Detection and Imaging of L-Glutamate Released from Mouse-Brain Slices with an Enzyme-Based Membrane

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A new and simple method for detecting and color imaging of L-glutamate released in mouse-brain slices is described based on an enzyme-coupling reaction. The method utilizes L-glutamate oxidase (GluOx) and horseradish peroxidase (HRP) immobilized in a bovine serum albumin (BSA)-glutaraldehyde matrix on a poly-L-lysine-coated glass cover slip. The enzymes are spontaneously and continuously supplied from the membrane into an extracellular solution of the brain slice placed on the membrane after dipping in a redox substrate (DA-64). The Bindschedler's Green (BG) converted from the DA-64 by the enzyme reaction gives green signals in the brain slice, which are related to the concentration of L-glutamate released in its regions. The first examples of color images for the spatial distribution of L-glutamate in the regions of adult mouse brain slices are demonstrated. The regions where the intense green signals were observed matched the reported distribution of mRNAs encoding the NMDA receptor in the mouse-brain slice.

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Imaging the spatial and time-resolved distribution of biologically active substances in cells and tissues has attracted much attention in view of obtaining direct information about intracellular and intercellular processes of biological signaling cascades.¹ Various imaging methods have been reported, including fluorescence spectroscopy,² atomic-force microscopy³ and electron microscopy.⁴

Many studies have been described on the imaging of dynamic changes in the intracellular concentration and spatial distribution of target molecules and ions which play key roles in signal cascades, such as Ca^{2+} ,⁵⁻⁷ adenosine 3',5'-cyclic monophosphate (cAMP),^{8,9} NO^{10} and protein kinase C.¹¹ The spatial distribution of membrane proteins, such as the N-methyl-D-aspartate (NMDA) receptor and its subunits in brain slices, has also been imaged by autoradiography¹² and *in situ* hybridization.¹³⁻¹⁵ Extracellular neurotransmitters having fluorescent groups, such as serotonin and catecholamines, have been imaged by native fluorescence microscopy.¹⁶⁻¹⁹ However, no studies have been reported on imaging of L-glutamate, an important neurotransmitter in the brain, because of a lack of highly sensitive and selective probes for the neurotransmitter. Recently, an enzymatic, fluorometric imaging of L-glutamate releases from retinal slices has been proposed based on the fluorescence of NADH converted from NAD by an enzyme reaction between L-glutamate and glutamate dehydrogenase.²⁰

In the present paper, we describe an enzyme-based method for color imaging of L-glutamate released in mouse-brain slices. Cells and tissues are optically transparent in visible regions and imaging with visible radiation provides a simple method for detecting signals from target substances. The principle of the present method is shown in Fig. 1. L-Glutamate oxidase (GluOx)²¹⁻²³ and horseradish peroxidase (HRP)^{22,23} are immobilized on a poly-L-lysine coated cover slip by the bovine

serum albumin (BSA)-glutaraldehyde method. Brain slices are immersed in a solution of the substrate, in the present case DA-64, and transferred onto the membranes. The enzymes spontaneously diffuse from the membrane into the extracellular solution, where L-glutamate triggers an enzyme reaction to convert DA-64 to Bindschedler's Green (abbreviated hereafter as BG). The amount of BG formed is in a stoichiometric relation with that of L-glutamate and, hence, the intensity of the green signal is proportional to the concentration of L-glutamate. In this approach, the membrane is a reservoir of enzymes, from which enzymes are continuously supplied, directed toward an extracellular solution of the brain slice. The first examples of color images for the spatial distribution of L-glutamate in

Fig. 1 Schematic representation for the principle of color imaging of L-glutamate released from mouse-brain slices. GluOx, L-glutamate oxidase; HRP, horseradish peroxidase; BSA, bovine serum albumin; DA-64, N-(carboxymethylaminocarbonyl)-4,4'-bis(dimethylamino)-diphenylamine sodium salt; BG, Bindschedler's Green.

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regions of adult mouse brain slices are reported based on studies using enzyme-based membranes.

Experimental

Materials

GluOx from *Streptomyces* sp. X-119-6 was obtained from Yamasa Shoyu (Choshi, Japan) and HRP from Wako Pure Chemicals Co. (Osaka, Japan). Poly-L-lysine, hydrobromide and BSA were purchased from Sigma Chemical Co. (St Louis, MO). 10% (w/v) Glutaraldehyde solution, *N*-(carboxymethyl-aminocarbonyl)-4,4'-bis(dimethylamino)-diphenylamine sodium salt (DA-64), L-glutamic acid and Triton X-100 were obtained from Wako Pure Chemicals Co. Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) was obtained from Dojindo Laboratories (Kumamoto, Japan). Other chemicals used were all of analytical reagent grade. Glass cover slips (18×24 mm, 0.17–0.25 mm in thickness) were obtained from Matsunami Glass Co. (Tokyo, Japan). Milli-Q water (Millipore reagent water system, Bedford, MA) was used throughout the experiments.

Preparation of GluOx-HRP membranes

Glass cover slips were first cleaned in SCAT 20-X (Nacalai Tesque, Inc., Kyoto, Japan) overnight. The cover slips were thoroughly rinsed with water and air-dried. A 5.0 μ l aliquot of the poly-L-lysine solution (5.0 μ g/ml) was dropped on the cover slip with a micropipet, on which another cover slip was placed. The cover slips were allowed to stand overnight for the adsorption of poly-L-lysine. Both cover slips were used for preparing GluOx-HRP membranes. A 30 μ l aliquot of GluOx-HRP solution (41 U/ml GluOx, 2.5 mg/ml HRP, 0.050% (w/v) glutaraldehyde, 0.50% (w/v) BSA, 38 mM NaCl and 6.3 mM $\text{K}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.4) was layered on the poly-L-lysine adsorbed cover slip and dried in air under darkness for one day. The GluOx-HRP solution was prepared just before use by mixing equal volumes of GluOx solution (1.6×10^2 U/ml GluOx, 0.15 M NaCl and 25 mM $\text{K}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.4), 10 mg/ml HRP solution, 0.20% (w/v) glutaraldehyde solution and 2.0% (w/v) BSA solution. The thus-prepared GluOx-HRP membranes were stored at room temperature under darkness until use.

The optimum concentration of BSA was in the range from 0.5 to 0.6% (w/v). When the BSA concentration was 0.8% or greater, color signals were very weak. At BSA concentrations lower than 0.3%, the membrane became pulpy upon placing L-glutamate solution on it.

The GluOx-HRP membranes were repeatedly usable several times, although in the present study newly prepared membranes were always used.

Imaging of L-glutamate release from brain slices

Adult male ddY mice were decapitated under ether anesthesia. Coronal and parasagittal slices (thickness 150 μ m) were cut using a Dosaka DTK-1000 microslicer (Kyoto, Japan) on an ice bath. The slices were incubated for 1 h in a recovery solution at 30–32°C and held at room temperature until use. The recovery solution contained 0.12 M NaCl, 1.5 mM KCl, 26 mM NaHCO_3 , 1.2 mM KH_2PO_4 , 2.4 mM CaCl_2 and 10 mM glucose, and was saturated with a 95% O_2 –5% CO_2 gas mixture. For imaging, the slice was immersed in a 5.3 mM DA-64 solution containing 0.050% TritonX-100 and 0.010 M PIPES/NaOH (pH 7.0) for 3 min and then transferred onto the GluOx-HRP membrane. The solution on the slice was sucked up with

Fig. 2 Coloring properties of PIPES buffer containing L-glutamate on GluOx-HRP membranes. (A) Absorption spectra of BG formed in 15 μ l PIPES buffer containing 0.25 mM L-glutamate on the GluOx-HRP membrane. Curve 1, after reaction of 5 min; Curve 2, after washing with Milli-Q water. (B) L-Glutamate concentration-dependence of absorbance of BG at 727 nm in 15 μ l PIPES buffer on the GluOx-HRP membrane. Reaction time, 5 min. (C) Time-resolved changes in absorbance of BG at 727 nm in 15 μ l PIPES buffer containing 0.25 mM L-glutamate on the GluOx-HRP membrane.

Kimwipe until the slice remained wet. The membrane was set on a slide glass on the stage of a microscope (BH-2, Olympus, Tokyo, Japan). Dark-field photographs of the slice were taken with 1× objective and 2.5× photographing lenses. After dropping a 5 μ l portion of 1 M KCl as a stimulant onto the slice, photographs of the slice were taken at a time interval of 2–3 min. For enlarged images, a 2× objective lens was used. All images were taken at room temperature (24–26°C).

Measurement of absorption spectra

A 15 μ l portion of PIPES buffer (74 mM NaCl, 1.4 mM KCl, 1.0 mM CaCl_2 , 5.0 mM glucose and 5.0 mM HEPES, 0.25% TritonX-100, 2.6 mM DA-64 and 0.050 M PIPES/NaOH, pH 7.2) containing different concentrations of L-glutamate was dropped and spread onto the GluOx-HRP membrane with a micropipet. After standing for 5 min, a cover slip was placed on the membrane to stop the enzyme reaction. The cover slip cut off the supply of oxygen, which is necessary for a GluOx-mediated reaction, leading to the suspension of the enzyme reaction. The GluOx-HRP membrane, together with the cover

Fig. 3 Schematic representation of coronal and parasagittal mouse-brain slices. A) Coronal slice. B) Parasagittal slice. Amd, amygdala; Cx, cerebral cortex; DG, dentate gyrus; Hi, hippocampus; Hyp, hypothalamus; Pir, piriform cortex; Th, thalamus; B, basal ganglia. The regions in the coronal and parasagittal slices were assigned according to "The Rat Brain in Stereotaxic Coordinates", compact third edition, by G. Paxinos and C. Watson, Academic Press, San Diego, 1997 and "Atlas of Prenatal Rat Brain Development", by J. Altman and S. A. Bayer, CRC Press, Boca Raton, 1995, respectively.

slip, was attached to the sample-side cell holder of a Shimadzu (Kyoto, Japan) UV-240 spectrophotometer. A blank was prepared with another GluOx-HRP membrane by dropping 15 μ l PIPES buffer without L-glutamate. The absorption spectra of BG produced in the PIPES buffer containing L-glutamate on the GluOx-HRP membrane were measured against the blank. For investigating the time course of the coloring, the GluOx-HRP membranes were treated with the PIPES buffer containing 0.50 mM L-glutamate for different periods (1 – 7 min).

Results and Discussion

Testing the principle of color imaging with the GluOx-HRP membrane

Color images of brain slices in the present system were obtained based on the enzyme reaction given in Fig. 1. The principle of the method was tested with droplets of L-glutamate solution in place of a brain slice. A 15 μ l of PIPES buffer (*vide supra*) containing 0.25 mM L-glutamate was placed on the membrane. The absorption spectra were measured after standing for 5 min, as described in the experimental section. As shown in Fig. 2A, the spectrum (curve 1) showed an absorption maximum at 727 nm, in accordance with that of BG in solution. When the membrane was washed with Milli-Q water, the absorption at 727 nm disappeared almost completely (curve 2). On the other hand, when a GluOx-HRP membrane was soaked in a DA-64 solution overnight and was washed with Milli-Q water, a droplet of L-glutamate solution placed on the membrane did not exhibit a green signal, indicating that DA-64 is hardly soluble in the membrane. These results prove that the product BG was formed in the solution on the membrane rather than in the membrane, suggesting that the enzymes (GluOx and HRP) spontaneously diffused from the membrane into the

solution. The absorbance at 727 nm increased with the concentration of L-glutamate up to 4.0 mM (Fig. 2B). Thus, the intensity of green signals due to BG is related to the concentration of L-glutamate, *i.e.*, the stronger is the green signal, the larger is the concentration of L-glutamate. Naturally, in the absence of L-glutamate, no color signals were detected. The detection limit for L-glutamate, defined as 3-times the background absorbance, was 0.13 mM for a reaction time of 5 min.

The time-resolved changes in the absorbance of BG produced in solution in contact with the GluOx-HRP membrane are shown in Fig. 2C. The absorbance at 727 nm started to increase several seconds after the L-glutamate solution was brought into contact with the membrane, and reached the maximum after 170 s. Saturation of the BG production is simply due to the consumption of L-glutamate initially present in the solution. It was calculated that if 0.2% of GluOx in the membrane diffuses into 15 μ l of a 0.50 mM L-glutamate solution, L-glutamate is almost completely consumed after 183 s. It is emphasized that the green signals of BG once formed do not vanish, even after the complete consumption of L-glutamate, owing to the irreversible enzyme reaction. This feature is advantageous for imaging low concentrations of L-glutamate, because the amount of BG is time-integrated and its green signals are enhanced. However, time-resolved color images for dynamic changes in L-glutamate concentration are of course not feasible.

The effects of some amino acids (glycine, D-glutamate, L-aspartate, L-glutamine and L-asparagine) and common compounds in an extracellular solution in the brain (ascorbate, γ -aminobutyric acid and dopamine) on the generation of color signals were investigated by placing 15 μ l of PIPES buffer containing 0.50 mM of each compound on the membranes. These compounds did not give any changes in absorbance at 727 nm, demonstrating that the green signals are specific to L-glutamate.

Imaging of L-glutamate release from mouse brain slices

Dark-field photographs of coronal slices (Fig. 3A) of an adult mouse brain on the GluOx-HRP based membrane before and after stimulation with 1 M KCl are shown in Fig. 4A. The brain slice was immersed in a DA-64 solution for 3 min beforehand, as described in the experimental section. The K⁺ ions evoke depolarization of the membrane potential, triggering L-glutamate release. The green signals, ascribable to the formation of BG, started 2 min after a KCl stimulation, especially in the thalamus (Th). After waiting for 16 min, intense green signals were locally seen in the CA1 and CA3 regions of hippocampus (Hi), dentate gyrus (DG), cerebral cortex (Cx), piriform cortex (Pir), thalamus and amygdala (Amd). Further waiting for coloring lead to a dimming of the locally distributed green signals, due to a lateral expansion of water-soluble BG over the whole slice. In the enlarged images for Hi in another coronal slice (Fig. 4B), intense signals in the hippocampal CA1 region and weak signals in the dentate gyrus and CA3 region were seen. Without KCl stimulation (Fig. 5A), green signals were observed only in the cerebral cortex and piriform cortex. These signals came from the background L-glutamate release. When the color images for the coronal slices with and without the stimulation of KCl were compared, it is clear that the intense green signals in the hippocampus (Hi) were observed only in the presence of the KCl stimulation.

The imaging of L-glutamate release in the parasagittal slices (Fig. 3B) of mouse brain was also examined. As shown in Fig. 5C, under KCl stimulation, distinct signals were observed in the dorsal hippocampus, ventral hippocampus, dentate gyrus and

Fig. 4 Color images for coronal mouse brain slices under 1 M KCl stimulation. (A) Images for whole slice and (B) enlarged images for Hi.

Fig. 5 Color images for mouse-brain slices. (A) Coronal slice, without KCl stimulation. (B) Color images for the coronal slice under stimulation of 1 M KCl obtained after dipping in a mixture of GluOx, HRP and DA-64. (C) Parasagittal slice, with KCl stimulation.

cerebral cortex. Green signals were also detected in the basal ganglia and amygdala. Importantly, the regions in the parasagittal slices, where the intense green signals were detected, were identical to those in the coronal slices. Also, without KCl stimulation, green signals were detected in the same regions, *i.e.*, in the cerebral cortex, for both the parasagittal and coronal slices.

The distribution of the intense green signals in the coronal slice was compared with the reported distribution^{13,14} of expression of the mRNAs encoding the $\epsilon 1$ and $\epsilon 2$ subunits of the NMDA receptor in the adult mouse brain slices (Fig. 3 in ref. 13 and Fig. 4 in ref. 14). The intensely colored regions closely matched to the regions where L-glutamate receptors are rich: the $\epsilon 1$ and $\epsilon 2$ subunit mRNAs are highly expressed in the CA1 and CA3 regions of the hippocampus, dentate gyrus and cerebral cortex and weakly expressed in the thalamus and amygdala. The matching of the L-glutamate-release and the NMDA receptor-rich regions suggests that the present approach gives an accurate method for imaging the spatial distribution of L-glutamate released in the regions of brain slices.

It should be noted that the imaging of L-glutamate release without the membrane was unsuccessful. When the coronal slice was dipped into a solution containing GluOx (41 U/ml), HRP (2.5 mg/ml) and DA-64 (5.3 mM) for 3 min and transferred onto a cover slip (without the GluOx-HRP membrane), very weak signals were detected only in the thalamus, and no notable signals were detected in other regions, even after waiting for 17 min (Fig. 5B).

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