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Controlled On-chip Stimulation of Quantal Catecholamine Release from Chromaffin Cells Using Photolysis of Caged Ca^{2+} on Transparent Indium-Tin-Oxide Microchip Electrodes

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Abstract

Photorelease of caged Ca^{2+} is a uniquely powerful tool to study the dynamics of Ca^{2+} -triggered exocytosis from individual cells. Using photolithography and other microfabrication techniques, we have developed transparent microchip devices to enable photorelease of caged Ca^{2+} together with electrochemical detection of quantal catecholamine secretion from individual cells or cell arrays as a step towards developing high-throughput experimental devices. A 100 nm - thick transparent Indium-Tin-Oxide (ITO) film was sputter-deposited onto glass coverslips, which were then patterned into 24 cell-sized working electrodes ($\sim 20 \mu\text{m}$ by $20 \mu\text{m}$). We loaded bovine chromaffin cells with acetoxymethyl (AM) ester derivatives of the Ca^{2+} cage NP-EGTA and Ca^{2+} indicator dye Fura-4F, then transferred these cells onto the working ITO electrodes for amperometric recordings. Upon flash photorelease of caged Ca^{2+} , a uniform rise of $[\text{Ca}^{2+}]_i$ within the target cell leads to quantal release of oxidizable catecholamines measured amperometrically by the underlying ITO electrode. We observed a burst of amperometric spikes upon rapid elevation of $[\text{Ca}^{2+}]_i$ and a “priming” effect of sub-stimulatory $[\text{Ca}^{2+}]_i$ on the response of cells to subsequent $[\text{Ca}^{2+}]_i$ elevation, similar to previous reports using different techniques. We conclude that UV photolysis of caged Ca^{2+} is a suitable stimulation technique for higher-throughput studies of Ca^{2+} -dependent exocytosis on transparent electrochemical microelectrode arrays.

Introduction

Neurons and neuroendocrine cells secrete signaling molecules via Ca^{2+} -dependent exocytosis, a process in which intracellular vesicles fuse with the cell membrane and release their contents of hormones and neurotransmitters into the extracellular space in response to an increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). Techniques that can measure “quantal” release, i.e., release of the contents of individual vesicles, are particularly powerful to study exocytosis at the molecular level. Among these, carbon fiber amperometry stands out as a high temporal-resolution technique for detecting quantal vesicle release events¹⁻³. In brief, a carbon fiber microelectrode is placed close to a cell that secretes electroactive substances such as dopamine, epinephrine, norepinephrine or serotonin, and is held at a constant potential. Upon stimulation

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of the cell, oxidative current spikes are measured, with each spike reporting a single-vesicle release event. Integration of the current spike indicates the amount of transmitter released by an individual vesicle. The time course of the spike also provides important information about vesicle release kinetics such as the initial flux of transmitter through a nanometer-scale fusion pore^{2, 4-6}. Vesicle release kinetics are altered upon activation of second-messenger systems, so quantal assays of exocytosis are used to understand regulatory mechanisms of the cell⁷⁻⁹. In addition, measurements of quantal exocytosis following experimental perturbations of relevant proteins provide detailed information about the molecules that trigger or regulate vesicle fusion^{10, 11}.

A drawback to the use of carbon-fiber electrodes to measure quantal exocytosis is that it is a low-throughput and labor-intensive technique. This is because carbon fiber microelectrodes are manually fabricated in small lots, must be manually positioned close to cells using micromanipulators, and can only record from one cell at a time. Application of microchip technology potentially offers compact, automated, high-throughput lab-on-a-chip solutions to biomedical studies at the single-cell level¹². Recent efforts to develop microfabricated devices to electrochemically measure quantal exocytosis include gold electrodes patterned in cell-sized wells etched in silicon to enable detection over a large area of the cell¹³, a glass microchip with 4 platinum electrodes surrounding a single cell to enable localization of the site of release on the cell surface^{14, 15}, and a carbon electrode on a silicon substrate to detect serotonin release from isolated vesicles¹⁶. Our group¹⁷ and another¹⁸ have recently demonstrated quantal release of catecholamines from chromaffin cells using transparent ITO electrodes patterned on a glass substrate to potentially allow simultaneous optical and amperometric measurements of cells using conventional inverted microscopy.

The most commonly used method to stimulate exocytosis for amperometric studies is perfusion of high- K^+ solution, which leads to cell membrane depolarization, opening of voltage-gated Ca^{2+} channel, influx of Ca^{2+} and then Ca^{2+} -triggered exocytosis^{13, 17, 19}. Although high- K^+ stimulation is an effective way to trigger exocytosis, it only allows indirect control of the $[Ca^{2+}]_i$ stimulus, which makes it difficult to resolve which step in the stimulus-secretion cascade is affected by an experimental perturbation. Perfusion of buffered Ca^{2+} solutions over permeabilized cells allows more direct control of $[Ca^{2+}]_i$ ⁸, but readily diffusible cytosolic components are lost upon membrane permeabilization. In addition, the rate that $[Ca^{2+}]_i$ can be elevated is limited by the solution exchange time, therefore rapid kinetic phases of Ca^{2+} -triggered exocytosis cannot be resolved.

Arguably the most powerful stimulation method for studying Ca^{2+} -secretion coupling with high temporal resolution is photorelease of “caged” Ca^{2+} within cells²⁰⁻²². Caged Ca^{2+} compounds are organic molecules that bind Ca^{2+} with a high affinity but undergo a large decrease in affinity for Ca^{2+} upon UV photolysis. Flash photolysis leads to rapid elevation of $[Ca^{2+}]_i$ to enable measurement of fast kinetic phases of exocytosis. In addition, photoelevation of $[Ca^{2+}]_i$ is believed to occur in a spatially uniform manner throughout the cell. In contrast, depolarization-evoked Ca^{2+} entry leads to steep near-membrane Ca^{2+} gradients so that the local $[Ca^{2+}]_i$ that is driving exocytosis is difficult to measure with fluorescent Ca^{2+} indicators. Thus combination of caged Ca^{2+} with fluorescent Ca^{2+} indicators allows fine control and accurate measurement of the $[Ca^{2+}]_i$ stimulus.

Here we report the development of an innovative microchip device, which integrates the caged Ca^{2+} technique with transparent ITO electrodes and fluorescence photometry, to perform controlled on-chip stimulation of Ca^{2+} -dependent exocytosis. By integrating the caged Ca^{2+} technique into our ITO microchip devices, we can elevate $[Ca^{2+}]_i$ to a targeted level to trigger and measure exocytosis. Our results demonstrate that, upon flash photolysis, a burst of

catecholamine release was detected from cells sitting above the transparent recording ITO electrodes, similar to previous reports using different techniques^{23, 24}.

Materials and methods

Design, microfabrication and testing of ITO microchip

ITO deposition—No. 2 borosilicate cover glass (thickness: 170 μm - 250 μm , Fisher Scientific, Pittsburgh, PA, USA) was used as the substrate for our biochip devices. A 110 nm-thick ITO film was sputtered onto the glass substrate using a magnetron sputtering system (ATC 2000-V, AJA international Inc. North Scituate, MA, USA) with an ITO target (90 wt% In_2O_3 and 10 wt% SnO_2 , Williams Advanced Materials Inc, Buellton, CA, USA). We optimized the fabrication parameters for ITO deposition based on two criteria. What we were most concerned with optimizing was the transmittance of the ITO film in the near UV range (wavelengths ~ 340 - 390 nm) so that we could pass light to photolyze the cage and excite the fluorescent Ca^{2+} indicators. Our secondary goal was to have a low sheet resistance. Since we are measuring small currents, the sheet resistance does not have to be extremely low to result in a negligible voltage drop within the ITO film. The parameters for the flow rate of Ar gas and O_2 and the DC sputtering mode were adapted from Deng. et al²⁵. We sputter-deposited ITO using different working pressures, substrate temperatures, gas flow rate combinations for Ar and O_2 , and deposition times. Typically we changed one parameter at a time and measured the transmittance spectrum and sheet resistance, and then decided on an optimal value for one parameter. By repeating this process for different parameters, we obtained the optimal parameters for our application. The final parameters we used were DC mode deposition at a power of 100 W, a working pressure of 2 mTorr, and a substrate temperature of 325°C. Ar gas was introduced at a flow rate of 20 sccm, and O_2 was introduced at 0.2 sccm. The deposition time was 600 s. The transmittance spectrum for ITO-coated cover glass was measured using a UV-VIS recording spectrophotometer (UV-2401PC, Shimadzu, Kyoto, Japan). The conductance of the deposited ITO film was measured using a 4-point probe (S302, Lucas Labs, Gilroy, CA, USA).

ITO electrode patterning and insulation—Transparent ITO electrodes were patterned on the ITO-coated cover glasses using photolithography and wet-etching techniques adapted from Sun and Gillis¹⁷. A schematic of the procedure is depicted in Fig. S1 (See online Electronic Supplementary Information). Briefly, we first rinsed the ITO-coated cover glass with 2-propanol, then sonicated for 20 min in acetone (Ultrasonic cleaner 1510, Branson, Danbury, CT, USA), then cleaned using air plasma (Plasma cleaner/sterilizer, PDC-32G, Harrick Scientific Corp., Pleasantville, NY, USA) for 1 min at the medium RF power level. Shipley S1813 positive photoresist (Rohm and Haas electronic materials, Philadelphia, PA, USA) was then spincoated (single-wafer spin processor, Laurell Technologies Corp., North Wales, PA, USA) onto the ITO-coated cover glass at 2500 rpm for 60 s to give a thickness of ~ 2 μm . The coated cover glass was baked on a hot plate at 115°C for 2 min. Photoresist-covered cover glass was exposed to UV light through a high-resolution (20,000 dpi) transparency mask (CAD Art Services, Bandon, OR, USA) and then developed in M351 (Rohm and Haas electronic materials) for ~ 1 min. It was then baked at 200°C on the hot plate for 10 min. An acidic solution composed of 6 M HCl and 0.2 M FeCl_3 was used to wet etch the portion of the ITO film that was not protected by photoresist for 30 min, leaving 20 μm -wide ITO stripes (vertical stripes in Fig. 1(B) bottom) that widen to form connecting pads on the edge of the chip (Fig. 1(B) top). The photoresist protection layer was then removed with an acetone wash. The cover glass with patterned ITO electrodes was rinsed with DD water and air-dried. Using similar photolithographic methods, we insulated the bulk of the ITO stripes with photoresist, leaving a 20 μm -wide opening orthogonal to the ITO stripes (horizontal stripe in Fig. 1(B) bottom) to result in an array of 24 working electrodes with dimensions of 20 μm by

20 μm . In some of our earlier experiments we used AZ P4620 positive photoresist (AZ Electronic Materials USA Corp, Branchburg, NJ, USA) to pattern ITO electrodes following a similar procedure.

ITO microchip assembly—A 10:1 ratio of polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning, Midland, MI, USA) monomer and cross-linking agent was used to make the PDMS gaskets for holding the cell-containing solution on the chip. The monomer and curing agent mixture was degassed in a vacuumed desiccator and poured into a plastic ring on a silicon wafer. After curing for 1 hr in a conventional oven at 80 °C, the PDMS slab was cut into 10 mm by 8 mm slabs with a 6 mm by 3 mm opening in the middle serving as the PDMS gasket (Fig. 1(B) top). It was then peeled off the wafer and sealed in the middle of the ITO microchip so that the 24 working ITO electrodes were within the opening of the PDMS gasket. A custom-built chamber was used to hold the ITO microchip device and provide electrical connection from the amplifier connection pin to the ITO connection pads at the edge of the chip.

Cell preparation and solutions

Cell preparation—Bovine adrenal chromaffin cells were prepared as described before²⁴. About 3×10^6 cells in 5 ml of culture media (Dulbecco's Modified Eagles Medium supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin) were seeded into a poly-L-lysine coated T25 polystyrene flask. Cells were kept in a humidified incubator at 37°C with 5% CO_2 and used 1-5 days after preparation.

Solutions—For the carbon fiber experiments to verify digitonin permeabilization efficiency, the bath solution consisted of (in mM): 160 K-Glutamate, 20 HEPES, 0.2 K-EGTA, 1 MgCl_2 , and 10 Glucose (titrated to pH 7.2 with KOH). The “ Ca^{2+} perfusion solution” had a free $[\text{Ca}^{2+}]_i$ of $\sim 20 \mu\text{M}$ and consisted of (in mM): 152 K-Glutamate, 20 HEPES, 10 DPTA (1,3-Diaminopropan-2-ol-tetraacetic acid), 2 Na_2ATP , 3 MgCl_2 , 2 CaCl_2 and 20 μM digitonin titrated to pH 7.2 with KOH.

For the caged Ca^{2+} experiments with digitonin-permeabilized cells on ITO electrodes, the “intracellular mimicking solution” consisted of (in mM): 100 K-glutamate, 20 DPTA, 6.49 CaCl_2 , 10 NP-EGTA, 30 HEPES (titrated to pH 7.2 with KOH), 2 Na_2ATP , 50 μM bisfura-2 and 50 μM fura-2FF. Cells were initially permeabilized in culture media containing 20 μM digitonin. The usual cell bathing solution consisted of (in mM): 150 NaCl, 5 KCl, 1.2 MgCl_2 , 2 CaCl_2 , 10 HEPES, 11 Glucose, titrated to pH 7.2 with NaOH.

For the experiments using AM ester cell loading, the “incubation solution” for loading cells consisted of 25 μM NP-EGTA (AM) and 5 μM fura-4F (AM) in Dulbecco's Modified Eagles Medium (DMEM). Stock solutions of NP-EGTA (AM) and fura-4F (AM) were dissolved in DMSO at a concentration of 5 mM with 10% w/v Pluronic F-127 to facilitate loading. For the “ Ca^{2+} priming effect” experiments, the bath solution was either the standard cell bathing solution or a solution with lower Ca^{2+} (1 mM). NP-EGTA (AM) and fura-4F (AM) were obtained from Invitrogen (Carlsbad, CA, USA). All other reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise stated.

$[\text{Ca}^{2+}]_i$ calibration, photorelease and measurement

$[\text{Ca}^{2+}]_i$ calibration—For the experiments using digitonin permeabilized cells on ITO electrodes, $[\text{Ca}^{2+}]_i$ was measured ratiometrically using an equimolar combination of Ca^{2+} indicators bisfura-2 and fura-2FF as previously described²⁶. For calibration, eight solutions with known free $[\text{Ca}^{2+}]$ of 0, 0.31, 1.2, 3.6, 7.2, 28, 127 μM and 10 mM were prepared using Ca^{2+} buffers EGTA (Ethylene lycol-bis(2-aminoethyl-ether)-N, N, N', N'-tetraacetic acid, $K_d=150 \text{ nM}$ at pH 7.2), HEDTA (N-(2-Hydroxyethyl)-ethylenediamine-triacetic acid, $K_d=3.6$

μM at pH 7.2) and DPTA ($K_d=8.1 \mu\text{M}$ at pH 7.2). A drop of each calibration solution was placed on an ITO-coated coverslip and the ratio of fluorescence emission ($535 \pm 25 \text{ nm}$) for 360 nm versus 380 nm excitation was recorded. The measured ratios were fitted by equation (1) to obtain the five free parameters²⁷. We then used the inversion of equation (1) to get $[\text{Ca}^{2+}]_i$ from the measured ratio in experiments.

$$R=R_0+R_1 \frac{[\text{Ca}^{2+}]_i}{[\text{Ca}^{2+}]_i+K_1}+R_2 \frac{[\text{Ca}^{2+}]_i}{[\text{Ca}^{2+}]_i+K_2}. \quad (1)$$

For experiments using AM ester loaded cells, we used the Ca^{2+} indicator dye fura-4F (AM). Since fura-4F (AM) is hydrolyzed to the free base fura-4F inside the cell, we performed calibrations by loading a solution containing the fura-4F (K^+ salt) into cells through a patch-clamp pipette during whole-cell recordings. The 4 calibration solutions we used contained 10 mM EGTA with either 0, 6.7 mM or 9 mM CaCl_2 to yield free $[\text{Ca}^{2+}]$ values “0”, 311 nM, or 1.2 μM . In addition, a solution containing 10 mM CaCl_2 without EGTA was used to measure the fluorescence of Ca^{2+} -saturated fura-4F. Fluorescent ratios obtained from 360 nm versus 380 nm stimulation were fitted by the equation from Grynkiewicz., et.al.,²⁸ to obtain K_{eff} , R_{min} , and R_{max} .

$[\text{Ca}^{2+}]_i$ measurement—We used in-house software written in the Igor macro language (Wavemetrics, Portland, OR, USA) to control the ITC-16 data acquisition interface (Instrutech Co., Long Island, NY, USA) for measuring $[\text{Ca}^{2+}]_i$ and triggering the firing of a UV flash lamp (SP20-1, Rapp OptoElectronic, Hamburg, Germany). A monochromator (Polychrome II, TILL Photonics, Munich BioRegio, Germany) was coupled to the epifluorescence port of an inverted Olympus IX50 microscope (Olympus America, Center Valley, PA, USA) with an optical fibre. A 40X 1.15 NA water immersion lens (U-APO, Olympus America) focused the excitation light and collected fluorescent light. The fluorescent light ($535 \text{ nm} \pm 25 \text{ nm}$) was measured using a photodiode mounted in a viewfinder (TILL Photonics). A two-port condenser (TILL Photonics) combined the monochromator excitation path with that of the UV flash lamp (illustrated in Fig. 1(A)). Fluorescent signals from Ca^{2+} indicator dyes within the cells were collected by the photodiode and then sampled by the ITC-16 computer interface. The software then calculated $[\text{Ca}^{2+}]_i$ from the 360 nm / 380 nm fluorescence ratio as previously described.

On-chip amperometry and photometry

For the experiments using photorelease of caged Ca^{2+} in digitonin-permeabilized cells, 5 μM digitonin in DMSO (0.1% after dilution in culture media) was added into a T25 flask of cultured chromaffin cells and incubated in a humidified incubator (37°C , 5% CO_2) for 2 min. Then the cells were vigorously washed off the flask with the media, transferred into a 15 ml conical tube, and centrifuged at 100 g for 4 min. The supernatant was aspirated out and 100 μl “intracellular mimicking solution” was added and the cell pellet was resuspended. $\sim 20 \mu\text{l}$ of the cell suspension was pipetted onto the ITO microchip. Brief exposures to 360 nm and 380 nm light were used to measure $[\text{Ca}^{2+}]_i$ whereas continuous 360 nm light from the monochromator was used to photorelease the caged Ca^{2+} . The amperometric current from quantal release of oxidative catecholamines was measured using a patch-clamp amplifier (EPC9, HEKA, Lambrecht, Germany) with a dc potential at 700 mV relative to a Ag/AgCl ground electrode placed in the drop of solution. The monochromator was dark while recording amperometric current from cells sitting on the ITO electrodes. We used the data acquisition interface built into the EPC9 patch-clamp amplifier and Pulse software (HEKA) to control the monochromator for these experiments.

For the experiments where cells were loaded with AM cage and indicator, chromaffin cells were washed off a T25 flask with 2 ml DMEM and transferred into a 35 mm tissue culture

dish. After adding 25 μM NP-EGTA (AM) and 5 μM fura-4F (AM), we incubated the cell suspension in a humidified incubator (37°C, 5% CO_2) for 60 min. Then the cell suspension was centrifuged at 100 g for 4 min. The supernatant was removed using a Pasteur pipette and discarded. The pellet was resuspended in 5 ml of the standard cell bathing solution. The cell suspension was again centrifuged at 100 g for 4 min and the supernatant was removed with Pasteur pipette and discarded. 700 μl cell bathing solution was added and the pellet was resuspended. About 20 μl of the cell solution was pipetted onto the ITO microchip.

Results

Design and fabrication of a biochip device with multiple cell-sized ITO electrodes

Effective use of caged Ca^{2+} requires use of a high-numerical-aperture lens both to focus the excitation light and to collect fluorescent light emitted by the Ca^{2+} indicator dye with high efficiency. The distance between the lens surface and the sample (working distance) is typically ~ 0.3 mm for common high numerical apertures objective lenses, therefore fluorescent microcopy is often carried out using an inverted microscope with cells visualized from below through a thin glass coverslip. Thus fluorescence measurement of cells on microchips is most conveniently carried out using a thin transparent substrate. Another important design principle for our application is that the working electrochemical electrodes should be small to minimize background noise so that $\sim$ pA-scale currents due to quantal exocytosis can be resolved. Finally, electrochemical electrodes need to be directly adjacent to cells (within several μm) so that released catecholamines diffuse to the electrode over a brief enough interval so that a recognizable “spike” of oxidative current is produced to indicate the time course of release from an individual vesicle.

Our solution to these design constraints is depicted in schematic fashion in Fig. 1(A). Use of transparent ITO electrodes allows us to visualize cells sitting directly on top of electrochemical electrodes, therefore we sputter-deposited a thin (~ 100 nm) ITO film on size 2 glass coverslips (nominal thickness ~ 220 μm). We used photolithography and wet etching to pattern the ITO into 24 cell-sized working electrodes (see Methods section for details). During experiments, one of the electrodes is connected to the headstage of the amplifier to serve as the working electrode. A Ag/AgCl wire is immersed in the drop containing the cell suspension to serve as the ground electrode. A computer controls the firing of a UV flash lamp to photolyze caged Ca^{2+} within cells. A monochromator is used to excite the Ca^{2+} indicator dye at a pair of wavelength (360/380 nm) and is also under computer control. A high numerical aperture objective lens on an inverted microscope is used to both focus the flash and monochromator excitation light on the cell and collect the emitted fluorescent light. A photodiode is used to measure the fluorescent light emitted by the Ca^{2+} indicator dye.

A photograph of our microchip device is shown in the upper panel of Fig. 1(B). The 24 ITO squares around the edge of the microchip serve as the connection pads to facilitate connection to the amplifier headstage. A PDMS gasket sealed around the 24 ITO working electrodes functions as a reservoir for the drop of solution containing the cell suspension. The lower panel of Fig. 1(B) is a photomicrograph showing 2 of the 24 working electrodes. The vertical stripes are 20 μm -wide ITO stripes patterned by wet etching whereas the 20 μm -wide horizontal stripe is the opening of the photoresist that serves to insulate portions of the ITO film that are not electrochemically active. Therefore the working electrodes are at the intersection of the vertical and horizontal stripes.

Characterization of ITO working electrodes

It is important for our deposited ITO film to pass both visible and near-UV light in order to photolyze the Ca^{2+} cage (< 400 nm), excite the Ca^{2+} indicators (360 nm, 380 nm) and pass

the fluorescent light (~500 nm). We optimized the fabrication parameters for ITO sputter-deposition on cover glass, so that 80% of light is transmitted for wavelengths longer than 360 nm (Fig. 2(A), see Methods section for deposition parameters). The transmittance spectrum of our ITO-coated cover glasses (ITO thickness: 110 nm) is comparable to commercially available ITO-coated glass slides with a thinner ITO coating (15-30 nm). The electrical conductance of the ITO electrodes was also optimized. The measured sheet resistance of ITO-coated cover glass is 5.38 Ω /square, with a resistivity of $0.59 \times 10^{-4} \Omega\text{cm}$.

We next characterized the electrochemical response of our patterned ITO electrodes using a standard reversible analyte: 1 mM $\text{K}_3\text{Fe}(\text{CN})_6$ in 0.5 M KCl, pH 3.0. Fig. 2(B) shows a sample voltammogram (scan rate: 1 V/s) that exhibits a sharp transition and flat diffusion-limited reduction current for potentials more negative than ~ -0.2 V versus Ag/AgCl, indicative of an appropriately low series resistance. The theoretical value of a diffusion limited current (i_{lim}) for a univalent reaction on a disk electrode in an infinite plane is given by:

$$i_{\text{lim}} = 4FDCr$$

where D is the diffusion coefficient, C is the analyte concentration, F is Faraday's constant and r is the radius of the electrode. For this experiment the working ITO electrode was rectangular with dimensions of $17.5 \times 25 \mu\text{m}$. If we assume the "effective radius" of our rectangular electrode is $\sim 12 \mu\text{m}$, (the radius of a circle with the same area), and a diffusion coefficient of $7.2 \times 10^{-6} \text{ cm}^2/\text{s}$ for $\text{Fe}(\text{CN})_6$, then the theoretical value of i_{lim} is 3.3 nA. Since the measured value in Fig. 2(B) is close to this theoretical value, we conclude that the working ITO electrode has the expected active area.

We also measured the specific capacitance of our deposited ITO film electrodes using sinusoidal voltage stimulation (frequency 1 kHz, peak amplitude 1 mV). The measured specific capacitance of a typical $20 \mu\text{m}$ by $20 \mu\text{m}$ ITO working electrode is $\sim 0.1 \text{ pF}/\mu\text{m}^2$, comparable to the value we measured from commercially available ITO slides¹⁷ and carbon fiber microelectrodes²⁹.

Photorelease of caged Ca^{2+} in digitonin-permeabilized cells

Our initial approach to stimulate cell secretion through photorelease of caged Ca^{2+} was to permeabilize cell membranes with digitonin to allow loading of the cage and Ca^{2+} indicators into cells. To confirm the efficacy of cell permeabilization by digitonin, we first positioned a carbon fiber electrode adjacent to a chromaffin cell in our standard cell bath solution lacking Ca^{2+} . A pipette containing $20 \mu\text{M}$ digitonin and $\sim 20 \mu\text{M}$ free Ca^{2+} was brought close to the cell and the pipette solution was perfused onto the cell membrane. Free Ca^{2+} diffused into the cell through the permeabilized cell membrane and triggered quantal catecholamine release, which was detected by the carbon fiber electrode (Fig. 3(A)).

We next loaded digitonin permeabilized chromaffin cells with the Ca^{2+} cage NP-EGTA and Ca^{2+} indicator dyes (an equimolar combination of bisfura-2 and fura-2FF). To minimize the impact of endogenous Ca^{2+} buffers, we designed an "intracellular mimicking solution" with very high Ca^{2+} buffer capacity. This solution was intended to confound the ability of intracellular Ca^{2+} buffers to reduce $[\text{Ca}^{2+}]_i$ within the cell (e.g., see ³⁰) in order to minimize the gradient of $[\text{Ca}^{2+}]_i$ between the inside and outside of the cells after photolysis of caged Ca^{2+} . Loaded cells were transferred to the microchip device. Those cells settling on top of the ITO microelectrodes were used for amperometric recordings. Upon UV photolysis of caged Ca^{2+} , we detected oxidative current spikes as released catecholamine molecules were oxidized on the surface of ITO microelectrodes (Fig. 3(B)). Surprisingly, we also detected a large background current that continued for tens of seconds even when the excitation light was terminated (Fig. 3(B)). This slowly varying current occurred whether cells were present or not

and resulted from photolysis of caged Ca^{2+} in the bath solution because it was absent when cage was removed (data not shown).

Use of membrane-permeant (AM) forms of the Ca^{2+} cage and Ca^{2+} indicator

The large current artifact due to photolysis of cage-containing solution over the electrochemical electrode led us to pursue an alternative approach whereby we used membrane-permeant acetoxymethyl (AM) ester derivatives of the Ca^{2+} cage (NP-EGTA) and the Ca^{2+} indicator (fura-4F). Modification of carboxylic acids with AM ester groups creates an uncharged molecule that can permeate cell membranes. Once inside the cell, the lipophilic blocking groups are cleaved by nonspecific esterases within the cell, resulting in a charged form that leaks out of cells much more slowly than its parent compound.³¹

Bovine adrenal chromaffin cells were incubated with NP-EGTA (AM) and fura-4F (AM) and then washed with a dye-free and cage-free bath solution. A drop of cell-containing solution was then transferred to the microchip device. Fig. 4(A) presents a photomicrograph of cells that settled randomly on the microdevice. In this example an individual chromaffin cell settled onto the working electrochemical electrode, which is at the intersection of the horizontal and vertical stripes in the center of the photo. We were able to verify the loading of fura-4F AM by measuring the green cell fluorescence in response to 360 nm excitation (Fig. 4(B), taken from a different experiment). We set the aperture defining the illuminated area and the aperture defining the area of collected fluorescent light equal to the location of the working electrode. This ensures that only the cell on the working electrode is stimulated upon photolysis of the cage and that the measured fluorescent light is reporting the $[\text{Ca}^{2+}]_i$ of the cell under study.

Upon flash photolysis of caged Ca^{2+} inside a cell sitting immediately above an ITO microelectrode, fura-4F reported a step-like increase of $[\text{Ca}^{2+}]_i$ to $\sim 3.5 \mu\text{M}$ (Fig. 4(D)(d)). Flash photoillumination did not result in an increase in $[\text{Ca}^{2+}]_i$ if cells were incubated in fura-4F AM without the cage (data not shown). Simultaneously the ITO microelectrode detected a wave of oxidative current consistent with a burst of catecholamine release from the cell (Fig. 4(D)(a))³². Note that current spikes are evident, which likely represent quantal release from individual vesicles fusing to the bottom surface of the cell immediately adjacent to the electrode. Note that the amperometric current diminishes despite the continuous elevation of $[\text{Ca}^{2+}]_i$. This is thought to be due to depletion of a readily releasable pool of vesicles that are docked to the plasma membrane and primed for release²².

We noticed that UV illumination creates a modest background current (Fig. 4(D) (b)) even in the absence of Ca^{2+} cage in the bath solution. In fact, this light-induced current is noted in the absence of cells. We and others have previously noted a small light-induced current in carbon-fiber electrodes also (data not shown). We scanned the wavelength of the monochromator to obtain an excitation spectrum of the light-induced current (Fig. 4(C)). The spectrum reveals that the current is prominent for UV to blue excitation wavelengths. This light-induced current produces small pulse-like current artifacts when the excitation wavelength is alternated between 360 nm and 380 nm for fluorescent $[\text{Ca}^{2+}]_i$ measurements (see inset of Fig. 4(D) (b)) and a large, rapidly decaying current artifact when the flash lamp discharges (Fig. 4(D) (b)). By subtracting the light-induced current (Fig. 4(D) (b)) from the amperometric current elicited from photorelease of caged Ca^{2+} in a cell (Fig. 4(D) (a)), we can eliminate the light-induced current artifact (Fig. 4(D) (c)).

“Priming” effect of sub-stimulatory $[\text{Ca}^{2+}]_i$ on catecholamine release reported by microchip devices

Most neuron and neuroendocrine cells secrete hormone and neurotransmitters via Ca^{2+} -dependent exocytosis, a process involving the fusion of vesicles with plasma membrane

triggered by a rise in $[Ca^{2+}]_i$. Vesicles undergo several steps, such as translocation, docking and priming, before they are ready to fuse with the plasma membrane and release their content into extracellular environment³³. Although the exact role of Ca^{2+} in every step of this vesicle cycle is not completely understood, experimental evidence suggests that a moderate increase in basal $[Ca^{2+}]_i$ facilitates priming vesicles to become fusion-competent³⁴⁻³⁶.

We performed a set of experiments using the ITO microdevices to correlate the response to flash photoelevation of $[Ca^{2+}]_i$ with the basal $[Ca^{2+}]_i$ level before the flash. In order to obtain a larger range of basal $[Ca^{2+}]_i$ we incubated cells in a bath solution containing either 1 mM or 2 mM Ca^{2+} . Fig. 5(B) depicts a typical robust response to photoelevation of $[Ca^{2+}]_i$ when basal $[Ca^{2+}]_i$ is relatively high (680 nM) whereas Fig. 5(A) depicts a much smaller response from a cell with a low initial $[Ca^{2+}]_i$ (243 nM). Note that, whereas the basal, pre-flash $[Ca^{2+}]_i$ varies ~3-fold between the 2 cells, the post-flash $[Ca^{2+}]_i$ that triggers release is similar (3.29 μ M versus 3.95 μ M). Therefore the difference in response is potentially due to Ca^{2+} -dependent “priming” of exocytosis. Whereas Fig. 5(B) depicts a response with few amperometric spikes, many cells with low basal $[Ca^{2+}]_i$ had no detectable amperometric response at all to photoelevation of $[Ca^{2+}]_i$. We found that out of 37 cells with basal $[Ca^{2+}]_i$ lower than 600 nM (mean $[Ca^{2+}]_i$ = 267 nM), only 9 cells showed detectable catecholamine release (defined as one or more amperometric spikes with an amplitude greater than 10 pA) in response to flash photoelevation of $[Ca^{2+}]_i$ (mean post-flash $[Ca^{2+}]_i$ = 3.11 μ M). In contrast, 17 out of 34 cells with basal $[Ca^{2+}]_i$ above 600 nM (mean basal $[Ca^{2+}]_i$ = 976 nM) respond with a burst of amperometric spikes to photoelevation of $[Ca^{2+}]_i$ (mean post-flash $[Ca^{2+}]_i$ = 3.21 μ M).

Discussion

We describe here a novel biochip device to perform on-chip optical stimulation and electrochemical detection of Ca^{2+} -dependent exocytosis at the single-vesicle level. Use of transparent ITO electrodes on a glass coverslip substrate allows us to use an inverted microscope with a high numerical aperture objective lens for high efficiency fluorescence measurement of cell $[Ca^{2+}]_i$ and on-chip photolysis of caged Ca^{2+} . Previous work in our lab has shown that transparent ITO microelectrodes have a similar electrochemical performance as carbon fiber electrodes¹⁷. Using the acetoxymethyl ester derivative of the Ca^{2+} cage NP-EGTA and the Ca^{2+} indicator dye fura-4F, we are able to deliver caged Ca^{2+} and Ca^{2+} indicator dye into a cell population in a much faster and less invasive manner than delivery through a patch-clamp pipette. Since we can restrict the light excitation aperture to cell-size, we can confine photorelease of caged Ca^{2+} and measurement of $[Ca^{2+}]_i$ to an individual cell (Fig. 4 (B)).

We encountered 2 different light-induced current artifacts in our recordings. A large light-induced current was produced only when the Ca^{2+} cage was in the bath solution and decayed for tens of seconds after illumination was discontinued (Fig. 3(B)). Possible sources for this current are acidification of the solution upon cage photolysis or electrochemical activity of one or more byproducts of NP-EGTA photolysis. This artifact was eliminated by preloading cells with the membrane-permeant AM form of NP-EGTA. The other light-induced artifact was smaller, decayed rapidly following a light pulse, and was prominent for wavelengths in the blue to UV range (Fig. 4(D)(a),(b)). Light effects on metal electrochemical electrode currents were observed as early as 1839 by M. Edmond Becquerel³⁷ and likely result from a low-efficiency water-splitting reaction at the metal-electrolyte junction³⁸. Unfortunately, this artifact is difficult to avoid in ratiometric measurement of $[Ca^{2+}]_i$ because fluorescent indicators in the fura-2 family are excited over this same range of wavelengths. The use of fluorescent Ca^{2+} indicators excited by longer wavelengths could minimize this problem, but the available indicators are not ratiometric so accurate calibration is problematic. Nevertheless,

we were able to eliminate this artifact by subtracting the current elicited by illumination of the electrode in the absence of a cell (Fig 5D(c)).

Our on-chip experiments show that this ITO biochip device is suitable for study of exocytosis. We observed a burst of amperometric spikes upon rapid elevation of $[Ca^{2+}]_i$ as shown in Fig. 4(D) and Fig. 5. A wave of oxidative current with superimposed spikes was observed following flash photolysis of caged Ca^{2+} , which is likely due to a burst of catecholamine release from the cell, similar to previous reports using carbon fiber electrodes³². Using our biochip device, we have also observed that the response to step elevation of $[Ca^{2+}]_i$ depends on the basal $[Ca^{2+}]_i$ level before the flash (Fig. 5). Higher basal $[Ca^{2+}]_i$ ($> \sim 600$ nM) appears to “prime” vesicles for exocytosis, similar to previous reports using different techniques^{34, 35}. Our microchip device should facilitate further study of this effect. For example, it would be straightforward to use a feed-back control system to control UV illumination to elevate $[Ca^{2+}]_i$ to different sub-stimulatory levels and study the dose response of the “priming” effect on a subsequent secretory response triggered by a higher $[Ca^{2+}]_i$ level.

Our approach allows very powerful control of the Ca^{2+} stimulus and measurement of quantal exocytosis. With our current approach of random placement of cells on electrodes we typically get 4 cells on electrodes per chip which allows slightly higher throughput than use of carbon-fiber electrodes which are manually positioned to record from one cell at a time. An area for future improvement to enable higher throughput would be to integrate approaches to automatically target cells to the electrochemical electrodes. Another opportunity for improvement would be to integrate on-chip amplifier arrays and a sensitive CCD camera to allow parallel stimulation and recording from many cells simultaneously in an electrode array.

Conclusion

We have designed and microfabricated a novel biochip device to integrate photolysis of caged Ca^{2+} , ITO amperometry, and fluorescence photometry together for higher-throughput on-chip stimulation and detection of Ca^{2+} -dependent exocytosis. This biochip device has potential applications for higher-throughput screening of drugs and toxins that target exocytosis. In addition, studies of exocytosis which use Green Fluorescent Protein as a reporter of gene expression could benefit from the transparent device.

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Fig. 1. Design of the instrument for on-chip photolysis of caged Ca^{2+} and electrochemical measurement of quantal exocytosis using transparent ITO microelectrodes. **(A)**. Schematic of the experimental system. The flash lamp is used for rapid (~ 1 ms) photolysis of the cage whereas the monochromator excites Ca^{2+} indicator dyes to measure $[\text{Ca}^{2+}]_i$. **(B)**. Top: Photo of microchip with 24 electrochemical ITO electrodes. Bottom: A DIC micrograph of 2 working ITO electrodes with dimensions of $20 \times 20 \mu\text{m}$.

Fig. 2. Optical and electrochemical characterization of ITO microelectrodes. **A.** The optical transmittance of our sputtered ITO film is comparable to a commercially available ITO film (solid line: our ITO-coated coverslip with a 110 nm-thick ITO coating; dotted line: commercial ITO-coated glass slide with a 15-30 nm-thick ITO coating). **B.** Cyclic voltammogram with test analyte (1 mM $\text{K}_3\text{Fe}(\text{CN})_6$ in 0.5 M KCl, pH 3, scan rate 1V/s). The ITO microelectrode had dimensions of 17.5 μm by 25 μm .

Fig. 3.

On-chip amperometric detection of quantal catecholamine release from digitonin-permeabilized cells. **(A)**. Catecholamine release from digitonin-permeabilized chromaffin cells detected by a carbon fiber electrode. **(B)**. Top: Typical amperometric spikes recorded from digitonin-permeabilized chromaffin cells sitting on top of ITO electrodes. The sloping baseline is a current artifact that resulted from cage photolysis. Bottom: Expanded view of the interval outlined by the gray box in the top panel.

Fig. 4.

On-chip amperometric exocytosis measurement from cells loaded with AM forms of the Ca²⁺ cage and Ca²⁺ indicator **A**. A DIC picture of an individual chromaffin cell sitting on top of an ITO working electrode (20 μm by 20 μm). **(B)**. A chromaffin cell loaded with Fura-4F (AM) sitting on top of a working ITO electrode (white square) fluoresces in response to UV excitation. **(C)**. Spectrum of light-induced current artifact at ITO electrochemical electrodes immersed in cell bathing solution. (red: light on; black: light off). **(D)**. Quantal catecholamine release from chromaffin cell induced by Ca²⁺ uncaging. **(a)**. A sample amperometric response to flash photolysis of caged Ca²⁺ from a cell sitting on an ITO electrode. **(b)**. Light-induced current artifact from an ITO electrochemical electrode. Discharge of the flash lamp produces a large, rapidly decaying current artifact. Illumination from the monochromator alternating between 360 nm and 380 nm to measure [Ca²⁺]_i with a fluorescent indicator produces small pulse-like current transients (inset). **(c)**. Faradaic current due to oxidation of catecholamines released from the cell obtained by subtracting the light-induced artifacts (b) from the recorded current (a). **(d)**. Fluorescent measurement of [Ca²⁺]_i using fura-4F (AM) reports rapid [Ca²⁺]_i elevation from 281 nM to 3.6 μM upon UV photolysis of caged Ca²⁺.

Fig. 5. Elevation of “basal” (pre-flash) $[Ca^{2+}]_i$ results in more catecholamine release in response to flash photoelevation of $[Ca^{2+}]_i$. **(A)**. Sample response of a cell with lower initial $[Ca^{2+}]_i$ (243 nM), to photo-elevation of $[Ca^{2+}]_i$ to several μM . **(B)**. Sample response of a different cell with higher initial $[Ca^{2+}]_i$ (680 nM).