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Photodynamic Cell Membrane Perforation for Intracellular Electrodes *

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Abstract— **We report a new aspect of rapid (<10 seconds) light-induced cell membrane perforation for intracellular electrode by Hematoporphyrin (HP), which is an original photosensitizer for photodynamic therapy concept. A microelectrode insertion process to cell could be improved by pinpoint use of the photosensitizing effect to degenerate cell membranes prior to insertion. According to the concept, simple electrode coating with photosensitizer will enable intracellular electrode array for neural engineering.**

I. Introduction

Electrodes for neural recording or stimulation are fundamental tools for biology [1][2], and are drawing much attention because of the growing interest in brain science and artificial sensory organ. Integrated extracelluler electrode is one of the most common devices for such purposes, but the performance are low – the sensitivity of monitoring action potentials is smaller than a hundred microvolts and few hundreds millivolts is required for the stimulation, and it gives low spatial resolution for the monitoring and stimulation. Intracellular electrode could be an ideal but conventional one is a glass capillary electrode, which is hard to integrate. Although current micro fabrication techniques are progressing, but simple micro electrode by such techniques could not be inserted into a cell. This problem comes from the fluidity and flexibility of the membrane. Although conventional mechanical microinjection processes could enable insertion of the capillary tip into a cell by shear force, this process exposes the cell to strong –nearly mortal– stress, or detaches from the culture dish by cell -capillary absorption, so physical[3] and electrical[4] cell membrane perforation methods which could be suited for integrated electrodes are in progress.

In contrast, we found that the local use of a photosensitizer [5] resulted in the recovery of treated cells within a few minutes even after the cells were damaged to the point that the electrical resistance of the cell membrane was reduced by half [6]; on the basis of this finding, we have attempted to apply the phenomenon to an intracellular sensor or method for intracellular delivery of molecules of interest. After collecting corroborative lines of evidence that the mechanism of this transient cell damage is mediated by photooxidation reactions on the lipid cell membrane [7], we applied the perforation to the microinjection method; as a result, we could obtain a cell viability of approximately 90% 3 days after the injection [8]. The outside diameter of the capillary was 1 μm and the supposed perforation size was at the submicron level. In addition, we also found that the injection of a functional dye, an antibody, and mRNA to rat neurons resulted in almost 100% survival as well as the successful manifestation of the functions of these injected molecules [9]. Recently, we have expanded the processing cell number with needle array like cell membrane perforator [10][11] and this structure will be applicable for integrated intracellular electrodes (Fig.1).

Under these background circumstances, we report on the photodynamic cell membrane perforation process for the intracellular electrode. We coated Hematoporphyrin dihydrochloride (HP) as a photosensitizer [12], on the tip of micro grass capillaries. HP is the original material for the photodynamic therapy concept and we employed HP as the following reasons: (1) the adequate excitation wavelength of HP as the photosensitizer is ca. λ=500nm and it is relative safer for the cell application than that of α-therthienyl and TiO_2, which were applied in our previous research and these materials require UV irradiation for inducing photosensitizing

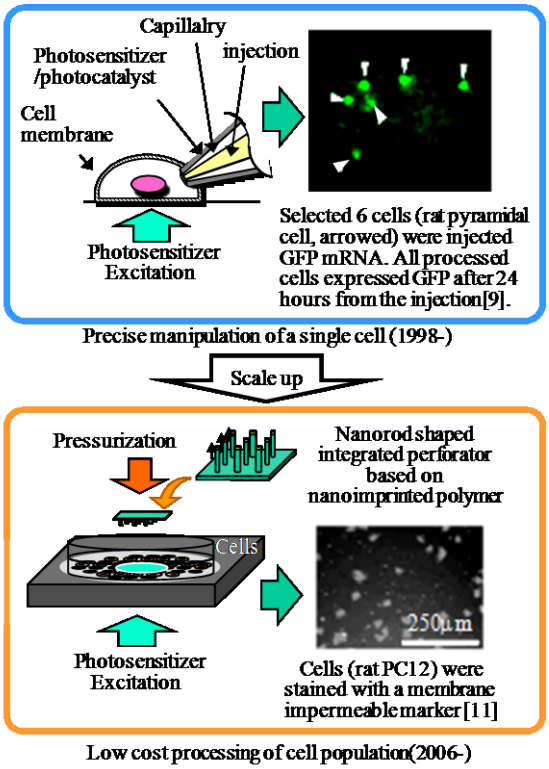

Figure 1. Former efforts on high performance cell membrane perforation

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effect; (2) Current pharmaceutical photosensitisers are designed to be hydrophilic but HP is hydrophobic, therefore HP coating on the cell membrane perforator is easy; (3) HP is a intermediate metabolite of heme synthesis for various species including human being, and is suited for future medical applications.

The evaluation methods of the capillary were microinjection and cell membrane potential recording. The sample cell line was rat pheochromocytoma PC12[13]. PC12 is a standard cell line for engineering trials and we have applied the membrane perforation concept to PC12 in our previous studies. Although PC12 hardly shows neuron cell like action potentials in general, but in this research we employed PC12 as target cell to compare the results to our previous researches. HP-coated capillaries were easy to apply because the perforation was carried out simply by making such tools come in contact with the target cells followed by exposure of both the cells and tools to the excitation light.

II. METHODOLOGY

A. Rat pheochromocytoma cell

PC12 was obtained from the Bio Resource Center of the Physics and Chemistry Research Institute (RIKEN, Tsukuba, Japan) and was cultured as described elsewhere [7]. The NeuroBasal™ (NB, Invitrogen) experimental medium preparation was as follows: 7.35 mg/L L-glutamic acid and 2 mM L-glutamine were added to the NB medium, which consisted of 5% B27 supplement (Invitrogen). The cell was cultured on the collagen I coated Φ35mm plastic dish (AGC).

B. Instruments

The common instruments for the microinjection and electrophysiology were follows: upright fluorescence microscope (BX51WI, Olympus); motorized microinjection system (NI and Femtojet, Eppendorf) . The light source for the HP excitation was blue light (λ=460-496nm) from 100W-mercury lamp of the microscope, which was transmitted with the blue excitation filter (U-MWIB3, Olympus). Each process was recorded by a digital camera (SP350, Olympus). During the both microinjection and electrophysiological experiment, 95% air - 5%CO_2 mixed gas was supplied at 210ml/min on the surface of NB medium in the dish, to keep the medium at neutral pH. The cell was kept at room temperature, ca. 20°C.

C. Microinjection

The HP coated injection capillary was prepared as follows: (1) The tip of ready-made capillary (Sterile Femtotips, Eppndorf) was immersed HP- methanol 16mM solution at 42mm from the capillary connecting end; (2) During the immersion process, pressurized air at 3kgf/cm^2 was supplied inside the capillary, to keep the HP solution outside of the capillary; (3) After the immersion, the capillary was stood vertically as tip-top and was dried up.

We evaluated the perforation as follows: (1) Lucifer Yellow CH (LY) (LY lithium salt for microinjection, Invitrogen) was injected to the cell. LY is a low toxic fluorescence dye that is used for microinjection[14] and could be a sensitive marker of a membrane penetration; (2) The injection pressure for cell searching was adjusted to the degree that LY was released from the capillary tip slightly; (3) The blue light from 100W-mercury lamp was used for both HP and LY excitation. The Z axis limit alignment of the capillary was adjusted accurately as possible at the same height of the cell nucleus on the dish; (4) The injection parameters were as follows: capillary speed: 3 μm/s; injection pressure: 150 hPa; injection time: 1s. The approaching speed is slow, hence the cell membrane is hardly penetrated by simple physical shear force by the tip. Under the condition, the perforation ratio in various conditions with/without photosensitizing effects can be compared.

D. Electrophysiology

The cell membrane voltage was measured with microelectrode amplifier (Axon Multiclamp 700B, Molecular Devices). The voltage recording software was programmed on LabVIEW 2011 (National Instruments) and recorded by a PC with an A/D converter, on differential input mode (NI USB-6212, National Instruments). From the measured voltage signals, we subtracted the power-line hum and non-neural frequency components: 47- 53 Hz and over 320Hz with software filters. The raw voltages from two input channel, differential-filtered voltage, and peak frequency data were analyzed and displayed simultaneously.

The experimental setup is shown in Fig.2. Glass capillary electrode was made from glass pipes (B100-75-10, Sutter) by a pipette puller (P-97IVF, Sutter). The capillary making parameters of the puller were adjusted to form the similar shape as Femtotips: HEAT: 550(RAMP); PULL: 100; VEL: 70; TIME: 200. The HP coating process of the capillary was the same as that for the microinjection. Finally, the glass capillary was filled with 3M potassium chloride (KCl) and prepared as Ag/AgCl wire inserted glass micro electrode with the preamplifier unit of the amplifier. We also prepared a Ag/AgCl wire as the counter electrode and immersed in the medium during the experiment.

We set the amplifier to the current clamp mode, to keep the current measured by the electrodes as zero for the cell membrane voltage measurement. The electrode operation protocol was the same as that for the microinjection.

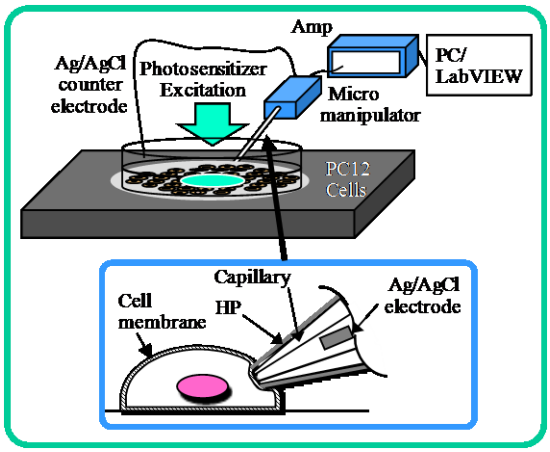

Figure 2. Instruments setup (electrophysiology)

III. RESULTS and DISCUSSION

A. Microinjection

With the HP coated capillary under the excitation light, the ratio of capillary insertion to the target cell was succeeded at 100% (n = 6, 6cells were succeeded), but the injection ratio was 50% (n = 6, 3cells were succeeded). The typical example of the successive injection is shown in Fig.3. This injection ratio reduction was caused by the choke of the capillary tip, we confirmed the choke by the LY releasing condition from the tip. The capillary retreat processes from the cell surface were smooth and no tight attachments with the cell and capillary were observed. With non HP coated capillary under the excitation light, both capillary insertion and injection ratios to the target cell were succeeded at about 16% (n = 12, 2cells were succeeded). Even the two case of successive injection, the capillary tip was tightly attached on the membrane, and could not be applied for another cell.

B. Electrophysiology

Under the condition of the blue light irradiation with the HP coated capillary, we could measure some voltage drop during the capillary tip approach to the cell. A typical example is shown in Fig.4. At 8sec, the capillary was started the approaching to the cell. A temporal voltage pulse was observed simultaneously at the movement, it is an artifact signal by the movement. After the capillary contact at 20 sec, the slant decay of voltage was observed and it is typical for chain oxidative cell membrane damage by photodynamic effect, which was measured by patch clamp method [6]. This dropped voltage would reveal the cell membrane voltage. The recorded cell membrane voltage was ca. -30-40mV and was relative depolarized than normal, it would caused by bad seal condition of the electrode or thermosensitiveness of the cell [15]. The noise signals and frequency analysis plots simultaneously recorded are shown in Fig. 5(a)(b) and Fig.6(a)(b). The both noise level was adjusted as the average

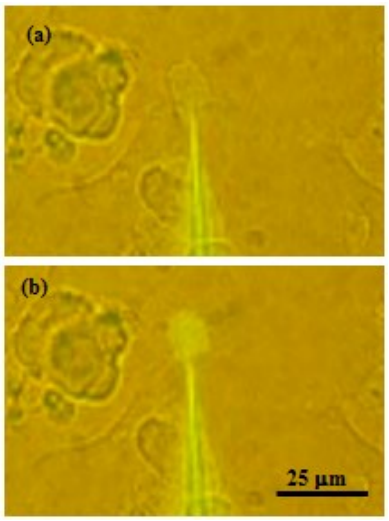

Figure 3. Typical example of the photodynamic microinjection
(a) before and (b) after the injection
with HP coated capillary, under excitation light.

The capillary was operated with motorized micromanipulator under the limited speed (< 3 µm/s), to prevent the membrane from the perforation by pure physical shear force. The cell shows yellow fluorescence of Lucifer Yellow dye after the photodynamic injection.

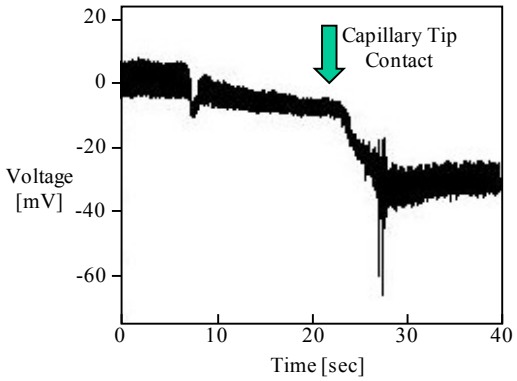

Figure 4. Voltage recording through the perforation process

Temporal voltage pulse at 8sec is an artifact signal with approaching start of the electrode. Slant decay of the voltage is typical signal for the chain oxidative cell membrane damage [6]. Whole process was performed under the light irradiation for the coated HP excitation to induce photodynamic reaction to the cell membrane.

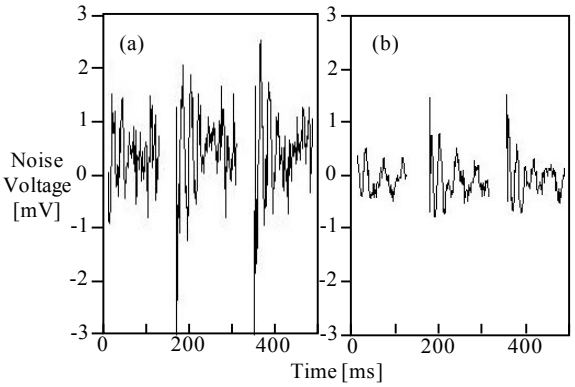

Figure 5. Noise signal of extra and intracellular voltage recordings
(a) extracellular voltage recording
(b) intracellular voltage recording

Data transfer protocol of the developed software has a problem and cyclic data lost was observed.

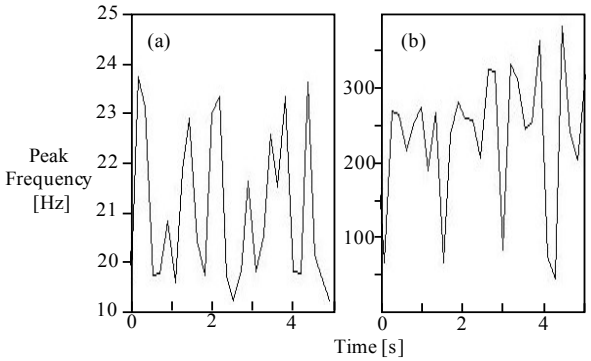

Figure 6. Peak frequency of extra and intracellular voltage recordings
(a) extracellular voltage recording
(b) intracellular voltage recording

voltages at ca. 0V. The noise level and the peak frequency were reduced in supposed intracellular recording state (b), these may reveal that the membrane resistance component were added to the electrophysiological circuit. These voltage drops were observed only 1-3 min, and returned to extracellular state gradually (data not shown).

In our previous report about the patch clamp recording and the photodynamic cell membrane damage[6], firstly the whole cell patch clamp recording was established by the breaking the patched membrane by suction. Later, the membrane perforation was achieved by the photooxidiation. Even after the various material introduction researches [8-11], the matter – photodynamic cell membrane perforation could be alternate method for establishing an electrical channel in the membrane or not - was still unclear, because it required the membrane perforation and sequential membrane-electrode sealing.

Our result in this research shows that pinpoint photooxidation could be compatible for the membrane break process by the suction of the patch clamp recording. We could not perform stable electrical sealing with the membrane and electrode and the sealing condition prevented us from continuous voltage measurement. Hence the cell membrane oxidation is a chain reaction, the membrane damage goes excessively even that we stopped the irradiation immediately after the voltage drop observation. We should add the membrane seal resistance measurement function on the neural recording software, and analyze qualitatively the seal condition.

We evaluated the new electrode concept but the structure is just based upon a conventional micro glass capitally. Like a unique shaped electrode for a pinpoint electroporation[16], we should modify the shape of electrode suited to the photodynamic cell membrane perforation process.

IV. Conclusion

We demonstrated a photodynamic cell membrane perforation process could be applied for intracellular electrode, with very simple “coating”-“irradiate”-“attach” processes. The result would provide a method which could lead to an improved technique for cell membrane penetration for e.g. multi-channel intracellular electrodes for nerve recording/stimulation.

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