Exocytosis of a Single Bovine Adrenal Chromaffin Cell: The Electrical and Morphological Studies

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Exocytosis of a single bovine adrenal chromaffin cell, triggered by histamine stimulation, was investigated via the electric responses detected with single-walled carbon-nanotube field-effect transistors (SWCNT-FET) and the morphological changes acquired by atomic force microscopy (AFM). Secretion of chromogranin A (CgA), stored in the vesicles of a single chromaffin cell, can be monitored in situ by the antibody against CgA (CgA-antibody) functionalized on the SWCNT-FET devices. The SWCNT-FET can further discriminate the amount of released CgA with different levels of histamine stimulations. The AFM morphological studies on a chromaffin cell indicate that the depression structures on the cell surface, caused by the histamine-evoked exocytotic fusion pores, appeared much more frequently than those without histamine stimulation or with the pretreatment of mepyramine before histamine stimulation. The vesicle diameters are about 50 nm calculated from the obtained three-dimensional AFM images. In comparison, the fusion pores of chromaffin cells stimulated by high-K⁺ buffer solution were also investigated to have a wider-ranging distribution of vesicle diameters of 60–260 nm. This work demonstrates that the combination of novel techniques, SWCNT-FET and AFM, can provide further insights into the fundamental properties of exocytosis in neuroendocrine cells.

1. Introduction

The development of nanotechnology offers novel tools to characterize the activities of single molecules and individual living cells with high spatial/temporal resolution. These tools allow researchers to obtain more detailed information than traditional approaches in life science research. Exocytosis is an important physiological process that a secretory vesicle (SV) undergoes trafficking, priming, docking, and fusion to the cell membrane.^{1,2} Thereafter, soluble proteins or bioactive molecules stored inside the vesicle are released to the extracellular environment for signal transmission. Biochemical studies have identified a set of proteins involved in this process; however, the kinetics for the formation of fusion pores is still obscure. To further characterize this mechanism, single-walled carbon-nanotube field-effect transistors (SWCNT-FET) and atomic force microscopy (AFM) have been applied in this study to investigate the exocytotic process in single chromaffin cells isolated from bovine adrenal gland.

CNT-FET^{3–5} and nanowire-based field-effect transistors (NW-FET)^{6–9} have been well developed for biosensory applications because of their selectivity, sensitivity, real-time, and label-

free detection capabilities. Binding of proteins to the corresponding binding receptors functionalized on the CNT-FET or NW-FET surface can cause prominent change in the FET conductance. Our previous study has demonstrated that the release of chromogranin A (CgA),¹⁰ a soluble protein stored in SV, from living neurons upon glutamate stimulation can be detected by SWCNT-FET modified with the antibody (Ab) against CgA (termed CgA-Ab/SWCNT-FET).⁵ The detection limit of the CgA peptides by CgA-Ab/SWCNT-FET can be as low as 1 nM. To further exploit the sensory applicabilities of the FET devices for biological research, we apply CgA-Ab/SWCNT-FET in this study to detect the release of CgA from cultured single bovine chromaffin cells.

In the study of cellular exocytosis, several biochemical methods have been widely used to investigate the exocytotic characteristics. For examples, electron microscopy provides high-resolution images about the vesicle fusion and recycling,¹¹ although the sample should be fixed in the examination. Amperometry,¹² which oxidizes the catecholamine (CA) released from single vesicles, reveals the kinetics of exocytosis, but little information about the fusion pore. Fluorescent proteins in combination with confocal microscopy¹³ or total internal reflection microscopy¹⁴ were applied recently to investigate the vesicle recycling process. However, because of the weak fluorescence signals, these fluorescent images are usually of low spatial and temporal resolutions. Although CNT-FET/NW-FET is a very sensitive biosensor with many merits in monitoring the proteins released from live cells, it can not be used to observe the fusion pore opening.

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Figure 1. Localization of CgA in a bovine adrenal chromaffin cell. The cell was fixed with formaldehyde and stained with (a) DAPI and (b) rhodamine-conjugated phalloidin to label the nucleus and F-Actin, respectively. (c) Distribution of CgA visualized with specific antibody. (d) Bright field image of the cell. The images were observed in a Leica epifluorescence microscope (100 $\times$, NA1.45, oil objective) equipped with a CCD camera. The scale bar indicates 5 μ m.

AFM, which has spatial resolution comparable to electron microscopy, can provide a true three-dimensional (3D) surface profile of a sample. Additionally, AFM has been improved to be able to monitor molecular interactions under normal physiological saline buffer and has been extensively applied to image many kinds of cells for morphological studies^{15–18} as well as electrophysiological¹⁹ and biochemical²⁰ investigations. With AFM imaging, Jena's group¹⁸ has demonstrated that there are lots of circular pits of 500–2000 nm in diameter, containing smaller depressions of 100–180 nm in diameter on the surface of pancreatic β -cells. Under electron microscopic investigations, the vesicle sizes of β -cells were revealed to range from 100 to 1000 nm. It has been reported that the depression-like structures of 120 nm in diameter were also found in living chromaffin cells.¹⁷ In this experiment, to further characterize the evoked exocytotic events in single bovine chromaffin cells under different stimulations, the surface of plasma membrane was scanned by AFM to identify the fusion pores formed by vesicles and plasma membrane.

2. Experimental Section

Cell Preparation. Chromaffin cells were prepared by digestion of bovine adrenal gland obtained from local slaughterhouses with collagenase (0.5 mg/mL) and purified by density gradient centrifugation, as previously described.²¹ In brief, the isolated cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% of fetal bovine serum and plated on poly-L-lysine-coated glass coverslips. Culture medium was replaced every other day. All experiments were carried out within 2–7 days after the cells were isolated.

Immunostaining. CgA has been shown to be stored in the SV close to the plasma membrane of a chromaffin cell.²² To confirm that CgA exists in the cells prepared in our laboratory, the distribution of CgA in a chromaffin cell was verified by antibody against CgA (Figure 1). As in the protocol described before,⁵ the chromaffin cells were immobilized by 3.7% formalin in phosphate buffer saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4 with NaOH) for 30 min and permeabilized with triton X-100 (0.5% in PBS) for another 15 min. Inside a cell, the nucleus was stained by DAPI (diamidino-2-phenylindole) (Sigma-Aldrich Co.) (Figure 1a) and the F-Actin under the plasmalemma was stained with rhodamine-phalloidin (Sigma-Aldrich Co.) (Figure 1b). To verify the distribution of CgA, cells were stained with goat anti-CgA antibody (Santa Cruz Biotechnology Inc., 1:200 dilution) and visualized by fluorescein isothiocyanate conjugated rabbit antigoat IgG (Sigma-Aldrich Co., 1:500 dilution) (Figure 1c). These results demonstrated that CgA is abundant in the cytosol, especially in a region close to the plasma membrane of a chromaffin cell.

Electrical Measurement. To measure cellular exocytosis by CgA-Ab/SWCNT-FET, a single bovine chromaffin cell was held by a micropipet with gentle suction and was moved to locate on the top of a CgA-Ab/SWCNT-FET device (Figure 2a). A real image acquired under this manipulation is shown in Figure 2b. The CgA-Ab/SWCNT-FET chip was placed on the stage of the upright microscope (Axioskop, Carl Zeiss, Inc.) alongside a micromanipulator (MO-103, Narishige Inc.). The microscope and micromanipulator were placed on an antivibration table and were covered with a Faraday cage to prevent electrical interfer-

Figure 2. In situ detection of the CgA released from a living chromaffin cell by CgA/SWCNT-FET. (a) Schematic illustration of using CgA/SWCNT-FET to monitor the CgA released from a single chromaffin cell in the histamine-evoked exocytosis. (b) Real image of a single chromaffin cell manipulated by a glass micropipet onto a CgA/SWCNT-FET device.

ence. A long-working-distance (7.9 mm) objective lens (LD PN 20 $\times$ /0.4 Corr, Carl Zeiss, Inc.) was used for ease of operation in the following experiments. In the experiment, a piece of coverslip (5 $\times$ 5 mm²) with some chromaffin cells cultured on it was put alongside the CgA/SWCNT-FET chip without touching the source-drain electrodes of the FET circuits. Chromaffin cells together with the CgA/SWCNT-FET were then bathed in 200 μ L normal bath buffer at room temperature (25 $^{\circ}$ C). The glass micropipets were pulled from thin-walled glass tubes (61700, A-M Systems, Inc.) using a pipet puller (P-97, Sutter Instrument Co.) and the tips of 1–2 μ m in diameter were fire-polished with a microforge (MF830, Narishige Inc.). The micropipet was backfilled with pipet buffer (130 mM K-aspartate, 20 mM KCl, 1 mM MgCl₂, 0.1 mM EGTA, 3 mM Na₂ATP, 0.1 mM Na₂GTP, and 20 mM HEPES, pH 7.3) and mounted on the holder attached to the headstage of EPC7 amplifier (HEKA GmbH). The whole system was controlled by pClamp 6 software (Axon Inc.) and the resistance was monitored every second by applying a 10 mV step potential command for 10 ms. The resistance of the micropipet ranges typically from 3 to 5 M Ω . The micropipet was moved to slightly touch a chromaffin cell and then sucked it gently until the resistance was over 1 G Ω . The chromaffin cell was lifted and moved onto a CgA/SWCNT-FET device (Figure 2), where the I_{sd} was recorded at V_{sd} = 10 mV by a lock-in amplifier (SR830, Stanford Research System Inc.) with modulation frequency of 17.77 Hz.

The manufacture of SWCNT-FET devices was carried out in a clean-room environment following the previous design.⁵ In brief, carbon nanotubes of 2 nm in diameter (Thomas Swan & Co. Ltd.) were electrically contacted with a double layer of 50 nm thick Cr and 50 nm thick Al leads, which were further passivated with a 10 nm thick Al₂O₃ layer to avoid electric leakage to the buffer solution. The Al₂O₃ layer was formed by blowing 600 Torr of oxygen gas into the thermal evaporation chamber for 10–15 min after the thermal evaporation of Al leads. The electric performances of the as-fabricated SWCNT-FET devices have been characterized by the source-drain current (I_{sd}) vs gate voltage (V_g) curves (not shown). All SWCNT-FET devices used in this work are p-type semiconductive. To functionalize CgA-Ab on the SWCNT-FET surface, the as-

fabricated SWCNT-FET devices were first treated in 7.5 mM 1-pyrenebutanoic acid succinimidyl ester for 1 h and then flushed with pure methanol for 15 min. In the surface functionalization, the solution containing 1-pyrenebutanoic acid succinimidyl ester was guided to flow through a microfluidic channel (6.25 $\times$ 0.5 $\times$ 0.05 mm³) to contact the SWCNT-FET devices driven by a syringe pump at 0.5–0.8 mL/h. Subsequently, the FET devices were incubated for 2 h in the PBS containing 0.2 μ g/mL of goat IgG antibody against CgA (Santa Cruz Biotechnology Inc.). After washing off extra CgA-Ab, CgA-Ab/SWCNT-FET were then incubated for 20 min in PBS containing 0.5% TWEEN-20, which serves as a blocking agent to prevent nonspecific bindings. The CgA-Ab/SWCNT-FET devices, finally washed with PBS buffer, were ready for the experiment of detecting cellular exocytosis.

Calcium Image. To measure cytosolic Ca²⁺ concentration ([Ca²⁺]_i), chromaffin cells were incubated in normal bath buffer containing 5 μ M fura-2 AM for 1 h at 37 $^{\circ}$ C. For the fura-2 excitation, a Lambda DG4 system (Sutter Instrument Co.) was used, which was controlled by Metafluor software (Molecular Devices Corp.). Ratiometric calcium estimates were made with the excitations at 340 and 380 nm by employing 10 nm wide filters centered at the wavelengths of interest (Chroma Technology Corp.). The emitted light at 485–540 nm was collected by a cooled CCD camera (CoolsnapFx, Roper Scientific Inc.) through a 20 $\times$ objective (Axiovert 200 microscope, Carl Zeiss, Inc.) at each excitation wavelength for 300 ms. The fluorescence ratio within each cell was computed every second from the images obtained at the excitation wavelengths of 340 and 380 nm by subtracting the appropriate background fluorescence at each wavelength.

AFM Imaging. AFM experiments were performed with the samples in PBS using the AFM equipment (Bioscope SZ, Digital Instruments, Inc.) combined with an inverted optical microscope (TE2000-U, Nikon Corp.). The whole system is placed on an antivibration table and housed in a sound-proof cage to eliminate vibration and acoustic noise, respectively. A patch ($\sim$ 1 $\times$ 1 μ m²) of plasma membrane was scanned by AFM in a tapping mode with 256 scan lines and 512 line samples at a scanning rate of 0.5 Hz, of which the resonant frequency was set at 17 kHz with the amplitude setpoint of 0.6 V, close to the target

Figure 3. (a)–(b) Electric responses of CgA/SWCNT-FET to the CgA released from a living single chromaffin cell with histamine stimulations. (a) Current–time plot of the in situ detection of CgA released from a single chromaffin cell when the cell was stimulated by 30 μM histamine. The inset depicts a control experiment without cell. Arrows indicate the addition of histamine. (b) Current–time plot of the concentration-dependent experiment with different histamine stimulations. (c)–(d) Changes in $[\text{Ca}^{2+}]_i$ represented by the fluorescence ratio of fura-2 with the 340 and 380 nm excitations. The traces were averaged from 10 cells. (c) Averaged $[\text{Ca}^{2+}]_i$ response stimulated with 30 μM histamine. (d) Averaged $[\text{Ca}^{2+}]_i$ responses with the same serial histamine stimulations as those in (b).

amplitude of 0.8 V. The integral and proportional gains of the scanning parameters were ~ 0.6 and ~ 1.2 , respectively. Silicon–nitride cantilevers with a normal force constant of 0.06 N/m (PNP-DB, NanoWorld) were mounted in the AFM fluid tip-holder and rinsed carefully with PBS before the tips were immersed into the cell samples in fluid.

Immunogold Labeling. Chromaffin cells were fixed by 3.7% formaldehyde in PBS for 30 min and then incubated in PBS containing 1% skim milk for 15 min. Rabbit IgG against the N-terminal of synaptotagmin I (SytI) (S2177, Sigma-Aldrich Co.) was diluted in PBS (1:200) to stain the exposed SytI for 1 h. After washing off extra IgG, cells were incubated in PBS containing 1% skim milk for another 15 min. PBS containing 30 nm gold particle tagged goat antirabbit IgG antibody (Ab27238, Abcam, 1:200 dilution) was used to label the stained SytI for 1 h. After extra antibodies were washed off, cells were then fixed by 3.7% formaldehyde in PBS for another 30 min for AFM imaging experiments.

3. Results and Discussion

Exocytotic Release Detected by FET. CgA has been shown to be costored and coreleased with CA in chromaffin cells²³ to control blood pressure.²⁴ It is well-known that Ca^{2+} is the most important messenger to evoke cellular exocytosis. At resting, $[\text{Ca}^{2+}]_i$ is at ~ 100 nM and can increase to be higher than 1 μM by different stimulations. The sources responsible for the elevation of $[\text{Ca}^{2+}]_i$ come from either intracellular Ca^{2+} stores or extracellular bath solution. In the exocytosis evoked with histamine, the activation of histaminergic H_1 G protein-coupled receptors on adrenal chromaffin cells will excite the phospho-

lipase C to release IP_3 (inositol 1,4,5-triphosphate) to the cytosol. The IP_3 then binds to a specific receptor on the endoplasmic reticulum, from which the stored Ca^{2+} is released into the cytosol to evoke exocytosis. This mechanism responsible for the histamine-evoked exocytosis has been widely discussed.^{25,26} Alternatively, opening the voltage-gated Ca^{2+} channels on the plasma membrane by action potential or by changing the K^+ gradient across the plasma membrane also allows the influx of Ca^{2+} into the cytosol to trigger exocytosis.²⁷

In the in situ detection of the CgA released from a single chromaffin cell upon histamine stimulation, the CgA-Ab/SWCNT-FET devices were covered with normal bath buffer (145 mM NaCl, 5 mM glucose, 10 mM Na-HEPES, 1 mM MgCl_2 , 5 mM KCl, and 2.2 mM CaCl_2 , pH 7.3 with NaOH), as schematically illustrated in Figure 2a. Figure 3a shows the electric response of CgA-Ab/SWCNT-FET in the cell experiment, where I_{sd} increased from 12.9 to 13.6 nA when 30 μM histamine was perfused into the chamber. This increase was not due to the nonspecific binding of histamine with CgA-Ab/SWCNT-FET, because histamine alone in the absence of chromaffin cell had no effect on the FET conductivity as demonstrated in the inset of Figure 3a. Although the immunostaining images shown in Figure 1c were not sufficient to demonstrate that CgA is stored in the SV, the increase in the conductivity of CgA-Ab/SWCNT-FET revealed that CgA was released from a stimulated chromaffin cell. These results indicate that the sensitivity of CgA-Ab/SWCNT-FET is high enough to detect the release of CgA from a single chromaffin cell. Furthermore, Figure 3b shows that the change of FET conductivity increases with the concentrations (18, 33, 46, 57, 66, and

Figure 4. (Upper) AFM image of the depression on the surface of a chromaffin cell under high- K^+ buffer stimulation. The chromaffin cell was fixed and stained with antibody against SytI, which was identified by AFM imaging with assistance of the labeling 30-nm gold particle tagged second antibody. Arrows indicate the gold nanoparticles, which help to locate a depression (in dark brown color). (Lower) Cross-section analysis, along the white line AB drawn in the upper AFM image, for the depression and a 30 nm gold particle. The width of the depression is 190 nm marked between two dash lines. The height (~ 30 nm) of a gold nanoparticle is pointed by an arrow.

75 μM) of added histamine, suggesting that our CgA-Ab/SWCNT-FET can discriminate the amount of CgA release depending on the strength of the stimulation.

Because of its low isoelectric point ($pI = 4.5-5$), CgA is negatively charged in normal bath buffer at pH 7.3. Therefore, the binding of CgA to CgA-Ab/SWCNT-FET should enhance the conductivity in the p-type semiconductive device due to an electrostatic gating effect. Furthermore, it has been reported that the amount of exocytotic CgA is proportional to the CA released from chromaffin cells.²⁸ Accordingly, the detected amount of CgA reflects that of CA release.²⁸ It was also shown in the dose response curve that the level of CA release, evoked with histamine, does not reach plateau even after 5 h stimulation, and the response is not saturated until 100 μM . In this study, we have tested the $[\text{Ca}^{2+}]_i$ responses in a chromaffin cell by fura-2 fluorescence ratio. In Figure 3c, the addition of 30 μM histamine to chromaffin cells quickly induced an elevation in $[\text{Ca}^{2+}]_i$, which then decreased slowly to a basal level. In comparison, Figure 3d shows the response of $[\text{Ca}^{2+}]_i$ to a series of histamine stimulations, where the addition of 18 μM histamine did not induce immediately a significant elevation in $[\text{Ca}^{2+}]_i$ but with a delay. Afterward, the $[\text{Ca}^{2+}]_i$ quickly reached a maximum and declined slowly but remained at a level higher than the basal no matter how high the concentration of histamine was further increased. These fura-2 fluorescence ratio experiments explain the FET results (Figure 3b) why there was no much CgA detected under 18 μM histamine stimulation; afterward, the $[\text{Ca}^{2+}]_i$ was kept at a high level to allow more

CgA release in the subsequent histamine stimulations. In view of the report that higher concentration of histamine can increase the rate of CA release,²⁹ we observed consistently the release of CgA in a faster rate to reach equilibrium under the continuous increases of histamine concentration (see the horizontal I_{sd} level is more quickly reached in the higher histamine concentrations as shown in Figure 3b), although the $[\text{Ca}^{2+}]_i$ was not significantly enhanced during the serial stimulations (Figure 3d). These results indicate that the CgA-Ab/SWCNT-FET can respond to different levels of CgA release.

Although the surface of nanotube is tiny and the interaction between CgA and CgA-Ab is strong, the FET device is not easily saturated as its conductance kept escalating when histamine concentration was increasingly added. On the basis of these tests, our system is well appropriate for detecting the release of proteins from a single excitable cell with high sensitivity. Furthermore, in the exocytosis of chromaffin cells with 100 μM histamine stimulation in 15 min,³⁰ it has been reported that 10% of CA stored in the cell can be released, which is equivalent to ~ 17 fmol.³¹ According to the study that the amount of CgA released is much less than CA,³² this hints that less than 1 fmol of CgA could be detected by our SWCNT-FET.

Fusion Pores Imaged by AFM. In the observation of possible fusion pores by AFM, the antibody against the N-terminal of SytI was chosen to label the formaldehyde-fixed chromaffin cells. SytI is a vesicle transmembrane protein with N-terminal in the lumen of the vesicle. When fused with

Figure 5. AFM images for fixed chromaffin cells with different stimulations. (a) Control cell without stimulation. (b) Cell stimulated with 30 μM histamine for 2 min and then fixed. (c) Cell pretreated with 10 μM mepyramine for 15 min and then stimulated with 10 μM histamine for 2 min. The scale bars are 100 nm. Shown on the right side are their corresponding 3D images.

plasma membrane, this N-terminal will be exposed to the extracellular surface and be recognized by this antibody. For ease of detection, the antibody against SytI was further conjugated with the second antibody tagged by 30 nm gold particle, which will later serve as a marker for the AFM imagings. To evoke cellular exocytosis, chromaffin cells were treated with high- K^+ buffer (54 mM) to open the voltage-gated Ca^{2+} channels and were subsequently labeled with the antibody against SytI. Figure 4 depicts a representative AFM image of a depression surrounded by three gold nanoparticles (marked by arrows). A cross-section analysis indicates that the width of this depression is ~ 190 nm and the gold nanoparticles are elevated by about 30–50 nm above the cellular surface. The gold nanoparticles are two-antibody away from the labeled SytI proteins; its location may not directly reflect the location of vesicle fusion but must be very close because the size of an antibody is no more than 10 nm. Furthermore, such elevations caused by immunogold could not be identified in the cells without high- K^+ stimulation (data not shown) and no such elevations accompanied the depression were found in scanned patches without immunogold labeling (Figures 5 and 6). These results suggest that the depression is where the fusion occurs.

To characterize how the fusion pores would be modulated by different secretagogues, chromaffin cells were first stimulated with histamine for 2 min and then fixed with 3.7% formaldehyde for AFM scanning (Figure 5). For a comparative study, Figure 5a shows the surface of a typical cell without stimulation, which is rough with small fluctuations. After histamine treatment, several deep depressions were frequently identified in each of the membrane patches scanned (Figure 5b). In contrast, the number of depressions was reduced by treating the cell with mepyramine, an H_1 receptor antagonist,³³ before histamine application (Figure 5c). These differences suggest that the surface depressions, stimulated with histamine, are due to the activation of H_1 G protein-coupled receptor pathway for the elevation of $[\text{Ca}^{2+}]_i$ to trigger exocytosis.

It is unlikely that such depressions were randomly produced. When the histogram of surface heights in all of the scanned membrane patches are plotted, either stimulated or untreated, there is no significant difference in the height distribution (Supporting Information S1 and S2). These measurements indicate that the overall membrane fluctuation was not altered regardless of the stimulation. Nevertheless, the depressions at some specific locations have big local

Figure 6. Different pore sizes with different stimulations obtained by AFM imaging. The cells were stimulated with histamine (group II), mepyramine-pretreated before histamine stimulation (group III), high- K^+ buffer (group IV), or without stimulation (group I), and then fixed for AFM scanning. (a) Group II cell with lots of depressions. The scale bar is 100 nm. (b) Group IV cell with 5 depressions indicated by arrows. The leftmost depression is estimated to be formed from a vesicle of 130 nm in diameter. The scale bar is 100 nm. (c) Averaged number of vesicles/depressions per AFM scanning frame. Only depressions with depth and width over 20 and 30 nm, respectively, were counted as fusion pores. Data presented are mean $\pm$ SEM from 10 scanning frames in each group. (d) Histogram of the estimated vesicle diameters. The data are fitted with Gaussian distribution functions to estimate the vesicle diameters (red, group II; green, group IV). The center of the distribution indicates the averaged vesicle diameter of 50 and 115 nm for groups II and IV, respectively.

fluctuations. In addition, such depressions could be blocked by mepyramine, which further confirms that the occurrence of such depressions is due to the evoked-exocytosis via the H_1 receptor pathway.

It has been suggested that the vesicle fusion onto the plasma membrane can be either kiss-and-run or full-collapse depending on the stimulation level. Both mechanisms have been identified in bovine chromaffin cells.³⁴ To characterize the difference in the depressions elicited by either histamine or high- K^+ stimulation, the width and depth of the depressions obtained from the AFM images were analyzed (Figure 6). Four groups of chromaffin cells were prepared for the AFM scans: group I, cells without any treatment; group II, cells were stimulated with 10 μ M histamine for 5 min; group III, cells were pretreated with 10 μ M mepyramine for 20 min and then stimulated by 10 μ M histamine for 5 min; group IV, cells were stimulated with high- K^+ buffer in the presence of 3.7% formaldehyde for 2 min. After these treatments, the cells of groups I–IV were all fixed with 3.7% formaldehyde in PBS for 30 min.

The AFM images (or frames) of each group were sampled in several randomly selected surface regions of $1.2 \times 1.2 \mu\text{m}^2$ from different cells. In the measurements, only the depressions with depth and width over 20 and 30 nm, respectively, were

analyzed. In Figure 6a,b, the representative images show that depressions could be identified in the cells of groups II and IV stimulated with histamine and high- K^+ buffer, respectively, but not in those of groups I and III (Figure 5a,b). Figure 6a shows a hot-spot-like region on the plasma membrane, in which more depressions (thirteen depressions were counted within the criteria that the depressions with depth and width over 20 and 30 nm, respectively) were observed than those in other images usually taken for group II. Figure 6c presents the averaged number of depressions per AFM scanning frame of 0.7 ± 0.37 , 4.1 ± 1.27 , 0.6 ± 0.27 , and 3.7 ± 0.72 , respectively, acquired from groups I–IV cells.

The observed concave depression patch is considered to come from the fusion of a spherical SV onto the plasma membrane. To estimate the vesicle diameter, a numerical volume integral is introduced

$$\iint \sum_i z_i dx dy = \frac{4}{3} \pi R^3$$

where $dx = (\text{scan size})/(\text{line samples}) = 2.34 \text{ nm}$, $dy = (\text{scan size})/(\text{scan line}) = 4.69 \text{ nm}$, z_i is the height for the area element of $dx \cdot dy$ within a depression, and R is the radius of the calculated vesicle. Figure 6b shows that there are five depres-

sions within a large "pit". The diameters calculated from the volume integral are 130 nm for the largest depression and ~60–90 nm for the others. Figure 6d displays Gaussian-profile fittings to the histograms of the estimated vesicle diameters with peak center (X_c) and peak width (w) of (X_c , w) = (49.9 ± 0.9 nm, 12.5 ± 0.9 nm) and (114.5 ± 3.9 nm, 27.6 ± 4.6 nm) for the cells stimulated with histamine and high- K^+ buffer, respectively. These results demonstrate that different stimulations evoke different size-distributions of vesicles in the exocytosis.

Our results reveal that AFM can be used to monitor the occurrence of exocytosis and to discriminate various pore-size distributions of the fused vesicles under different stimulations. Previous studies suggest that the Ca^{2+} influx coming through Ca^{2+} channels has a better efficacy to induce vesicle fusion than those from intracellular Ca^{2+} stores³⁵ and the vesicles tend to proceed full-collapse fusion under high $[Ca^{2+}]_i$.³⁴ The $[Ca^{2+}]_i$ elevated by high- K^+ buffer remains at a higher level compared to that stimulated by histamine (data not shown). Therefore, the difference in size-distributions of the estimated vesicle sizes (Figure 6) may reflect different modes (full-collapse or kiss-and-run) involved in the vesicle fusion depending on the level of $[Ca^{2+}]_i$ elevation.

There are two main vesicle types verified by electron microscopy in chromaffin cells, large dense core vesicle (LDCV) and SV with diameters of ~200 and ~50 nm, respectively.^{11,36,37} The vesicle sizes we estimated are close to those obtained from electron microscopic results. However, the fused vesicles might not have fully dilated and are still in Ω -shape, suggesting that the observed AFM images may underestimate the vesicle sizes. Alternatively, the depressions obtained by AFM might be endocytotic pits, because the cells used in this study were fixed. This is difficult to discriminate unless the experiments were done in live cells. However, the results obtained from live pancreatic β -cells have also shown similar depression sizes,¹⁸ supporting that the depressions we observed should be due to exocytosis. In the report using capacitance measurement to reflect the changes in cell size due to the occurrence of exocytosis and endocytosis which increase and decrease the cell size, respectively, the capacitance continues increasing during the period of histamine stimulation; in contrast, the capacitance increases during the membrane depolarization and recovers to resting level afterward.²¹ This measurement elucidates that endocytosis is not significant or may not even occur during histamine stimulation. Comparatively, endocytosis occurs when the cells are depolarized by high- K^+ stimulation but might be blocked by the addition of formaldehyde, which was used to fix the cells in this experiment. Therefore, it is unlikely that the depressions observed are due to endocytosis.

4. Conclusions

In summary, we have demonstrated the successful applications of SWCNT-FET and AFM to detect, respectively, the tiny amount of CgA release and the small fusion pores in single chromaffin cells. Furthermore, CgA-Ab/SWCNT-FET can discriminate the amounts of CgA release evoked with different levels of stimulation. The membrane surfaces obtained by AFM imaging indicate that depressions occur much more frequently in the stimulated cells than those without stimulation. The SV sizes estimated from the AFM images are different with high- K^+ buffer and histamine stimulations. This work demonstrates that AFM can provide insights into the fundamental properties of exocytosis in neuroendocrine cells. In addition to many merits of SWCNT-

FET and AFM as powerful techniques, the most advantage of applying these tools for biological research is that they are noninvasive to the cells, which can be kept intact with minimal perturbation during the investigations. In the future, live-cell monitoring will be adopted to capture the exocytotic scenes in real time and to characterize how this pore formation is modulated by different proteins.

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Supporting Information Available: Discussion of the surface roughness of cells is available free of charge via the Internet at <http://pubs.acs.org>.

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Supporting Information

Exocytosis of a Single Bovine Adrenal Chromaffin Cell: The Electrical and Morphological Studies

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S1. No significant difference in the surface roughness of cells

Figure S1 shows the height distributions analyzed from the acquired AFM images (or frames) for Groups I–IV. The horizontal axis indicates the height (z) of an area pixel ($\Delta x \cdot \Delta y$) in each scanned AFM 3D image, where the height was divided into 500 categories between the highest and lowest pixels. Statistically, height = 0 corresponds to the average surface roughness of the cell. The vertical axis represents the number percentage of each area pixel (256×512 pixels in total) that falls into the 500 height categories. In the AFM imaging experiments, ten frames have been scanned for each Group. In Figures S1a–d, ten curves with different colors depict the height distributions analyzed from the ten obtained AFM frames. The results from statistical analysis show that the height distributions in the acquired AFM images for Groups I–IV are not significantly different as will be discussed in the following section S2.

Figure S1. Height distributions analyzed from the obtained AFM images (or frames) of Groups I–IV.

S2. Statistic analysis for the surface roughness of cells

The height distributions in Figure S1 were further fitted into Gaussian distribution function:

$$y = y_0 + Ae^{-\frac{(x-X_c)^2}{2W^2}},$$

where X_c represents the peak center and W is the peak width of the Gaussian profile. The mean values of X_c and W per AFM image for Groups I–IV were analyzed as displayed in Figure S2. The error bars indicate the standard errors. According to the results of one-way ANOVA analysis, the height distributions for Groups I–IV have no significant difference.

Figure S2. Statistic analysis for the peak centers (X_c) and peak widths (W) averaged from the obtained AFM images of Groups I–IV. The histograms show the mean values of (a) peak center and (b) peak width in the Gaussian distribution.