Acta Physiol 2008, **192**, 173–184

REVIEW

Measuring secretion in chromaffin cells using electrophysiological and electrochemical methods

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Received 2 June 2007, accepted 2 June 2007
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Abstract

Our present understanding of exocytosis of catecholamines has benefited tremendously from the arrival of single-cell electrochemical methods (amperometry and voltammetry), electrophysiological techniques (whole-cell and patch capacitance) and from the combination of both techniques (patch amperometry). In this brief review, we will outline the strengths and limitations of amperometric and electrophysiological methods and highlight the major contribution obtained with the use of these techniques in chromaffin cells.

Keywords adrenal, amperometry, catecholamine, cell capacitance, exocytosis, patch amperometry.

The theory for quantal release of transmitter received the most direct confirmation when electrophysiological and electrochemical techniques for single-cell recording of exocytosis became available. The cell of choice for these studies has been the adrenal chromaffin cell starting from the seminal papers first describing patch-clamp capacitance measurements (Neher & Marty 1982), amperometry (Leszczyszyn *et al.* 1990) and patch amperometry (Albillos *et al.* 1997). Our current understanding of exocytosis includes the concept of both partial and full fusion, identification and characterization of cytosolic and integral membrane proteins that participate in exocytosis and unravelling of some of the regulatory mechanisms of the stimulus–secretion cascade. Here, we will describe strengths and weaknesses of these techniques and highlight some of the major findings that have been obtained with these approaches over the last two decades.

Patch-clamp capacitance measurements as an assay of exocytosis and endocytosis

The electrical capacitance of the cell membrane is proportion to its area; therefore, insertion of membrane during exocytosis leads to an increase in membrane capacitance; conversely, endocytosis leads to a decrease in membrane capacitance. Whereas experiments describing increases in membrane capacitance associated with exocytosis date back to experiments by K.S. Cole in the 1930s (Cole 1935), the modern era of high-resolution measurements began in 1982, only several years after the invention of the patch-clamp gigaohm seal. In their landmark paper, Neher & Marty (1982) described changes in membrane capacitance associated with exocytosis and endocytosis in chromaffin cells. They found that, just as patch-clamp techniques can resolve the currents because of the opening and closing of individual ion channels, capacitance measurements can resolve the fusion and fission of individual vesicles with millisecond time resolution (Debus & Lindau 2000). Unlike single-channel recording, however, capacitance measurements were slow to be adopted and only a handful of laboratories used the technique in the 1980s. Capacitance measurements blossomed in the 1990s, partly because of the emergence of powerful software packages that greatly simplified application of the method. The capacitance technique continues to make a large contribution towards a mechanistic understanding of exocytosis and endocytosis in chromaffin cells and currently

 doi: 10.1111/j.1748-1716.2007.01814.x

requires nothing more than a patch-clamp amplifier and one of several commonly used software packages.

Strengths and applications of the technique

Table 1 summarizes some of the applications of the technique with emphasis on work performed using chromaffin cells. A great strength of the technique is that, under appropriate conditions, *the time course of individual 'quantal' fusion and fission events can be resolved*. Step-like increases in capacitance serve as an important confirmation of the quantal hypothesis. The reversibility of the fusion event is demonstrated by step decreases of the same size as immediately preceding step increases. The kinetics of rapid fusion/fission events ('kiss and run' exocytosis) can be resolved. In addition, impedance measurements from membrane patches can be used to follow the formation and expansion of the 'fusion pore' conductance that results from the nanometer-sized initial connection between the vesicle lumen and the outside of the cell (Breckenridge & Almers 1987, Zimmerberg *et al.* 1987) (also see references in Table 1, line 1).

A feature that is both a strength and a weakness is that capacitance measurements *uniquely monitor the process of membrane fusion* independently from the discharge of vesicle contents. Carbon fibre amperometry and styryl dye (e.g. FM1-43) fluorescent measurements are powerful techniques for measuring the efflux of material from vesicles during exocytosis. However, the steps of vesicle membrane fusion cannot unambiguously be distinguished from transmitter efflux steps with these assays. Isolating the fusion step allows development of quantitative models relating $[Ca^{2+}]_i$ to exocytosis (Table 1, line 2). As discussed below, patch amperometry is a powerful combination of techniques to allow correlation of single-vesicle fusion kinetics with the discharge of vesicle contents (Albillos *et al.* 1997).

An important positive feature of patch-clamp capacitance measurements are that they *allow a high level of control over parameters that trigger and modulate exocytosis*. Voltage clamp control allows one to depolarize the cell in a controlled manner and measure the relationship between Ca^{2+} influx and exocytosis (Table 1, line 3). Application of toxins that block specific Ca^{2+} channels can be used to determine the coupling between a particular Ca^{2+} channel type and exocytosis or endocytosis. In general, it is possible to determine which steps in the stimulus–secretion cascade, (membrane depolarization leading to Ca^{2+} influx leading to Ca^{2+}-triggered exocytosis) are affected by an experimental manoeuvre such as application of a drug or activation of a receptor or second messenger system (Table 1, line 4). References to work investigating

Table 1 Applications of membrane capacitance measurements in chromaffin cells

Applications of membrane capacitance measurements	References
Analysis of single-vesicle fusion/fission events	Neher & Marty (1982), Chow *et al.* (1996), Albillos *et al.* (1997a), Moser & Neher (1997), Ales *et al.* (1999), Henkel *et al.* (2001a,b), Dernick *et al.* (2003), Elhamdani *et al.* (2006a) and Neef *et al.* (2007)
Coupling of $[Ca^{2+}]_i$ to exocytosis	Augustine & Neher (1992), Neher & Zucker (1993), von Ruden & Neher (1993), Chow *et al.* (1994), Heinemann *et al.* (1994), Smith *et al.* (1998), Voets (2000), Voets *et al.* (2001a) and Yang *et al.* (2002)
Coupling of Ca^{2+} channels to exocytosis or endocytosis	Artalejo *et al.* (1994), Horrigan & Bookman (1994), Engisch and Nowycky (1996), Moser and Neher (1997), Engisch *et al.* (1997), Fomina and Nowycky (1999), Lukyanetz and Neher (1999), Powell *et al.* (2000), Ulate *et al.* (2000), Albillos *et al.* (2000), Aldea *et al.* (2002), Xu *et al.* (2005), Polo-Parada *et al.* (2006), Rosa *et al.* (2007) and Wykes *et al.* (2007)
Receptor or second-messenger mediated regulation of exocytosis	Gillis *et al.* (1996), Lim *et al.* (1997), Liu and Misler (1998), Smith (1999), Powell *et al.* (2000), Ulate *et al.* (2000), Teschemacher and Seward (2000), Harkins and Fox (2000), Nagy *et al.* (2002), Brede *et al.* (2003), Carabelli *et al.* (2003), Ennion *et al.* (2004), Fulop and Smith (2006), Polo-Parada *et al.* (2006), Bauer *et al.* (2007), Yang *et al.* (2007) and Zeniou-Meyer *et al.* (2007)
Regulatory mechanisms in endocytosis	Artalejo *et al.* (1995, 1996), Smith & Neher (1997), Engisch & Nowycky (1998), Henkel *et al.* (2000), Chan & Smith (2001, 2003) and Elhamdani *et al.* (2006b)
Role of specific proteins in exocytosis or endocytosis	Penner *et al.* (1986), Schweizer *et al.* (1989), Johannes *et al.* (1994), Kibble *et al.* (1996), Johannes *et al.* (1998), Xu *et al.* (1998), Xu *et al.* (1999), Elhamdani *et al.* (1999), Wei *et al.* (2000), Voets *et al.* (2001a), Xu *et al.* (2002), Sorensen *et al.* (2002), Pan *et al.* (2002), Nagy *et al.* (2002), Sorensen *et al.* (2003), Yizhar *et al.* (2004), Henkel *et al.* (2004), Speidel *et al.* (2005), Nagy *et al.* (2006), Bauer *et al.* (2007) and Yang *et al.* (2007)

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regulatory mechanisms of endocytosis using capacitance measurements in chromaffin cells are summarized in Table 1, line 5.

Whole-cell patch-clamp capacitance measurements also allow easy manipulation of the intracellular environment of the cell. In particular, fluorescent indicators, drugs, peptides and soluble second messengers can be loaded into a cell by diffusion from the pipette solution. For example, Ca^{2+} bound to a high-affinity photo-labile chelator (cage) can be introduced into cell. Photolysis of the cage with ultraviolet light can be used to elevate $[Ca^{+}]_i$ uniformly throughout the cell, while the time course of exocytosis is measured using the capacitance technique (e.g. Heinemann *et al.* 1994, reviewed by Sorensen 2004). This approach is the most direct way currently employed to measure the relationship between $[Ca^{2+}]_i$ and exocytosis. Here, the high temporal resolution of capacitance measurements is critical because the most rapid phases of Ca-triggered release, thought to reflect fusion of the most physiologically relevant 'primed' pool of vesicles, are complete within a fraction of a second.

An important attribute shared by the capacitance technique and carbon fibre amperometry are that they are *single-cell assays*. This allows one to tease apart cell-to-cell variability from the overall response of a cell population. This also facilitates study of the effects of protein expression on exocytosis or endocytosis even under conditions where only a small fraction of a cell population expresses the gene of interest. Cells expressing a fluorescent marker protein, such as GFP, can be individually selected for patch-clamp and/or amperometric recordings. This approach has allowed detailed study of numerous proteins that play a role in exocytosis and endocytosis. Table 1, line 6, lists a sampling of work that uses capacitance measurements to assay the role of specific protein perturbations on exocytosis from chromaffin cells.

Weaknesses of the technique

The greatest weakness of the capacitance technique is that *it is not simple to separate exocytosis from endocytosis* if the two processes occur simultaneously. This is less of an issue in recordings from membrane patches where step changes in capacitance because of fusion or fission of single vesicle can be resolved. By contrast, in whole-cell or perforated patch recordings, unitary events cannot be resolved and increases in capacitance may underestimate the rate of exocytosis if endocytosis is occurring at the same time. Under certain experimental conditions, such as following mild stimulation during whole-cell recording, the separation of exocytosis and endocytosis is relatively clear because the rate of exocytosis is much faster than endocytosis. On the other hand, endocytosis is more rapid in chromaffin cells when the stimulus elevates $[Ca^{2+}]_i$ to greater than $\sim$30 μM or when the cellular contents are better preserved such as during perforated patch recording (Smith & Neher 1997). Under these conditions great caution must be applied in quantitatively relating capacitance changes to exocytosis and endocytosis. Here, dual application of carbon fibre amperometry can be used to confirm the relationship between capacitance increases and exocytosis.

Increases in membrane capacitance may result from membrane insertion processes other than fusion of transmitter-containing vesicles. Experiments using photorelease of caged Ca^{2+} have shown Ca^{2+}-induced increases in capacitance in cell types, such as epithelial cells and fibroblasts, that do not contain transmitter-laden vesicles (Coorssen *et al.* 1996, Chen *et al.* 2001). In addition, even in chromaffin cells, photorelease of caged Ca^{2+} to high levels leads to increases in capacitance without a corresponding release of catecholamine detected with a carbon fibre electrode (Oberhauser *et al.* 1996, Xu *et al.* 1998). These Ca^{2+}-induced increases in capacitance unrelated to transmitter release are not prominent unless $[Ca^{2+}]_i$ is elevated to high levels ($> \sim 50$ μM) and are generally slower and thus can be distinguished kinetically from the signal of interest (Xu *et al.* 1998, Chen *et al.* 2001). Nevertheless, it is important to confirm that capacitance increases correspond to transmitter release using an independent method, especially when applying the technique to a new preparation.

Capacitance changes may result from phenomena other than changes in membrane surface area. The capacitance of a biological membrane is usually assumed to be a constant with a value of $\sim$10 fF μm^{-2}. However, the exact value of the specific capacitance varies with the density of mobile charges in integral membrane proteins such as voltage-gated ion channels. For example, recovery of Na^{+} channels from inactivation can produce a voltage- and time-dependent change in capacitance following steps in membrane potential that is unrelated to exocytosis or endocytosis (Horrigan & Bookman 1994). This ion channel-related capacitance change can be eliminated from the capacitance trace by subtraction. In addition, large changes in membrane resistance can cause artefacts in capacitance measurements unless great care is taken (Chen *et al.* 2001).

The capacitance technique is limited to cell types that can be modelled with a simple equivalent circuit. The usual application of the capacitance technique is based on a simple, three-component equivalent circuit representation depicted in Figure 1 and thus can only be

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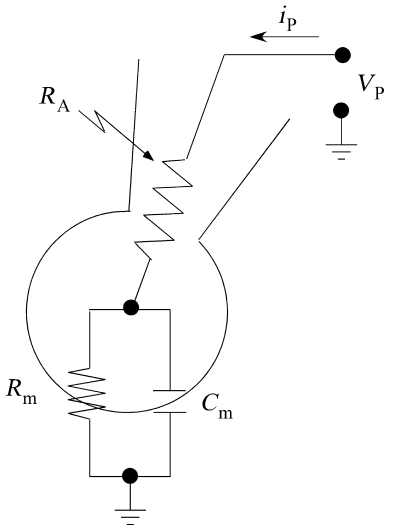

Figure 1 The equivalent circuit commonly used as a basis for capacitance measurements during patch-clamp recordings. The whole-cell patch-clamp configuration is illustrated, but the same equivalent circuit, with different parameter values, applies to on-cell recordings from membrane patches. R_A is the 'access' resistance through the patch-clamp pipette, whereas R_m and C_m are the membrane resistance and capacitance respectively.

applied to preparations, such as chromaffin cells and other endocrine cells, that have a single membrane compartment. Special neural cells, such as cochlear hair cells and retinal bipolar nerve terminals (von Gersdorff & Matthews 1994), are also amenable to the technique. Capacitance measurements have recently been made in pre-synaptic terminals at the calyx of Held (Sun *et al.* 2004) and in hippocampal mossy fibre terminals (Hallermann *et al.* 2003). These preparations are not always well described by a single membrane compartment model; however, simulations indicate that the estimated capacitance change faithfully follows changes in the terminal capacitance (Hallermann *et al.* 2003, Sun *et al.* 2004). The extension of the capacitance technique to explicitly account for more complex equivalent circuits than represented in Figure 1 is likely to be an important future direction for increasing the applicability of this powerful technology.

Variations of the technique

We present here only a very brief survey of techniques for estimating changes in membrane capacitance related to surface area changes; see Gillis (1995, 2000) for a detailed treatment. All current techniques are based on the three-component equivalent circuit of the recording configuration depicted in Figure 1. In this circuit, we neglect the pipette capacitance because the current passing through this pathway is electronically subtracted upon proper adjustment of the pipette capacitance compensation circuitry of the patch-clamp amplifier. During whole-cell recording of a chromaffin cell, typical parameters are $C_m \sim 6$ pF, $R_A \sim 8$ MΩ and $R_m > 2$ GΩ. For on-cell recording, R_A is somewhat smaller and C_m is a fraction of a pF.

The most common approach to estimating capacitance changes is to apply a sinusoidal voltage stimulus and analyse the resulting sinusoidal current. The amplitude of the stimulus sinusoid is usually less than 50 mV, whereas the frequency ranges from ~1 kHz for whole-cell recordings to 50 kHz or higher for on-cell measurements. A significant limitation is that a single sinusoid provides only two pieces of information (magnitude and phase), whereas there are three unknown components of the equivalent circuit. We describe next two approaches used to obtain the additional information needed.

In the '*sine + dc' approach*, the dc (average) current is measured and used, together with an estimate of the extrapolated zero-current potential, to estimate the dc resistance ($R_A + R_m$) (Lindau & Neher 1988, Pusch & Neher 1988). This approach is incorporated in the 'PULSE' and 'PATCHMASTER' software packages (HEKA, Lambrecht, Germany) and is currently the most widely used capacitance method for whole-cell recording. In the simplest version of the sine + dc approach the assumed reversal potential does not change during the recording, but it is not difficult to extend the technique to account for slow zero-current potential changes because of activation of a membrane conductance (Okada *et al.* 1992, Chen *et al.* 2001). The sensitivity of capacitance estimates to errors in the value of the assumed reversal potential is small if R_m is high (GΩ range). More complicated multi-sinusoid or square-wave approaches are necessary if R_m is both small and rapidly changing.

The '*piecewise linear' approach* is the original implementation of the patch-clamp capacitance technique developed by Neher & Marty (1982). A sinusoid voltage stimulus is applied and a phase-sensitive detector (lock-in amplifier) is connected to the patch-clamp amplifier to extract the component of the sinusoidal current that is proportional to changes in membrane capacitance. The output signal of the lock-in amplifier, when set to an appropriate phase, is directly proportional to changes in membrane capacitance but has little sensitivity to changes in R_A or R_m. Dithering of the membrane capacitance compensation knob of the patch-clamp amplifier is used both to find the appropriate phase setting of the lock-in amplifier and to calibrate the capacitance signal. A subsequent variant of the technique (phase tracking) uses computer-controlled dithering of series resistance introduced between the bath and ground to find the

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appropriate phase setting (Fidler & Fernandez 1989). The piecewise-linear approach was initially quite popular because it can be implemented entirely in hardware (e.g. in the Cairn Optopatch patch-clamp amplifier), however, it has largely been replaced by more powerful (and less *ad hoc*) computational approaches for whole-cell capacitance measurements. Nevertheless, the use of this approach with a hardware lock-in amplifier is still commonly used for on-cell capacitance measurements because there are technical difficulties in using software-based approaches for the high-frequency sinusoids (>20 kHz) necessary for low-noise on-cell recordings (Debus & Lindau 2000). A recent report, however, has demonstrated the ability of the PATCHMASTER software package to perform high-frequency, low-noise, on-cell capacitance measurements (Neef *et al.* 2007).

A number of *multi-sinusoid approaches* are also used. In the case of two sinusoids, there are four pieces of information to determine the values of the three unknown parameters. Optimal use of the information to 'fit', i.e. estimate the parameters with the minimal variance 'noise', is a complex problem that is thoroughly addressed in a study by Barnett & Misler (1997). A number of suboptimal, but computationally simpler, *ad hoc* approaches to estimate the equivalent circuit parameters from dual sinusoid excitation have also been implemented (Donnelly 1994, Rohlicek & Schmid 1994, Santos-Sacchi 2004). A challenging problem with all multi-sinusoid approaches is optimizing the choice of amplitudes and frequencies of the stimuli and therefore these approaches tend to give noisier estimates of membrane capacitance than single-sinusoid techniques. In addition, the non-ideal frequency dependence of C_m results in an underestimate of R_m (K.D. Gillis, unpublished observations).

Approaches using *square-wave stimuli* are also used. In response to a square step in pipette potential, the equivalent circuit depicted in Figure 1 will respond with a transient current that decays with an exponential time course. Fit of the current transient to an exponential function can be used to produce estimates of the three circuit parameters. An excellent implementation of this approach is described in (Thompson *et al.* 2001). This approach can be quite robust in that estimates of C_m can be generated that are quite insensitive to changes in R_A and R_m and C_m estimates can be produced with nearly as low a noise as sinusoidal techniques (Thompson *et al.* 2001). This method requires a high bandwidth setting of the patch-clamp amplifier, a high sampling rate and is computationally intense. Nevertheless, an efficient algorithm running on a modern computer can generate estimates at $\sim$ 100 Hz (Thompson *et al.* 2001).

Concluding remarks concerning the capacitance technique

Any of the above techniques, when carefully applied, can produce valid estimates of changes in membrane capacitance related to exocytosis and endocytosis; so, the choice of technique often depends on finding an attractive software package. In general, for capacitance techniques to be truly useful, they must be embedded within a powerful, flexible software package that is capable of executing complex stimulus protocols while recording and displaying multiple data streams in real time.

Electrochemical methods

Single-cell amperometry is the simple miniaturization of electrochemistry (Kissinger *et al.* 1974) commonly implemented using carbon fibre microelectrodes (Ponchon *et al.* 1979) placed onto a cell to act as sensor. It allows detecting several biological amines with high sensitivity and time resolution. Amperometry is a very powerful tool for the *in vitro* study of the mechanisms underlying secretion from single cells or tissues and it is relatively simple and inexpensive to use.

What can and cannot be measured with electrochemical methods in chromaffin cells

Electrochemistry is limited to detecting those substances that can be oxidized or reduced in a range of voltage of –400 to +1450 mV. Higher potentials will produce the electrolysis of water, thus creating oxygen bubbles on the surface of the electrode. Within this range, a large number of biological substances can be theoretically analysed by electrochemistry including certain neurotransmitters such as biological amines (adrenaline, noradrenaline, dopamine, histamine and serotonin), some peptides, ascorbic and uric acids and some of the metabolic products derived from these substances (Pihel *et al.* 1994). The principle electroactive products released by chromaffin cells are catecholamines (CA). Although adrenaline and noradrenaline can be separated by cyclic voltammetry (Ciolkowski *et al.* 1992) for most experimental purposes, it is not crucial to know which CA is being released.

Amperometry with carbon fibre microelectrodes allows the detection of the released CA during a single exocytotic event. The time course of the amperometric 'spike' event, is thought to reflect the kinetic of release from an individual secretory vesicle that fuses near the electrode tip (Chow *et al.* 1992a). Amperometry, however, is blind to all processes occurring before vesicle unloading and cannot detect exocytosis from empty vesicles, or from vesicles with very low content of amines.

A detailed description of the equipment needed and general advice for application of the technique are covered in a recent review (Machado *et al.* 2008). For the analysis of amperometrical spikes, there is a number of commercially available software or free shareware (Segura *et al.* 2000, Mosharov & Sulzer 2005). The former can be downloaded free of charge from http://webpages.ull.es/users/rborges.

The potentiostats used in amperometry maintain a fixed potential (≈0.7 V) at the tip of the electrode that provides enough energy to force oxidation of CA. Amperometry offers a unique time resolution that provides kinetic information as well as the total amount of CA released from a vesicle during a fusion event. However, it has two major limitations: it does not distinguish the substances oxidized and it does not measure the real concentrations of amines.

Voltammetry has been used in single cell measurements to detect ascorbic acid (Cahill & Wightman 1995) or to distinguish between adrenaline and noradrenaline (Pihel *et al.* 1994). However, this technique is slower than amperometry in that samples are generally obtained at a maximum rate of ~50–60 Hz. Thus, amperometry is generally preferred for studying single-vesicle fusion events. Cyclic voltammetry has recently been applied to quantify electroactive species within individual cells (Mosharov *et al.* 2003) where a slow time course is not a serious limitation. In addition, it is worth noting that cyclic voltammetry allows the measurement of the concentration of oxidized amines, which cannot be performed by amperometry because the latter consumes the products thus forcing the concentration of CA at the surface of the carbon electrode to be zero. Conversely, fast cyclic voltammetry reconverts the oxidized amine (orthoquinones) into CA, thus maintaining and measuring the real concentration of amines at the electrode tip.

Measuring secretory spikes

Amperometric recordings offer a direct view of single secretory events. The usual way to analyse secretory spikes is to extract several parameters from the record (Gomez *et al.* 2002) (Fig. 2).

The Q is generally used as synonymous of vesicle size or vesicle content, although it has not been firmly demonstrated that all of the vesicular CA content is released upon fusion. In any case, it seems that the physiological relevant CA are those that are released during exocytosis. In addition, foot parameters have been happily interpreted as a direct measure of the fusion pore kinetics. However, patch-amperometry studies, where fusion pore conductance can be directly measured from the membrane impedance, do not support a direct correlation. In particular, the interval

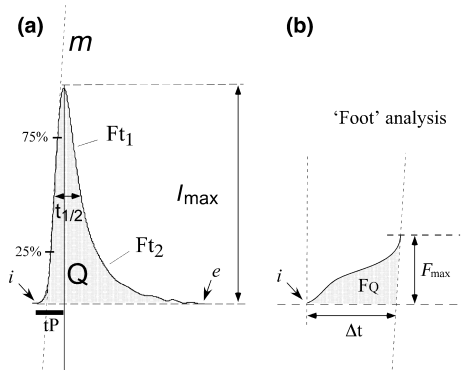

Figure 2 Kinetic parameters extracted from secretory spikes. (a) The initial (*i*) and ending (*e*) points of the spike are located following the algorithm described (Segura *et al.* 2000, Mosharov & Sulzer 2005). The slope of the ascending part (*m*) is determined by fitting a line to the spike between 25% and 75% of the peak amplitude (I_{max}). $t_{1/2}$ is calculated as the duration that the spike exceeds 50% of I_{max}, and is a more robust measure than the total spike duration. The integration of the current gives the net charge of the spike (Q). The time to peak (t_p), also called rising time, is measured from point *i* to the peak maxima; this parameter is not robust because it is strongly dependent of the presence of noise at the top of the spike. Two time measurements Ft_1 and Ft_2 can be taken from the decaying phase. (b) Several parameters can also extracted from the pre-spike features (feet) present in 30–60% of the spikes (see also Mosharov & Sulzer 2005). Feet are delimited by the interval between point *i* and the intercept of the line fit to the rising phase with Δt describing the foot duration, F_{max} the foot maxima and F_Q as the foot charge.

between the initiation of the fusion pore conductance and the earliest detectable amperometric foot current is highly variable. Nevertheless, the foot signal gives an idea about the stability of the fusion pore and perhaps the concentration of free amines inside the vesicle.

The peak amplitude of the spike (I_{max}) indicates approximately the concentration of amines that reaches the electrode surface, although it cannot be directly transformed into concentration because the electrode oxidizes all of the molecules near its tip thus creating a chemical sink. The initial slope (m) gives an idea about the release of the free catecholamines escaping from the vesicle matrix once the fusion pore is fully dilated (Segura *et al.* 2000, Mosharov & Sulzer 2005).We determine m from the linear part of the spike (Fig. 2).

It is virtually impossible to precisely measure the total spike duration because the beginning and end of the spike gently slope to the baseline value and thus are buried within the noise. Instead, the time interval that the spike exceeds the half-maximal value ($t_{1/2}$) is often used as a metric of spike duration in analogy with the analysis of chromatographic recordings. The time to peak (t_p) has been used in some studies; however, the robustness of this metric is questionable, particularly

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with slow spikes, because of the presence of noise at the top of spikes.

Most parameters, such as I_{max} and Q, are positively correlated. By contrast, I_{max} and $t_{1/2}$ are often negatively correlated in that a rapid spike will have a short $t_{1/2}$ and a large I_{max} (Machado *et al.* 2000) and vice versa (Machado *et al.* 2002a) to result in a similar total release of CA (Q). However, this relationship is violated when intravesicular factors are altered, such as in chromaffin vesicles lacking chromogranins, where the vesicular matrix cannot efficiently retain catecholamines. In these spikes, the rate of decay is also clearly accelerated (Montesinos *et al.*, unpublished observations). Some of the major contributions of amperometry, to our knowledge, of exocytosis are summarized in Table 2.

Patch amperometry

The first use of the combination of patch clamp with amperometry to directly correlate fusion pore conductance increases and capacitance steps with amperometric spikes was by the laboratory of Alvarez de Toledo *et al.* (1993). This seminal paper demonstrated release of serotonin from beige mast cells during transient fusion events and established that the 'foot signal' feature of amperometric spikes represents slow flux of transmitter through a fusion pore, verifying the hypothesis initially proposed by Chow *et al.* (1992b). Direct measurement of individual capacitance steps and fusion pore conductance during whole-cell recording, however, is limited to special preparations, such as beige mast cells, that have extraordinary large vesicles.

Simultaneous whole-cell capacitance measurements and amperometric measurements in chromaffin cells can be carried out to determine the relative timing and amplitude of the capacitance jump that results from fusion of a single vesicle by averaging many hundreds of capacitance sweeps in register with detected amperometric spikes to reduce the noise of the capacitance signal (Chow *et al.* 1996). By contrast, the lower noise of impedance measurements in membrane patches allows direct measurement of single-vesicle fusion events. Thus, insertion of a carbon fibre electrode *inside* a patch-clamp pipette potentially allows simultaneous resolution of the time course of impedance changes associated with fusion pore opening and membrane fusion together with amperometric detection of CA flux. The technical feat of combining amperometry and patch clamp within the same pipette was achieved through collaboration between Álvarez de Toledo and Lindau in a seminal paper to demonstrate the presence of transient fusion events in chromaffin cells (Albillos *et al.* 1997a). More recently, these laboratories have also shown stimulus-induced fusion of empty vesicles (Tabares *et al.* 2001) and have demonstrated that a high Ca^{2+} concentration on the extracellular side of the membrane promotes the kiss-and-run mode of exocytosis (Ales *et al.* 1999). An additional finding from patch amperometry is that the concentration of CA in chromaffin vesicles is independent of vesicle size and is ~1 M (Gong *et al.* 2003).

Table 2 Major findings obtained with amperometry

Concept	References
The exit of catecholamines is slower than predicted by diffusion. The fusion pore is permeable to catecholamines	Schroeder *et al.* (1992) and Chow *et al.* (1992a)
The quantum size is regulated by VMAT and autoceptors	Pothos *et al.* (1998, 2000) and Colliver *et al.* (2000)
Catecholamines escape through fusion pore 'flickers'	Zhou *et al.* (1996)
PKG, PKA and PKC modulate the kinetics of exocytosis	Machado *et al.* (2000) and Graham *et al.* (2000)
Estrogens slow exocytosis	Machado *et al.* (2002)
Ca^{2+} affects the fusion event	Elhamdani *et al.* (1998, 2001) and Mundorf *et al.* (2000)
Sizes and characteristics of the different vesicle pools	von Ruden & Neher (1993)
Finding of 'hot spots' of exocytosis	Schroeder *et al.* (1994), Robinson *et al.* (1995) and Gutierrez *et al.* (1998)
Drugs sequestered into vesicles change the quantum size	Mundorf *et al.* (1999) and Machado *et al.* (2002a,b)
Vesicular pH regulates the kinetics and the quantal size	Camacho *et al.* (2006)
Effects of SNARE and SNARE-related proteins over-expression on the kinetics of exocytosis or quantal size	Criado *et al.* (1999), Graham & Burgoyne (2000), Graham *et al.* (2001, 2004) and Voets *et al.* (2001a,b)
Effects of SNARE and SNARE-related proteins over-expression on fusion pore characteristics	Archer *et al.* (2002) and Barclay *et al.* (2004)
Cytoskeletal proteins affect the kinetics of exocytosis	Graham *et al.* (2002) and Neco *et al.* (2004)
Physico-chemical characterization of exocytosis	Jankowski *et al.* (1994), Borges *et al.* (1997) and Amatore *et al.* (2000)

 doi: 10.1111/j.1748-1716.2007.01814.x

Two new technical modifications of the technique have been recently implemented. First, patch amperometry was developed in excised (inside out) membrane patches to elucidate the role of extracellular vs. intracellular calcium in the kinetics of fusion and fission (Dernick *et al.* 2003). Secondly, the use of patch amperometry under the whole-cell configuration allowed the electrochemical analysis of the intracellular environment. In this paper, the authors use cyclic voltammetry for the identification of several intracellular species (Mosharov *et al.* 2003). A review describing details of implementing patch amperometry has recently been published (Dernick *et al.* 2005).

New approaches

Recent efforts have been made to use microfabricated microelectrodes rather than carbon fibres to detect quantal fusion events (Dias *et al.* 2002, Chen *et al.* 2003, Hafez *et al.* 2005, Amatore *et al.* 2006, Sun & Gillis 2006, Spegel *et al.* 2007). These microchip approaches offer the potential for highly automated and thus faster and cheaper measurement of quantal exocytosis.

Concluding remarks

The use of electrochemical and impedance methods, alone or in combination, has provided direct information about secretory processes at the single-vesicle level. Application of these techniques over the last 20 years to the chromaffin cell work has changed our current view of exocytosis and endocytosis.

Conflict of interest

The authors have no conflict of interest to declare.

We are indebted to Dr R. Mark Wightman and Dr Tim J. Schroeder for teaching us the technique of amperometry. This work was supported by the Spanish Ministry of Education (MEC, grant no. BFU2004-08038) and the US National Institutes of Health (NS48826).

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Journal compilation © 2008 Scandinavian Physiological Society, doi: 10.1111/j.1748-1716.2007.01814.x