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Real-Time Amperometric Measurements of Zeptomole Quantities of Dopamine Released from Neurons

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Amperometry with carbon-fiber microelectrodes provides a unique way to measure very small chemical concentration changes at the surface of biological cells. In this work, an investigation of dopamine release from individual neurons isolated from the mouse retina is described. The mice were genetically modified so that, in cells that expressed the protein responsible for catecholamine synthesis, tyrosine hydroxylase, the marker protein, placental alkaline phosphatase, was also expressed. This modification allowed for identification of the dopamine-containing cells among the many present in the freshly dissociated retina. Release of dopamine was evoked by chemical secretagogues delivered from micropipets that were calibrated with respect to response time and concentration delivered. Amperometric measurements were recorded with low-noise patch clamp amplifiers, and the primary noise source was found to be the electrode capacitance. Dopamine release occurred in the form of transient concentration spikes, consistent with release from small intracellular vesicles. With optimized filtering of the data, the quantity secreted during each release event could be determined. The average quantity determined at one cell was 52 zmol. However, the spikes were quite variable in size and the amount released per event ranged from 8 to 170 zmol. These measurements allow an estimation of the concentration of released transmitter in a synapse.

Exocytosis is a biological process by which neurons and neuroendocrine cells release individual packets of chemical messengers. Because this process is key to intercellular communication, it has been the subject of considerable investigation.[1] Exocytosis can be measured as an increase in whole-cell capacitance that results from the increased surface area upon cell membrane fusion.[2,3] Alternatively, direct chemical measurements of the extrusion process can be made with a carbon-fiber electrode placed adjacent to the cell.[4] Initial electrochemical measurements were at chromaffin cells, neuroendocrine cells isolated from the adrenal gland.[5] The average content of each vesicle in these cells is approximately 5 amol or 3×10^6 molecules. Measurement of this small quantity by electrooxidation can be achieved when the electrode is placed adjacent to or firmly against the cell so that dilution into the bulk solution does not occur.[6]

Since the initial measurements at chromaffin cells, there have been continued efforts to measure exocytosis from cells that contain vesicles with even lower amounts. Ewing and co-workers measured release of catecholamines from the dopamine neuron of the snail, *Planorbis corneus*, that contains vesicles with a mean content of 800 000 molecules[7] and from PC12 cells that have an approximately 115 000 molecules/vesicle.[8] Zhou and Misler examined release from superior cervical ganglion neurons in culture, which release only 30 000 molecules per exocytotic event.[9] Real-time amperometric measurements of exocytotic release of 5000 5-hydroxytryptamine molecules (8 zmol or 10^{-21} mol) from leech neurons have been reported during exocytosis.[10] Measurement of dopamine release from mammalian neurons has also been recently reported.[11,12] The amount released during exocytosis at dopamine neurons in culture had a mean of 3000 molecules. Thus, in less than a decade, electrochemical measurements of exocytosis have shown a 3 orders of magnitude difference in the amount of material exocytotically released from different cells.

Electrooxidation of zeptomole quantities released by exocytosis leads to picoampere currents that occur on a time scale of less than 1 ms. A high-quality current transducer is required for such measurements, and these are available from manufacturers of patch clamp instrumentation. The patch clamp technique is an electrophysiological technique in which the potential-dependent currents that flow through cell membranes can be measured. Thus, its instrumental requirements are very similar to those of

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(1) Zucker, R. S. *Neuron* **1996**, *17*, 1049−1055.
(2) Neher, E.; Marty, A. *Proc. Natl. Acad. Sci. U.S.A.***1982**, *79*, 6712−6716.
(3) Fernandez, J. M.; Neher, E.; Gomperts, B. D. *Nature* **1984**, *312*, 453−455.
(4) Travis, E. R.; Wightman, R. M. *Annu. Rev. Biophys. Biomol. Struct.* **1998**, *27*, 77−103.
(5) Leszczyszyn, D. J.; Jankowski, J. A.; Viveros, O. H.; Diliberto, E. J., Jr.; Near, J. A.; Wightman, R. M. *J. Neurochem.* **1991**, *56*, 1855−1863.
(6) Wightman, R. M.; Schroeder, T. J.; Finnegan, J. M.; Ciolkowski, E. L.; Pihel, K. *Biophys. J.* **1995**, *68*, 383−390.
(7) Chen, G.; Gavin, P. F.; Luo, G.; Ewing, A. G. *J. Neurosci.* **1995**, *15*, 7747−7755.
(8) Chen, T. K.; Luo, G.; Ewing, A. G. *Anal. Chem.* **1994**, *66*, 3031−3035.
(9) Zhou, Z.; Misler, S. *Proc. Natl. Acad. Sci. U.S.A.* **199.5**, *92*, 6938−6942.
(10) Bruns, D.; Jahn, R. *Nature* **1995**, *377*, 62−65.
(11) Pothos, E. N.; Davila, V.; Sulzer, D. *J. Neurosci.* **1998**, *18*, 4106−4118.
(12) Jaffe, E. H.; Marty, A.; Schulte, A.; Chow, R. H. *J. Neurosci.* **1998**, *18*, 3548−3553.

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amperometry. The patch clamp method employs a glass micropipet attached to an individual cell that measures ionic currents. For recordings of single membrane channels, the magnitude of the current is extremely small, on the order of 1 pA, and transient, occurring in a few milliseconds. Thus, the current transducers and techniques used in such experiments were designed to minimize amplifier noise.[13]

We are initiating a study of exocytotic release from mammalian dopamine neurons. The neurons are isolated from a genetically altered mouse that expresses placental alkaline phospatase (PLAP) in all cells that express tyrosine hydroxylase. Thus, cells that can synthesize catecholamines also have PLAP on their surface, and this can be identified with a specific antibody tagged with a fluorophore.[14] In this work we describe an evaluation of analytical approaches and noise sources in the measurement of vesicular release of dopamine from neurons isolated from the retina. As will be shown, the implementation of these methods has allowed quantitative measurement of dopamine release from individual vesicles.

EXPERIMENTAL SECTION

Retina Dissociation and Dopaminergic Amacrine Cell Identification. Dopaminergic amacrine cells (DA cells) were isolated from the retina of transgenic mice as described by Gustincich and co-workers.[15,16] In these mice, catecholaminergic cells express the gene for human placental alkaline phosphatase because PLAP cDNA[17] was linked to the promoter sequence of tyrosine hydroxylase, the rate-limiting enzyme for the synthesis of catecholamines. Adult retinas were dissected, enzymatically digested with papain, and mechanically triturated with fire-polished Pasteur pipets. DA cells were identified by labeling them with the monoclonal antibody to PLAP, E6,[18] conjugated to the fluorochrome, Cy3. The cells were stored in a 95:5 CO_2/O_2 incubator for at least 1 h before use.

Electrochemical Procedures. Glass-encased electrodes were constructed as previously described[19,20] with 5 μm diameter carbon fibers (Thornel T650, Amoco Corp., Greenville, SC). Electropainted electrodes were constructed by modifying a procedure for preparing microcylinder electrodes.[21] Lengths of 100−500 μm of the carbon fibers were left protruding beyond the cut glass prior to sealing the electrodes with epoxy (Epon 828 with 14% w/w *m*-phenylenediamine; Miller-Stephenson Chemical Co., Danbury, CT). After the electrodes were removed from the epoxy, they were immediately rinsed with acetone for 2−3 s to remove residual epoxy from the carbon surface. The electrodes were then stored for 16 h at room temperature before placing them in a 100 °C oven for 1 h and then into a 150 °C oven for 3 h. The electrodes were then coated with an approximately 1 μm thick insulating layer of electrodeposition paint (BASF, Münster, Germany) as described elsewhere.[22,23] Immediately prior to use, both glass-encased and electropainted electrodes were beveled at a 45° angle with a diamond-coated polishing wheel (Sutter Instrument Co., Novato, CA) and then cleaned by ultrasonication in neat 2-propanol for 3 s. Electrodes were backfilled with a solution containing 4 M potassium acetate/150 mM KCl, and electrical connection to the head stage (CV201A/Axopatch 200A, Axon Instruments, Foster City, CA) was made by a chlorided silver wire. Electrode precalibration was accomplished with slow-scan cyclic voltammetry in an extracellular solution containing 50 μM dopamine. All electrochemical potentials were applied versus a Ag/AgCl reference electrode.

Pressure Ejection Micropipet Calibration. Micropipets were used to deliver secretagogues to individual cells. They were pulled with a Sutter PC84 micropipet puller (Sachs/Flaming type, Sutter Instrument Co.) to yield a low aspect ratio geometry so that 10 psig applied by a pressure ejection system (Picospritzer, General Valve Corp., Fairfield, NJ) gave consistently rapid responses. Typically, micropipet tip openings were 6 μm and the distance from the full diameter portion of the capillary tube to the tip opening was 5−6 mm. Rise times were measured with amperometry by placing a micropipet 25 μm from the face of a carbon-fiber microelectrode while the oxidation of pressure-ejected epinephrine (50 μM) was monitored.. Fast-scan cyclic voltammetry (FSCV) was used to determine the concentrations of pressure-ejected epinephrine that reached the electrode tip by comparison of the response to that measured in a flow injection analysis stream. This technique was used because it is relatively immune to the effects of convection.

Isolated Neuron Experiments. DA cells were located on the culture dish by detecting the fluorescence from the Cy3 fluorescent probe attached to the E6 antibody during raster-scanning of the dish over a 20× objective mounted on an inverted microscope (Diaphot 300, Nikon, Inc.) while exciting with 535 ± 25 nm light from a 100 W mercury lamp. Amperometric data were obtained with a CV201A head stage attached to an Axopatch 200A amplifier (Axon Instruments). The output of the Axopatch 200A was filtered at 10 kHz by its internal four-pole low-pass Bessel filter and digitized at 44 kHz onto a DAT recorder (Biologic, Claix, France). The applied potential (+0.650 mV for catecholamine oxidation) was controlled by the Axopatch 200A (after appropriate modification to the holding potential circuitry as outlined by Axon Instruments Hardware Modification Note 22).

Data Analysis. Amperometric data were played back from the DAT recorder through a signal conditioner (CyberAmp 320, Axon Instruments) and digitized (TL-1 DMA Interface, Axotape version 1.3.0, Axon Instruments) at a rate of 50 kHz. The resulting files were subsequently low-pass filtered (eight-pole Bessel, Clampfit 8, Axon Instruments). Transient spike statistics were obtained with MiniAnalysis version 4.1.1 (Synaptosoft Inc., Leona, NJ). The threshold for event beginning detection was set to 4.5 times the

(13) Sherman-Gold, R., Ed. *Axon Guide*: 1993; http://www.axon.com.
(14) Jaffe, E.; Schulte, A.; Marty, A.; Chow, R. H. *Biophys. J.* **1997**, *72*, MPO95−MPO95
(15) Gustincich, S.; Feigenspan, A.; Wu, D. K.; Koopman, L. J.; Raviola, E. *Neuron* **1997**, *18*, 723−736.
(16) Feigenspan, A.; Gustincich, S.; Bean, B. P.; Raviola, E. *J. Neurosci.* **1998**, *18*, 6776−6789.
(17) Kam, W.; Clauser, E.; Kim, Y. S.; Kan, Y. W.; Rutter, W. J. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 8715−8719.
(18) De Waele, P.; De Groote, G.; Van De Voorde, A.; Fiers, W.; Franssen, J.-D.; Herion, P.; Urbain, J. *Arch. Int. Physiol. Biochim. Biophys.* **1982**, *90*, B21.
(19) Kawagoe, K. T.; Zimmerman, J. B.; Wightman, R. M. *J. Neurosci. Met.* **1993**, *48*, 225−240.
(20) Hochstetler, S. E.; Wightman, R. M. Detection of secretion by electrochemical methods. In *On-Line Biophysics Textbook*; Bloomfield, V., De Felice, L., Eds.; Biophysical Society: Bethesda, MD, 1998; http://biosci.umn.edu/biophys/OLTB/Textbook.html.
(21) Cahill, P. S.; Walker, Q. D.; Finnegan, J. M.; Mickelson, G. E.; Travis, E. R.; Wightman, R. M. *Anal. Chem.* **1996**, *68*, 3180−3186.
(22) Schulte, A.; Chow, R. H. *Anal. Chem.* **1996**, *68*, 3054−3058.
(23) Schulte, A.; Chow, R. H. *Anal. Chem.* **1998**, *70*, 985−990.

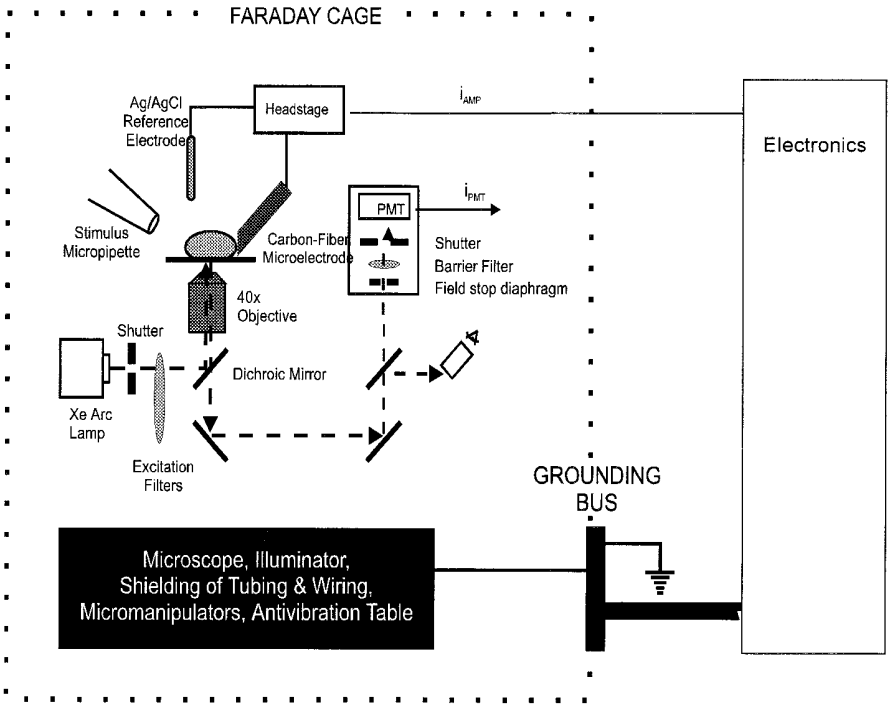

Figure 1. Diagram of apparatus for measurements of zeptomole quantities released from neurons. The microscope, head stage, cell culture, and other equipment immediately adjacent to the preparation are enclosed in a Faraday cage (represented by a dotted line). The fluorescent system can be used to make direct measurements of intracellular Ca^{2+}, the trigger for exocytosis. Electronic equipment that resides outside of the Faraday cage is grounded by individual wires to a central grounding point near the Faraday cage to minimize line noise.

root mean square (rms) of the baseline noise and the event ending detection was set at 10% of the event's maximal amplitude.

Solutions. Extracellular buffer solution consisted of (mM) NaCl (137), KCl (5), CaCl2 (5), $MgCl_2$ (1), HEPES (5), and glucose (20) and was adjusted to pH 7.4 with NaOH. All solutes were obtained from Sigma (St. Louis, MO). Epinephrine and dopamine were also from Sigma.

RESULTS

System Hardware. Figure 1 shows a typical instrumental setup used in both electrochemical and patch clamp measurements. The amplifier used should have a head stage placed adjacent to the preparation. This arrangement ensures minimization of line noise pickup and also can provide a rigid mechanical support for the electrode.[24] Because of the large number of electrical components needed, line noise also can be introduced into the system by improper grounding. All equipment that is connected to the preparation must be grounded at the same point as the signal ground of the amplifier to reduce this noise source, as indicated in Figure 1. In addition to proper grounding of all equipment, any liquid-containing tubes entering the Faraday cage were enclosed in a metal shield and connected to the central ground.

Secretagogue Delivery. Exocytotic events are often chemically evoked by secretagogues such as elevated K^+ or drugs and are typically applied to an individual cell by pressure ejection from a micropipet. The effect of a stimulant on secretion is usually concentration dependent, and in addition, it is important to know the time course of secretagogue delivery. Previous work with fast local superfusion techniques[25−27] involved a delivery pipet and a suction pipet operating in tandem. Although this method provides rapid solution changes, it requires two micromanipulators, thus complicating the technical requirements of the experiment, especially when simultaneous amperometric and patch clamp measurements are desired. We chose to use pressure ejection of secretagogues with a single pipet to simplify the experiment. To ensure the cell is stimulated with the concentration that is placed in the micropipet, consistent micropipet geometry is necessary. This was obtained with a programmable micropipet puller. Previously in our laboratory, construction of secretagogue micropipets used a single-stage pull which resulted in a high aspect ratio tip that was sealed (full capillary diameter to tip distance was typically 12−15 mm). A tip opening was created by cutting the micropipet with a scalpel. This procedure yields micropipets that have jagged, asymmetric tips with inconsistent flow profiles. Also, because of the high aspect ratio, rise times of secretagogue concentration were slower than those presented in this work.

To determine the performance of pipet tips with a low aspect ratio and preformed tip, they were filled with buffer solutions containing 5, 10, or 25 μM epinephrine, an oxidizable substance, and a carbon-fiber microelectrode was placed 25 μm from the micropipet tip and with its active surface normal to the flow (Figure 2A). Using FSCV, a technique that at sufficiently fast potential scan rates is essentially immune to convection-enhanced Faradaic currents, the response was measured and compared to that obtained in a flow injection apparatus. The voltammetric

(24) Sachs, F. *Pflugers Archiv.* **1995**, *429*, 434−435.

(25) Streit, J.; Lux, H. D. *J. Neurosci.* **1989**, *9*, 4190−4199.
(26) Lessmann, V.; Dietzel, I. D. *J. Neurosci.* **1995**, *15*, 1496−1505.
(27) Veselovsky, N. S.; Engert, F.; Lux, H. D. *Pflugers Archiv.* **1996**, *432*, 351−354.

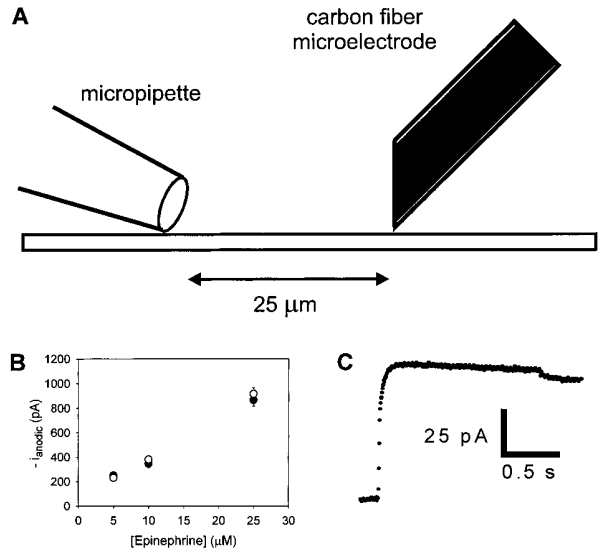

Figure 2. Calibration of pressure ejection micropipets. (A) Arrangement of microelectrode and micropipet on culture plate. (B) Comparison of the anodic current for the oxidation of epinephrine at a carbon-fiber microelectrode with FSCV (300 V/s, 100 ms between scans) during flow injection analysis (filled circles) with that measured following pressure ejection (open circles). Each data point represents an average of two separate trials using the same electrode in all measurements. Ten cyclic voltammograms were averaged for each trial, and the anodic current reported is obtained from an average of the current response from a 100 mV window centered at the anodic E_p. (C) An amperometric temporal profile of a bolus of epinephrine that was applied to a microelectrode.

signals from FSCV in both the pressure ejection configuration and the flow injection analysis system were essentially equal (Figure 2B). Thus, the concentration that reaches the cell is the same as in the pipet. In the Figure 2C is shown a temporal profile of epinephrine delivered to an amperometric microelectrode. Such concentration rise times (0−50%) were consistently less than 50 ms. To circumvent dilution of the secretagogue at the micropipet's tip, the micropipet was discharged in a cell-free region for several seconds before use.

Amplifier Specifications. Current amplification is typically accomplished with a large resistor (∼500 MΩ) in the feedback loop of an operational amplifier located in the head stage (Figure 3A). The rms voltage noise associated with a large resistor is termed Johnson noise and is given by

$$\nu_{\mathrm{rms}} = \sqrt{4kTRB} \qquad (1)$$

where k is Boltzmann's constant, T is absolute temperature, R is resistance, and B is the bandwidth. The calculated ν_{rms} for a temperature of 298 K, a 500 MΩ feedback resistor, and 5 kHz bandwidth is 2.03×10^{-4} V, or with a gain of 1 mV/pA, 0.20 pA rms current noise. For these conditions, the open loop rms noise of the Axopatch 200A is comparable to this value (Table 1) and has a negligible amount of line noise.

To circumvent Johnson noise, amplifiers based on integration amplification have been developed.[28−30] In this approach, the current is integrated in the head stage (Figure 3B). Compared to

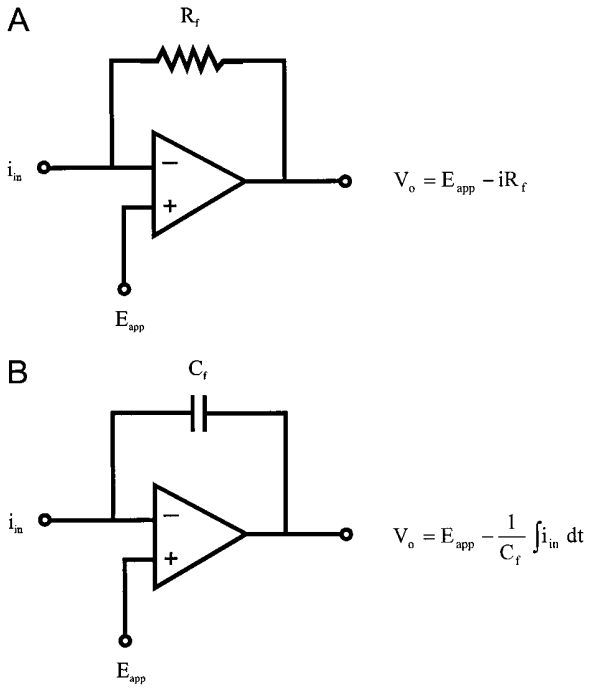

Figure 3. Types of amplification in low-noise current measurements. A resistive feedback amplifier utilizes the circuit shown in (A) and an integration amplifier utilizes the circuit shown in (B). In both circuits, E_{app} is removed with a subtractor circuit (not shown). To recover the actual signal in (B), a differentiator circuit follows the integration circuit (not shown).

Table 1. Noise Characteristics of the Axopatch 200A Amplifier[a]

		amplification type	
condition	N	capacitive rms noise ± sem (pA)	resistive rms noise ± sem (pA)
open loop	3	0.059 ± 0.0015	0.543 ± 0.0088
dummy cell	3	0.523 ± 0.0088	0.740 ± 0.0153
GE-T650	7	1.06 ± 0.142	1.31 ± 0.11
EP-T650	7	1.20 ± 0.075	1.38 ± 0.05

[a] Measurements were made in a Faraday cage containing the apparatus shown in Figure 1. An internal low-pass filter (5 kHz, four-pole Butterworth) conditioned the signal, and the rms noise was read directly off the front panel display of the Axopatch 200A. Open loop measurements were performed with no connections to the head-stage input. The dummy cell (Electrochemistry Model Cell, Axon Instruments) was a 15 kΩ resistor in series with a 20 pF capacitor encased in an aluminum housing mounted directly to the head stage. The electrodes were glass-encased (GE-T650) and electropainted (EP-T650) carbon-fiber microelectrodes beveled at 45°.

resistive feedback amplifiers, the amplifier noise with an integrating head stage is 10-fold lower, an advantage achieved with the Axopatch 200A (Table 1). The chief disadvantage to an integrating amplifier is that once the capacitor is fully charged, it must be discharged. A sample and hold circuit retains the value for 50 µs while the capacitor is discharged. Due to limitations of the reset

(28) Rae, J. L.; Levis, R. A. *Pflugers Archiv.* **1992**, *420*, 618−620.

(29) Sigworth, F. J. Electronic design of the patch clamp. In *Single-Channel Recording*; Sakmann, B., Neher, E., Eds.; Plenum Press: New York, 1995; pp 95−127.

(30) Finkel, A. S. Progress in instumentation technology for recording from single channels and small cells. In *Cellular and Molecular Neurobiology: A Practical Approach*; Chad, V., Wheal, H., Eds.; Oxford University Press: New York, 1991; pp 3−25.

circuitry, there is usually a current deflection in the baseline of the recorded signal during the reset. The deflection is consistently shaped and may be digitally removed after the experiment is performed.

In electrochemical terms, the low noise of the patch clamp amplifier allows the possibility of detection of extremely small amounts of material. For example, an rms noise of 45 fA corresponds to an rms fluctuation of electrons of 280/ms—the time scale of many exocytotic events. For exocytotic events that yield an average current amplitude of 10 pA for a period of 1 ms, the corresponding number of electrons is 62 000, yielding a theoretical signal-to-noise ratio of 222. This would correspond to 31 000 molecules, or 52 zmol for the two-electron oxidation of a catecholamine, the mean value obtained in this work (vide infra). Clearly, the intrinsic noise of the amplifier does not have to be the limiting factor in real-time measurements of small numbers of molecules.

Electrode Capacitance and Noise. With the use of a well-designed amplifier of either type, capacitance is the major source of noise in patch clamp[28] or electrochemical measurements.[22,23] Capacitance on the input reacts with the input voltage noise of the head-stage amplifier (Johnson noise of the input transistor) to produce frequency-dependent current noise. The power spectral density of the input noise current is given by

$$S_{I,1}(f) = 4\pi^2 C^2 f^2 S_V \qquad (2)$$

in which C is the total capacitance connected to the operational amplifier's input JFET including the JFET's intrinsic capacitance, f is the frequency, and S_V is the spectral density of the voltage noise. Thus, the capacitance due to the input JFET, the head-stage assembly, the electrode holder, and the electrode assembly sum to increase capacitance and hence the noise at the output of the current-to-voltage converter. In amperometric measurements with carbon-fiber microelectrodes, the capacitance due to the exposed carbon surface is the dominant source of noise at high frequencies. Polishing an electrode as opposed to cleaving the fiber with a scalpel, another method commonly employed,[31] yields a surface that has a similar sensor radius, but less electroactive area. Thus, the capacitive noise due to the exposed carbon fiber should be less for polished electrodes than for cleaved electrodes.

At lower frequencies, another important noise source is dielectric noise whose frequency dependence is given by

$$S_{I,2}(f) = 8\pi kTDCf \qquad (3)$$

in which k is the Boltzmann constant, T is the absolute temperature, D is the dielectric loss factor, and C is the capacitance of all parts of the electrode that are submerged in solution, including the insulation. Because the capacitance of an exposed carbon fiber is dependent on the diameter and geometry of the fiber, the frequency at which dielectric noise becomes a significant contributor to the current output noise is electrode-type dependent. It is important to note that aside from constructing microelectrodes with the smallest useful area, head-stage materials, the electrode holder, and the construction of the electrode should be carefully chosen to ensure the lowest possible capacitance in the system. To minimize input capacitance and dielectric losses, materials such as polycarbonate and poly(tetrafluoroethylene) (PTFE) are typically used in electrode holder construction.

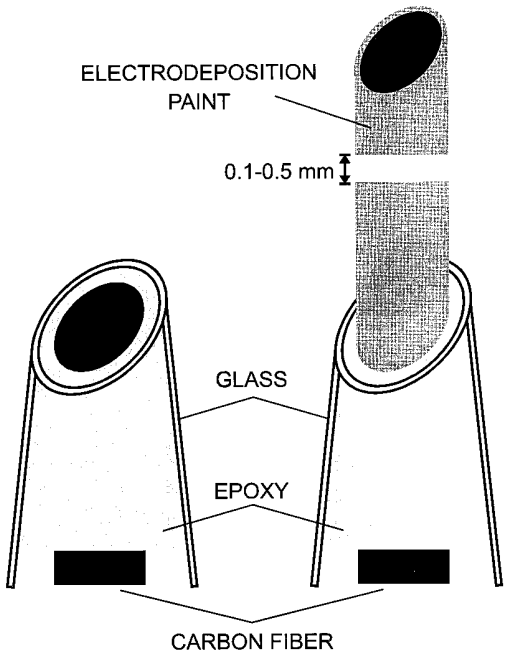

Figure 4. Depiction of glass-encased and electropainted carbon fiber microelectrodes. On the left is a diagram of a glass-encased microelectrode constructed by using previously published methods.[18,19] On the right is an electropainted microdisk electrode. Both types of electrodes were constructed with 5 μm T650 carbon fibers.

Table 1 compares rms noise values for the Axopatch 200A using capacitive or resistive feedback when the instrument is in four different configurations: open loop, a "dummy" cell attached, and a glass-encased electrode (GE-T650) or electropainted electrode (EP-T650), both polished and submerged in extracellular solution. The two electrodes differ in their insulation and thus their capacitance. The GE-T650 has the glass of the micropipet flush with the elliptical surface of the exposed carbon fiber (Figure 4). The EP-T650 has a protruding length of carbon fiber that is insulated with a layer of electrodeposited paint (Figure 4).

Although the difference in rms noise at a 5 kHz filter frequency is not statistically significant between the two electrodes, the GE-T650 electrodes tend to have lower noise characteristics than the EP-T650 electrodes. This difference is likely due to dielectric losses of the electropainted coating. However, the EP-T650 electrodes have two practical advantages. First, the overall tip diameter is less than that of a GE-T650 electrode because the thickness of the electrodeposited layer is ~1 μm whereas the glass thickness typically about 1−2 μm. In addition, there is a ~1 μm epoxy layer between the glass and the carbon fiber. The smaller tip dimensions of EP-T650 electrodes allow the electrode to be more accurately positioned near a cell. The second advantage is that the EP-T650 may be repolished without an increase in tip diameter. Theoretically, this enables an electrode to be used many times before it must be discarded. In practice, however, the rms noise of an electropainted electrode increases with solution

(31) Chow, R. H.; Schulte, A.; Shipston, M. J. *J. Physiol. (London)* **1996**, *495P*, 4−4.

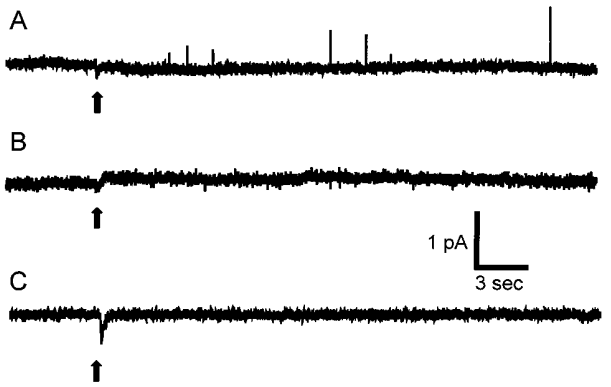

Figure 5. Applied electrode potential and Ca^{2+} dependence of exocytosis amperometric measurements. A carbon-fiber microelectrode was positioned adjacent to the membrane of a DA cell body. In (A), the cell was stimulated with a high-K^+ solution containing 5 mM Ca^{2+}. In (B), the same cell was stimulated with a high-K^+ solution containing 5 mM Ca^{2+} with the applied potential at 0 mV vs Ag/AgCl reference electrode. With an electrode applied potential of +650 mV vs a Ag/AgCl reference electrode, a cell was stimulated with a Ca^{2+}-free high-K^+ solution (C). All signals were low-pass filtered at 40 Hz.

immersion time, thus limiting the practical reuse of such an electrode.

A Biological Example of Real-Time Detection of Zeptomole Quantities. Freshly dissociated neurons from the retina adhere to polylysine-coated plates within 1 h, allowing their study before significant changes in protein expression occur. Exocytotic events from the DA cells in this preparation were examined using the low-noise system. In these experiments, the amperometric electrode was placed gently against the cell body. Pressure ejection of a buffer solution containing elevated K^+ onto a cell caused a series of current spikes (Figure 5A). Several pieces of evidence indicate that the spikes are exocytotic events that release dopamine. First, we selected cells that synthesize catecholamines on the basis of the presence of genetically inserted PLAP.[15] In the mammalian retina, dopamine can be secreted[32,33] and the only catecholamine-synthesizing cells are dopaminergic.[34,35] Second, the stability of the background electrode current indicates that the spikes evoked by stimulating the cell are of biological origin. Third, consistent with the $E°$ of dopamine ($E° = +250$ mV vs Ag/AgCl), exocytosis in response to high K^+ is not observed when the applied potential is 0 mV vs a Ag/AgCl reference electrode (Figure 5B). The occurrence of evoked spikes is restored, however, when the potential of the carbon-fiber electrode is restored to 650 mV (data not shown). Fourth, the current spikes that result from application of elevated K^+ are Ca^{2+}-dependent, a common feature of all exocytotic events.[36] When the cells are stimulated in the absence of external Ca^{2+}, no spikes are observed (Figure 5C). Taken together, these data indicate that the small quantities of secreted material are dopamine released by exocytosis.

Effects of Low-Pass Filtering on Spike Characteristics. Recordings such as those in Figure 5 contain a large amount of

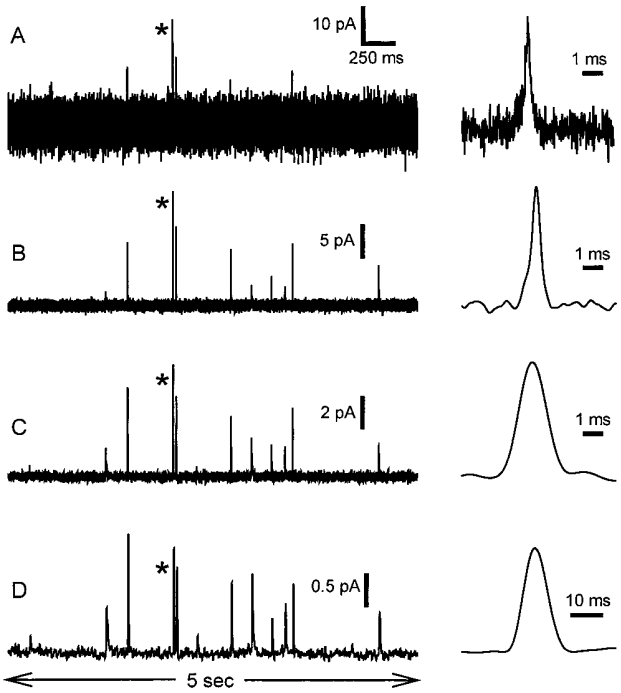

Figure 6. Effects of low-pass filtering on amperometric spikes. A 5 s segment of amperometric data collected from a DA cell is presented at four4 low-pass filter rates: 10 kHz (analog four-pole Bessel), 1 kHz (digital eight-pole Bessel), 200 Hz (digital eight-pole Bessel), and 40 Hz (digital eight-pole Bessel) (A–D, respectively). The spike marked with an asterisk is shown on an expanded time scale to the right of each trace. Note that, for each spike, the amplitude decreases while the full width at half-height increases with each filter rate.

information concerning the secretory process. These include the frequency of release, the amount released per exocytotic event, the latency following stimulation, and the time course of extrusion from the vesicle. The latter process is extremely rapid, occurring over a time scale of hundreds of microseconds to a few milliseconds. Clearly, to examine the true extrusion time course, minimal filtering must be used. We examined the effects of filter frequency cutoff to improve the measure of spike frequency and to determine the amounts they contain. A low-pass digital filter with corner frequencies of 1 kHz, 200 Hz, and 40 Hz (Figure 6B–D, respectively) was used to evaluate a trace that was collected from a stimulated single DA cell at an initial filter rate of 10 kHz (Figure 6A). With decreasing filter frequency, the number of spikes that are detected by the analysis software increases significantly. However, as shown in the right panels of Figure 6, the time course of individual spikes dramatically increases (by 40-fold in this example) while the amplitude decreases.

The number of molecules released per exocytotic event is obtained by integration of the current as a function of time, which yields charge. Charge is coulometrically related to the number of molecules oxidized. An amperometric trace, filtered at 40 Hz, obtained at a cell exposed to high K^+ is shown in Figure 7A. This trace was also filtered at 200 and 1000 Hz. Of the 95 spikes that were detectable in the 40 Hz-filtered trace, 59 were detected in both the 200 Hz and 1 kHz traces. In Figure 8 the average fractional area of spikes is 0.92 ± 0.02 for the 200 Hz-filtered trace and 0.80 ± 0.02 for the 1 kHz trace, both relative to the 40 Hz filtered trace. Close inspection of the data revealed two reasons

(32) Ehinger, B. *Retina* **1982**, *2*, 305–321.
(33) Nguyen-Legros, J.; Moussafi, F.; Simon, A. *Vision Neurosci.* **1990**, *4*, 547–553.
(34) Djamgoz, M. B.; Wagner, H. J. *Neurochem. Int.* **1992**, *20*, 139–191.
(35) Witkovsky, P.; Dearry, A. *Prog. Retinal Res.* **1991**, *11*, 247–292.
(36) Voets, T.; Neher, E.; Moser, T. *Neuron* **1999**, *23*, 607–615.

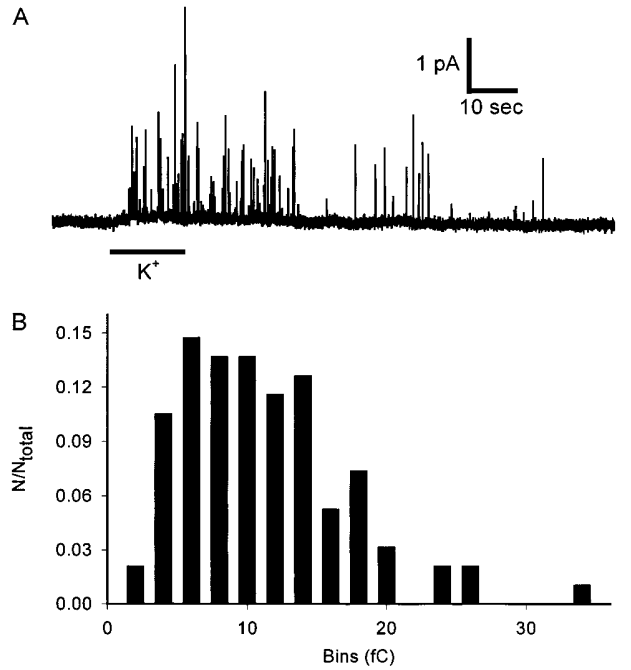

Figure 7. Exocytotic current spikes from DA cells. (A) Application of an elevated K^+ solution to a DA cell results in a series of sharp amperometric spikes. Trace filtered at 40 Hz. (B) Charge distribution from the spikes in (A).

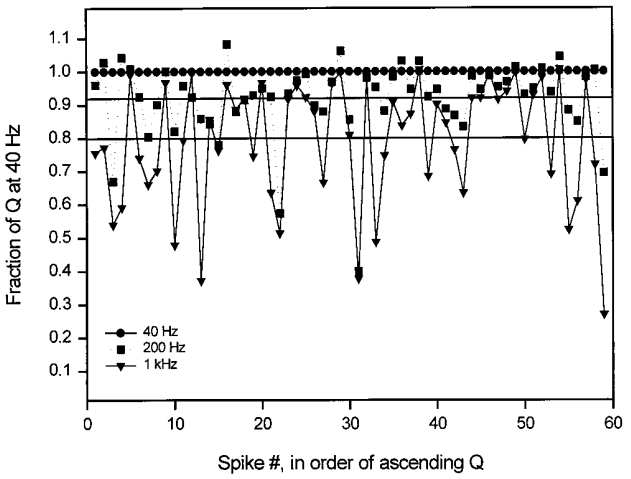

Figure 8. Effect of various filter rates on determined spike area. Of the 95 spikes shown in, 59 of them were detected in separate traces that which had been low-pass filtered at 40 Hz, 200 Hz, and 1 kHz. Areas are shown as a fraction of that obtained for the same spike filtered at 40 Hz. Linear regression lines are overlaid on the 200 Hz and 1 kHz filtered data to demonstrated the fractional area trend.

for this trend. First, when two events occur very close to one another, overfiltering will merge them, yielding a falsely identified single event. Fortunately, with the low frequency of events elicited from DA cells, this is not a significant problem. Second, noise causes uncertainty in the integration limits resulting in an underestimation of the spike area, especially for spikes that emerge slowly from the baseline. A similar error occurs for spikes that tail. Thus, it is imperative that data are filtered at the same cutoff frequency when different data sets are compared.

The histogram of the spike areas (Figure 7B) reveals that the amounts released vary over more than 1 order of magnitude. The mean of the data corresponds to 32 000 molecules with a minimum detected of 4910 molecules (8 zmol) and a maximum of 100 910 (168 zmol). Far fewer spikes would have been detected without low-pass filtering of the amperometric trace.

DISCUSSION

The use of high-quality current amplifiers and appropriate grounding enables extremely small quantities of material to be detected with an amperometric electrode. Theory indicates that the major noise source is the capacitance associated with the electrode, and the experimental measurements verify that this is the case. With a low-noise system, release of dopamine in a spikelike fashion from a single neuron can be detected. The secretory events reported here have the characteristics expected for neuronal exocytosis: small packets of molecules released rapidly and having an absolute dependence for Ca^{2+} in the extracellular fluid. The number of detected exocytotic events increases with increased filtering. With such overfiltering, the temporal information contained in a single spike is severely compromised but the quantity released appears to be more accurately determined. The spikes from the retinal cell shown had a broad distribution and a mean value 32 000 dopamine molecules/spike. Other dopamine cells from the retina examined during this study released similar amounts.

The exocytotic process has evolved into a highly efficient way to deliver chemical signals between neurons. Transmitter substances are stored within intracellular vesicles that are quite small (~50 nm diameter[10,37]). Fusion of the cell and vesicular membranes during exocytosis leads to the extrusion of the vesicle contents that relay information to an adjacent chemical sensor. In vivo, the sensor is a receptor, a specialized protein whose function is altered by the binding of a transmitter. In the experiments reported here, the chemical sensor is the carbon-fiber microelectrode. The concentrations stored within the vesicle are thought to be quite high.[38] The measurements reported here confirm this since the packaging of ~50 zmol, the mean quantity of material detected per spike, into a spherical container the size of a vesicle would result in a concentration of greater than 0.8 M.

Once released, the packet of dopamine molecules diffuses into the extracellular space. In the experiments described here, the contents are confined to a small region between the cell and the electrode, which is firmly placed against it. We estimate this distance to be 50 nm, the approximate roughness of the electrode surface. This separation is greater than that between neurons as seen by electron microscopy.[15]

The flux measured at the electrode can be used with the estimated cell−electrode spacing to estimate the transient dopamine concentration at the cell surface immediately after its exocytotic release. The amperometric current (i) is related to concentration via Fick's first law:

$$i/nFA = D(\mathrm{d}C/\mathrm{d}x) \qquad (4)$$

where n is number of electrons per mole, F is Faraday's constant,

(37) Henderson, L. P.; Kuffler, D. P.; Nicholls, J.; Zhang, R. *J. Phys.* **1983**, *340*, 347−358.

D is the diffusion coefficient of dopamine, and A is the area of the electrode, all of which is in contact with the cell. Approximation of the derivative by the differential and rearrangement yield

$$\Delta C = (i/nFAD)\Delta x \quad (5)$$

where ΔC is the difference in concentration between the cell membrane and the electrode surface and Δx is the distance between them. Assuming D is 5×10^{-6}, the value in free solution,[39] the maximal concentration during a spike of 10 pA maximal amplitude is 90 nM. Since, in amperometric experiments the concentration at the electrode surface is zero, this value is the average concentration distributed over the cell surface. The high concentration in the vesicle rapidly dilutes to a low value in this situation.

The situation in vivo is somewhat different in that contacts between adjacent neurons, i.e., the synapse, have much smaller areas. For dopamine synapses in the rat brain, synapses have typical diameters of 300 nm. Thus, an equivalent release into a space with an area similar to that of a synaptic contact and a spacing identical to the cell−electrode experiments would lead to an instantaneous concentration of 70 μM. Unlike the amperometric experiment, however, synaptic dopamine is not consumed by the opposing cell, but rather binds to its receptors and other proteins such as transporters or diffuses into the extracellular space. In either case, the initially high vesicular concentration is essential so that the released dopamine reaches its receptor site at an effective concentration, thought to be in the nanomolar range.[40] In the specific case of the cells examined in this work, presynaptic dopaminergic contacts do not occur on the cell body,[15] thus long-range diffusion of the released dopamine is the dominant mode of its transference of information. Future studies with these dopaminergic neurons will examine this mode of neurotransmission in greater detail.

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(38) Kandel, E. R., Schwartz, J. H., Jessell, T. M., Eds. *Principles of Neural Science*, 3rd ed.; Elsevier Science Publishing Co., Inc.: New York, 1991.
(39) Gerhardt, G.; Adams, R. N. *Anal. Chem.* **1982**, *54*, 2618−2620.
(40) Richfield, E. K.; Penney, J. B.; Young, A. B. *Neuroscience* **1989**, *30*, 767−777.