# The protease thrombin is an endogenous mediator of hippocampal neuroprotection against ischemia at low concentrations but causes degeneration at high concentrations

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**We have considered the extracellular serine protease thrombin and its receptor as endogenous mediators of neuronal protection against brain ischemia. Exposure of gerbils to prior mild ischemic insults, here two relatively short-lasting occlusions (2 min) of both common carotid arteries applied at 1-day intervals 2 days before a severe occlusion (6 min), caused a robust ischemic tolerance of hippocampal CA1 neurons. This resistance was impaired if the specific thrombin inhibitor hirudin was injected intracerebroventricularly before each short-lasting insult. Thus, efficient native neuroprotective mechanisms exist and endogenous thrombin seems to be involved therein. *In vitro* experiments using organotypic slice cultures of rat hippocampus revealed that thrombin can have protective but also deleterious effects on hippocampal CA1 neurons. Low concentrations of thrombin (50 pM, 0.01 unit/ml) or of a synthetic thrombin receptor agonist (10 $\mu$M) induced significant neuroprotection against experimental ischemia. In contrast, 50 nM (10 units/ml) thrombin decreased further the reduced neuronal survival that follows the deprivation of oxygen and glucose, and 500 nM even caused neuronal cell death by itself. Degenerative thrombin actions also might be relevant *in vivo*, because hirudin increased the number of surviving neurons when applied before a 6-min occlusion. Among the thrombin concentrations tested, 50 pM induced intracellular $Ca^{2+}$ spikes in fura-2-loaded CA1 neurons whereas higher concentrations caused a sustained $Ca^{2+}$ elevation. Thus, distinct $Ca^{2+}$ signals may define whether or not thrombin initiates protection. Taken together, *in vivo* and *in vitro* data suggest that thrombin can determine neuronal cell death or survival after brain ischemia.**

The extracellular serine protease thrombin, a well known, key player in blood coagulation and platelet activation, has been found to be expressed in different brain regions (1, 2). Its physiological importance in the central nervous system is emphasized further by the parallel expression of the highly specific thrombin inhibitor protease nexin-1 (3, 4) and PAR-1, the classical thrombin receptor (2, 5–7). Some recent evidence indicates that thrombin and its receptor might be involved in neurodegenerative processes observed after different insults such as stroke, traumatic brain injuries, and heart arrest or as a frequent consequence of bypass surgeries (8–11). Normal brain function depends critically on a permanent supply of glucose and oxygen. Depending on its source, a disruption of the cerebrospinal blood flow leads to global or focal ischemia (hypoxia/hypoglycemia) and irreversible neuronal damage. Prothrombin as well as the classical thrombin receptor are expressed in brain regions that are particularly vulnerable to ischemia, e.g., neocortex, cortex, striatum, hypothalamus, hippocampus, and cerebellum (2). Furthermore, studies performed on isolated cells (neurons, astrocytes) have demonstrated that nanomolar concentrations of thrombin exert cytotoxic effects (12–14). However, it has been discussed controversially whether thrombin represents a general death signal or whether this protease also can initiate neuroprotection (8, 12, 13, 15).

To examine the role of thrombin in ischemia-induced neurodegeneration, we employed *in vivo* and *in vitro* models of experimental ischemia: (*i*) transient global ischemia by occlusion of both common carotid arteries in Mongolian gerbils and (*ii*) exposure of organotypic rat hippocampal slice cultures to oxygen and glucose deprivation (OGD).

## Methods

**Materials.** Recombinant hirudin (10,000 antithrombin units/mg protein) and high-activity thrombin [3,000 National Institutes of Health (NIH) units/mg protein] from human plasma were purchased from Calbiochem and Sigma, respectively. NIH units are defined by Sigma by direct comparison with a specific NIH thrombin reference standard lot (manufacturer information). Thrombin was essentially free of other known nonactivated and activated clotting factors as well as of plasminogen and plasmin (manufacturer information). All thrombin treatments were performed by using units per milliliter assuming that 200 units/ml thrombin is approximately equivalent to 1 $\mu$M thrombin (9). The synthetic thrombin receptor agonist peptide Ala-pFluoro-Phe-Arg-Cha-HomoArg-Tyr-$NH_2$ was obtained from Neosystem (Strasbourg). If not stated otherwise, all other compounds used were from Sigma. Experiments involving animals have been approved by the author's institutional review board. Statistical analysis was performed by using the heteroscedastic *t* test.

***In Vivo* Ischemia.** Male Mongolian gerbils (*Meriones unguiculatus*, Tumblebrook farm strain; Charles River Breeding Laboratories) *ca.* 3 months old, weighing 60–80 g, were used. Global cerebral ischemia was performed by transient occlusion of both common carotid arteries. Briefly, 7 days before the final experiment, an injection cannula (0.6-mm diameter) was implanted into the lateral ventricle (0.6 mm posterior to bregma, 1.2 mm lateral to midline) of anaesthetized animals (80 mg/kg pentobarbital). Before the ischemic procedure, gerbils were anaesthetized with 3% halothane in 30% $O_2$/70% $N_2O$. Drugs or vehicle was applied intracerebroventricularly (ICV) 20 min before the occlusion. During all operations, the rectal temperature was maintained at 37 ± 0.5°C by using a heating pad. Surgical clips were used to occlude transiently both common carotid arteries. To

Abbreviations: $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; ICV, intracerebroventricularly; OGD, oxygen and glucose deprivation; PI, propidium iodide.

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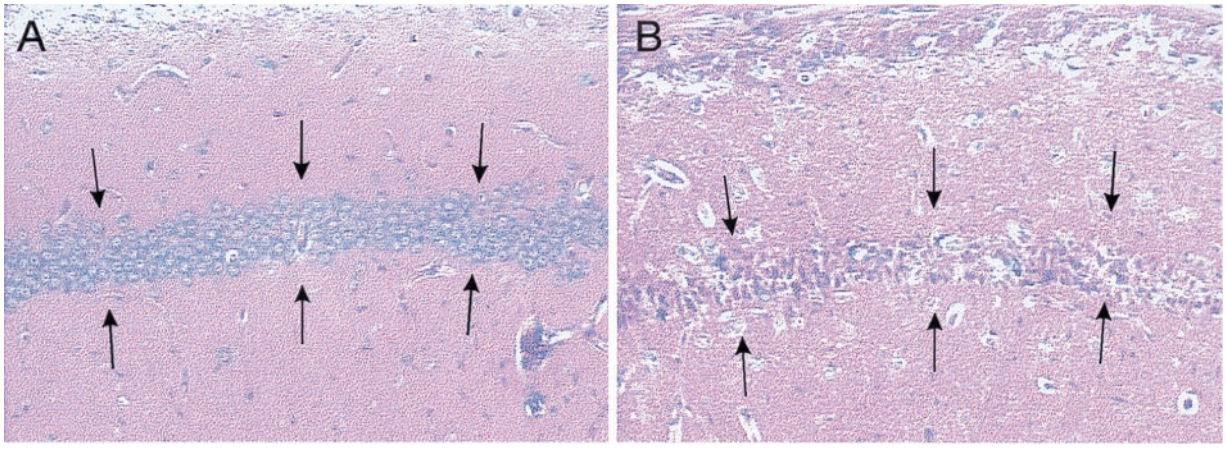


**Fig. 1.** Global ischemia causes a massive loss of pyramidal cells in the hippocampal CA1 region of Mongolian gerbils. Cellular survival was visualized by staining of paraffin-embedded brain slices with toluidine blue/fuchsin acid 7 days after a sham operation (*A*) or a transient occlusion (6 min) of both common carotid arteries (*B*). A layer of intact pyramidal cells can be identified easily within the CA1 region of the control animals (*A*) whereas most neurons disappeared after ischemia (*B*). The pyramidal cell layers are indicated by arrows in *A* and *B*.

avoid anesthetic side effects, halothane was reduced to 0.75% during ischemia. After removing the clips, arteries were inspected for immediate reperfusion and the wound was treated with lidocaine and sutured. Subsequently, the animals were kept at 30°C environmental temperature until recovery from anesthesia. After 7 days, the gerbils were decapitated and the brains were taken out, fixed by immersion in Carnoy's solution, dehydrated with alcohol, transferred into chloroform, and embedded in paraffin. Paraffin sections (10-$\mu$m thick) were cut by using a microtome (Leica, Deerfield, IL) and stained with toluidine blue/fuchsin acid. Stained sections were examined by using a microscope (Diaphot; Nikon). Intact pyramidal cells were counted within a 400-$\mu$m section of the hippocampal CA1 region. Three slices per animal were evaluated.

In the first series of experiments, animals were divided into three groups: sham-operated group ($n$ = 5), hirudin-treated animals ($n$ = 9), and vehicle-treated animals ($n$ = 11). The arteries either were not clamped (sham operation) or clamped for 6 min (severe ischemia). In a second series, two animal groups were exposed to two short-lasting and, therefore, mild occlusions (2 min) at a 1-day interval. A severe occlusion (6 min) was applied 48 h later. Before each mild occlusion, both of these groups received hirudin ($n$ = 20) and vehicle ($n$ = 19) ICV injections, respectively.

**Organotypic Hippocampal Slice Cultures.** Organotypic hippocampal slice cultures were prepared and maintained according to Stoppini *et al.* (16). Briefly, hippocampi were prepared from male Wistar rats (approximately 10 days old, 18–20 g) after decapitation. Hippocampal slices (350 $\mu$m) were cut by using a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall, Surrey, U.K.) and transferred to ice-cold MEM-Hanks' solution (pH 7.35 at 6°C, saturated with 100% $O_2$) containing 25 mM Hepes and 2 mM L-glutamine (GIBCO). Subsequently, undamaged slices were selected by using a binocular microscope and transferred onto Anopore membranes (25-mm diameter, 0.02-$\mu$m pore size) of cell and tissue culture inserts (Nunc). Usually, four to six slices per membrane were cultured. The inserts were set into Nunclon cell and tissue culture six-well plates (Nunc) containing 1.2 ml of culture medium per well. The culture medium (pH 7.35 at 37°C) was 50% MEM-Hanks' solution (Biochrom, Berlin)/25% HBSS (GIBCO)/25% heat-inactivated horse serum (GIBCO)/350 mg/ml $NaHCO_3$/12.5 mM Hepes. During culture, the slices were exposed to 95% air/5% $CO_2$ (36°C). The medium was changed every 2–3 days.

**OGD Experiments.** After 10–13 days in culture, the quality of the organotypic slices was evaluated by transmission microscopy. Only slices of good quality, identified by a bright transmission image, were used for experiments. Slices were washed twice in Ringer's buffer (pH 7.4) containing 10 mM mannitol instead of glucose (Ringer's-mannitol) for 1 min immediately before the experiment. To induce OGD, the slices were transferred into another six-well plate containing 1 ml of Ringer's-mannitol per well and incubated under 95% $N_2$/5% $CO_2$ at 36°C for 5 min (mild OGD) or 30 min (severe OGD). After OGD, the organotypic slices were cultured in the presence of glucose and oxygen for another 24 h. The fluorescence of propidium iodide (PI), which has been shown to enter exclusively damaged cells (17) was used for quantification of cellular survival. The PI fluorescence observed 24 h after 30-min OGD correlates with a complete collapse of synaptic transmission in the hippocampal CA1 region (K.G.R., unpublished data). Thus, the fluorescence of PI was assumed to reflect neuronal cell damage. For PI staining, the slices were incubated in the culture medium containing additionally 0.1 mg/ml PI for 2 h and transferred into Ringer's buffer (pH 7.4) containing 0.05 mg/ml PI. The PI fluorescence was monitored by using an inverted fluorescence microscope (Diaphot; Nikon). Excitation and emission wavelengths were 545 nm and 610 nm, respectively. Both transmission and fluorescence images were captured by using a ×4 objective and a charge-coupled device camera (Visitron Systems, Puchheim, Germany). The fluorescence images were analyzed by using the LUCIA M software package (Laboratory Imaging, Prague). The neuronal damage within each organotypic slice was calculated as the area of PI fluorescence in the hippocampal CA1 and CA4/CA3 region in relation to the total slice area.

**Intracellular $Ca^{2+}$ Imaging.** Organotypic hippocampal slice cultures were loaded with 50 $\mu$M fura-2/AM at 27°C for 45 min. Slice cultures then were transferred into a perfusion chamber (vol = 3 ml) mounted on the stage of an Axiovert inverted florescence microscope with a Fluar objective (×20, 0.75) (Zeiss). An artificial cerebrospinal fluid medium containing 124 mM NaCl, 4.9 mM KCl, 1.3 mM $MgSO_4$, 2.0 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 25.6 mM $NaHCO_3$ and 10 mM glucose (pH 7.4) saturated with 95% $O_2$/5% $CO_2$ was used for perfusion (1 ml/min). The chamber temperature was adjusted to 27 ± 1°C. The fura-2 fluorescence of single CA1 neurons was captured by using a cooled IMAGO charge-coupled device camera and the Polychrome II System (TILL Photonics, Planegg, Germany). Exci-

tation and emission wavelengths were 340/380 nm and ≥500 nm, respectively. Thrombin additions were performed by switching to another medium reservoir containing the appropriate concentration of the protease. The TILLVISION software package (TILL Photonics) was used for data analysis.

## Results

**Hirudin Increases the Survival of Hippocampal CA1 Neurons After Ischemia *in Vivo*.** The number of surviving pyramidal cells in the hippocampal CA1 region was determined 7 days after transient occlusion of both carotid arteries. An occlusion maintained for 6 min caused a massive loss of CA1 neurons. Compared with sham-operated animals, only 6.3 ± 1.1% of the neurons survived (Figs. 1 and 2*A*). To investigate whether endogenous thrombin is involved in ischemia-induced degeneration, we injected the specific thrombin antagonist hirudin ICV (final concentration about 0.1 unit/ml) 20 min before the onset of carotid occlusion. As in the animal group that did not receive hirudin, the survival of hippocampal CA1 cells was determined 7 days after the ischemia. In the hirudin-treated animals, the number of intact CA1 neurons increased about 5-fold to 30.3 ± 8.3% ($P = 0.019$, Fig. 2*A*). Thus, hirudin was able to mediate a significant, albeit only partial, protection against ischemia-induced neuronal degeneration.

**Hirudin Impairs the Ischemic Preconditioning Effect Observed *in Vivo*.** It has been reported previously that prior mild ischemic insults can cause tolerance to an otherwise severe ischemia, an effect that has been termed ischemic preconditioning (18–20). As shown in Fig. 2, exposure of animals to two mild occlusions (each 2 min, 1-day interval) 2 days before a severe occlusion (6 min) indeed caused a robust resistance of the hippocampal CA1 neurons, of which 64.0 ± 5.7% survived compared with the *ca.* 6% in the animals that did not receive the prior mild occlusions. To elucidate whether endogenous thrombin, besides its role in ischemia-induced degeneration, is also involved in this kind of ischemic tolerance, hirudin again was employed. For these experiments, hirudin (0.1 unit/ml) was applied 20 min before each short-lasting but not before the long-lasting occlusion. This treatment decreased significantly the number of neurons surviving in the hippocampal CA1 region—from 64% to 43.8 ± 6.6% ($P = 0.016$, Fig. 2*B*).

**Experimental Ischemia *in Vitro*.** The *in vivo* experiments presented above indicate that endogenous thrombin might be involved in both ischemia-induced degeneration and ischemic preconditioning. Conceivably, opposite thrombin effects might be caused by different concentrations of the protease present in the brain after mild or severe ischemic conditions. Unfortunately, the thrombin concentration hardly can be controlled *in vivo*. Therefore, to manipulate the thrombin concentration, we used organotypic hippocampal slice cultures. Because the intercellular communication network remains at least partly intact, slices resemble the *in vivo* situation better than the dissociated cells in culture used frequently. The organotypic cultures were subjected to experimental "ischemia" by transient OGD. After another 24 h of normal culture conditions, i.e., in the presence of glucose and oxygen, the neuronal survival within each organotypic slice was quantified by the uptake of the fluorescent PI into damaged cells. As shown in Fig. 3, 30 min of OGD induced a widespread neuronal cell death throughout the hippocampal CA1 region. Other regions such as CA4/CA3 and the dentate gyrus usually were less affected (Fig. 3*D*). In contrast, an OGD lasting for only 5 min was tolerated by the cells in all areas, including the particularly sensitive CA1 region (not shown).

To try to imitate the preconditioning effect observed *in vivo*, organotypic hippocampal slices were subjected to a mild OGD (5 min), which was followed by a severe OGD (30 min) applied 24 h later. We were not able to detect any protection by preconditioning *in vitro* (not shown). We have also tested whether thrombin is able to assist a mild OGD in inducing ischemic tolerance *in vitro*. For that purpose, organotypic hippocampal slice cultures were coexposed to a prior mild OGD (5 min) and different concentrations of thrombin 24 h before applying the severe OGD (30 min). Notwithstanding, even this combined treatment failed to improve neuronal survival.

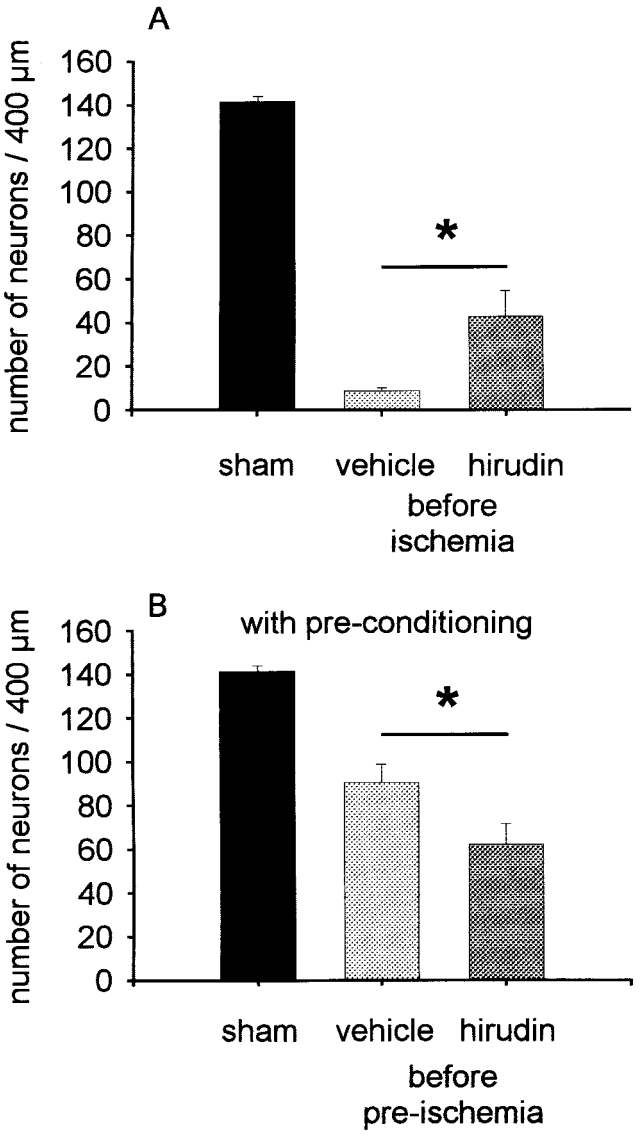


**Fig. 2.** Effects of ischemia and hirudin on the survival of hippocampal CA1 neurons. After staining of brain slices, as shown in Fig. 1, intact pyramidal cells were counted within a 400-$\mu$m section of the hippocampal CA1 region. A severe ischemia (6-min occlusion) caused a massive neuronal cell death, which was decreased if hirudin (≈0.1 unit/ml) was injected ICV 20 min before the occlusion (*A*). If the animals were "preconditioned" by prior mild ischemic insults (two 2-min occlusions at 1-day interval 2 days before a severe ischemia), a marked ischemic tolerance was observed (*B*). Hirudin, applied before each mild insult but not before the severe occlusion, impaired the resistance. Each bar represents the mean ± SEM of *n* animals (*n* is between 5 and 20). *, $P <$ 0.025.

**Effect of Thrombin Treatment on the Viability of Organotypic Hippocampal Slice Cultures.** Considering the possibility that ischemia-induced neurodegeneration and protection might be regulated

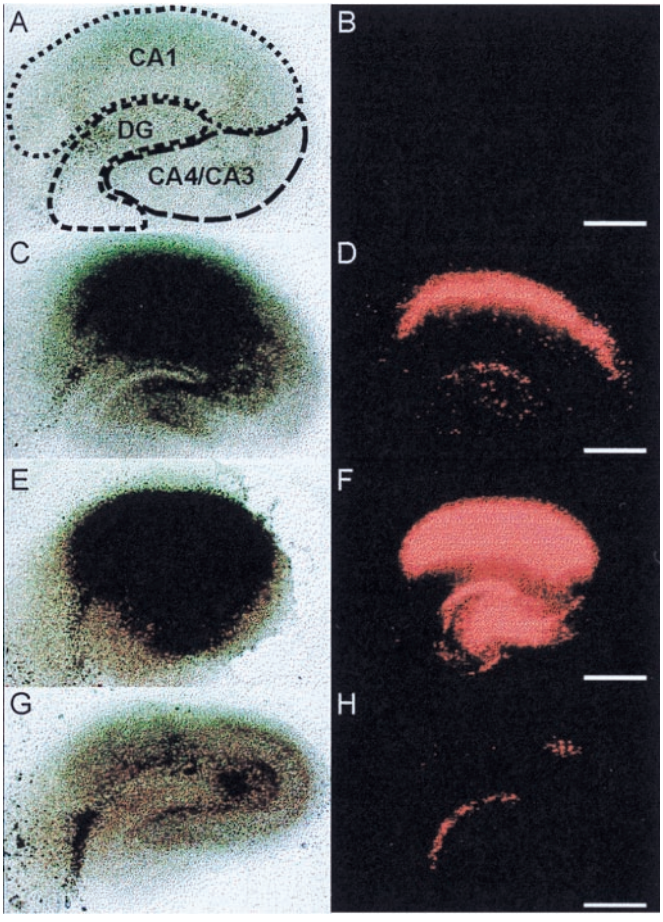


**Fig. 3.** Influence of OGD and/or thrombin on neuronal survival in organotypic hippocampal slice cultures. Transmission images (*Left*) and the uptake of fluorescent PI (*Right*) are shown. Neuronal cell damage can be recognized by dark slice areas and red PI fluorescence in the transmission and fluorescence images, respectively. A control slice is shown in *A* and *B*. Transient OGD (30 min) was followed by a pronounced neuronal damage in the CA1 region 24 h later (*C* and *D*). Exposure to a relatively high thrombin concentration (500 nM, 1-h incubation) in the presence of oxygen and glucose caused even greater damage (*E* and *F*). In contrast, thrombin at a concentration as low as 50 pM, given immediately before and during the OGD, induced significant neuroprotection (*G* and *H*). (Bars = 500 $\mu$m.)

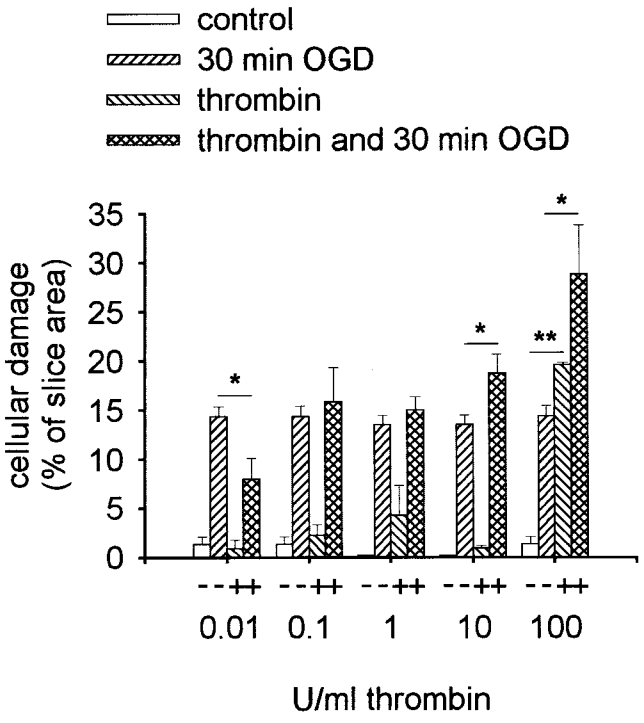


**Fig. 4.** Thrombin can perform either protective or deleterious effects in a concentration-dependent manner. Organotypic hippocampal slice cultures were exposed to OGD and/or thrombin as indicated. The presence or absence of thrombin is indicated by (+) and (−), respectively. At 50 pM (0.01 unit/ml), thrombin caused protection against experimental ischemia, whereas at 500 nM (100 units/ml), thrombin was cytotoxic *per se*. Note that even 50 nM (10 units/ml) thrombin was deleterious when applied in combination with OGD. Intermediate thrombin concentrations of 500 pM–5 nM (0.1–1 unit/ml) did not affect the survival of the slice cultures. For each experiment, the mean ± SEM with *n* between 3 and 22 is shown. *, $P < 0.025$; **, $P < 0.001$.

by different concentrations of endogenous thrombin *in vivo*, organotypic hippocampal slice cultures were exposed to different concentrations of thrombin. At first, we were interested to see whether thrombin *per se* affects the viability of organotypic hippocampal slices. For this reason, slices were transiently exposed to 50 pM, 500 pM, 5 nM, 50 nM, or 500 nM thrombin in the presence of oxygen and glucose. In each experiment, the incubation with thrombin was maintained for 1 h. In analogy to the former experiments, the extent of neuronal degeneration was quantified by the uptake of PI into damaged cells 24 h later. We found that thrombin concentrations up to 50 nM did not affect the neuronal survival (Fig. 4). The outcome, however, was changed dramatically if the concentration of the protease was increased further. At 500 nM, the neuronal cell death exceeded even that induced by 30 min of OGD (Figs. 3 and 4). Similar to the effect seen after a severe OGD, the neuronal cell death occurred mainly in the hippocampal CA1 region with variable damage within the CA4/CA3 region (Fig. 3*F*).

After finding that low and intermediate concentrations of thrombin are well tolerated, we proceeded to examine whether low concentrations of thrombin alter the vulnerability of hippocampal slice cultures to OGD. Therefore, the slices were exposed to different doses of thrombin (50 pM–50 nM, 1-h incubation) and a severe OGD (30 min) was applied after 24 h of normal, thrombin-free conditions. This did not improve neuronal cell survival.

A further experiment was designed to identify possible protective, short-term effects of thrombin. Hippocampal slice cultures were exposed to different thrombin concentrations 30 min before and during a severe OGD (30 min). The protease was added before the OGD to ensure that it had reached all cells within a slice when the OGD was induced. Thrombin was able to induce significant neuroprotection if applied at the lowest concentration used here, namely, 50 pM, 30 min before and during OGD (Figs. 3 and 4). This effect was concentration-dependent because we did not observe neuroprotection at thrombin concentrations of ≥500 pM. At 500 pM or 5 nM, thrombin neither improved nor impaired the neuronal survival of organotypic slices that follows a severe OGD (Fig. 4). In contrast, the extent of neuronal cell death after such an OGD was exacerbated by 50 or 500 nM thrombin (Fig. 4). It should be emphasized here that 50 nM thrombin was "nontoxic" if applied without OGD (Fig. 4).

**Thrombin-Induced Neuroprotection Is Caused by Specific Receptor Activation.** To test whether the protective effect caused by 50 pM thrombin was due to specific activation of the thrombin receptor, we used the synthetic receptor-activating peptide Ala-pFluoro-Phe-Arg-Cha-HomoArg-Tyr-$NH_2$. This peptide has been designed as a specific activator of the classical thrombin receptor

PAR-1 (21). The experiment was performed as that which revealed neuroprotection by 50 pM; but, instead of the protease, the agonist peptide was added 30 min before and during the severe OGD (30 min). The agonist peptide exerted significant neuroprotection at a concentration of 10 $\mu$M (Table 1). Moreover, the extent of protection was similar to that obtained by 50 pM thrombin (compare Table 1 and Fig. 4). Because of the relatively low efficiency of artificial receptor-activating peptides (21), it was not possible to apply an agonist concentration that could mimic the degenerative effect seen at 500 nM thrombin.

**Thrombin-Induced $Ca^{2+}$ Signals in Organotypic Hippocampal Slice Cultures.** The finding that 50 pM thrombin induced neuroprotection against OGD whereas concentrations of ≥500 pM were not effective might be due to distinct signaling cascades that follow receptor activation. Thrombin initiates the generation of inositol 1,4,5-trisphosphate, which regulates the intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) (11). To explore the possibility that specific $Ca^{2+}$ signals are involved in thrombin-induced neuroprotection, hippocampal CA1 neurons were loaded with fura-2. As shown in Fig. 5, the two thrombin concentrations tested, 50 and 500 pM, affected $[Ca^{2+}]_i$ of CA1 neurons in a characteristic manner. The protective concentration of thrombin (50 pM) induced single or repetitive $Ca^{2+}$ spikes (Fig. 5*C*). On the other hand, thrombin concentrations that had failed to mediate neuroprotection against ischemia (≥500 pM) caused a delayed single spike of $[Ca^{2+}]_i$ followed by a sustained plateau phase (Fig. 5*D*). Moreover, in the latter case, the spike occurred synchronously among the different cells of a slice.

## Discussion

One of the central findings of this work is that the thrombin antagonist hirudin protected hippocampal CA1 pyramidal cells *in vivo* when applied before the onset of a severe global ischemia. Because hirudin is said to be highly thrombin-specific and pharmacodynamically inert (22, 23), it appears that its protective effect is caused by inhibition of endogenous thrombin. This, in turn, strongly suggests that endogenous thrombin participates in ischemia-induced neurodegeneration in the hippocampus. An involvement of thrombin in neurodegeneration is supported further by the *in vitro* experiments that showed that 500 nM exerted degenerative effects whereas concentrations ≤50 nM were tolerated. Such a concentration dependence is in accordance with previous studies performed on primary cultures of astrocytes and neurons (12–14). It may be questioned whether thrombin concentrations of 500 nM are reached in the brain, even under pathophysiological conditions. However, it is pertinent to mention here that 50 nM thrombin, although nontoxic *per se*, already impaired further the neuronal survival of the cultures when applied in combination with a 30-min OGD. This combination of factors may reflect the situation encountered during a stroke, where the cells might be exposed simultaneously to OGD and to increased levels of thrombin. That is, under ischemic conditions, thrombin does not need to reach very high concentrations to unleash its deleterious effects.

The development of ischemic tolerance after a prior mild ischemia *in vivo* indicates the existence of endogenous mecha-

**Table 1. Effect of the thrombin receptor agonist peptide on OGD-induced degeneration**

| | | Cellular damage | | | |
|---|---|---|---|---|---|
| Agonist, $\mu$M | OGD, min | % | SEM | *n* | *P* |
| Control | 30 | 100 | 0.0 | 7 | |
| 1 | 30 | 82 | 13.0 | 4 | 0.263 |
| 10 | 30 | 61* | 6.6 | 3 | 0.027 |

Hippocampal slice cultures were exposed to different agonist concentrations 30 min before and during OGD. The neuronal survival was quantified 24 h after OGD. *, $P < 0.05$.

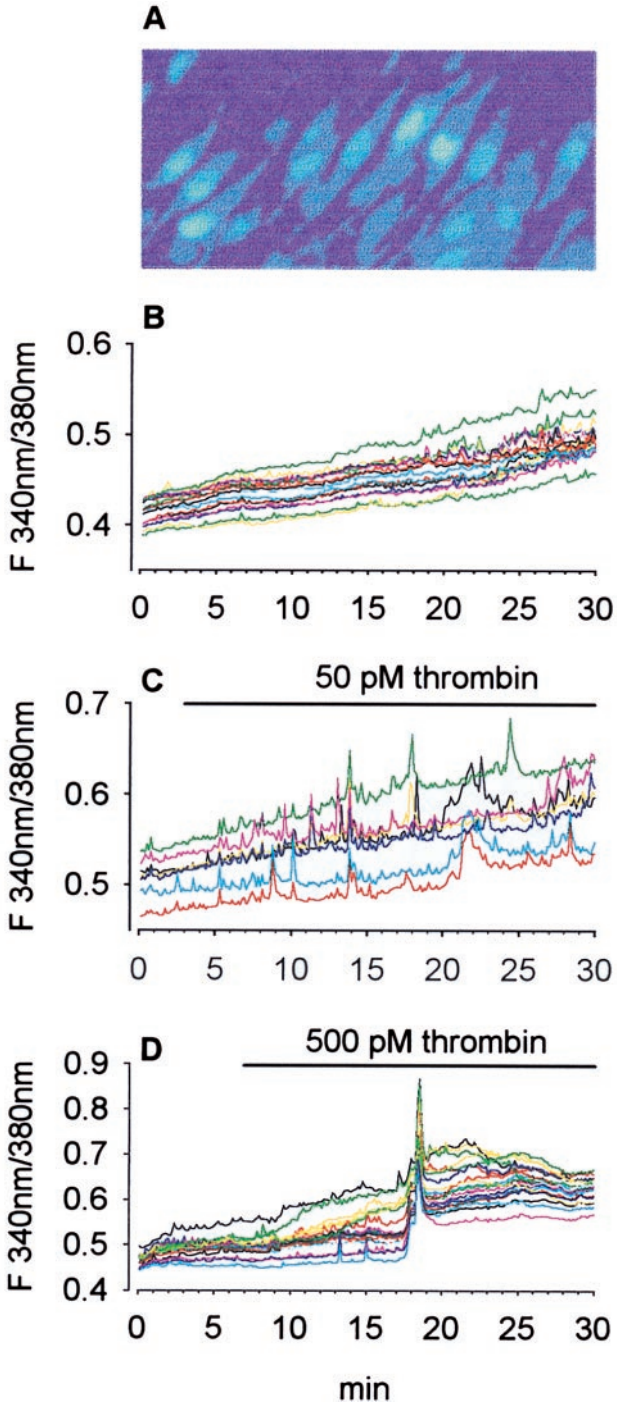


**Fig. 5.** Thrombin-induced intracellular $Ca^{2+}$ elevations in hippocampal CA1 neurons. After loading of organotypic slice cultures with fura-2, individual pyramidal cells in the CA1 region can be identified easily (*A*). Only their soma was used for $Ca^{2+}$ measurements. Slices were exposed either to a thrombin-free (*B*) or thrombin-containing (*C* and *D*) perfusion medium. Both thrombin concentrations raised $[Ca^{2+}]_i$ albeit with different characteristics. Single or repetitive $Ca^{2+}$ spikes were observed with 50 pM thrombin, whereas a sustained $Ca^{2+}$ elevation was induced by 500 pM. For each agonist concentration, a typical experiment (of 11) is shown. Each line illustrates the response of an individual cell within the same slice. Fluorescence images were captured every 8 s.

nisms mediating long-term protection against ischemia (18–20). This fact is of paramount importance for the development of new strategies to alleviate the disabling consequences of stroke. Unfortunately, apart from the suggestion that different heat-shock proteins play a role in ischemic preconditioning (24), its mechanism remains widely unknown. In this respect, that hirudin impairs ischemic preconditioning when injected immediately before prior mild ischemic insults is of special interest, because it implies that endogenous thrombin may be a necessary factor. However, because we failed to reproduce the preconditioning effect in the slices *in vitro*, it is likely that an increase in thrombin is necessary but not sufficient for an efficacious preconditioning.

Nevertheless, we were able to induce significant neuroprotection by thrombin *in vitro* when the protease was applied immediately before and during OGD, suggesting that thrombin, besides its involvement in long-lasting ischemic preconditioning, also can mediate rapid neuronal protection against ischemic insults. It turns out that thrombin receptor activation as well as possibly a distinct intracellular $Ca^{2+}$ signal, namely, single or repetitive spikes of $[Ca^{2+}]_i$, underlie this effect. Thrombin-induced neuroprotection was obtained with very low concentrations (*ca.* 50 pM), whereas higher doses either did not affect the neuronal survival or further impaired it. Therefore, it appears that thrombin has a biphasic effect on neuronal cells, mediating neuroprotection against ischemia at very low concentrations and inducing cell death at higher concentrations. It already has been shown that *in vitro* low thrombin concentrations protect isolated astrocytes or neurons from cell death induced by glucose deprivation, oxidative stress, growth supplement deprivation (8, 25), or $\beta$-amyloid peptide (26). Thus, thrombin might mediate neuroprotection against a variety of degenerative insults.

Taken together, several lines of evidence imply a role of thrombin in neuronal cell death but also in neuronal survival after brain ischemia. Based on the findings presented here, we suggest the following model. After a mild ischemia, low levels of thrombin participate in manifesting a long-lasting, certainly limited, ischemic tolerance. After a severe ischemia, high thrombin concentrations induce cell death within the infarct center, whereas low levels of thrombin may rescue neuronal cells in the periphery.

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