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Original Article

Scanning and Transmission Electron Microscopic Observation of Changes in Cylindrical Cytoplasmic Processes of Isolated Single Merkel Cell

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Abstract

The aim of the present study was to determine the reason isolated single Merkel cells do not respond to mechanical stimulation by fluorescent or histological techniques. Cells identified as Merkel cells by quinacrine fluorescence and measurement of intracellular calcium concentration were observed by transmission electron and scanning electron microscopy. Observations elucidated that the cylindrical cytoplasmic processes of single Merkel cells disappeared with time shortly after isolation. Transmission electron microscopy revealed the presence of numerous dense-cored granules, which may function as sensory receptors in the cytoplasm of the isolated single Merkel cell. Disappearance of the cylindrical cytoplasmic processes impeded reception of mechanical stimulation. The results suggest that an isolated single Merkel cell continues to function as a sensory receptor cell due to the presence of numerous dense-cored granules. Furthermore, the results show that an isolated single Merkel cell is not an appropriate specimen for investigation of mechanically-gated channels.

Key words: Merkel cell—Mechanoreceptor—Cylindrical cytoplasmic process—Scanning electron microscopy—Touch dome

Introduction

Merkel cells in the epidermis of vertebrates are believed to function as mechanoreceptors, making synaptic contacts with nerve fibers to form Merkel cell-neurite complexes[8,13,15]. Previous electrophysiological studies have reported that Merkel cell-neurite complexes are slowly adapting type I mechanoreceptors[2,10,20,24]. It has also been proposed that Merkel cells are

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mechano-electric transducers[9]. According to this hypothesis, mechanical stimulation causes bending of the cylindrical cytoplasmic processes of Merkel cells. Mechanically-gated channels located in the basal area of these cylindrical cytoplasmic processes might be opened as a result of mechanical stimulation, allowing extracellular calcium ions to flow into the cells through these channels. The calcium flowing into the cell subsequently produces a signal, causing a release of transmitters within the dense-cored granules, resulting in generation of action potential on a connected nerve fiber. There is some evidence to support this hypothesis: the presence of voltage dependent Ca^{2+} channels[4,29]; chemical dependent channels[4]; and stretch activated ion channels[23,25] in Merkel cells. This suggests, therefore, that Merkel cells are not only mechanoreceptors, but also mechano-electric transducers. Morphological studies using transmission electron microscopy (TEM), have revealed that there is close contact between Merkel cells and nerve endings, and the presence of numerous dense-cored granules in the cell cytoplasm, as well as numerous cylindrical cytoplasmic processes[21]. Moreover, the morphology of Merkel cells has been observed by immunostaining[28] and the morphology of the Merkel cell and its cylindrical cytoplasmic processes by scanning electron microscopy (SEM)[22,30]. Some of these earlier studies support the hypothesis that these cylindrical cytoplasmic processes function as mechanoreceptors[20,27,30]. However, it was not possible to elicit mechanically-gated currents in Merkel cells in response to vibratory stimulation of Merkel cell-neurite complexes[7] or mechanical stimulation by means of perfusion with Krebs solution[16]. Therefore, it has been suggested that Merkel cells are not actually sensory receptor cells[7,16].

The aims of this study were to 1) determine why an isolated Merkel cell does not respond to mechanical stimulation by perfusion with Krebs solution, and 2) demonstrate that, from a morphological point of view, the function of an isolated single Merkel cell as a sensory receptor cell is sustained due to the presence of dense-cored granules in its cytoplasm.

Materials and Methods

Cheek pouches from 30 male golden hamsters aged 3–5 weeks were used. As specimens, touch domes were isolated from the cheek pouch mucosa under sodium pentobarbitone anesthesia (50 mg/kg *i.p.*, Dainihonseiyaku, Japan). All animals were handled in strict accordance with the Guidelines for the Treatment of Experimental Animals of Tokyo Dental College.

1. Isolation and identification of single Merkel cells

Quinacrine dihydrochloride was used to identify Merkel cells[3,5,14,19]. Quinacrine dihydrochloride (10–15 mg/kg, Sigma Chemical Co., MO, U.S.A.) was administered intraperitoneally 12–24 hr prior to commencement of experiments. To facilitate separation of epithelium from lamina propria, excised mucosa was treated with Krebs solution containing DL-Dithiothreitol (2 mg/ml, Sigma Chemical Co.) at 31°C for 10 min. The epithelium was then treated with a Ca^{2+}-free Krebs solution containing collagenase (0.25 mg/ml, type I, Sigma Chemical Co.) and trypsin (0.1 mg/ml, Sigma Chemical Co.) at 31°C for 8 min. Epithelial cells were transferred to a tissue culture dish with normal Krebs solution containing (mM): NaCl, 136; $CaCl_2$, 2.5; KCl, 5; $MgCl_2$, 0.5; $NaHCO_3$, 12; $C_6H_{12}O_6$, 11; $NaH_2PO_4 \cdot 2H_2O$, 1.2; pH 7.4. The cells were incubated in darkness at 37°C for 30 min. Quinacrine fluorescent cells were identified as Merkel cells under a fluorescent microscope (Multiple Microfluorescence Analyzer Attofluor System)[1,4,20,25,26,29,30]. Positions of isolated single Merkel cells were marked on the tissue culture dish.

2. Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

Merkel cells were incubated in fura 2-AM (10 μM, Dojindo, Japan) at 37°C for 45 min.

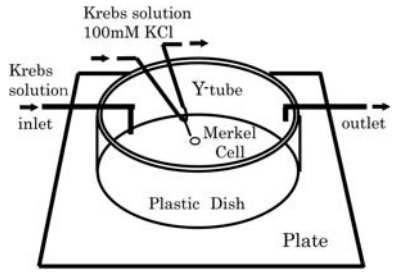

Fig. 1 Y-tube method
Krebs solution flowed from inlet to outlet (3 ml/min). Krebs or 100 mM KCl stimulation solution was applied to single Merkel cell using Y-tube (1 ml/min).

The dual-excitation ratio of fluorescence intensity (500 nm) induced by 344- and 380-nm excitation wavelengths from the Merkel cells was calculated using the Zeiss Attofluor Ratio Vision[6]. For the experiments, the cells were maintained in a culture dish continually perfused with normal Krebs solution. To obtain mechanical stimulation at a high concentration of KCl (KCl, 100 mM; NaCl, 4.1 mM; CaCl, 2.5 mM; $MgCl_2$, 0.5 mM; $NaHCO_3$, 12 mM; $C_6H_{12}O_6$, 11 mM; $NaH_2PO_4 \cdot 2H_2O$, 1.2 mM), single Merkel cells were subjected to depolarized simulation with normal Krebs solution using a Y-tube (Fig. 1), which allows exchange of whole external solution within 10–20 msec[1,20,25,29]. The presence of voltage-dependent Ca^{2+} channels was confirmed in almost all the fluorescent cells.

3. Scanning electron microscopy

Single Merkel cells were maintained in the tissue culture dish at room temperature for 1, 2 or 5 hr after isolation. The position of each cell was established in the tissue culture dish. The Krebs solution contained in the tissue culture dish was then replaced with a buffer solution which included 1% glutaraldehyde and 1% formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). Epithelial cells and Merkel cells in the tissue culture dish were fixed at 4°C for 15 min. After fixation, they were dehydrated through a graded series of ethanol and dried by the t-butyl alcohol freeze-drying method[12]. They were then coated with Au-Pt and examined under SEM (JSM-6340F; JEOL, Japan).

4. Transmission electron microscopy

After measurement of $[Ca^{2+}]_i$ concentration, the cells were fixed in a solution of 1% glutaraldehyde and 1% formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) at 4°C for 15 min. Cells were then postfixed in 1% osmium tetroxide in the same buffer at 4°C for 2 hr, dehydrated through a graded ethanol series and embedded in Epon 812 (Nissin EM, Japan). After staining with uranyl acetate-lead citrate, they were examined under an electron microscope (H-7100, HITACHI, Japan).

Results

Epithelial cells on the plastic dish are shown in Fig. 2A–E. The quinacrine fluorescent cell indicated by an arrow in Fig. 2B is the same cell indicated in Fig. 2A, C and D. Numerous cylindrical cytoplasmic processes were observed on the cell surface cell (Fig. 2D). Numerous cylindrical cytoplasmic-like processes were also observed on the plastic dish (Fig. 2D, asterisks). The quinacrine non-fluorescent cell (Fig. 2B, arrowhead) is the same cell as that indicated by an arrowhead in Fig. 2A and C and Fig. 2E. The surface of the quinacrine non-fluorescent cell was rough and no cylindrical cytoplasmic processes were present. No cylindrical cytoplasmic-like processes were observed (Fig. 2E).

Application of 100 mM KCl (clear bar in Fig. 3A; for depolarizing stimulation) induced an increase in $[Ca^{2+}]_i$ in isolated single Merkel cells due to an increase in the fura 2 fluorescence. However, mechanical stimulation by perfusion with fluid (black bar in Fig. 3A; for mechanical stimulation) induced no increase in $[Ca^{2+}]_i$ in isolated single Merkel cells. After confirmation of presence of voltage-dependent Ca^{2+} channels, isolated single fluorescent cells were fixed and observed under SEM (Fig. 3B). All cell surfaces were smooth and numerous cylindri-

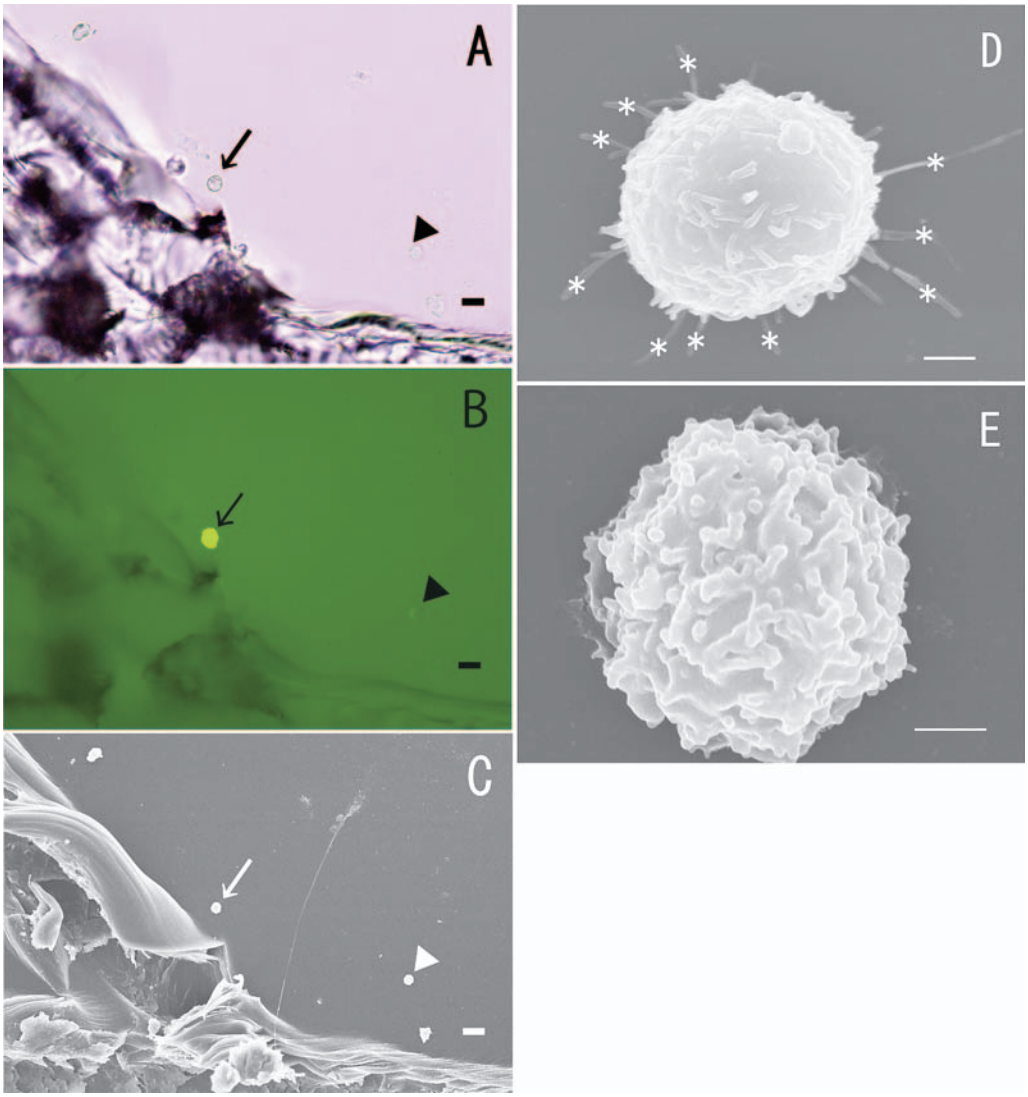

Fig. 2 Epithelial cells on plastic dish

(A) Light microscopic photograph of epithelial cells. (B) Fluorescent photograph of epithelial cells. (C) SEM photograph (low magnification) of epithelial cells. (D) SEM photograph (high magnification) of quinacrine fluorescent cells. Asterisk indicates cylindrical cytoplasmic-like process. (E) SEM photograph (high magnification) of non-quinacrine fluorescent cells. Cell indicated by arrow in A, B and C and D is same cell. Arrow in A, B and C indicates quinacrine fluorescent cell. Arrowhead in A, B and C indicates non-quinacrine fluorescent cell. Scale bar of A to C is 10 μm. Scale bar of D and E is 1 μm.

cal cytoplasmic-like processes were observed on the plastic dish.

The quinacrine fluorescent cell surface at each hour after single cell isolation was observed using SEM, and the results are shown in Fig. 4. Cylindrical cytoplasmic processes were observed on the upper side of the cell surface after 1 hr of single cell isolation (Fig. 4 A and B). The condition of the cylindrical cytoplasmic processes was better maintained at 4°C (Fig. 4A). One hour after single cell isolation at room temperature,

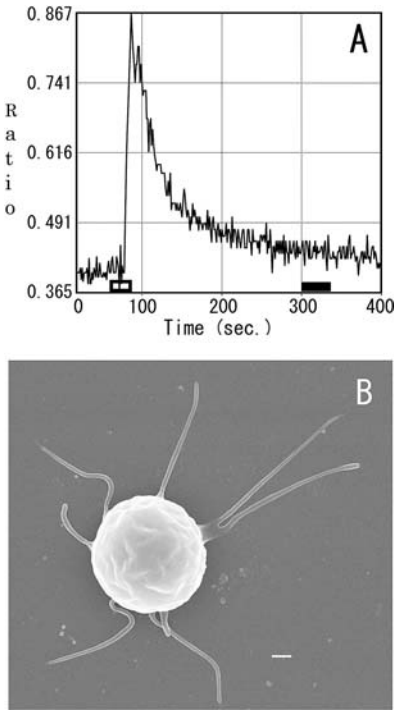

Fig. 3 Graph showing changes in fura2-AM 334/380 nm excitation ratio in isolated single Merkel cell and SEM

(A) Ca^{2+} increase at fura 2-AM 334/380 nm excitation ratio was induced by depolarizing Krebs solution (100 mM KCl; clear bar). Black bar indicates application of Krebs solution. (B) SEM view of Merkel cell after measurement of fura 2-AM 334/380 nm excitation ratio (A). Scale bar of B is 1 μm.

the cylindrical cytoplasmic processes on the upper side of the cell surface were bent or flush with the cell surface (Fig. 4B). Few cylindrical cytoplasmic processes were observed on the upper side of the cell surface after 2 hr of single cell isolation (Fig. 4C). Vestiges of the cylindrical cytoplasmic processes were also observed on the upper side of the cell surface. After 5 hr single cell isolation, the cell surface was smooth (Fig. 4D), showing a balloon like shape.

The mean ± standard error and minimum-maximum in number of cylindrical cytoplasmic processes after single cell isolation was as follows: after 1, 2 and 5 hr it was 3.8 ± 0.7 and 0–19 (n = 28), 2.6 ± 0.8 and 0–12 (n = 18) and 1.0 ± 0.4 and 0–6 (n = 18) per Merkel cell on the upper side of the cell surface, respectively. We did not count the cylindrical cytoplasmic-like processes on the plastic dish, short cylindrical cytoplasmic processes and/or vestiges of the cylindrical cytoplasmic processes. On the other hand, the number of balloon-like cells, which did not have cylindrical cytoplasmic processes on the cell surface, was as follows: after 1, 2 and 5 hr it was 1 cell (3.6%), 7 cells (38.9%) and 11 cells (61.1%), respectively. The diameter of the cylindrical cytoplasmic processes after single cell isolation is shown in Fig. 5. The mean ± standard error and minimum-maximum in diameter of the cylindrical cytoplasmic processes after single cell isolation was as follows: after 1, 2 and 5 hr it was 239.5 ± 3.8 nm and 160.7–356.3 nm (n = 98), 247.3 ± 5.0 nm and 207.3–353.7 nm (n = 40) and 262.2 ± 9.4 nm and 163.9–363.6 nm (n = 22), respectively.

Figure 6 shows the ultrastructure of an isolated single Merkel cell (M) in which an increase in $[Ca^{2+}]_i$ was induced by 100 mM KCl. The shape of this cell was spherical, and serial section revealed no cylindrical cytoplasmic processes. However, numerous dense-cored granules (arrows) and vacuoles were observed in the cytoplasm.

Discussion

Since it was not possible to record an intracellular ionic current after mechanical stimulation by means of perfusion with Krebs perfusion, Nurse and Cooper[16] concluded that Merkel cells were not mechanosensory transducers. Yamashita *et al.*[29] were also unable to elicit a mechanically-gated current in an isolated single Merkel cell under a whole cell patch clamp. They suggested that the application of small pressure pulses of perfusion fluid to an isolated single Merkel cell might be inadequate to bend its cylindrical cytoplasmic processes. Furthermore, in a similar preliminary experiment, we were unable to elicit any increase in $[Ca^{2+}]_i$. In the present study, we clarified the reason no increase in $[Ca^{2+}]_i$ occurred with stimulation using fluid perfu-

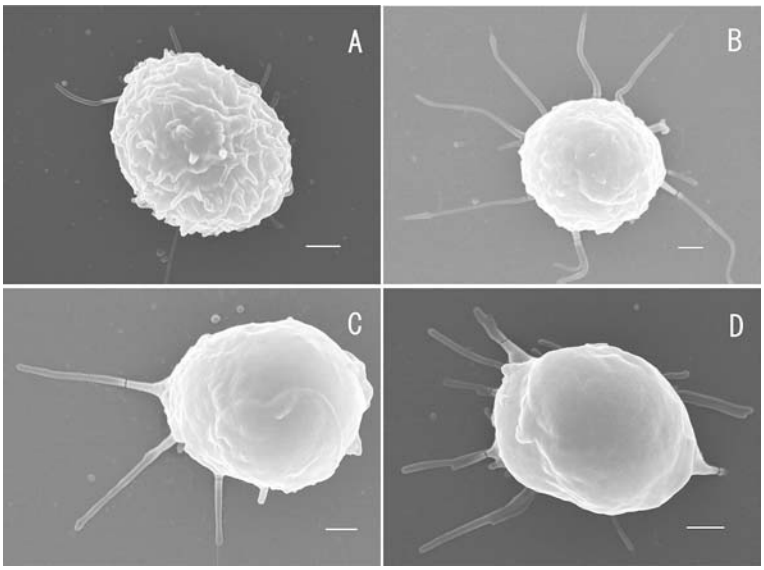

Fig. 4 SEM photograph of cell surface after single Merkel cell isolation
(A) 1 hr at 4°C after isolation. (B) 1 hr at room temperature after isolation. (C) 2 hr at room temperature after isolation. (D) 5 hr at room temperature after isolation. Scale bar of A to D is 1 μm.

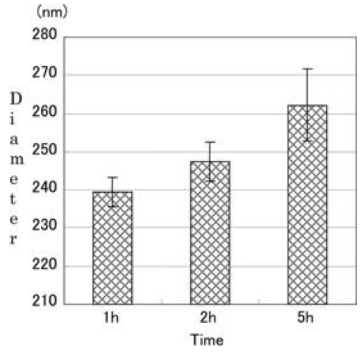

Fig. 5 Diameter of cylindrical cytoplasmic processes after single Merkel cell isolation

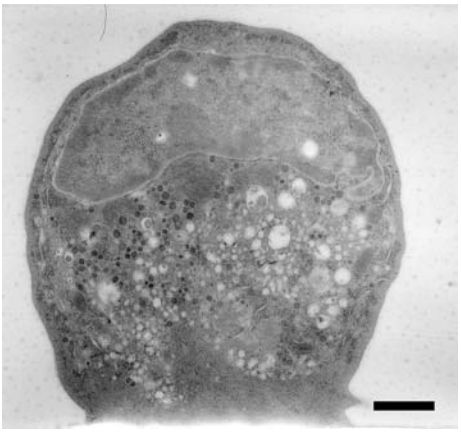

Fig. 6 TEM photographs of isolated single Merkel cell in hamster cheek pouch mucosa. Scale bar is 1 μm.

sion morphologically.

Quinacrine fluorescence is required to identify Merkel cells, as it does not cause any impairment of cellular function[5,17–19,29,30]. When an isolated single Merkel cell is used as a specimen, it is necessary to commence a functional experiment within 1 hr of cell isolation. In quinacrine fluorescence cells which had been stimulated by fluid perfusion, few cylindrical cytoplasmic processes were observed in surfaces where there was no increase in $[Ca^{2+}]_i$; and where they were

observed, most of them showed a balloon-like shape. Yamashita *et al.*[30] have reported that the cylindrical cytoplasmic processes were bent on the exposed side of the cell surface, but lay straight, or sometimes bifurcated, where in contact with a plastic dish. Distortion of the processes may possibly be ascribed to enzymatic and mechanical treatment during isolation. The results show that the morphology of an isolated Merkel cell changes with time. The cylindrical cytoplasmic processes on the exposed side of the cell surface are long and thin. After cell isolation, these processes begin to bend, finally lying flush with the cell membrane until disappearing altogether. As a consequence, the cell surface becomes smooth and spherical in appearance. In this study, the diameter of the cylindrical cytoplasmic processes remaining on the exposed side of the cell surface became thicker with time. A previous study reported that an increase in $[Ca^{2+}]_i$ occurred when a Merkel cell was directly stimulated with a thin glass stick[26]. The findings of the present study suggest that mechanical stimulation by means of fluid perfusion of an isolated single Merkel cell is inadequate to elicit an opening of mechanically-gated channels.

In this study, it was confirmed that the cylindrical cytoplasmic processes in the Merkel cell disappeared within a short period following isolation. In experiments involving mechanical stimulation of Merkel cells, an isolated Merkel cell might not be an appropriate specimen.

However, numerous dense-cored granules, which are involved in transduction[11], were also recognized in the spherical Merkel soma, in addition to the surface cylindrical cytoplasmic processes, suggesting that changes in soma shape do not lead to disappearance of cell function. In other words, it is possible that the dense-cored granules in Merkel cells are released by an appropriate extracellular stimulus.

Our results support the hypothesis that Merkel cells are mechano-electric transducers. Further study, however, is required to clarify various factors in regard to mechanically-gated channels and using a more appropriate method of stimulation to elucidate the role of mechanically-gated channels in isolated single Merkel cells.

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