Role of Thrombin in Activation of Neurons in Rat Hippocampus

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The effect of thrombin, an agonist of proteinase-activated receptor (PAR) family, was studied on cultured rat hippocampal neurons. Thrombin in a concentration range of 1 pM — 10 nM induced a transitory dose-dependent increase in intracellular free calcium concentration. Involvement of PAR1 in neural response to thrombin was corroborated in experiments with TFLLRN, a selective synthetic peptide agonist of these receptors. In a calcium-free medium and after treatment with cyclopiazonic acid (inhibitor of Ca^{2+}-ATPase in the endoplasmic reticulum) activation of PAR not only mobilized Ca^{2+} from intracellular stores, but also induced Ca^{2+} entry into the cells. Thrombin decreased Ca^{2+} signal triggered by activation of NMDA-subtype glutamate receptors.

Key Words: *thrombin; PAR; neurons; hippocampus; fluorescent microscopy; intracellular calcium*

Serine proteinases, *e.g.* thrombin, play an important role in the death of cerebral neurons during hemorrhagic strokes, Alzheimer's disease, posttraumatic epilepsy, arteriovenous bypass surgery, and other pathological states [14,15]. Expression of prothrombin mRNA was detected in the brain, hence thrombin can appear in the nervous tissue even under conditions of intact blood-brain barrier [9]. The functions of thrombin are mediated via proteinase-activated receptors PAR1, PAR3, and PAR4, which are coupled to G protein family. These receptors are expressed in the thalamus, hypothalamus, hippocampus, cerebral cortex and cerebellum [15].

The effect of thrombin on brain cells qualitatively depends on its concentration. At high concentrations (>100 nM) thrombin induces degradation of neural processes and apoptosis of cultured neuronal cells [2, 8,15]. By contrast, at low concentrations thrombin protects the cells during cerebral damage caused by β-amyloid, intracerebral hemorrhages, and focal ischemia [9,10]. This diversity of the effects of thrombin is not clearly understood.

The most important element in the chain of neurodegenerative processes in damaged CNS is long-term elevation of cytosolic calcium concentration $[Ca^{2+}]_i$ followed by destabilization of calcium homeostasis [1,11].

Our aim was to study the mechanisms of thrombin effect on $[Ca^{2+}]_i$ in cultured rat hippocampal neurons.

MATERIALS AND METHODS

The study was carried out on 8-10-day-old primary culture of hippocampal neurons isolated from 1-3-day-old Wistar rat pups and on 7-10-day-old primary culture of cerebellar neurons isolated from 5-7-day-old Wistar rat pups [1].

Measurements of $[Ca^{2+}]_i$ was performed on a Spex microfluorimeter (Spex) combined with an inverted microscope (Nikon) and image analysis system (Diamorph). Cultured cells were loaded with Ca^{2+}-sensitive probe Fura-2/AM (5 μM) for 40 min, and then the

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culture medium was replaced with a buffer containing (in mM): 130.0 NaCl, 5.6 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 20.0 HEPES (pH 7.4), 5.0 glucose, and tetrodotoxin (0.5 μM). Glass coverslips with cell culture were placed into a perfused chamber (200 μl) on microscopic stage. Fluorescence of Ca^{2+}-bound Fura-2 was recorded at emission wavelength λ_{em}=505 nm and two excitation wavelengths (λ_{ex}) of 340 and 380 nm. Cytosolic calcium concentration was calculated by the formula:

$$[Ca^{2+}]_i=[(R-R_{min}-R_{max})/(R_{max}-R)]\times K_d\times S,$$

where R is F_{340}/F_{380} ratio in each point of the curve, K_d is dissociation constant of Fura-2/AM (225 nM), and $S=F_{min}$ (at 380 nM)/F_{max} (at 380 nM) [5].

For measuring maximum (R_{max}) and minimum (R_{min}) fluorescence, the cells were successively incubated in the following calibration solutions (in mM): 130 KCl, 5 NaCl, 0 Mg^{2+}, 5 $CaCl_2$, 5 HEPES, ionomycin (5 μM, for R_{max}), and in Ca^{2+}-free buffer with 5 mM EGTA (for R_{min}).

RESULTS

Stimulation of the cells with thrombin induced a rapid transitory increase in $[Ca^{2+}]_i$, which peaked after 20-30 sec and then returned to the baseline value despite the presence of agonist in the incubation medium (Fig. 1, *a*). This rise was dose-dependent: in concentrations of 10 pM and 10 nM thrombin increased $[Ca^{2+}]_i$ by 40 nM and 750 nM, respectively (Fig. 1, *b*). By contrast to hippocampal neurons, in rat cerebellar granular cells thrombin had no effect on $[Ca^{2+}]_i$. Since PAR1 mRNA is expressed in the cerebellum [15], the absence of the effect of thrombin can be explained by poor development of endoplasmic reticulum (EPR) in the cerebellar granular cells [3].

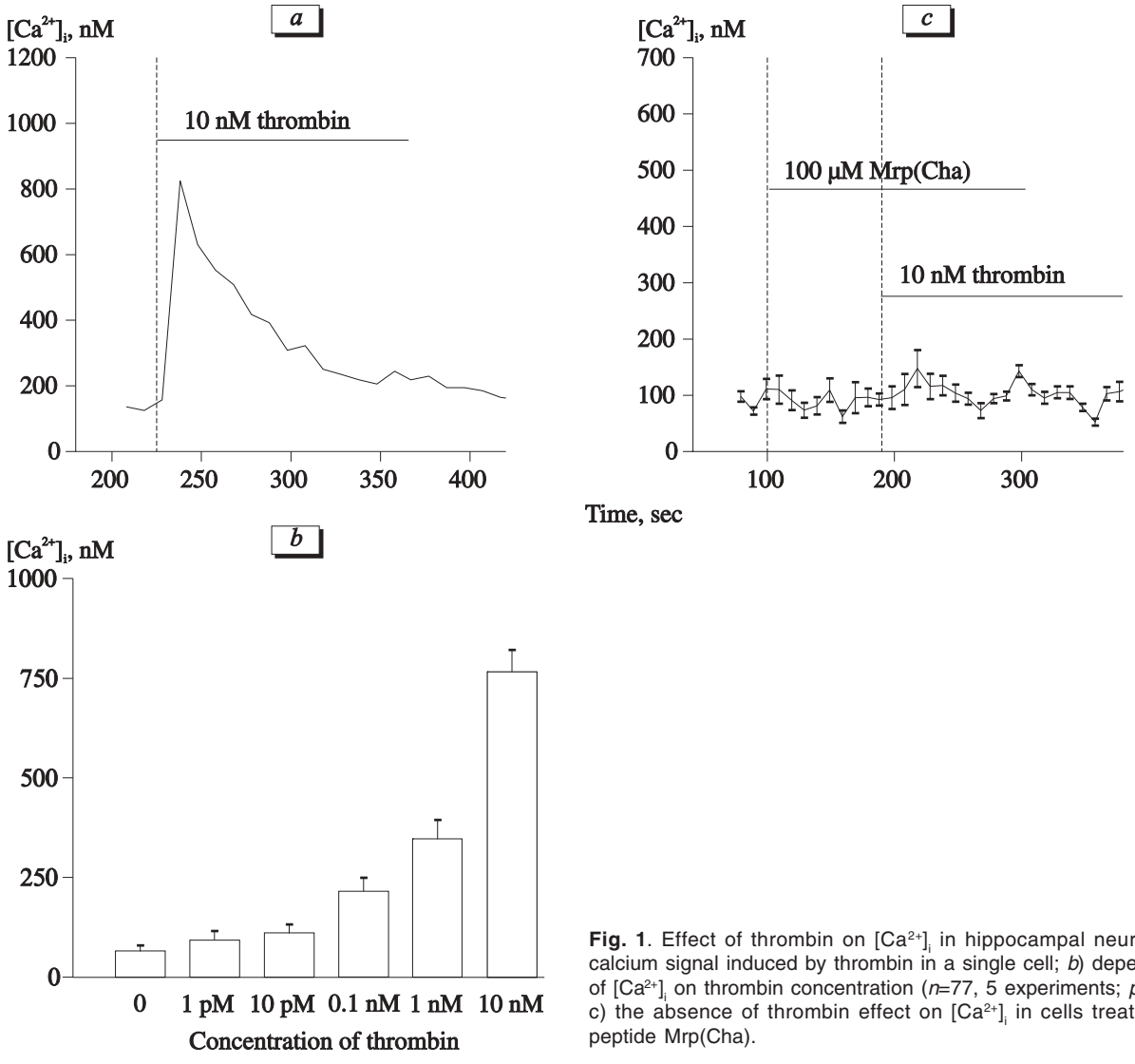

Fig. 1. Effect of thrombin on $[Ca^{2+}]_i$ in hippocampal neurons. *a*) calcium signal induced by thrombin in a single cell; *b*) dependence of $[Ca^{2+}]_i$ on thrombin concentration (n=77, 5 experiments; p<0.05); c) the absence of thrombin effect on $[Ca^{2+}]_i$ in cells treated with peptide Mrp(Cha).

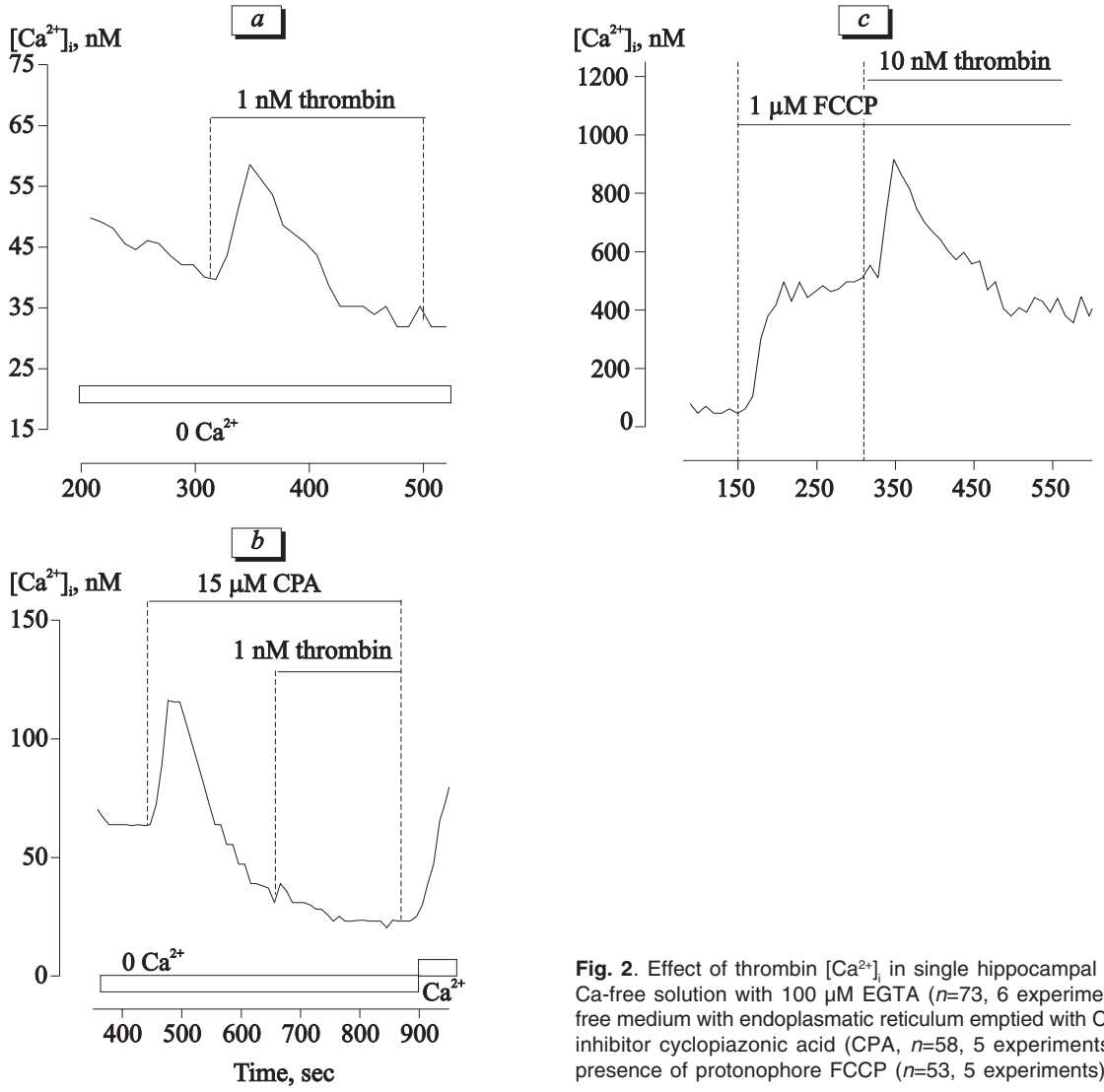

Fig. 2. Effect of thrombin $[Ca^{2+}]_i$ in single hippocampal neurons: *a*) Ca-free solution with 100 μM EGTA (*n*=73, 6 experiments); *b*) Ca-free medium with endoplasmatic reticulum emptied with Ca^{2+}-ATPase inhibitor cyclopiazonic acid (CPA, *n*=58, 5 experiments); *c*) in the presence of protonophore FCCP (*n*=53, 5 experiments).

For evaluation of the involvement of PAR1 into thrombin-induced activation of neurons, we used TFLLRN, a synthetic modified peptide selectively activating PAR1. Similarly to thrombin, this agent (10 μM) elevated $[Ca^{2+}]_i$, by on average 70 nM. PAR is characterized by rapid desensitization after activation with proteinases. This desensitization is determined by internalization of cleaved receptor and cell tolerance to subsequent application of the enzyme [9]. Preliminary activation of thrombin-sensitive PAR in hippocampal neurons with thrombin (1 nM) prevented elevation of $[Ca^{2+}]_i$ in response to TFLLRN (data not shown). For verification of the hypothesis on the involvement of PAR1 in thrombin-induced activation of neurons, we also used Mpr(Cha), a synthetic peptide antagonist of PAR1 on platelets and embryonic cells of human kidney [7]. Incubation with Mpr(Cha) peptide (100 μM) had no effect on $[Ca^{2+}]_i$ in resting neurons. Stimulation of Mpr(Cha)-incubated neurons with thrombin (1 nM) or TFLLRN (100 μM) did not increase $[Ca^{2+}]_i$ (Fig. 1, *c*). Thus, our data agree with the hypothesis on the involvement of PAR1 in the realization of the effect of thrombin on neurons, although other thrombin receptors can also take a part in this process.

The increase in $[Ca^{2+}]_i$ in response to thrombin or TFLLRN can be related to Ca^{2+} entry into cells and/or mobilization of Ca^{2+} from intracellular stores. To examine the mechanisms of this elevation, we used two paradigms: 1) activation of neurons in a Ca^{2+}-free medium (0 Ca^{2+}+100 μM EGTA) and 2) application of thrombin to neurons with emptied EPR calcium depots in a Ca^{2+}-free medium. Replacement of calcium medium with Ca^{2+}-free solution slightly decreased $[Ca^{2+}]_i$. Under these conditions, thrombin increased $[Ca^{2+}]_i$ almost in 70% neurons (48 of 73 cells), but the amplitude of calcium signal was twofold lower than in the calcium-containing medium (Fig. 2, *a*).

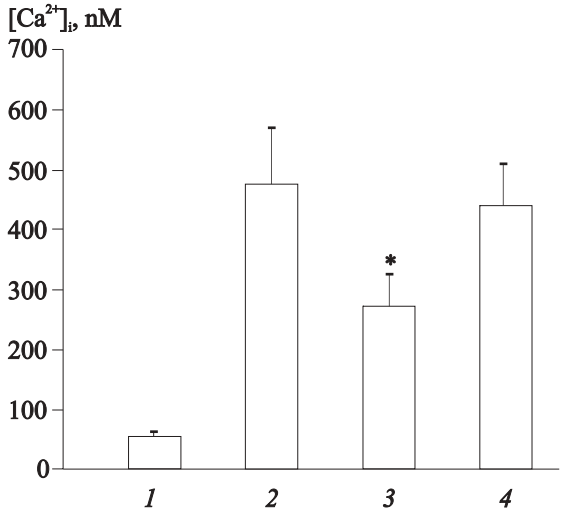

Fig. 3. Effect of thrombin and TFLLRN on maximum changes in $[Ca^{2+}]_i$ induced by NMDA (n=31, 5 experiments). *1*) basal level; *2*) 10 μM NMDA; *3*) 10 μM NMDA and 1 nM thrombin; *4*) 10 μM NMDA and 100 μM TFLLRN. *p<0.05 in comparison with *2*.

In the next experimental series with calcium-free medium, we first emptied EPR in neurons with cyclopiazonic acid (CPA, 15 μM), an inhibitor of Ca^{2+}-ATPase in EPR. CPA induced a transitory increase in $[Ca^{2+}]_i$ caused by calcium release from EPR [6]. Then $[Ca^{2+}]_i$ returned to the baseline level. Subsequent application of thrombin did not increase $[Ca^{2+}]_i$ (Fig. 2, *b*). Peptide TFLLRN also increased $[Ca^{2+}]_i$ in the calcium-free medium (in 60% neurons), but was ineffective after emptying of EPR. These data show that activation of PAR not only mobilizes Ca^{2+} from the intracellular depots via activation of IP_3 receptors of EPR [9], but also induces Ca^{2+} entry into cells, probably, via store-operated channels [13].

For evaluation of the role of mitochondria in thrombin-induced elevation of $[Ca^{2+}]_i$, we used carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP), a protonophore depolarizing mitochondrial membrane and preventing Ca^{2+} uptake by mitochondria via potential-operated uniporter channel. FCCP (1 μM) slightly increased $[Ca^{2+}]_i$ due to calcium release from mitochondria and minor increase in plasmalemma permeability [14]. For evaluation of the dynamics of $[Ca^{2+}]_i$ recovery after application of thrombin, we measured the time of $[Ca^{2+}]_i$ decrease to a half-maximum level ($t_{1/2}$). Application of thrombin in the presence of FCCP resulted in a more prolonged rise of $[Ca^{2+}]_i$. During this process, the amplitude of Ca^{2+}-signal did not significantly change, although the half-time of calcium response increased from $t_{1/2}$=25±5 sec (n=37, 7 experiments, thrombin) to $t_{1/2}$=45±9 sec (n=18, 4 experiments, p<0.05, Fig. 2, *c*). Thus, the experiments showed that Ca^{2+} ions released from EPR under the action of thrombin are captured by mitochondria.

In organotypic hippocampal slice cultures, thrombin increases ionic current via NMDA-subtype glutamate channel-receptor complexes of CA1-pyramidal cells via PAR1 activation [4]. At high concentrations (30-300 nM) thrombin can cleavage a subunit of NMDA-receptor, but the above potentiation occurred independently on receptor cleavage [4]. In our experiments we used N-methyl-D-aspartate (10 μM), a synthetic agonist to NMDA-receptors, which did not induce maximum rise in $[Ca^{2+}]_i$. Preliminary 5-min incubation of neurons with thrombin (1 nM) decreased the amplitude of intracellular calcium signals to NMDA by 1.7 times over 1 min (Fig. 3). However, TFLLRN (10-100 μM) did not exert such an effect on $[Ca^{2+}]_i$. The observed effect of thrombin is probably mediated via activation of other members of PAR receptor family (PAR3 or PAR4), or requires cooperative activation of these receptors. Therefore, our data disagree with [4], but suggest the possibility of cytoprotective effect of thrombin via moderation of $[Ca^{2+}]_i$-signal induced by activation of glutamate receptors.

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REFERENCES

1. B. I. Khodorov, T. P. Storozhevykh, A. M. Surin, *et al.*, *Biol. Membr.*, **18**, 421-432 (2001).
2. D. D. Cunningham and F. M. Donovan, *Adv. Exp. Med. Biol.*, **425**, 67-75 (1997).
3. S. Eimerl and M. Schramm, *J. Neurochem.*, **65**, 739-743 (1995).
4. M. B. Gingrich, C. E. Junge, P. Lyuboslavsky, and S. F. Traynelis, *J. Neurosci.*, **20**, 4582-4595 (2000).
5. G. Grynkiewicz, M. Poenie, and R. Y. Tsien, *J. Biol. Chem.*, **260**, 3440-3450 (1985).
6. M. Hoth, C. M. Fanger, and R. S. Lewis, *J. Cell Biol.*, **137**, 633-648 (1997).
7. A. Kawabata, M. Saifeddine, B. Al-Ani, *et al.*, *J. Pharmacol. Exp. Ther.*, **288**, No. 1, 358-370 (1999).
8. K. R. Lee, I. Drury, E. Vitarbo, and J. T. Hoff, *J. Neurosurg.*, **86**, 272-278 (1997).
9. S. R. Macfarlane, M. J. Seatter, T. Kanke, *et al.*, *Pharmacol. Rev.*, **53**, No. 2, 245-282 (2001).
10. T. Masada, G. Xi, Y. Hua, and R. F. Keep, *Brain Res.*, **867**, 173-179 (2000).
11. A. Ogura, M. Miyamoto, and Y. Kudo, *Exp. Brain Res.*, **73**, 447-458 (1988).
12. Z. Suo, M. Wu, B. A. Citron, *et al.*, *J. Biol. Chem.*, **278**, No. **39**, 37,681-37,689 (2003).
13. G. Szabadkai, A. M. Simoni, and R. Rizzuto, *Ibid.*, No. 17, 15,153-15,161.
14. L. Tretter, C. Chinopoulos, and V. Adam-Vizi, *Mol. Pharmacol.*, **53**, No. 4, 734-741 (1998).
15. G. Xi, G. Reiser, and R. F. Keep, *J. Neurochem.*, **84**, No. 1, 3-9 (2003).