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Enzyme-Linked Sensitive Fluorometric Imaging of Glutamate Release from Cerebral Neurons of Chick Embryos

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This paper describes a method for imaging the endogenous release of glutamate from cerebral neurons. This method is based on the reactions of glutamate oxidase and peroxidase, and on the detection of hydrogen peroxide by a fluorescent substrate of peroxidase. Glutamate has been sensitively measured *in vitro* in the range of 20 nM to 1 μM. We used two types of Ca^{2+} channel inhibitors, MK-801 and ω-Conotoxin GVIA, which act to suppress Ca^{2+} transport at postsynaptic and presynaptic neurons, respectively. MK-801 did not inhibit the increase in glutamate release after KCl stimulation, while there was no increase in glutamate release after KCl stimulation when ω-Conotoxin GVIA was used, probably due to the inhibition of voltage-activated Ca^{2+} channels in the presynapse. Glutamate release and Ca^{2+} flow in the synaptic regions were imaged using a laser confocal fluorescence microscope. KCl-evoked glutamate release was localized around cell bodies linked to axon terminals. This procedure allows imaging that can be sensitively detected by the fluorometric enzymatic assay of endogenous glutamate release in synapses.

Key words: bioimaging, confocal microscope, enzyme sensor, fluorescence, glutamate, neuronal network.

Glutamate is a kind of major excitatory neurotransmitter in the central nervous system. The long-term potentiation (*1*, *2*) or depression of excitatory synaptic transmission (*3*, *4*) is considered to be the neuronal basis for learning and memorizing. Glutamate also plays an important role in synaptic plasticity; the quantitative and special analysis of glutamate is required in order to study neuronal system mechanisms and neuron functioning. Endogenous excitoxins such as glutamate are believed to be involved in the pathogenesis of neurodegenerative brain diseases, endogenous excitoxins such as glutamate: Alzheimer's disease, Parkinson's disease, and cerebral ischemia. The elevation of extracellular glutamate concentrations under ischemia has been reported (*5*). Glutamate might be released from neurotransmitter pools in neurons depolarized by ischemia. Another explanation is that glutamate from metabolic pools in neurons and astroglia may diffuse out of cells due to altered cell membrane permeabilities, possibly induced under ischemia.

Various *in vivo* and *in vitro* methods have been developed for detecting glutamate. Many studies have employed the nonmetabolizable "false substrate," D-[^{3}H] aspartate (*6–10*), to elucidate the general parameters for the uptake and release of glutamate. But the ability of D-aspartate to access synaptic vesicles and to be released by exocytosis is still controversial. On the other hand, liquid chromatography (*11*), flow injection analysis combined with a microdialysis sampling electrochemical microsensor (*12*), wall-jet type flow cell with interdigitated microarray electrode (*13*), and on-line sensors (*14–16*) for real-time measurement have been used to measure transmitters. Electrochemical glutamate sensors consist of electrodes and glutamate oxidase, which oxidizes glutamate efficiently in the presence of oxygen (*17–23*), and the generated hydrogen peroxide can be detected with a platinum or mediator-modified electrode. However, electrochemical methods are not suitable for spatial imaging of the distribution of glutamate release from the neuronal cell network.

Direct fluorometric assays of specific neurotransmitters are selective and respond only to the specific amino acid examined (*24*). They are also sensitive to micromolar levels of the tested amino acids. In this case, an amino acid transmitter is acted upon enzymatically, resulting in the production of a fluorescent NADH molecule. By modifying such an assay so that it can be applied directly to living neuronal cells, endogenous glutamate can be monitored as it is released from cells. The application of a glutamate-specific microassay thus amplifies the localization of glutamate release sites. However, the sensitivity of this method is not sufficient for imaging our primary cultures of cerebral cortex from chick embryos (*25*). Therefore, we have tried to develop more sensitive fluorescence imaging of glutamate release from synapses. On the other hand, the dual whole-cell recording technique indicated that glutamate synapses are selectively present at synaptic sites during the early development of postsynaptic neurons (*26*). These cerebral cells when developed in primary culture appear to constitute a suitable model in which to study glutamate release mechanisms during the neuronal differentiation process.

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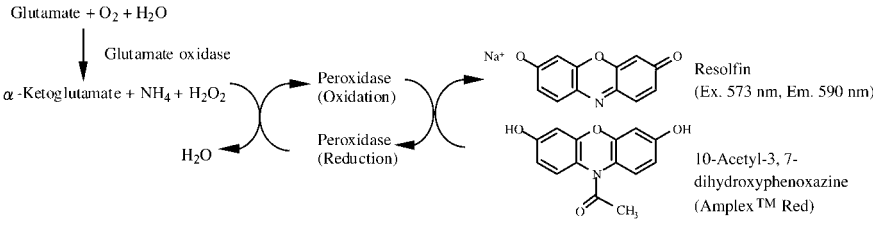

Fig. 1. **Principle of glutamate detection.**

In this study, we have developed a glutamate oxidase and peroxidase-linked fluorometric assay for the selective and sensitive imaging of glutamate, and applied it to monitoring neurotransmitter release from cerebral neuronal cells. Especially, we succeeded in the sensitive determination of nanomolar levels of glutamate. The effects of KCl stimulation and Ca^{2+} channel inhibitors were also examined with our newly developed system and the results are discussed.

EXPERIMENTAL AND METHODS

Materials—Glutamate oxidase was purchased from Seikagaku (Japan). Peroxidase and glutamate were purchased from Wako Pure Chemical (Japan). 10-Acetyl-3,7-dihydroxyphenoxazine (Amplex™ Red) and Flou3-AM and *N*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino) styryl) pyridimium dibromide (FM®1-43) were purchased from Molecular Probes (USA). ω-Conotoxin GVIA was purchased from Peptide Institute (Japan). Dulbecco's Modified Eagle Medium, penicillin-streptomycin liquid and trypsin were purchased from Gibco BRL® (USA). Fetal Bovine Serum was purchased from Cosmobio. Insulin. Poly-L-Lysine and MK-801 were purchased from Sigma–Aldrich (USA). Glass bottom culture dishes were purchased from Mat Tek (USA).

Instrumentation—The imaging system consists of a microscope (IX70, OLYMPUS®, Japan) attached to a confocal unit (CSU10, Yokogawa Electric, Japan) and elective cooled CCD camera (Quantix™, Photometrics, USA). The fluorescence signal was collected by an Olympus objective: 20X (N/A 0.40), 40X (N/A 0.85). The laser system was an Argon/Argon–Krypton laser (Omnichrome®, USA) and it exchanged exiting waves. For monitoring the glutamate fluorescence signal, it was a 530 nm Argon-Krypton laser ion was used. For the simultaneous monitoring of the calcium-Fluo-3 fluorescence signal, a 488 nm Argon ion laser was coupled to this system. The fluorescence intensity of the supernatants of cell cultures was measured with a spectrofluorometer (FP-777, JASCO, Japan).

Principles of Glutamate Detection—The glutamate sensing scheme involves the use of glutamate oxidase, which catalyzes the oxidation of glutamate as shown in Fig. 1. A highly sensitive and stable probe for hydrogen peroxide was used as a fluorogenic substrate for peroxidase detection. In the presence of horseradish peroxidase, the Amplex™ Red reagent reacts with hydrogen peroxide (1:1) to produce highly fluorescent resorufin (*19–20*) (Ex. 573 nm, Em. 590 nm).

Cell Culture—Cell cultures were prepared following the same method as described previously (*25*). In brief, cerebral hemispheres were dissected from chick embryos on embryonic day 8, and treated 0.025 % trypsin in Ca^{2+}, Mg^{2+}-free phosphate buffered saline containing 10 mM glucose. Dissociated neurons (5–10 × 10^5 cell/ml) were cultured for 3–5 days on 35-mm in diameter glass bottom tissue culture dishes that had been coated with 0.25 mg/ml poly-L-lysin. The neuronal cells were cultured in Dulbecco's Modified Eagle Medium with 10% Fetal Bovine Serum, 1% penicillin-streptomycin and 5 mg/ml insulin at 37.0°C in 5% CO_2 with saturated humidity.

In Vitro Glutamate Monitoring—All experiments were performed at 37°C. Four kinds of incubation medium were prepared: (i) basal buffer (170 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 20 mM TAPS [*N*-tris(hydroxymethyl) methyl-3-aminoethanasulphonic acid], 5 mM $NaHCO_3$, 5 mM glucose, 1.2 mM Na_2SO_4, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$), (ii) high K^+ buffer (53.5 mM KCl, 120 mM NaCl, 20 mM TAPS, 5 mM NaHCO3, 5 mM glucose, 1.2 mM Na2SO4, 1.2 mM MgCl2, 1.3 mM CaCl2), (iii) low Ca^{2+} buffer (170 mM NaCl, 3.5 mM KCl, NaCl, 20 mM TAPS, 5 mM $NaHCO_3$, 5 mM glucose, 1.2 mM Na_2SO_4, 10 mM $MgCl_2$, 50 μM $CaCl_2$ 100 mM EGTA), and (iv) high K^+/low Ca^{2+} buffer (120 mM NaCl, 53.5 mM KCl, NaCl, 20 mM TAPS, 5 mM $NaHCO_3$, 5 mM glucose, 1.2 mM Na_2SO_4, 10 mM $MgCl_2$, 50 μM $CaCl_2$ 100 mM EGTA). The culture medium was removed from each well, and then the new medium was added. The cells were incubated in one of the above incubation media containing 0.05 U/ml glutamate oxidase at 37°C. After 2 min, the incubation medium was collected and 1 U/ml peroxidase and 50 μM Amplex™ Red were added. Then, fluorescence intensity was measured by spectrofluorometry. Neurotoxic tests were performed with 1 mM ω-Conotoxin GVIA.

Image Analyses—Glutamate release was monitored using Amplex™ Red in chick neuronal cells cultured for 3–6 days. Amplex™ Red (50 μM), glutamate oxidase and peroxidase were mixed and added to the culture dish, and glutamate release was measured after 1 min. Intracellular Ca^{2+} was monitored by fluorescence of the indicator Fluo-3. The loading solution was prepared to dissolve solid Fluo3-AM into DMSO, which was then diluted in basal buffer to the desired final concentration. Chick neuronal cells were loaded with Fluo3-AM (1 μM) for 30 min at 37°C. Neuronal cells were loaded with FM®1–43 (15 mM) by KCl stimulation for 60 s after washing with basal buffer. Images were analyzed using IPLab® (Solution systems, Japan) and NIH image (National Institutes of Health, USA) picture analysis software.

RESULTS AND DISCUSSION

Glutamate Determination—We measured fluorescence intensity images under a microscope linked to a CCD camera with glutamate oxidase, peroxidase, Amplex™ Red and glutamate at 1 U/ml, 0.025 U/ml, 50 μM and 100 μM, respectively. A time course of histograms of fluores-

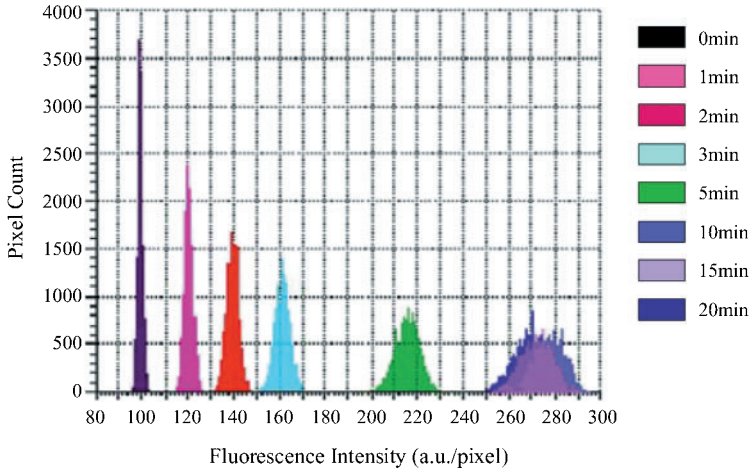

Fig. 2. **Time couse of the histogram of fluorescence distribution obtained with a CCD camera linked to a microscope.** 50 μM Amplex™ Red, 1 U/ml glutamate oxidase and 0.025 U/ml peroxidase were used.

cence distribution is shown in Fig. 2. The *x*-axis and *y*-axis indicate fluorescence intensity and the distribution of the number of pixels with the same fluorescence intensity, respectively. Figure 3 shows the relationship between reaction time and total fluorescence intensity as calculated from integration of the pixel count profile in Fig. 2. Total integrated intensity increased with reaction time until 5 min and then remained steady. Figure 4 shows the calibration of our glutamate imaging system based on fluorescence intensity obtained by steady state measurement for 5 min. A linear relationship was obtained between fluorescence intensity and glutamate concentration in the range from 20 nM to 1 μM. According to the reports of Zhou (*27*) and others, hydrogen peroxide concentrations of 5 pM are detectable with 50 μM of Amplex™ Red and 1 U/ml of peroxidase. When the level of glutamate oxidase decreased to 0.2 U/ml, the detection limit for glutamate became 100 nM even at high concentrations of peroxidase (1 U/ml). The relative activity of glutamate oxidase may indicate a rate determining step with total reaction system.

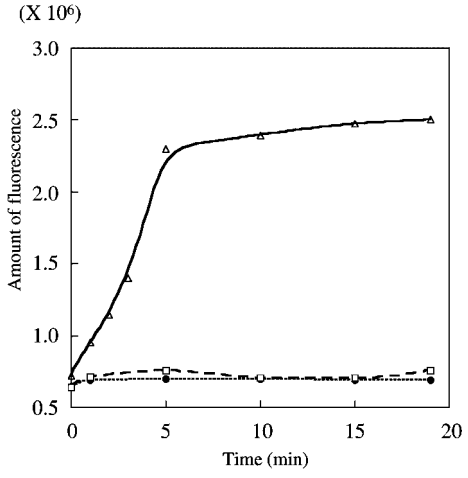

Fig. 3. **Relationship between reaction time and fluorescent intensity. Experimental conditions are described in the text.** Amount of fluorescence was calculated from the following equation: $F = \Sigma(N_i \cdot X_i)$; F, amount of fluorescence; N_i, density of fluorescence; X_i, pixel counts. (solid circles) Amplex™ Red, (open squares) Amplex™ Red + glutamate oxidase, (open triangles) Amplex™ Red + glutamate oxidase + peroxidase. 50 μM of Amplex™ Red, 1 U/ml of glutamate oxidase and 0.025 U/ml of peroxidase were used.

Application to Monitoring Glutamate Release from Neuronal Cells—Glutamate release from neuronal cells (10^5 cells/ml) was monitored in the basal buffer containing high or low Ca^{2+} concentrations (Table 1). KCl stimulation resulted in a significant increase in glutamate release (1.5-fold over basal release). However, it was found that a background level of glutamate was detected even in basal buffer without KCl stimulation. Glutamate release is dependent on Ca^{2+} concentration, and the difference in glutamate release between the basal and low Ca^{2+} buffer is believed to reflect spontaneous exocytosis from neuronal cells without KCl stimulation (*28*). The reverse of the glutamate transporter may be quantified as the difference in the amount of glutamate release with and without KCl stimulation in low Ca^{2+} buffer. We used two types of Ca^{2+} channel inhibitors, MK-801 and ω-Conotoxin GVIA, which act to suppress Ca^{2+} transport at postsynaptic and presynaptic neurons, respectively. Table 2 shows the different responses obtained with MK-801 and ω-Conotoxin GVIA. In the presence of ω-Conotoxin GVIA, there was no increase in glutamate release

Table 1. **Effects of KCl stimulation and Ca^{2+} flow on glutamate release.** Four kinds of buffer are described in the experimental section. Standard deviations were calculated from 6 measurements.

	Relative activity (%)
Basal buffer	100 ± 45
High KCl buffer	160 ± 42
Low Ca2+ buffer	65 ± 42
Low Ca2+/high KCl buffer	94 ± 45

Table 2. **Effect of Ca^{2+} channel inhibitors under KCl stimulation.** Two kinds of buffer and Ca^{2+} channel inhibitors are described in the experimental section. The standard deviations were calculated from 6 measurements.

	Relative activity (%)
Basal buffer	100 ± 45
High KCl buffer	160 ± 42
MK-801/high KCl buffer	65 ± 42
ω-Conotoxin GVIA/high KCl buffer	94 ± 45

after KCl stimulation, probably due to the inhibition of voltage-activated Ca^{2+} channels in presynapses. This result is in accord with a report (*29*) that exocytosis and Ca^{2+} influx from voltage-activated Ca^{2+} channels in the presynapse are closely associated. On the other hand, MK-801 does not inhibit the increase in glutamate release after KCl stimulation. MK-801 inhibits signal transmission to postsynaptic neurons by binding to the NMDA receptor in postsynapses. Therefore, glutamate release from presynapses is not inhibited by MK-801.

Imaging of Glutamate Release from Primary Cultured Chick Neuronal Cells—The release of glutamate from the chick the neuronal network was observed using this fluorometric enzymatic assay for glutamate with a cooled CCD camera. Figure 5, a and b, are glutamate release images that consist of fluorescence images overlayed on phase contrast images. Amplex™ Red, peroxidase, and glutamate oxidase were added to the chick neuronal network after 6 days of incubation, and fluorescence was measured. Before and after KCl stimulation, the increase in fluorescence was observed especially in the regions where the neuronal cell body connect to axons. Fluores-

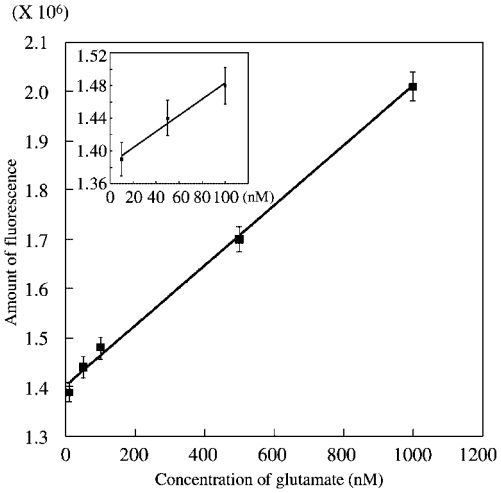

Fig. 4. **Calibration range of glutamate.** Experimental conditions are described in the text. The amount of fluorescence is the same as in Fig. 3.

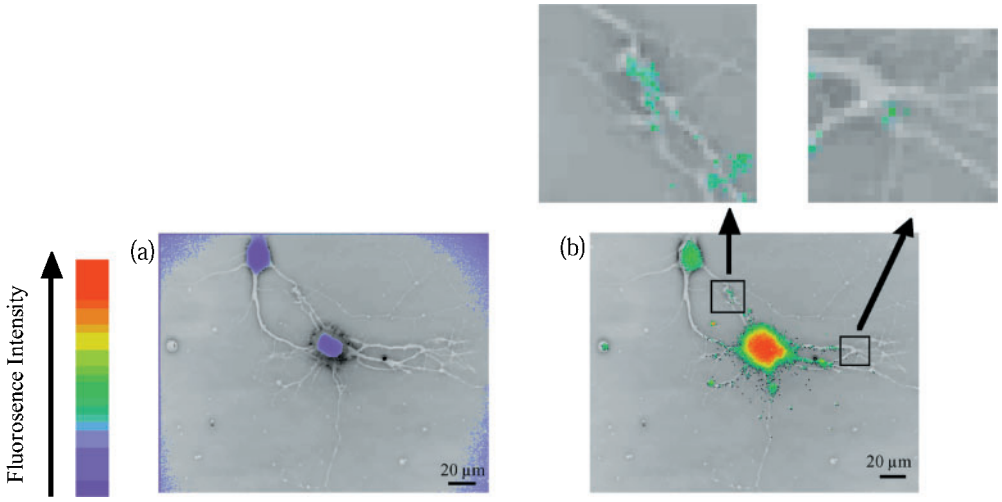

Fig. 5. **Imaging of glutamate release from chick neuronal cells before (a) and after (b) KCl stimulation.** The fluorescence images were overlaid onto the phase contrast images.

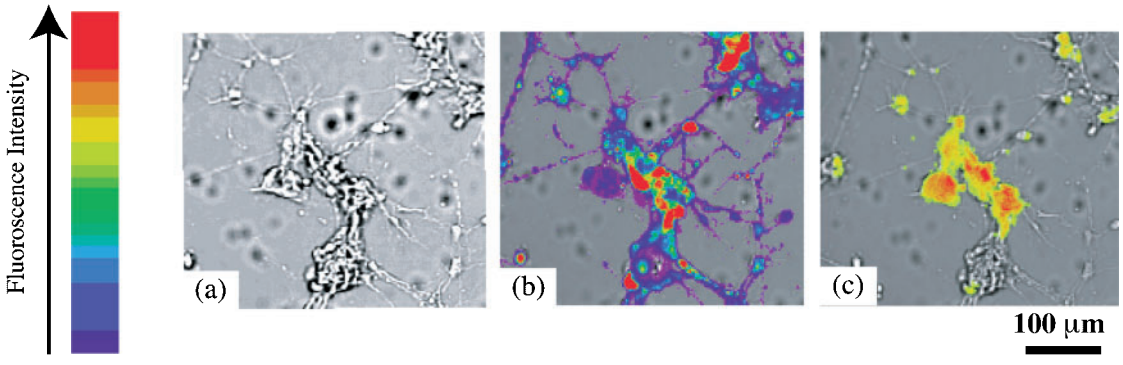

Fig. 6. **Fluorescent Ca^{2+} (b) and glutamate (c) images were overlaid onto the phase contrast images (a).** Experimental conditions are described in the text.

cent resolfin generated from Amplex™ Red was expected to be adsorbed to the cell surface and to emit a strong fluorescence at the neuronal cell and synaptic regions that cause glutamate release. KCl stimulation was performed by adding a 1 M KCl solution. A KCl concentration of 53.5 mM KCl, the same as in Table 1. The center of the cell body appears brighter than the peripheral parts probably due to the presence of dense synaptic junctions. Synaptic sites apart from the cell body also produced a detectable signal as shown in Fig. 5b.

Figure 6 shows microscopic images of neuronal cells showing the localization of Ca^{2+} and glutamate. The activities of both glutamate release and Ca^{2+} flow can be measured and discussed in terms of the correlation between the two signals. KCl-evoked Ca^{2+} flow plays a role in triggering glutamate release, which is thought to be related to the interconnection between neuronal cells and axons.

FM®1–43 is widely used as a fluorescent dye for staining synaptic vesicles. By simultaneous imaging with Amplex™ Red and FM®1–43 (*30–32*), KCl-evoked glutamate release and synaptic exocytosis can be demonstrated to occur in the same regions (data not shown). According to Nicholl's (*33*) review, glutamate is normally released by Ca^{2+}-independent exocytosis and may be reaccumulated directly into the nerve terminal or into an adjacent glia. Under resting conditions, glutamate concentrations in the extracellular space, the presynaptic cytoplasm and the lumen of glutamatergic synaptic vesicles are on the order of 1 μM, 10 mM, and 100 mM, respectively. However, there has been no report of glutamate concentrations around glutamatergic synapses in primary cultures of dissociated cerebral cortex neurons differentiated from chick embryos. Based on the calibration graph shown in Fig. 4, the glutamate concentration was roughly estimated to be 10–100 nM around bright synaptic regions. This system will be followed by simulation of the dilution by diffusion of glutamate linked with the quantitative estimation of glutamate molecules released from neurons. The relative intensity imaging demonstrated in Fig. 6, b and c, will be discussed taking into consideration synapse density and location.

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