Physiological stimulation regulates the exocytic mode through calcium activation of protein kinase C in mouse chromaffin cells

Tiberiu FULOP and Corey SMITH¹

Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH 44106, U.S.A.

Adrenal medullary chromaffin cells release catecholamines and neuropeptides in an activity-dependent manner controlled by the sympathetic nervous system. Under basal sympathetic tone, catecholamines are preferentially secreted. During acute stress, increased sympathetic firing evokes release of both catecholamines as well as neuropeptides. Both signalling molecules are co-packaged in the same large dense core granules, thus release of neuropeptide transmitters must be regulated after granule fusion with the cell surface. Previous work has indicated this may be achieved through a size-exclusion mechanism whereby, under basal sympathetic firing, the catecholamines are selectively released through a restricted fusion pore, while less-soluble neuropeptides are left behind in the dense core. Only under the elevated firing experienced during the sympathetic stress response do the granules fully collapse to expel catecholamines and neuropeptides. However, mechanistic description and physiological regulation of

this process remain to be determined. We employ electrochemical amperometry, fluid-phase dye uptake and electrophysiological capacitance noise analysis to probe the fusion intermediate in mouse chromaffin cells under physiological electrical stimulation. We show that basal firing rates result in the selective release of catecholamines through an Ω -form ‘kiss and run’ fusion event characterized by a narrow fusion pore. Increased firing raises calcium levels and activates protein kinase C, which then promotes fusion pore dilation until full granule collapse occurs. Our results demonstrate that the transition between ‘kiss and run’ and ‘full collapse’ exocytosis serves a vital physiological regulation in neuroendocrine chromaffin cells and help effect a proper acute stress response.

Key words: capacitance, chromaffin, endocytosis, exocytosis, fusion pore, noise analysis.

INTRODUCTION

Adrenal chromaffin cells are a major output of the sympathetic nervous system. Under most conditions the sympathetic nervous system fires at a low rate, setting the sympathetic tone and placing the organism into a ‘breed and feed’ status of energy storage. Under these conditions, chromaffin cells release modest amounts of catecholamine into the circulation to regulate physiological parameters, including vascular tone and insulin release. Acute stress, due to external threat or injury, initiates the sympathetic ‘fight or flight’ response and causes chromaffin cells to fire at a rate up to 30-fold greater than basal tone [1,2]. This elevated activity, and thus elevated cytosolic Ca^{2+} , greatly increases catecholamine release and also evokes the release of neuropeptides (including neuropeptide Y, chromogranins and enkephalin) [3]. Physiological consequences include increased cardiac output, shunting of blood flow to skeletal muscle, increased circulating glucagon levels and altered neuronal activity. Thus chromaffin cells differentially release catecholamine and neuropeptide transmitters in an activity-dependent manner [4,5] to contribute to the acute stress response. Yet catecholamines and neuropeptides are co-packaged within the same secretory granule [6], therefore selective catecholamine and neuropeptide release is regulated at a step after granule fusion with the cell surface.

Previous studies have shown that the chromaffin granule exocytotic cycle differs with stimulus intensity [7,8]. Briefly, granule fusion evoked under basal firing rates results in selective release of catecholamines through a narrowed fusion pore, while larger less-soluble neuropeptides are retained in a gel-like granule core. Conversely, the acute stress response causes granules to expel both catecholamine and neuropeptides [6–8]. Thus selective release of catecholamine versus neuropeptides in an activity-

dependent manner may be achieved by a size-dependent filtering mechanism. This scenario predicts a physiological shift from a ‘kiss and run’ exocytic path to a ‘full collapse’ mode of granule fusion. In the present study we employ electrochemical, fluorescence imaging and computational electrophysiological analysis to measure fusion pore dilation and transmitter release. Stimulation paradigms are designed to mimic physiological firing patterns of the ‘breed and feed’ as well as ‘fight or flight’ sympathetic states. With this approach we provide data supporting the hypothesis that, in chromaffin cells, basal firing results in an Ω -form ‘kiss and run’ exocytosis [9]. Enhanced firing, mimicking acute stress, results in elevated Ca^{2+} influx that activates conventional isoforms of PKC (protein kinase C). This, in turn, causes fusion pore dilation and drives the granule to collapse into the membrane. Thus a post-fusion control of pore dilation and granule collapse may regulate release of catecholamines versus neuropeptide transmitters in an activity-dependent manner.

EXPERIMENTAL

Cell preparation

Chromaffin cells were isolated from the adrenal medullae of adult C57/BL6 mice as described previously [8]. All anaesthesia and euthanasia protocols were reviewed and approved by the IACUC (Institutional Animal Care and Use Committee) of Case Western Reserve University, an accredited oversight body (Federal Animal Welfare Assurance no. A3145-01). During recording, the cells were superfused in a Ringer solution of the following composition (in mM): 150 NaCl, 10 Hepes-H, 10 glucose, 2.8 CaCl_2 (or adjusted to 0.5 or 10 as indicated in text), 2.8 KCl and 2 MgCl_2 . Mannitol was added to all Ringer solutions to adjust the final

Abbreviations used: fura 2/AM, fura 2 acetoxymethyl ester; PKC, protein kinase C.

¹ To whom correspondence should be addressed (email corey.smith@case.edu).

osmolarity to 320 mOsm. For experiments in which pharmacological agents were used (as indicated in the text), cells were superfused with control Ringer until 5 min before the recording, at which time the superfusion was switched to a Ringer solution containing the agent indicated. In such experiments only one recording was made per culture dish to guard against non-specific long-term pharmacological effects. Additionally, both control and experimental protocols were alternated so that experiments for several data sets were run on a single cell preparation.

Electrophysiology and electrochemistry

Electrophysiological recordings were performed in the perforated-patch configuration as previously described [8]. Briefly, cells were held at a -80 mV potential. The internal pipette solution was of the following composition (in mM): 135 caesium glutamate, 10 Hepes (free acid), 9.5 NaCl, 0.5 tetraethylammonium chloride and 0.53 amphotericin B. Cell capacitance was calculated by the 'sine + D.C.' method [10] using a 650 Hz sine wave of ± 20 mV amplitude about the holding potential. Cells with greater than 30 pA leak current or series resistance greater than 30 M Ω were discarded from analysis. All experiments were performed at room temperature (25 °C). Amperometric recordings were also performed as previously described [8]. Amperometric current was recorded using a dedicated amplifier (VA-10x; ALA Scientific, Longneck, NY, U.S.A.) with a head stage modified with a 1 G Ω feedback resistor to minimize noise. The signal was filtered through a 4-pole analogue Bessel filter at a cut-off frequency of 1.3 kHz and sampled at 20 kHz through an ITC-1600 A/D board (Instrutech, Port Washington, NY, U.S.A.) into IGOR Pro (WaveMetrics Inc., Lake Oswego, OR, U.S.A.). Spike analysis was performed in IGOR Pro using a macro modified from the original 'Spike' package [11].

Imaging and fluorimetry

Fluorescence images were acquired on an Olympus IX70 inverted microscope with a 100 $\times$ oil-immersion objective (numerical aperture 1.3). Cells were superfused with the above-described Ringer solution. The Ringer solution additionally contained 1 μ M Texas Red-conjugated 40 kDa dextran, as indicated in the text. Images were captured with a cooled CCD (charge-coupled-device) camera (QImaging Corporation, Burnaby, BC, Canada) at a set exposure time of 5 ms to minimize photobleach during the illumination, and stored at 12-bit resolution. Image acquisition and data analysis were performed using custom-written macros in IGOR Pro. Dextran internalization was measured from an image taken at the end of the stimulus train. Cell intensity was background-subtracted and internalized dye was calculated as the average intensity inside the cell cytosol (see the dotted line in Figure 2b below). Dye uptake was normalized to exocytic activity by counting the number of amperometric spikes recorded during stimulation and dividing the fluorescence intensity by the number of spikes.

Calcium measurements were made in cells pre-loaded with fura 2/AM (fura 2 acetoxymethyl ester; Teflabs, Austin, TX, U.S.A.). fura 2/AM stock was prepared fresh daily in DMSO at 1 mM. Cells were loaded by incubation in normal Ringer solution containing 2 μ M fura 2/AM for 15 min at 37 °C. Resting and activity-evoked Ca^{2+} influx were measured by excitation at 360 and 390 nm, and emission intensity was measured through a photomultiplier tube (TillPhotronics, Pleasanton, CA, U.S.A.). Excitation illumination as well as acquisition of emission intensity were both controlled by PulseTM Software (HEKA, Lambrecht, Germany). A variable aperture acquisition window was set to collect light from only the cell being stimulated and excluded other cells in the visual field as

well as the recording pipette. Following the experimental protocol, the perforated patch was ruptured, causing the intracellular fura 2 to dialyse out of the cell and allowing for measurement of the autofluorescence of the cell. Autofluorescence values were similar to those measured in unloaded cells and thus probably did not include a significant amount of compartmentalized fura-2 (results not shown). The autofluorescence values at 360 and 390 nm excitation were subtracted from values measured during the experiment. Ca^{2+} data are reported as the ratio of emission intensity at F_{360}/F_{390} over the final 50 % of the stimulus train. While no absolute estimate for the amount of fura 2 loaded into each cell was made, it was noted that, after breaking into the whole-cell configuration to allow fura to dialyse out, cell fluorescence decreased only by 50 % (fura fluorescence was only as bright as autofluorescence). Comparison with emission intensity at 360 nm excitation in whole-cell experiments where a known concentration of fura was used predict a fura 2 concentration of perhaps only a few tens of micromolarity in the present study. These concentrations of fura would be unlikely to significantly alter the cytosolic Ca^{2+} -buffering and the time course of post-stimulus Ca^{2+} clearance.

Capacitance noise analysis

A capacitance-noise-analysis model for detecting Ω -form 'kiss and run' granule fusion in adrenal chromaffin cells has been developed. This assay is based on thermal noise of the cell equivalent circuit [10] and extended to include fusion-pore conductance. Addition of Ω -figures during exocytosis introduces an additional equivalent circuit term that increases the noise of the signal. [The term ' Ω -figure' is used to imply a morphology of the fused granule intermediate in that from the side the fused (but not collapsed) granule makes an ' Ω '-shape in the membrane; this is a key difference when compared with granules that fully collapse into the cell surface.] Thus noise is quantified as the variance of the capacitance signal. A model of the cell circuit is utilized to fit capacitance variance under differing stimulation conditions in order to differentiate between 'kiss and run' versus 'full collapse' exocytosis. Below is a step-by-step development of the model used in this technique.

In a perforated-patch voltage-clamp configuration the dominant source of noise is generated from the thermal (Johnson) noise of the resistive elements in the equivalent electrical circuit of the cell (see Figure 3a below). The variance (σ^2 , S.D. squared) of the current fluctuation in a circuit is given by eqn (1):

$$\sigma_i^2 = 4kTB_n Re Y(f) \quad (1)$$

where k is the Boltzmann constant, T is the absolute temperature, B_n is the bandwidth of the measurements (in Hz) and $Re Y(f)$ is the real component of the admittance of the equivalent electrical circuit. The real part of the admittance is represented as the resistive element of the electrical circuit:

$$Re Y_{\text{cell}} = \frac{1 + \omega^2 C_M^2 R_p R_M}{R_t (1 + \omega^2 C_M^2 R_p^2)} \quad (2)$$

where $R_t = R_A + R_M$ and $R_p = (R_A R_M)/(R_A + R_M)$. R_A and R_M are the access and membrane resistances respectively. C_M is cell membrane capacitance and ω is the angular frequency of the sine wave. From this analysis, an increase in the admittance is expected with granule fusion as described by:

$$Y_{\text{cell}+\Omega} = Y_{\text{cell}} + nG_{\text{fp}} \frac{(G_g + i\omega C_g)}{G_{\text{fp}} + G_g + i\omega C_g} \cdot \frac{R_M}{R_t(1 + i\omega C_M R_p)} \quad (3)$$

where n is the number of granules fused, $Y_{\text{cell}+\Omega}$ is the admittance after fusion of n granules, Y_{cell} is the admittance of a circuit without any fused granule, G_{fp} is the fusion-pore conductance, G_{g} the granule membrane conductance and C_{g} the granule capacitance.

An increase in the real part of the admittance is given by:

$$Re \Delta Y = \frac{nG_{\text{fp}}R_{\text{M}}}{R_{\text{t}}} \cdot \frac{\frac{G_{\text{g}}}{\omega C_{\text{g}}} \left(\frac{G_{\text{fp}} + G_{\text{g}}}{\omega C_{\text{g}}} \right) + 1 + R_{\text{p}}G_{\text{fp}} \frac{C_{\text{M}}}{C_{\text{g}}}}{\left[\left(\frac{G_{\text{fp}} + G_{\text{g}}}{\omega C_{\text{g}}} \right)^2 + 1 \right] \cdot (1 + \omega^2 C_{\text{M}}^2 R_{\text{p}}^2)} \quad (4)$$

The relation that links the noise from the current to the capacitance is given by:

$$\sigma_{C_{\text{M}}}^2 = \frac{\sigma_i^2}{\left| \frac{\partial Y}{\partial C_{\text{M}}} \right|^2 U^2} \quad (5)$$

where $\sigma_{C_{\text{M}}}^2$ is capacitance variance, σ_i^2 is current variance and U is the amplitude of the sinusoidal voltage used for the lock in amplifier detection.

The variance in the capacitance signal can be expressed as previously described [12]:

$$\sigma_{\text{cell}}^2 = \frac{4kTB_n R_{\text{t}}^3}{R_{\text{M}}^4} \cdot \frac{(1 + \omega^2 C_{\text{M}}^2 R_{\text{p}} R_{\text{M}})(1 + \omega^2 C_{\text{M}}^2 R_{\text{p}}^2)}{\omega^2 U^2} \quad (6)$$

The variance in capacitance with the fusion of n granules that remain in Ω -figures ($G_{\text{fp}} > 0$) can be expressed as:

$$\sigma_{\text{cell}+\Omega}^2 = \frac{4kTB_n Re Y_{\text{cell}+\Omega}}{\left| \frac{\partial Y_{\text{cell}+\Omega}}{\partial C_{\text{M}}} \right|^2 U^2} = \frac{4kTB_n (Re Y_{\text{cell}} + Re \Delta Y)}{\left| \frac{\partial Y_{\text{cell}+\Omega}}{\partial C_{\text{M}}} \right|^2 U^2} \quad (7)$$

Accumulating Ω -figures contribute a predicted increase in capacitance variance [scaled by the number (n) of events]:

$$\sigma_{\text{cell}+\Omega}^2 = \sigma_{\text{cell}}^2 + \frac{4kTB_n}{\omega^2 U^2} \cdot \frac{nG_{\text{fp}}R_{\text{t}}^3}{R_{\text{M}}^3} \cdot \frac{\left[\frac{G_{\text{g}}}{\omega C_{\text{g}}} \left(\frac{G_{\text{fp}} + G_{\text{g}}}{\omega C_{\text{g}}} \right) + 1 + R_{\text{p}}G_{\text{fp}} \frac{C_{\text{M}}}{C_{\text{g}}} \right]}{\left(\frac{G_{\text{fp}} + G_{\text{g}}}{\omega C_{\text{g}}} \right)^2 + 1} (1 + \omega^2 R_{\text{p}}^2 C_{\text{M}}^2) \quad (8)$$

Corrections for the capacitance variance were introduced to compensate for the '1/f-like current noise' [12]. The estimated noise at lower frequencies is somewhat smaller than the measured noise. Because our measurements were made at 650 Hz, a correction factor is added.

$$\sigma_{\text{cell}+\Omega \text{ corrected}}^2 = \sigma_{\text{cell}+\Omega}^2 \cdot \left[1 + \frac{2R_{\text{M}}^2 C_{\text{M}} f_c (1 - e^{-1/f_c R_{\text{p}} C_{\text{M}}})}{(1 + \omega^2 R_{\text{p}}^2 C_{\text{M}}^2) R_{\text{t}} (1 + \omega^2 R_{\text{M}} R_{\text{p}} C_{\text{M}}^2)} \right] + A \frac{(1 + \omega^2 R_{\text{p}}^2 C_{\text{M}}^2) R_{\text{t}}^4}{\omega^2 U^2 R_{\text{M}}^4} \quad (9)$$

where $A = 4 \times 10^{-26}$ is a constant, as previously defined [12], and f_c is the frequency of the line wave in Hz.

RESULTS

Fusion-pore size is dependent on Ca^{2+} influx

Carbon-fibre amperometry has proven a valuable tool to measure the quantal size of exocytic catecholamine release from chromaffin cells [13,14]. Using this technique it has been shown that quantal size is smaller under light stimulation than under heavy stimulation [8,15]. However, morphological studies ([16], but see [16a]), and electrochemical amperometric analysis in calf [15] as well as in mouse [17] chromaffin preparations show that large dense-core granules fall into a single size distribution (but see also [18]). Lastly, it has been shown that chromaffin cells tightly regulate the concentration of catecholamine within granules [19,20]. Thus the rate of catecholamine release scales with the size of the fusion pore; secretion through a smaller-diameter pore will release catecholamine at a lower rate than secretion through a larger-diameter pore. We measured the rate of catecholamine release (determined as the slope of the current spike between 25 and 75 % of its amplitude [11]; foot current omitted if present) under physiological firing conditions. Cells were stimulated with action potential trains to mimic basal and acute stress conditions [1,21]. In these recordings, Ca^{2+} influx, and therefore cytosolic Ca^{2+} , was separated from firing frequency by altering external Ca^{2+} . Cells were bathed in 0.5 mM, 2.8 mM and 10 mM external Ca^{2+} ($[\text{Ca}^{2+}]_{\text{o}}$), while held in perforated-patch voltage clamp, and stimulated with action potentials at 0.5 Hz. Measured Ca^{2+} currents followed external Ca^{2+} concentration (mean $\pm$ S.E.M.; Ca^{2+} influx 545 ± 64 , 895 ± 91 and 1042 ± 74 pA for 0.5, 2.8 and 10 mM $[\text{Ca}^{2+}]_{\text{o}}$ respectively). fura measurements confirmed that relative cytosolic Ca^{2+} measured during the stimulus train scaled with current flux under 0.5 Hz conditions and that cytosolic Ca^{2+} under 0.5 Hz stimulation in elevated (10 mM) $[\text{Ca}^{2+}]_{\text{o}}$ approached that measured under 15 Hz stimulation in normal Ca^{2+} (Figure 1a). The mean rate of catecholamine release (nA/s, ■) as well as mean quantal size (pC, □) were measured under each condition and are plotted in Figure 1(b). Both parameters increase with cell activity or with increased Ca^{2+} influx. The increased rate of catecholamine release is consistent with a larger fusion pore under elevated cell firing, and the increased quantal size under these conditions is consistent with the findings of previous studies [15]. These results also show that the shift toward larger, more rapid, amperometric spikes associated with increased cell firing could be mimicked by stimulating at 0.5 Hz, and by increasing Ca^{2+} influx. Thus the stimulus-dependent increase in the rate of catecholamine release from single granules as well as quantal size is encoded by Ca^{2+} influx and not by the rate of firing. Representative spikes are provided above each recording condition (note the different scales) and show that, under low Ca^{2+} influx, spikes were quite small and prolonged. They often had complex and multiple peaks as if the fusion pore were flickering [22]. At the same frequency, but with elevated Ca^{2+} influx, stimulation evoked more conventional, large and rapid amperometric events, often preceded by a foot current. These data are consistent with the hypothesis that fusion-pore dilation in chromaffin cells is positively regulated by Ca^{2+} and are in line with previous studies carried out in other neuroendocrine systems [23,24].

Stimulus-dependent uptake of fluid-phase fluorescent molecules has been used to probe fusion pore size in neuroendocrine cells. Dextran with a molecular diameter near to that of the fusion pore act as a probe for pore dilation (see Figure 2a, i and ii, for a cartoon representation of this assay). This approach has been successfully utilized to probe fusion-pore diameter in pancreatic β -cells [25], as well as chromaffin cells [8]. We measured dextran uptake to probe Ca^{2+} -dependent fusion-pore dilation. Fusion pores are dynamic structures expanding or contracting during

Figure 1 Amperometric spike amplitude and charge increase in elevated external calcium

(a) Chromaffin cells were pre-loaded with fura, bathed in Ringer solutions with low (0.5 mM), normal (2.8 mM) and high (10 mM) Ca²⁺ content and stimulated with trains of action potentials at 0.5 Hz or at 15 Hz in normal Ca²⁺. Relative cytosolic Ca²⁺ levels were determined as the average value over the final 50% of the stimulus train (after they reached a steady value) and are reported as the ratio of light emitted under 360 and 390 nm excitation. (b) The mean rate of catecholamine release (slope of the spike rising phase; nA/s, black bars) as well as mean spike charge (pC, white bars) for each condition are shown in the category plot. Example single amperometric spikes for each Ca²⁺ condition are shown above each category. [Ca²⁺]_o values are indicated below each category. Sample numbers from left to right (numbers of spikes from cells/number of cells): 99/4, 1301/15, 1143/7, 2819/14. These data show that mean spike size and charge grow with calcium influx.

Figure 2 Endocytic uptake of 40 kDa dextran is blocked under low Ca²⁺

(a) A cartoon representation of the protocol is provided. A fluorescence-based size-exclusion assay was used to probe the fusion pore diameter by determining fluid-phase uptake of fluorescently labelled 40 kDa dextran. The predicted molecular diameter of this marker is approx. 7 nm. (i) Under conditions of 'kiss and run' exocytosis, the dextran molecules are too large to fit through the fusion pore, (ii) but are readily incorporated into endosomes formed under 'full collapse'. (b) Cells were bathed in a Ringer solution containing 1 μM Texas Red-labelled 40 kDa dextran and either 0.5, 2.8 or 10 mM Ca²⁺ and stimulated. Following stimulation the cells were imaged and internalized fluorescence was measured as the mean image brightness within the cell cytosol (broken white line). (c) Mean dye uptake was measured during 0.5 Hz stimulation under various [Ca²⁺]_o values and at 15 Hz normal Ca²⁺. Sample numbers were from left to right (number of cells/number of spikes): 7/99; 18/1301; 7/1143; 13/2819. The fluorescence signal is expressed as a function of amperometric spike number (i.e. dye uptake per fusion event). These data indicate that dye is excluded from endosomal uptake under low Ca²⁺ influx, but is readily internalized under higher Ca²⁺ influx.

exocytosis [26], and no quantitative estimates are available for mean pore diameter in chromaffin cells under physiological electrical stimulation. However, estimates are available in other neuro-endocrine systems that indicate that mean diameters of < 12 nm can be expected under moderate stimulation [25,27]. For this reason we chose to probe pore diameter with a 40 kDa dextran, which has a predicted molecular diameter of 7 nm [28]. Thus exocytosis through a fusion pore less than the diameter of 40 kDa dextran is predicted to exclude dye uptake into newly forming endosomal compartments, whereas dilation of the pore beyond the predicted 7 nm dextran diameter should allow endosomal dye internalization. To test this prediction, cells were bathed in a Ringer solution including 1 μM Texas Red-labelled 40 kDa dextran, held under perforated-patch voltage clamp and stimulated with action potentials at 0.5 Hz. After stimulation, internalized dextran was quantified as the average fluorescence increase within the cytosol (Figure 2b). As above, cells were stimulated in Ringer solution containing 0.5, 2.8 and 10 mM Ca²⁺. Fewer amperometric events were measured under low Ca²⁺ influx than under higher Ca²⁺ conditions, this resulting in less potential total dye uptake in low-Ca²⁺ Ringer solution. In order to compare dye uptake under various [Ca²⁺]_o conditions, the internal fluorescence signal was normalized to the number of amperometric events measured for each condition. This normalization therefore reports dye uptake per exocytic event and represents the efficiency with which dye passes through the fusion pore rather than the number of exocytic events. Data from this analysis are plotted in Figure 2(c) and show the dye was excluded from endosomes under 0.5 Hz stimulation in 0.5 mM Ca²⁺. However, dye was readily internalized in cells stimulated at 0.5 Hz in 2.8 and 10 mM Ca²⁺ or in cells stimulated at 15 Hz in 2.8 mM Ca²⁺. These data indicate that the fusion pore was smaller than the diameter of the 40 kDa dextran (~7 nm) under the condition with the lowest Ca²⁺ influx, but that the fusion pore was greater than this size under all other conditions.

Cell-capacitance noise analysis as an assay for 'kiss and run' exocytosis

Previous studies have relied on ultrastructural electron-microscopy-based studies for detecting 'kiss and run' Ω-form exocytosis [9]. However, these approaches have not provided information regarding the initial stages of pore dilation [26] and cannot provide time-resolved signals. In addition the limited number of fusion events expected at any given time under 0.5 Hz stimulation along with the difficulty in processing and analysing serial planes of single cells with electron microscopy led us to choose an alternative strategy. We developed a cell-capacitance noise-analysis method for detecting Ω-figures and subsequent dilation of the fusion pore. Cell capacitance is proportional to cell surface area, and thus changes in capacitance can provide an index of granule fusion and fission during the exocytic cycle. In our implementation, cell capacitance is measured using the 'sine + D.C.' method [10] in the perforated-patch voltage-clamp configuration. After minimizing other sources of system noise, the major source of signal variance in frequency-domain capacitance measurements is introduced as thermal (Johnson) noise through the conductive elements of the equivalent circuit [12] (Figure 3a; see the Experimental section for a full description of the variance model). As evoked granule fusion decorates the cell surface with Ω-figures, an extra conductance (G_{Ω}) is added by the fusion pore and contributes to the total thermal noise of the equivalent circuit (Figure 3a, shaded box). We set out to measure the activity-dependent changes in capacitance noise (measured as the

Figure 3 Cell-capacitance noise analysis predicts Ω -figures under 0.5 Hz stimulation

(a) The equivalent electrical circuit of a patch-clamped cell and the equation predicting the capacitance variance (σ_{cell}^2 ; see the Experimental section for a description of the terms). 'Kiss and run' exocytosis results in accumulation of Ω -figures and additional electrical elements: granule capacitance (C_g), resistance (R_g) and fusion-pore conductance (G_{fp} , shaded box on circuit). These elements contribute to the variance equation (σ_{Ω}^2 , shaded box in lower equation) to increase capacitance noise by a factor scaled by the number of Ω -figures present (' n '). (b) Capacitance variance predicted for the equivalent circuit is plotted against command sine-wave frequency under full granule collapse (σ_{cell}^2) and 'kiss and run' fusion ($\sigma_{cell+\Omega}^2$). The balance between exocytosis and endocytosis is predicted to result in the equilibrium accumulation of 2.88 granules in the cell membrane during stimulation (as described in the text). Therefore the $\sigma_{cell+\Omega}^2$ calculations include the accumulation of 2.88 Ω -figures. The contribution of the Ω -figures (σ_{Ω}^2) is the difference between the continuous and dotted lines. (c) The variance of the capacitance signals calculated at the 650 Hz are plotted for σ_{cell}^2 and $\sigma_{cell+\Omega}^2$ against fusion-pore conductance. Cartoons represent the status of granule fusion at various pore conductances.

variance of the capacitance signal) and to use it to determine G_{fp} and, ultimately, to detect 'kiss and run' exocytosis. Two basic assumptions were made in the variance calculations: (1) specific resistance of all membrane is equal; absolute granule membrane conductance is far smaller than cell membrane conductance and therefore makes a non-significant contribution to the equivalent circuit; (2) voltage-gated ion channels, which also are predicted to contribute to thermal noise when open, are in a non-conductive state before and after cell stimulation (a limitation common to 'sine + D.C.' capacitance measurements). The model requires an estimate of the number of Ω -figures present in the membrane during stimulus trains. Briefly, to estimate this value we measured the signal-averaged capacitance increase due to action potential stimulation. Capacitance was averaged over a 50 ms window immediately before and after depolarization to provide the stimulus-evoked signal. Non-secretory contributions (i.e. gating charge movements from Na^+ channels) were subtracted from this value as previously described [29], providing only Ca^{2+} -dependent secretory capacitance signals (the quantal content). From that number we dynamically subtracted membrane internalization due to endocytosis using a previously described method [8,29]. This difference provides the average amount of granule membrane resident at any time in the cell surface. For example, 0.5 Hz stimulation in 2.8 mM external Ca^{2+} predicts an equilibrium value of 2.88 Ω -figures present in the membrane at any time. This protocol was repeated for all Ca^{2+} conditions and stimulus frequencies.

We then applied the variance model to match the recording conditions in the present study. The expected variance of the

capacitance signal was calculated using equivalent circuit values measured during experiments (R_a , R_m and $C_m = 23.1 \pm 2.0 \text{ M}\Omega$, $8.5 \pm 1.7 \text{ G}\Omega$ and $8.1 \pm 0.5 \text{ pF}$ respectively (mean $\pm$ S.E.M.; $n = 20$) combined with the calculated steady-state accumulation of 2.88 Ω -figures under 0.5 Hz stimulation (see above). Figure 3(b) shows a plot of expected cell variance without Ω -form (σ_{cell}^2) and with Ω -form ($\sigma_{cell+\Omega}^2$) exocytosis at different sine-wave frequencies. As expected, this plot shows that Ω -figure contributions to cell variance is greatest at lower sine frequencies [12]. However, low sine frequencies also practically limit the sample frequency available for variance measurements. For this reason we chose the intermediate sine frequency of 650 Hz for all experiments in the present study. This frequency represents a trade-off, allowing for an adequate cell voltage clamp and variance signal due to Ω -figure accumulation while affording sufficient sample frequency to allow analysis during 15 Hz stimulation. An arrow points to the variance contribution expected from 'kiss and run' exocytosis under 0.5 Hz at 650 Hz sine frequency (Figure 3b). Next, we calculated the expected mean capacitance variance as a function of fusion-pore conductance. When fusion-pore conductance is very small, little or no current passes through the fusion pore and the noise contribution is small. As the pore dilates, conductance increases, more current flows through and the noise contribution grows. Finally, as the pore dilates to the point where electrical access to the granule membrane is no longer limited, and the Ω -figure become indistinguishable from the rest of the cell surface, its contribution to capacitance variance decreases. Previous reports utilizing capacitance [30] or amperometric [19,26] measurements find the fusion pore to dilate with time after

initial opening. During this dynamic process, pore conductances are estimated to vary anywhere between tens of picosiemens to tens of nanosiemens, depending on cell and stimulus type [26]. Our model predicts the variance signal at 650 Hz sine frequency to be sensitive to changes in this range and is thus expected to provide an adequate sensitivity to differentiate 'kiss and run' from 'full collapse' exocytosis (Figure 3c).

Variance analysis predicts a Ca^{2+} -dependent shift from 'kiss and run' to 'full collapse'

The variance model was tested against measured data to calibrate its use as a qualitative assay for 'kiss and run' exocytosis. Chromaffin cells were stimulated and cell capacitance was recorded (Figure 4a). Pre- and post-pulse capacitance were fitted by a linear function (continuous line), and the S.D.s of the mean (σ) are co-plotted (broken lines). Mean capacitance variance (σ^2) was measured over a 50 ms period after each action potential stimulation by squaring the S.D. of the mean. Thus, when no Ω -figure accumulation occurs, the variance is expected to remain as in unstimulated cells (σ^2_{cell}), while Ω -figure accumulation ($\sigma^2_{\text{cell}+\Omega}$) due to 'kiss and run' exocytosis will increase the post-stimulus variance. Cell-capacitance variance data were collected in this manner from cells stimulated at 0.5 and 15 Hz frequencies and are plotted as a function of stimulus number (Figure 4b). They and show that both stimulation paradigms initially increase capacitance variance. However, with further stimulation the 15 Hz condition results in the variance falling back to pre-stimulus control values, presumably as cytosolic Ca^{2+} increases to drive granules to fully collapse. Continued 0.5 Hz stimulation, however, does not result in a relaxation in the variance signal; rather the signal remains elevated, indicating the persistence of the 'kiss and run' mode of exocytosis. These data show 0.5 Hz stimulation resulted in an increased capacitance variance, while 15 Hz stimulation, despite robust catecholamine release (see Figure 1) did not change variance above unstimulated controls. Next we used equivalent circuit parameters (R_A , R_M and C_M) measured during the experiment to calculate the expected variance signal under all conditions (Figure 4c). C_g was obtained for our cell type from the literature [17]. Accumulated Ω -figures were added to the modelled equivalent circuit as described above. We then used this parameter set to fit the fusion-pore conductance (the only free variable; eqn 8) and found that 0.5 Hz stimulation predicted Ω -figure accumulation with a mean conductance value of 3.26 nS, while 15 Hz stimulation did not predict any additional components added to the equivalent circuit (i.e. no Ω -figures). Thus, using the equivalent circuit noise model, we can explain the increased capacitance variance measured under 0.5 Hz stimulation (black bars) with the accumulation of Ω -figures during stimulation (white bars). Overall, the results from this variance analysis draw the same conclusion as the amperometric and dye-exclusion data, namely that granule fusion and catecholamine release from Ω -figures occurs under 0.5 Hz stimulation but not under 15 Hz stimulation. Thus it may provide a valuable and accessible qualitative assay for 'kiss and run' versus full-collapse forms of exocytosis.

Next, capacitance variance was utilized to probe for Ca^{2+} -dependent dilation of the fusion pore. Capacitance noise was measured under conditions that led to differential amperometric spike amplitude and selective internalization of 40 kDa dextran presented previously. Variance was determined in cells stimulated at 0.5 Hz under 0.5, 2.8 and 10 mM $[\text{Ca}^{2+}]_o$ and, for comparison, variance measured in cells stimulated at 15 Hz in 2.8 mM Ca^{2+} is re-plotted from Figure 4(c). The results from these experiments are presented in Figure 4(d) and show that variance predicts

Figure 4 Capacitance variance predicts a Ca^{2+} -dependent dilation of the fusion pore

(a) Cells held in the perforated-patch configuration were stimulated with action-potential waveforms and evoked cell capacitance is plotted. Mean cell capacitance (continuous lines), and the standard deviation (σ , broken lines) before and after the pulse are shown. (b) Stimulus-dependent development of the cell variance signal is shown for 0.5 and 15 Hz stimulation. For clarity, variance data are plotted against stimulus number. (c) Capacitance variance (σ^2) measured under control, 0.5 and 15 Hz stimulation ($n = 7, 19$ and 13 cells respectively) is plotted along with variance fitted by the model, incorporating accumulation of 2.88 'kiss and run' Ω -figures under 0.5 Hz, but no Ω -figures under 15 Hz stimulation. Broken lines represent variance from unstimulated (σ^2_{cell}) and Ω -figure-decorated ($\sigma^2_{\text{cell}+\Omega}$) cells. Cartoons below each category show the hypothesized fusion mode. (d) Cell capacitance variance was measured at 0.5 Hz stimulation under various values of $[\text{Ca}^{2+}]_o$ and at 15 Hz normal Ca^{2+} . As in (c), dotted lines indicate smooth (σ^2_{cell}) versus Ω -figure-decorated cell membrane ($\sigma^2_{\text{cell}+\Omega}$). Cartoon representations of the hypothesized fusion mode are shown below each category. Sample sizes from left to right ($n = \text{cells}$): 13, 19, 17, 13.

accumulation of Ω -figures in cells stimulated at 0.5 Hz at low and normal Ca^{2+} , but not in cells stimulated at 0.5 Hz in high Ca^{2+} . Data combined from Figures 1(b), 2(c) and Figure 4 show that the chromaffin cells release catecholamine through a 'kiss and run' exocytic mode under conditions that match basal sympathetic firing. Additionally, these data also show that increased cell firing shifts the exocytic mode to 'full collapse' in a Ca^{2+} -regulated manner.

Ca^{2+} acts through PKC to shift the exocytic mode

Previous studies have shown that stimulating chromaffin cells in elevated $[\text{Ca}^{2+}]_o$ increases the quantal size [15], and pharmacological activation of PKC increases the rate of catecholamine release [31]. It has also been shown that physiological stimulation is capable of raising cytosolic Ca^{2+} to levels sufficient to activate conventional isoforms of PKC, causing its translocation to the cell membrane, where it acts to alter the kinetics and magnitude of exocytosis [32–34]. We tested the possibility that the cellular signalling pathway that controls the Ca^{2+} -dependent shift from 'kiss and run' to 'full collapse' incorporates a PKC-dependent step. We treated cells with inhibitors of conventional, Ca^{2+} -dependent PKC isoforms [Ro-31-8220 (100 nM) and Gö 6983 (100 nM), both more potent members of the bisindolylmaleimide family] or with PMA, a potent PKC activator (100 nM). Under these pharmacological conditions we measured the slope of evoked amperometric spikes, 40 kDa dextran uptake and capacitance variance. The summary of these experiments is provided in Figure 5. Data obtained under both PKC blockers were statistically identical and are therefore pooled under 'PKC-Inh.'. Action potential stimulation at 0.5 Hz resulted in low amperometric-spike-slope values and high capacitance variance, indicating relatively-small-diameter fusion pores and the accumulation of Ω -figures. The 40 kDa dextran was internalized under control conditions, confirming that the fusion pore is slightly larger than the predicted 7 nm molecular diameter of the dextran, a result consistent with the variance analysis presented in Figure 4(c). Next we tested the same three parameters in cells treated with the PKC inhibitors. Pretreatment with Ro-31-8220 and Gö 6983 resulted in slow catecholamine release (shallow spike slope) and elevated capacitance variance, matching control data. However, this treatment blocked uptake of 40 kDa dextran. These data indicate that there is a basal PKC activity in the control condition that, when inhibited, results in a narrowing of the mean fusion-pore diameter. Furthermore, they show that the mean pore diameter under control conditions is near in size to the diameter of the 40 kDa dextran (i.e. a decrease from control results in total loss of dye uptake).

Phorbol ester pretreatment converted all parameters measured under 0.5 Hz stimulation to match those observed under 15 Hz stimulation. Bath treatment of cells with PMA caused faster amperometric currents, low cell variance and left 40 kDa dextran internalization unchanged. This effect was not due to an enhanced Ca^{2+} influx, as determined by measured current amplitudes and fura ratio analysis (results not shown). These data combine to form a profile whereby PMA treatment by-passes Ca^{2+} -dependent processes and drives exocytosis through a dilated fusion pore. Furthermore, cells bathed in PMA and stimulated at 15 Hz did not show further increases in the rate of catecholamine release or quantal size. Conversely, pretreatment of cells with PKC inhibitors and stimulation at 15 Hz caused a decrease in amperometric spike slope, blocked uptake of 40 kDa dextran and increased cell variance. These data match the values measured under 0.5 Hz stimulation in low Ca^{2+} and suggest a shift from 'full collapse' to 'kiss and run' exocytosis. Lastly, in control experiments to confirm

Figure 5 Activation or inhibition of conventional PKC shifts the mode of granule fusion

Cells were stimulated at 0.5 Hz or 15 Hz in 2.8 mM Ca^{2+} under conditions in which PKC activity was pharmacologically blocked or activated. Top panel: analysis of evoked rates of catecholamine release (initial spike slope) are provided for all conditions. Pretreatment of cells with PMA increased the rate of catecholamine release measured under 0.5 Hz stimulation to that measured under 15 Hz stimulation, while inhibition of PKC with Gö 6983 and Ro-31-8220 (pooled together as 'PKC Inh.') acted to decrease the 15 Hz-evoked rate of catecholamine release to that measured under 0.5 Hz. Sample numbers from left to right (number of spikes/number of cells): 1301/15, 821/13, 209/4, 2819/14, 3940/20. Middle panel: endocytic uptake of fluorescent 40 kDa dextran was measured and normalized to the number of amperometric spikes for each condition. Dye uptake was blocked by inhibition of PKC. In all other conditions, dye was internalized equally efficiently on an 'all-or-none' basis when normalized to the number of release events. Sample numbers from left to right (n = cells) 18, 10, 9, 13, 18. Bottom panel: capacitance variance measured under control, PKC-blocked and PKC-activated conditions. As in Figure 3, dotted lines represent variance values from smooth (σ^2_{cell}) and Ω -figure-decorated ($\sigma^2_{\text{cell}+\Omega}$) membranes. Cell variance was higher under 0.5 Hz than 15 Hz stimulation. A block of PKC under 0.5 Hz stimulation had no effect on variance. A block of PKC under 15 Hz stimulation raised cell variance to the level of 0.5 Hz control conditions. Pre-treatment of cells with PMA and stimulation at 0.5 Hz resulted in decreased variance matching the 15 Hz control condition. Sample numbers for these data were from left to right (n = cells) 19, 36, 15, 13, 58. Control data are re-plotted from previously shown Figures. Summary cartoon: taken together, all three techniques predict that granule fusion occurs through a 'kiss and run' mechanism under modest Ca^{2+} influx, or when PKC is inactive.

that the target of the PMA was indeed PKC and not other potential phorbol-ester-sensitive molecules such as munc13 (a mammalian homologue of the *Caenorhabditis elegans* unc-13 gene product), we co-treated cells with PKC inhibitors and PMA. The resulting amperometric spikes were statistically identical with those of cells stimulated in the presence of PKC inhibitors alone (Table 1) and confirm that the PMA-dependent shift in spike parameters is mediated by PKC. Thus manipulation of PKC supersedes cell firing frequency as well as cytosolic Ca^{2+} in its ability to regulate fusion-pore dilation and ultimately in the physiological control of activity-dependent transmitter release.

Table 1 Summary of amperometric data under PKC activation and inhibition

Basic parameters of amperometric spikes are reported for control cells and cells that were pretreated with 100 nM PMA or with 100 nM PMA and 100 nM Ro-31-8220. Cell numbers are presented for each data set in parentheses. '+ PKC Inh.' Refers to both PKC inhibitors (see the text).

Frequency	Control	+PMA	+PMA, +PKC Inh.
0.5 Hz			
Slope (nA/s)	3.20 ± 0.37 (14)	9.57 ± 1.23 (7)	2.24 ± 0.36 (6)
Charge (pC)	0.43 ± 0.01	0.62 ± 0.05	0.40 ± 0.40
15 Hz			
Slope (nA/s)	11.15 ± 0.53 (14)	9.74 ± 0.05 (5)	1.94 ± 0.15 (6)
Charge (pC)	0.73 ± 0.01	0.71 ± 0.02	0.31 ± 0.01

DISCUSSION

Chromaffin cells release transmitter molecules under two different physiological conditions. Basal sympathetic firing evokes catecholamine release and places the organism into a 'breed and feed' state of energy storage. The 'fight or flight' stress response, on the other hand, is characterized by elevated catecholamine release and also evokes the release of neuropeptides into the circulation. Previous studies had shown that the differential transmitter release is due to an activity-dependent selection between two modes of granule exocytosis and subsequent endosomal trafficking [8]. In the present study we refine these observations to provide resolution of the fusion-pore behaviour under both modes. We provide quantification of the process shift, as well as propose a cellular mechanism for its control under physiological conditions. We show that, under basal firing rates, catecholamines are released through a narrow fusion pore of an Ω -figure characteristic of 'kiss and run' exocytosis. Increased firing rates that match the sympathetic acute stress response raise intracellular Ca^{2+} , activate PKC and cause fusion-pore dilation until the granule becomes electrically indistinguishable from the rest of the cell (i.e. full collapse).

Previous studies have provided a quantitative analysis of cell capacitance noise in chromaffin cells and showed that its major source is the thermal noise in the cell membrane resistance [12,35]. Moser and Neher [17] used a non-stationary noise analysis to estimate the mean capacitance of secretory granules in mouse adrenal chromaffin cells. In the present study we extend capacitance noise analysis to develop a method for assaying the mode of granule fusion in chromaffin cells. We use this tool to resolve the relative difference in the capacitance variance signal, depending on whether granules fully collapse into the cell surface or if they maintain an Ω -form fusion intermediate. This technique is based upon the fortunate characteristic that accumulating Ω -figures contribute an extra electrical element to the cell's equivalent circuit that contributes substantially to the Johnson noise of the capacitance signal, which, in a well-grounded set-up, is the dominant source of capacitance noise [12]. These data complement the well-established electrochemical amperometric technique (Figure 1) as well as the fluorescence fluid-phase dye uptake presented in Figure 2. The capacitance noise analysis, however, has the advantage that it does not require additional hardware or manipulation beyond standard voltage-clamp recording conditions. Analysis of the capacitance noise is easily accomplished off-line. In addition, it is possible that the variance technique could easily be applied to studies of exocytosis and endocytosis in tissue slice preparations where quantum-level

amperometric measures are clouded by release of catecholamine by nearby chromaffin cells and the use of fluorescence techniques are hindered by non-specific background signals from the rest of the tissue. A second likely application would be for the study of exocytosis in cells that do not release oxidizable transmitters and where amperometry cannot be used (i.e. peptidergic nerve terminals or pancreatic β -cells). Further studies will be required to determine the general usefulness of this approach. Despite these points, several potential limitations of the variance approach are immediately apparent. Currently the variance analysis serves to provide a qualitative and relative read-out of the accumulation of Ω -figures. It is limited to recording conditions where external sources of noise (i.e. radio and instrumentation noise) are kept to a minimum. Additionally, as inherited from the capacitance technique, any change in cell membrane conductance due to series resistance changes, active gating of ion channels or other alterations in the cell equivalent circuit, will all minimize the usefulness of the noise analysis. Lastly, it would be theoretically possible to utilize the noise-analysis technique to measure the diameter of the fusion pore and quantitatively resolve its dilation. However, that would require specific knowledge of the physical characteristics of the pore at all stages of dilation in order to convert conductance into a geometry. One would need to know how long the pore was, whether it spanned both granule and cell lipid bilayers, or simply spanned a single bilayer, in order to make any estimate of pore diameter.

We propose a cellular mechanism whereby increased cell firing, as experienced under the acute stress response, increases cytosolic Ca^{2+} that then activates PKC and drives granules from 'kiss and run' to full collapse exocytosis. This mode shift forms the basis for activity-dependent differential transmitter release. Previous studies have shown that conventional PKC isoforms are activated in chromaffin cells by the Ca^{2+} concentrations expected in the present study [32,33,36]. Our data complement these studies; we show that both elevations in cytosolic Ca^{2+} as well as phorbol-ester treatment results in a dilation of the fusion pore and exocytosis through a full-collapse mode. Phorbol-ester treatment can regulate exocytosis in chromaffin cells through two different pathways – one through the activation of PKC and another through activation of munc13-1 [37]. Like PKC, munc13-1 has been shown to bind diacylglycerol and phorbol ester [38], whereby it translocates to the cell periphery and promotes granule priming, increasing the readily releasable granule pool and thus facilitates evoked secretion. We isolate the phorbol-ester effect in our study to a PKC rather than munc13-mediated pathway. We pretreated cells with both PMA as well as with Ro-31-8220, a member of the bisindolylmaleimide family of PKC blockers that acts by occupying the ATP-binding pocket on PKC [39]. Munc13-1 activation by PMA has been shown to be insensitive to block by the bisindolylmaleimides [38], thus this class of PKC blockers act to differentiate the action of PMA on PKC versus munc13. The fact that the PKC inhibitor reversed the effects of PMA treatment isolated PKC as the effector molecule. However, the downstream substrate for PKC has not yet been identified. A potential candidate may be found in the literature. PKC substrates that have been shown to be involved in chromaffin cell exocytosis include munc18-1. One study has shown munc18-1 to play a role in vesicle recruitment prior to docking, but not a role in the final stages of catecholamine release [40]. However, work by the Burgoyne group [41–43] provides data showing that munc18-1, in conjunction with syntaxin, plays a role in the regulation of fusion-pore dilation. The potential source for this discrepancy may lie in species or stimulus protocol differences. Further experiments utilizing native physiological electrical stimuli and diverse assays for exocytosis and fusion pore status may resolve this point.

We thank Dr Shyue-An Chan (Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH, U.S.A.) and Professor Uziel Landau (Department of Chemical Engineering, Case Western Reserve University, Cleveland, OH, U.S.A.) for helpful comments during the preparation of this manuscript. This work was supported by grants from the NSF (National Science Foundation) (IBN-0344768) and the NIH (National Institutes of Health) (1R01NS052123) to C. S. and (T32 HL 07653) for support of T. F.

REFERENCES

- Brandt, B., Hagiwara, S., Kidokoro, Y. and Miyazaki, S. (1976) Action potentials in the rat chromaffin cell and effects of acetylcholine. *J. Physiol. (London)* **263**, 417–439
- Kidokoro, Y. and Ritchie, A. K. (1980) Chromaffin cell action potentials and their possible role in adrenaline secretion from rat adrenal medulla. *J. Physiol. (London)* **307**, 199–216
- Winkler, H. and Westhead, E. (1980) The molecular organization of adrenal chromaffin granules. *Neuroscience* **5**, 1803–1823
- Takiyuddin, M. A., Cervenka, J. H., Sullivan, P. A., Pandian, M. R., Parmer, R. J., Barbosa, J. A. and O'Connor, D. T. (1990) Is physiologic sympathoadrenal catecholamine release exocytotic in humans? *Circulation* **81**, 185–195
- Giampaolo, B., Angelica, M. and Antonio, S. (2002) Chromogranin 'A' in normal subjects, essential hypertensives and adrenalectomized patients. *Clin. Endocrinol.* **57**, 41–50
- Rahamimoff, R. and Fernandez, J. M. (1997) Pre- and postfusion regulation of transmitter release. *Neuron* **18**, 17–27
- Elhamdani, A., Azizi, F. and Artalejo, C. R. (2006) Double patch clamp reveals that transient fusion (kiss-and-run) is a major mechanism of secretion in calf adrenal chromaffin cells: high calcium shifts the mechanism from kiss-and-run to complete fusion. *J. Neurosci.* **26**, 3030–3036
- Fulop, T., Radabaugh, S. and Smith, C. (2005) Activity-dependent differential transmitter release in mouse adrenal chromaffin cells. *J. Neurosci.* **25**, 7324–7332
- Ceccarelli, B. and Hurlbut, W. P. (1980) Vesicle hypothesis of the release of quanta of acetylcholine. *Physiol. Rev.* **60**, 396–441
- Gillis, K. D. (1995) Techniques for membrane capacitance measurements. In *Single-Channel Recording*, 2nd edn (Sakmann, B. and Neher, E., eds.), pp. 155–198, Plenum Press, New York
- Gomez, J. F., Brioso, M. A., Machado, J. D., Sanchez, J. L. and Borges, R. (2002) New approaches for analysis of amperometrical recordings. *Ann. N.Y. Acad. Sci.* **971**, 647–654
- Chen, P. and Gillis, K. D. (2000) The noise of membrane capacitance measurements in the whole-cell recording configuration. *Biophys. J.* **79**, 2162–2170
- Wightman, R. M., Jankowski, J. A., Kennedy, R. T., Kawagoe, K. T., Schroeder, T. J., Leszczyszyn, D. J., Near, J. A., Diliberto, E. J. and Viveros, O. H. (1991) Temporally resolved catecholamine spikes correspond to single vesicle release from individual chromaffin cells. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10754–10758
- Chow, R. H., von Ruden, L. and Neher, E. (1992) Delay in vesicle fusion revealed by electrochemical monitoring of single secretory events in adrenal chromaffin cells. *Nature (London)* **356**, 60–63
- Elhamdani, A., Palfrey, H. C. and Artalejo, C. R. (2001) Quantal size is dependent on stimulation frequency and calcium entry in calf chromaffin cells. *Neuron* **31**, 819–830
- Plattner, H., Artalejo, A. R. and Neher, E. (1997) Ultrastructural organization of bovine chromaffin cell cortex – analysis by cryofixation and morphometry of aspects pertinent to exocytosis. *J. Cell Biol.* **139**, 1709–1717
- Erratum (1998) *J. Cell Biol.* **140**, 973
- Moser, T. and Neher, E. (1997) Estimation of mean exocytic vesicle capacitance in mouse adrenal chromaffin cells. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 6735–6740
- Grabner, C. P., Price, S. D., Lysakowski, A. and Fox, A. P. (2005) Mouse chromaffin cells have two populations of dense core vesicles. *J. Neurophysiol.* **94**, 2093–2104
- Albillos, A., Dernick, G., Horstmann, H., Almers, W., Alvarez de Toledo, G. and Lindau, M. (1997) The exocytotic event in chromaffin cells revealed by patch amperometry. *Nature (London)* **389**, 509–512
- Gong, L. W., Hafez, I., Alvarez de Toledo, G. and Lindau, M. (2003) Secretory vesicles membrane area is regulated in tandem with quantal size in chromaffin cells. *J. Neurosci.* **23**, 7917–7921
- Kidokoro, Y. (1980) Role of action potentials in hormone secretion. *Biochem. Res.* **1**, 117–123
- Zhou, Z., Misler, S. and Chow, R. H. (1996) Rapid fluctuations in transmitter release from single vesicles in bovine adrenal chromaffin cells. *Biophys. J.* **70**, 1543–1552
- Hartmann, J. and Lindau, M. (1995) A novel Ca^{2+} -dependent step in exocytosis subsequent to vesicle fusion. *FEBS Lett.* **363**, 217–220
- Fernandez-Chacon, R. and Alvarez de Toledo, G. (1995) Cytosolic calcium facilitates release of secretory products after exocytotic vesicle fusion. *FEBS Lett.* **363**, 221–225
- Takahashi, N., Kishimoto, T., Nemoto, T., Kadowaki, T. and Kasai, H. (2002) Fusion pore dynamics and insulin granule exocytosis in the pancreatic islet. *Science* **297**, 1349–1352
- Lindau, M. and Alvarez de Toledo, G. (2003) The fusion pore. *Biochim. Biophys. Acta* **1641**, 167–173
- Klyachko, V. A. and Jackson, M. B. (2002) Capacitance steps and fusion pores of small and large-dense-core vesicles in nerve terminals. *Nature (London)* **418**, 89–92
- Ioan, C., Aberle, T. and Burchard, W. (2000) Structure properties of dextran. 2. Dilute solution. *Macromolecules* **33**, 5730–5739
- Chan, S. A. and Smith, C. (2003) Low frequency stimulation of mouse adrenal slices reveals a clathrin-independent, protein kinase C-mediated endocytic mechanism. *J. Physiol. (London)* **553**, 707–717
- Breckenridge, L. and Almers, W. (1987) Currents through the fusion pore that forms during exocytosis of a secretory vesicle. *Nature (London)* **328**, 814–817
- Graham, M. E., Fisher, R. J. and Burgoyne, R. D. (2000) Measurement of exocytosis by amperometry in adrenal chromaffin cells: effects of clostridial neurotoxins and activation of protein kinase C on fusion pore kinetics. *Biochimie* **82**, 469–479
- TerBush, D. R., Bittner, M. A. and Holz, R. W. (1988) Ca^{2+} influx causes rapid translocation of protein kinase C to membranes. Studies of the effects of secretagogues in adrenal chromaffin cells. *J. Biol. Chem.* **263**, 18873–18879
- Smith, C., Moser, T., Xu, T. and Neher, E. (1998) Cytosolic Ca^{2+} acts by two separate pathways to modulate the supply of release-competent vesicles in chromaffin cells. *Neuron* **20**, 1243–1253
- Smith, C. (1999) A persistent activity-dependent facilitation in chromaffin cells is caused by Ca^{2+} activation of protein kinase C. *J. Neuroscience* **19**, 589–598
- Debus, K. and Lindau, M. (2000) Resolution of patch capacitance recordings and of fusion pore conductances in small vesicles. *Biophys. J.* **78**, 2983–2997
- Chan, S. A., Chow, R. and Smith, C. (2003) Calcium dependence of action potential-induced endocytosis in chromaffin cells. *Pflügers Arch.* **445**, 540–546
- Ashery, U., Varoqueaux, F., Voets, T., Betz, A., Thakur, P., Koch, H., Neher, E., Brose, N. and Rettig, J. (2000) Munc13-1 acts as a priming factor for large dense-core vesicles in bovine chromaffin cells. *EMBO J.* **19**, 3586–3596
- Betz, A., Ashery, U., Rickmann, M., Augustin, I., Neher, E., Sudhof, T. C., Rettig, J. and Brose, N. (1998) Munc13-1 is a presynaptic phorbol ester receptor that enhances neurotransmitter release. *Neuron* **21**, 123–136
- Davis, P. D., Elliott, L. H., Harris, W., Hill, C. H., Hurst, S. A., Keech, E., Kumar, M. K., Lawton, G., Nixon, J. S. and Wilkinson, S. E. (1992) Inhibitors of protein kinase C.2. Substituted bisindolylmaleimides with improved potency and selectivity. *J. Med. Chem.* **35**, 994–1001
- Voets, T., Toonen, R. F., Brian, E. C., de Wit, H., Moser, T., Rettig, J., Sudhof, T. C., Neher, E. and Verhage, M. (2001) Munc18-1 promotes large dense-core vesicle docking. *Neuron* **31**, 581–591
- Graham, M. E., Barclay, J. W. and Burgoyne, R. D. (2004) Syntaxin/Munc18 interactions in the late events during vesicle fusion and release in exocytosis. *J. Biol. Chem.* **279**, 32751–32760
- Barclay, J. W., Craig, T. J., Fisher, R. J., Ciufu, L. F., Evans, G. J., Morgan, A. and Burgoyne, R. D. (2003) Phosphorylation of Munc18 by protein kinase C regulates the kinetics of exocytosis. *J. Biol. Chem.* **278**, 10538–10545
- Fisher, R. J., Pevsner, J. and Burgoyne, R. D. (2001) Control of fusion pore dynamics during exocytosis by Munc18. *Science* **291**, 875–878

Received 2 May 2006/2 June 2006; accepted 20 June 2006

Published as BJ Immediate Publication 20 June 2006, doi:10.1042/BJ20060654