MALC, a novel microinjection method for loading of macromolecules into cultured neurons

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Microinjection is one of the most useful and important methods in cellular neurobiology. However, direct insertion and retraction of a capillary produce physical stresses inside cells that make it difficult to apply on small and fragile cells, especially the central nervous system (CNS) neurons. In this study, we developed a novel method called MALC (microinjection method assisted with laser activated chromophore) to avoid this disadvantage of the conventional method. In MALC, a capillary is just attached on cell surface, and combination of a newly developed photosensitizer with a laser pointing system makes the plasma membrane permeable to molecules. This novel method can expand the application of the microinjection method and realize molecular manipulations inside CNS neurons. *NeuroReport* 13:1263–1266 © 2002 Lippincott Williams & Wilkins.

Key words: BAT; Ca^{2+} indicator; chromophore; laser; MALC; microinjection

INTRODUCTION

Microinjection is a convenient and useful method for introducing molecules such as dyes, DNAs, RNAs and antibodies, into cells and monitoring their intracellular functions [1]. However, some disadvantages in the method have narrowed the range of its application. In the conventional microinjection procedure, a capillary is directly inserted into the cell body, then pressure is applied to inject molecules, and the capillary is retracted from the plasma membrane. This exposes the cell to strong physical stress during the process, and cells with small size and fragile structure, such as CNS neurons, this procedure reduces cells, making it difficult to use this method.

To overcome this disadvantage, a novel microinjection method using a femtosyringe was developed several years ago [2]. This method used a syringe with a small diameter ($\sim$0.1 μm) tip to decrease the physical stress during its maneuver. The authors succeeded in injecting a dye and DNA into small cells or organelles. However, making the femtosyringe needed special skills and devices, and the amount of injected molecules was limited by the small diameter of the tip.

Recently we proposed a novel approach to improve the efficiency of microinjection of dyes into cultured cells [3]. In this procedure, a newly developed photosensitizer was used to make the plasma membrane permeable for molecules. The newly developed photosensitizer, BAT (5,5′-bis(aminomethyl)-2,2′:5′,2′-terthiophene dihydrochloride), is a derivative of α-terthienyl (αT), which is a photo-toxic oligothiophene trimer of plant origin [4]. αT and its derivatives in excited state generate reactive oxygen species, i.e. singlet oxygen and superoxide anion radical, which attack phospholipids and lipoproteins to make the membrane permeable for ions and other molecules [5,6]. However, the low water solubility of αT (0.42 μM) limits its usefulness. Recently BAT was developed to expand its applications with high solubility in water (0.35 mM) [7]. Application of the photosensitizer improved efficiency of injection of lucifer yellow into PC12 cells [3].

Based on this observation, we developed a novel microinjection method called MALC to avoid the disadvantage of the conventional method. In this method, the photosensitizer was combined together with a laser pointing devise to reduce physical stress and cytotoxicity of the compound for the injected cells. The small diameter of the laser light ($\sim$0.5 μm) at the capillary tip and short time of the exposure ($<$ 10 pulses) minimized the side effects on injected molecules and intracellular structures.

MATERIALS AND METHODS

BAT: BAT was synthesized as described previously [7]. In brief, the amino group of 2-aminomethylthiophene was protected by treatment with 1,1,4,4-tetramethyl-1,4-dichlorodisilethylene to obtain 2-[(2,2,5,5-tetramethyl-1-aza-2,5-disila-1-cyclopentyl)methyl]thiophene. After treatment with

butyl-lithium, Grignard coupling of the thiophene rings was carried out on the compound to give the intermediate, 5,5′-bis[(2,2,5,5-tetramethyl-1-aza-2,5-disila-1-cyclopentyl)-methyl]-2,2′-terthiophene. After removing the protecting groups, we obtained 5,5′-bis(aminomethyl)-2,2′:5′,2′-terthiophene dihydrochloride (BAT).

Tissue culture of hippocampal neurons: Hippocampal neurons were prepared from E19 (embryonic day 19) rats (Wistar/ST). After dissection, cells were plated on surface of glass bottom dishes (MS coated; Matsunami Glass Co. Ltd., Japan). These cells were cultured in neuro-basal medium with B27 supplement (Gibco-BRL; Life Technologies Inc., MD) and L-glutamine (0.5 mM). At 7 days in culture (DIV 7), they were used for the experiments.

The microscope-laser pointing system: An inverted microscope system, Olympus IX-70 (Olympus Optical Co., Ltd., Japan), was used for all microinjection experiments. Laser pulses (4 ns, 2 Hz, 370 nm) were emitted from a nitrogen laser (VSL-337ND-S; Laser Science, Inc., MA) coupled with a DUO series dye laser (Laser Science, Inc.). After passing through a beam-expander and ND filters (ND25 × 2), the laser pulses were introduced into the center of the objective (UplanApo 40 ×; Olympus Optical Co., Ltd) with a dichroic mirror (400 nm). At the tip of the capillary, estimated power and diameter of the laser pulse were 0.2 μJ/pulse and 0.53 μm, respectively. A combination of filters (excitation 520–550 nm and emission 580 nm) was used for monitoring of Alexa568 dye (Molecular Probes, Inc., OR) infusion.

Microinjection procedure: Capillaries (Femtotips I; Eppendorf-Netheler-Hinz GmbH, Germany) were filled with injecting solution (KCl 140 mM, NaCl 8 mM, HEPES (pH 7.3) 10 mM, $MgCl_2$ 0.5 mM, BAT 0.1 mM and Alexa568 0.5 mM), and handled by a motor-driven micromanipulator (InjectMan; Eppendorf-Netheler-Hinz GmbH) to access the upper side of a cultured cell. After attaching of the capillary on the cell surface, air pressure (20 hPa) was applied inside the capillary with a Transjector 5246 (Eppendorf-Netheler-Hinz GmbH) for 15 s. Simultaneously the tip of the capillary was exposed to the laser pulses (2 Hz, 370 μm). The laser pulses were cut off from the light-path with an electric shutter immediately after starting of Alexa568 infusion into the cytoplasm monitored under the fluorescent microscope. The entire operation was finished in one minute and the capillary was detached from the cell surface. In some experiments, injected solution included these molecules: GFP mRNA (67 μg/ml; synthesized by T7 RNA polymerase *in vitro*), Alexa488 conjugated anti-mouse IgG antibody (0.2 mg/ml; Molecular Probes, Inc.) or Calcium Green-1 (0.387 μM; Molecular Probes, Inc.).

Intracellular Ca^{2+} measurement: Three days after injection, the neurons loaded with Alexa568 and Calcium Green-1 were perfused with artificial cerebrospinal fluid (ACSF (pH 7.4): NaCl 125 mM, KCl 5 mM, $CaCl_2$ 2.4 mM, $MgCl_2$ 1.2 mM, KH_2PO_4 5 mM, $NaHCO_3$ 25 mM, D-glucose 11 mM) bubbling with O_2 95% and CO_2 5%. The images (100 ms exposure) were taken every 0.4 s for 2 min with a cooled CCD camera (PXL37; Photometrix, Inc., AZ) on an inverted fluorescent microscope (Axiovert S100; Carl Zeiss Co. Ltd., Germany). The filters used in the experiment: for Alexia568, excitation 500–570 nm, emission 515–565 nm, and for Calcium Green-1, excitation 450–490 nm, emission 555–625 nm.

RESULTS AND DISCUSSION

Using MALC, we injected Alexa568, a fluorescent dye, into cultured hippocampal pyramidal neurons. The capillary filled with Alexa568 (0.5 mM) and BAT (0.1 mM) solution was attached on the cell surface with weak force enough to seal the edge of the tip preventing leaks of the dye solution.

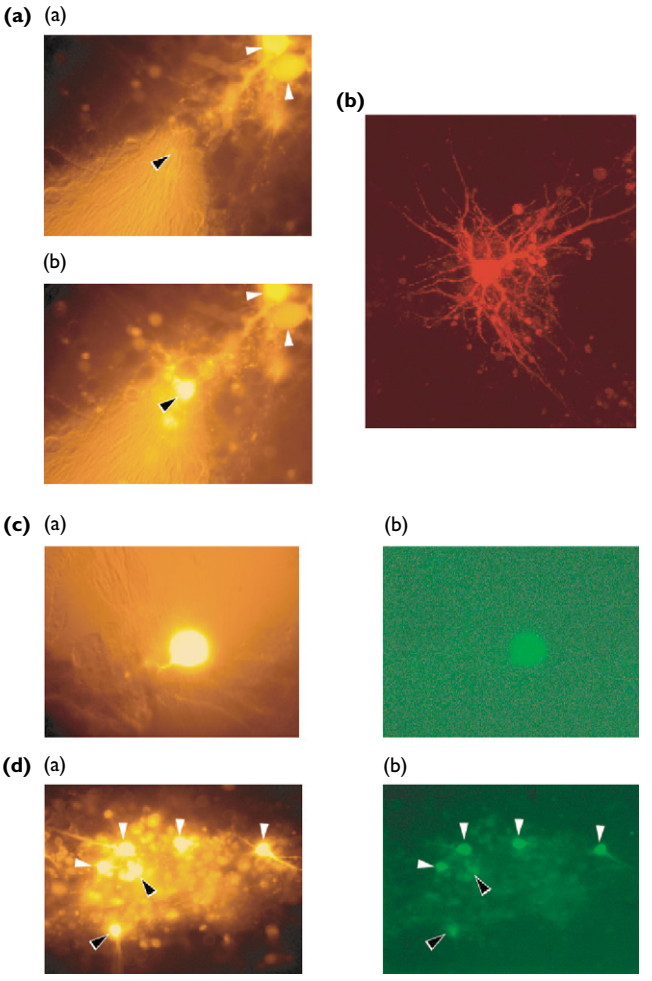

Fig. 1. Loading of Alexa568 dye and macromolecules (antibody and mRNA) into the CNS neurons. (**a**) Loading of Alexa568 dye into the cultured neuron (black arrowhead) without (a) or with (b) exposure to the laser pulses. Previously injected cells are shown in the field (white arrowheads). (**b**) Staining of soma and dendrites was observed in the hippocampal pyramidal cell three days after injection. (**c**) Injection of Alexa488-conjugated antibodies. Loading of Alexa568 dye (a) and the antibody (b) was observed in the cell soma. (**d**) Injection of GFP mRNA and expression of GFP proteins. Six cells in the field were injected with Alexa568 dye (a) and GFP mRNA. One day after the injection expression of GFP proteins was observed in all of the cells (b), however, intensity of the signal was various in each cell (range of relative intensity 0.21–1.0). Four showed strong expression (white arrowheads), while two had only weak expression (black arrowheads).

Application of air pressure alone (20 hPa, 15 s) inside the capillary did not inject the dye into the cell (Fig. 1a,a). After short exposure to the laser light (0.2 μJ/pulse, < 10 pulses), however, the dye went into the cell and staining of the cell soma and dendrites was detected under the fluorescent microscope (Fig. 1a,b). After stopping exposure to the laser light, plasma membrane was quickly resealed in 1 min to prevent leakage of intracellular materials. Fine structure of the injected neuron was observed three days after the injection (Fig. 1b). The injected cells did not change their morphology for ≥ 1 week after the injection. Without BAT the dye could not be injected into cells by the same procedure (data not shown). The injection succeeded in almost 100% of the cells, and we injected 10–20 cells with a single capillary sequentially in the same field. With this injection condition, the intensity of dye fluorescence was mostly homogeneous among injected cells. Increasing of air pressure inside of the capillary enhanced the intensity of fluorescence inside the injected cells. However, this strong condition might be harmful for the cells, because changes in the cell shape were frequently observed after injection with increased pressure.

In the next experiments, macromolecules, i.e. DNA, mRNA and antibody, were injected into neurons with MALC. Alexa488-conjugated anti-mouse IgG antibody (0.2 mg/ml) was injected into neurons (Fig. 1c). The antibody readily entered into the cell body and Alexa488 signals were detected in the soma. However, the signal was hardly detected in processes including dendrites, suggesting that most of the injected antibodies stayed inside the cell body (Fig. 1c,b). When a plasmid DNA coding the green fluorescent protein (GFP) gene was injected into cells, it was difficult to detect GFP signals inside the cells including soma and dendrites (data not shown). Using this method, most of the injected DNA might be kept in the cytoplasm and not being transcribed in the cell nucleus. On the other hand, injection of the GFP mRNA (67 μg/ml) produced expression of GFP proteins inside the cells (Fig. 1d). Although the intensity of GFP signals varied in each cell, most of the injected neurons expressed GFP proteins (Fig. 1d,b). The variation of expression intensity (range of relative intensity 0.21–1.0) might be caused by the difference in the cell condition.

Monitoring of cellular activity, such as changes of membrane potentials or intracellular calcium concentration, by indicator dyes is one of the important technologies in cellular neurobiology. Usually, to record activity of the particular cell, membrane-impermeable dye is directly injected into the cell by microinjection. In this type of experiment, capillary is left inserting into the cell body during the recording to prevent leakage of intracellular materials or cellular damages by incomplete resealing of the plasma membrane after retraction of the capillary. Therefore, such recording is restricted to short-term experiments. To avoid this restriction, indicator dyes may be injected into the cells with the MALC method that may enable long-term changes of cellular activities be monitored. To examine this possibility, Calcium Green-1 (0.387 μM), a membrane-impermeable calcium indicator dye was injected into the hippocampal neurons with the MALC method. Three days

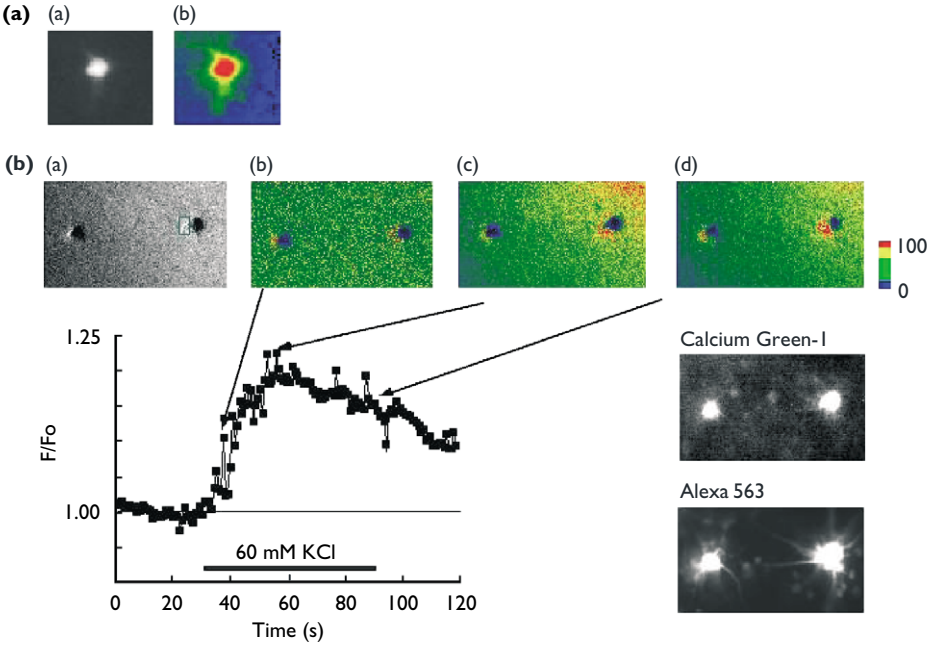

Fig. 2. Intracellular Ca^{2+} measurement with injection of Calcium Green-1 indicator dye. (**a**) Increase of Ca^{2+} concentration was observed in soma of the first cell. (a) Fluorescence of Calcium Green-1 at the peak. (b) The increment of Calcium Green-1 signal is shown by subtraction of the basal from the peak signals. (**b**) The time course of Ca^{2+} increase in dendrite of the second cell. (a) Ca^{2+} increase is shown by subtraction of the average value at the basal points from the peak value. The rectangle shows region of interest (ROI) for the observation. (b–d) Subtraction images at each time point. The time course of Ca^{2+} increase in the ROI normalized to the average of the basal level (F/F_0) is shown in the graph. The horizontal bar indicates time of application of high K^+ solution (60 mM KCl). The right figures show the initial images of Calcium Green-1 and Alexa568 fluorescence, respectively.

after injection, changes in the intracellular calcium concentration were observed with a fluorescent microscope (Fig. 2). Application of higher potassium concentration in extracellular solution increased fluorescent intensity of Calcium Green-1 in the soma and dendrite of some neurons, corresponding to increase in calcium concentration inside cells (Fig. 2a). In other neurons, the increase of fluorescent intensity was observed only in their dendrites (Fig. 2b). The fluorescence began to increase 5 s after starting application of high potassium solution (60 mM) (Fig. 2b,b), then peaked at 20 s (c), and decreased gradually (d). The peak level showed 20% increase over the basal level. The time course of the increment was well correlated to the application of high potassium solution, suggesting that it is a normal response of the neurons to the stimulation. These results showed that cellular activities could be monitored for a long-term range with injection of indicator dyes by MALC.

CONCLUSION

In this study, we developed MALC, a novel microinjection method without direct insertion of the capillary into cells. The conventional microinjection method is a useful and important procedure in cell biology but frequently damages the cells during manipulation of a capillary. MALC overcomes problems of the conventional method using a different strategy to access inside of cells, and expands the range of application of the microinjection method. As we showed in this study, we can monitor activities of intact cells for a long period combining together with extrinsic gene expression, and also apply antibodies locally inside cells to interfere with molecular functions using this method.

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