35th Annual International Conference of the IEEE EMBS
Osaka, Japan, 3 - 7 July, 2013

Interfacing neurons through the patch membrane pierced with single-walled carbon nanotubes*

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Abstract—**The usability of single-walled carbon nanotubes (CNTs) as electrically conductive channels across the cell membrane was examined at the submicroscopic level. By using the patch-clamp technique, we found the surface-modified single-walled CNTs dispersed in the micropipette solution can provide an electrical access to the intracellular space across the patch of cell membrane in dissociated mammalian neurons, thereby enabling us to measure the membrane excitabilities in the 'pierced-patch' whole-cell mode.**

I. Introduction

Biological applications of carbon nanotubes (CNTs) have been investigated in recent years. Particularly, the possibilities of the electrical interface with neural tissues have been demonstrated in physiological experiments [1][2][3]. Because of the high electron conduction and charge transfer of CNTs, neuronal spiking could be extracellularly monitored and/or elicited with using CNT-based electrodes, e.g. a film of randomly oriented CNTs [4], an island of vertically aligned CNTs [5], or a CNT-coated metal electrode [6]. Moreover, since CNTs are known to exhibit the strong van der Waals interaction with each other, a bundle of CNTs can be formed with parallel alignment of the individual nanotubes [7], to be used as an intracellular probe for single-cell experimentation [8]. Indeed, we were able to fabricate the needle-shaped CNT bundles with the lengths ranging from a few microns to millimeters and the tip diameters of less than a micron [9]. Moreover, such a CNT bundle with an electrical insulation except for the tip was usable as a membrane-penetrating intracellular electrode for stimulating a mammalian neuron to induce action potential firings [10]. Also, other studies have suggested that the intracellular access across the cell membrane can be made by a single multi-walled CNT in non-neuronal cells [11][12]. These findings suggested that the CNTs as an assemblage are useful for extra/intra-cellularly interfacing neurons.

As a different usability of CNTs, it has been reported that the neurites of cultured neurons might be electrically short-circuited via the CNT film in which some of individual CNTs spontaneously penetrate the cell membrane [13]. Previous simulation studies suggested that individual CNTs dispersed in physiological saline can spontaneously enter into, and align across, the lipid bilayer [14][15], so that various chemical molecules might be able to permeate the cell membrane via the CNTs. However, there has been little direct evidence whether CNTs internalized in the lipid bilayer can function as electrically conductive channels in native neurons. Therefore, in the present study, we examine this possibility by utilizing the patch-clamp technique [16] in acutely dissociated mammalian neurons. Our experimental data show, for the first time, that single-walled CNTs dispersed in the intra-pipette solution provide an electrical conductance across the patch of cell membrane.

* Resrach supported partly by KAKENHI, Grant-in-Aid for Young Scientists (B), 22700472, from Japan Society for the Promotion of Science.

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II. Methods

A. Dispersed-CNT solution

We tested single-walled CNTs and those functionalized with either OH or COOH (1-2 nm in outer diameter, 0.5-2 microns in length), although any significant difference among those could not be detected in the present experiments. To prepare a dispersed-CNT solution [17], first, either type of the CNTs and surfactant, Pluronic F127, were mixed in dimethyl-sulfoxide (DMSO) at 10-20 and 25-50 mg/ml, respectively, and this mixture was vortexed and sonicated for 10-30 min each. Then, this was added at 0.4-0.8 % (v/v) to "pipette" solution consisted of (in mM): 110 K-$_D$-gluconic acid, 15 KCl, 15 NaOH, 2.6 $MgCl_2$, 0.34 $CaCl_2$, 1 EGTA, 10 HEPES (pH adjusted to 7.2 with methanesulfonic acid), which was further vortexed, sonicated for 30-60 min each, and centrifuged for 30 min. The supernatant of this solution was extracted and used as a dispersed-CNT pipette solution for the electrophysiological experiments described below. The degree of the dispersion of CNTs in this solution was quantified by the transmittance of light (645 nm, LED) through the solution in a dispenser; the transmittance ratio was in a range of 60-90 % in the present experiments.

For comparison, we prepared also two other pipette solutions: 1) CNT-free solution in which no CNTs, but Pluronic F127 and DMSO were contained; 2) antibiotic-containing solution in which amphotericin B as a perforating agent was contained, together with Pluronic F127 and DMSO [18]. The amounts of Pluronic F127 and DMSO in those three solutions before the centrifugation were the same.

B. Cell preparation

All animal care and experimental procedures in this study were approved by the committee for animal researches of Kumamoto University, and performed in accordance with the guidelines from The Physiological Society of Japan.

Neurons were acutely dissociated from the ganglion-cell layer of rat retinas by the non-enzymatic method we previously proposed [19]. In brief, the retinas without the inner limiting membrane were isolated from a rat (Wistar, postnatal 7-25 days), and the retinal piece was transferred to a cell culture dish, flattened with the ganglion-cell layer facing up, and then held with a tissue holder. The inter-cellular connections were weakened by incubating the retinal piece in low-Ca^{2+} solution consisted of (in mM); 140 sucrose, 2.5 KCl, 70 CsOH, 20 NaOH, 1 NaH_2PO_4, 15 $CaCl_2$, 20 EDTA, 11 $_D$-glucose, 15 HEPES (pH 7.2; 290-300 mOsmol/kg; estimated free Ca^{2+} concentration 100-200 nM) for 3-5 minutes [18]. Then, the surface of the retinal piece was scanned with the fire-fused tip (a few microns in diameter) of a glass micropipette vibrating horizontally (amplitude of 0.2-0.5 mm, frequency of 50-100 Hz) for 2-5 minutes, so that the cells were dissociated from the surface of the ganglion-cell layer. After removing the tissue holder and remaining retinal piece, the culture dish was filled with bath solution consisted of (in mM); 140 NaCl, 3.5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 10 $_D$-glucose, 5 HEPES (pH 7.3; 280-290 mOsmol/kg), left untouched for 15-40 min, rinsed and refilled with a fresh aliquot of the bath solution supplemented with 1 mg/ml bovine serum albumin, and then kept on a vibration-isolation table for >2 hours at room temperature before measurements. The ganglion cells were identified by combining two or three criteria based on the soma size [20], the rising slope of action potentials as well as voltage-gated Na^+ current density [21][22], and retrograde labeling with dextran-conjugated Alexa Fluor 488 [23].

C. Experimental setup

The bath in the recording chamber (i.e. the cell culture dish) was grounded via an agar bridge (150 mM NaCl in 2 % (w/v) agar) with an Ag/AgCl pellet. Micropipettes were fabricated from thick-walled borosilicate glass capillaries (0.9 and 1.5 mm in inner and outer diameters, respectively), to the tip resistance of 6-9 MΩ in saline (approximately one micron in inner diameter, Fig. 1 lower inset), by using a micropipette puller (P-97, Sutter instrument, CA; or PC-10, Narishige, Tokyo). The tip of the micropipette was filled with the supplement-free pipette solution and the shank was backfilled with either one of the above-described three solutions (i.e. dispersed-CNT, CNT-free, and antibiotic-containing solutions). The solution in the micropipette was connected to the signal line of the amplifier via an Ag/AgCl wire, for use as the patch electrode. Voltage- and current-clamp recordings were made using a patch-clamp amplifier (EPC9 or EPC10, Heka Elektronik, Lambrecht) controlled by computer software (Pulse ver.8.65-8.8, Heka Elektronik, Lambrecht). The output signals from the amplifier were low-pass filtered by the two or one built-in analog filters (3-pole Bessel with 10 or 30 kHz cutoff followed by 4-pole Bessel with 2-5 kHz cutoff) and digitally sampled (5-50 kHz). To reduce noise, the recorded signals were digitally filtered off-line (non-lagging Gaussian with 0.2 kHz cutoff) in some instances.

D. Electrophysiological measurements

As in the conventional patch clamp technique, a giga-ohm seal between the electrode tip and the cell membrane of a target retinal neuron was formed in voltage-clamp mode. After the baseline current at a holding voltage (−70 mV) was stabilized, a biphasic negative-first voltage pulse (5-mV amplitude, and 10-ms duration, per phase) was applied every five seconds and the resulting current was recorded. Fig. 1 illustrates a general model of the electrical circuit for the cell-attached/whole-cell configuration of the patch-clamp technique. When the electrical impedance across the patch membrane is approximated as a pure resistance, the time course of the current ('$I(t)$') in response to a voltage step (amplitude of 'E') can be described as follows:

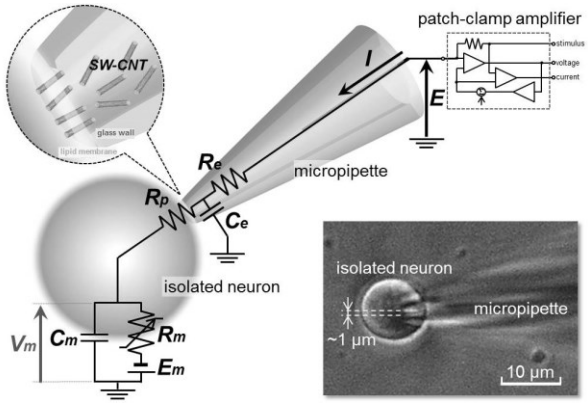

Figure 1. An electrical circuit model for the cell-attached/whole-cell configuration of the patch-clamp technique [16][25]. Upper left inset, an illustration of the electrode-cell interface in which single-walled CNTs are internalized in the lipid membrane. Lower right inset, a phase-contrast micrograph of the dissociated retinal neuron with the micropipette attached.

$$I(t) = E/R_e * \exp\{-t/(C_e R_e)\} + E/(R_e+R_p) * \exp\{-t/(C_m R_e + C_m R_p)\} + E/(R_e+R_p+R_m(V_m,t)) , \quad (1)$$

where R_e is the electrode tip resistance, C_e is the electrode capacitance, R_p is resistance across the patch membrane, C_m is the membrane capacitance, and R_m is the membrane resistance which is a function of the membrane potential V_m and time t. In the right of this equation, the first and second terms indicate capacitive transients with fast and slow time constants, respectively. Thus, the series conductance, $G_{series}=1/(R_e+R_p)$ can be estimated from the peak amplitude of the slow capacitive transients, as similar to the case of the perforated-patch configuration [24][25]

When the estimated values of the series resistance, $R_{series}=R_e+R_p$ reached a plateau, the membrane potential recording was attempted in "fast" current-clamp mode [26]. The series resistance and the electrode capacitance were compensated by a built-in circuitry of the patch-clamp amplifier. The membrane potentials reported here have been corrected for the liquid junction potential between the bath and pipette solutions (approx. −3 mV as measured). All recordings were made in the cells within 24 hours after dissociation, at room temperature (22-27 ^oC), to compare with results of previous studies [19][20][21].

III. Results

A. Electrical conduction across the cell membrane

By the patch-clamp technique, we first examined the electrical conduction across the patch of cell membrane with

using the dispersed-CNT solution in the micropipette. Fig. 2 superimposes two traces of the current (lower) in response to the voltage pulse (upper), recorded at the immediate time point (*a*) and at five minutes (*b*) after the seal was stabilized in the cell-attached configuration (see METHODS). In the beginning of recordings (trace *a*), a fast component of the capacitive transients at the onsets of voltage steps was apparent, and then, a relatively slow component of the capacitive transients became significant over time, as seen in the trace *b*. By subtracting the trace *a* from the other current traces recorded at later time points, the slow capacitive transients were extracted, as shown in Fig. 3 inset. The peak amplitude of those extracted transients was increased with time. Fig. 3 plots the amount of change in the series conductance (G_{series}, see METHODS) against the time of recording. The series conductance increased within several minutes, and then remained at the similar level, with a small drift, as long as the recordings were held. Among different trials/cells, although the series conductance was increased with different time course and often in step-wise fashion, the estimated value of the series conductance reached a plateau within a few to ten minutes. In some instances, when the degree of the CNT dispersion in the pipette solution was relatively high (~60 % in the light transmittance ratio, see METHODS), the patch membrane was spontaneously ruptured and the cells died afterwards. Excepting those cases, the estimated series resistance at the plateau was 60.5±14.2 MΩ (mean±SEM, n=17 cells).

Consistent with previous studies [18][19][25], a slow component of the capacitive transient was observed also when the antibiotic-containing solution was used in micropipettes (data not shown, n=6). In contrast, a slow capacitive transient was hardly detected with using micropipettes filled with the CNT-free solution (Fig. 3, open rhombuses; n=3). Since the ionic composition and the amounts of supplements (i.e. Pluronic F127 and DMSO) in the three pipette solutions were the same, the increase of the series conductance shown in Fig. 3 was unequivocally attributed to effects of the dispersed CNTs on the lipid bilayer in the patch membrane.

B. Whole-cell recording with the dispersed-CNT solution

Having found that the electrical conduction across the patch membrane is attained by the CNTs in the micropipette, we next tested if intracellular recordings can be made from a dissociated neuron patched with the same pipette solution. Fig. 4 superimposes five traces of the voltage in response to constant current pulses of different amplitudes (–20 to +60 pA with 20-pA increment). These recordings were obtained at 10 (*i*), 30 (*ii*), and 50 (*iii*) minutes after the estimated series resistance reached a plateau of approximately 65 MΩ. After the recordings in *iii*, the series resistance was estimated to be slightly higher, ~70 MΩ, although the voltage traces were little changed. As found in the Fig., the voltage changes recorded here were indeed the membrane potential responses to the current pulses in this neuron, and the recordings were stable over time. The membrane potential was hyperpolarized from –70 to –105 mV by the negative current injection, and was depolarized to fire action potentials, peaking around +25 mV from the threshold of –50 to –45 mV, in response to the positive current injections. In this cell, the single action potentials were evoked during the current pulses, and this was consistent with the previous recordings from a certain type of juvenile rat retinal ganglion cells in the perforated-patch whole-cell configuration, as shown in Fig. 4 inset [19].

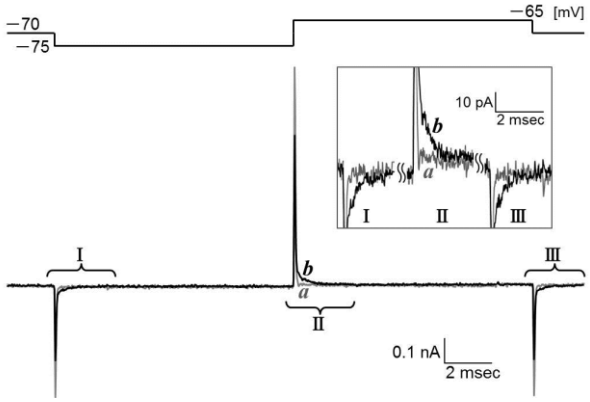

Figure 2. Capacitive transient currents in response voltage steps. Two traces recorded at the immediate time point (*a*, gray) and five minutes (*b*, black) after the seal was stabilized in the cell-attached configuration were superimposed. Inset, the current traces near the onsets of voltage steps (I, II, III), in an expanded scale.

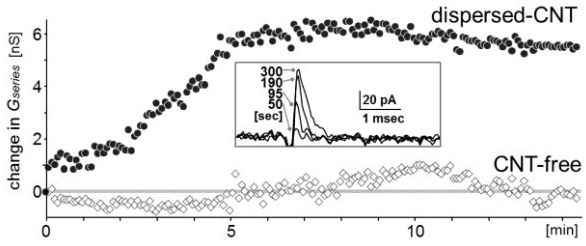

Figure 3. Change in the series conductance over time. The micropipette was filled with the dispersed-CNT solution (filled circles) or CNT-free solution (open rhombuses). Inset, the extracted traces showing a slow component of the capacitive transient near the onset of the voltage step from −75 to −65 mV, recorded at different time points.

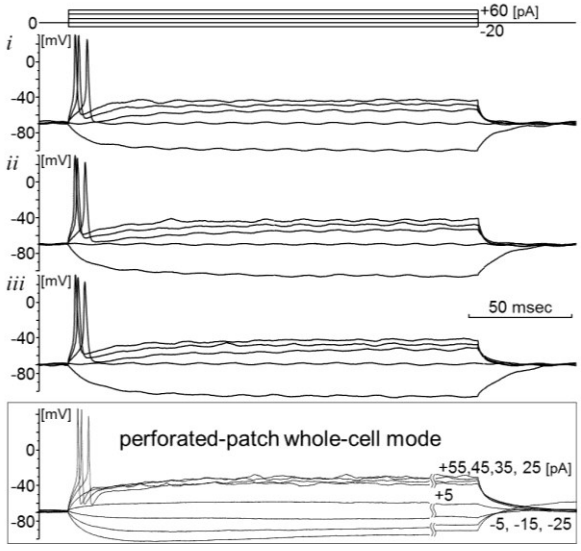

Figure 4. Membrane potential responses to constant current pulses in a retinal ganglion cell patched with the dispersed-CNT pipette solution. The traces in *i*, *ii* and *iii* show the recordings at 10, 30 and 50 minutes, respectively, after the estimated series resistance reached a plateau. Inset, an example of normal recording in the perforated-patch whole-cell mode from a different retinal ganglion cell.

IV. CONCLUSIONS

We demonstrated, for the first time to our knowledge, that the single-walled CNTs dispersed in the micropipette solution spontaneously form electroconductive paths/channels across the lipid bilayer at the membrane patch in native mammalian neurons. Moreover, taking advantage of the patch-clamp technique, we were able to estimate the electrical conductance gained by the "CNT-channels" to be around 20 nS/μm^2, which was enough for providing an intracellular electrical access to stimulate/record the neuronal activity. This implies a potential usage of CNTs as artificial inorganic ionic channels formed on the cell membrane [27] with their various inner diameters and abilities of being functionalized on their surfaces.

We can assume that the patch membrane conductance was increased as the number of the CNT insertion in the lipid bilayer is increased [13], as similar to the situation of the perforated-patch mode [24][25]. We refer the new whole-cell configuration presented here to as "pierced-patch" mode.

Although we were able to obtain the stable electrical conduction across the patch membrane in many cells, it should be noted that the patch membrane was abruptly ruptured in some other cells. This could be due, for instance, to re-aggregation of CNTs in the lipid bilayer, or to relatively long CNTs entering into the cytoplasm. The former possibility, however, seemed less likely since the degree of CNT dispersion (see METHODS) unchanged for, at least, two months after preparation. Further study remains to more precisely control the state of CNTs inside the lipid bilayer as well as to optimize the functionalization [14].

ACKNOWLEDGMENT

The authors are grateful to Prof. Nobuki Murayama of Kumamoto University for invaluable comments on the present experiments, and also to Prof. Norio Akaike of Kumamoto Health Science University for providing us some of the experimental equipment used in this study.

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