In situ visualization of caspase-1-like activity associated with promotion of hippocampal cell death

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Abstract To clarify the function of caspase-1-like proteases in neuronal cell death, it is important to be able to detect the activity in living organs by microscopic visualization. In the present study, we synthesized a novel fluorescent substrate sensitive to the caspase-1-like activity, which is easily introduced into cells constituting living organs by extracellular application. As a result, the substrate was shown to be useful in imaging the caspase-1-like activity in rat hippocampal slice cultures. After induction of cell death with glutamate, a significant increase in the activity was observed, especially in the pyramidal cells, suggesting the association of the activity with promotion of cell death.

Key words: Caspase-1; Cell death; Fluorescent substrate; Fluorescence resonance energy transfer; Glutamate; Hippocampus; Visualization

1. Introduction

Cell death in the nervous system is a physiologically important process in the development and in various neuronal diseases [1]. Such cell death is known to be regulated by a cysteine protease family called caspases. Among them, caspase-3 and related caspases are thought to be important in the initiation and execution of neuronal apoptosis and their molecular mechanisms have been relatively well understood [2]. On the other hand, the function of caspase-1 and the closest relatives, caspases-4 and -5, in the nervous system has not been well characterized. The caspase-1 group is assumed to function primarily in activation of proinflammatory cytokines [3]. However, activation of caspase-1-like proteases has also been reported in several models of neuronal apoptosis [4–8].

To clarify the details of the activation mechanism in vivo of caspase-1-like proteases in the nervous system, microscopic visualization of the activity in living cells would provide much information. Along this line, we synthesized a novel fluorescent substrate sensitive to the caspase-1-like activity, which is easily introduced into cells by extracellular application. Indeed, the substrate could be introduced not only into single cells but also into organs constituted by many cells. Furthermore, using this substrate, we were able to visualize intracellular caspase-1-like activity in rat hippocampal slice cultures. The result clarified time-dependency and subcellular localization of the caspase-1-like activity in vivo, indicating the implication of the activity in promotion of glutamate-stimulated cell death. This novel approach using cultured slices may be applicable in general to analyses for other intracellular proteolytic activities in living cells.

2. Materials and methods

2.1. Synthesis of TMR-YVADAC(AD)

10 μl of 50 mM Tyr-Val-Ala-Asp-Ala-Cys, synthesized by a conventional Fmoc solid-phase peptide synthesis method, in dimethylsulfoxide and 1 ml of 50 mM HEPES buffer, pH 6.9, were mixed and to this was added 100 μl of 10 mM 6-acryloyl-2-dimethylaminonaphthalene (acrylodan; Molecular Probes) in dimethylformamide and 400 μl of acetonitrile. After reaction for 5 h at room temperature, the product was purified by high-performance liquid chromatography (HPLC) (LC-10 system, Shimadzu) on a TSKgel ODS-120T column (4.6×250 mm, Tosoh) and lyophilized. Then, 10 μl of the product (33 mM) in dimethylsulfoxide, 1 ml of 100 mM sodium bicarbonate buffer, pH 8.6, and 100 μl of 10 mg/ml tetramethylrhodamine-5-succinimidyl ester (Molecular Probes) in dimethylsulfoxide were mixed. After reaction for 5 h at room temperature, the product TMR-YVADAC(AD) was purified by HPLC, lyophilized and dissolved in dimethylsulfoxide to an appropriate concentration. Matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS): *m/z* 1375.49 (theoretical MH^+, 1375.57). Quantitative analysis was done by an amino acid analyzer (model 421, Applied Biosystems) after acid hydrolysis.

2.2. Protease assay in vitro

36 pmol of TMR-YVADAC(AD) and 1 U of recombinant human caspase-1 (Biomol Research Laboratories) in 600 μl of 50 mM HEPES buffer, pH 7.5, containing 100 mM sodium chloride, 20% glycerol and 10 mM dithiothreitol, were incubated at 37°C. The reaction mixture was then analyzed by a fluorescence spectrophotometer (F2000, Hitachi) (Fig. 2) or by HPLC (Fig. 3). The kinetic parameters of the substrate toward caspase-1 were determined by a Hanes–Woolf plot.

2.3. Preparation of rat hippocampal slice cultures and stimulation by glutamate

Organotypic slice cultures of rat hippocampus were prepared as described [9]. The hippocampi were removed from Wistar rats 5–7 days after birth and 300-μm slices prepared using a McIlwain tissue chopper (The Mickle Laboratory Engineering). Each slice was then

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Abbreviations: acrylodan, 6-acryloyl-2-dimethylaminonaphthalene; FRET, fluorescence resonance energy transfer; HPLC, high-performance liquid chromatography; YVAD, Tyr-Val-Ala-Asp; Z-DEVD-AFC, N^{α}-bezyloxycarbonyl-Asp-Glu-Val-Asp-4-trifluoromethylcoumaryl-7-amide; Z-YVAD-AFC, N^{α}-benzyloxycarbonyl-Tyr-Val-Ala-Asp-4-trifluoromethylcoumaryl-7-amide

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placed in a six-well microplate (code 3810-006, Iwaki Glass) containing 1.0 ml of culture medium (Eagle's minimal essential medium, Gibco BRL Life Technologies) and cultured at 37°C for 1 week. Then, cell death was induced in them by treatment with 10 mM glutamate in the medium for 1 h and they were further cultured for appropriate periods.

2.4. Detection of dead cells by propidium iodide (PI) staining

Dead or seriously damaged cells in the cultures were stained by treatment with 2 μM PI in the medium for 1 h before the analyses at various time points (2.5, 5, 7.5 and 10 h), as described [9]. Then the cultures were mounted on the stage of a fluorescence microscope (IX70, Olympus). The PI fluorescence (excitation at 520–550 nm, emission at >580 nm) of the culture was detected by using an SIT camera (C2400/8, Hamamatsu Photonics) set at constant sensitivity and quantified as the average intensity using an image analyzer (Argus 50, Hamamatsu Photonics). The intensity of the fluorescence for given areas (each about 300 pixels), clipped from a total of 510×480 pixels, was calculated by digitalizing the intensity by frame memory, and the average fluorescence intensity was obtained by dividing the intensity by the total pixels.

2.5. Loading TMR-YVADAC(AD) and detection of fluorescence change

The cultures were treated with 37.5 μM TMR-YVADAC(AD) in the medium for 1 h before the analyses at various time points (2.5, 5, 7.5 and 10 h). The microscopic observation was done as described above (excitation at 375–385 nm, emission at 510–530 nm).

3. Results

3.1. Design and characteristics of a fluorescent substrate for detecting caspase-1-like activity

It is known that caspase-1 cleaves very specifically after the YVAD sequence in intracellular proteins and synthetic peptides [3,10,11]. Based on an oligopeptide containing this sequence, we designed and synthesized a novel double-fluorescence-labeled substrate, N^{α}-5-tetramethylrhodaminyl-Tyr-Val-Ala-Asp-Ala-Cys(*S*-acrylodan) (TMR-YVADAC(AD)), for detecting caspase-1-like activity (Fig. 1). When we measured the fluorescence spectra (excitation at 380 nm) of the substrate before and after incubation with recombinant human caspase-1 in vitro, fluorescence intensity at 525 nm was increased time-dependently (Fig. 2). Upon HPLC analysis, two products were detected after the incubation (Fig. 3) and identified to be N^{α}-5-tetramethylrhodaminyl-Tyr-Val-Ala-Asp and Ala-Cys(*S*-acrylodan) by MALDI-TOF-MS analyses (*m/z*, 879.58 and 515.38, respectively; theoretical MH^+, 879.36 and 515.23, respectively). The K_m, k_{cat} and k_{cat}/K_m values for the substrate were calculated to be 14.1 μM, 1.2 s^{-1} and 8.5×10^{-2} μM^{-1} s^{-1}, respectively, under the present assay conditions.

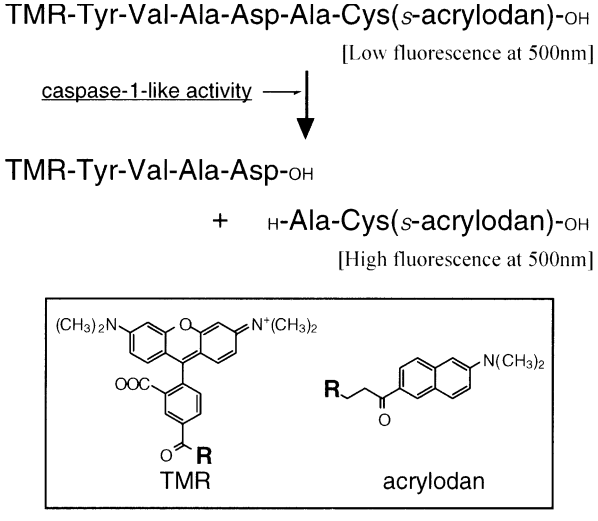

Fig. 1. The structure of TMR-YVADAC(AD) and detection of caspase-1-like activity. TMR, 5-tetramethylrhodaminyl.

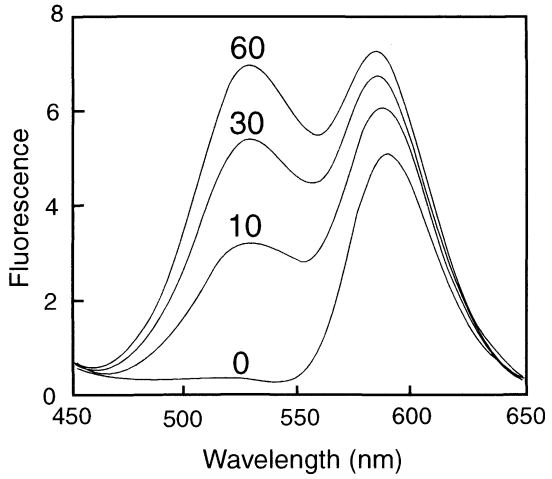

Fig. 2. Fluorescence profile of TMR-YVADAC(AD) (excitation, 380 nm). The time (min) of incubation with recombinant human caspase-1 is indicated above each curve.

3.2. Induction of cell death in hippocampal cultures by glutamate

In the present study, we used rat hippocampal slice cultures as a target organ for the synthetic substrate. Cell death was

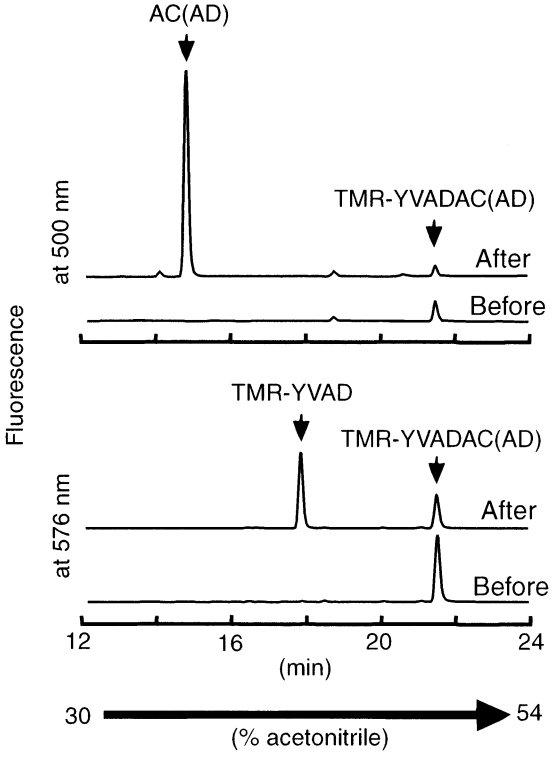

Fig. 3. HPLC profiles of TMR-YVADAC(AD) before and after incubation. Incubation time was 60 min. Excitation and emission wavelengths were 391 nm and 500 nm, respectively, for the upper panel, and 544 nm and 576 nm, respectively, for the lower panel. AC(AD), Ala-Cys(*S*-acrylodan); TMR-YVAD, N^{α}-5-tetramethylrhodaminyl-Tyr-Val-Ala-Asp.

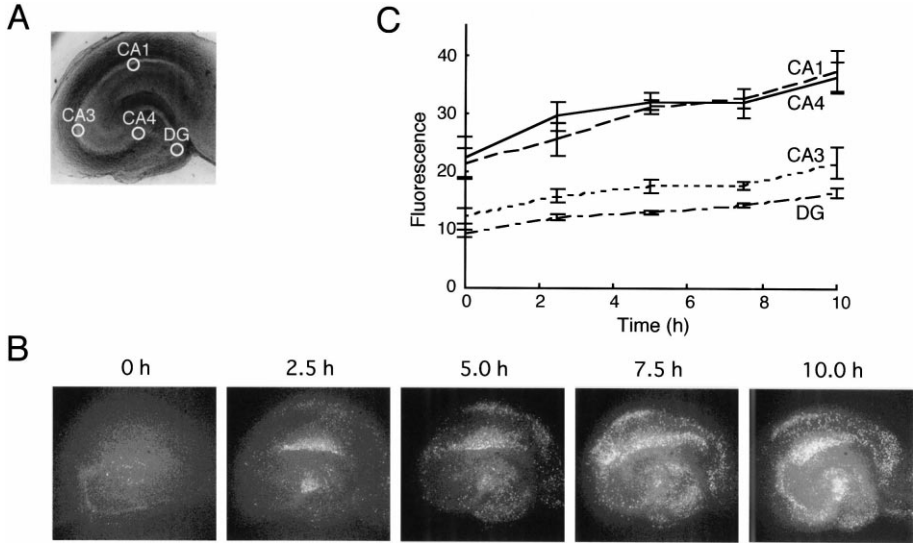

Fig. 4. Detection of dead cells by PI staining in rat hippocampal slice cultures. A: A microscopic image of a rat hippocampal slice culture (not stimulated with glutamate). Areas shown with open circles were used for evaluating fluorescence intensity. B: Images of the cultures stained by PI at indicated times after the start of the glutamate treatment. C: Change of the fluorescence in each area. Error bars indicate ±S.D. ($n=6$). DG, dentate gyrus.

induced in the culture by incubation with 10 mM glutamate for 1 h. After further cultivation for appropriate periods, we treated the culture with PI and observed stained dead or seriously damaged cells by using a fluorescence microscope with an imaging apparatus (Fig. 4). As a result, the number of stained cells increased time-dependently after the glutamate treatment. The increases in number of stained pyramidal cells, especially in the CA1 and CA4 areas, were apparently much more marked than those in CA3 and dentate gyrus.

3.3. Application of TMR-YVADAC(AD)

We applied TMR-YVADAC(AD) to the glutamate-stimulated hippocampal slice cultures to detect caspase-1-like activity in vivo. The cultures were treated with a 37.5 μM substrate solution in the medium for 1 h before analyses to allow the substrate to penetrate into cells and then examined them under the fluorescence microscope with 380 ± 10 nm excitation. For image processing of the fluorescence, we used a bandpath filter (520 ± 10 nm) to detect cleavage of the substrate. We tested three slice cultures, I, II and III, which were prepared from three individual rats, (Fig. 5). At 0 h (no stimulation with glutamate), almost no fluorescence was observed in the three cultures. In the case of culture I, the fluorescence in the CA1 and CA4 areas increased significantly, peaked at 7.5 and 5 h, respectively, after the start of the glutamate treatment and then decreased until 10 h. The increase in the CA3 area, which also peaked at 7.5 h, was less marked. On the other hand, the fluorescence in dentate gyrus scarcely increased. The results with culture II were similar to those with culture I; fluorescence in the CA1, CA3 and CA4 areas increased, peaked at 7.5 h and then decreased until 10 h, whereas that in dentate gyrus increased only slightly. In the case of culture III, the fluorescence in the CA1, CA3 and CA4 areas increased more markedly than that in dentate gyrus, similar to the cases of cultures I and II; however, maximum fluorescence was observed at 10 h in all the area.

4. Discussion

In the present study, we designed and synthesized a novel fluorescent substrate, TMR-YVADAC(AD), based on an oligopeptide containing YVAD sequence for detecting caspase-1-like activity. According to the classification of caspases by Nicholson et al., caspase-1 and its relatives, such as caspases-4 and -5, are categorized to group I caspases of which substrate specificity is distinctly different from those of group II (caspases-2, -3 and -7) and group III (caspases-6, -8 and -9) caspases [11]. The YVAD sequence is highly specific for group I but not for the groups II and III caspases. Thus the above substrate was thought to be fairly specific to caspase-1-like activity. Indeed the substrate was efficiently cleaved by caspase-1 in vitro at the peptide bond between Asp and Ala residues as expected. The K_m, k_{cat} and k_{cat}/K_m were comparable to those of Ac-YVAD-AFC (11.5 μM, 1.0 s^{-1} and 9.1×10^{-2} μM^{-1} s^{-1}, respectively) and DABCYL-YVADAPV-EDANS (N^{α}-4-(4′-dimethylaminophenylazo)benzoyl-Tyr-Val-Ala-Asp-Ala-Pro-Val-5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid; 13.4 μM, 0.41 s^{-1} and 3.1×10^{-2} μM^{-1} s^{-1}, respectively) [11], which are among the most generally used substrates for detecting caspase-1-like activity in vitro. Upon cleavage of the substrate, the fluorescence at 525 nm apparently originating from the acrylodan moiety, which had been quenched by fluorescent resonance energy transfer (FRET) to the tetramethylrhodamine moiety before cleavage, was greatly increased. These features of the substrate should be certainly useful for detecting caspase-1-like activity.

So far, several groups reported on microscopic visualization of activities of intracellular proteases in living cells by using FRET-based fluorescent substrate [12–15]. Such substrates are categorized to two types; one type of the substrates includes peptide substrates similar to that used in the present study and the other involves green fluorescent protein-based protein substrates. The former have been introduced to cells by microinjection and the latter expressed into cells by transfected vectors. In both cases, substrates are applied to each single

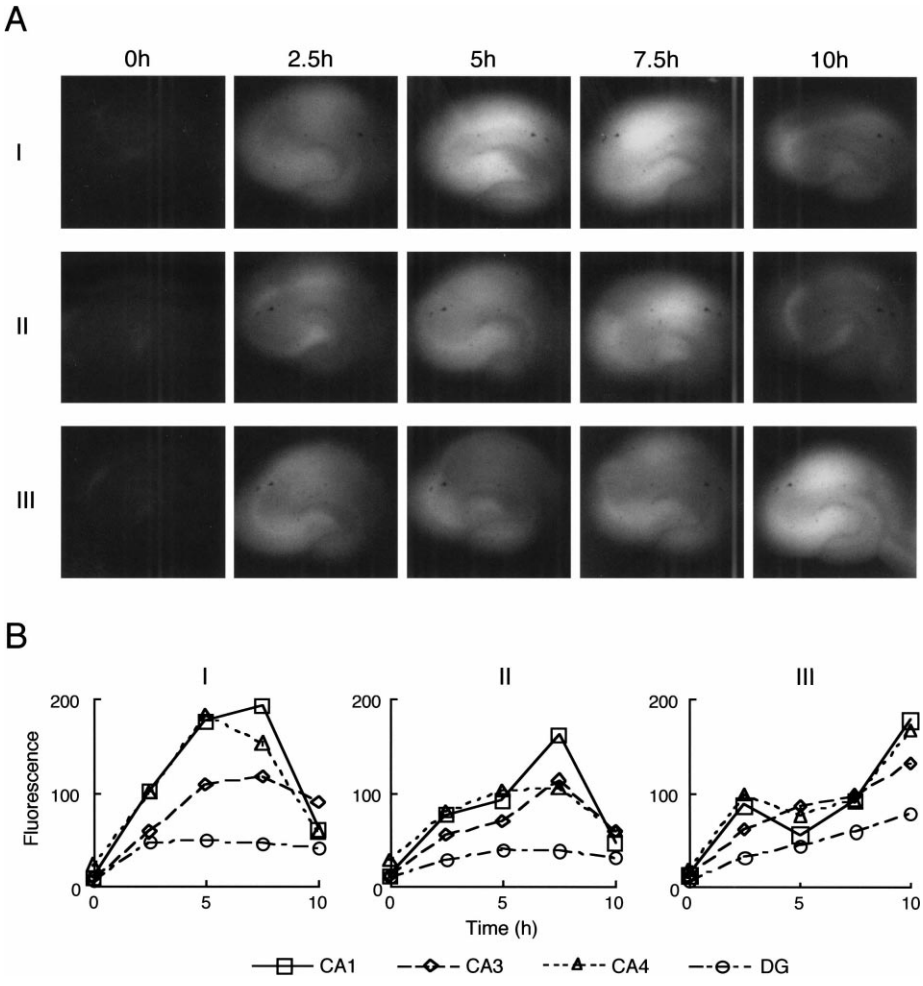

Fig. 5. Application of TMR-YVADAC(AD) to the hippocampal slice cultures (I–III). A: Images of the cultures loaded with TMR-YVADAC(AD) at indicated times after the start of the glutamate treatment. B: Change of the fluorescence in each area (see Fig. 4A).

cell and so cannot be applied to organs constituted by many cells. In the present study, we presumed that the substrate could be introduced into cells by extracellular application because of its relatively high hydrophobicity. Thus this method is easily applicable to organs that contain many cells. As a result, the substrate could indeed be introduced into the cells of cultured rat hippocampus slices. On the other hand, neither of the commercially available substrates, Z-YVAD-AFC (N^{α}-benzyloxycarbonyl-Tyr-Val-Ala-Asp-4-trifluoromethylcoumaryl-7-amide) and Z-DEVD-AFC (N^{α}-bezyloxycarbonyl-Asp-Glu-Val-Asp-4-trifluoromethylcoumaryl-7-amide) (Calbiochem, CA, USA), which are used to detect in vitro caspases-1 and -3 activities, respectively, could be introduced into cells by the same procedure (data not shown). Furthermore, such a good permeability has not been reported for other substrates used so far. With the present substrate at hand, it should be possible to analyze the caspase-1-like activity in living organs.

Glutamate receptor-mediated toxicity is an important mechanism of neuronal cell death under various pathogenic conditions [16–20]. Neurotoxicity initiated by overstimulation of glutamate receptors has been known to induce subsequent influx of Ca^{2+} in several neural systems including hippocampal neurons [21]. This causes cell death by leading to an intracellular cascade of cytotoxic events. In the present study, we detected the dead cells in the hippocampal slice culture by PI staining after glutamate treatment. As a result, the number of dead cells increased time-dependently after the stimulation as expected, at least for 10 h, and the increase was more marked in CA1 and CA4 than in CA3 and dentate gyrus (Fig. 4).

Based on these results, we then applied substrate TMR-YVADAC(AD) to the hippocampal slice cultures and visualized caspase-1-like activity. We tested three cultures that were prepared from three individual rats. In cultures I and II, fluorescence intensity representing the caspase-1-like activity was significantly increased in pyramidal cells, especially in the CA1 and CA4 areas, peaked at 5–7.5 h and then decreased (Fig. 4), whereas little change in the fluorescence occurred in dentate gyrus. The areas with caspase-1-like activity well corresponded to those where dead cells were detected. It is worth noting that the cell death still progressed after the decrease in the caspase-1-like activity. On the other hand, in culture III, maximum fluorescence intensity was observed at 10 h in every area, though the areas where high fluorescence was observed well corresponded to those of cultures I and II. It seems that this might be caused by delay of the temporal activation of the caspase-1-like activity observed in cultures I and II and that the time course of activation might be slightly variable from each other. These results suggest the possibility that the caspase-1-like activity might play a certain role at a certain

stage of an intracellular cascade of cytotoxic events in hippocampus after stimulation by glutamate. It remains to be clarified how the caspase-1-like activity is concerned in the cascade in each area and whether it acts as a direct executioner or as a modulator of cell death through activation of proinflammatory cytokines or other functions.

In the present study, we demonstrated a novel approach for investigating intracellular caspase-1-like activity in a living organ and showed a temporal increase in the activity in certain areas of hippocampus. It should be possible to apply this method to detection of other proteolytic activities in living organs and such analyses will provide novel information to clarify functions of intracellular proteases.

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