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Detection of Glutamate in Optically Trapped Single Nerve Terminals by Raman Spectroscopy

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Glutamate is the major excitatory neurotransmitter and is of particular interest in light of current models of memory and learning. The paper describes the first in situ detection of glutamate in single nerve terminals (synaptosomes), which is achieved by using laser trapping Raman spectroscopy. The near-infrared laser light captures a single synaptosome obtained from a Wister rat brain. The release of glutamate in a single laser-trapped synaptosome was detected by subtracting the Raman spectrum before depolarization from that after depolarization with the addition of the K^+-channel blocker, 4-aminopyridine. The result indicated that the single synaptosome released ~3 amol of glutamate and that the release rate depended on the 4-aminopyridine concentration.

Amino acids, such as glutamate, γ-aminobutyrate, and glycine, are the most familiar neurotransmitters utilized at neuronal synapses. Glutamate is the major excitatory transmitter and is of particular interest in light of current models of memory and learning. Various biosensors have been developed for glutamate detection with a view to revealing the role of glutamate in neural cells.[1−5] Another approach designed to provide a detailed understanding of the uptake, storage, and exocytosis mechanisms of glutamate at a presynapse involves the use of nerve terminals, called synaptosomes, which have been used as the simplest presynapse system.[6−9] The synaptosomes of a rat brain are 500−700 nm in diameter and contain many synaptic vesicles ~50 nm in diameter, which remain viable for several hours after isolation from a neuron and have the ability to release neurotransmitters. The glutamate released from aggregated synaptosomes has been studied by fluorescence spectroscopy.[10−12] However, this technique is incapable of measuring glutamate at the single-synaptosome level because of the difficulty involved in isolating a single synaptosome. To reveal the glutamate release process in a single synaptosome, we have combined the laser trapping technique with Raman spectroscopy. This method provides information about the molecular vibration of glutamate without the need for a staining process.

The laser trapping technique, also called optical tweezers, has been used to manipulate single microparticles such as microdroplets, aerosol particles, microcapsules, and biological cells. This technique was first reported in 1970 by Ashkin, who used two laser beams,[13] and then a more practical method employing one laser beam and an objective lens was introduced by Chu and coworkers in 1986.[14] With the latter approach, a microparticle is captured by the force of the radiation pressure gradient generated by the tightly focused laser beam under the objective lens. Many biological applications using the laser trapping technique, such as the manipulation of bacteria, blood cells, and organelles in cells, have been reported since the adoption of near-infrared (NIR) laser light in the 700−1100-nm range rather than visible light to eliminate any photochemical damage to the samples.[15]

The combination of laser trapping with various spectroscopic methods, including fluorescence, absorption, and Raman techniques, enables us to study chemical reactions in single microdroplets, microcapsules, and other micrometer-sized particles.[16−21] Although laser trapping Raman spectroscopy (LTRS) is especially advantageous for obtaining various kinds of information about species and structures, and the conformations of molecules in laser-trapped microparticles, its biological application has remained limited by lower sensitivity in the NIR light region rather than

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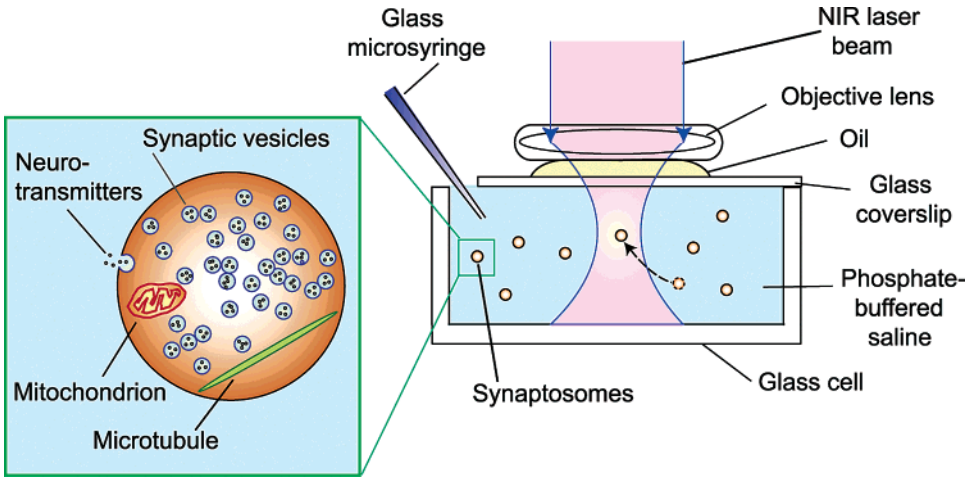

Figure 1. Experimental setup with a glass cell for laser trapping synaptosomes in solution and for detecting neurotransmitters.

the visible one. Our previous report showed that the LTRS system could capture a single synaptosome in solution and also provide the Raman peaks of lipids and proteins in the synaptosome.[22] The use of NIR laser light in Raman spectroscopy is advantageous because it causes less photochemical damage to the biological samples and results in much less fluorescence background in the Raman spectrum. Li's group also reported a biological application using LTRS for the analysis of red blood and yeast cells and showed the Raman peaks of lipids and proteins.[23] The Raman peaks of lipids and proteins are much larger than those of amino acids in the spectra of synaptosomes because their molecules are present in greater numbers and they have larger Raman cross sections. This makes it very difficult to observe glutamate directly in a single synaptosome.

In this paper, we describe the in situ detection of glutamate released from a single synaptosome using LTRS by subtracting the Raman spectrum before depolarization from that after depolarization. The depolarization was induced by the addition of the K^+-channel blocker, 4-aminopyridine (4-AP), which indicates that the glutamate was released by exocytosis. The result constitutes the first application of LTRS to neurotransmitter detection and also shows the advantage of using LTRS for the noninvasive analysis of molecules contained in single nanometer-sized biological samples.

EXPERIMENTAL SECTION

Synaptosome sample preparation has been described previously[22] and is the same as Shira's method.[9] Simply, the cerebral cortex of a 7- or 8-day-old Wister rat brain is isolated. Then homogenization and centrifugal separation processes are performed using the Percoll gradient method. Cerebral cortex in an aqueous sucrose solution (0.32 M) is mixed with a homogenizer (Hom, Iuchi). Then, a synaptosome layer is isolated in the aqueous sucrose solution by using an ultracentrifuge (Optima TLX, Beckman) and purified in Percoll (Amersham Pharmacia Biotech) gradient solution (23, 15 10, and 3 vol %). Finally, the synaptosomes are dispersed in phosphate-buffered saline (D-PBS 14040, Gibco) including 2 mM calcium hydrochloride for the Raman measurement. Figure 1 shows the experimental setup with a glass cell that we used for laser trapping synaptosomes in solution. Phosphate-buffered saline containing synaptosomes was placed in the glass cell, and then the glass cell was covered with a 0.17-mm-thick glass coverslip (No. 1-S fluorescence-free type, Matsunami Glass Industries). For laser trapping and Raman measurements, an NIR laser beam was focused on the sample solution in the glass cell through immersion oil and a glass coverslip. The 4-aminopyridine (98%, Sigma) was diluted to 0.8−2 mM without further purification and added to the solution in the glass cell by using a glass microsyringe. L-Glutamate solutions of 50 mM comprised a mixture of glutamic acid monosodium salt (Sigma) and the phosphate-buffered saline. The pH of each glutamate solution was adjusted with hydrochloric acid solution.

The LTRS laser Raman trapping system is described in detail elsewhere.[18,21] Briefly, a Raman microprobe spectrometer (Ramascope, Renishaw) containing two holographic notch filters and a single-grating polychromator was specially modified for NIR laser light. The excitation light source was a continuous-wave, single-frequency Ti:sapphire ring laser (Titan-cw, Schwartz Electro-Optics) tuned to 730 nm in the TEM_{00} mode pumped by the 532-nm line of a solid-state visible continuous-wave laser (Millennia, Spectra-Physics Lasers). The laser beam (power ~150 mW) passes through an optical microscope (BH-2, Olympus) and is focused onto the sample by an oil-immersed objective lens with a numerical aperture of 1.3. The objective lens is also used to collect light scattered from the sample at 180° with respect to the incident light. A charge-coupled device (CCD) camera (02-06-1-225, Wright Instruments) containing a Peltier-cooled (200 K) CCD chip and a two-slit confocal arrangement, which reduces the background Raman scattering from the region outer trapped particles, are used for the Raman measurement. An additional CCD camera and a holographic notch filter are fitted to the optical microscope to provide optical images.

RESULTS AND DISCUSSION

Figure 2 shows a sequence of time-dependent images of light reflected from a single synaptosome in the phosphate-buffered saline. The image at 0 min was obtained just after the single synaptosome was laser trapped. The bright circular pattern in each image was made by the interference between the top and the

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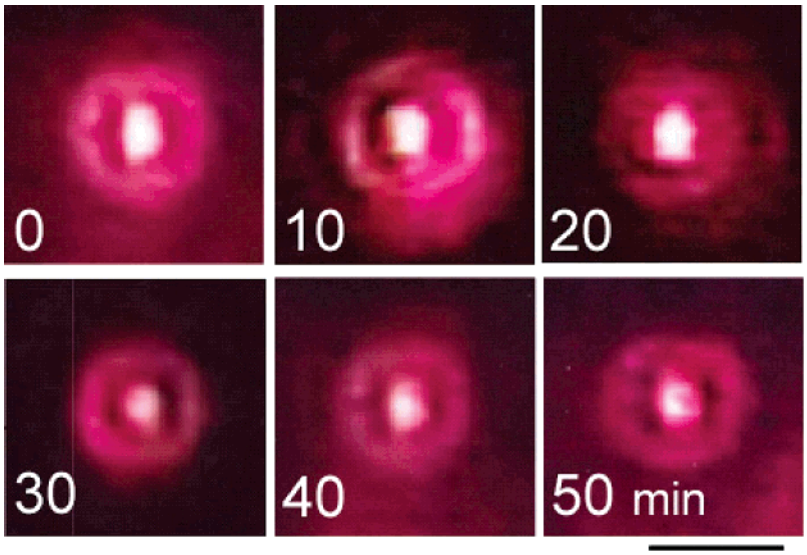

Figure 2. Time-dependent laser light interference images of a single laser-trapped synaptosome. The scale bar denotes 2 μm. The 4-aminopyridine was added to the solution just after the image at 10 min was recorded.

bottom of the single laser-trapped synaptosome, which is determined by the wavelength of the laser light and the refractive indexes of the synaptosome and the buffered saline solution.[24] The image becomes fuzzy when two or more synaptosomes are trapped;[22] therefore, the clear circular pattern in the 0-min image indicates that a single synaptosome is stably trapped in the focal spot of the laser beam. The K^+-channel blocker 4AP was added to the solution after the image at 10 min had been recorded. The final concentration of 4AP in the glass cell was 4 μM. It is well known that the addition of 4AP induces repetitive firing of the Na^+-channel in a synaptosome, thus releasing glutamate by exocytosis.[10−12] The circular patterns in the images before and after the addition of 4AP are very similar in size; this indicates that the change in refractive index of the synaptosome by 4AP is very small. The brightness of the circular patterns became slightly weaker after the addition of 4AP. This is probably related to morphological changes, for example, changes in the size or surface roughness of the synaptosome, or the distribution of the synaptic vesicles in the synaptosome.

Figure 3a shows the time-dependent Raman spectra of a synaptosome corresponding to the images in Figure 2. The exposure time for the Raman spectrum was 10 min. The background spectrum of the saline solution is subtracted from each spectrum. Two main peaks are observed in this spectral region: one at 1445 cm^{-1} and another at 1657 cm^{-1}. The former is assigned to the CH_2 deformation mode, indicating that the trapped synaptosome contains lipids of cell membranes, and the latter is assigned to the amide-I mode, suggesting the presence of various proteins.[25,26] The spectra are very similar even after the addition of 4AP at 20 min. The difference between the spectra is very small because the other Raman peaks are weaker than the larger Raman peaks of the lipids and proteins.

The peaks of glutamate are seen more clearly in Figure 3b when the peaks of the lipids and proteins are subtracted as a baseline. The calculation was performed using the spectrum at 50 min as the baseline and subtracting this value from each Raman spectrum in Figure 3a. Four additional broad peaks appeared after the subtraction, and they are denoted by α, β, γ, and δ. The γ peak is considered to be that of the COO^- stretching mode of the $NH_3^+COO^-$ group at 1616 cm^{-1}. The α peak at 1344 cm^{-1} and the δ peak at 1727 cm^{-1}, respectively, are considered to be those of the glutamate of the CH_2 deformation mode and the C═O stretching mode in the COOH group from their Raman peak positions in bulk solution.[27,28] Furthermore, the β peak can presumably be attributed to a mixture of the COO^- symmetrical stretching mode, the CH_2 deformation mode, and the symmetrical NH_3^+ deformation mode, which correspond respectively to the peaks at 1415, 1422, and 1445−1450 cm^{-1} in the reference, which cannot be separated because of the insufficient signal-to-noise ratios of the spectra. The total amount of glutamate (about 50−100 mM) in the synaptic vesicles far exceeds that of other neurotransmitters,[6,29,30] which enables us to determine glutamate Raman peaks using our system. The four peaks in the spectrum 10 min before the 4AP addition are very similar to those in the spectrum at 0 min, which indicates that the synaptosomes were noninvasively trapped by NIR laser light. They have completely disappeared in the spectrum at 40 min after the 4AP addition, which indicates the release of glutamate from a single synaptosome. The addition of 4AP induces repetitive firing of the Na^+-channel in a synaptosome thus releasing glutamate for a lengthy period, because after exocytosis the synaptic vesicles are regenerated within the synaptosome and reloaded with neurotransmitters.[31]

Figure 4a shows the time-dependent Raman spectra of a synaptosome before and after 4AP addition. The measurement

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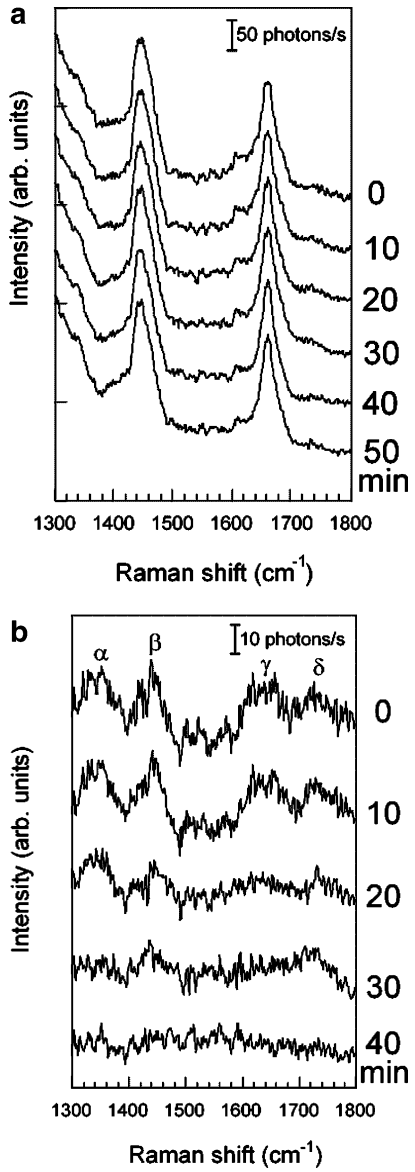

Figure 3. (a) Time-dependent Raman spectra of a single synaptosome with the addition of 4-aminopyridine (4 μM), which correspond to the images in Figure 2. The spectra were recorded every 10 min. The spectrum of phosphate-buffed saline was subtracted from each spectrum as a baseline. (b) The Raman spectra after the spectrum at 50 min in (a) subtracted from the other spectra in (a).

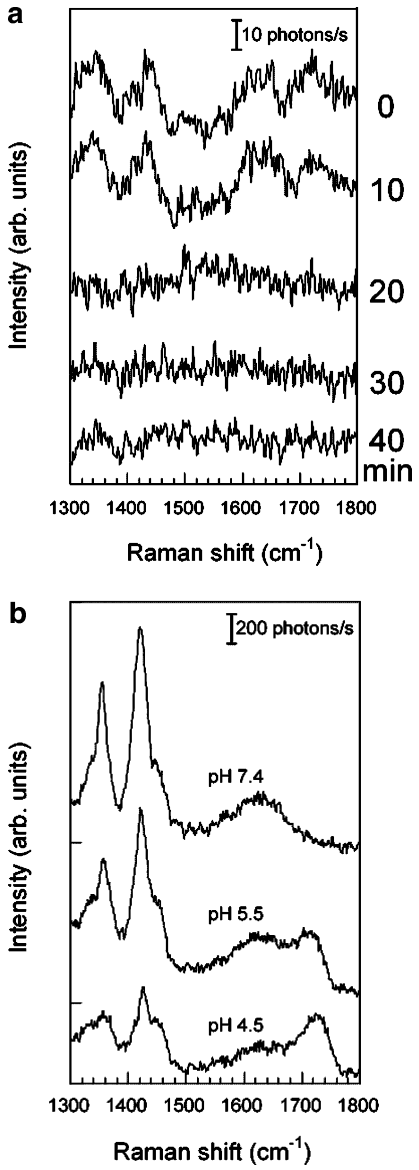

Figure 4. (a) Time-dependent Raman spectra of a single synaptosome with the addition of 4-aminopyridine (10 μM). The measurement condition was same as those for the spectra in Figure 3b except for the 4-aminopyridine concentration. (b) Raman spectra of 50 mM glutamate solutions at pH values of 4.5, 5.5, and 7.4.

conditions and spectrum subtraction method are the same as those in Figure 3b except for the 4AP concentration. The final concentration of 4AP in the glass cell was 10 μM, namely, 2.5 times higher than that in Figure 3b. The four peaks seen at 0 min did not change after 10 min. After the addition of 4AP, the glutamate peaks rapidly disappeared from the spectrum at 20 min, which was faster than in Figure 3b. The two Raman spectra of a 50 mM L-glutamate aqueous solution at pH values of 4.5, 5.5, and 7.4 are shown in Figure 4b to allow us to compare the neurotransmitter and bulk solution spectra. The exposure time for each spectrum was 2 min. It is known that the pH of ordinary synaptic vesicles is 5.5 and is called internal pH.[32−35] The solution in the synaptic vesicles is acidic because a proton pump, Mg^{2+}-activated vacuolar ATPase, injects protons into the vesicles. Therefore, the glutamate contained in the synaptosomes is acidic and the released glutamate has a pH of 7.4, the same as that of the phosphate-buffered saline outside the trapped synaptosome. The ratios of the peak intensities in the spectrum at 0 or 10 min appear to be closer to that in the bulk solution spectrum for pH 5.5 than those for pHs of 4.5 and 7.4. In particular, the peak of the C═O stretching mode in the COOH group at 1727 cm^{-1} does not appear in the bulk solution spectrum at pH 7.4. However, the signal-to-noise ratio of the glutamate peaks in a single synaptosome is insufficient to discuss

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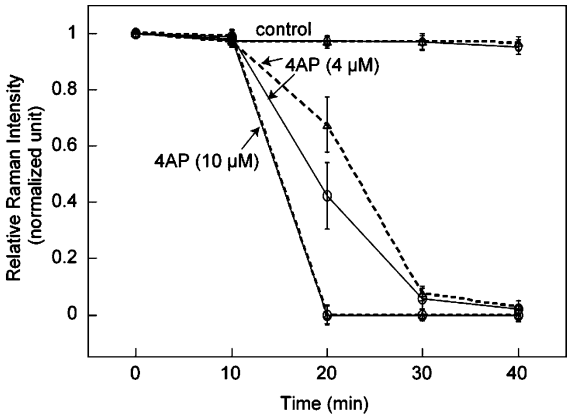

Figure 5. Time-dependent relative Raman intensities of glutamate released from single synaptosomes, the CH_2 deformation mode at 1344 cm^{-1} (dashed lines), and the COO^- stretching mode of the $NH_3^+COO^-$ group at 1616 cm^{-1} (solid lines). 4-Aminopyridine with concentrations of (1) 4 and (2) 10 μM (final concentrations) was added to the sample solutions after 10 min, which are same conditions as those in Figures 3b and 4b, respectively, and (3) 10 μM was added to the sample solution after 40 min as a reference The normalized intensity of each point from 0 to 40 min is calculated from the spectra of five samples with standard deviation when the minimum value is set at 0 and the maximum value at 1.

the broadness of the peaks or determine the glutamate pH accurately. To obtain more detailed information regarding the internal pH, it is necessary to investigate the uptake or reuptake of glutamate into the synaptic vesicles. Bafilomycin A1, a V-ATPases inhibitor, has been used to control the uptake or reuptake of glutamate into synaptic vesicles;[31,36] however, the background peaks of lipids and proteins fluctuated slightly with the addition of bafilomycin A1 in our experiment, which made it difficult to isolate the glutamate peaks.

The time-dependent relative Raman peak intensities are shown in Figure 5. The integrated peak intensities of the CH_2 deformation mode at 1344 cm^{-1} and the COO^- stretching mode of the $NH_3^+COO^-$ group at 1616 cm^{-1} were calculated using spectroscopic analysis software (Grams/32, Thermo Galactic). Each of the five samples was measured under three conditions, namely, the addition of 4AP after 10 min under the same conditions as those in Figures 3b and 4a, respectively, and the addition of 4AP after 50 min (final concentration of 10 μM), as a reference experiment without 4AP from 0 to 40 min. The intensities of the two peaks of single trapped synaptosomes without exposure to 4AP (control) remained very stable during laser trapping for 40 min. The addition of 4AP led to a rapid decrease in the peak intensities at a 10 μM concentration, but the decrease was slow with a concentration of 4 μM. The release rate observed for lower 4AP concentrations is slightly slower than in other reports,[10−12] which is presumably related to the slow diffusion rate of 4AP in the glass cell. The difference between the reduction speeds of the peak intensities of the CH_2 deformation mode and the COO^- stretching mode at lower 4AP concentrations is probably related to the pH of glutamate in the synaptosome, which indicates that some glutamate is more acidic during depolarization-induced release. This phenomenon is consistent with the model in which the pH of the glutamate in the vesicles can be less than 5.5 because the isoelectric point of glutamate is much lower than that of other neurotransmitters.[37,38] We estimated the amount of released glutamate by using the peak intensities of the CH_2 deformation modes, which are at 1344 cm^{-1} in synaptosomes and 1349 cm^{-1} in bulk solution with a pH of 5.5, respectively. The volume of the detection area of our confocal Raman system is ~1.6 fL when the laser spot is 1 μm in diameter and 2 μm high. The calculated average concentration of glutamate in the detection area of our Raman system is $\sim 2.0 \times 10^{-3}$ mol·dm^{-3}, which corresponds to ~3.2 amol. Therefore, ~3.2 amol of glutamate is released from a single laser-trapped synaptosome by 4AP.

In the paper, we showed that the LTRS system has sufficient sensitivity for us to observe the release of glutamate in single synaptosomes; however, the sensitivity is insufficient for identifying other neurotransmitters such as γ-aminobutyrate and glycine because of their low concentrations. Therefore, the sensitivity of the LTRS system must be improved if we are to study the mechanism of memory and learning at the single-synapse level.

CONCLUSION

LTRS was used to observe neurotransmitters in single nerve terminals (synaptosomes). The results show the ability of the laser trapping Raman spectroscopic technique to trap a single synaptosome without causing damage and to allow the observation of amino acid neurotransmitter release without the use of a staining process for the first time. By subtracting the spectrum before the addition of 4-aminopyridine, K^+-channel blocker, from that after its addition, we were able to eliminate the large peaks of lipids and proteins and detect a glutamate transmitter. The glutamate was released with the addition of 4AP at a concentration of 4−10 μM. The estimated amount of glutamate released from a single synaptosome was ~3 amol. Further developments in the sensitivity of the LTRS system may provide Raman spectra with higher signal-to-noise ratios and enable us to understand the mechanism of neurotransmitter release from single synapses in greater detail. Moreover, we believe that the analysis of the Raman bands of membrane proteins will reveal the uptake, storage, and exocytosis of neurotransmitters in the synapse at the molecular level.

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