興奮性神経細胞死における神経細胞核DNA損傷・修復の1分子イメージングによる評価

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研究代表者　山本　清二
（浜松医科大学・光量子医学研究センター・助教授 ）

は し が き

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研究組織

研究代表者：山本清二（浜松医科大学・光量子医学研究センター・助教授）

研究分担者：寺川　進（浜松医科大学・光量子医学研究センター・教授）

櫻井孝司（浜松医科大学・光量子医学研究センター・助手）

研究協力者：范　文英（浜松医科大学・COE研究員）

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Inositol trisphosphate pathway as an excitotoxic death signal to the nucleus in hippocampal neurons

Summary

We sought to determine the mechanism underlying the rapid nuclear change that corresponds to DNA fragmentation during excitotoxicity in hippocampal neurons. We observed: 1) glutamate or N-methyl-D-aspartate (NMDA) increased cytoplasmic and nucleoplasmic inositol trisphosphate (IP_3) concentrations detected with green fluorescent protein, and increased nucleoplasmic Ca^{2+} concentration ($[Ca^{2+}]_n$) that was consistently associated with the nuclear changes, 2) IP_3 induced an increase in $[Ca^{2+}]_n$ and the nuclear changes in permeabilized neurons, and 3) a blocker of IP_3-mediated Ca^{2+} release significantly inhibited the nuclear changes induced by glutamate or by NMDA. Glutamate activates NMDA and metabotropic receptors both linked to the IP_3 pathway, increases $[Ca^{2+}]_n$ persisting above the physiological level, and rapidly damages the nuclear DNA in excitotoxicity.

Key words:

excitotoxicity, DNA fragmentation, inositol trisphosphate, nuclear calcium signal

Introduction

Excitotoxicity, a neuronal cell death induced by overactivation of glutamate receptors, is the major pathological basis for neurodegenerative diseases, stroke, and trauma (Choi, 1988a). In our laboratory, a video-enhanced contrast-differential interference contrast (VEC-DIC) microscope revealed that glutamate induces a rapid nuclear change in the cultured cortical neurons in rats (Ikeda et al., 1996). Under the VEC-DIC microscope, the nuclei of the neurons gain abnormal granularity within 20 min following glutamate exposure, a change corresponding to nuclear DNA fragmentation (Ikeda et al., 1996). Interestingly, this nuclear change is a leading step in both acute and delayed neuronal cell death (Ikeda et al., 1996). However, the mechanism underlying this early nuclear change induced by glutamate remains unknown.

Ca^{2+} has been thought to be involved in causing glutamate-induced neuronal death (Choi, 1988b). By activation of the N-methyl-D-aspartate (NMDA) receptor with glutamate, the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) rises over the capacity of the cell regulatory mechanism, driving a cascade of calcium-dependent processes all the way to the neuronal death. However, the trigger for the Ca signal may not be so simple. In hippocampal neurons of the gerbil, for example, two-thirds of the Ca^{2+} surge induced by ischemia are released from the internal stores (Mitani et al., 1993). Glutamate activates metabotropic-, G-protein linked receptors, as well, and induces Ca^{2+} release from intracellular organelles. The participation of Ca^{2+} released from internal stores may explain the slow progress in clinical use of the antagonist to ionotropic glutamate receptors for the treatment of excitotoxic diseases.

Nuclear Ca^{2+} signals regulate gene transcription, DNA synthesis, DNA repair, and other nuclear functions (Bachs et al., 1992; Karin, 1992), while the signal source remains under debate. The increase in the nucleoplasmic Ca^{2+} concentration ($[Ca^{2+}]_n$) persisting above the physiological level may result in the damage of nuclear DNA Since the nuclear envelope is recently considered as an internal Ca^{2+} store (Gerasimenko et al., 1995; Stehno-Bittel et al., 1995), glutamate can release Ca^{2+} directly from the nuclear envelope, activates nuclear signals, and induces DNA damage in the process of

excitotoxicity. To test the hypothesis, we examined: 1) the increase in $[Ca^{2+}]_n$ associated with the nuclear change, 2) the effects of inositol trisphosphate (IP_3) on the nuclear structure in permeabilized neurons, 3) IP_3 generation upon glutamate receptor activation, and 4) the effects of IP_3-cascade blocker on the nuclear changes during excitotoxicity. This study may contribute to the understanding of the mechanisms underlying DNA damage during the early process of excitotoxicity and thereby contribute to the treatment of neuronal death. This study can also contribute to the understanding of the source of the nuclear Ca^{2+} signals. We report that glutamate stimulates the IP_3 system, increases $[Ca^{2+}]_n$, and induces rapid changes in intranuclear properties. We will demonstrate that NMDA receptor activation alone also stimulates the IP_3 pathway.

Methods

Primary culture of hippocampal neuron

Hippocampal neurons were prepared from one-day-old Wistar rats. After removal of the brain under brief anesthesia, the hippocampus was dissected out, and dissociated. Cells were placed on collagen-coated culture dishes (Iwaki 35 mm/Glass Base Dish, Asahi Techno Glass Co., Tokyo, Japan). The culture medium was a mixture of DMEM (50%; Gibco No. 31600, Invitrogen Japan K.K., Tokyo, Japan), HBSS (25%; Gibco No. 11201), and horse serum (25%). Glucose and HEPES were added at a final concentration of 36.1 mM and 23.7 mM, respectively. The pH of the medium was adjusted to 7.2 by adding NaOH. Penicillin-G, streptomycin, and cytosine arabinoside were added to the culture medium. The dish was incubated for a week at 37°C with a 5% CO_2-containing atmosphere, and was then subjected to the experiment.

Morphological observation

A cultured dish containing cells was placed on the stage of an inverted microscope (Axiovert 10, Zeiss, Oberkochen, Germany) equipped with a 100x DIC lens. The DIC image was obtained with a charge-coupled device (CCD) camera (C6489, Hamamatsu Photonics K.K., Hamamatsu, Japan). The video image was contrast-enhanced digitally in real time by using an image processor (ARGUS 20, Hamamatsu Photonics K.K.), then monitored and recorded on videotape. Artificial cerebrospinal fluid (aCSF) was used as a recording medium, and its composition (mM) was 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 10 HEPES, and 10 glucose with a pH of 7.2. In case of NMDA challenge, aCSF containing 50 μM glycine was used. Throughout the experiment, the temperature of the medium was maintained at 34 ± 2 °C.

Ca^{2+} imaging

The neurons were loaded with a Ca^{2+}-indicating dye by incubation in aCSF (see morphological observation) containing 4.4 μM fluo-3/AM (Dojindo, Kumamoto, Japan) in 5% CO_2 and 95% air at 37 °C for 20 min. After washing, the neurons were observed under a confocal laser microscope (IX 70, Olympus, Tokyo, Japan) equipped

with a microlens-attached Nipkow-disk scanner (CSU-10, Yokokawa Electric Co., Tokyo, Japan). The confocal fluorescence images were recorded at video rate with a CCD camera (C2400, Hamamatsu Photonics K.K., Hamamatsu, Japan) combined with an image intensifier (C2400-21SV, Hamamatsu Photonics K.K.), and analyzed with a software, MetaMorph (Universal Imaging Co., Downingtown, PA, USA). Before and after the application of drugs, the DIC images of the same neurons were also examined.

Cell permeabilization

The cultured cells were washed three times with Ca^{2+}-free aCSF (pH 7.2), and then washed three times with Ca^{2+}-free aCSF (pH 6.8) containing 0.2 mM ethyleneglycoltetracetic acid (EGTA, Dojindo, Kumamoto, Japan), and then incubated for 5 min at 37°C. Subsequently, the cells were permeabilized with a permeabilizing buffer (pH 6.8) containing sodium glutamate (140 mM), glucose (5 mM), PIPES (20 mM), EGTA (5 mM), and β-escin (1 μM, Sigma, St. Louis, MO, USA). In order to examine the effect of IP_3, 10 μM IP_3 (Dojindo, Kumamoto, Japan) was added to the Ca^{2+}-free aCSF.

Observation of IP_3 signal in living cells

To analyze the effects of glutamate on the IP_3 system, we used GFP-PHD. The plasmid of GFP-PHD was kindly provided by Dr. Andreas Jeromin (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, University of Toronto, Toronto, Canada). Cells were transiently transfected with plasmid GFP-PHD using a LipofectAMINE 2000 (Invitrogen Japan K.K., Tokyo, Japan). At 36 to 48 h after transfection, the cells were observed under a confocal laser microscope (see *Ca^{2+} imaging*). After recording the control images, the cells were exposed to the medium containing glutamate or NMDA.

Statistics

We conducted each experiment at least three times using a completely different preparation each time (the number of trials may not be noted in the text). Data were analyzed by one-way analysis of variance (ANOVA), and two group comparisons were

made by using the least significant difference test. We set statistical significance to be $p < 0.05$.

Results

1. Glutamate-induced granulation of the nucleus

Under the VEC-DIC microscope, the hippocampal neurons in control showed large nuclei containing one or two nucleoli and otherwise an amorphous nucleoplasm (**Fig 1-a**). An application of glutamate (100 μM - 1 mM) rapidly induced granulation inside the nucleus within 10 min (**Fig. 1-b**) corresponding to DNA fragmentation (Ikeda et al., 1996). Upon continuous exposure to glutamate, these nuclear changes were followed by nuclear and cellular swelling indicating the process of necrosis (**Fig. 1-c**), which was fully consistent to our previous observations in cortical neurons (Ikeda et al., 1996). The necrosis was further confirmed by observing the neurons under a fluorescent microscope after staining them with propidium iodide and annexin V-FITC (data not shown). Notably, the changes in morphological property of the nucleus occurred at first before all other morphological changes revealed by these staining.

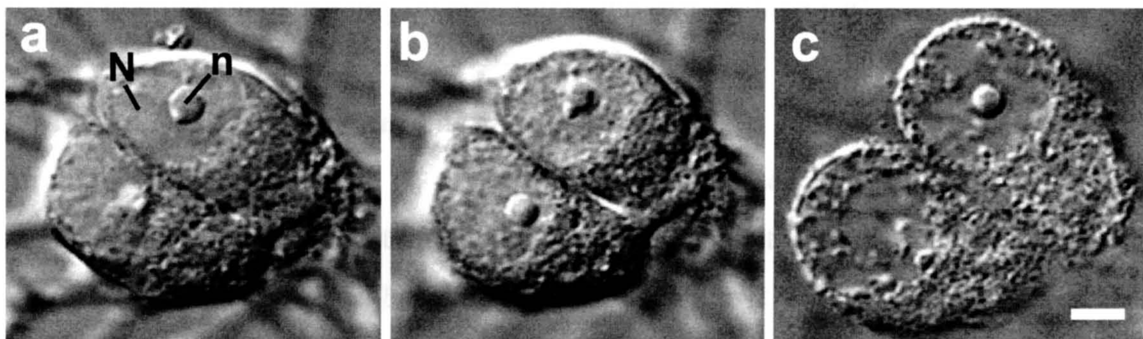


Fig. 1
Sequential changes of rat hippocampal neurons following glutamate exposure. Neurons were observed under the VEC-DIC microscope before (a), and at 10 min (b), and 40 min (c) after an application of glutamate (1 mM). Before an application of glutamate, the neurons had large nuclei (N) containing a smooth and amorphous nucleoplasm and one or two nucleoli (n). Note that glutamate rapidly induced granulation inside the nucleus followed by nuclear and cellular swelling, indicating the character of necrosis. Scale Bar = 5 μm

The morphological changes induced by glutamate depend upon the concentration of glutamate and upon the duration of exposure. A hundred micro molar of glutamate required longer exposure to induce nuclear changes than did 1 mM glutamate. The morphological changes also depended on the period of the culture. In 3 or 4-day-cultured neurons, an application of 100 μM - 1 mM glutamate sometimes did not induce rapid nuclear changes. Therefore, in this study, the neurons were cultured for a week, and then used for the experiments.

2. Dependence on extra- and intracellular Ca^{2+}

Pre-incubation (for 20 min) of neurons in the presence of NMDA receptor antagonist, MK-801 (20 μM), followed by its co-administration with glutamate (100 μM) partially and never completely inhibited the granular changes [the time at which granulation became noticeable (see Fig.1) in the nucleus: from 284.2 ± 164.8 s (glutamate) to 680.9 ± 399.8 s (glutamate with MK-801), $p<0.005$, $n=12$]. Removal of Ca^{2+} from the medium also significantly delayed but did not completely inhibit the responses (to 725.0 ± 403.6 s, $p<0.005$, $n=12$). These results indicated that glutamate could induce nuclear granulation depending on both intra- and extracellular Ca^{2+}.

3. $[Ca^{2+}]_n$ elevation before nuclear granulation

An application of glutamate (1 mM) persistently increased $[Ca^{2+}]_n$ as well as $[Ca^{2+}]_c$ observed with a Ca^{2+}-indicator, fluo-3 (4.5 μM), under the time-lapse confocal laser microscope (**Fig. 2-A**). It has been found that fluo-3 shows higher fluorescence intensity in the nucleoplasm homogenates that in the cytosolic hemogenates (Perez-Terzic et al., 1997). Therefore, the fluorescence intensities between the cytoplasm and the nucleoplasm may not be compared each other even in the same neurons. However, the qualitative determinations of nuclear and cytosolic Ca^{2+} signals can be analyzed. When we observed the changes in $[Ca^{2+}]_n$ and nuclear morphology almost simultaneously in the same neuron, the increase in $[Ca^{2+}]_n$ always preceded the nuclear granulation (data not shown). This increase in $[Ca^{2+}]_n$ was consistently associated with rapid nuclear changes when we observed the same neurons under the VEC-DIC microscope. In the absence of increase, no morphological changes were seen

in the nucleus (**Figs. 2-B, C**).

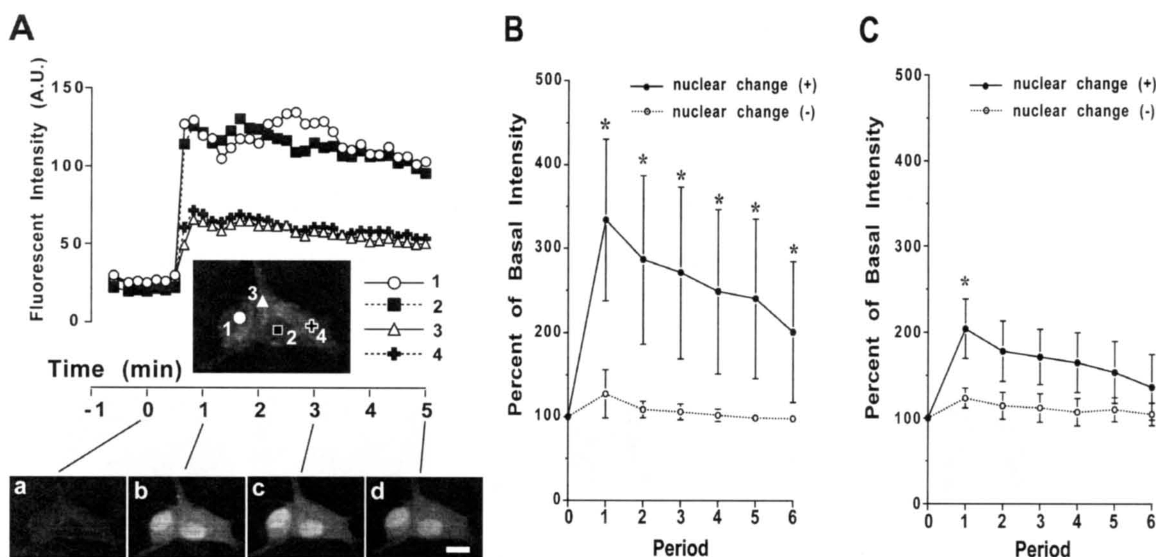


Fig. 2

A. Ca^{2+} images following glutamate exposure observed under a time lapse confocal laser microscope. The fluorescence images of Ca^{2+} obtained by using fluo3/AM were observed every 10 s, and the fluorescence intensity (arbitrary unit, A.U.) was measured in the representative four areas of the nucleoplasm (1, 2) and the cytoplasm (3, 4) in the two hippocampal neurons. The representative images before (a), and at 1 min (b), 3 min (c), and 5 min (d) after an application (Time = 0) of glutamate (1 mM) are shown below the graph. Note that glutamate persistently increased fluorescence intensity in the nucleoplasm as well as in the cytoplasm. Scale bar = 10 μm. **B. Changes in nucleoplasmic Ca^{2+} intensity.** The fluorescence images of Ca^{2+} were observed by using fluo3/AM. In 3 or 4-day-cultured neurons, an application of glutamate at 100 μM-1 mM sometimes did not induce rapid nuclear changes. To examine the correlation between Ca^{2+} increase and the morphological changes, we compared the change in the fluorescence intensity of the Ca^{2+}-indicator in the neurons with (closed circle, n=15, exposure to 1 mM glutamate) or without (open circle, n=10, exposure to 100 μM-1 mM glutamate) nuclear change. The basal intensity was measured before glutamate exposure, and the percent of basal intensity was calculated at the initial peak (Time = 0) following glutamate exposure, and every 1 min after the initial peak. Note that the nucleoplasmic intensities in the neurons with nuclear changes were significantly higher than those in the neurons without nuclear changes in any periods. **C. Changes in cytoplasmic Ca^{2+} intensity.** The cytoplasmic intensities in the neurons with nuclear changes also significantly increased as compared with those in the neurons without nuclear changes, but it did not do so persistently. * $p<0.05$ different from nuclear change (-). Data represent as mean ± SD.

4. IP_3-induced $[Ca^{2+}]_n$ rise and nuclear change

In the neurons permeabilized with β-escin (1 μM) (Konishi and Watanabe, 1995) and treated with ethyleneglycoltetracetic acid (EGTA, 5 mM), a Ca^{2+} chelating agent, an application of IP_3 (1-10 μM) induced the rapid granulation of nucleus (**Figs. 3-A, B**),

which were comparable to those induced by glutamate (see Fig. 1). Addition of IP_3 to the external medium also induced a persistent increase in $[Ca^{2+}]_n$ in the permeabilized neurons (**Fig. 3-D**). Since EGTA was present in the medium, an external application of IP_3 did not increase $[Ca^{2+}]_c$. However, IP_3 increased $[Ca^{2+}]_n$ in the same preparation, indicating that the nucleus alone can exhibit its own Ca^{2+} transient in response to IP_3. The nuclear granulation and persistent increase in $[Ca^{2+}]_n$ induced by IP_3 were inhibited by co-administration of heparin (10 U/ml), an IP_3 receptor blocker (**Figs. 3-C, E**).

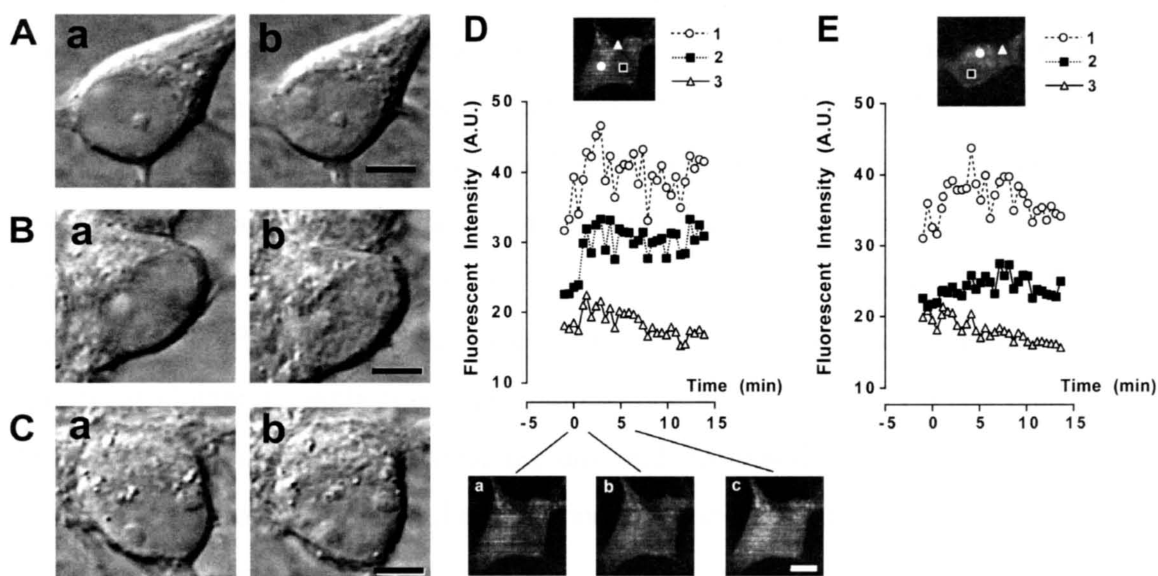


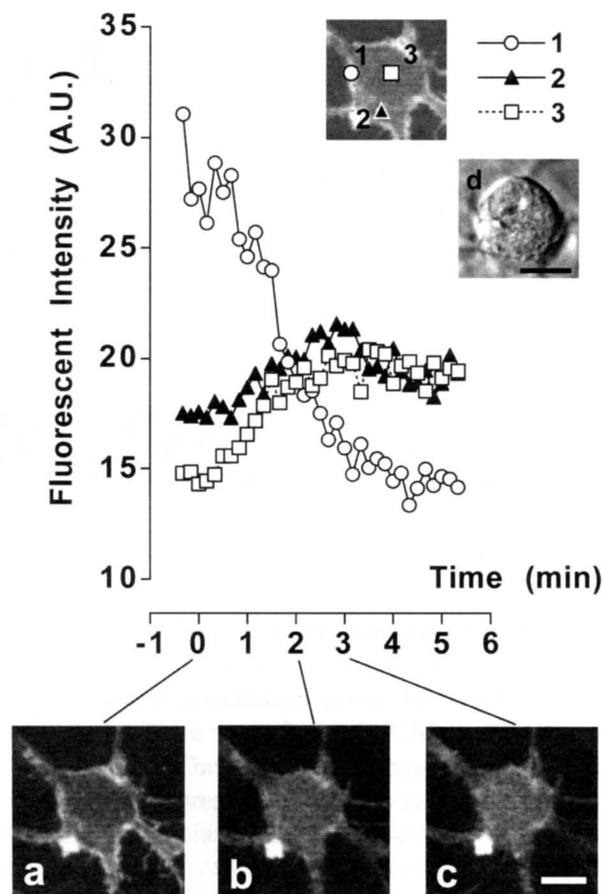
Fig. 3
DIC images of permeabilized neurons following an application of vehicle (A), IP_3 (10 μM) (B), and IP_3 with heparin (10 U/ml) (C). The neurons were pemeabilized with β-escin (1 μM), and a Ca^{2+} chelator, ethyleneglycoltetracetic acid (EGTA). The neurons were observed under a DIC microscope before (a) and at 10 min (b) after vehicle or IP_3 application. Vehicle alone (**A**) did not induce nuclear changes in the permeabilized neuron. Note that an application of IP_3 (**B**) induced rapid granular changes inside the nucleus which were identical to those induced by glutamate (see Fig. 1), and that co-administration of heparin (**C**), an IP_3 receptor blocker, inhibited the morphological changes induced by IP_3. Scale bar = 10 μm. **Changes in the fluorescence intensity of Ca^{2+} images following IP_3 exposure in the pemeabilized neuron with (E) or without (D) heparin.** The fluorescence images of Ca^{2+} obtained by using fluo3/AM were observed, and the fluorescence intensity (arbitrary unit, A.U.) was measured every 10 s in the representative three areas; one was the high intensity area in the cytoplasm (area 1, open circle) which was probably mitochondria, one was the nucleoplasmic region (area 2, closed square), and the other was the low intensity area in the cytoplasm (area 3, open triangle) in the permeabilized hippocampal neuron. The images before (a), at 1.5 min (b) and 6 min (c) after IP_3 application (Time = 0) are shown together below the graph (**D**). Since EGTA chelates Ca^{2+}, an application of IP_3 did not increase the intensity of area 3. IP_3 increased the $[Ca^{2+}]_n$ intensity of area 2 in **D** that was inhibited by co-administration of heparin (area 2 in **E**). In area 1, fluorescent intensity varies during an application of IP_3. Scale bar = 10 μm.

To distinguish the contribution of ryanodine-sensitive Ca^{2+} release, we examined the effect of caffeine (10 mM), which stimulates ryanodine-sensitive receptors and increases $[Ca^{2+}]_c$ in the neurons (Irving et al., 1992; Seymour-Laurent and Barish, 1995), on the nuclear changes observed under the VEC-DIC microscope. During a period of 30 min, caffeine did not induce the typical granular changes of the nucleus in the neurons (data not shown).

5. Glutamate-induced rise in IP_3 concentration

To analyze the effects of glutamate on the IP_3 system, we used an endogenous fluorescent indicator: green fluorescent protein (GFP) tagged to the pleckstrin homology domain (PHD) of phospholipase C (PLC) δ1 (GFP-PHD) (Stauffer et al., 1998). This indicator binds to inositol bisphosphate in the plasma membrane and translocates to the cytoplasm as receptor stimulation brings about IP_3 liberation, and thus allows us to detect the IP_3 signal (Hirose et al., 1999; Okubo et al., 2003). After transfection, GFP-PHD was expressed mainly in the plasma membrane (**Fig. 4a**). Following glutamate application, the fluorescence intensity of GFP-PHD increased in the cytoplasmic and nucleoplasmic regions and decreased in the plasma membrane region (**Fig. 4**), indicating that glutamate increased cytoplasmic and nucleoplasmic IP_3 concentrations during the same period in which we observed nuclear granulation.

Fig. 4
Translocation of GFP-PHD observed under the confocal laser microscope following glutamate exposure in the hippocampal neuron. The neurons were transfected with green fluorescent protein (GFP)-tagged the pleckstrin homology domain (PHD) of phospholipase C (PLC) δ1 (GFP-PHD), an in vivo fluorescent indicator for IP_3, which locates within the plasma membrane and translocates to the cytoplasm after receptor stimulation, and allows the detection of increased concentrations of IP_3. Under a confocal laser microscope, prior to an application of glutamate (a), GFP-PHD was expressed in hippocampal neurons, and was concentrated at the plasma membrane (area 1, open circle). Following glutamate application (Time = 0), the fluorescence intensity of GFP-PHD in the cytoplasmic (area 2, closed triangle) and nucleoplasmic (area 3, open square) regions increased, whereas that at plasma membrane (area 1) decreased, indicating that glutamate increased cytoplasmic and nucleoplasmic IP_3 concentration. The images (a, b, c) are shown together at the time points indicated. After observing fluorescent images, DIC image of the same neuron indicated nuclear granulation (d). Scale bar = 10 μm.



6. NMDA receptor-linked IP_3 system

Activation of NMDA receptor alone with a specific agonist induced a nuclear granulation in the neuron observed comparable to those induced by glutamate (**Fig. 5-A**, and see Fig. 1). The selective activation increased $[Ca^{2+}]_n$ as well as $[Ca^{2+}]_c$ as observed with fluo-3 under the time-lapse confocal laser microscope (**Fig. 5-B**). The increase in $[Ca^{2+}]_n$ but not in $[Ca^{2+}]_c$ was consistently associated with the rapid nuclear changes observed under the VEC-DIC microscope (**Fig. 5-C, D**). In the neurons transfected with GFP-PHD, an application of NMDA (1 mM) induced translocation of the fluorescence from the plasma membrane to the cytoplasm and the nucleoplasm, showing an IP_3 signal (**Fig. 6**).

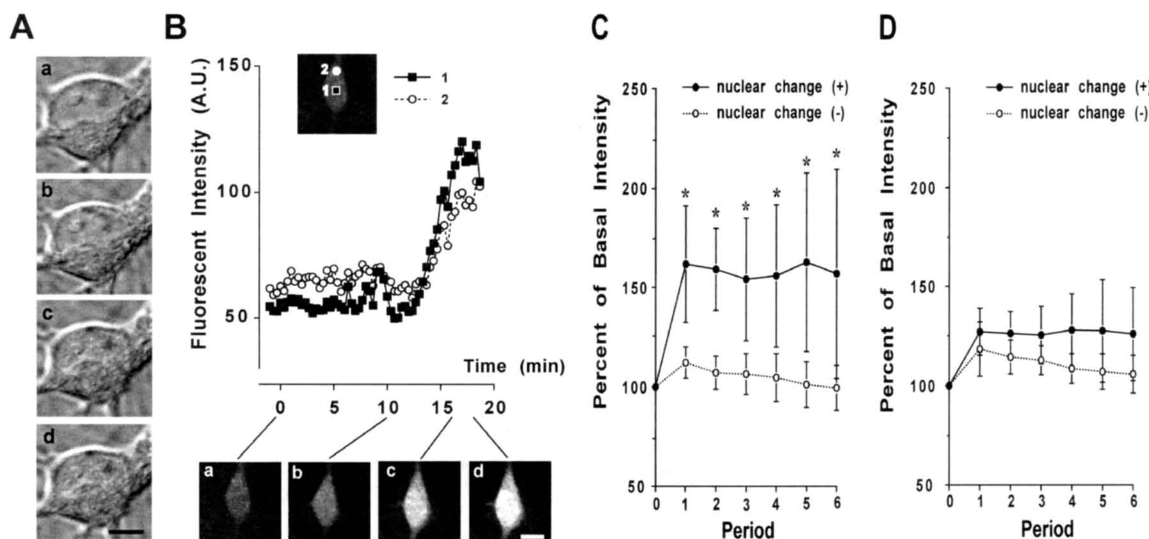


Fig. 5

Sequential changes of the hippocampal neuron exposed to NMDA. The neuron was observed under a DIC microscope before (a), and at 8 min (b), 15 min (c), and 20 min (d) after NMDA (1 mM) application. NMDA induced granulation inside the nucleus, which was identical to that induced by glutamate (see Fig. 1). Scale bar = 5 μm. **B. Changes in fluorescence intensity of Ca^{2+} image following NMDA exposure.** The fluorescent images of Ca^{2+} obtained by using fluo3/AM were observed every 10 s, and the fluorescence intensity (arbitrary unit, A.U.) was measured in the representative two areas of the nucleoplasm (area 1, closed square) and the cytoplasm (area 2, open circle) in the hippocampal neuron. The representative fluorescence images before (a) and at 10 min (b), 16 min (c), and 18 min (d) after NMDA (1 mM) exposure (Time = 0) are indicated below the graph. Note that NMDA slightly increased fluorescence intensity in the cytoplasm, and markedly increased it in both nucleoplasm and cytoplasm. The increase in $[Ca^{2+}]_n$ was consistently associated with the nuclear changes. Scale bar = 10 μm. **Changes in nucleoplasmic (C) and cytoplasmic (D) intensity of Ca^{2+} image following NMDA exposure.** In 3 or 4-day-cultured neurons, an application of 1 mM NMDA sometimes did not induce rapid nuclear changes. To examine the correlation between Ca^{2+} increase and morphological changes, we compared the change in fluorescence intensity of Ca^{2+}-indicator in the neurons with (closed circle, n=8) or without (open circle, n=10) nuclear change. The basal intensity was measured before NMDA (1 mM) exposure, and the percent of basal intensity was calculated at the initial peak (Time = 0) following NMDA exposure, and every 1 min after the initial peak. Note that the nucleoplasmic intensities in the neurons with nuclear changes were significantly higher than those in the neuron without nuclear changes in any periods. The cytoplasmic intensities in the neurons with nuclear changes did not statistically differ from those in the neurons without nuclear changes. * $p<0.05$ different from nuclear change (-). Data represent as mean ± SD.

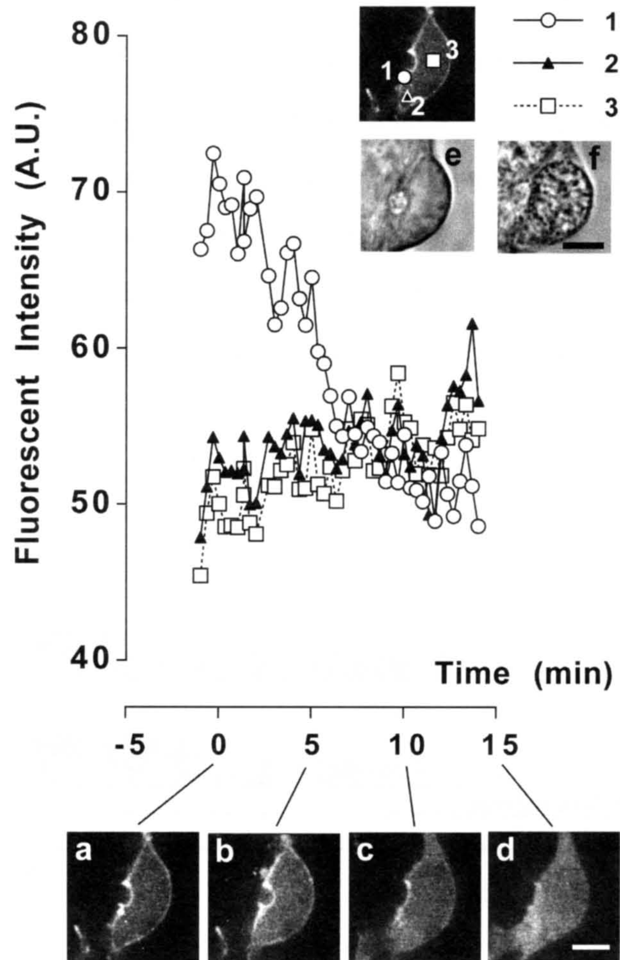


Fig. 6

Translocation of GFP-PHD following NMDA exposure in the hippocampal neuron. Before an application of NMDA (1 mM) (a), GFP-PHD was expressed in hippocampal neurons, and was concentrated at the plasma membrane (area 1, open circle). Five minutes after NMDA application (Time = 0), fluorescence intensity of GFP-PHD in the cytoplasmic (area 2, closed triangle) and the nucleoplasmic (area 3, open square) regions increased, whereas that at the plasma membrane (area 1) decreased, indicating that NMDA increased cytoplasmic and nucleoplasmic IP_3 concentrations. The representative fluorescence images of GFP-PHD before (a), and at 5 min (b), 10 min (c) and 15 min (d) after NMDA application are indicated. The DIC images of the same neuron before (e) and at 20 min after (f) NMDA exposure indicate the marked changes in the nucleus. Scale bar = 10 μm.

7. Effects of IP_3-mediated Ca^{2+} release blocker

To determine the roles of IP_3-mediated Ca^{2+} release in the process of nuclear changes, we examined the effects of Xestospongin C (XeC, Calbiochem, La Jolla, CA, USA), a membrane-permeable inhibitor of IP_3-mediated Ca^{2+} release. Since the high concentration (10 μM) of XeC decreased [^{3}H]ryanodine binding by 22% and caffeine-induced Ca^{2+} release by 46% (Gafni et al., 1997), 1 μM of XeC (IC_{50} = 358 nM) was used to specifically inhibit the IP_3-mediated Ca^{2+} release. This treatment significantly inhibited the granular changes induced by glutamate and by NMDA (**Fig. 7**).

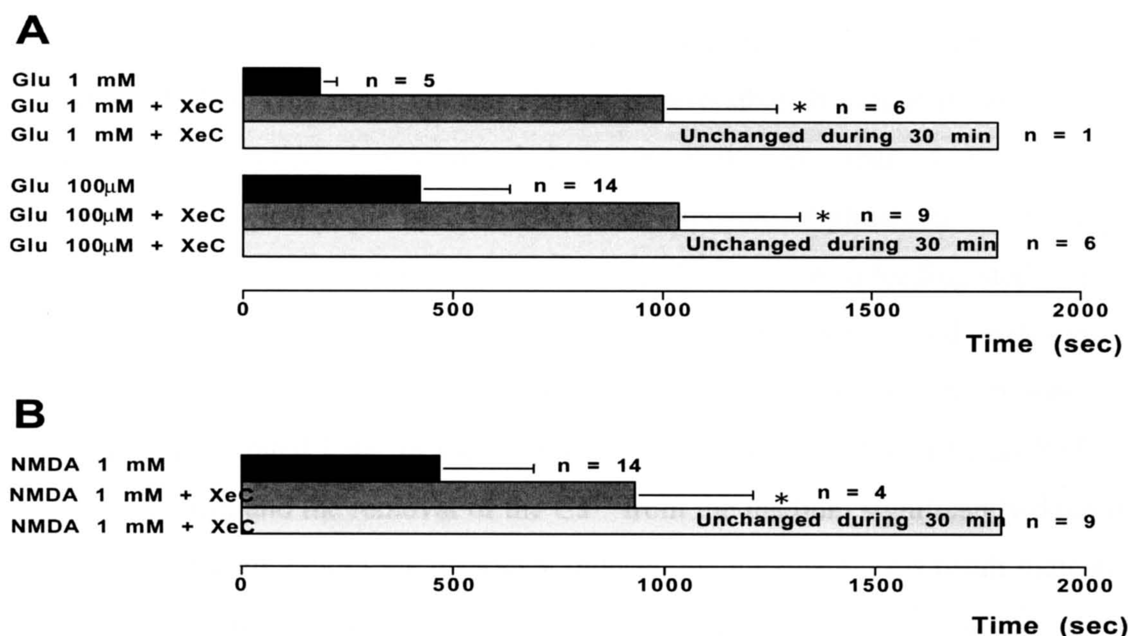


Fig. 7
The effects of Xestospongin C (XeC) on the nuclear changes induced by glutamate (A) and by NMDA (B). The graphs show the time at which granulation was appearing inside the nucleus (see Fig.1-b) following glutamate (100 μM and 1 mM) and NMDA (1 mM) exposure with or without the treatment with XeC (1 μM), an IP_3-mediated Ca^{2+} release inhibitor. XeC was resolved in dimethyl sulfoxide (DMSO = vehicle). Since DMSO alone sometimes induced the nuclear changes, the treatment with XeC did not completely inhibit the responses in some cases. However, even in these cases, XeC significantly delayed the responses. * $p<0.001$ from vehicle. Values represent mean ± SD

Discussion

In the present study, we observed in hippocampal neurons that: 1) glutamate activated the IP_3 system, increased $[Ca^{2+}]_n$, and induced granulation in the nucleus, all of which are very early events leading to the DNA fragmentation and the necrosis (Ikeda et al., 1996), 2) NMDA alone activated IP_3 system and induced large nuclear changes, and 3) a blocker of IP_3-mediated Ca^{2+} release inhibited the rapid nuclear changes induced by glutamate and by NMDA. These findings indicated that glutamate stimulates the IP_3 pathway through NMDA and metabotropic receptors, increases $[Ca^{2+}]_n$, and induces DNA fragmentation during the early process of excitotoxicity.

The earlier study using the in situ end-labeling method suggested that the rapid granulation of the nucleus in cortical neurons is associated with DNA fragmentation (Ikeda et al., 1996). This rapid nuclear change is reversible, if glutamate exposure is limited to a short period. It was noted, however, that many neurons, though once recovered morphologically, die by 24 h after brief exposure of glutamate (Ikeda et al., 1996). This suggests that the rapid nuclear change also involves a leading step toward the delayed neuronal death induced by glutamate. In this study, we focused on this rapid DNA fragmentation, and sought to determine the mechanism underlying this process.

We demonstrated here, in the hippocampal neurons, that MK-801, an NMDA receptor antagonist, and the removal of the Ca^{2+} from the medium significantly delayed but did not completely inhibit the responses induced by glutamate. This result indicates that the rapid granular change still occurred independently of Ca^{2+} influx in the hippocampal neurons, while the earlier study showed that, in cortical neurons, MK-801 inhibits the nuclear changes (Ikeda et al., 1996). We also observed that glutamate increased $[Ca^{2+}]_n$ before inducing rapid nuclear changes. Moreover, this increase was consistently associated with nuclear changes. These results suggest that glutamate increases $[Ca^{2+}]_n$ and induces nuclear changes by mobilizing extra- and intracellular Ca^{2+}. Glutamate receptors are divided into two main groups: ionotropic and metabotropic receptors. Among ionotropic receptors, NMDA receptor is much permeable to Ca^{2+} and induces Ca^{2+} influx. One of the metabotropic receptors, Group I,

is linked via G-protein to PLC. PLC activation results in IP_3 formation, which causes the release of Ca^{2+} from intracellular Ca^{2+} stores (Murphy and Miller, 1988). Consistently with these reports, we observed by using GFP-PHD in the hippocampal neurons that glutamate rapidly increased IP_3 formation.

The mechanism underlying the IP_3-mediated persistent increase in $[Ca^{2+}]_n$ has not yet been elucidated. There are three possible mechanisms that might account for this: 1) IP_3 activates its receptor, releases Ca^{2+} from the endoplasmic reticulum, increases $[Ca^{2+}]_c$, and then increases $[Ca^{2+}]_n$ by diffusion of Ca^{2+} from the cytoplasm to the nucleoplasm through the nuclear pores (Allbritton et al., 1994; Shirakawa and Miyazaki, 1996); 2) IP_3 passes the nuclear pore, directly activates the receptor of nuclear membrane, and increases $[Ca^{2+}]_n$; and 3) the IP_3-related message following glutamate exposure may activate the IP_3 system inside the nucleus and increases $[Ca^{2+}]_n$, since the nucleus itself has a property to produce IP_3 (Malviya and Rogue, 1998). Our results favor the second possibility. This is because 1) we observed that, in the permeabilized neurons immersed in a Ca^{2+}-free medium, an application of IP_3 increased $[Ca^{2+}]_n$ that was inhibited by heparin, indicating the increase in $[Ca^{2+}]_n$ induced by IP_3 occurs independently of extranuclear Ca^{2+}, 2) we observed that GFP-PHD translocated from the plasma membrane to the nucleus, indicating the diffusion of cytoplasmic IP_3 and the increase in intranuclear IP_3 concentration following glutamate exposure, and 3) the inner nuclear membrane has IP_3 receptors (Humbert et al., 1996), which has not been yet proven in the neurons. However, our results cannot completely exclude the possibility that glutamate directly stimulates the IP_3 system inside the nucleus.

The nuclear pore allows to pass all molecules with masses as large as 30-60 Kdalton (Gerace and Burke, 1988). Therefore, IP_3 (MW = 543) can pass through the nuclear pore easily, activates IP_3 receptors on the inner nuclear membrane, and increases $[Ca^{2+}]_n$ by releasing Ca^{2+} from the nuclear membrane compartment (Gerasimenko et al., 1995; Stehno-Bittel et al., 1995). We observed in this study that this process in permeabilized neurons was inhibited by heparin, an IP_3 receptor inhibitor (5-30 KDaltons), permeative through the nuclear pore. The increase in $[Ca^{2+}]_n$ must therefore be induced by IP_3 receptor activation inside the nucleus. We observed that GFP-PHD translocated from the plasma membrane to the nucleus under the confocal

laser microscope. Since the molecular weight of GFP-PHD is 48 KDaltons (Stauffer et al., 1998), this protein is permeative through the nuclear pore. The translocation means that GFP-PHD passes the nuclear pore according to increase in IP_3 concentration in the nucleus.

Glutamate activates NMDA receptor and induces Ca^{2+} influx. Since, in our study, NMDA receptor antagonist significantly delayed the process of rapid nuclear changes induced by glutamate, NMDA receptor must contribute in the process of persistent increase in $[Ca^{2+}]_n$, and thus nuclear changes. We observed that NMDA receptor activation increased $[Ca^{2+}]_c$ and induced a persistent increase in $[Ca^{2+}]_n$. We also observed that NMDA receptor activation increased IP_3 concentration in the neurons. These results support the hypothesis that NMDA receptor activation alone stimulates the IP_3 system. The activities of all PLC isozymes isolated from the brain are potentiated by Ca^{2+} in vitro (Ryu et al., 1987). The NMDA-induced long-term potentiation requires IP_3 activation (Berridge, 1993), and NMDA receptor activation upregulates PLC in the neurons (Shimohama et al., 1995). These reports also support our hypothesis. There remains the possibility that an increase in $[Ca^{2+}]_c$ stimulates the ryanodine sensitive receptor, and induces rapid nuclear changes. However, this possibility is not likely, since we observed that an application of caffeine, a stimulant of ryanodine-sensitive Ca^{2+} release (Henzi and MacDermott, 1992; Irving et al., 1992; Carlson et al., 1997; Seymour-Laurent and Barish, 1995), did not induce rapid nuclear changes. The fact that in the hippocampus IP_3 receptor but not ryanodine receptor is predominantly expressed in the CA1 region (Furuichi et al., 1994) may explain the vulnerability against ischemia.

Taken together, IP_3 system activation is a final common pathway to induce rapid granular changes of the nucleus, i.e., DNA fragmentation, induced by glutamate receptor activation. In fact, we observed that a blocker of IP_3-mediated Ca^{2+} release inhibited rapid nuclear changes induced by glutamate and by NMDA. Nuclear calcium signals control a variety of nuclear functions, including gene transcription, DNA synthesis, and DNA repair (Santella and Carafoli, 1997). Probably, the increase in $[Ca^{2+}]_n$ persisting above the physiological level results in the damage of nuclear DNA. There are two types of neuronal death: one is necrosis and the other is apoptosis. The

way in which neurons die depends upon the severity and the duration of excitotoxicity (Bonfoco et al., 1995). Recent studies show that breaks in DNA strands may be involved in triggering apoptosis (Enoch and Norbury, 1995; Engelward et al., 1998). Probably, when endogenous systems correctly repair DNA breaks, neurons will survive. If they are incorrectly repaired, they may be excluded, resulting in apoptosis or delayed neuronal death. When excitotoxicity is severe enough to damage the all repair process, it can induce necrosis. The very early processes of DNA change should, therefore, be extensively studied further in order to understand the mechanism of neuronal death. At present, the following questions remain to be answered: 1) how does $[Ca^{2+}]_n$ elevation induce DNA damage, and 2) how do NMDA receptors activate the IP_3 system?

In conclusion, glutamate activates the IP_3 pathway, increases $[Ca^{2+}]_n$, and causes DNA fragmentation during the acute phase of excitotoxicity. During this process, NMDA receptor activation alone also participates in activating the IP_3 system, and aggravates the excitotoxicity.

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2

Advanced optical imaging reveals that glutamate rapidly induces DNA fragmentation in excitotoxicity

Summary

We previously observed, under the video enhanced contrast-differential interference contrast (VEC-DIC) microscope, that glutamate produces granulation inside the nucleus within 20 minutes. Although the granular changes were thought to be DNA fragmentation, direct evidence remained to be established. We attempted to directly analyze the DNA extracted from a few neurons undergoing the nuclear change under the VEC-DIC microscope. In cultured rat hippocampal neurons, following the nuclear changes, 3-5 neurons were aspirated with micropipette. Their DNAs were extracted with proteinase K and phenol, stained with a nucleic acid dye, and observed under the total internal reflection fluorescence (TIRF) microscope, which allows us to observe the single molecules stained with fluorescent dye. Glutamate rapidly induced granulation inside the nucleus followed by nuclear and cellular swelling, and by Brownian motion of the granules inside nucleus indicting the process was necrosis. While DNAs extracted from the neurons undergoing the nuclear granulation showed Brownian motion on the non-coated glass, they showed various types of fragmentation on the poly-D-lysine coated glass, which was positively charged. Glutamate significantly increased TUNEL-positive neurons in 1h. Three dimensional confocal imaging also revealed that DNA *in situ* showed fragmentation in the neuron with nuclear granulation. The advanced optical imaging for nuclear DNA reveals that glutamate induced DNA fragmentation in the early phase of necrosis in neurons.

Key words:

total internal reflection fluorescence microscope, single molecule imaging, DNA fragmentation, excitotoxicity, necrosis

Introduction

We previously observed, under the video enhanced contrast-differential interference contrast (VEC-DIC) microscope, that the intact cultured neurons showed large nuclei containing an amorphous nucleoplasm except for nucleoli, and that glutamate produces granulation inside the nucleus within 20 minutes (Ikeda et al., 1996; Yamamoto et al., 1996). Although the granular changes were thought to be DNA fragmentation, direct evidence remained to be established.

The total internal reflection fluorescence (TIRF) microscope allows us to observe the single molecules of λ-phage DNA stained with fluorescence dye (unpublished data). In this study, by using the TIRF microscope, we attempted to analyze directly the single DNA extracted from a few neurons undergoing the nuclear changes under the VEC-DIC microscope.

Methods

Primary culture of hippocampal neuron

Hippocampal neurons were prepared from one-day-old Wistar rats. After removal of the brain under brief anesthesia, the hippocampus was dissected out, and dissociated by repeated trituration with re-polished Pasteur pipettes. Cells were placed on collagen-coated culture dishes (Iwaki 35 mm/Glass Base Dish, Asahi Techno Glass Co., Tokyo, Japan). The culture medium was a mixture of DMEM (50%; Gibco No. 31600, Invitrogen Japan K.K., Tokyo, Japan), HBSS (25%; Gibco No. 11201), and horse serum (25%). Glucose and HEPES were added at a final concentration of 36.1 mM and 23.7 mM, respectively. The pH of the medium was adjusted to 7.2 by adding NaOH. Penicillin-G, streptomycin, and cytosine arabinoside were added to the culture medium. The dish was incubated for a week at 37°C with a 5% CO_2-containing atmosphere, and was then subjected to the experiment.

Morphological observation

A cultured dish containing cells was placed on the stage of an inverted DIC microscope (Axiovert 10, Zeiss, Oberkochen, Germany). The microscope was equipped with a 100x DIC lens. The DIC image was obtained with a charge-coupled device (CCD) camera (C6489, Hamamatsu Photonics K.K., Hamamatsu, Japan). The video image was contrast-enhanced digitally in real time by using an image processor (ARGUS 20, Hamamatsu Photonics K.K., Hamamatsu, Japan), then monitored and recorded on videotape. Artificial cerebrospinal fluid (aCSF) was used as a recording medium, and its composition (mM) was 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 10 HEPES, and 10 glucose with a pH of 7.2. Throughout the experiment, the temperature of the medium was maintained at 34 $\pm$ 2 °C.

DNA extraction and staining with fluorescent dye

Following the nuclear changes under the VEC-DIC microscope, 3-5 neurons were aspirated with micropipette, and put into DNA-extraction buffer (150 mM NaCl, 10 mM Tris-HCl, 10 mM EDTA, pH = 8.0) in a polypropylene tube. 10% Sodium dodecyl

sulfate (SDS) was add at 1% volume of total cell suspension. Proteinase K was added at the final concentration of 100 μg /ml, and incubated at 42°C for 1 h. The same volume of TE-saturated phenol (pH = 8.0) was added, gently mixtured, and then centrifuged for 10 min at 3000 round per minute (rpm) at room temperature. The superficial layer was carefully aspirated, the same volume of phenyl-chloroform-isoamylalcohol (PCI) was added and centrifuged or 10 min at 3000 rpm at room temperature. The superficial layer was removed to another polypropylene tube, 400 μl of 100% ethanol added gently, and DNA was extracted with capillary tube. The DNAs were stained with 10 nM YOYO-1 (Molecular Probes Inc, Eugene, OR, USA) for 10 min.

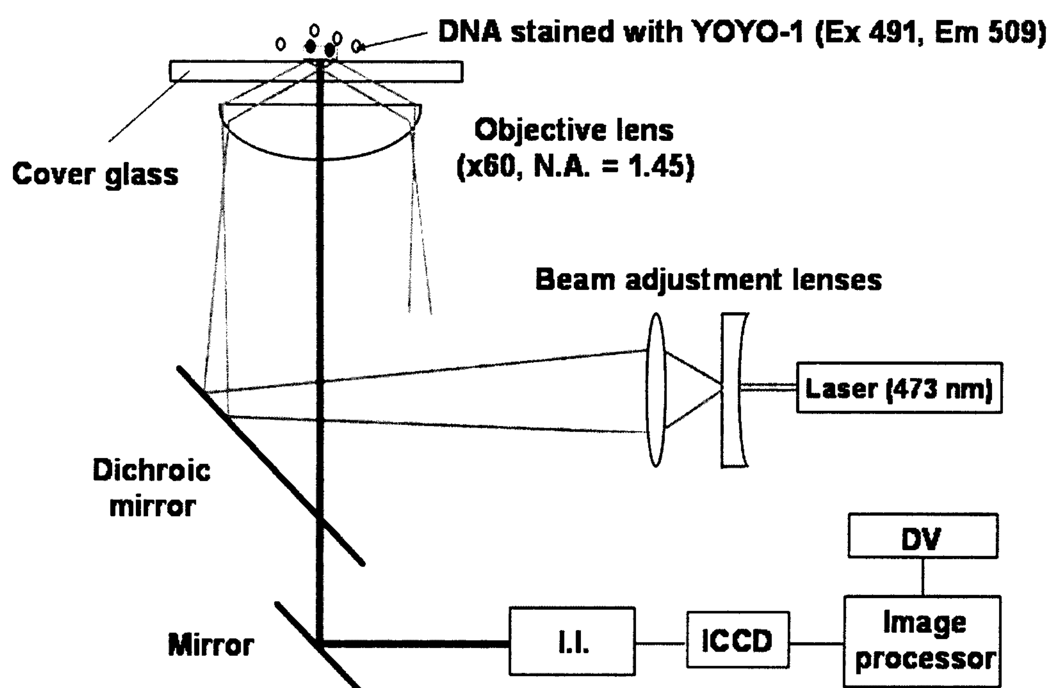

Fig. 1
Observation of DNA under the TIRF microscope.
I.I; image intensifier, ICCD; intensified charged couple device, DV; digital video.

Single molecule imaging

To observe the single molecule of DNA, the total internal reflection fluorescence (TIRF) microscope (IX 70, Olympus, Tokyo, Japan) was employed. The incident light for evanescent illumination was introduced from the objective lens (PlanApo 60 x, 1.45 NA, Olympus). The fluorescence images were recorded with a CCD camera (C2400, Hamamatsu Photonics K.K., Hamamatsu, Japan) combined with an image intensifier (C2400-21SV, Hamamatsu Photonics K.K.), and analyzed with MetaMorph software (Universal Imaging Co., Downingtown, PA, USA).

Three dimensional (3D) fluorescence imaging

To analyze the nuclear DNA *in situ*, DNA was stained with membrane permeable fluorescent dye, and observed under a 3D confocal laser microscope. The neurons were loaded with a nucleic acid dye by incubation in aCSF (see morphological observation) containing 1 μM SYTO-24 (Molecular Probes Inc, Eugene, OR, USA) in 5% CO_2 and 95% air at 37 °C for 15 min. After washing, the neurons were observed under the 3D confocal laser microscope (IX 70, Olympus, Tokyo, Japan) equipped with a microlens-attached Nipkow-disk scanner (CSU-10, Yokokawa Electric Co., Tokyo, Japan). The confocal fluorescence images were recorded with a CCD camera (C2400, Hamamatsu Photonics K.K., Hamamatsu, Japan) combined with an image intensifier (C2400-21SV, Hamamatsu Photonics K.K., Hamamatsu, Japan), and analyzed with a software, IPlab (Scanalytics Inc., Fairfax, VA, USA). After recording the DIC images, the cells were exposed to the medium containing glutamate, and fluorescence images of nuclear DNA of the same neurons were examined under the 3D confocal microscope.

Results

1. Glutamate induces nuclear changed during necrosis.

Glutamate induced granulation rapidly inside the nucleus followed by cellular swelling (**Fig. 2**), and by Brownian motion of the granules in the nucleus. In one hour, propidium iodide, a cell-impermeant fluorescent dye, stained the nucleus, indicating the necrotic process.

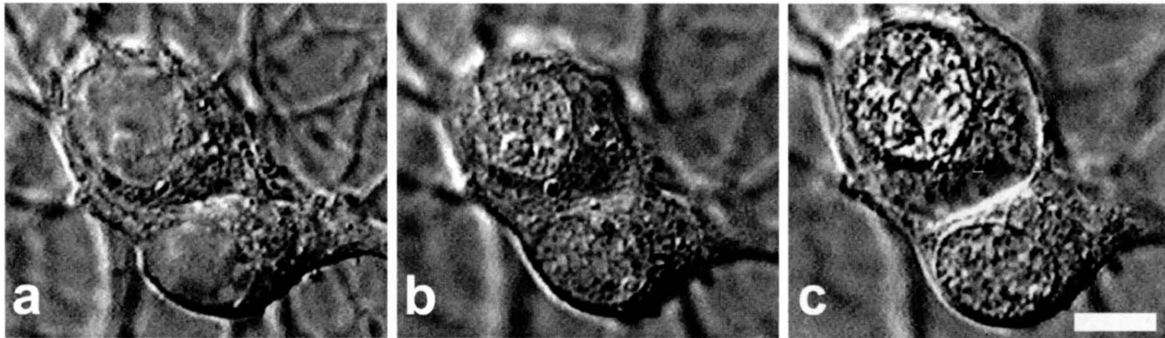

Fig. 2
Sequential changes of rat hippocampal neurons following glutamate exposure. Neurons were observed under the VEC-DIC microscope before (a), and at 2.5 min (b) and 20 min (c) after an application of glutamate (1 mM). Before an application of glutamate, the neurons had large nuclei containing a smooth and amorphous nucleoplasm and one or two nucleoli. Note that glutamate rapidly induced granulation inside the nucleus followed by nuclear and cellular swelling, indicating the character of necrosis. Scale Bar = 5 μm

2. DNAs extracted from the neurons with the nuclear granulation showed fragmentation.

DNAs of intact neurons showed straight and long strands under the TIRF microscope (**Fig. 3a**). After treatment with DNase I (10 U), the fragmented DNAs were observed (data not shown). DNAs extracted from the neurons undergoing the nuclear granulation showed Brownian motion on the non-coated glass (data not shown). They showed various types of fragmentation including small spots (**Fig. 3c**), short strands (about 10

μm) (**Fig. 3d**), and clusters of fragments of a variable length (**Fig. 3e**) on the poly-D-lysine (5 μg/ml) coated glass, which was positively charged. Glutamate significantly increased the TUNEL-positive neurons in one hour (**Fig. 4**).

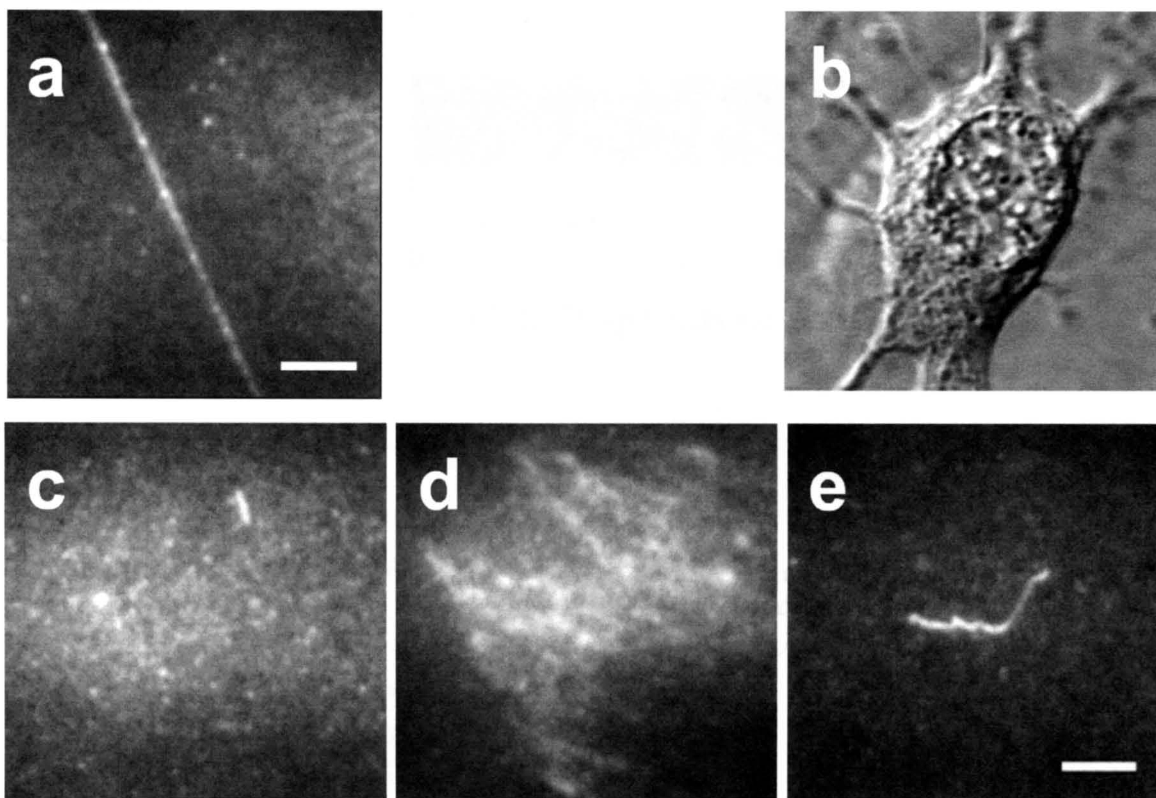

Fig. 3
Intact DNA (a) and DNA extracted from the neuron (b) undergoing nuclear granulation. DNA was stained with YOYO-1, and observed under TIRF microscope. Note that DNAs extracted damaged neuron showed various types of fragmentation. Bar = 5 μm

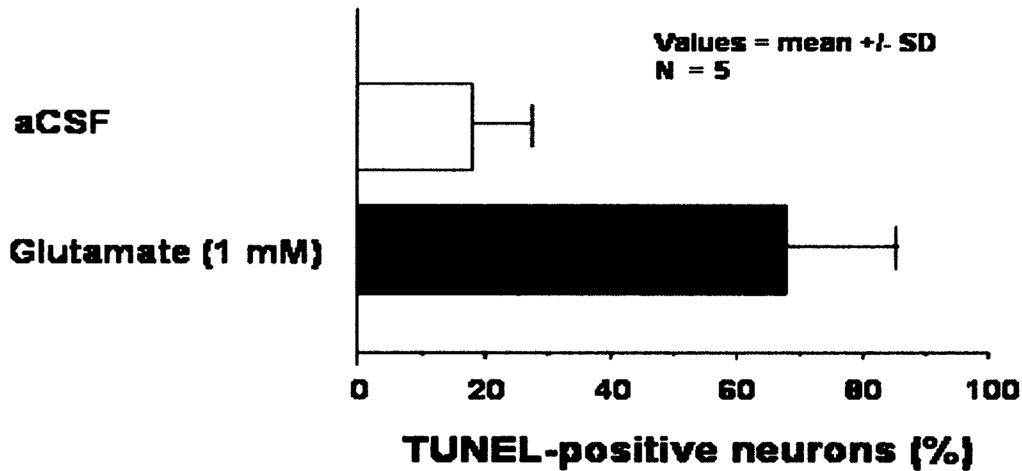

Fig. 4
TUNEL stain 1h after continuous exposure to 1 mM glutamate. Glutamate significantly increased the number of TUNEL-positive neurons ($p < 0.01$), indicating DNA fragmentation.

3. DNAs stained with a nucleic acid dye in the neurons undergoing the nuclear granulation showed fragmentation.

While reconstructed 3D images of DNA stained with SYTO-24 in the intact neuron showed homogenous structure (**Fig. 5a, b**), those in the neurons undergoing nuclear granulation did show fragmented structure (**Fig. 5c, d**).

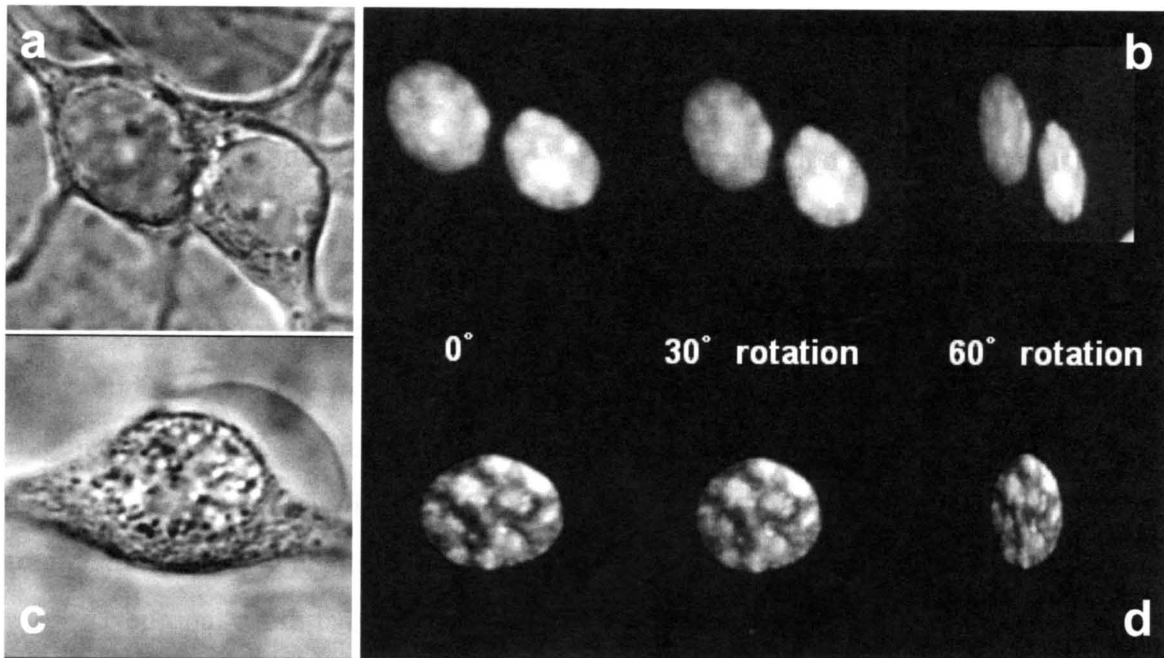

Fig. 5

DIC images (a, c) and fluorescence images of DNA *in situ* with (d) or without (b) glutamate exposure. DNA was stained with membrane permeable fluorescent dye, and observed under a 3D confocal laser microscope. DIC images of the neuron exposed to aCSF (a) showed large nuclei containing an amorphous nucleoplasm except for nucleoli. Reconstructed 3D images of DNA (b) in the same neuron showed homogenous structure. On the contrary, DIC images of the neuron exposed to 1 mM glutamate for 20 min (c) showed granulation inside the nucleus. Reconstructed DNA images of the same neuron (d) showed heterogeneous structure. Note the blank space of fluorescence in DNA of the neuron exposed to glutamate.

Discussion

We have observed, in this study, that: 1) 1 mM glutamate induced nuclear granulation under the VEC-DIC microscope during necrosis within 1 h; 2) DNAs extracted from the neuron undergoing nuclear change showed fragmentation under the TIRF microscope; 3) 1 mM glutamate significantly increased the TUNEL-positive neurons in 1 h; and 4) DNAs, *in situ*, in the neurons with nuclear granulation showed fragmentation when stained with nucleic acid dye under the fluorescence microscope. These results clearly indicated that high concentration of glutamate induces DNA fragmentation during necrosis, i.e. acute excitotoxicity.

Neuronal death is commonly divided into two types, i.e. apoptosis and necrosis (Choi, 1988; Bonfoco et al, 1995; Du et al., 1997). Recent studies suggested that whether neurons die by apoptosis or necrosis, it depends on exposure duration and concentration; exposure to brief and lower concentration of glutamate produces delayed and apoptotic neuronal death; whereas exposure to continuous and higher concentration may result in acute and necrotic neuronal death (Ankarcrona et al., 1995; Bonfoco et al, 1995; Larm et al., 1997). Our morphological observations, in the present study, agree with the above reports indicating that continuous exposure to higher concentration of glutamate induces acute necrosis. On the other hand, apoptosis is characterized by the shrunk cell body as well as nuclear DNA fragmentation. Interestingly, however, our results demonstrated that, even in the process of necrosis, nuclear DNA fragmentation occurred following exposure to high concentration of glutamate. While the differences between the two types of DNA fragmentation remain unclear at present, it should be noticed that some extracellular signal including stimulation of glutamate receptors induces nuclear signals and the morphological changes in nuclear DNA even in the necrosis (Yamamoto et al., 2000).

A further implication of this study should be considered. The results showed that TUNEL-positive neurons increased due to DNA fragmentation during necrosis. Since the cell membrane ruptured eventually, the necrosis induced TUNEL-positive and shrunken cells in the late phase. These cells should be distinguished from the apoptotic neurons in excitotoxicity.

In conclusion, in the present study, advanced optical imaging revealed that glutamate induces DNA fragmentation during acute process of necrotic excitotoxicity in cultured hippocampal neurons, indicating that nuclear signals exists following glutamate stimulation. The result should be taken into account in analyzing the excitotoxic neuronal death.

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3

Caspase 3 activation induced by glutamate in hippocampal neurons

Summary

Excitotoxic neuronal death induced by glutamate is generally divided into two types; one is acute and the other is delayed. The mechanisms underlying the delayed neuronal death involve apoptotic process. Since caspase 3 is a central downstream effecter of caspase cascade executing the apoptosis, glutamate may activate caspase 3. However, the time-course of activation remains unclear. In the present study, we examined the effects of brief (20 min) and continuous exposure to glutamate on the activation of caspase 3 in rat cultured hippocampal neurons. Neurons were prepared from 1-day-old rats, and cultured on the glass-based dishes. Morphological changes in the neurons were observed with a video-enhanced contrast-differential interference contrast microscope. Activations of caspase 3 were analyzed under a confocal fluorescence microscope using a cell-permeable caspase 3 substrate FAM-DEVD-FMK. Our results showed that 20-min exposure to glutamate induced delayed neuronal death and activated caspase 3 significantly in 6 h in a dose-dependent manner. Interestingly, the continuous exposure for 1 h, which induced necrosis, also significantly activated caspase 3. Our data indicated that caspase 3 pathway is activated in the process of acute as well as delayed neuronal death induced by glutamate. Even in the necrotic process, apoptotic signal pathway is already activated but covered with the acute cell death.

Key words:
excitotoxicity, apoptosis, caspase

Introduction

The excessive release of glutamate in extracellular space has been postulated to result in excitotoxic neuronal death that implicated in the pathogenesis of stroke, trauma and neurodegenerative diseases (Choi 1988; Lipton et al., 1994). Based on biochemical and morphological criteria, glutamate-induced excitotoxic neuronal death is usually classified into two types; one is acute and the other is delayed (Choi 1988). The former is considered to be necrosis showing rapid cellular swelling and disintegration of the cells and nuclei, while the latter is apoptosis presenting cellular shrinkage, cytoplasmic and nuclear fragmentation, and formation of apoptotic bodies (Ankarcrona et al., 1995; Du et al, 1997; Tenneti and Lipton, 2000).

Caspase 3 is known as a major executioner during apoptosis, which activation is considered to be a key step that switches execution of excitotoxic neuronal death induced by glutamate (Du et al, 1997; Tenneti and Lipton, 2000; Brecht et al, 2001). However, little information is available on time course of caspase 3 activation during this process. On the other hand, several studies reported that whether neurons die by apoptosis or necrosis, it depends the duration of exposure and the treatment concentration (Ankarcrona et al., 1995; Bonfoco et al, 1995; Larm et al, 1997). Mild insults lead to delayed apoptotic neuronal death; whereas intense insults produce acute necrotic neuronal death.

In our earlier studies, using a video-enhanced contrast-differential interference contrast (VEC-DIC) microscope, we have previously reported that a continuous exposure to high concentration (1mM) glutamate induced a rapid granulation inside the nucleus within 20 min, and followed by nuclear and cellular swelling, as well as eventual necrosis in rat cultured cortical and hippocampal neurons (Ikeda et al, 1996; Yamamoto et al., 2000; Yamamoto et al, 2001). In addition, the identical granular change of nucleus was also found in the early process of apoptosis induced by staurosporine, an apoptosis inducer, but did not follow by cellular swelling (Nakahara et al, 2002). These findings strongly suggest that 20-min exposure to glutamate, inducing such a morphological feature, may be enough to induce neuron undergo delayed neuronal death. In the present study, we investigated that whether a brief (20 min)

exposure to glutamate may induce caspase activation, a trigger of apoptosis, in rat cultured hippocampal neurons.

Methods

Primary culture of hippocampal neurons

Hippocampal neurons were prepared from 1-day-old Wistar rats. Briefly, under anesthesia, freshly isolated hippocampi were removed, dissected and dissociated. Neurons were plated on poly-D-lysin coated culture dishes (IWAKI 35 mm/Glass Base Dish, Asahi Techno Glass Co., Tokyo) in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 Ham (DMEM/F-12 1:1; Gibco BRL, Invitrogen Japan KK., Tokyo) supplemented with 10% fetal bovine serum. The pH of medium was adjusted to 7.4. Cytosine arabinoside were added to the culture medium at 72 h after incubation, to prevent the growth of non-neuronal cells. All neurons were maintained at 37 °C with a 5% CO_2-containing atmosphere for 8 to 10 days, and then used for experiments.

Detection of caspase-3 activation

Activated caspase 3 was detected in living cells by using CaspaTag Caspase-3 Activity Kit (Intergen Company, Oxford). Briefly, neurons were rinsed 3 times with artificial cerebrospinal fluid (aCSF, PH 7.2; 140 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Glucose, and 10mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid), and incubated at 37 °C with aCSF-containing a fluorescent caspase 3 inhibitor FAM-DEVD-FMK, a carboxyfluorescein-labeled fluoromethyl ketone tetrapeptide inhibitor of caspase-3. After incubation for 30 min, neurons were washed three times with aCSF and twice with washing buffer. aCSF was used as a recording medium for observation of neurons. Morphological changes in the neurons were observed with a VEC-DIC microscope. Activations of caspase 3 were observed by an Olympus IX70 inverted fluorescence microscope equipped with a confocal scanner of a microlens-attached Nipkow-disk (CSU-10, Yokokawa Electric Co), with excitation at 485 nm, and emission above 520 nm. Neuron exhibiting a feature of condensed brightly staining in the fluorescence images was defined as caspase 3-positive neuron. The percentage of caspase 3-positive neuron in the total neuron number was used to evaluate the activation of caspase-3.

Data analysis

Data were obtained from three independent experiments, and expressed as mean ± SEM. The average of three randomly selected microscopic fields per cultured dish was used as one data point per experiment. Data were analyzed by One-way analysis of variance (ANOVA) followed by Fisher's protected least-squares difference test. A P value < 0.05 was considered statistically significant.

Results

Staurosporine (1 μM) activated caspase 3

Previously we have demonstrated that exposure of the rat hippocampal neurons to 1 μM staurosporine induced apoptosis in dose- and time-dependent manner (Nakahara et al, 2002). In the present study, we examined whether caspase 3 activity can be detected in neuronal death induced by 1 μM staurosporine. As shown in **Fig. 1**, the clear fluorescence image of caspase 3-positive neurons was observed. The application of 1 μM staurosporine significantly activated caspase 3 in both 6-h and 24-h exposure (data not shown).

Brief (20 min) exposure to glutamate activated caspase 3 in 6 hours

Caspase 3 activity could also be detected in the process of neuronal death induced by 20-min exposure to glutamate (**Fig. 1**). **Fig. 2-A, B** showed activation of caspase 3 at 6 h and 24 h after a 20-min exposure to glutamate. At 6 h, percentages of caspase 3-positive neuron in all groups exposed to glutamate (1μM -1 mM) showed a dose-dependent increase compared with those in control. At 24 h, percentages of caspase 3-positive neuron were also increased significantly in groups exposed to high concentration (100μM and 1 mM) of glutamate but not in groups exposed to low concentration (1 μM and 10 μM) glutamate.

Continuous exposure (1 h) to glutamate activated caspase 3

Finally, we examined whether caspase 3 is activated during the process induced by continuous (1 h) exposure to high concentration (100 μM and 1 mM) glutamate, which induces necrosis. Interestingly, activation of caspase 3 was also detected in such a necrotic process (**Fig. 2-C**).

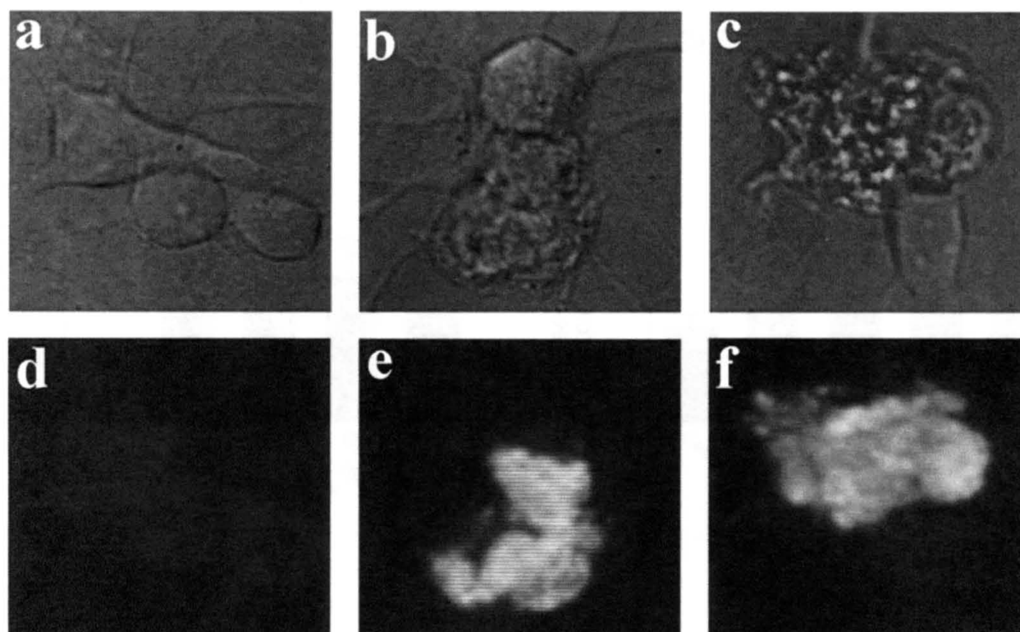

Fig. 1

Differential interference contrast (DIC) and fluorescence images of rat cultured hippocampal neurons. Neurons were observed under both VEC-DIC microscope and confocal laser microscope. The images showed neurons in (A) control, (B) at 6 h after exposure to 1 μM staurosporine, and (C) at 6 h after a 20-min exposure to 1 mM glutamate. Activation of caspase 3 was detected in living cells using a carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of caspase 3 (FAM-DEVD-FMK). Note that caspase 3-positive neurons exhibit a feature of condensed brightly staining in the fluorescence images.

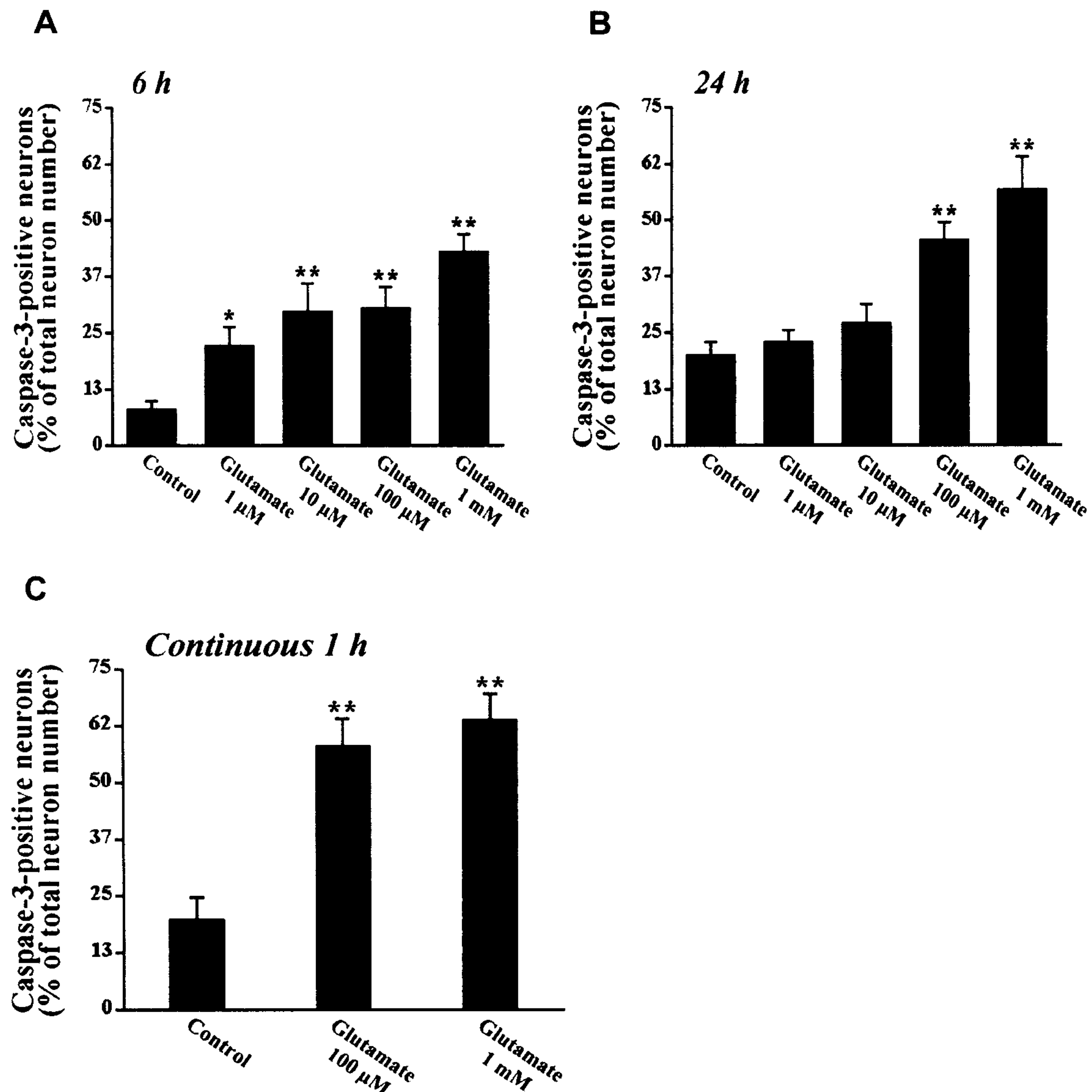

Fig. 2

Brief (20 min) exposure to lower concentrations of glutamate activated caspase 3 in a dose-dependent manner. 20-min brief exposure to glutamate (1 μM - 1 mM) induced a dose-dependent increase in caspase 3 activation at 6 h (A) and 24 h (B). Caspase 3 activation was detected as described in Fig. 1. Data are presented as percentage (%) of caspase 3-positive neuron in total neuron number from three randomly chosen fields in each culture, and mean ± SEM from n = 6-7 cultures in three separate experiments. * $P < 0.05$ and ** $P < 0.01$ compared with the respective control. **Continuous (1 h) exposure to higher concentrations of glutamate activated caspase 3** (C). Continuous (1 h) exposure to glutamate (1 mM) activated caspase 3 and induced a markedly increased caspase 3 activation. Data are presented as percentage (%) of caspase 3-positive neuron in total neuron number from three randomly chosen fields in each culture, and mean ± SEM from n = 6 cultures in three separate experiments. ** $P < 0.01$ compared with the respective control.

Discussion

In the present study, we demonstrated that a 20-min exposure to glutamate activated caspase 3 significantly in 6 h in a dose-dependent manner in rat cultured hippocampal neuron. We found that the most neurons without caspase 3 activation showed normal appearance and clear cell structure in their DIC images. However, caspase 3-positive neurons, exhibited shrunken and rough bodies with indistinguishable cell structure in their DIC images, indicating a morphological feature of apoptosis. In addition, at 1 h after glutamate exposure for 20 min, we did not find marked morphological changes of neuron similar to the changes observed at 6 h (unpublished observation). These findings indicated that rat cultured hippocampal neuron exposed to glutamate (1 μM to 1 mM) for 20 min activated caspase 3 and underwent delayed neuronal death between 1 and 6 h.

Secondly, in the present study, we demonstrated that caspase-3 activation was also detected after continuous exposure to high concentration glutamate for 1 h. We have previously observed that a continuous exposure to high concentration glutamate for 1 h induced necrosis in rat cultured cortical and hippocampal neurons (Ikeda et al, 1996; Yamamoto et al., 2000; Yamamoto et al, 2001). Therefore, the result indicated that casepase-3 was activated in the necrosis, while it is considered that caspase 3 is generally activated only in apoptotic neurons but not in necrotic neurons (Armstrong et al, 1997; Du et al, 1997; Tenneti and Lipton, 2000). Our result suggests that in the necrotic process, apoptotic signal may be already activated but covered with the acute cell death.

In summary, our data indicated that caspase 3 is activated in the process of acute as well as delayed neuronal death induced by glutamate. We speculate that even in the necrotic process, apoptotic signal pathway may be already activated but covered with the acute cell death.

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