Chapter 13

Membrane Capacitance Measurements of Stimulus-Evoked Exocytosis in Adrenal Chromaffin Cells

Elizabeth P. Seward and Robert C. Wykes

Abstract

Research using membrane capacitance (C_m) measurements in adrenal chromaffin cells has transformed our understanding of the molecular mechanisms controlling regulated exocytosis. This is in part due to the exquisite temporal resolution of the technique, and the possibility of combining quantification of exo-/endocytosis at the whole-cell level, with the ability to simultaneously monitor and control the calcium signals triggering vesicle fusion. In this regard, experiments performed with C_m measurements complement amperometry experiments that give a measure of secreted transmitter and the behavior of the fusion pore, and fluorescent microscopy studies used to monitor vesicle and protein dynamics in imaged regions of the cell. In this chapter, we provide a detailed account of the methodology used to perform whole-cell patch clamp measurements of C_m in combination with voltage-clamp recordings of voltage-gated calcium channels to quantify stimulus-secretion coupling in chromaffin cells. Stimulus protocols developed for investigation of functionally distinct releasable vesicle pools are also described.

Key words Exocytosis, Endocytosis, Chromaffin cells, Capacitance measurements, Calcium channels, RRP, IRP, SRP

1 Introduction

The ability to measure secretion has been an important advancement in our understanding of the mechanisms underlying stimulus-coupled exocytosis. Biochemical methods like spectrophotometric detection (a chemical reaction between the released product and an extracellularly applied molecule leading to formation of a colored or fluorescent signal) or radiochemical detection (radiolabeling of the released product) have been useful but offer poor time resolution and report secretion from a population of cells. Biophysical methods like amperometry (electrochemical detection) or membrane capacitance measurements (C_m) have much higher temporal resolution. These techniques allow the kinetics of release to be studied in detail at the single-cell level.

Ricardo Borges (ed.), *Chromaffin Cells: Methods and Protocols*,
Methods in Molecular Biology, vol. 2565, https://doi.org/10.1007/978-1-0716-2671-9_13,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2023

Exocytosis involves the fusion of a secretory vesicle (a membrane-enclosed packet of neurotransmitter) with the cell plasma membrane to release its contents to the outside. The mixing of vesicle and cell membranes leads to an increase in the surface area of the plasma membrane. This increase in cell surface area can be measured by determining the cell membrane capacitance C_m since the value of a capacitor is directly proportional to its surface area [1].

The specific membrane capacitance of almost all biological membranes can be calculated (and experimentally measured using a modified Wheatstone bridge current balance) to be roughly 1 μF/cm^2:

$$C_m = (\varepsilon\varepsilon_0/d)A$$

(ε = dielectric constant, ε_0 = polarizability of free space, d = membrane thickness, A = area).

Therefore, a cell of 13 μm diameter will have a C_m of about 5.0 pF [2].

If the size of the vesicle can be calculated and a change in capacitance is measured, then it is possible to determine how many vesicles have fused in response to a given stimulus [3]. For adrenal chromaffin granules, the estimated increase in C_m resulting from fusion of single vesicle has been estimated to be in the range of 1.3 fF [4].

Measurements of C_m with whole-cell patch clamp recordings essentially apply a voltage and separate a capacitative current from a resistive current.

The current that flows across a capacitor is given by:

$$I_C = C \times dV/dt$$

Thus, if the voltage does not change, there is no capacitative current. Membranes also have resistive properties, and the current that flows across a resistor is given by Ohm's law:

$$I_R = V/R$$

Several distinct techniques have been developed for detecting C_m changes resulting from vesicle fusion. Time domain-based techniques involve applying square voltage pulses (at hyperpolarized voltages that will not activate nonlinear membrane conductances). The capacitive transients that charge the membrane capacitance are then canceled. Subsequent voltage steps to depolarized potentials evoke a nonlinear ionic current of interest. This current declines with an exponential time course. Fitting this exponential can be used to evaluate ΔC_m. A problem associated with this technique is that it relies on an instantaneous voltage step, which experimentally is difficult to achieve (pipette resistance can slow the voltage step, etc.), and the interval between

voltage steps must allow for complete charging/discharging of C_m. This therefore limits the resolution of this technique to estimate C_m to a frequency of 1 Hz (for further details, *see* refs. 2 and 5). The PRBS technique, similar to the time domain technique square-wave voltage pulses, is applied; however, the duration of the voltage step is now a random variable. The resulting stimulus spectrum approximates white noise. The spectrum of the resulting current signal is directly related to the admittance spectrum, from which C_m can be derived [6]. This method relies on fitting the admittance to theoretical algorithms to determine C_m; if these are not accurate, then the estimation of the cellular parameters will be inaccurate [7].

Sinusoidal excitation is the most commonly used technique and involves applying a sine wave about a hyperpolarized potential. If a sine wave is applied ($V_{Command}$) to a cell, then the resulting sinusoid current (I_m) will contain both a resistive and capacitative component. It is possible to split these components. When there is no change in voltage (peak of sine wave), there is no capacitative current. Therefore, all the current will be resistive and we know the voltage, allowing the determination of R_m. Likewise, when the voltage changes most (at the inflection point of the sine wave), there is no net applied voltage and thus no resistive current. At this point, all the current will be capacitative. As the voltage and current are known, it is possible to calculate membrane capacitance. Since the sinusoid current is shifted with respect to the voltage stimulus, the magnitude and degree of phase shift can be analyzed using a phase-sensitive detector to produce estimates of C_m. The first studies using a hardware-based phase-sensitive detector (lock-in amplifier) were developed by [1]. Since then, software-based phase detectors and C_m measurement techniques have been developed [8–10]. Exocytosis may be triggered by interrupting the sinusoidal stimulus and applying variable depolarizing steps to stimulate calcium influx through voltage-gated calcium channels [11–14], ligand-gated and receptor-operated channels [15, 16], or photolysis of caged Ca^{2+} [17, 18]. Combining measurements of the calcium signals controlling exocytosis with C_m measurements of vesicle fusion has transformed our understanding of the molecular mechanisms regulating exocytosis in chromaffin cells [19] and crucially allows the researcher to quantify and distinguish between effects mediated through the calcium signals and calcium-sensitive proteins that regulate vesicle priming and fusion and calcium-independent molecular interactions that also control stimulus-secretion coupling.

2 Materials

2.1 Solutions and Small Parts

All solutions should be prepared using ultrapure water and analytical grade reagents. For convenience, we recommend making a 10X stock of the "external" solution (without added glucose or HEPES) to inhibit bacterial growth; this solution can be stored at room temperature for several months. 1X "external" solution with added glucose and HEPES and pH adjusted are made fresh on the day of the experiment. The pipette filling solutions are filter sterilized (0.4-μm filter) and stored frozen in aliquots at −20 °C.

1X external solution: 150 mM NaCl; 2 mM KCl; 5 mM $NaHCO_3$; 1 mM $MgCl_2$; 2.5 mM $CaCl_2$; 10 mM glucose; 10 mM HEPES; pH adjusted to 7.3 with NaOH; osmolarity adjusted with 1 M sucrose to ~310 mOsm.

Whole-cell patch pipette solution: 145 mM D-Cs-glutamate; 10 mM HEPES; 8.5 mM NaCl; 2.0 mM ATP-Mg, 0.1 mM GTP, 0.3 mM BAPTA, adjusted to pH 7.3 with CsOH; osmolarity ~290 mOsm (*see* **Note 1**).

For perforated whole-cell patch recordings, ATP and GTP are omitted from the pipette solution (*see* **Note 2**).

Borosilicate glass capillaries (such as those available from Multichannel Systems or Warner Instruments) with external and internal diameters of 1.65 (or as required to fit the electrode holder supplied with headstage of patch clamp amplifier) and 1.3 mm, respectively.

Patch pipette filling needle, such as MicroFil™ (World Precision Instruments) nonmetallic syringe needle.

A 1-mL plastic syringes and 0.22-μm syringe filters.

Sylgard™ 184 Elastomer kit.

2.2 Equipment

Inverted microscope on a vibration-isolation table with micromanipulator.

Patch clamp amplifier such as HEKA EPC-10 or Sutter dPatch (*see* **Note 3**).

Pipette holder suitable for patch clamp headstage.

Computer for controlling amplifier and data recording.

Pipette puller (such as Narishige PC-100 or Sutter Instruments P1000).

Microforge for polishing pipette tip and curing Sylgard (such as MF2 from Narishige or CPM-2 from ALA Scientific).

Vortex and sonicator (if using perforated patch method; *see* **Note 2**).

3 Methods

3.1 Fabrication of Patch Pipettes

1. Custom-made capillary borosilicate glass microelectrodes are pulled in two stages on a commercial pipette puller, and the second heat setting is adjusted produce a pipette with a tip diameter of ~1–2 μm and resistances of 1.5–2.0 mΩ for perforated patch or 2–5 mΩ for whole cell recordings.
2. To reduce stray capacitance (*see* **Note 4**), coat the shank of the pipette up to the tip with a hydrophobic substance (wax or Sylgard) to prevent liquid from "climbing" up and wetting the pipette due to surface tension. Sylgard is prepared by mixing nine parts resin to one part catalyst oil. The mixture may be placed and stored in a small volume syringe and stored at −20 °C until needed. Sylgard is "painted" around the shank of each electrode up to the tip using a hypodermic needle (19-gauge needle) attached to the syringe. The Sylgard must be cured quickly by placing the electrode between the coils of a fine wire heater for ~30 s. Alternatively, pipettes may be dipped in Sticky Wax (Kerr Inc., Orange, CA, USA, or similar) [20].
3. Fire polishing is the final stage of pipette fabrication and used to remove any contaminants and smooth the edges of the pipette to aid "giga seal" formation with the membrane. A microforge is used to visualize and fire polish the pipette; a platinum filament lightly coated with melted electrode glass provides the heat source. A reduction in pipette tip diameter and a faint darkening of the tip indicate that polishing has occurred. Once fabricated, patch pipettes are stored on a layer of BluTack™ stuck on the base of large petri dish; keep the dish closed at all times to protect the pipettes from accidental damage and to avoid contamination with dust. We find pipettes work best if made on the same day of the experiment and fire polished shortly before the experiment. For further discussions on making patch electrodes, *see* [21].

3.2 Whole-Cell Patch Clamp Recording and C_m Measurements of Exocytosis

1. Set up the patch clamp amplifier as per manufacturer instructions, mount the headstage on a micromanipulator located near the recording chamber, and attach the manufacturer-recommended patch pipette holder with mounted Ag/AgCl wire to the headstage.
2. Set up voltage stimuli protocols on the data acquisition software, and activate the software-implemented lock-in amplifier (e.g., *see* Fig. 2). We routinely use the Sine + DC method available with Patchmaster software (HEKA). For whole-cell recordings of C_m in chromaffin cells, we add a 1-kHz sinusoidal voltage (30–35 mV amplitude; *see* Table 1) to the holding membrane potential, typically set at −80 mV. Estimates of

Table 1
Sine wave parameters used for software lock-in measurements of C_m

Peak amplitude (mV)	35
Frequency	1.0 kHz
Points per cycle	25
Cycles to skip	1
Cycles to average	1
Total cycles	250

C_m are generated at one point per cycle; for these settings, the time resolution will be 1 ms per point. Avoid setting the amplitude of the sinusoid in a range where activation of voltage-dependent channels may occur.

3. Set the gain of the patch clamp amplifier between 5 and 10 mV/pA, and adjust as needed to avoid saturation by current transients. Set the current Bessel filters: filter 1–10 kHz and filter 2–3 kHz.
4. Isolated chromaffin cells need to be plated in such a way that they are easily visualized on an (inverted) microscope and accessible with a patch electrode (*see* **Note 5**). Continuous or frequent perfusion of the cells with external solutions at 1–5 mL/min is recommended to avoid autocrine and paracrine regulation of calcium channels and exocytosis [22, 23]. Figure 1 illustrates a simple gravity-fed system that may be used to perfuse cells with external solution and apply drugs in a time- and concentration-dependent manner.
5. After placing the recording chamber and cells on the stage of the microscope, place a Ag/AgCl pellet, which is connected to the ground pin of the headstage, in the external solution perfusing the cells (*see* **Note 6**). Identify the cell you wish to record from before proceeding to fill the patch pipette.
6. Once a cell has been identified for patching, proceed with filling the patch pipette with solution. If using non-capillary glass or perforated patch methods, pipette tips are first dipped into filtered internal solution for a few seconds to induce uptake by capillary action and then backfilled using a MicroFil™ nonmetallic syringe needle. Gentle shaking and flicking of the pipette will remove any air bubbles. In perforated patch recordings, the same procedure is applied although the tips are dipped in amphotericin B-free internal for 10 s prior to backfilling with internal containing amphotericin B (*see* **Note 2**).
7. The filled pipette is attached to the headstage of the amplifier using the pipette holder supplied with the amplifier, making

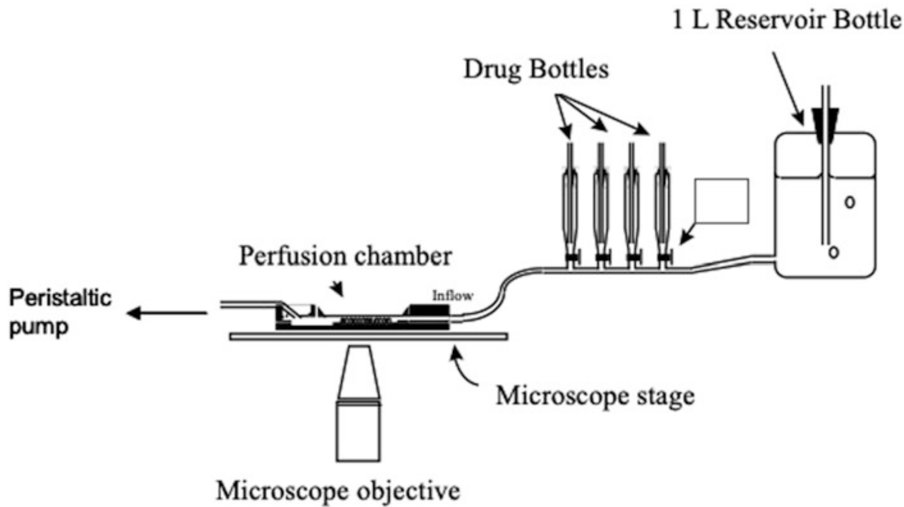

Fig. 1 A schematic representation of a gravity-controlled perfusion system. Cells were continually superfused (~1–2 mL/min) with external solution to remove effects of released endogenous modulators and remove traces of culture media and cell debris, which impede gigaohm seal formation. All solutions may be kept at room temperature or passed through an in-line controllable perfusion heating system placed in front of the inflow (such as the TC02 from MultiChannel Systems or Warner Instruments). Superfusion was obtained by use of gravity flow from a 1-liter reservoir bottle. Solutions containing drugs or altered external composition were stored in 10-, 20-, or 50-mL syringes connected in series with the main reservoir bottle. The solutions can be changed by switching a three-way stopcock/taps (such as those available through Cole-Parmer) connected to the bottom of the syringes; numerous stopcocks may be connected in series to allow easy switching between multiple different solutions. The height of the syringes is adjusted so that the flow rate from them is matched to the flow rate from the reservoir bottle. Additionally, stoppers containing a fixed length of plastic tubing are placed into the syringes to ensure a constant flow rate; aligning the bottom of the tubing in the bottle and syringes will ensure even flow rates. The addition of the stoppers and plastic tubing is important; otherwise, the flow rate will alter as the fluid levels in the syringes and bottles change. The outflow may be controlled by a peristaltic pump, vacuum, or gravity. A narrow bore metal tube, bent at 45° and beveled at one end, is attached to the outflow tubing (a low-gauge needle (e.g., 19 G) may be used for this purpose). This metal tube is placed in the superfusion chamber and held in place by either a small magnet or a piece of BluTack™. The tip of this metal tube is placed just below the meniscus of the desired bath volume. The outflow tube contained a small piece of silver wire and was passed through a bubble trap to reduce noise. A steady rate (inflow matching outflow) is achieved by optimizing the diameter of tubing flowing in and out of the system

sure that the Ag/AgCl wire in the holder is in contact with the solution in the pipette. Positive and negative pressure is applied to the pipette through tubing attached at one end to the holder and at the other end to a three-way stopcock and 1-mL syringe. With the aid of the micromanipulator, lower the pipette into the bath solution with positive pressure applied to prevent the tip being clogged before it reaches the cell membrane. Avoid applying much positive pressure when using the perforated patch experiments as the aim is to achieve giga seal formation

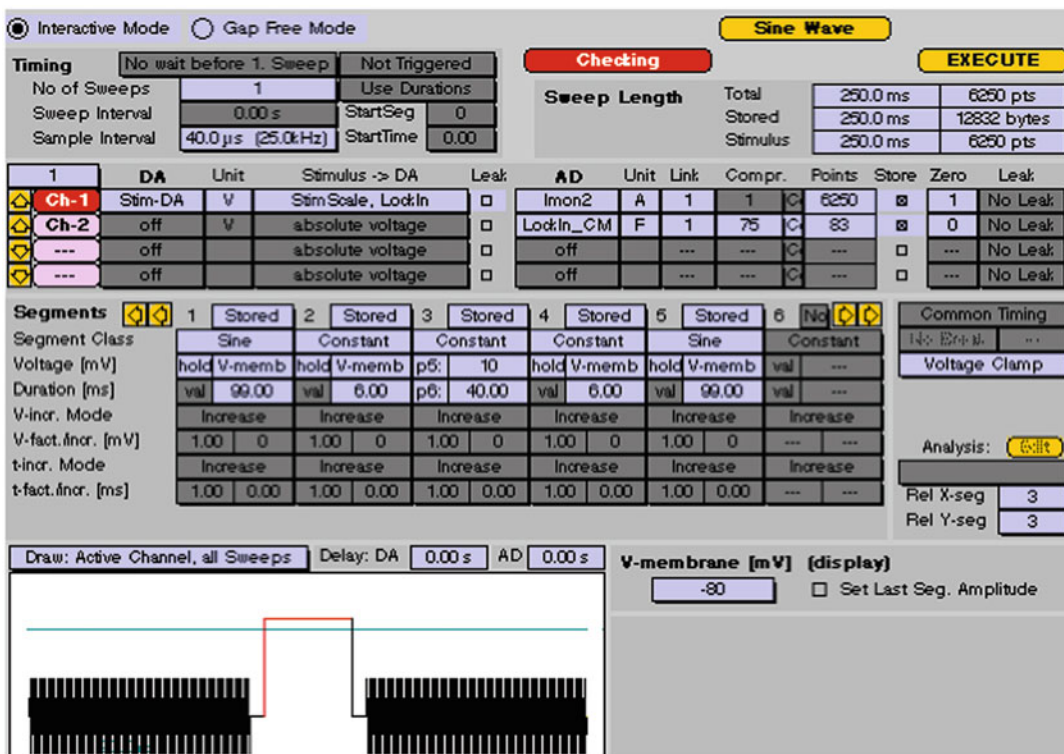

Fig. 2 Setting up a simple voltage-stimulus protocol for quantifying exocytosis using C_m measurements in response to a step depolarization. The image above shows a sample pulse generator file setup using Patchmaster software to perform C_m measurements with an EPC-10 amplifier. Activation of voltage-gated calcium channels and calcium influx to trigger exocytosis is accomplished through delivery of a single step in the membrane potential from a holding potential of −80 to +10 mV for the specified time, in the case shown here 40 ms. A sinusoidal voltage is added to membrane potential (100 ms duration in this case) before and after the step depolarization. A short segment of constant potential (6 ms in the example) at the holding potential is inserted between the step and sinusoidal segment to allow for measurements of the holding current and settling of any tail currents evoked by the step. A cartoon view of the stimulus can be seen at the bottom of the image. Currents recorded during the step depolarization are used to measure calcium influx across the membrane, while positive differences in C_m (ΔC_m) measured before and after the voltage step are used to quantify exocytosis. Parameters of the sine wave used can be seen in Table 1

before the perforant (amphotericin B) reaches the tip and perforation commences.

8. Once the electrode is in contact with the external solution, the command potential at the electrode is set to 0 mV and the current zeroed by canceling any junction potential differences using the offset on the amplifier (*see* **Note 7**). A small seal test voltage step (5–10 mV, 5 ms duration) is applied at high frequency (10 Hz) to monitor the electrode resistance. The electrode is then manipulated until its shadow can be seen under high magnification and then placed directly above a target cell; positive pressure is released. The electrode is then

slowly lowered onto the cell using the fine control on the micromanipulator; contact with the cell membrane can be observed by an increase in the input resistance. Before going whole cell, or obtaining electrical assess with the perforated patch method, it is necessary to obtain a giga seal (a seal whose electrical resistance is >1 GΩ) between the electrode tip and cell; this is achieved by application of gentle negative pressure on the pipette. Formation of the giga seal is monitored by observing a decrease the current evoked by the test seal voltage step as the resistance at the electrode tip increases. As the seal is forming and the resistance of the pipette is observed to be increasing, the holding potential may be hyperpolarized from 0 to −80 mV. A giga seal is usually obtained within seconds of touching a cell. Diagrammatic representations of the whole-cell and perforated patch recording configuration can be seen in Fig. 2 of Chapter 9 of this book.

9. Once a stable giga seal is established, use the fast capacitance compensation on the amplifier to eliminate the electrode current transients elicited by the test seal voltage steps.
10. To establish the whole-cell recording configuration, apply further gentle suction (negative pressure) to rupture the patch of membrane under the pipette; this leads to a sudden increase in capacitive transients; *see* ref. [24] for further details. This increase in current reflects the addition of the whole cell membrane to the pipette input capacitance. These transients at the start and end of a pulse should now be nulled by use of the patch amplifier capacitance compensation and series resistance compensation. For chromaffin cells, typically whole-cell capacitance is ~6 pF and series resistance <10 mΩ. If using the perforated patch recording method, do not apply any further pressure after establishing the giga seal, but wait for the perforant to enter the membrane and monitor perforation of the patch by observing changes in the transients evoked by the seal test voltage steps, the gradual appearance of the slow capacitance transients, and the series resistance to drop <15 mΩ. When stable (usually after ~20 min), use the patch amplifier slow capacitance compensation and series resistance compensation to null the transients (*see* **Note 8**).
11. Once voltage clamp of the cell has been established, the external solution may be exchanged for one in which the [NaCl] is reduced by 10 mM and replaced with 10 mM TEA-Cl. TEA will block calcium-dependent outward potassium currents that could contaminate calcium current traces elicited with long depolarizations. The solution should only be exchanged after establishing voltage clamp to avoid uncontrolled depolarization of the cell (due to block of potassium channels by TEA-Cl) stimulating secretion prior to the start of the experiment.

Tetrodotoxin (1 μM) may also be added to the external solution to block voltage-gated sodium current contamination of voltage-gated calcium current recordings.

12. After a suitable delay (~3 min) to allow the pipette solution to dialyze the cell, exocytosis may be triggered by application of a stimulus that causes sufficient rise in intracellular [Ca^{2+}].-Application of depolarizing voltage steps of varying amplitude and duration to stimulate Ca^{2+} influx through voltage-gated calcium channels is easily implemented. C_m is measured before and after each depolarization using the software lock-in and addition of a sinusoidal voltage to the holding potential (*see* Figs. 2, 3, 4, and 5 and **Note 9** for a description of commonly used stimulus protocols).

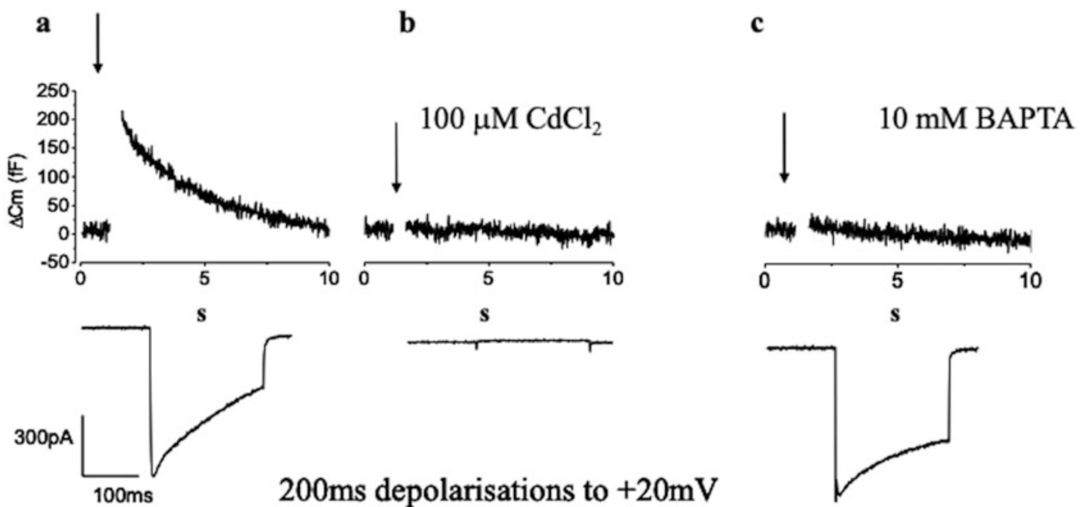

Fig. 3 C_m increases caused by exocytosis are triggered by a rise in intracellular Ca^{2+} in chromaffin cells. Exocytosis may be inhibited either by blocking calcium channels with 100 μM $CdCl_2$ or by addition of a high concentration (10 mM) of the fast calcium chelator BAPTA to the pipette solution. (**a**) Sample capacitance (top) and current (bottom) traces evoked by 200-ms depolarizations from −80 to +20 mV in bovine adrenal chromaffin cells. Arrows above the traces indicate when C_m measurements were interrupted (indicated by gaps) to apply the depolarizing voltage stimulus. Increases in C_m due to exocytosis can be clearly seen to occur on a much faster timescale than compensatory endocytosis, observed as a subsequence decline in C_m back to baseline. (**b**) Application of 100 μM $CdCl_2$ to the extracellular solution abolishes both ΔC_m and calcium entry in response to a 200-ms depolarization. (**c**) In whole-cell patch clamp experiments, increasing intracellular [BAPTA] from 0.3 to 10 mM increases calcium entry by inhibiting calcium channel inactivation [14], but exocytosis and ΔC_m increases are inhibited due to calcium chelation
Performing a simple experiment such as this is recommended for researchers setting up C_m measurements of exocytosis for the first time to validate their method

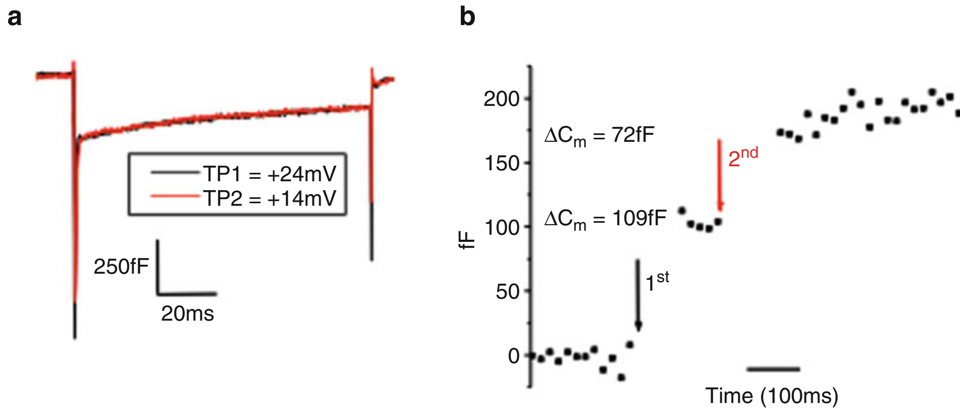

Fig. 4 Using C_m measurements to quantify the readily releasable pool of vesicles in chromaffin cells. (**a**) Sample current traces from a pair of 100-ms depolarizations given 100 ms apart to different membrane potentials to ensure identical calcium entry during the depolarization. (**b**) Sample capacitance points, displaying the ΔC_m in response to each depolarization. To determine the size of the RRP, a pair of 100-ms depolarizations are applied with a 400-ms interval, and the step potentials for the two pulses are adjusted to ensure that equivalent calcium influx is delivered in response, as shown. Using this protocol, we have estimated the RRP in bovine chromaffin cells using the perforated patch configuration at 193 $\pm$ 27 fF, $n = 9$. To measure the IRP, a similar approach is taken, but the step depolarizations are reduced to 10 ms duration and given with a 100-ms interval, giving an estimated pool size of 40 $\pm$ 10 fF, $n = 18$

3.3 Data Analysis

Calcium currents may be analyzed by determining the maximum "peak" current detected within predefined limits. Limits are usually set between 3 ms after the start of the step depolarization and 1 ms from the end to avoid contamination from sodium and tail currents, respectively (*see* **Note 10**). Additionally, calcium influx may also be quantified determined by integration of the voltage-gated calcium currents, again between the defined limits.

The total "synchronous" ΔC_m triggered by each voltage step in a protocol is calculated as the difference between an averaged value before depolarization and an averaged value after depolarization; typically, we average between 3 and 5 points. With trains of depolarizations, positive drifts in C_m between pulses, referred to as "asynchronous" exocytosis, are quantified by averaging the first 3–5 points acquired immediately after the voltage step and subtracting them from the average of 3–5 points taken immediately before the next voltage pulse. Summing the synchronous and asynchronous C_m increases provides a total measure of exocytosis evoked by a given protocol. Exocytotic efficiency may be determined by dividing the size of the C_m increase by the value obtained for integrating the corresponding calcium current.

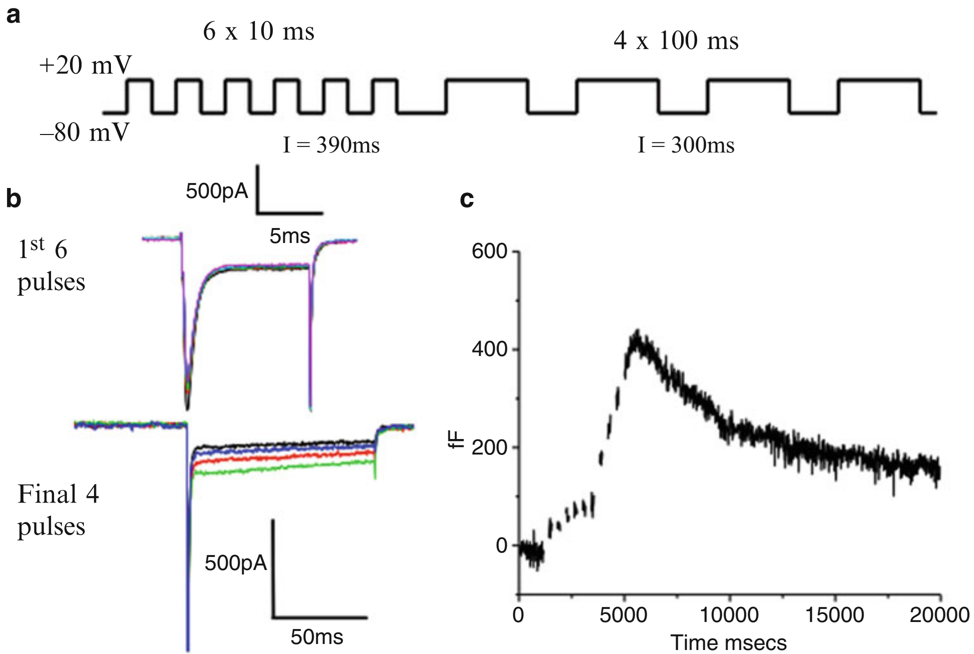

Fig. 5 Exocytosis evoked by a train of depolarizing voltage steps shows depletion of the immediately releasable pool of vesicles. (**a**) The protocol is shown schematically. Six 10-ms depolarizations with an interpulse interval of 390 ms are delivered immediately followed by four 100-ms depolarizations with an interpulse interval of 300 ms. (**b**) Representative calcium currents in response to this protocol, top (6 × 10 ms), bottom (4 × 100 ms). (**c**) Corresponding capacitance trace to the calcium currents shown in (**b**). The trace is biphasic with release in response to the first six short pulses becoming depressed as the IRP is depleted and then increasing again in response to the last four longer pulses as secretion from the RRP and SRP is triggered. Asynchronous release can be observed as a slow upward drift in the C_m after the last depolarization, and this is then followed by a slow decline as endocytosis takes over

4 Notes

1. Note the concentration of BAPTA or EGTA and free [Ca^{2+}] in the internal patch electrode solution impacts exocytosis when using the whole-cell configuration [11, 25, 26]. In perforated patch experiments [27], intracellular [Ca^{2+}] is not directly manipulated but controlled by endogenous buffering mechanisms [28].
2. For perforated whole-cell patch recordings, ATP and GTP are omitted from the pipette solution, and amphotericin B (Sigma, A4888) at a final concentration of 250 μg/mL (made freshly from a stock of 10 mg/mL in tissue culture grade DMSO, Sigma, D2650) is added to an aliquot of the pipette solution, vortexed (~30 s), sonicated (~30 s), and then vortexed again immediately before use. Stock is kept at room temperature in the dark and can be used for several days. The pipette solution

is made fresh on the day of the experiment and also stored in the dark; we find making fresh amphotericin pipette solution every few hours improves perforation.

The electrode tip is dipped for a few seconds into amphotericin-free internal and then backfilled with internal containing the perforation reagent. Internal solution is briefly vortexed before pipette filling. The 1-mL syringe used to backfill the pipette with the amphotericin-containing pipette solution must not be fitted with any filters. For further details on the perforated patch method for establishing whole-cell voltage-clamp, please *see* ref. 27.

3. Patch clamp amplifiers suggested both have facilities to combine whole-cell voltage-clamp recordings with the software-based sinusoidal excitation and C_m measurements described in this chapter.

4. It is essential when making capacitance recordings that there are no stray capacitance changes associated with the pipette. Most stray capacitance arises across the pipette wall between the pipette and bath [29]. Pipette capacitance can be reduced using the pipette capacitance cancellation circuitry on the patch clamp amplifier. It is possible (and necessary) to reduce stray capacitance further by using a small perfusion chamber, keeping the depth of the bathing solution to a minimum, and only filling the electrode with enough internal solution to make contact with the silver wire. Also, stray capacitance is attenuated further by coating the shank of the pipette up to the tip with a hydrophobic substance to prevent liquid from "climbing" up and wetting the pipette due to surface tension. To do this, we coat our electrodes with Sylgard, which also has the advantage of thickening the wall of the pipette, reducing capacitive coupling between the bath and pipette solutions.

5. Isolated chromaffin cells may be plated on 13- or 16-mm collagen type VII (rat tail), poly-D lysine or Matrigel-coated glass coverslips in 24- or 12-well plates at either 100,000 or 200,000 cells/well and covered with 1 or 2 mL of feeding media (10% fetal calf serum, 90% DMEM supplemented with 44 mM $NaHCO_3$, 15 mM HEPES, 0.1 mg/mL penicillin/streptomycin solution, 0.05 mg/mL gentamicin, 2.5 mg/mL 5′-fluorodeoxyuridine, 0.5 mg/mL cytosine-β-γ-arabinofuranoside, and 1% glutamine). Cells are kept in a humid incubator (5% CO_2 at 37 °C). At 24 h post preparation, ~ 70–80% of the feeding media is exchanged with fresh media. Cells are typically used 48–120 h post preparation.

For each experiment, a coverslip with isolated adrenal chromaffin cells is transferred to a perfusion chamber (such as Warner instruments, model P3/P4), placed on the stage of an inverted microscope, and viewed under phase contrast optics at

320–400× magnification using a long-working distance objective.

6. In experiments where the ionic composition of the external solution is altered in such a way that a change in the liquid junction potential will occur [30], use of a salt bridge to connect the ground electrode/pellet to the recording chamber is recommended. *See* [31] and https://www.warneronline.com/tutorials-method for further details.
7. Since the external and patch pipette contain solutions of different ionic composition, a liquid junction potential will exist at the pipette tip. An error in membrane voltage measurement will arise because of this liquid junction potential of the pipette tip [29]. The voltage error induced by liquid junction potentials is fairly easy to measure. With the patch clamp amplifier set to current clamp, a pipette containing internal solution is placed into the bath, which also contains internal solution. Any tip potential should then be zeroed before exchanging the bath solution for external solution. A liquid junction potential between internal and external will be displayed on the amplifier, which can be noted. This potential difference can be applied to the voltage command when performing experiments or corrected for when analyzing results if desired. For the solutions given here, the liquid junction potential between the patch pipette solution and external solutions is +15 mV. If the bath solution is changed for one with a different ionic composition, a further liquid junction will develop between the new bath solution and the reference (ground) electrode. In this case, an agar or salt bridge should be employed to keep the reference electrode zero.
8. Exocytosis and endocytosis have been shown to run down in the whole-cell configuration [11, 32]; therefore, if the experiment requires prolonged recordings, for example, for testing the effects of pharmacological agents or activation of receptor signaling pathways [16, 33], then use of the perforated configuration is recommended as this prevents rundown, allowing reproducible responses for periods of several hours.
9. In chromaffin cells across species, capacitance jumps evoked in response to a train of brief (10–25 ms) depolarizations [17, 34] or photoreleased Ca^{2+} are biphasic [18], which are interpreted as representing fusion of vesicles from functionally distinct "releasable" pools. Vesicles in the immediately releasable pool (IRP) fuse in response to very brief depolarizations in which the required elevation of intracellular calcium for exocytosis is restricted to the immediate proximity of the calcium channels [35, 36], while vesicles in the readily releasable pool (RRP) fuse in response to longer depolarizations but are rapidly depleted.

It is possible to quantitate the size of the IRP and RRP using a dual-pulse protocol [17, 37] (*see* Fig. 4) and using the equation $B_{\max} = S/(1 - R^2)$, where S represents the capacitance sum of the first (ΔC_{m1}) and second (ΔC_{m2}) and R is the ratio $\Delta C_{m2}/\Delta C_{m1}$. The upper limit of the pool size is assumed to be the value derived for $B_{\max}$, and a lower pool size estimate is given by S.

An alternative protocol used to measure vesicle pools with C_m measurements is the "6 + 4" train of depolarizations (*see* Fig. 5). This protocol consists of a train of six 10-ms pulses followed by four 100-ms pulses. With this protocol, the 10-ms stimuli will result in a ΔC_m that reflects fusion of the IRP, and the further four longer pulses result in a second bout of secretion that reflects fusion of the remainder of the RRP and a fraction of the SRP [38].

10. It is not always necessary to block sodium currents in chromaffin cells if step depolarizations used to trigger calcium influx and exocytosis are to potentials greater than 0 mV. This is because sodium currents in adrenal chromaffin cells inactivate by 3 ms after a depolarizing pulse to potentials greater than 0 mV and may be easily distinguished from the slower activating voltage-gated calcium channels (*see* Fig. 4).

References

1. Neher E, Marty A (1982) Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells. Proc Natl Acad Sci U S A 79:6712–6716
2. Gillis K (1995) Techniques for membrane capacitance. In: Sakmann B, Neher E (eds) Single channel recording. Springer, pp 155–198
3. Penner R, Neher E (1989) The patch-clamp technique in the study of secretion. Trends Neurosci 12(4):159–163. https://doi.org/10.1016/0166-2236(89)90059-3
4. Moser T, Neher E (1997) Estimation of mean exocytic vesicle capacitance in mouse adrenal chromaffin cells. Proc Natl Acad Sci U S A 94:6735–6740
5. Lindau M, Neher E (1988) Patch-clamp techniques for time-resolved capacitance measurements in single cells. Pflugers Arch 411(2):137–146
6. Fernandez JM, Bezanilla F, Taylor RE (1982) Effect of chloroform on charge movement in the nerve membrane. Nature 297(5862):150–152
7. Neher E, Zucker RS (1993) Multiple calcium-dependent processes related to secretion in bovine chromaffin cells. Neuron 10(1):21–30
8. Joshi C, Fernandez J (1988) Capacitance measurements. An analysis of the phase detector technique used to study exocytosis and endocytosis. Biophys J 53:885–892
9. Filder N, Fernandez J (1989) Phase tracking: an improved phase detection technique for cell membrane capacitance measurements. Biophys J 56:1153–1162
10. Gillis KD (2000) Admittance-based measurement of membrane capacitance using the EPC-9 patch-clamp amplifier. Pflugers Arch 439(5):655–664. https://doi.org/10.1007/s004249900173
11. Seward EP, Nowycky MC (1996) Kinetics of stimulus-coupled secretion in dialyzed bovine chromaffin cells in response to trains of depolarizing pulses. J Neurosci 16(2):553–562
12. Moser T, Neher E (1997) Rapid exocytosis in single chromaffin cells recorded from mouse adrenal slices. J Neurosci 17:2314–2323
13. Lukyanetz EA, Neher E (1999) Different types of calcium channels and secretion from bovine chromaffin cells. Eur J Neurosci 11(8):2865–2873
14. Wykes RC, Bauer CS, Khan SU et al (2007) Differential regulation of endogenous N- and P/Q-type Ca2+ channel inactivation by Ca2+/calmodulin impacts on their ability to support

exocytosis in chromaffin cells. J Neurosci 27(19):5236–5248

15. Mollard P, Seward EP, Nowycky MC (1995) Activation of nicotinic receptors triggers exocytosis from bovine chromaffin cells in the absence of membrane depolarization. Proc Natl Acad Sci U S A 92:3065–3069
16. Teschemacher AG, Seward EP (2000) Bidirectional modulation of exocytosis by angiotensin II involves multiple G-protein-regulated transduction pathways in adrenal chromaffin cells. J Neurosci 20:4776–4785
17. Voets T, Neher E, Moser T (1999) Mechanisms underlying phasic and sustained secretion in chromaffin cells from mouse adrenal slices. Neuron 23:607–615
18. Houy S, Martins JS, Mohrmann R et al (2021) Measurements of exocytosis by capacitance recordings and calcium uncaging in mouse adrenal chromaffin cells. Methods Mol Biol 2233:233–251. https://doi.org/10.1007/978-1-0716-1044-2_16
19. Marengo FD, Cárdenas AM (2018) How does the stimulus define exocytosis in adrenal chromaffin cells? Pflugers Arch 470(1):155–167. https://doi.org/10.1007/s00424-017-2052-5
20. Dernick G, de Toledo GA, Lindau M (2007) The patch amperometry technique: Design of a Method to study exocytosis of single vesicles. In: Michael AC, Borland LM (eds) Electrochemical methods for neuroscience, Frontiers in neuroengineering. CRC Press, Boca Raton
21. Ogden D, Stanfield P. (1994). Patch clamp techniques for single channel and whole-cell recording. In “Microelectrode Techniques: The Plymouth Workshop Handbook” editor David Ogden, published by The Company of Biologists, 2nd edition
22. Hernandez-Guijo JM, Gandia L, Lara B et al (1998) Autocrine/paracrine modulation of calcium channels in bovine chromaffin cells. Pflugers Arch 437:104–113
23. Powell AD, Teschemacher AG, Seward EP (2000) P2Y purinoceptors inhibit exocytosis in adrenal chromaffin cells via modulation of voltage-operated calcium channels. J Neurosci 20:606–616
24. Yawo H (2012) Whole-cell patch method. In: Okada Y (ed) Patch clamp techniques: from beginning to advanced protocols. Springer, Tokyo, pp 43–69
25. Augustine GJ, Neher E (1992) Calcium requirements for secretion in bovine chromaffin cells. J Physiol Lond 450(1):247–271. https://doi.org/10.1113/jphysiol.1992.sp019126
26. Smith C, Moser T, Xu T et al (1998) Cytosolic Ca2+ acts by two separate pathways to modulate the supply of release-competent vesicles in chromaffin cells. Neuron 20:1243–1253
27. Linley JE (2013) Perforated whole-cell patch-clamp recording. Humana Press, pp 149–157
28. Zhou Z, Neher E (1993) Mobile and immobile calcium buffers in bovine adrenal chromaffin cells. J Physiol (Lond) 469:245–273
29. Ogden D, Stanfield P (eds) (1994) Patch clamp techniques for single channel and whole cell recording, Microelectrode techniques, 2nd edn. The Company of Biologists, Cambridge
30. Neher E (1992) Correction for liquid junction potentials in patch clamp experiments. Methods Enzymol 207:123–131. https://doi.org/10.1016/0076-6879(92)07008-c
31. Barry PH, Lewis TM, Moorhouse AJ (2013) An optimised 3 M KCl salt-bridge technique used to measure and validate theoretical liquid junction potential values in patch-clamping and electrophysiology. Eur Biophys J 42(8):631–646. https://doi.org/10.1007/s00249-013-0911-3
32. Smith C, Neher E (1997) Multiple forms of endocytosis in bovine adrenal chromaffin cells. J Cell Biol 139:885–894
33. Bauer CS, Woolley RJ, Teschemacher AG et al (2007) Potentiation of exocytosis by phospholipase C-coupled G-protein-coupled receptors requires the priming protein Munc13-1. J Neurosci 27(1):212–219. https://doi.org/10.1523/JNEUROSCI.4201-06.2007
34. Horrigan FT, Bookman RJ (1994) Releasable pools and the kinetics of exocytosis in adrenal chromaffin cells. Neuron 13:1119–1129
35. Neher E (1998) Vesicle pools and Ca2+ microdomains: new tools for understanding their roles in neurotransmitter release. Neuron 20:389–399
36. Marengo FD, Monck JR (2000) Development and dissipation of Ca(2+) gradients in adrenal chromaffin cells. Biophys J 79(4):1800–1820
37. Gillis KD, Mossner R, Neher E (1996) Protein kinase C enhances exocytosis from chromaffin cells by increasing the size of the readily releasable pool of secretory granules. Neuron 16(6):1209–1220
38. Voets T, Moser T, Lund PE et al (2001) Intracellular calcium dependence of large dense-core vesicle exocytosis in the absence of synaptotagmin I. Proc Natl Acad Sci U S A 98(20):11680–11685