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Detection of Secretion with Electrochemical Methods

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Introduction

In the 1950s, Fatt and Katz and Del Castillo and Katz showed that the delivery of neurotransmitters in the frog neuromuscular junction was quantal (Fatt and Katz, 1952; Del Castillo and Katz, 1954). These results contributed to a growing body of evidence that the extrusion of chemical messenger-containing vesicles was by an exocytotic process (Martin, 1966). Development of the patch clamp technique by Neher and Sakmann (Neher and Sakmann, 1976; Hamill et al., 1981) led to the first measurements of membrane capacitance changes believed to be caused by the exocytotic and endocytotic processes (Neher and Marty, 1982). Direct measurement of catecholamines from bovine adrenal chromaffin cells by cyclic voltammetry provided further support for exocytosis (Leszczyszyn et al., 1990). Further study with cyclic voltammetry revealed that the temporally resolved current spikes were due to exocytosis of individual vesicles (Wightman et al., 1991).

Electrochemical techniques such as cyclic voltammetry and amperometry that employ carbon fiber microelectrodes are becoming recognized as the tools of choice for answering questions about exocytosis at the single cell level. Both techniques have been used to characterize exocytosis at bovine adrenal chromaffin cells (Chow et al., 1992; Jankowski et al., 1993; Zhou et al., 1996). Cyclic voltammetry has been shown to be useful to measure release and uptake in the extracellular fluid of the intact brain (Millar et al., 1985; Kuhr and Wightman, 1986; Bunin and Wightman, 1998). Many neuronal and neuroendocrine systems utilize chemical messengers that are easily oxidized. These include epinephrine, norepinephrine, dopamine, serotonin, and histamine, as well as smaller tryptophan and tyrosine containing polypeptide chains such as α-

melanocyte stimulating hormone (α-MSH). Other compounds such as insulin, glutamate, acetylcholine, and choline may also be detected with the use of electron transfer mediators or enzyme modified electrodes. The advantages of electrochemical techniques for the study of exocytosis include submillisecond temporal resolution, chemical identification of the substance being detected, nonintrusive measurements at the single cell level, high sensitivity (detection of a few thousand molecules has been achieved for a single vesicular event in neurons (Bruns and Jahn, 1995)), and high spatial resolution (multiple membrane regions have been examined simultaneously at an individual cell (Schroeder et al., 1994; Robinson et al., 1995)). The purpose of this article is to not only inform readers about practical aspects of electrochemical techniques for use at the single cell level, but also to present general concepts of how electrochemical processes arise and factors that contribute to sensitivity and selectivity of these types of measurements.

Electrochemistry

General Principles

The basic requirements for the types of electrochemical experiments that are used at single cells are two electrodes placed in a common solution, a voltage source, and a picoammeter. One of the electrodes, the reference electrode, should be low impedance and capable of maintaining a constant potential. Early in the development of electrochemical techniques, the normal hydrogen electrode (NHE) was accepted as a standard. For this reason, reference tables of standard potentials are given versus this electrode. In practice, the NHE is difficult to use and, more commonly, the Ag/AgCl electrode is used. By convention, the NHE has a standard potential, or

E^o, of 0 V. and other types of reference electrode's potentials are commonly compared to the NHE. For example, the Ag/AgCl electrode has an E^o of 0.197 V versus the NHE, or $E^o = 0.197$ V vs. NHE, in electrochemical terminology. The Ag/AgCl electrode consists of metallic Ag coated with AgCl in contact with an aqueous solution containing a fixed concentration of chloride ion. This electrode configuration generates a stable potential and is insignificantly perturbed when small currents flow through it. The other electrode in biological applications is typically a carbon fiber microelectrode and it is referred to as the working electrode. By controlling the surface potential of the working electrode and simultaneously measuring the amount of current passing through it, information concerning the charge transfer processes that occur at the interface of the carbon and solution is obtained.

One of the charge transfer processes that can occur is the oxidation or reduction of molecules in the solution phase. Oxidation occurs when the potential of the working electrode is sufficiently positive that a chemical species in solution (e.g. dopamine) donates electrons to the electrode when the species comes in contact with the electrode. Conversely, reduction occurs when the electromotive force (potential) of the working electrode is sufficiently negative that a chemical species in solution (e.g. oxidized form of dopamine) accepts electrons from the electrode when the species comes in contact with the electrode. Molecules that can be oxidized predominate in biological applications, thus the remainder of this discussion will be written in oxidative terms, although the reductive case could be deduced from opposite reasoning.

Another source of current in electrochemical experiments arises from the capacitive nature of the electrode-solution interface. When a positive potential with respect to the reference electrode is applied to the working electrode in the absence of oxidizable species, negative charge in the

form of ions in solution accumulates at the electrode surface. This region of excess charge that extends a few Angstroms into solution is referred to as the diffuse layer. Figure 1 depicts the double layer at a charged electrode.

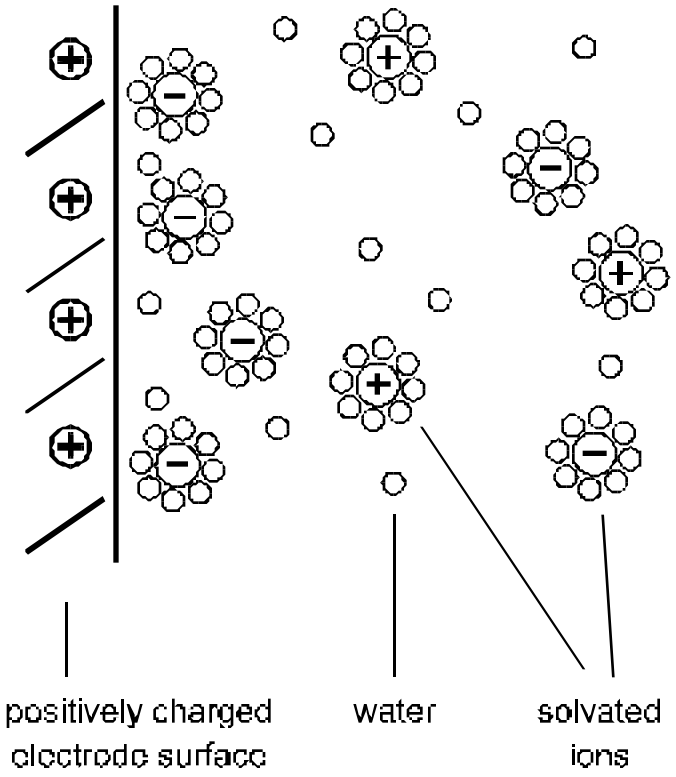

Figure 1

Figure 1. Depiction of the electrical double layer found at a positively charged electrode.

Because the electrode/solution interface has a separation of charge, it acts as a capacitor. However, because of the spatial distribution of the ionic charge on the solution side, the apparent capacitance varies with the concentration and identity of ionic species in solution as well as the potential applied to the working electrode. These are processes that can be described by

sophisticated models of the double layer such as the Gouy-Chapman model (Bard and Faulkner, 1980). In physiological electrolytes where Na^+ and Cl^- are the most concentrated ions with a concentration above 10^{-1} M, the electric field resulting from the potential applied to the working electrode decays through the diffuse layer in a very short distance, about 1 nm (Bard and Faulkner, 1980). For a molecule to "feel" the potential of a charged electrode, it must approach the electrode surface to within a few angstroms to undergo electron transfer. Therefore, electron transfer with an easily oxidized or reduced molecule will not occur until it physically reaches the electrode surface.

There are two major factors that affect the current magnitude arising from electrooxidation of a solution species: mass transport and the rate of electron transfer at the electrode surface. In an unstirred solution, mass transport usually occurs in the form of diffusion that can be described by Fick's first and second laws. (Stirring and convection are other sources of mass transport). Once mass transport brings a molecule up to the potential gradient that exists in the double layer, electron transfer can occur. For oxidation, consider the generalized reaction:

$$\text{Red} \rightleftharpoons \text{Ox} + ne^- \qquad (1)$$

where "Red" is the reduced form of an electroactive molecule, "Ox" is the oxidized form, and ne^- is the number of electrons donated to the working electrode by "Red" for each "Ox" generated. Thermodynamically, the probability that a molecule in reaction scheme (1) will be electrolyzed is given by the Nernst equation:

$$E = E^{o} + \frac{RT}{nF} \ln \frac{C_{Ox}}{C_{Red}} \quad (2)$$

In this equation, E is the potential applied to the carbon fiber surface, E^o is the standard potential which can be found in tables, R is the gas constant, T is the temperature, n is the number of electrons transferred, and F is the Faraday constant. By convention, E^o is measured versus the NHE with the activities of the electrode constituents at standard states. Reducible compounds with more negative E^os are harder to reduce than those with less negative E^os. Correspondingly, oxidizable compounds with more positive E^os are more difficult to oxidize than those with less positive E^os. We consider C_{Ox} and C_{Red} (the surface concentration of Ox and Red) as concentrations rather than activities because normally dilute solutions are measured. To properly account for observed electron transfer processes we need to incorporate the kinetic as well as thermodynamic concept of electron transfer. This can be given by the Butler-Volmer equation for the effect of potential on electron transfer kinetics:

$$j = -k^{\circ} C_{Red}(0,t) \exp\left[\frac{(1-\alpha) n_a F \left(E - E^o\right)}{RT}\right] \quad (3)$$

where j is flux of Red (i.e. epinephrine, dopamine, etc.), $k°$ is the standard rate constant for electron transfer, α is the transfer coefficient, n_a is the number of electrons passed in the rate limiting step in the electrochemical reaction, F is the Faraday constant, E is electrode potential, and C_{Red} is expressed as a function of time and distance. In words, this states that flux is proportional to the electron transfer rate, concentration of unoxidized species, and the amount of overpotential (E-E^o) applied to the electrode surface. At any single potential we can write

$$k_a = -k^\circ \exp\left[\frac{(1-\alpha)n_a F\left(E-E^o\right)}{RT}\right] \quad (4)$$

where k_a is the anodic, or oxidative, electron transfer rate.

To relate $C_{\mathrm{Red}}(0,t)$ to the bulk concentration (C^*_{Red}) we have to consider mass transport. For the diffusion component of current flux, Fick's law describes diffusion in one dimension:

$$j_{\mathrm{Red}} = -D_{\mathrm{Red}}\left[\frac{\partial C_{\mathrm{Red}}(x,t)}{\partial x}\right]_{x=0} \approx -D_{\mathrm{Red}}\frac{C^*_{\mathrm{Red}} - C_{\mathrm{Red}}(0,t)}{\delta} \quad (5)$$

where D_{Red} is the diffusion coefficient of Red, C_{Red} is the concentration of Red at the electrode surface, x is the distance from the electrode surface, t is time, C^*_{Red} is the concentration of Red in the bulk solution, C_{Red} is the concentration of Red at the electrode surface, and δ is a function of the distance the depletion layer of Red extends into solution. Described with words, Fick's first law states that flux is proportional to the concentration gradient of Red at the electrode surface. Under steady state conditions Fick's second law, which describes the time dependence of the current, can be ignored and thus the value of δ remains constant. Then, by combining equations (3) through (5) , and noting that the same flux is described by equations (3) and (5), the following relationship is obtained:

$$j_{\mathrm{Red}} = -\frac{k_a C^*_{\mathrm{Red}}}{1+\frac{k_a \delta}{D_{\mathrm{Red}}}} \quad (6a)$$

Equation (6a) shows that when E, and thus k_a is large the flux of electrons, i.e. the current through the electrode, will be controlled by the rate of diffusion of Red to the electrode surface. Then:

$$j_{\text{Red}} = -\frac{D_{\text{Red}} C_O^*}{\delta} \quad \text{with units of} \quad \frac{\left(cm^2/\sec\right)\left(mol/cm^3\right)}{cm} \qquad \frac{mol}{cm^2 \cdot \sec} \tag{6b}$$

Thus the current is proportional to the bulk concentration through a knowledge of D_{Red} and δ.

For a fixed value of δ, that is one which is time independent, equation (6a) predicts that the current will be time independent, but voltage dependent. In practice, the precise value of δ and its degree of time independence are dictated by the experimental conditions and thus the boundary conditions used to solve Fick's laws of diffusion. However, all solutions to the flux equations lead to a similar form. For time dependent situations, the time dependence appears in δ. For further details of electrochemical theory and methods, see (Bard and Faulkner, 1980). The relation of δ to the experimental conditions for the specific cases of amperometry and cyclic voltammetry are described below.

Description of Electrochemical Techniques

The above concepts apply to all electrochemical techniques in which potential is controlled and the current is measured. In this section, these concepts will be considered with respect to two techniques, amperometry and cyclic voltammetry. Amperometry is a technique in which the electrode is held at a constant potential. The potential is selected so that it is sufficiently positive that the oxidation of the molecule of interest is driven as fast as possible and is therefore diffusion controlled. Current is then measured with respect to time to observe changes in concentration that occur at the electrode surface. Experimentally, the potential at which the

oxidation is diffusion controlled may be determined by increasing the potential of the electrode until a limiting current is reached, i.e., the current is independent of potential.

In cyclic voltammetry, the potential is changed, or swept, in a linear fashion from potentials where no oxidation reactions occur to more positive potentials until the current measured is stable. Potential sweep rates are dependant on both the experimental conditions and the information that is desired. Sweep rates ranging from μVs^{-1} to MVs^{-1} have been employed. The potential sweep is then reversed and allowed to return to the original potential, where the potential remains between scans. On the positive going scan, a cyclic voltammogram has oxidative features and on the negative going scan, reductive features are generated. If oxidized product remains near the electrode surface (i.e., does not completely diffuse away) and is reducible, then a current will flow on the reverse scan when its redox potential is reached.

Diffusion and Amperometry

As an electrode oxidizes solution species at its surface, a concentration gradient of this species is generated. New material is brought to the electrode surface by diffusion from the bulk solution. The region where the concentration gradient exists is termed the diffusion layer and has dimensions given by δ. With micron-scale diameter microelectrodes, the shape of the diffusion layer can varies with the method in which the potential is applied to the electrode. In amperometry, the electrode potential is held constant and diffusion of molecules can occur from regions directly adjacent to the electrode surface and outside the perimeter of the electrode. The latter type of diffusion, termed radial diffusion predominates at long (seconds) time scales and is

depicted in the left portion of Figure 2A. Because of the greater accessibility of molecules to the perimeter of the electrode, current flux at the edge of the electrode is higher than at the center. On the other hand, if the current is observed at μs timescales after a potential step, the diffusion layer is much smaller than the electrode dimensions. Under these conditions, the current is time-dependent because δ is expanding.

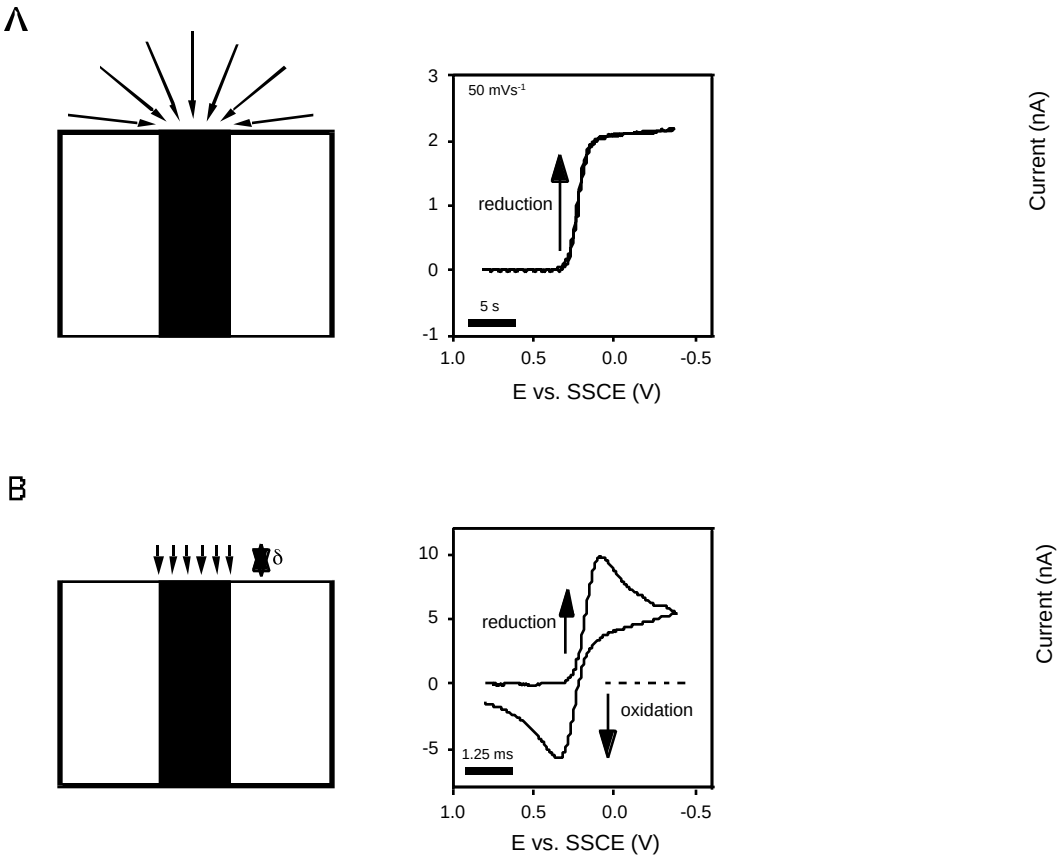

Figure 2

Figure 2. At microelectrodes, two types of diffusion exist. Radial diffusion (A) occurs when the electrode voltage is scanned slowly (generally millivolts to a few volts per second) or is held constant as it is in amperometric mode. On the right hand side is a reduction cyclic voltammogram of 1 mM $K_3Fe(CN)_6$, pH 3, 0.5 M KCl, 50 mVs^{-1}. Note that the text describes oxidation voltammograms with a reversed potential scan, i.e. the resting potential is more negative than switching potential. Linear diffusion (B) occurs at microelectrodes when the timescale of

voltage application is short compared to the case of radial diffusion. Depth of the diffusion layer is given by δ. A corresponding reduction cyclic voltammogram is shown on the right (1 mM $K_3Fe(CN)_6$, pH 3, 0.5 M KCl, 200 Vs^{-1}). In both voltammograms, the reference electrode was a sodium-saturated calomel electrode (SSCE).

Diffusion and Cyclic Voltammetry

Similar diffusion processes occur during cyclic voltammetry. When scanning slowly (on the order of 1 V/sec), radial diffusion occurs and diffusion layer extends significantly beyond the perimeter of the electrode surface. A diagram of radial diffusion and a resulting voltammogram are shown in Figure 2A. (Note that in cyclic voltammetry, electrochemical custom in the United States (Bard and Faulkner, 1980) places more positive potentials to the left on the X-axis, oxidative current as a negative deflection, and reductive current as a positive deflection.) Alternatively, when the timescale of the experiment is significantly reduced by scanning the potential rapidly (on the order of 100 V/s), most of the current that is passed is due to molecules very near the electrode and that diffuse in a perpendicular direction to its plane. This is referred to as linear diffusion. A diagram of linear diffusion and a resulting voltammogram is shown in Figure 2B. In the linear diffusion controlled voltammogram, the potential-dependent rise in current results from a steady increase in the driving force for electron transfer. Eventually, the depletion of material directly adjacent to the electrode surface results in the decay of current, a result that is a time dependent process. This indicates diffusion of unoxidized species is the current limiting process. In the radial diffusion case, diffusion still occurs perpendicular to the electrode surface and the depletion layer has time to extend beyond the volume perpendicular to the electrode surface. This provides an extra volume of “accessible” unoxidized species and prevents a decrease in current even after the maximum electron transfer rate is achieved. As in

the amperometric case, current flux is greatest at the edge of the electrode because of radial diffusion.

Fast Scan Cyclic Voltammetry at Individual Cells

Fast Scan Cyclic Voltammetry (FSCV) is usually employed in biological systems when chemical identification of the substance being detected is required, thus FSCV is a complementary technique to amperometric detection of exocytosis in which the potential of the working electrode is held constant and no species identity information is obtained. (Note: Before the advent of microelectrodes, the fastest rate at which an electrode potential could be feasibly swept was typically limited by the extremely large amplitudes of charging current. At potential sweep rates greater than the V/s range, charging current overwhelms Faradaic current. Therefore, "slow" scan rates refer to those typically employed when using electrodes with electroactive surface areas that are many times larger than those of microelectrodes.) FSCV was used to help verify that catecholamines were responsible for the electrochemical signals in the early papers describing electrochemical detection of exocytotic events from chromaffin cells (Leszczyszynet al., 1990; Leszczyszyn et al.,6-1991). A diagram of a typical experimental arrangement is shown in Figure 3. A carbon fiber microelectrode with micron-scale dimensions is placed adjacent to a cell's plasma membrane with a micropositioner situated on top of an inverted stage microscope. [See the following section titled "Effect of Cell-Electrode Spacing" for a discussion of practical considerations of electrode positioning] Current that arises at the carbon microelectrode is amplified, filtered, and recorded digitally for later analysis. Secretion may be induced chemically via a micropipette or electrically with a variety of methods including the application of the

appropriate agonists, elevation of appropriate extracellular ion concentrations (K^+, Ba^{2+}, etc. for depolarization or direct activation of exocytosis), patch clamp depolarization, and introduction of Ca^{2+} via intracellular injection, among others.

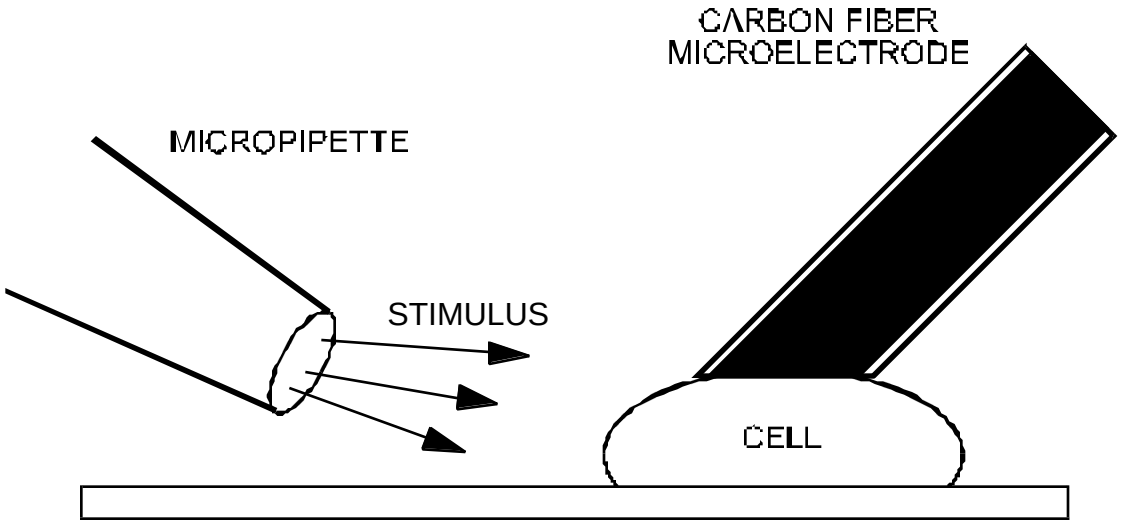

Figure 3

Figure 3. Diagram of a general experimental setup for the electrochemical detection of exocytosis with a carbon fiber microelectrode.

FSCV is implemented by repetitively scanning the potential of an electrode in a triangular fashion with a period of resting time between each scan (Figure 4A). When no electroactive

species is present, the current that is recorded is due to charging of the electrical double layer as described above and depicted by the solid line in Figure 4B. In addition, the carbon surface itself contains moieties that may exist in protonated or deprotonated forms depending upon the applied electrode potential. Surface activity such as proton transfer yields reproducible features in a background voltammogram. For this reason, carbon fiber microelectrodes may be used to detect changes in solution pH by measuring changes in the background current (Jones et al., 1994; Rice and Nicholson, 1989). When an oxidizable species is present at the electrode surface (in this case, epinephrine, inset-Figure 4C), a small additional current passes due to electron transfer and is superimposed on the background current (dashed line in Figure 4B). Because the background current of a carbon fiber microelectrode is extremely stable, it can be subtracted from the current obtained with an oxidizable species present to yield a background-subtracted fast scan cyclic voltammogram as shown in Figure 4C.

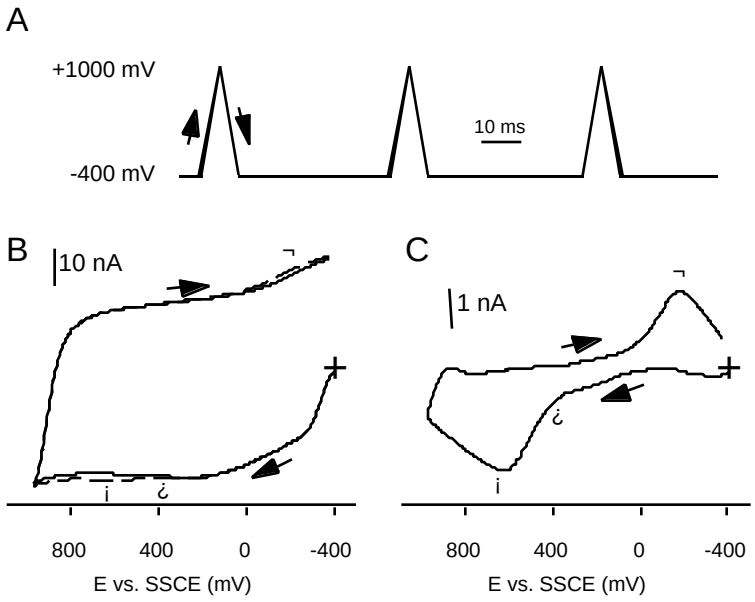

Figure 4

Figure 4. A diagram of the repetitive voltage waveform applied to an electrode for cyclic voltammetric detection of time dependent release (A). The solid line in (B) is the background current trace of the microelectrode with no analyte present. When an analyte (epinephrine in this example) is present, a small observable current is seen overlayed on the background (dashed line in B). Subtracting the background trace from the signal trace yields a background subtracted cyclic voltammogram of epinephrine (C). Scan rate was 300 Vs^{-1} every 100 ms.

The maximum frequency of repetition of scans is the time required for the collapse of the depletion layer such that the next scan "sees" an unperturbed solution phase at the beginning of the scan. The depth of the depletion layer is approximated by

$$\delta = \sqrt{D_{\text{Red}} t} \tag{7}$$

where δ is the depth of the depletion layer, D_{Red} is the diffusion coefficient of unoxidized species, and t is time in which oxidation is occurring.

For example, consider the oxidation of epinephrine at a scan rate of 800 V/s as shown in Figure 4. In the background-subtracted cyclic voltammogram in Figure 4C, the potential sweep (300 Vs^{-1}) begins at the plus symbol. The oxidizing "power" of the electrode increases with positive-going potential and at approximately +400 mV oxidation of epinephrine begins ( ). Oxidation current continues to increase and reaches a maximum at +650 mV ( ). The oxidation current begins to decay thereafter because of depletion of unoxidized epinephrine at the electrode surface. After the maximum oxidation current, oxidation continues and the reaction rate at the electrode surface is limited by the diffusion of unoxidized epinephrine to the electrode surface. At +1000 mV, the potential sweep direction is reversed. The potential is swept in a negative direction and the product that was generated by the forward scan is reduced back to its original form ( ). The total time that oxidation occurs is related to the sweep rate and the time during which an oxidation current is produced. Because oxidation occurs over a range of 600 mV, we can divide this value by the sweep rate to determine total oxidation time. In this example, the total oxidation time is 2 ms. The time necessary for diffusion to restore a uniform concentration of unoxidized dopamine near the electrode surface is approximately ten times the duration of electrolysis, in this example, approximately 20 ms. This number has been determined empirically with the aid of computer modeling. To measure release from chromaffin cells in which the width at half-maximum of the current transient due to exocytosis is on the order of 100 ms, this scan interval is sufficient to quantitate the majority of release events. Higher scan rates and repetition rates would have to be employed to measure very fast exocytotic events such as those found in neurons.

A history of the concentration changes occuring adjacent to the electrode is obtained by examining the current in successive voltammograms at the peak potential of the species of interest. Because all the cyclic voltammograms are stored on a computer, it is an easy matter to examine the current changes that occur at a single potential. Such a trace is shown for release events measured at a mouse mast cell in Figure 5. In the absence of a release event, the measured current would be zero and when an exocytotic event occurs, the measured current would depend on the concentration of neurotransmitter stored in the vesicle. In the case of histamine released from mouse mast cells, the oxidative wave occurs at +1400 mV vs. the sodium saturated calomel reference electrode and the data points are measured at this potential.

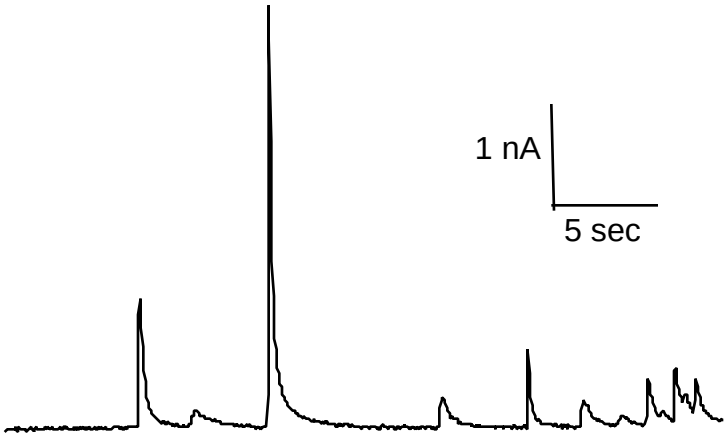

Figure 5

Figure 5. Current spikes resulting from the exocytosis of individual vesicles containing 5-hydroxytryptamine from mouse peritoneal mast cells are shown. Application of a 0.5 μM solution of the Ca^{2+} ionophore A23187 was applied to induce secretion. Current was recorded at the oxidation peak potential of +550 mV vs. SSCE at every 33 ms. The scan rate was 800 Vs^{-1} and the resulting current trace was low pass filtered at 5 kHz.

Effects of Varying Potential Scan Rate

After a molecule is oxidized at the electrode surface it can undergo one or more following behaviors: diffusion away from the electrode surface, reaction with another species in solution, or reaction with itself. By manipulating the voltage scan rate, and thus the time scale of the measurement, one or more of these behaviors can be controlled or eliminated. For example, in the presence of ascorbic acid (AA) the ortho-quinone oxidation product (DOQ) of dopamine (DA) can react with AA to be reduced back to DA. This is the case in the mammalian brain. Schematically, the reaction is

$$\mathrm{DA} \underset{-2\mathrm{H}^+}{\overset{-2\mathrm{e}^-}{\rightleftharpoons}} \mathrm{DOQ} \tag{8}$$

$$\mathrm{DOQ} + \mathrm{AA} \xrightarrow{k_C} \mathrm{DA} + \mathrm{DHA} \tag{9}$$

where DHA is the oxidized form of AA. The dopamine produced in the reaction of DOQ and AA can subsequently be oxidized by the electrode, thus giving rise to a catalytic current due to DA. This results in a catalytically enhanced signal that is difficult to account for with “in the beaker” calibration methods because the exact concentration of AA in the extracellular fluid may vary with time and location. Because the catalytic signal enhancement cannot be accurately determined, *in vivo* DA concentrations may be erroneous. However, in FSCV, the potential of the electrode can be scanned sufficiently rapid such that k_c is not large enough to convert DOQ to DA on the timescale of a complete voltammetric sweep and the catalytic current is eliminated (Dayton et al., 1980).

Norepinephrine (NE) and epinephrine (E) are two compounds that are released from chromaffin cells. To discriminate between norepinephrine (NE) and epinephrine (E) from the same cell, the use of a slow scan rate can be helpful. The methyl group attached to the amine group of E allows intracyclization to occur at a significantly faster rate than NE. By choosing a scan rate that is slow enough to allow intracyclization to occur in E, but not in NE, discrimination between the two compounds is possible. After intracyclization occurs, the resulting product gives rise to a reduction wave on the negative going scan which allows chemical identification. Using a slow scan rate for the detection of single vesicles has the disadvantage of reduced temporal resolution. Despite this disadvantage, some individual exocytotic events can be resolved and it was found that a bovine adrenal chromaffin vesicle may contain either E or NE, and in a few instances, both molecules are present (Ciolkowski et al., 1992).

Amperometry at Individual Cells

Amperometry allows submillisecond release events to be measured. This time resolution is necessary to measure rapid events during exocytosis such as fusion pore flicker (Zhouet al., 1996) and the release of small neuronal vesicles (Bruns and Jahn, 1995). Another feature of amperometry is that the electrode potential is held constant which minimizes capacitive background current. This reduces noise due to small fluctuations in the background that are sometimes observed in FSCV. While low noise and high speed are gained with amperometry, the technique perturbs cellular environments more than FSCV because of the constant presence of an oxidizing potential and its resulting large diffusion layer. The concentration gradient that results from a fixed oxidizing potential leads to an increase in the rate at which the exocytosed vesicular

contents will dissipate into the extracellular space (Schroeder et al., 1996). Although the concentration gradient decreases the measured spike widths due to molecule consumption by the electrode, comparison of spike characteristics under different conditions may be made if electrode size and cell-electrode spacing is the same. It is also important to realize that upon oxidation, catecholamines are converted to a quinone form that is quite reactive. In addition, the quinone form is unable to participate in the hydrogen-bonding that appears to be so important in the interaction of catechols with chromogranin in chromaffin cells.

Jankowski and coworkers found that when release from chromaffin cells into an external medium of pH 5.5, the intravesicular pH, was measured with cyclic voltammetry very few release events occurred (Jankowskiet al., 1993). However, when amperometry was used to measure exocytosis, current spikes where observed when the recording electrode was near the cell membrane. The transient concentration gradient associated with FSCV at the cell's membrane in the absence of driving forces produced by a pH gradient thus were not sufficient to cause release. In contrast with amperometry, the large, constant concentration gradient produced by the electrode was sufficient to "force" release to occur even in the absence of a pH gradient.

The chief disadvantage of amperometry compared to FSCV is the loss of chemical identity information. In any electrochemical experiment, the measured current can arise from oxidation or reduction reactions of either surface-bound or solution phase species as well as capacitance changes. With FSCV, the measured current, when plotted versus potential, gives an identifier in the form of a voltammogram. For amperometry, however, chemical identification of transient current spikes is not possible.

Effect of Cell-Electrode Spacing

When an isolated cell undergoes exocytosis, the vesicular contents are released and are free to diffuse into the extracellular space and are diluted into the external media. For a chromaffin cell vesicle with a radius of 150 nm and a concentration of 0.5 M catecholamine, the concentration on the cell surface immediately following exocytosis will be 0.5 M. However, after diffusion of the catecholamine into a culture medium volume of 1 mL the final concentration will be about 7×10^{-15} M, a very low concentration. Thus, it is important that the volume in which the molecules are allowed to diffuse is kept small. This is accomplished by placing the microelectrode very close to the cell surface. It has been shown theoretically (Schroeder et al., 1992; Wightman et al., 1995) and experimentally (Jankowskiet al., 1993; Wightmanet al., 1995) that placing electrodes more than several microns away from the cell surface results in a significant decrease in signal and spatio-temporal resolution. The best scenario for detecting exocytotic events without diffusional dilution is to actually touch the cell surface with the electrode. Touching the cell is possible without the electrode itself inducing release because the electric field at the electrode surface decays rapidly with distance from the electrode surface. However, close proximity of the electrode to the cell has calibration implications. In amperometry, the diffusion layer, δ, extends several times the radius of the electrode into solution. By placing an electrode near the cell membrane, the cell-electrode spacing restricts the size of δ. Thus, calibration of amperometric measurements at cells is not useful for determination of concentrations of vesicular contents.

Rather, the signal is a measure of the flux of molecules from the cell. However, concentrations can be measured with cyclic voltammetry, because the diffusion layer is small and transient.

Microelectrode Construction

Design Considerations

The criteria for electrodes for use of making exocytotic measurements at the single cell level include a micron scale size measurement area, low-noise, stable in biological extracellular solutions, and facile handling. Carbon is the material of choice for constructing microelectrodes for biological measurements because it is electrochemically stable in biological environments. Also, it can be obtained in the form of carbon fibers with diameters in the micron range, thus meeting the requirement of small size. Encasement of individual carbon fibers in glass or plastic tubing yields facile handling properties. Ideally, the surface area of carbon fiber that is used to make a measurement at a single cell should be approximately equal to the area from which the measurement is made. This is crucial for several reasons. If the experimenter desires to measure exocytosis from an isolated area of the cell, excess electroactive surface area would reduce spatial resolution. Also, if the sides of the electrode are exposed, diffusion of vesicular contents from other regions of the cell or even from other nearby cells could possibly be detected, thus further reducing spatial resolution. More critically, any electroactive surface area that is not useful for the detection of exocytosis increases the electrical capacitance of the electrode which in turn increases the level of baseline noise. Unutilized surface area may result from small cracks in the seal between the carbon fiber and the electrode insulation (i.e. glass or plastic tubing). For this reason, obtaining a robust seal between the electrode insulation and the carbon fiber is extremely

important when constructing carbon fiber microelectrodes. At slow scan rates, the apparent capacitance is larger than at fast scan rates (Wehmeyer and Wightman, 1985).. Thus, if amperometry is chosen for measurements of exocytosis, the construction of low-capacitance electrodes is crucial for low-noise measurements.

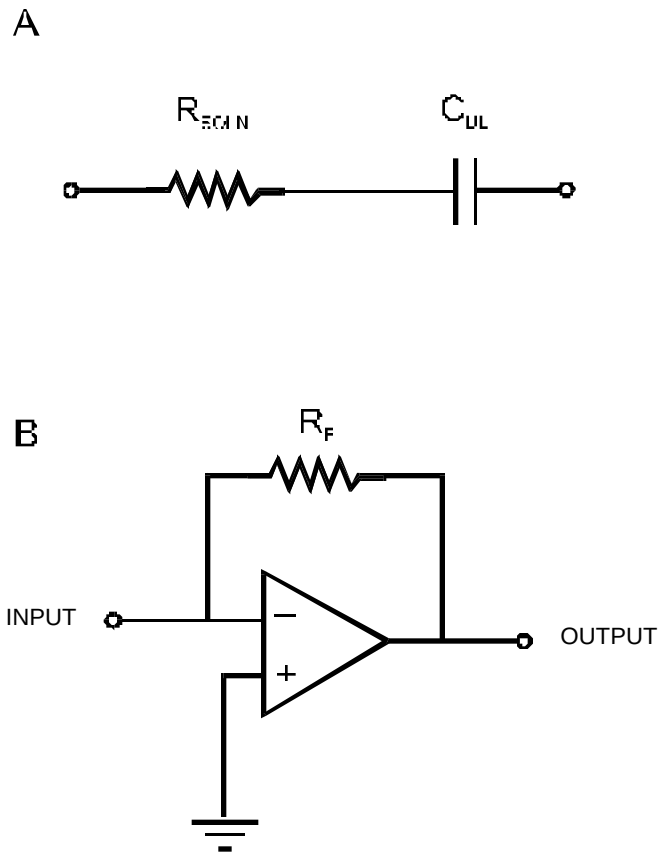

Figure 6

Figure 6. An electrical circuit representation of an electrochemical cell is shown in (A). R_{CT} is the resistance to charge transfer, C_{DL} is the capacitance of the electrical double layer, R_{SOLN} is the solution resistance, and C_{REF} is the capacitance of the reference electrode. In (B), a operational amplifier differentiator circuit is shown.

The effect of electrode capacitance on electrochemical signals can be understood by considering the basic electronic circuitry of a electrochemical measurement. An equivalent circuit of a typical experimental setup is shown in Figure 6A. R_{SOLN} is electrical resistance due to the extracellular solution and C_{DL} is the capacitance associated with the electrode double layer. Figure 6B shows the simplified amplification circuit used to boost the small measured currents before digitization and storage. When the circuit in Figure 6A is placed before the amplification circuit in 6B, the resulting circuit functions as a differentiator. The differentiated input signal appears at the output. To minimize this, C_{DL} should be as small as possible. For more information about the electronic behavior of electrochemical measurement systems, see (Bard and Faulkner, 1980). A simple way to check for a good seal at an electrode is to plot normalized capacitance as a function of potential scan rate (Wehmeyer and Wightman, 1985). If the slope of the line is negative, then leaks or cracks in the seal exist. A perfect seal would yield a slope of zero, implying that normalized capacitance is independent of scan rate.

Materials and Procedure

Several methods and materials are used to construct microelectrodes for electrochemical measurements at single cells. A common characteristic that they all share is the use of carbon fibers as the electrode conductor. In the amperometric mode, low-drift baselines are normally obtained under physiological experimental conditions with carbon fiber electrodes. The choices for insulators include glass (Kawagoe et al., 1993), polymers such as polyethylene (Zhou and Misler, 1995; Chow and Von Ruden, 1995) or polypropylene (Zhou and Misler, 1996), electrodeposited paints (Schulte and Chow, 1996) or polymers (Strein and Ewing, 1992), or

vapor deposited silicon oxide (Zhao et al., 1994). Several methods of microelectrode construction exist including carbon fibers that are encased in glass, polypropylene, or polyethylene. The authors' method of choice is covered in detail in this review. Other methods are described in (Chow and Von Ruden, 1995; Zhou and Misler, 1996).

Glass Microelectrodes

Materials

carbon fibers (Thornel P55, diam. 10 μm, Amoco Oil Products, Greenville, SC USA)
glass capillaries (1.2 mm o.d. x 0.68 mm i.d. x 10 cm, Corning #7740 borosilicate, A-M Systems, Everett, WA USA)
aspirator device (0.25" i.d. Tygon tubing, small rubber septum)
single-stage micropipette puller (Model PF-2, Narishige, Japan)
microscope with calibrated reticle (~100X magnification; Olympus BH-2)
heat plate with stirrer
150 mL beaker
thermometer
oven (Isotemp 500 series, Fisher Scientific, Pittsburgh, PA USA)
backfilling compound (colloidal graphite, Polysciences, Inc., Warrington, PA USA or an aqueous solution of 4 M potassium acetate/150 mM potassium chloride)
nichrome wire (0.4 mm diam.)
sealant (Torr-Seal; Varian Vacuum Products, Lexington, MA USA or white Elmer's glue)
isopropanol (Mallinckrodt Baker, Inc., Paris, KY USA)
foam (0.5" x 1" x 1" pieces)
scintillation vials (20 mL)

Procedure

Carbon fibers (approximately 6 to 8 inches in length) are laid out onto a white surface to aid in the visualization of the individual strands. A light positioned nearby also assists in viewing the fibers. One end of a glass capillary is inserted into Tygon tube fitted with a small rubber septum. The tubing is connected to a water aspirator or other low vacuum generating device and individual fibers are pulled into the capillaries. The capillary is carefully removed from the tubing

when the majority of the fiber has been pulled in the capillary and the ends of the fiber are broken off at the capillary openings. The fiber-loaded capillaries can be easily stored on tape strips or slotted foam blocks throughout the construction procedure. The glass capillaries are then placed into a single stage micropipette puller and are drawn to a fine taper. Taper lengths are typically 10-15 mm. Care must be taken so that a sufficient length of fiber is left in the capillary to make contact with a conductive backfilling compound. Approximately 1 inch of gap between the tapered glass capillaries typically leaves plenty of fiber in the capillary barrel. The carbon fiber is cut in the center to create two electrodes out of one capillary before they are removed from the puller. Next, each electrode is mounted onto a microscope slide with modeling clay that is flattened out on the end of a microscope slide affixed in the stage of a microscope. The tip of the electrode (where the pulled glass meets exposed carbon fiber) should be lying flat on the microscope slide. A surgical scalpel handle fitted with a fresh No. 11 blade is then used to cut the tip where the inner diameter of the capillary is slightly larger than the diameter of the carbon fiber. If the fiber recesses into the capillary, then a gentle bounce (dropping the electrode on its lengthwise side onto a hard surface from about 1 inch) one to several times usually works the fiber out past the capillary opening. The end of the carbon fiber is positioned approximately 10 microns beyond the end of the capillary by carefully pushing it with the tip of the scalpel blade under microscope magnification.

The next step of microelectrode construction is to cement the carbon fiber in place by using a two component epoxy system (Shell Epon 828 resin and MPDA hardener). This resin/hardener combination was chosen because it is inviscid, has extremely good bonding characteristics between the glass and carbon, and its inert behavior in a variety of aqueous and nonaqueous

solvent systems, thus extending the versatility of these electrodes to more general chemical measurements. 3.5 g of the epoxy resin is weighed into a small glass vial (15 mm x 45 mm) and the vial is placed into a stirred water bath with a temperature of 90°C. With a wooden applicator stick, the resin is occasionally stirred as it comes to the desired temperature. Upon reaching the 90°C, 0.5 g epoxy hardener (14% by weight) is added to the vial while stirring the resin. After 2 to 5 minutes, the epoxy mixture will acquire a light tan to brown color at which time one may begin dipping groups (tips first) of 5 or 6 electrodes at a time into the hot epoxy. Approximately 5 mm of the electrode tips should be immersed into the epoxy for 20 seconds. (Make sure the epoxy does not make contact with the electrodes at areas where the multiple electrodes being dipped touch each other.) Slowly, the electrodes are removed, thus not allowing air bubbles to form at the tips, and are place onto a tape strip or other holding apparatus and allowed to set at room temperature overnight. The next day, the electrodes are first placed into an oven heated to 100°C. After one hour, the electrodes should be transferred to an oven heated to 150°C and allowed to cure at least overnight. In our laboratory, longer curing times (3-5 days) at 150°C are used which result in improved electrode stability and response times under experimental conditions.

After curing the epoxied electrodes, easily accessible external contact must be made with the carbon fiber. Several substances may be used to make the electrical connection including colloidal graphite and nichrome wire or a concentrated potassium acetate/KCl solution with a chlorided silver wire as a connection wire (Rice and Nicholson, 1989). To chloride-coat the silver connecting wire, it is partially submerged in a household bleach solution (Clorox, Oakland, CA) for 20-30 minutes, removed, and allowed to dry. Injecting colloidal graphite into the electrode is

the backfilling method most often employed by the authors. A spinal needle (25 gauge x 3.5 inches) is used to inject the isopropanol/graphite suspension into the electrode capillary. If a preamplifier which has an alligator clip as the connection method, a length of nichrome wire (5 cm x 0.4 mm) is secured in the back end of the electrode capillary with Elmer's white glue or Torr Seal to prevent evaporation. If a pipette holder similar to the type used in patch clamp experiments is used, the electrode is backfilled with either colloidal graphite or potassium acetate/KCl solution and is inserted into the holder which contains a bare silver or nichrome wire for the colloidal graphite backfill or a chlorided silver wire for the potassium acetate solution backfill.

Electrode Surface Preparation

After the microelectrode is fabricated, the surface must be treated so that the carbon fiber is clean and easily positionable around a cell. Some researchers trim the glass and fiber tip with a scalpel. This procedure is simple, but a few caveats exist: the glass may not break exactly where the scalpel is positioned leading to larger than desired overall electrode diameters, or the glass/epoxy/fiber seal may be compromised which can lead to increases in baseline electrical noise of electrode. The method preferred by our laboratory is to use an electrode beveler fitted with a polishing wheel coated with appropriately sized diamond particles (Model BV-10-B with 104D diamond abrasive plate, Sutter Instrument Co., Novato, CA USA). The electrode is polished at 45 degrees and a white line is painted on one side of the electrode with white correction fluid for positioning guide. This procedure produces a clean and consistent electrode tip surface as shown in Figure 7.

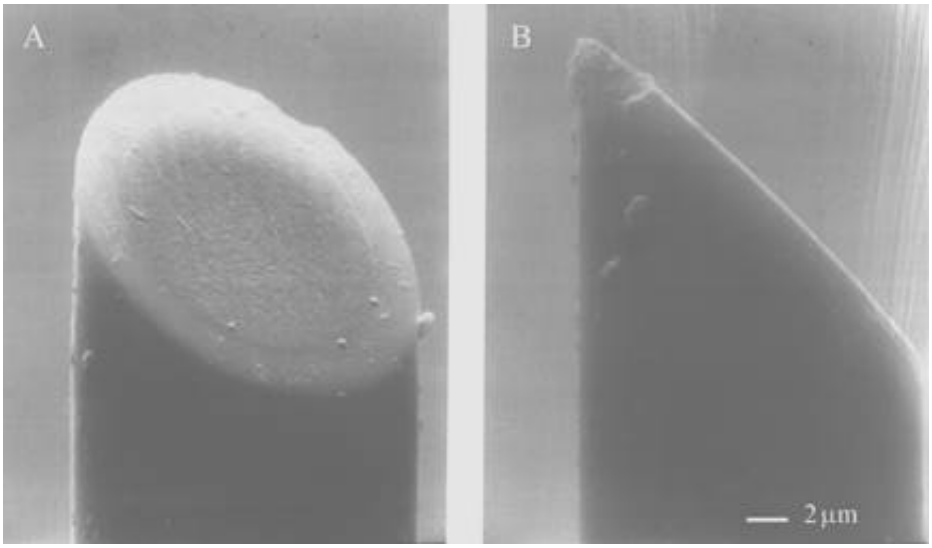

FIGURE 7

Figure 7. A scanning electron microscopic (SEM) surface view of a glass-encased carbon fiber microelectrode beveled at 45° is shown in (A). The carbon fiber was Thornel P55, Amoco, Greenville, SC, USA. An SEM side-view of the same electrode is shown in (B).

Another advantage of a smooth surface is that surface area in contact with a cell membrane is maximized and diffusion of exocytosed vesicular contents from other areas of the cell are not detected, thus increasing spatial resolution of the measurement. To clean debris left by the polishing process or other obstructing matter from the electrode surface, the electrode tip should be soaked in a neat isopropanol solution for 15 minutes prior to use. Slits cut into a small piece of square-shaped foam are adequate at holding electrodes in a 20 mL scintillation vial of isopropanol. The authors have found that this is an important step for consistent performance characteristics of microelectrodes. Electrodes may be repolished after using them for single cell measurements of exocytosis, however, the overall tip dimensions increase with successive polishing rendering the microelectrode more difficult to position near an isolated cell. Also,

increases in the baseline noise level of electrodes that have been repolished when compared to the noise level of the electrode during its initial use have been observed by the authors.

Other Electrode Geometries: Cylinder and Flame-etched Microdisk

For certain *in vivo* applications where sensitivity of the carbon fiber microelectrode is of great concern, an electrode geometry which increases electroactive surface area is desired. Cylinder-type electrodes increase surface area while maintaining a small diameter and are easily constructed with a few modifications to the above protocol. After the capillary glass and fiber are cut with a scalpel, the desired length (usually 50 to 500 μm) of carbon fiber is allowed to protrude beyond the capillary opening. Then, when the electrodes are removed from the epoxy solution, they are immediately rinsed with acetone for several seconds to remove epoxy from the fiber, while allowing the epoxy to remain at the capillary/fiber junction. Upon curing and backfilling, the electrodes are ready for use (Cahill et al., 1996).

Electrodes with extremely small diameters (~500 nm to 2 μm) may be constructed using a variation of the cylinder construction method. A 500 μm length of fiber is allowed to protrude beyond the capillary surface and the electrode is subsequently epoxied. Electrodes are cured for 1 h in a 100°C oven and 2 hr in a 150°C oven. To etch the electrode to a conical shape, the protruding fiber is passed through the base of a laboratory burner flame for a few seconds. Care should be taken that excessive melting of the glass capillary does not occur. A poly(oxyphenylene) polymer can then be used to insulate the electrode surface. A detailed description of the insulating procedure may be found in (Kawagoe et al., 1991) with modified

procedures found in (Strein and Ewing, 1992). Briefly, several etched electrode tips are connected in parallel and placed into 50:50 methanol:water solution containing 2-allylphenol (0.23 M), allylamine (0.4 M), and 2-butoxyethanol (0.23 M). A platinum wire is also placed in solution and 4 V is placed between the electrodes and the platinum wire with the platinum wire held at ground. Electropolymerization occurs and after 2 to 4 minutes the electrodes are removed and placed into an oven at 150°C for 1 h for polymer crosslinking to take place. To remove the insulation at the tip, the electrode is connected to one lead of an ohmmeter (in the hundreds of megaohm range) and is placed in the carriage of an electrode beveler that has a polishing wheel fitted with a cloth wick attached to a wire. The other lead of the ohmmeter is connected to the wick's wire. Water is used to wet the polishing wheel. The electrode is slowly lowered onto the polishing surface and when the insulation is polished away, a finite resistance is measured and the electrode is backed away from the polishing wheel surface within a few seconds of tip breakthrough. Electrodes polished in this manner typically have diameters on the order of 1 μm and may be used in applications where subcellular surface measurements are desired. An example of how flame-etched electrodes have been used is the discovery of zones of exocytosis in bovine adrenal medullary cells (Schroederet al., 1994).

Instrumentation

General considerations

Of key importance in making equipment-related decisions is quality of the equipment. Mechanical stability, easy employment, low-drift, low-noise, high-bandwidth, vibration free are terms that describe a well-implemented experimental setup for the measurement of the minute

electrochemical signals generated at the single cell level. Brand names suggested by the authors for the various components of a single-cell measurement setup will be listed at the end of this section. This does not mean that there are not other brands suited to the task of single cell measurements, they are the manufacturer's in which the authors are familiar and use on a regular basis.

Microscope

The inverted-stage microscope should have enough room on its stage to mount at least two micromanipulators, one for holding the working electrode/headstage assembly and the other to hold a micropipette or other device for stimulating the cells. Because the microscope provides the foundation for the measurement equipment, its stage (including the culture dish holder) should be solidly built so that its attachments are held firmly in place. Magnification ranges from 300X to 600X are adequate for the visualization of electrode positioning. Phase contrast, bright field, and differential interference contrast (DIC, or sometimes called Nomarski) illumination optics are helpful in different circumstances. Sometimes, when bright field illumination doesn't yield the desired image contrast, phase contrast optics may be employed. DIC illumination is a high-resolution, adjustable contrast illumination method that can be helpful in visualizing small cells and/or neuronal processes. The microscope should be placed on a vibration isolation table to eliminate vibration at the electrode tip. Usually these tables are constructed of a heavy plate resting on pneumatic balloons. If there is little "traffic" in the area of the microscope and/or the microscope is in a basement of a quiet building where building vibrations are less prevalent, a thick steel plate resting on a partially inflated tire inner tube may suffice in eliminating vibrations.

Micromanipulators

Micromanipulators should be solidly built, have low-drift characteristics and be smoothly adjustable. Adjustments in position are made several ways including micrometer-like control, hydraulic, and piezoelectric. Coarse adjustments are usually made with a micrometer-like control and remote-controlled piezoelectric tubes are used for fine electrode positioning. Hydraulic controls are very smooth, but sometimes are prone to drift. When mounting micromanipulators on the microscope stage, care should be taken that the base of the manipulator to the electrode tip distance is as short as possible to prevent excessive vibration at the electrode tip.

Data acquisition

To analyze and further manipulate acquired data, it is most convenient for the data to be stored digitally. The digitizing board should have 16-bit resolution and should be capable of sampling in the kilohertz range. An economical yet reliable data storage method that meets the above criteria is the use of a A/D VCR adapter and VCR. The model the author's use is a Medical Systems Corp. PCM-2 and it allows two channels to be simultaneously digitized at 44 kHz with 16-bit voltage resolution onto a standard videocassette (6 hours of data per tape depending on the tape length and VCR speed). Some experiments may be computer controlled and digitizing occurs under software control. It may be desirable to have a "raw" copy of the data alongside the computer acquired data to have a high bandwidth backup copy in case the filter rates and/or gain settings were improperly chosen at the time of recording. The data from the VCR can then be played back through the VCR A/D adapter and further analyzed.

Electrical noise

To shield the experimental setup from electromagnetic fields emanating from sources such as lights and other nearby electrical equipment, the entire system should be housed in a grounded Faraday cage. The Faraday cage may be constructed simply out of a wood or metal frame that is wrapped in a contiguous conductive screen. Copper is the metal of choice for the screen, but it is relatively expensive compared to common aluminum window and door screen found in local hardware stores. Noise with a frequency of 60 Hz is common and can be nearly eliminated by simple measures. Equipment power cords should be kept apart from signal carrying wires to prevent crosstalk. The power supply for the illuminator should be a DC power supply. Even DC power supplies can have small amounts of "ripple" in them, so sometimes it may be best to turn the illuminator off while recordings are being made. All conductive equipment inside the Faraday cage including the Faraday cage should be grounded to a common connection point that exists at signal ground to prevent "hum" caused by ground loops. Some experiments are computer controlled and in these cases the monitor should be placed as far from the microscope as possible because a computer monitor produces high electric fields which can cause noise during measurements. In some cases, because the electric field emanates from the sides of the monitor, turning the monitor 45 or 90 degrees may reduce noise. The connection between the headstage and electrode should be kept as short as possible because the assembly can act as an antenna and even minute amounts of noise will affect the unamplified signal. All connections between the electrode and digitizing equipment should be in pristine condition to avoid noise introduced by "stray capacitance". Finally the potentiostat and headstage should be well designed for low

noise, high bandwidth measurements. Some manufacturers electrically cool the headstage to reduce fundamental noise (Johnson noise) that exists in the large feedback resistor of the headstage. Because the signals measured in patch clamp recordings also require low noise and high bandwidths, the headstages and potentiostats that have been designed for those types of experiments work extremely well in amperometric measurements.

Manufacturers

This list of manufacturers includes only those used by the authors.

Inverted-stage microscopes: Zeiss
Micromanipulators: Newport (micrometer), Narishige (micrometer), Kopf (hydraulic), Burleigh (piezoelectric)
Potentiostats: Axon Instruments Axopatch 200B for amperometry, Ensman Instrumentation EI-400 for cyclic voltammetry (now distributed by Cypress Systems)
Oscilloscopes: Nicolet, Tektronix
VCR based data recording: Medical Systems PCM-2 (A/D VCR adapter), Panasonic Pro Line (VCR)
Vibration isolation tables: Newport

Techniques in Conjunction with Amperometry

Amperometry by itself is a sensitive technique for quantitating various aspects of cellular exocytosis including easy screening for release, quantal size, temporal spike distribution, temporal characteristics of the exocytotic event, and fusion pore dynamics. Amperometry has been used in conjunction with other electrophysiological techniques to further the range of biological questions that can be answered. Patch clamp (Chowet al., 1992; Robinsonet al., 1995), ratiometric dye measurements of Ca^{2+} (Finnegan and Wightman, 1995), and patch amperometry

(Albillos et al., 1997) are examples of techniques which have been combined with electrochemical measurements.

Electrically excitable cells are conveniently and routinely studied with the patch clamp technique. In the voltage clamp mode, stepwise depolarization of the cell membrane yields a temporally discrete trigger for exocytosis which can be measured with the patch clamp electrode itself or with a carbon fiber microelectrode operated in the amperometric mode. When a vesicle fuses to the cell membrane an increase in whole-cell membrane capacitance is observed. However, the size of the capacitive transient is proportional to vesicle size and vesicles found in most neuronal cell types are very small (tens to hundreds of nanometers) and are therefore difficult to detect individually with patch clamp. With a second micromanipulator, an amperometric electrode may be positioned near the cell to detect exocytotic events. In 1992, Chow and coworkers measured average spike latency after membrane depolarization with a patch clamp electrode in chromaffin cells (Chowet al., 1992). Robinson and coworkers combined patch clamp stimulation, intracellular Ca^{2+} indicator, and amperometry with flame etched electrodes to provide evidence of localization of exocytosis with Ca^{2+} influx in chromaffin cells (Robinsonet al., 1995). By compiling spike latency histograms, Chow and coworkers back-calculated the time course of intracellular Ca^{2+} concentration during stimulation (Chow et al., 1994).

Other forms of discrete electrical depolarization methods such as intracellular (non-carbon fiber) microelectrode implantation have also been used in concert with amperometry in serotonin-containing Retzius cells by Bruns and Jahn in 1995. They observed two pools of vesicles with differing spike latencies and half-widths and postulated that the pools correspond to the small clear vesicles and large dense core vesicles commonly observed by electron microscopy in

Retzius cells and other neuronal systems. The smaller amperometric spikes were calculated to have resulted from the oxidation of only 4,700 molecules of 5-hydroxytryptamine, demonstrating the capability to characterize small and fast neuronal-type exocytotic events with amperometry.

A recently developed technique which combines patch clamp in the cell-attached mode with an amperometric electrode positioned within the patch clamp electrode was described (Albilloset al., 1997). With the patch pipette in the cell-attached configuration on bovine adrenal medullary cells, the capacitive transients due to transient and/or irreversible vesicular fusion are readily detectable. The carbon fiber subsequently detected any transient or full-fusion exocytotic event that occurred thus further characterizing the fusion pore and release of chemical messenger by allowing release to be correlated with changes in the patch capacitance and conductivity.

The use of fluorescent chelators such as fura-2, fluo-3, and indo-1 have been used to quantify intracellular concentrations of Ca^{2+}, an important trigger for exocytosis. With the fluorescent method of real-time Ca^{2+} observation combined with amperometry, independent observation of intracellular Ca^{2+} activity and exocytosis can be made. A successful union of temporally (millisecond timescale) and spatially (micron dimensions) correlated fluo-3 dye detection of intracellular Ca^{2+} with amperometric detection of exocytosis in chromaffin cells is demonstrated in the previously mentioned work of Robinson et al. (1995). A novel approach of simultaneously detecting vesicular stores of Ca^{2+} and epinephrine was recently developed (Xin and Wightman, 1998). A bead-bound form of the fluorescent Ca^{2+} indicator Calcium Green was coated on a carbon fiber microelectrode and was positioned near a bovine adrenal medullary cell. Caffeine, which raises internal stores of Ca^{2+} was used as the stimulant so that external Ca^{2+}

could be eliminated. Ca^{2+} and epinephrine where detected on a cellular level, i.e. individual events were not observed.

Other Examples of Electrochemical Monitoring of Exocytosis

Structure and subsequent mechanisms of the vesicle-plasma membrane machinery are of current interest. Evidence of fusion pore formation has been collected by amperometry in the form of a pre-spike feature (Chowet al., 1992; Wightmanet al., 1995), or foot, and small, rapid fluctuations in amperometric signal, or flicker (Zhouet al., 1996). Small amounts of unbound transmitter are believed to freely diffuse through the fusion pore that has a calculated average diameter of a few nanometers. A pre-spike feature is shown in Figure 8a and a flicker trace is shown in Figure 8b.

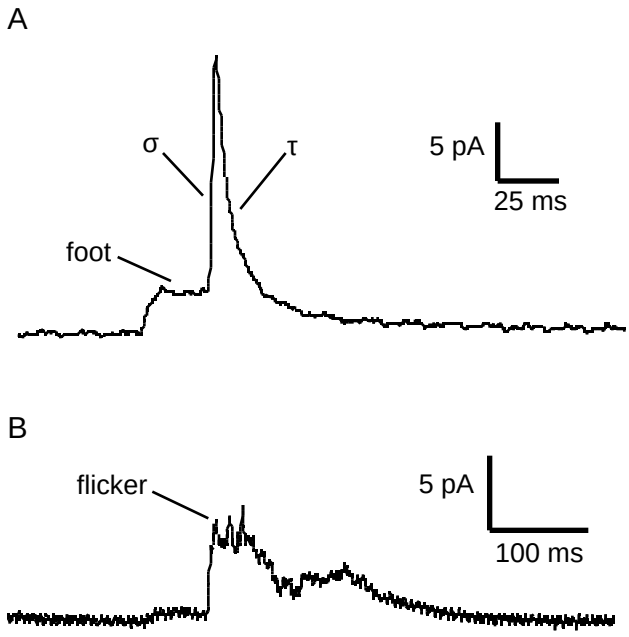

Figure 8

Figure 8. An example of a exocytotic current spike with a pre-spike feature, or foot, is shown in (A). Characteristics of release can be quantified by σ and τ, which represent extrusion and unraveling of the vesicle into extracellular space, respectively. Specifically, exocytotic spikes have an exponentially-modified Gaussian shape; σ is the standard deviation of the Gaussian component of the spike and τ is the decay time constant for the exponential component of the spike. The fusion pore, which gives rise to the foot shown in (A), can sometimes open and close rapidly without undergoing full fusion and release, this is termed flicker and is shown in (B). Both current traces were collected from bovine adrenal medullary chromaffin cells.

The values of σ and τ are other parameters that have been used to characterize kinetics of the exocytotic process and correspond to the expulsion and subsequent dissociation of vesicular contents. Specifically, exocytotic spikes have an exponentially modified Gaussian shape; σ is the standard deviation of the Gaussian component of the spike and τ is the decay time constant for

the exponential component of the spike. A proposed model for the exocytotic process is shown in Figure 9 (Schroederet al., 1996).

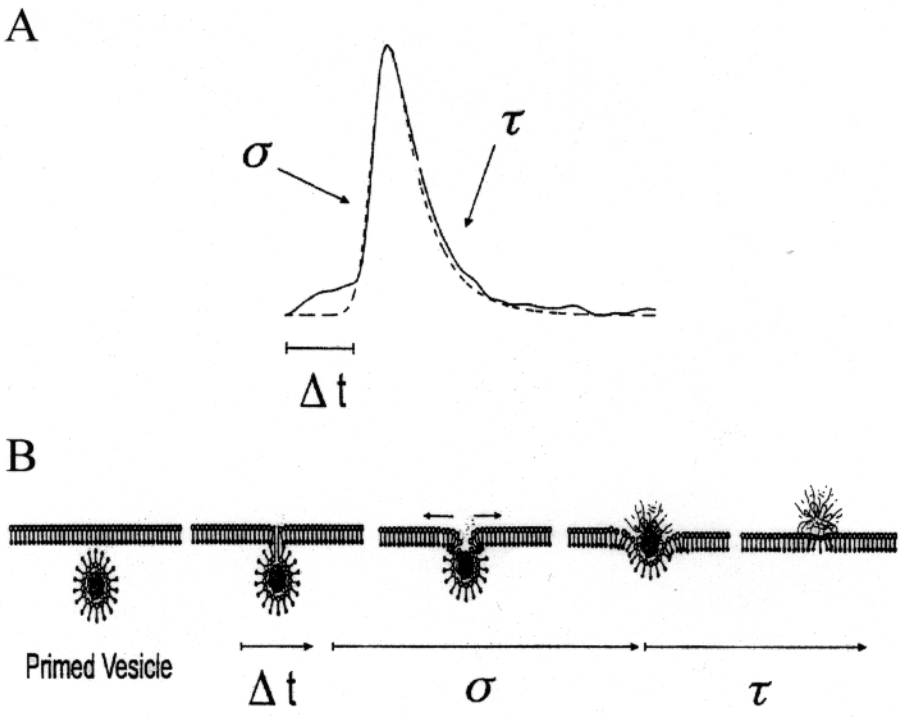

Figure 9

Figure 9. An exocytotic spike with a foot is shown in (A). A cartoon of a proposed model for exocytosis is shown in (B). A primed vesicle approaches the plasma membrane. Formation of a fusion pore can allow free intravesicular transmitter to diffuse out of the vesicle-fusion pore complex yielding the signal labeled Δt. One interpretation of the spike shape is that expansion of the fusion pore gives rise to the signal designated by σ and dissociation of chemical messenger from the vesicular matrix is depicted by τ. The solid line is an actual recorded spike and the dashed line is the exponentially-modified Gaussian fit. ((Schroeder et al., 1996), used by permission)

Osmotic gradients have been shown to play a role in the dissociation of vesicular contents into the extracellular space (Borges et al., 1997). Amperometry was used to measure spike shape characteristics such as risetime and width at half-height to compare hypo-, hyper-, and iso-

osmotic extracellular conditions. Intravesicular osmolarity is higher than that normally found in the extracellular solution and upon reducing the osmotic gradient by elevating extracellular osmolarity. After stimulation by increasing Ca^{2+} and Ba^{2+} (triggers for exocytosis) dissociation of vesicular contents can be slowed or even shut down as determined by amperometry. Extrusion of vesicular contents may subsequently occur with no further stimulation by restoring an osmotic gradient by the application of isotonic buffer. Thus, temporal separation of divalent cation entry from the exocytotic event was permitted.

Simultaneous detection of the co-release of 5-hydroxytryptamine (5-HT) and histamine from rat mast cell vesicles was accomplished with FSCV (Pihel et al., 1995). Because the oxidation waves for 5-HT and histamine are located at significantly different electrode potentials, detection of each compound is made possible during the same scan. By manipulating cell content of either 5-HT or histamine or their precursors, it was shown that the dissolution of the heparin sulfate-based vesicular matrix is dependent upon the concentration ratios of 5-HT and histamine found in the vesicle. Similarly, FSCV may be used to differentiate between epinephrine and norepinephrine in chromaffin cells (Ciolkowskiet al., 1992; Pihel et al., 1994). It was found that cells may contain either epinephrine or norepinephrine with a small fraction of cells containing both compounds that sometimes were coreleased from the same vesicle (Pihelet al., 1994).

Electrode Surface Modifications

Some compounds that are not directly oxidizable may be electrochemically detected by making appropriate electrode modifications. Histamine detection is made possible by electrochemically pretreating the electrode surface (Pihelet al., 1995). A triangular voltage

waveform from +0.1 to +1.4 volts was applied at 800 Vs^{-1} every 30 ms to an electrode immersed in a solution of a 0.1 M NaOH for 15s. This treatment does not preclude fast electrode reactions and has been used to detect the corelease of histamine and 5-HT from rat mast cells in the experiments described in the previous section.

A chemically modified microelectrode to detect insulin was developed by Kennedy et al (Kennedy et al., 1993). The electrode surface was electrochemically coated with a polynuclear ruthenium oxide/cyanoruthenate film that allowed catalytic oxidation of surface-oriented disulfide bonds of insulin. Insulin is secreted from pancreatic β-cells and upon culturing, individual islets of Langerhans may be isolated. When a modified electrode was placed 1 μm from a cell and held at a potential of +0.85 V vs. a sodium-saturated calomel electrode (SSCE), time-resolved current spikes were observed after stimulation with either high K^+ or glucose. Insulin was thought to be the main component that gave rise to the spikes. Temporal dynamics of insulin release at the individual cell level had not previously been recorded.

Conclusions

In this review, we have briefly described electrochemical concepts such as electron transfer and diffusion and how they affect the signal that arises at microelectrodes. Electrochemical techniques such as amperometry and cyclic voltammetry have been described. The application of these two techniques at single cells for the detection of exocytosis is becoming popular due to their ability to unintrusively detect quantal release, sensitivity, and temporal resolution. Cell types which have been studied include: chromaffin (Wightmanet al., 1991), mast (Pihelet al.,

1995), pancreatic β (Kennedyet al., 1993), pheochromocytoma (PC-12) (Chen et al., 1994), pituitary melanotrophs (Paras and Kennedy, 1995), giant dopamine neurons of the *Planorbis corneus* (Chen et al., 1995), Retzius cells of the leech *Hirudo medicinalis* (Bruns and Jahn, 1995), and superior cervical ganglion (SCG) neurons (Zhou and Misler, 1995). Advancements in cell culture and dissociation techniques will undoubtedly contribute to additional types of cells and neurons that can be studied by electrochemical methods. In the future, electrochemical methods combined with spectroscopic and other electrophysiological techniques will certainly play a role in solving unanswered questions of the exocytotic and neural transmission process.

Acknowledgement

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Bibliography

Albillos, A., G. Dernick, H. Horstmann, W. Almers, G. Alvarez de Toledo, and M. Lindau. 1997. The exocytotic event in chromaffin cells revealed by patch amperometry. *Nature* 389:509-512.

Bard, A.J. and L.R. Faulkner. 1980. Electrochemical Methods: Fundamentals and Applications. John Wiley and Sons, Inc., New York.

Borges, R., E.R. Travis, S.E. Hochstetler, and R.M. Wightman. 1997. Effects of external osmotic pressure on vesicular secretion from bovine adrenal medullary cells. *Journal of Biological Chemistry* 272:8325-8331.

Bruns, D. and R. Jahn. 1995. Real-time measurement of transmitter release from single synaptic vesicles. *Nature* 377:62-65.

Bunin, M.A. and R.M. Wightman. 1998. Measuring Uptake Rates in Intact Tissue. In Neurotransmitter Transporters (A Volume of Methods in Enzymology). S.G. Amara, editor. Academic Press, New York.

Cahill, P.S., Q.D. Walker, J.M. Finnegan, G.E. Mickelson, E.R. Travis, and R.M. Wightman. 1996. Microelectrodes for the measurement of catecholamines in biological systems. *Analytical Chemistry* 68:3180-3186.

Chen, G., P.F. Gavin, G. Luo, and A.G. Ewing. 1995. Observation and quantitation of exocytosis from the cell body of a fully developed neuron in Planorbis corneus. *Journal of Neuroscience* 15:7747-7755.

Chen, T.K., G. Luo, and A.G. Ewing. 1994. Amperometric monitoring of stimulated catecholamine release from rat pheochromocytoma (PC12) cells at the zeptomole level. *Analytical Chemistry* 66:3031-3035.

Chow, R.H., J. Klingauf, and E. Neher. 1994. Time course of Ca^{2+} concentration triggering exocytosis in neuroendocrine cells. *Proceedings of the National Academy of Sciences of the United States of America* 91:12765-12769.

Chow, R.H., L. von Ruden, and E. Neher. 1992. Delay in vesicle fusion revealed by electrochemical monitoring of single secretory events in adrenal chromaffin cells. *Nature* 356:60-63.

Chow, R.H. and L. Von Ruden. 1995. Electrochemical Detection of Secretion from Single Cells. In Single Channel Recording. B. Sakmann and E. Neher, editors. Plenum Press, New York. 245-275.

Ciolkowski, E.L., B.R. Cooper, J.A. Jankowski, J.W. Jorgenson, and R.M. Wightman. 1992. Direct Observation of Epinephrine and Norepinephrine Cosecretion from Individual Adrenal Medullary Chromaffin Cells. *Journal of the American Chemical Society* 114:2815-2821.

Dayton, M.A., A.G. Ewing, and R.M. Wightman. 1980. Response of Microvoltammetric Electrodes to Homogeneous Catalytic and Slow Heterogeneous Charge-Transfer Reactions. *Analytical Chemistry* 52:2392-2396.

Del Castillo, J. and B. Katz. 1954. Quantal Components of the End-Plate Potential. *Journal of Physiology* 124:560-573.

Fatt, P. and B. Katz. 1952. Spontaneous Subthreshold Activity at Motor Nerve Endings. *Journal of Physiology* 117:109-128.

Finnegan, J.M. and R.M. Wightman. 1995. Correlation of real-time catecholamine release and cytosolic Ca^{2+} at single bovine chromaffin cells. *Journal of Biological Chemistry* 270:5353-5359.

Hamill, O.P., A. Marty, E. Neher, B. Sakmann, and F.J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Archiv - European Journal of Physiology* 391:85-100.

Jankowski, J.A., T.J. Schroeder, E.L. Ciolkowski, and R.M. Wightman. 1993. Temporal characteristics of quantal secretion of catecholamines from adrenal medullary cells. *Journal of Biological Chemistry* 268:14694-14700.

Jones, S.R., G.E. Mickelson, L.B. Collins, K.T. Kawagoe, and R.M. Wightman. 1994. Interference by pH and Ca^{2+} ions during measurements of catecholamine release in slices of rat amygdala with fast-scan cyclic voltammetry. *Journal of Neuroscience Methods* 52:1-10.

Kawagoe, K.T., J.A. Jankowski, and R.M. Wightman. 1991. Etched carbon-fiber electrodes as amperometric detectors of catecholamine secretion from isolated biological cells. *Analytical Chemistry* 63:1589-1594.

Kawagoe, K.T., J.B. Zimmerman, and R.M. Wightman. 1993. Principles of voltammetry and microelectrode surface states. [Review] [51 refs]. *Journal of Neuroscience Methods* 48:225-240.

Kennedy, R.T., L. Huang, M.A. Atkinson, and P. Dush. 1993. Amperometric monitoring of chemical secretions from individual pancreatic beta-cells. *Analytical Chemistry* 65:1882-1887.

Kuhr, W.G. and R.M. Wightman. 1986. Real-time measurement of dopamine release in rat brain. *Brain Research* 381:168-171.

Leszczyszyn, D.J., J.A. Jankowski, O.H. Viveros, E.J.J. Diliberto, J.A. Near, and R.M. Wightman. 1991. Secretion of catecholamines from individual adrenal medullary chromaffin cells. *Journal of Neurochemistry* 56:1855-1863.

Leszczyszyn, D.J., J.A. Jankowski, O.H. Viveros, E.J. Diliberto, Jr., J.A. Near, and R.M. Wightman. 1990. Nicotinic receptor-mediated catecholamine secretion from individual chromaffin cells. Chemical evidence for exocytosis. *Journal of Biological Chemistry* 265:14736-14737.

Martin, A.R. 1966. Quantal Nature of Synaptic Transmission. *Physiological Reviews* 46:51

Millar, J., J.A. Stamford, Z.L. Kruk, and R.M. Wightman. 1985. Electrochemical, pharmacological and electrophysiological evidence of rapid dopamine release and removal in the rat caudate nucleus following electrical stimulation of the median forebrain bundle. *European Journal of Pharmacology* 109:341-348.

Neher, E. and A. Marty. 1982. Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells. *Proceedings of the National Academy of Sciences of the United States of America* 79:6712-6716.

Neher, E. and B. Sakmann. 1976. Single-channel currents recorded from membrane of denervated frog muscle fibres. *Nature* 260:799-802.

Paras, C.D. and R.T. Kennedy. 1995. Electrochemical detection of exocytosis at single rat melanotrophs. *Analytical Chemistry* 67:3633-3637.

Pihel, K., S. Hsieh, J.W. Jorgenson, and R.M. Wightman. 1995. Electrochemical detection of histamine and 5-hydroxytryptamine at isolated mast cells. *Analytical Chemistry* 67:4514-4521.

Pihel, K., T.J. Schroeder, and R.M. Wightman. 1994. Rapid and Selective Cyclic Voltammetric Measurements of Epinephrine and Norepinephrine as a Method To Measure Secretion from Single Bovine Adrenal Medullary Cells. *Analytical Chemistry* 66:4532-4537.

Rice, M.E. and C. Nicholson. 1989. Measurement of Nanomolar Dopamine Diffusion Using Low-Noise Perfluorinated Ionomer Coated Carbon Fiber Microelectrodes and High-Speed Cyclic Voltammetry. *Analytical Chemistry* 61:1805-1810.

Robinson, I.M., J.M. Finnegan, J.R. Monck, R.M. Wightman, and J.M. Fernandez. 1995. Colocalization of calcium entry and exocytotic release sites in adrenal chromaffin cells. *Proceedings of the National Academy of Sciences of the United States of America* 92:2474-2478.

Schroeder, T.J., R. Borges, J.M. Finnegan, K. Pihel, C. Amatore, and R.M. Wightman. 1996. Temporally resolved, independent stages of individual exocytotic secretion events. *Biophysical Journal* 70:1061-1068.

Schroeder, T.J., J.A. Jankowski, K.T. Kawagoe, R.M. Wightman, C. Lefrou, and C. Amatore. 1992. Analysis of diffusional broadening of vesicular packets of catecholamines released from biological cells during exocytosis [published erratum appears in Anal Chem 1993 Oct 1;65(19):2711]. *Analytical Chemistry* 64:3077-3083.

Schroeder, T.J., J.A. Jankowski, J. Senyshyn, R.W. Holz, and R.M. Wightman. 1994. Zones of exocytotic release on bovine adrenal medullary cells in culture. *Journal of Biological Chemistry* 269:17215-17220.

Schulte, A. and R.H. Chow. 1996. A Simple Method for Insulating Carbon-Fiber Microelectrodes Using Anodic Electrophoretic Deposition of Paint. *Analytical Chemistry* 68:3054-3058.

Strein, T.G. and A.G. Ewing. 1992. Characterization of Submicron-Sized Carbon Electrodes Insulated with a Phenol-Allylphenol Copolymer. *Analytical Chemistry* 64:1368-1373.

Wehmeyer, K.R. and R.M. Wightman. 1985. Scan Rate Dependence of the Apparent Capacitance at Microvoltammetric Electrodes. *Journal of Electroanalytical Chemistry* 196:417-421.

Wightman, R.M., J.A. Jankowski, R.T. Kennedy, K.T. Kawagoe, T.J. Schroeder, D.J. Leszczyszyn, J.A. Near, E.J. Diliberto, Jr., and O.H. Viveros. 1991. Temporally resolved catecholamine spikes correspond to single vesicle release from individual chromaffin cells. *Proceedings of the National Academy of Sciences of the United States of America* 88:10754-10758.

Wightman, R.M., T.J. Schroeder, J.M. Finnegan, E.L. Ciolkowski, and K. Pihel. 1995. Time course of release of catecholamines from individual vesicles during exocytosis at adrenal medullary cells. *Biophysical Journal* 68:383-390.

Xin, Q. and R.M. Wightman. 1998. Simultaneous Detection of Catecholamine Exocytosis and Ca^{2+} Release from Single Bovine Chromaffin Cells Using a Dual Microsensor. *Analytical Chemistry* 70:

Zhao, G., D.M. Giolando, and J.R. Kirchhoff. 1994. Fabrication of Silica-Coated Carbon-Fiber Ultramicroelectrodes by Chemical Vapor Deposition. *Journal of Electroanalytical Chemistry* 379:505-508.

Zhou, Z. and S. Misler. 1995a. Action potential-induced quantal secretion of catecholamines from rat adrenal chromaffin cells. *Journal of Biological Chemistry* 270:3498-3505.

Zhou, Z. and S. Misler. 1995b. Amperometric detection of stimulus-induced quantal release of catecholamines from cultured superior cervical ganglion neurons. *Proceedings of the National Academy of Sciences of the United States of America* 92:6938-6942.

Zhou, Z. and S. Misler. 1996a. Amperometric detection of quantal secretion from patch-clamped rat pancreatic beta-cells. *Journal of Biological Chemistry* 270:270-277.

Zhou, Z., S. Misler, and R.H. Chow. 1996b. Rapid fluctuations in transmitter release from single vesicles in bovine adrenal chromaffin cells. *Biophysical Journal* 70:1543-1552.