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Thrombin regulation of synaptic plasticity: Implications for physiology and pathology

Nicola Maggio [a,b,*], Zeev Itsekson [b], Dan Dominissini [c,d], Ilan Blatt [b,e], Ninette Amariglio [c], Gideon Rechavi [c,d], David Tanne [b,e], Joab Chapman [b,e]

[a] Talpiot Medical Leadership Program, The Chaim Sheba Medical Center, 52621 Tel HaShomer, Israel
[b] Department of Neurology, The J. Sagol Neuroscience Center, The Chaim Sheba Medical Center, 52621 Tel HaShomer, Israel
[c] Cancer Research Center, The Sheba Medical Center, 52621 Tel HaShomer, Israel
[d] Sackler School of Medicine, Tel Aviv University, 69978 Tel Aviv, Israel
[e] Department of Neurology, Sackler School of Medicine, Tel Aviv University, 69978 Tel Aviv, Israel

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ABSTRACT

Thrombin, a serine protease involved in the coagulation cascade has been recently shown to affect neuronal function following blood–brain barrier breakdown. Several lines of evidence have shown that thrombin may exist in the brain parenchyma under normal physiological conditions, yet its role in normal brain functions and synaptic transmission has not been established. In an attempt to shed light on the physiological functions of thrombin and Protease Activated Receptor 1 (PAR1) in the brain, we studied the effects of thrombin and a PAR1 agonist on long term potentiation (LTP) in mice hippocampal slices. Surprisingly, different concentrations of thrombin affect LTP through different molecular routes converging on PAR1. High thrombin concentrations induced an NMDA dependent, slow onset LTP, whereas low concentrations of thrombin promoted a VGCCs, mGluR-5 dependent LTP through activated Protein C (aPC). Remarkably, aPC facilitated LTP by activating PAR1 through an Endothelial Protein C Receptor (EPCR)-mediated mechanism which involves intracellular calcium stores. These findings reveal a novel mechanism by which PAR1 may regulate the threshold for synaptic plasticity in the hippocampus and provide additional insights into the role of this receptor in normal and pathological conditions.

Introduction

Cerebrovascular events induced by either ischemia or hemorrhage lead to blood–brain barrier (BBB) breakdown and exposure of the brain to blood constituents (Yang and Rosenberg, 2011). Among others, thrombin, a serine protease involved in the coagulation cascade, has been shown to contribute to the stroke pathology following these conditions (Chen et al., 2012; Wang et al., 2012a, 2012b). High concentrations of thrombin in the brain also perturb normal physiology by saturating synaptic plasticity and inducing seizures (Isaeva et al., 2012; Maggio et al., 2008, 2013). These effects depend on the activation of the thrombin receptor, the Protease Activated Receptor 1 (PAR1) and consequent potentiation of NMDA receptor functions (Gingrich et al., 2000; Maggio et al., 2008). Previous studies, however, have shown that thrombin may exist in the brain parenchyma in normal physiological conditions (Turgeon et al., 2000). Indeed, the mRNAs for both the thrombin precursors prothrombin and factor Xa, the enzyme converting prothrombin into thrombin, have been detected in several areas of the forebrain (Dihanich et al., 1991; Shikamoto and Morita, 1999). Nevertheless, both the mechanisms leading to the thrombin production in the brain under physiological conditions as well as its roles in normal brain functions and synaptic transmission have not yet been completely clarified.

PAR1 belongs to a family of seven transmembrane domains, G protein-coupled receptors whose activation requires the cleavage of a peptide bond at the N-terminal extracellular side which binds the second extracellular loop of the same receptor thus activating it (Sokolova and Reiser, 2008). In the brain PAR1 is expressed both in neurons and astrocytes (Junge et al., 2004; Luo et al., 2007). Its activation has been shown to modulate synaptic transmission and plasticity (Lee et al., 2007), yet the specific contribution of the astrocytic vs. the neuronal receptor remains under investigation. In addition, while in peripheral organs PAR1 has been shown to be activated by a pool of proteases, e.g. activated Protein C (aPC), leading to different outcomes (Mosnier et al., 2007), the role of other PAR1 agonists in the brain has not been fully investigated.

In an attempt to shed light on the physiological vs. pathological functions of thrombin and PAR1 in the brain, we studied the effects of different concentrations of thrombin and PAR1 agonist (PAR1-AP) on long term potentiation (LTP) using hippocampal slices. Surprisingly, we found that diverse thrombin concentrations differently regulate the threshold for synaptic plasticity in the hippocampus. These data provide additional insights into the role of this receptor in normal and pathological conditions.

* Corresponding author at: Department of Neurology, The Chaim Sheba Medical Center, 52621 Tel HaShomer, Israel. Fax: +972 35304752.
E-mail address: nicola.maggio@sheba.health.gov.il (N. Maggio).

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Methods

Drugs

The following drugs were prepared from frozen stocks: thrombin (Sigma Aldrich, Rehovot, Israel and Enzyme Research Laboratories, Swansea, England); PAR1 agonist (PAR-1AP, SFLLRN, Sigma Aldrich, Rehovot, Israel); PAR1 antagonist (SCH79797, Tocris Bioscience, Bristol, United Kingdom); plasmin (Sigma Aldrich, Rehovot, Israel); activated Protein C (aPC, Sigma Aldrich, Rehovot, Israel); inactivated Protein C (iPC, Enzyme Research Laboratories, Swansea, England); APV (Sigma Aldrich, Rehovot, Israel); nifedipine (Sigma Aldrich, Rehovot, Israel); MPEP (Tocris Bioscience, Bristol, United Kingdom); thapsigargin (Alomone Labs, Jerusalem, Israel); cyclopiazonic acid (CPA, Alomone Labs, Jerusalem, Israel); α-NAPAP (Sigma Aldrich, Rehovot, Israel). As the specific activity of the α-thrombin from various vendors ranged between 2700 and 3200 NIH U/mg by comparison to Lot K of the NIH standard, we estimated the concentration of active α-thrombin that corresponds to 1 U/ml activity, by calculating a conversion factor using pure α-thrombin (3200 U/mg), as previously described (Gingrich et al., 2000). In this lot, the manufacturer reported this protein to be >95% α-thrombin as determined by gel electrophoresis, hence a solution with 1 U/ml α-thrombin should be 9 nM by molecular weight for thrombin of 36.7 kDa. For the sake of simplicity, we used a conversion factor of 1 U/ml = 10 nM α-thrombin throughout the text to estimate the concentration of active α-thrombin (henceforth referred to as thrombin) from various vendors.

Electrophysiology

Animal handling was approved by the Institutional Animal Care and Use Committee, which adheres to the national law, and NIH rules. Briefly, 4–5 months old male C57BL/6 mice were rapidly decapitated and 350 μm coronal dorsal hippocampal slices were used. Slices were incubated for 1.5 h in a humidified, carbogenated (5% CO_2 and 95% O_2) gas atmosphere at 33 ± 1 °C and were perfused with artificial CSF [containing (in mM) 124 NaCl, 2 KCl, 26 $NaHCO_3$, 1.24 KH_2PO_4, 2.5 $CaCl_2$, 2 $MgSO_4$, and 10 glucose, pH 7.4] in a standard interface chamber. Recordings were made with a glass pipette containing 0.75 M NaCl (4 MΩ) placed in the stratum radiatum CA1. Stimulation was evoked using a Master 8 pulse stimulator (A.M.P.I., Jerusalem, Israel) and was delivered through two sets of bipolar nichrome electrodes placed on either side of the recording electrode such that two independent stimulation channels were used for each slice. The use of two parallel pathways allowed comparison of the effects of drug application in the same slice (Maggio and Segal, 2007a, 2007b). LTP was induced by high-frequency stimulation consisting of 100 pulses at twice the test intensity, delivered at a frequency of 100 Hz (HFS; 100 Hz, 1 s). Before applying the tetanic stimulation, baseline values were recorded at a frequency of 0.033 Hz. Responses were digitized at 5 kHz and stored on a computer. Off-line analysis and data acquisition were performed using Spike 2 software (CED, Cambridge, England). All numerical data are expressed as mean ± SEM, and EPSP slope changes after tetanic stimulation were calculated with respect to baseline. There were no systematic differences in the magnitudes of the baseline responses in the different conditions. All values reported refer to 30 min before tetanic stimulation. Unless otherwise indicated, statistical evaluations were performed by applying Student's *t* test for paired and unpaired data, as the case may be (Origin 8.0). *P* values of <0.05 were considered a significant difference between means.

Immunohistochemistry

The following antibodies were used for immunodetection: mouse antibodies raised against Neuronal nuclear antigen (NeuN) (1:100; Millipore, Billerica, MA, USA); rabbit antibodies to Protease Activated Receptor-1 (PAR1) (1:50; Abcam, Cambridge, UK); goat antibodies to Endothelial Protein C Receptor (EPCR) (P20, 1:100; Santa Cruz Biotech, CA, USA). Hippocampal sections (25 μm) were blocked in 10% normal serum in 0.01 M PBS/0.25% Triton for 1 h at room temperature (RT). After 48 h incubation at 4 °C with the primary antibody (NeuN, PAR-1, and EPCR with 2% normal serum), sections were exposed to the appropriate secondary antibody (1:500, DyLight flourophores—594; 488; 633, Thermo-Scientific, Rockford, IL, USA) for 1 h and finally mounted and coverslipped with Flouromount (Southern Biotechnology). Slides were imaged with a Zeiss LSM 510 confocal microscope and data were acquired and analyzed using a computer assisted image analysis system.

Intracerebroventricular (ICV) injections

Mice were anesthetized with an intraperitoneal (IP) injection of ketamine (100 mg/kg) and xylazine (20 mg/kg). The skull was carefully exposed, and a small hole was drilled above the right lateral ventricle (2 mm lateral to the midline and 2.5 mm posterior to the bregma). A rat monoclonal antibody blocking EPCR (R252 clone, Sigma Aldrich, Rehovot, Israel) was injected at a concentration of 30 μg/ml (2 mm depth) using a 27-gauge needle attached to a Hamilton syringe. Following the slow infusion of 1 μl of antibody solution, the needle was withdrawn, and the skin over the scalp was sutured. Control mice were injected with a vehicle solution. Hippocampal slices were cut 36 h following the procedure.

Results

Thrombin induces slow onset LTP in CA1

Thrombin concentrations rise following BBB breakdown (Chen et al., 2010, 2012). Exposure of mice hippocampal slices to high concentration of thrombin (1 U/ml thrombin; $[Thrombin]_{high}$) for 12 min produced a gradual increase in population EPSP recorded in the stratum radiatum of region CA1 of the hippocampus. This gradual change was specific to the EPSP, because no parallel change was noticed in the presynaptic volley produced in response to the stimulation (Fig. 1A). The increase in EPSP slope rose gradually over 40 min of recording, reaching a plateau level, which was 73% above control (n = 11 slices; 1.73 ± 0.78; $P < 0.01$), similar to that induced by tetanic stimulation (HFS) of the alternate pathway prior to drug application. Application of 2, 3, 5 and 10 U/ml thrombin produced the same effect (data not shown). A comparable, slow-rising, persistent increase in population EPSP was seen after bath application of 1 μM SFLLRN, a PAR1 receptor agonist ($[PAR1\text{-}AP]_{high}$; n = 11 slices; 1.62 ± 0.071; $P < 0.01$; Fig. 1B). To verify that the effect of thrombin was mediated through activation of a genuine PAR1 receptor, the selective PAR1 antagonist SCH79797 (SCH) was used. SCH had no effect on established tetanic LTP (Fig. 1C) (n = 11; 1.84 ± 0.079), yet the response to thrombin, tested in the non tetanized pathway, was completely abolished (0.99 ± 0.066). In order to address whether thrombin-induced slow onset LTP shared a downstream mechanism with the conventional LTP, thrombin was applied in the presence of the NMDA receptor antagonist 2(R)-APV (APV, 50 μM). Under these conditions, thrombin was unable to produce LTP (0.97 ± 0.062 at 25 min following thrombin application; n = 11 slices) (Fig. 1D). Likely, the effect of thrombin was abolished in presence of its specific inhibitor, α-NAPAP (Suppl. Fig. 1A). These experiments confirm the results we previously obtained in rats (Maggio et al., 2008), namely that the slow onset LTP induced by $[Thrombin]_{high}$ involves NMDA receptors.

The effects of thrombin and PAR-1AP are concentration dependent

Thrombin produces a variety of effects in the brain being detrimental at high concentrations and protective at low concentrations (Hua et al., 2009; Striggow et al., 2000). In order to test whether the