ M), and that of the

L-type channel blocker nifedipine, on thrombin-induced Glu release. Figure 4C shows that the inhibition of NCX by both tested inhibitors completely prevented the release of Glu, whereas nifedipine had no effect on thrombin-induced glutamate release Figure 4D, supporting the requirement of extracellular Ca^{2+} entry through NCX activity for a thrombin-induced effect.

Signaling pathways involved in thrombin-induced glutamate release: Thrombin promotion of phospholipase C- β (PLC- β) activity and the consequent release of calcium from the ER through IP₃ receptors, has been reported [43]. To analyze this pathway, cells were stimulated with 10 μ M SFLLRN in the presence of the PLC- β inhibitor U73122 (2.5 μ M). Figure 5A shows that the inhibition of PLC- β decreases PAR-1-mediated Glu release by about 50%, suggesting the participation of ER-IP₃ calcium channels in SFLLRN-induced Glu release. Moreover, the inhibition of ryanodine receptor by 10 μ M ryanodine or 30 μ M dantrolene did not modify thrombin-induced Glu release (data not shown).

PLC- β activity generates diacyl-glycerol (DAG), a known activator of c/n PKC. Figure 5B shows that preincubation of

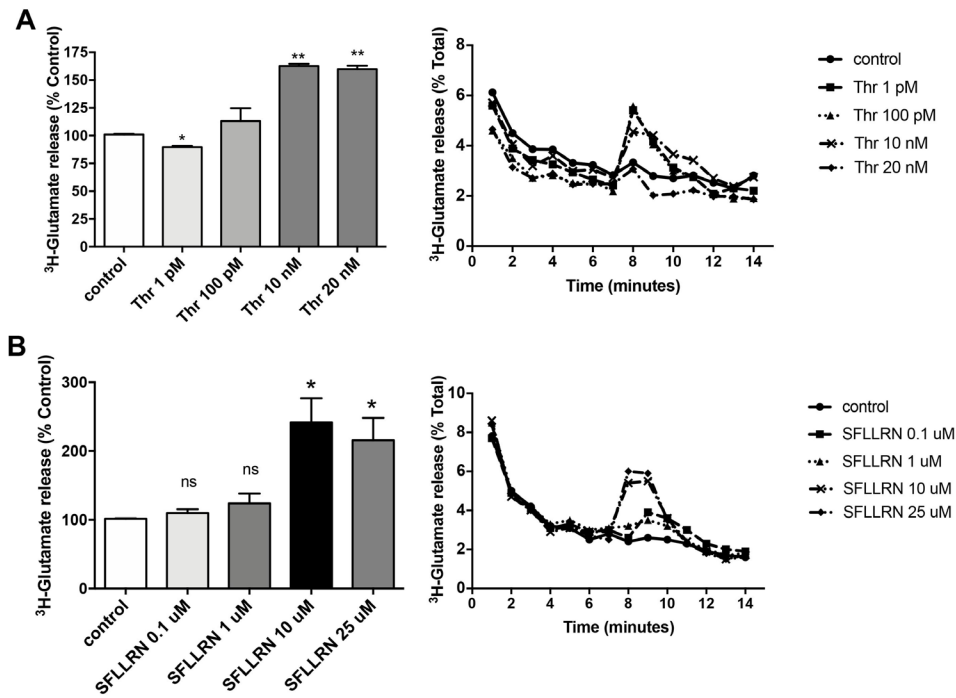


Figure 3. Thrombin-induced glutamate release is mediated by PAR-1. Primary cultures of rat RPE cells were loaded with 1 μ Ci/ml 3 H-glutamate and superfused with Krebs ringer bicarbonate (KRB; negative control) as described in Methods. Stimulation was carried in KRB containing increasing concentrations of thrombin (A) or of the specific protease-activated receptor 1 (PAR-1) agonist peptide Ser-Phe-Leu-Leu-Arg-Asn-amide trifluoroacetate salt (SFLLRN) (B), as described for Figure 2. Values are the mean \pm standard error of the mean (SEM) of three independent experiments. The Student *t* test with respect to negative control was statistically significant (* p <0.05).

RPE cells with 20 μ M of the c/n PKC-specific inhibitor Ro 32-0432 inhibits PAR-1 stimulation of glutamate release by about 80%. This result further confirmed the participation of the PLC- β /DAG/c/n PKC signaling pathway in thrombin-induced Glu release.

We previously demonstrated that thrombin activates $G_{12/13}$, known to activate phosphoinositide-3 kinase (PI3K) via the dissociated G-protein $\beta\gamma$ subunit in a Ca^{2+} -independent manner [44,45]. In turn, PI3K may lead to the stimulation of mitogen-activated protein kinases pathway (MEK/ERK) [44,46]. We tested the effect of the PI3K inhibitor wortmannin (2.5 μ M) and of the MEK inhibitor U0126 (20 μ M) on PAR-1-stimulated Glu release, and showed that neither the inhibition of PI3K nor that of MEK prevented PAR-1-induced Glu release. This result indicated that activation of the $G\beta\gamma$ signaling pathway is not involved in thrombin-induced glutamate release.

PAR-1-induced glutamate release is not mediated by vesicular transport or the reverse activity of GLAST: Control of the extracellular glutamate concentration is a crucial mechanism in the prevention of excitotoxic cell death. Among the mechanisms involved in this process, Glu released by the reverse activity of the reverse activity of the Glutamate Aspartate Transporter (GLAST) or through Ca^{2+} -dependent vesicular release in a neurotransmitter-like mode could contribute to the thrombin effect. To test this possibility, we used the

vesicular release inhibitor bafilomycin (Figure 6A) and the reverse transport inhibitor DL-threo- β -Benzyloxyaspartic acid (TBOA; Figure 6B). Neither had an effect on PAR-1-induced glutamate release.

DISCUSSION

Glutamate is the excitatory transmitter in the main signaling pathway of the retina from photoreceptors to ganglion cells. Because the elevation of the Glu concentration in pathological conditions has been shown to induce the excitotoxic death of retinal neurons, the extracellular Glu concentration must be tightly regulated [47]. Glutamate has also been shown to promote RPE cell proliferation by activating distinct signaling pathways linked to selective glutamate receptor subtypes [46,48,49]; thus, Glu release induced by thrombin may autoactivate Glu receptors on RPE cells, resulting in the alteration of RPE physiology, and the consequent death of retinal neurons.

The RPE is separated from the photoreceptor outer segments by the sub-retinal space. Although the actual volume of this space is minimal, the communication that occurs across this microenvironment is important to the visual process. In pathological conditions that alter the BRB, exposure of RPE cells to blood-contained thrombin promotes the EMT of RPE cells, including the transformation, increased proliferation, and migration to the vitreous which ultimately

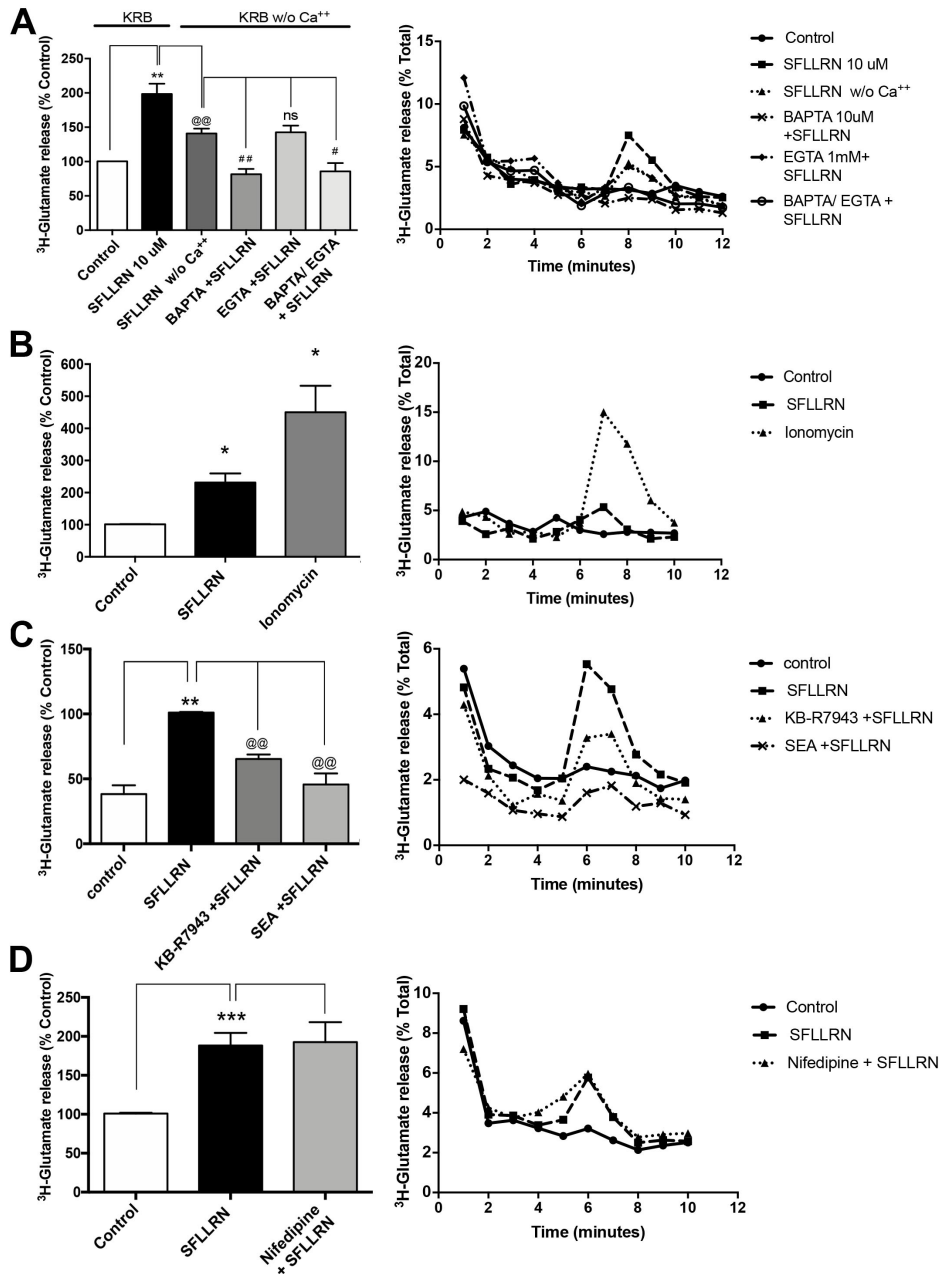


Figure 4. SFLLRN-induced glutamate release is calcium-dependent. Primary cultures of rat RPE cells loaded with 1 μCi/ml ³H-glutamate in Krebs ringer bicarbonate (KRB) for 30 min and stimulated with 10 μM protease-activated receptor 1 (PAR-1) AP (Ser-Phe-Leu-Leu-Arg-Asn-amide trifluoroacetate salt [SFLLRN]). A: SFLLRN-induced glutamate release is calcium-dependent. 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA AM) and/or EGTA were included in Ca²⁺-free KRB for 15 min in the presence or absence of the Ca²⁺ chelators BAPTA-AM (10 μM) and/or EGTA (1 mM) before stimulation. Values are the mean ± standard error of the mean (SEM) of three independent experiments. The Student *t* test with respect to negative control was statistically significant (**p<0.005); the Student *t* test with respect to SFLLRN w/ Ca²⁺ was statistically significant (@@p<0.005); the Student *t* test with respect to SFLLRN w/o Ca²⁺ was statistically significant (#p<0.05, ##p<0.005). B: Ca²⁺ release from intracellular stores is the main contributor to PAR-1 induced glutamate release. Cells were stimulated with 5 μM ionomycin to further demonstrate the requirement for intracellular Ca²⁺. Values are the mean ± SEM of three independent experiments. The Student *t* test with respect to

negative control was statistically significant (*p<0.05). C: Extracellular Ca²⁺ contributes to PAR-1-induced glutamate release. Cells were incubated with the Na⁺/Ca²⁺ exchanger (NCX) inhibitor SEA 0400 (50 μM), and of the inhibitor of the exchanger reverse activity KB-R7943 (75 μM) before stimulation with 10 μM of the PAR-1 agonist peptide SFLLRN. Results showed that PAR-1-induced release was statistically significantly decreased by these compounds. Data are the mean of three independent experiments. The Student *t* test with respect to negative control was statistically significant (**p<0.005); the Student *t* test with respect to SFLLRN was statistically significant (@@p<0.005). D: PAR-1-induced glutamate release is independent of L-type calcium channel activation. Thrombin stimulation in the presence of the L-type calcium channel blocker nifedipine (5 μM) showed that Glu release is unrelated to L-calcium channel activity. Values are the mean ± SEM of three independent experiments. The Student *t* test with respect to negative control was statistically significant (***)p<0.005). The Student *t* test with respect to SFLLRN was not statistically significant.

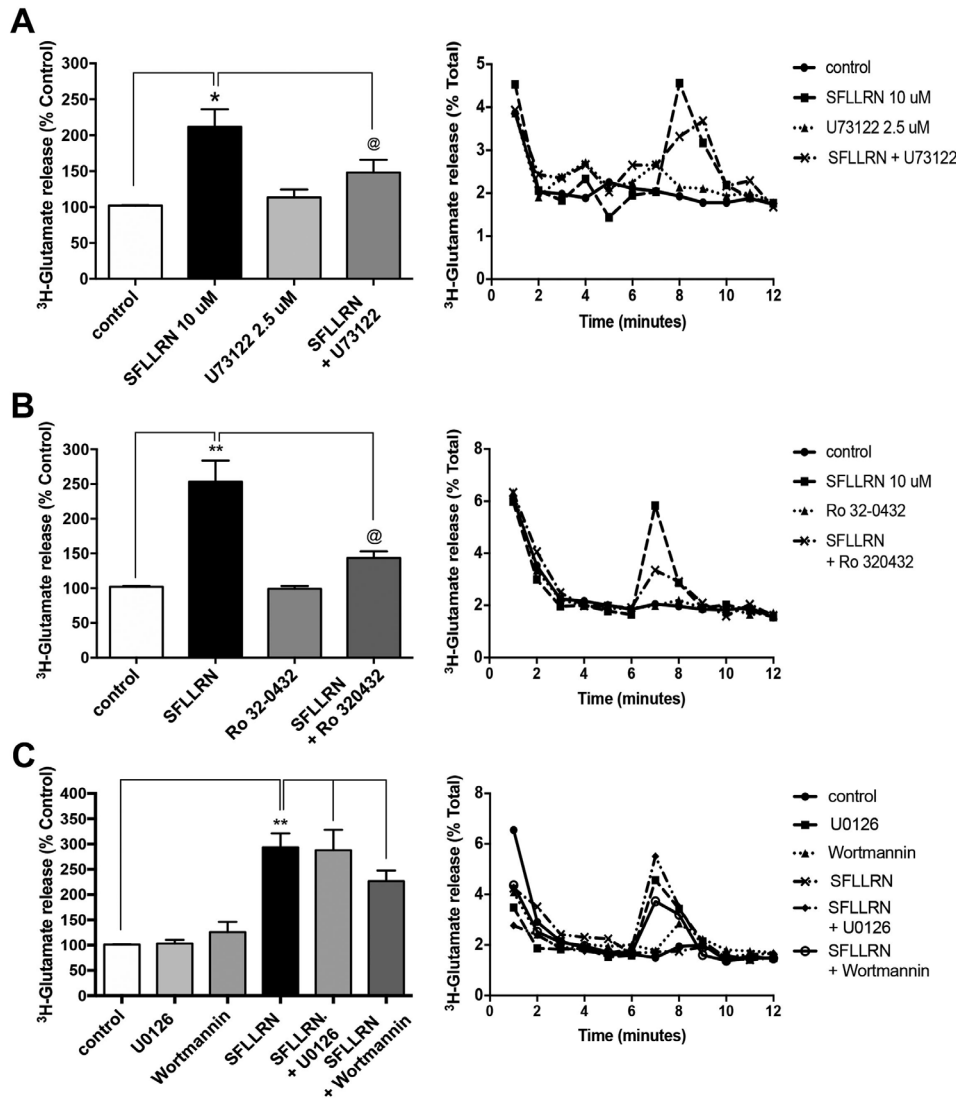


Figure 5. Signaling pathways involved in thrombin-induced glutamate release. **A:** Protease-activated receptor 1 (PAR-1)-induced glutamate (Glu) release requires phospholipase C- β (PLC- β) activity. Cells were incubated in the presence of the PLC- β inhibitor U73122 (2.5 μ M) for 30 min. The inhibitor was removed before stimulation with 10 μ M PAR-1 AP. Results showed that Ser-Phe-Leu-Leu-Arg-Asn-amide trifluoroacetate salt (SFLLRN) induced Glu release which was prevented by the inhibition of PLC- β . Results are the mean of three independent experiments. The Student *t* test with respect to negative control was statistically significant (* p <0.05). The Student *t* test with respect to SFLLRN was statistically significant (@ p <0.05). **B:** Inhibition of c/n protein kinase C (PKC) isoforms prevents SFLLRN-induced Glu release. Cells were incubated with the c/n PKC inhibitor Ro 32–0432 (20 μ M) for 30 min, and removed before stimulation with 10 μ M PAR-1 AP. Data are the mean \pm standard error of the mean (SEM) of three independent experiments. The Student *t* test with respect to negative control was statistically significant (** p <0.005). The Student *t* test with respect to SFLLRN was statistically significant (@ p <0.05). **C:** Thrombin-induced Glu release is independent of phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase kinase (MEK) activity. The MEK inhibitor U0126 (20 μ M) and the PI3K inhibitor wortmannin (1 μ M) were included in KRB for 30 min and removed, before stimulation. Data are the mean \pm SEM of three independent experiments. The Student *t* test with respect to negative control was statistically significant (** p <0.005). The Student *t* test with respect to SFLLRN was not statistically significant.

SFLLRN was statistically significant (@ p <0.05). **C:** Thrombin-induced Glu release is independent of phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase kinase (MEK) activity. The MEK inhibitor U0126 (20 μ M) and the PI3K inhibitor wortmannin (1 μ M) were included in KRB for 30 min and removed, before stimulation. Data are the mean \pm SEM of three independent experiments. The Student *t* test with respect to negative control was statistically significant (** p <0.005). The Student *t* test with respect to SFLLRN was not statistically significant.

contributes to neuronal death and the development of blindness. Despite the close apposition of the photoreceptors and RPE cells, the possible release and diffusion of glutamate from RPE, and its influence on retinal signaling, have not been examined. The present results showed that thrombin induces the fast, specific, dose-dependent release of Glu from RPE cells, suggesting that under pathological conditions or surgical procedures in which the BRB is compromised, the elevation of extracellular Glu induced by thrombin activation of PAR-1 may contribute to the long-term increase in

RPE cell proliferation, a main feature in the development of fibroproliferative eye diseases.

Numerous factors seem to be involved in the proliferation and transformation of RPE cells in PVR [50,51]. Although the glutamate concentration in PVR tissue has not been determined, an increase in glutamate levels in the vitreous cavity of patients is increased by retinal detachment and hypoxia [52], due to a decrease in the expression of glutamate transporters in retinal glia [35,53-55]. Additionally, glutamate from serum or from damaged retinal cells which

contain millimolar concentrations may reach the RPE [56]. Thus, under pathologic conditions in vivo, elevation of glutamate could contribute to the development of PVR through the activation of specific receptors on RPE cells.

Activation of PAR-1 by endogenous agonists, including thrombin and plasmin, or by specific agonist peptides (TFLLR and SFLLRN) has been shown to elevate intracellular Ca^{2+} in RPE cells [57,58] and astrocytes [59]. Consistent with these data, the present results showed that removal of intracellular calcium by BAPTA-AM completely prevents PAR-1-induced Glu release, clearly indicating that downstream pathways associated with Ca^{2+} release from internal stores are involved. This was further confirmed by the robust induction of Glu release by ionomycin (Figure 4B), known to deplete ER Ca^{2+} pools. Because EGTA modestly decreased PAR-1-induced Glu release, a contribution of extracellular calcium is also required, possibly aimed to the refilling of ER intracellular calcium stores. Therefore, it is tempting to speculate that the thrombin-triggered rise in $[\text{Ca}^{2+}]_i$ primes cells for glutamate release, as it has been shown for the release of ATP [37].

There are several mechanisms that trigger the elevation of intracellular Ca^{2+} levels. One mechanism for Ca^{2+} release from internal stores involves the activation of inositol 1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} channels on the ER

membrane. PAR-1 coupling to the GPCR G_{q11} subunit activates PLC- β , which catalyzes the formation of inositol 1,4,5-trisphosphate (IP_3) and DAG, leading to an increase in the intracellular Ca^{2+} concentration and DAG activation of c/n PKC isoforms [2]. We examined the contribution of this pathway to thrombin/PAR-1-induced Ca^{2+} -dependent glutamate release from RPE cells, and demonstrated that the pharmacologic inhibition of PLC- β by U73122, or that of its downstream target c/n PKC by Ro-32-0432, statistically significantly decreased PAR-1-induced Glu release, further confirming that the intracellular Ca^{2+} increase required for glutamate release is induced by activation of this pathway. Although PAR-1 is coupled to $G\beta\gamma$ subunits, essential for glutamate efflux from cultured astrocytes [34], inhibition of PI3K by wortmannin and of MEK/Erk by U0126 (Figure 5C) had no effect on PAR-1 evoked glutamate release, indicating that signaling through $G\beta\gamma$ subunits is not involved in this process.

Additionally, we examined the contribution of the caffeine/ryanodine-sensitive ER Ca^{2+} store to thrombin-induced Ca^{2+} -dependent glutamate release. Unexpectedly, the results showed that neither the pharmacological blockage of these pathways nor the inhibition of the thapsigargin-sensitive ER Ca^{2+} ATPase had an effect on thrombin-induced Glu release (data not shown). On this matter, RPE cells and

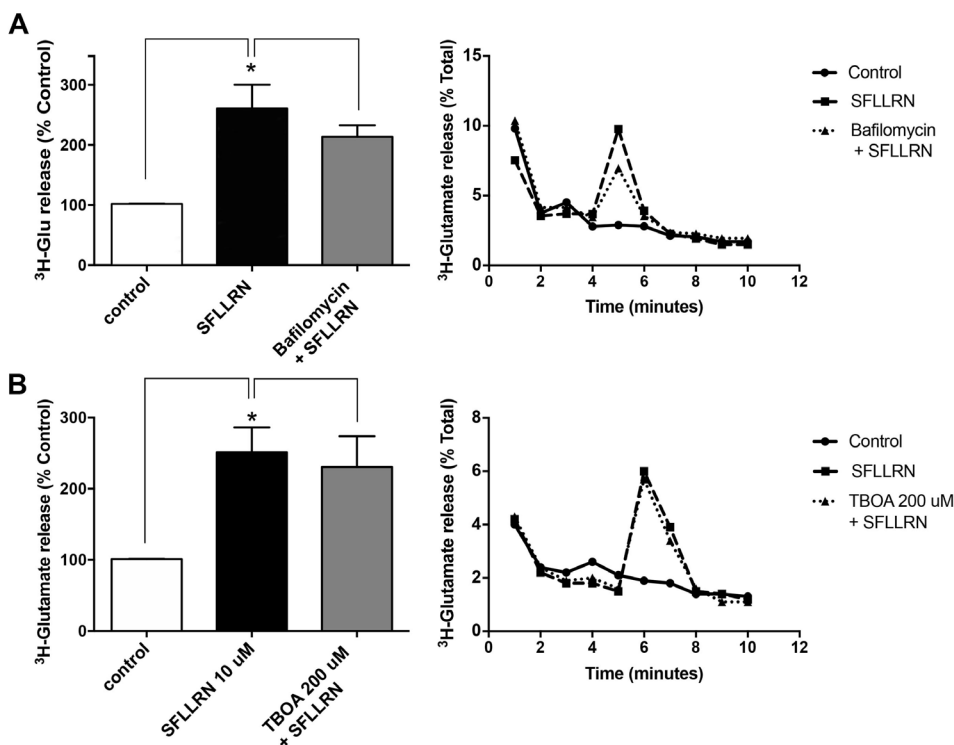


Figure 6. Thrombin induces glutamate release in a non-vesicular mode. The participation of vesicular mechanisms (Figure 6A) or reverse activity of Glutamate Aspartate Transporter (GLAST; Figure 6B) in thrombin induction of glutamate (Glu) release was tested. Results showed that neither GLAST inhibition by DL-threo- β -Benzyloxyaspartic acid (TBOA) nor that of vesicular release by bafilomycin prevented thrombin induction of Glu release. Data are the mean \pm standard error of the mean (SEM) of three independent experiments. The Student *t* test with respect to negative control was statistically significant (* $p < 0.05$). The Student *t* test with respect to Ser-Phe-Leu-Leu-Arg-Asn-amide trifluoroacetate salt (SFLLRN) was not statistically significant.

astrocytes express several types of transient receptor potential (TRP) channels [60,61] which contribute to modulate Ca^{2+} -dependent vesicular glutamate release by the promotion of store-operated Ca^{2+} entry (SOCE) [62,63]. However, bafilomycin, a macrolide that specifically targets vacuolar-type H^{+} -ATPase (V-ATPase), therefore inhibiting vesicular release, had no effect on thrombin-induced Glu release (Figure 6A).

Interestingly, recent evidence suggested that L-type calcium channels present in RPE are linked to intracellular signaling pathways by protein kinase-dependent regulation. RPE L-type Ca^{2+} channels can be activated by protein kinase C, protein tyrosine kinases of the Src-subtype [64,65], and receptor tyrosine kinases [66]. Although we tested this possibility, the lack of effect of nifedipine indicates that these channels are not involved in thrombin-induced release of glutamate.

The deregulation of the thrombin-PAR system in pathological conditions results in the loss of barrier function [67]. Thrombin is capable of modifying endothelial cell permeability [12], and it has been suggested to play a role in retinal disease [67,68]. Recent reports on this matter showed that the ion transporter NCX operating in the Ca^{2+} -influx (reverse) mode promotes endothelial barrier dysfunction in response to thrombin, which is prevented by the reverse-mode NCX inhibitor (KB-R7943) or NCX1 knockdown in vitro [69]. Ca^{2+} entry by this exchanger has been identified as a mechanism for inducing amplification of Ca^{2+} signals that occur during conditions of agonist activation. Thus, we investigated whether Ca^{2+} influx through NCX was involved in Glu release in response to thrombin, and showed that the specific inhibition of this process by KB-R7943 completely blocked thrombin-induced glutamate release.

In conclusion, the present study demonstrated that contact of RPE cells with thrombin, which may be present in pathological conditions, promotes the release of glutamate in a dose-dependent-specific manner. This effect is mediated by an increase in the intracellular Ca^{2+} concentration induced by the activation of the PLC- β /PKC pathways, and by the reverse activity of the $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger. Therefore, the deregulation of this system may allow glutamate diffusion to the inner retina, thus promoting the excitotoxic death of neurons.

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