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Electroporation followed by electrochemical measurement of quantal transmitter release from single cells using a patterned microelectrode

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Abstract

An electrochemical microelectrode located immediately adjacent to a single neuroendocrine cell can record spikes of amperometric current that result from exocytosis of oxidizable transmitter from individual vesicles, i.e., quantal exocytosis. Here, we report the development of an efficient method where the same electrochemical microelectrode is used to electroporabilize an adjacent chromaffin cell and then measure the consequent quantal catecholamine release using amperometry. Trains of voltage pulses, 5–7 V in amplitude and 0.1–0.2 ms in duration, were used to reliably trigger release from cells using gold electrodes. Amperometric spikes induced by electroporabilization had similar areas, peak heights and durations as amperometric spikes elicited by depolarizing high K^+ solutions, therefore release occurs from individual secretory granules. Uptake of trypan blue stain into cells demonstrated that the plasma membrane is permeabilized by the voltage stimulus. Voltage pulses did not degrade the electrochemical sensitivity of the electrodes assayed using a test analyte. Surprisingly, robust quantal release was elicited upon electroporation in the absence of Ca^{2+} in the bath solution (0 Ca^{2+} /5 mM EGTA). In contrast, electroporabilization-induced transmitter release required Cl^- in the bath solution in that bracketed experiments demonstrated a steep dependence of the rate of electroporabilization-induced transmitter release on $[Cl^-]$ between 2 and 32 mM. Using the same electrochemical electrode to electroporate and record quantal release of catecholamines from an individual chromaffin cell allows precise timing of the stimulus, stimulation of a single cell at a time, and can be used to load membrane-impermeant substances into a cell.

Introduction

Neurons and endocrine cells carry out cell-to-cell communication by secreting neurotransmitters and hormones. Vesicles laden with transmitters are contained within the cytosol of specialized cells. On application of an appropriate stimulus, the vesicles fuse to the cell membrane and release their contents to the outside of the cell in a process called exocytosis. Since transmitter is released in packets, this process is referred to as quantal exocytosis. In excitable cells, exocytosis is triggered by a rise in intracellular Ca^{2+} concentration. Exocytosis is inhibited by clostridial neurotoxins and targeted for therapeutic interventions,^{1, 2} thus it is important to develop robust platforms that can be used to study this mechanism in detail. Carbon-fiber microelectrodes (CFEs) have been commonly used to study quantal exocytosis of electroactive transmitters such as catecholamines from

individual cells^{3–6}. The CFE is placed adjacent to the cell so that the released transmitter is oxidized on the electrode surface to produce a spike of amperometric current. This powerful technique provides high sensitivity and temporal resolution but is a low-throughput process involving positioning of an electrode under a microscope to record one cell at a time.

Recently, in an effort to enable high-throughput experiments, our group and others have developed microfabricated devices with arrays of microelectrodes to measure the release of electroactive transmitter at the single-cell and single-vesicle level^{7–14}. With existing systems the most common way of stimulating exocytosis is by perfusion of cells with a secretagogue^{7, 11} leading to depolarization of the cell membrane, opening of voltage-gated Ca^{2+} -channels, Ca^{2+} influx and Ca^{2+} -triggered exocytosis. Perfusion systems, however, often stimulate multiple cells and do not allow precise timing on the stimulus. In contrast, extracellular electrodes can also be used to directly depolarize cells and can potentially stimulate individual cells with very precise timing^{10, 15, 16}. Significantly, Dittami and Rabbitt demonstrated cells undergo exocytosis when forced through a narrow constriction in a microfluidic channel in proximity to a recording electrode, perhaps due to cell depolarization¹⁰.

Another approach to stimulate exocytosis in neuroendocrine cells is by permeabilizing the cell membrane using either high electric fields or chemical agents that disrupt the integrity of the cell membrane^{17, 18}. Following permeabilization, exocytosis can be elicited by including Ca^{2+} or other secretagogues in the bath solution that subsequently diffuse into the cell. Permeabilizing the plasma membrane bypasses the normal excitation pathway and allows one to sort out whether a drug or protein mutation affects exocytosis through a direct effect on exocytosis as opposed to an effect on the membrane potential mediated by ion channels. It also allows one to introduce membrane-impermeable substances into the cell, such as peptides or antibodies, to modulate protein function or second-messenger cascades.

A number of groups have implemented cell electroporation on a microchip, usually by applying a high electric field in a microfluidic channel or through a narrow aperture (reviewed in¹⁹). Recently the Orwar group has induced electroporation at the single-cell level using carbon-fiber electrodes, thus demonstrating the feasibility of electroporation using polarizable microelectrodes suitable for electrochemistry²⁰.

We cultured cells on an open-volume microelectrode array and selected electrodes with a single adherent cell. We then tested the feasibility of using the same electrode to stimulate a cell and subsequently record release from the cell using amperometry. This stimulus-recording electrode approach allows selective stimulation of a single cell, measurement of quantal transmitter release within 1 s after permeabilization in order to capture rapid kinetic features of exocytosis, and can distinguish quantal release of transmitter from vesicles (evident as amperometric spikes) from release of cytosolic transmitter or other electroactive compounds (which result in a slowly changing background faradaic current)²¹.

Materials and Methods

Cell preparation and solutions

Chromaffin cells were isolated from bovine adrenal glands as described previously²². To make it easier to detach cells from the flask and to reduce cell clumping we cultured cells in Hibernate A media with calcium (BrainBits LLC, Springfield, IL, USA) in a refrigerator (4°C) and used them 1~6 days after preparation. In preparation for an experiment, cells were spun down at 100 g for 4 min. The supernatant was discarded and the cells were suspended and triturated in 5 ml standard bathing solution followed by a second pelleting. The supernatant was again discarded and the cells were re-suspended in 1 ml standard bathing solution resulting in a typical cell density of $\sim 2 \times 10^6$ cells/ml. We loaded 100 μ l of the cell solution on the microchip device and waited for 15–20 min to allow cell settling. During loading, the device was gently shaken sideways a few times to aid gravity-driven settling of the cells in the SU-8 microwells. We then gently exchanged the standard bathing solution for the experimental solution.

The pipette solution used for the whole cell patch clamp experiments consisted of (in mM): 150 K-Gluconate, 1 EGTA, 10 HEPES, 4.6 MgCl₂, and 4 Na⁺-ATP, pH 7.2. The standard cell bath solution consisted of (in mM): 150 NaCl, 5 KCl, 2 CaCl₂, 1.2 MgCl₂, 10 HEPES, and 10 glucose, pH 7.2. The Ca²⁺-free solution was made with 0 CaCl₂ and 5 EGTA. The Na⁺-free, Ca²⁺-free solution consisted of (in mM): 135 KCl, 5 EGTA, 1.2 MgCl₂, 10 HEPES, and 10 Glucose, pH 7.2. Similarly, the Cl⁻-free, Ca²⁺-free solution comprised of (in mM): 135 Na-glutamate, 5 EGTA, 1.2 MgCl₂, 10 HEPES, and 10 Glucose, pH 7.2. For the Cl⁻-dependency experiments we used Ca²⁺-free solutions with varied Cl⁻-concentrations by equimolar substitution of Cl⁻ salts with glutamate salts. The free Ca²⁺ concentration in the bath solution was varied using the Ca²⁺ chelator DPTA (diethylenetriaminepentaacetic acid, K_d = 81 μ M at pH 7.2²³) titrated with Ca acetate rather than CaCl₂ to keep the concentration of Cl⁻ fixed. We tested electrodes as described previously¹¹ by performing cyclic voltammetry with a ferricyanide solution that consisted of 1 mM K₃Fe(CN)₆ in 0.1 M KCl, pH 3.0.

For measurement of the intracellular Ca²⁺ concentration, cells were loaded with the ester form of ratiometric indicator Fura-4F (Invitrogen). The dye-loading solution contained Dulbecco's Modified Eagles Medium (DMEM) (Gibco, Invitrogen). The hibernate media was replaced with loading solution containing 10 μ M of Fura-4F AM (stock – 5.0 mM in DMSO). Cells were incubated with the dye-containing solution for 45 min at 37°C, washed, and then resuspended in regular bath solution containing 2 mM Ca²⁺. A monochromator (Polychrome II, TILL Photonics, Munich, Germany) was coupled to the epifluorescence port of an inverted Olympus IX50 microscope (Olympus America, Center Valley, PA, USA) with an optical fiber. A 40X 1.15 NA water immersion lens (U-APO, Olympus America) focused the excitation light and collected fluorescent light. The Fura-4F fluorescence (535 nm $\pm$ 25 nm) was monitored using a photodiode mounted in a viewfinder (TILL Photonics). Calculations for [Ca²⁺]_i were done by inputting the raw 360 and 380 data into the equation from Grynkiewicz, et. al.,²⁴ and using the K_{eff}, R_{min}, and R_{max} obtained from previous calibrations.

Device fabrication

Device fabrication was carried out using a two-mask photolithographic process. Microscope slides ($25 \times 75 \times 1$ mm, Fisherbrand, Fisher Scientific, Pittsburgh, PA, USA) were used as a transparent substrate and gold was used as the electrode material.

A semi-transparent 30 nm Au film was sputtered on top of an adhesion promoting Ti film (10 nm) using an RF magnetron sputtering system (ATC 2000-V, AJA international Inc., North Scituate, MA, USA, targets obtained from Kurt J. Lesker Company, Clairton, PA, USA). The deposition was performed at room temperature with a power of 100 W and a working pressure of 4 mTorr with an Argon gas flow rate of 20 sccm. The deposition rate for Ti and Au was 2 nm/min and 9 nm/min, respectively. The Au film was patterned using photolithography and wet etching into 72 conductive traces (36 on each half of the glass slide), 60 μ m wide, with 2 mm diameter connection pads arranged around the circumference of the chip. The Au/Ti coated substrate was first cleaned with 2-propanol, then rinsed with deionized water and air-dried. The slide was then cleaned with air plasma at 10.5 W RF power level for 1 min (plasma cleaner/sterilizer, PDC-32G, Harrick Scientific Corp., Pleasantville, NY, USA). Shipley S1813 positive photoresist (Rohm and Haas electronic materials, Philadelphia, PA, USA) was spin-coated (Single-wafer spin processor, Laurell Technologies Corp., North Wales, PA, USA) onto the Au/Ti substrate at 2500 rpm for 1 min resulting in a ~ 2 μ m thick layer. The photoresist-covered Au/Ti slide was pre-baked on a hot plate at 115 $^{\circ}$ C for 2 min. Then it was exposed to UV light (15 mW/cm²) through a high-resolution (20,000 dpi) transparency mask (CAD/Art Services Inc., Bandon, OR, USA) and developed in diluted M351 (4:1 dilution with deionized water) (MicroChem Corp, Newton, MA, USA) for 1 min. Then the Au/Ti slide was post-baked at 150 $^{\circ}$ C on the hot plate for 10 min. Gold etchant solution (Sigma, St. Louis, MO, USA) was used for wet-etching the portion of Au/Ti film that was not protected by photoresist for ~ 10 s. TFTN solution (Transene Company Inc., Danvers, MA, USA) was then used to etch the Ti film at 85 $^{\circ}$ C for ~ 20 min. The S1813 photoresist was finally removed using PRS-3000 stripper (J.T. Baker, Phillipsburg, NJ, USA). Finally, the patterned device was rinsed with deionized water and dried with compressed air.

We used the thick photoresist SU-8 2025 (MicroChem Corp, Newton, MA, USA) both to fabricate microwells and to insulate non-active areas of the conductive film. SU-8 was spin coated at 4000 rpm for 1 min to yield a film thickness of ~ 16 μ m measured with a Tencor Alphastep 200 Profiler (San Jose, CA, USA). It was subsequently baked at 65 $^{\circ}$ C for 3 min and 95 $^{\circ}$ C for 5 min on hot plates. A second high-resolution (20,000 dpi) transparency mask was used to define a single 20 μ m diameter microwell/electrode opening over each conductive trace as well as openings over each connection pad. Following mask alignment using a microscope, the photoresist was exposed to UV light (11.7 mW/cm²). Then it was post-baked at 65 $^{\circ}$ C for 1 min and 95 $^{\circ}$ C for 5 min on hot plates. It was then developed with SU-8 Developer (MicroChem Corp, Newton, MA, USA) for 10 min with mild agitation. Finally, the device was baked on a hot plate at 150 $^{\circ}$ C for 2 hrs to seal small cracks on the surface of the SU-8 film.

Next, a polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning, Midland, MI, USA) gasket was placed on top of the device to confine the drop of solution containing cells to the

part on the device where the 36 working electrodes are located. The assembly was treated with air plasma for 1 min at 10.5 Watts power setting to enhance the sealing of the gasket to the chip. This air plasma also helped clean the device surface.

Electrical stimulation on microchip and action potentials

Fabricated devices were cleaned with 2-propanol, then rinsed with deionized water, air-dried and then sterilized with exposure to UV light. Finally chromaffin cells were plated and incubated at 37 °C for up to a few hours. Whole cell patch clamp recordings of membrane potential were made in current-clamp mode using an EPC9 amplifier (HEKA, Lambrecht, Germany). Patch pipettes were fabricated from Kimax glass (Kimax-51, Kimble Glass Inc., Vineland, NJ) using a Flaming/Brown micropipette puller (Model P-97, Sutter instrument Co., Novato, CA). The pipette resistance typically ranged from 2 Ω 3 to 5 Ω 3 when filled with intracellular solution and was typically 5 Ω 3 to 10 Ω 3 in the whole-cell configuration. Voltage pulses were applied to patterned gold electrode adjacent to the chromaffin cell to facilitate transient depolarization of the plasma membrane thus inducing action potentials in the cell.

Electropermeabilization and corresponding amperometric measurements from single chromaffin cell

Electropermeabilization of the plasma membrane of cells was demonstrated by staining with trypan blue and propidium iodide (data not shown for the latter). Trypan blue solution (0.4%, Sigma, St. Louis, MO, USA) was mixed with regular chromaffin cell medium at a ratio of 1:4. Following cell loading, 10–20 μ l of this solution was added to 100 μ l of cell solution on the device. Uptake of dye was evaluated 10 min after staining.

Neurotransmitter release from a single chromaffin cell was induced as well as measured by the same planar Au working electrode. While applying the voltage pulse, the surface of the electrode was visually monitored to detect formation of bubbles in the solution, presumably due to hydrolysis of water. As the microelectrode passed current, a voltage drop occurred in the electrolytic solution surrounding the cell and this voltage gradient led to changes in the cell's membrane potential that produced electroporation upon dielectric breakdown of the cell membrane. For the stimulus-recording electrode approach to work, we needed the dynamic range of the potentiostat to be sufficient to supply a large current to induce electroporation, yet also be capable of resolving the picoamp-level currents associated with amperometric events. This was achieved by having a VA-10 two-electrode potentiostat modified in collaboration with the manufacturer, NPI Inc (Tamm, Germany). The modification added a diode network in parallel with the op-amp feedback resistor such that the diodes “turned on” to bypass the feedback resistor to allow a large transient current to be supplied to the electrode in response to a voltage step. Once the capacitive current settled, the diodes “turned off” to allow low-noise I-V conversion by the feedback resistor²⁵. The output of the VA-10 was sampled by the analog-to-digital converter of the EPC9 amplifier and recorded using Pulse software (HEKA, Lambrecht, Germany). During amperometric recording, the VA-10 potentiostat was held at a potential of 600 mV relative to an Ag/AgCl wire inserted in the solution that served as the counter/reference electrode. Amperometric signals were low-pass filtered with a cut-off frequency of 2.9 kHz and sampled at a rate of

10 ksamples/s. Amperometric spikes were analyzed using the software of Segura et al²⁶ running on Igor Pro (Wavemetrics, Lake Oswego, OR, USA). Spikes were not included in the analysis if the peak current was less than 2 pA, had negative charges, multiple peaks or other irregular shapes, or 10~90% rise times of greater than 20 ms.

Results and Discussion

Action potentials can be elicited by pulsing an adjacent electrochemical microelectrode

Triggering action potentials in excitable cells with a transient electric field is a widely used technique in neuroscience and can be implemented on microchips^{15, 16}. To our knowledge, however, patterned microelectrodes have not been demonstrated to elicit action potentials in small spheroidal neuroendocrine cells. We therefore sought to determine experimental conditions whereby action potentials could be elicited in an individual chromaffin cell by an adjacent electrochemical microelectrode as a method to stimulate, and subsequently record, exocytosis.

Voltage pulses were applied to planar electrodes with adherent chromaffin cells while simultaneously monitoring the cell's membrane potential using a patch-clamp pipette in the whole-cell current-clamp configuration. Fig. 1a shows a schematic of a typical chip device with an array of Au microelectrodes. The working electrode regions are small openings in the patterned SU-8 insulation layer. The PDMS gasket is designed to hold the solution with cells on top of the working electrodes. The schematic in Fig. 1b illustrates a voltage stimulus being applied to a cell patch clamped in whole cell mode. Fig. 1c shows the action potentials that are induced by the transient voltage pulses. Note that action potentials were induced in an all-or-none manner in that pulses with amplitudes of 2.4 or 2.3 V elicited action potentials whereas pulses to 2.0 V failed to induce an action potential. We found that the threshold for eliciting action potentials was typically between 2.0 and 3.0 V for cells that were well adhered to Au electrodes (2.23 ± 0.49 V, 0.2 ms pulse duration, $n=20$ cells). Since a train of action potentials is required to induce an appreciable rate of exocytosis^{27, 28}, protocols with trains of voltage pulses were tested. However, a precipitous drop in membrane resistance was often observed on repeated stimulation or application of higher voltages, (Fig. S1 in Supplement). This suggests that electropermeabilization of the cell membrane is occurring with these types of stimuli. Since electropermeabilization achieves our goal of inducing robust quantal release from individual cells, we turned our attention to optimizing this approach.

The same electrochemical electrode can be used to stimulate and record quantal transmitter release

The same planar electrochemical electrode was used to stimulate the cell and record the resulting electrochemical signal that results from oxidation of released transmitter. Fig. 2 depicts a representative trace with amperometric spikes resulting from quantal transmitter release induced by electropermeabilization of a single cell on a gold microelectrode. The amperometric spikes have amplitudes ($I_{\max}$), durations ($t_{1/2}$) and areas (Q) that are consistent with previous reports of quantal exocytosis (Table 1^{3, 5, 22, 29}). Thus the

electrochemical signal that follows stimulating pulses is consistent with quantal exocytosis of chromaffin granules.

A range of stimuli are able to elicit electroporation and quantal release. Pulses with durations less than 0.2 ms were used in order to prevent bubbles from forming on the surface of electrodes. Individual or trains of voltage pulses from the holding potential used for amperometry (~ 0.6 V) to test potentials of 4–7 V were found to trigger massive quantal release from single chromaffin cells. Steps to lower potentials led to transient release i.e. secretion that terminated within 10s of seconds. On the other hand, larger voltage steps or pulse trains led to release lasting for minutes. Figure 3a provides a sample response to a train of seven pulses to +3V, 0.1 ms in duration. Note that only a handful of release events are elicited before the response terminates. A subsequent train of pulses to +6V in the same cell elicits a massive response (Fig 3b) that lasts for a number of minutes (data not shown). Note that both modest and massive stimulation can be useful for studies of exocytosis. Whereas transient electroporation could leave the cell more intact to allow probing the cell over prolonged intervals, prolonged permeabilization would be necessary to study the dependency of exocytosis on external factors like changing concentrations of a molecule of interest in the external solution.

Trypan blue staining demonstrates selective electroporation of the cell docked to an electrode

The electroporation of the plasma membrane was validated next by observing the uptake of a membrane-impermeant stain. Trypan blue is a commonly used membrane-impermeant stain that is excluded from viable cells. Since it cannot cross the plasma membrane before electroporation, viable cells remained unstained. However, following stimulation, permeabilization of the plasma membrane allows the dye to enter the cell where it accumulates in the nucleus giving it a blue color. Fig 4a depicts two chromaffin cells; one directly over the electrode (white dashed circle) and another cell not adjacent to an electrode (green dashed circle). Neither cell took up the trypan blue present in the bath, indicating that they are viable cells. After application of the voltage pulse (Fig 4b), the cell over the electrode takes up the stain, indicating electroporation has occurred, whereas the control cell remains unstained. This indicates that the stimulation is specific and addressable to the single-cell level. Fig. 4c indicates amperometric spikes recorded from the stained cell in Fig. 4b in response to the voltage pulse and demonstrates that the cell remains viable enough to secrete transmitter via quantal exocytosis.

Voltage pulses do not degrade the electrochemical electrodes

The experiments depicted in Figs 2, 3, and 4 demonstrate that, following a voltage pulse, the electrochemical electrode could recover within several seconds and remain sensitive enough to record amperometric spikes with typical features. We next sought to determine if the voltage pulses alter the sensitivity of the electrodes or increase measurement noise. Our experiments indicated no change in the noise (standard deviation) resulting from brief (sub ms) pulses to +10V (data not shown). In order to test for changes in electrode sensitivity, we recorded cyclic voltammograms (CVs) using the test analyte ferricyanide before and after applying voltage pulses (Fig. 5). During the CV the voltage is scanned from +0.7 V to -0.5

V and a reduction peak is observed at $\sim +0.1$ V as the ferricyanide is reduced to ferrocyanide. This reaction is reversed as the voltage is scanned in the positive direction, resulting in an oxidation peak. The voltage difference between the oxidation and reduction peaks (E_p) is a measure of electron-transfer kinetics. Note in Fig. 5 that applying several 0.2 ms pulses to +10 V in between CV measurements has little effect on E_p or other features of the CVs, therefore there is no apparent degradation to the electrochemical sensitivity of the electrode. In fact, E_p decreases slightly after application of a voltage pulse, perhaps because the voltage pulse oxidizes, and thus removes, a thin layer of gold on the surface of the working microelectrode, thus cleaning the electrode surface.

Electropermeabilization-induced quantal release is dependent on $[Cl^-]_e$, but not $[Ca^{2+}]_e$

Under physiological conditions, exocytosis is triggered in excitable cells by entry of Ca^{2+} into the cell through ion channels. Therefore it would seem likely that electropermeabilization of the plasma membrane triggers exocytosis by providing an alternative pathway for Ca^{2+} to enter cells from the extracellular solution. In order to test this hypothesis we performed experiments using extracellular solutions with no added Ca^{2+} together with 5 mM of the Ca^{2+} chelator EGTA to buffer the free Ca^{2+} concentration into the subnanomolar range. Experiments were also carried out using 2 mM BAPTA instead of EGTA as the Ca^{2+} chelator. We were surprised to find that robust exocytosis can be elicited with voltage pulses applied to cells bathed in this “ Ca^{2+} -free” solution (Fig. 6a). In contrast, Ca^{2+} -free solutions do not support substantial exocytosis when chromaffin cells undergo large-scale electropermeabilization in a cuvette³⁰ or are permeabilized with chemical agents such as digitonin³¹. Cell depolarization also fails to elicit exocytosis in Ca^{2+} -free solutions³².

Whereas extracellular Ca^{2+} is not essential, we found that mM concentrations of Cl^- are necessary to support robust quantal transmitter release in response to our electropermeabilization protocol. Fig. 6a depicts a sample experiment where robust release is elicited in a “ Ca^{2+} free” solution containing 137 mM Cl^- . However, subsequent wash with a solution containing only 2 mM Cl^- (with equimolar substitution of glutamic acid for Cl^-) leads to a much lower rate of release (Fig. 6b). Subsequent wash with a solution containing 32 mM Cl^- restores a robust rate of secretion (Fig. 6c). In experiments from 5 cells following the same protocol, the rate of quantal release was reduced from 4.1 ± 1.2 to 0.11 ± 0.05 spikes/s upon reduction of Cl^- from 137 to 2 mM. Subsequent wash with 32 mM Cl^- returned the rate of release to 0.53 ± 0.49 spikes/s. The lower rate of release at 32 mM Cl^- compared to 137 mM Cl^- may be partially or completely explained by a slow “rundown” of exocytosis following electropermeabilization (also see³⁰), nevertheless, these bracketed experiments demonstrate a steep dependence of the rate of transmitter release on Cl^- between 2 and 32 mM using our approach to stimulate exocytosis.

A possible explanation for why extracellular Ca^{2+} is not needed to stimulate exocytosis in our experiments is that the pulse protocol leads to release of Ca^{2+} from intracellular stores, either through direct intracellular electroporation, heating, or other mechanisms. In order to address this we performed preliminary experiments measuring changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to voltage pulses. Supplement Fig. S2a presents a sample

experiment, representative of three cells, where $[Ca^{2+}]_i$ increases in response to the voltage pulses when the extracellular solution contains 2 mM $CaCl_2$. This is consistent with the hypothesis that electroporation allows substantial influx of Ca^{2+} from the bath solution. An additional two cells, however, did not show a prominent $[Ca^{2+}]_i$ response, perhaps because the electroporation was transient. In contrast, Fig. S2b shows a sample trace, representative of 5 cells, where a voltage pulse protocol that reliably evokes exocytosis does not lead to a discernable increase in $[Ca^{2+}]_i$ if the bath solution contains 0 Ca^{2+} /5 mM EGTA. This suggests that release of Ca^{2+} from internal stores is not prominent under these experimental conditions, although we cannot exclude the possibility that spatially local release of Ca^{2+} occurs that escapes detection in our system.

Whereas the absolute dependence of quantal transmitter release on Ca^{2+} appears to be lost following electroporation under our experimental conditions, we next sought to determine if Ca^{2+} can modulate the rate of Cl^- -dependent release. We therefore set the Cl^- concentration to 30 mM, which is a concentration where the rate of release is roughly half maximal in a low Ca^{2+} solution. Keeping $[Cl^-]_e$ fixed to 30 mM, cells were first electroporated in a 0 Ca^{2+} , 5 mM EGTA solution and then the cells were washed with a solution containing 97.7 μM free Ca^{2+} buffered with DPTA. The spike frequency was 1.90 ± 0.42 in the Ca^{2+} -free solution and 1.93 ± 0.50 in the Ca^{2+} -containing solution ($n=7$ cells). Thus, surprisingly, Ca^{2+} plays little role in triggering or modulating quantal transmitter release when electroporation is induced under our experimental conditions.

As noted previously, the amperometric spikes obtained under Ca^{2+} -free conditions have amplitudes and time courses that are typical for exocytosis events from chromaffin cells (Table 1). Thus Cl^- -stimulated, electroporation induced “quantal” catecholamine release almost surely occurs from individual chromaffin granules. Our experiments cannot resolve, however, whether the release occurs via bona-fide exocytosis or by lysis of secretory granules as they approach the membrane/electrode surface.

Of further note is the fact that the dependence of transmitter release on $[Cl^-]_e$ occurs after the electroporation step since manipulation of $[Cl^-]_e$ after electroporation modulates the rate of ongoing transmitter release in a reversible manner (Fig. 6). We speculate that the dependence of vesicle release on Cl^- may be mediated by ClC Cl^- channels on the vesicle membrane³³. It is interesting to note that Cl^- -containing solutions have previously been reported to support lysis of secretory granules isolated from chromaffin cells³⁴.

Conclusions

We have developed an approach where the same electrochemical electrode located immediately below a cell can be used to stimulate and record quantal release of catecholamines from an individual chromaffin cell via electroporation. The voltage-pulse protocol leads to selective permeabilization of an individual cell with precise timing. The amperometric spikes elicited by electroporation have magnitudes and kinetics similar to previous studies of depolarization-evoked exocytosis and therefore likely reflect quantal release of catecholamines. Mild stimuli support reversible electroporation, which

may in the future allow transfection of cells to express proteins of interest followed by on-chip measurement of exocytosis from transfected cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

An individual voltage pulse from an adjacent electrode can elicit all-or-none action potentials. Action potentials were stimulated with planar chip electrodes and recorded using whole cell patch clamp recordings of membrane potential in current clamp mode. (a) Schematic illustration of a chip device with an array of working microelectrodes. The patterned array consists of 36 parallel electrode traces in the center of the chip. 2 mm diameter connection pads are arranged around the circumference of the chip. A PDMS gasket placed on top confines the drop of solution containing cells to the middle of the device where the electrodes are located (b) Chromaffin cells were cultured on patterned Au microelectrodes and action potentials were induced by transient voltages, 0.2 ms in duration, applied to the planar electrode. (c) Arrows indicate transient voltages of 2.4 V, 2.0 V and 2.3 V.

Note that action potentials are stimulated in an all-or-none manner with a threshold stimulus between 2.0 and 2.3 V.

Fig. 2.

Trains of voltage pulses result in robust quantal transmitter release. Representative amperometric spikes following a train of voltage pulses that permeabilize the cell membrane of a single chromaffin cell on a Au microelectrode. The inset (b) is an expanded view of the amperometric spikes within the dashed box in part (a).

Fig. 3.

Increasing the amplitude of the stimulus voltage train leads to a greater frequency and duration of quantal transmitter release. (a) Representative trace showing infrequent spikes over a 30 s recording interval when the cell is stimulated by voltage pulse to 3V (7 pulses, 0.1 ms in duration). (b) A subsequent stimulation train to 6 V leads to massive quantal release that continues for minutes.

Fig. 4.

Uptake of the cell-impermeant dye Trypan blue demonstrates that the stimulation protocol leads to selective permeabilization of the cell adjacent to the electrode. (a) Photomicrograph with two viable chromaffin cells (encircled) sitting in a standard bath solution containing trypan blue before electroporation. The upper cell encircled in green is sitting on the SU-8 insulation layer whereas the cell encircled in white is sitting on the working electrode. (b) A few minutes after application of the stimulating pulse to the working electrode, the cell sitting on the electrode has taken up the dye, indicating electroporation, whereas the green encircled cell not directly adjacent to the electrode remains intact. (c) amperometric spikes resulting from electroporation of the cell adjacent to the electrode.

Fig. 5.

Voltage pulses do not reduce the sensitivity of the electrochemical electrode to a test analyte. Cyclic voltammograms were acquired at 1 V/s using a 1 mM ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$) solution in 0.1 M KCl, pH 3. The Au electrode was 20 μm in diameter. In between CVs the solution was exchanged to a normal extracellular solution and voltage pulses (+10 V for 0.2 ms) were applied.

Fig. 6.

Quantal transmitter release is dependent on the Cl^- concentration in the bath. Representative bracketed experiment from the same cell. (a) A high spike frequency is observed following electroporation in a solution containing 132 mM Cl^- . (b) Wash with a solution containing 2 mM Cl^- greatly reduces the rate of quantal transmitter release. (c) Exchange of the bath solution for one containing 32 mM Cl^- restores a high rate of transmitter release. Changes in Cl^- concentration were made by equimolar substitution of glutamic acid for Cl^- .

Table 1

Mean $\pm$ standard deviation of four descriptive spike parameters: half width ($t_{1/2}$), time to peak (t_p), charge (Q) and amplitude ($I_{\max}$). Data obtained from cells electroporated on gold microelectrodes in solutions containing a range of chloride concentrations

$t_{1/2}$ (ms)	t_p (ms)	Q (pC)	$I_{\max}$ (pA)
31.4 $\pm$ 11.5	11.0 $\pm$ 2.9	2.2 $\pm$ 1.6	64.1 $\pm$ 39.5
# of Cells = 9, # of Spikes = 771.			