High calcium concentrations shift the mode of exocytosis to the kiss-and-run mechanism

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Exocytosis, the fusion of secretory vesicles with the plasma membrane to allow release of the contents of the vesicles into the extracellular environment, and endocytosis, the internalization of these vesicles to allow another round of secretion, are coupled. It is, however, uncertain whether exocytosis and endocytosis are tightly coupled, such that secretory vesicles fuse only transiently with the plasma membrane before being internalized (the 'kiss-and-run' mechanism), or whether endocytosis occurs by an independent process following complete incorporation of the secretory vesicle into the plasma membrane. Here we investigate the fate of single secretory vesicles after fusion with the plasma membrane by measuring capacitance changes and transmitter release in rat chromaffin cells using the cell-attached patch-amperometry technique. We show that raised concentrations of extracellular calcium ions shift the preferred mode of exocytosis to the kiss-and-run mechanism in a calcium-concentration-dependent manner. We propose that, during secretion of neurotransmitters at synapses, the mode of exocytosis is modulated by calcium to attain optimal conditions for coupled exocytosis and endocytosis according to synaptic activity.

After fusion of secretory vesicles with the plasma membrane, the excess of membrane is retrieved by endocytosis[1,2]. However, the mechanism and location of endocytosis are still a matter of debate. The classical model of synaptic-vesicle recycling after neurotransmission involves the retrieval of membrane at a distant location from the active zone (which is where secretion of neurotransmitters occurs), and fusion of the internalized membrane with an endosome-like compartment[1,3]. An alternative model, the 'kiss-and-run' hypothesis, proposes that synaptic vesicles may transiently fuse with the plasma membrane and release their contents through a partially open fusion pore without merging of vesicle and plasma membrane[2,4–6]. Recent experiments[7] using styryl-FM fluorescent dyes have provided evidence that synaptic vesicles can be internalized much faster than was previously thought, in the order of few seconds, and that synaptic vesicles are recycled without passing through an endosomal compartment[7,8]. However, no direct measurements of the life cycle of a single synaptic vesicle on the plasma membrane have been made.

In addition to optical techniques, patch-clamp capacitance measurements are a powerful tool with which to monitor changes in the plasma-membrane area that are attributable to exocytosis and endocytosis[9]. Single fusion events and the associated release of neurotransmitter have been measured simultaneously in mast cells using whole-cell recording and an extracellular amperometric detector[10], or, more recently and at higher resolution, in chromaffin cells using patch amperometry[11]. In these patch-clamp studies, kiss-and-run events were rare and neurotransmitter release occurred slowly because of low fusion-pore conductance[10,11]. The protein machinery that mediates exocytosis in chromaffin cells resembles that operating in synapses[12]. However, an important difference between synapses and chromaffin cells is that the local calcium concentrations near the sites of exocytosis are much higher in synapses than in chromaffin cells[13,14]. We therefore tried to mimic the conditions present in the synapse by using raised concentrations of extracellular calcium in the pipette solution in cell-attached patch-amperometry studies of rat chromaffin cells. This should presumably lead to increased calcium concentrations near the submembraneous fusion sites under the patch.

Results

Patch amperometry combines high-resolution cell-attached capacitance measurements with electrochemical detection of secretory products released from a patch of membrane[11]. Cell-attached capacitance measurements allow us to resolve both single-vesicle fusion events on the same size scale as synaptic vesicles[9,15], and the kinetics and dimensions of the water channel connecting the vesicle lumen with the external medium, the so-called fusion pore[16,17]. The arrangement of the carbon-fibre electrode inside the patch pipette restricts the detection of catecholamines (the neurotransmitter being measured) to the plasma membrane under the pipette (an area of 3–5 μm^2). This arrangement also offers the possibility of subjecting the patch of membrane to different ionic environments with minimal interference in normal cell excitability or function (Fig. 1a).

Fast kiss-and-run events predominate at high Ca2+ concentrations. Depending on the pipette Ca^{2+} concentration (Fig. 1a), increments in patch-membrane capacitance, reflecting exocytosis of single chromaffin granules, were preferentially either permanent or transient. In the presence of 5 mM Ca^{2+}, step increases in capacitance were mainly permanent (Fig. 1b, top). These events were accompanied by amperometric spikes, indicating rapid release of catecholamines following fusion (Fig. 1b, bottom). In the presence of 90 mM Ca^{2+}, most events were transient, as reflected by the brief flickers in the capacitance trace superimposed on a constant capacitance level, showing no net increase in capacitance (Fig. 1c, top). However, rapid amperometric spikes, indistinguishable from those measured at 5 mM Ca^{2+}, were still observed, indicating rapid catecholamine release (Fig. 1c, bottom). We term these fast transient events 'fast kiss-and-run events'.

Fast kiss-and-run and permanent events could occur in the same patch, although their relative abundance varied with the Ca^{2+} con-

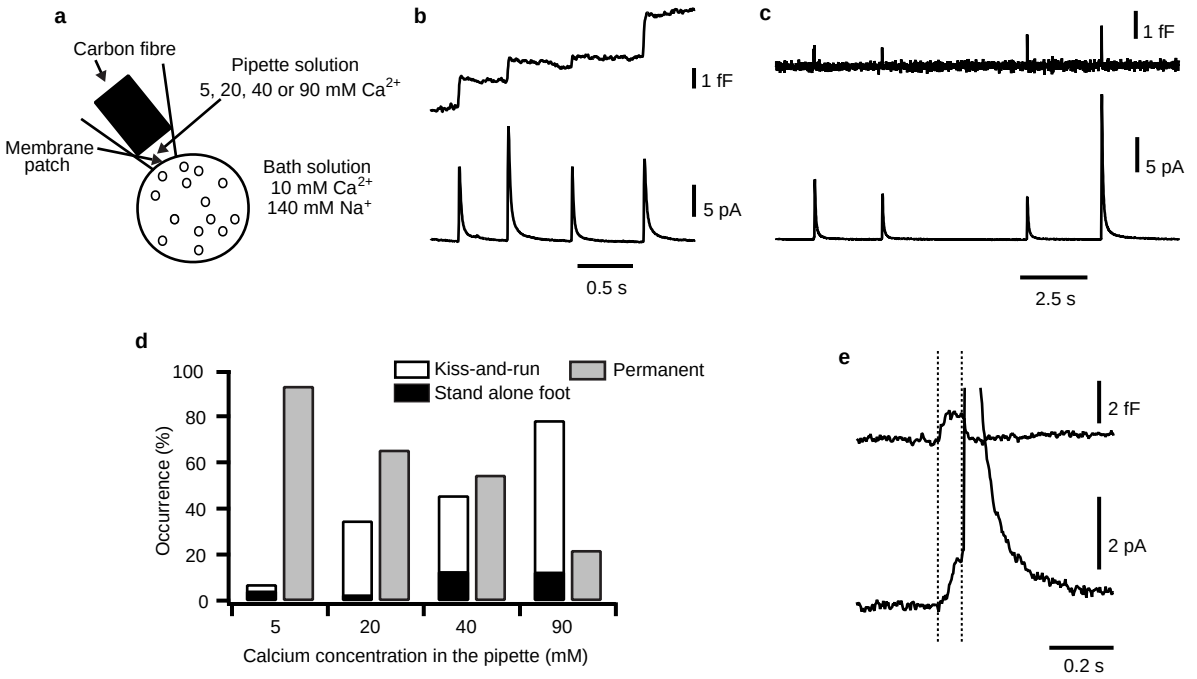

Figure 1 **Catecholamine release during fast kiss-and-run and permanent fusion events in rat chromaffin cells, recorded by patch amperometry. a**, The patch-amperometry technique. **b**, Irreversible fusion events, recorded with 5 mM Ca^{2+} in the patch pipette. Top, capacitance trace; bottom, electrochemical detection of catecholamines by amperometry. **c**, Fast kiss-and-run fusion events, recorded at 90 mM Ca^{2+}. **d**, Percentages of transient fusion events and irreversible events depending on patch-pipette Ca^{2+} concentration. **e**, Fast kiss-and-run event shown at an expanded timescale. Top, transient increase in capacitance; bottom, amperometric signal, showing a foot followed by a amperometric spike.

centration in the pipette solution (Fig. 1d). The relative frequency of fast kiss-and-run events increased from 7% at 5 mM Ca^{2+} (10 out of 142 events, 3 cells) to 35% at 20 mM Ca^{2+} (17 out of 49 events, 5 cells), 54% at 40 mM (43 out of 79 events, 5 cells) and 78% at 90 mM (104 out of 134 events, 12 cells). Ca^{2+} obviously shifts the mode of exocytosis to the kiss-and-run form, in a concentration-dependent manner.

A fast kiss-and-run fusion event is shown on an expanded timescale in Fig. 1e. The change in capacitance (flicker) lasted 90 ms. Transmitter release occurred in two stages. First there was a small amperometric signal ('foot'), indicating the trickle of transmitter from the granule. This foot signal had a duration of ~60 ms and thus lasted for most of the transient fusion; it resembled those described previously in bovine chromaffin cells by amperometric measurements[18,19]. The foot was followed by an amperometric spike, indicating rapid transmitter release. The offset of capacitance, indicating the closure of the pore, occurred shortly after the onset of the amperometric spike.

This release pattern is qualitatively different from the previously reported release pattern occurring during 'flicker'[10,11] and from the 'stand-alone' foot events recorded amperometrically in bovine chromaffin cells[20]. Stand-alone foot events are characterized by a slow release rate because of the small size of the fusion pore during flicker[10,11]. In our experiments, 3–13% of the exocytotic events were stand-alone foot events, and this percentage increased slightly when the pipette Ca^{2+} concentrations were higher, from 4.2% stand-alone foot events with 5 mM Ca^{2+} (6 out of 142 events, n=3) to 12.7% with 90 mM Ca^{2+} (17 out of 134 events, n=12) (Fig. 1d). These values are less than those reported in ref. 20 (up to 20%), but the cells that we used here were from a different species to those used in ref. 20. We thus consider the difference in the occurrence of such events to be insignificant. The kiss-and-run events that we report here, which occur preferentially at high Ca^{2+} concentrations, are qualitatively different to those described previously[10,11,20]. During these fast kiss-and-run events, the fusion pore appears to expand briefly to quite a large size, allowing for rapid transmitter release.

Similarities between fast kiss-and-run and permanent events. Fig. 2 shows a comparison of the properties of fast kiss-and-run events (Fig. 2a–c) and permanent fusion events (Fig. 2d–f). The step-size distributions for both types of event are very similar (Fig. 2a, d). The mean (±s.e.m.) flicker size (for fast kiss-and-run events) was 1.23±0.09 fF and ranged from 0.25 to 4.25 fF. In irreversible fusion events, step sizes averaged 1.13±0.03 fF with a range of 0.2 to 2.8 fF. These values are also similar to those estimated for mouse chromaffin granules using noise analysis in whole-cell capacitance measurements21. Transformation of vesicle capacitance to vesicle radius predicts a vesicle radius of 92±22 nm (mean ±s.d.) for permanent events and 94±28 nm for fast kiss-and-run events. These values were well fitted by a gaussian distribution (Fig. 2b, e, curved line), indicating that the sizes of rat chromaffin granules are normally distributed. The sizes of vesicles releasing neurotransmitter by the kiss-and-run method are thus indistinguishable from the sizes of vesicles that are undergoing permanent fusion.

We estimated the amount of catecholamine release in single events from the time integral of the amperometric spikes (charge). The charge distribution for both types of event was very similar; the mean charge (±s.e.m.) for transient fusion events was 1.38±0.17 pC, compared with 1.51±0.1 pC for irreversible events. From the step-size distribution and the amperometric charge we estimated an average (±s.e.m.) concentration of catecholamine-containing vesicles of 2.5±0.15 M for irreversible events and 2.59±0.24 M for transient fusion events. These results indicate that neurotransmitter release during fast kiss-and-run events is complete. The kinetics of release signals, as measured by the time course of the amperometric spikes for fast kiss-and-run and irreversible fusion events, were also indistinguishable (data not shown).

The fusion pore expands during fast kiss-and-run events. When a sine-wave voltage is applied to the patch membrane, the resulting current may change either because of opening and closing of ion channels or because of changes in membrane capacitance (vesicle fusion). The ohmic current flowing through ion channels is in phase with the applied sine-wave voltage, whereas the current

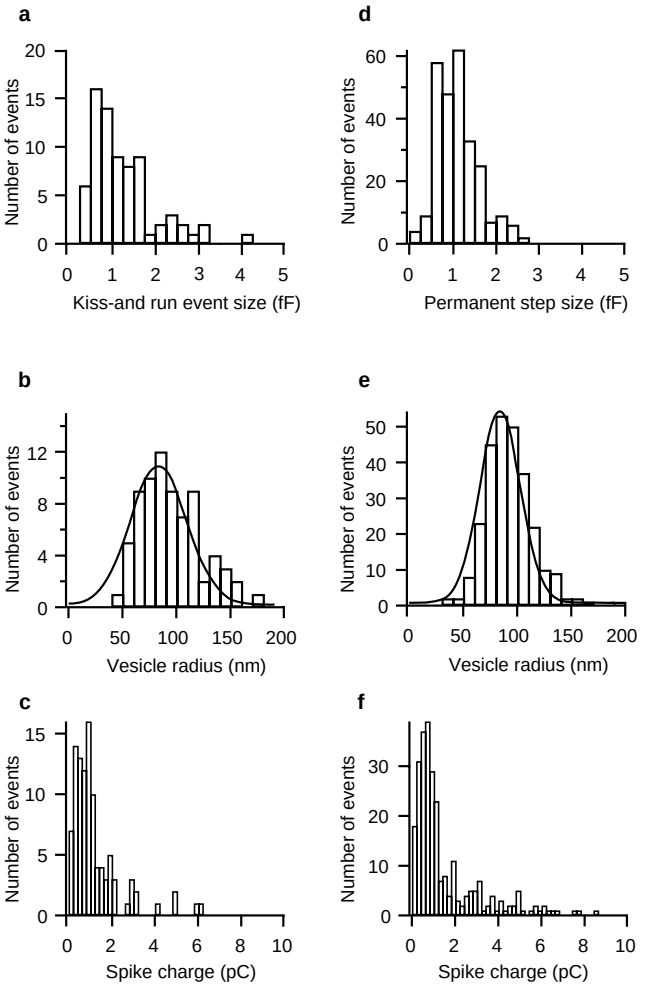

Figure 2 **Comparison of fast kiss-and-run and permanent fusion events. a–c**, Fast kiss-and-run events. **d–f**, Permanent events. **a**, Distribution of capacitance flicker sizes of fast kiss-and-run events. **b**, Transformation of capacitance flicker sizes to granule radii. **c**, Charge distribution of amperometric spikes detected during fast kiss-and-run events. **d**, **e**, Capacitance step-size distribution for permanent fusion events and its transformation to vesicle radii. **f**, Charge distribution for permanent fusion events.

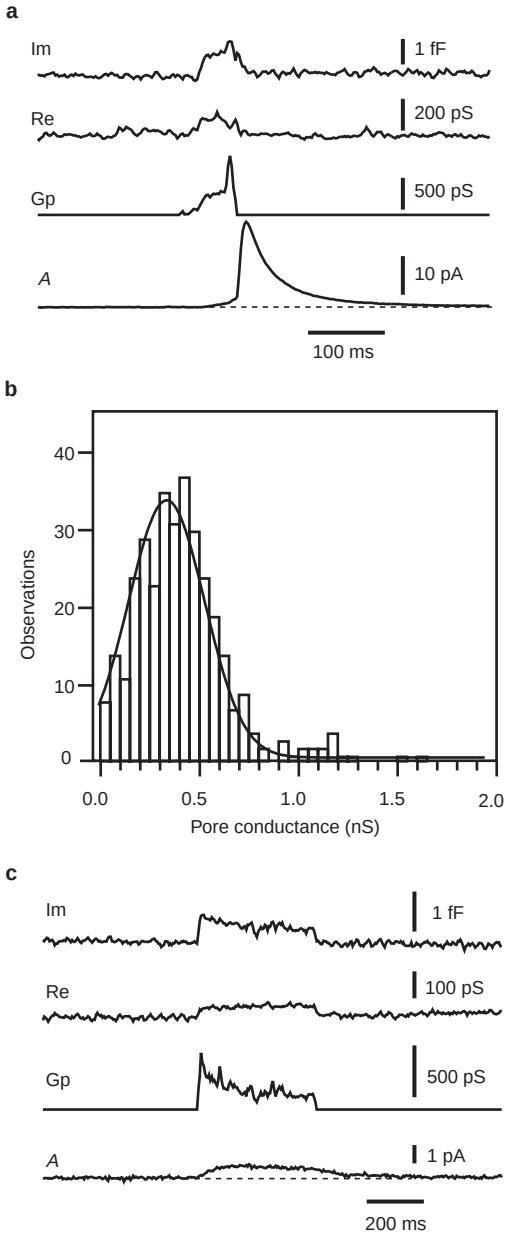

Figure 3 **Kiss-and-run kinetics in fast and slow fusion events.** Pore conductance (Gp) is calculated from the real (Re) and imaginary (Im) traces. **a**, Analysis of Gp during a fast kiss-and-run event. The amperometric foot followed by a spike-like signal developed during the expansion of the pore. **b**, Pore conductance distribution during the expansion of the exocytotic fusion pore in fast kiss-and-run events that elicit amperometric spikes. **c**, Analysis of Gp during a slow kiss-and-run event accompanied by a stand-alone foot signal. *A*, amperometric trace.

charging and discharging the patch capacitance is phase-shifted by 90°. To describe the changes in electrical properties of the patch, the conductance change is replaced by the complex admittance change. For the simple case of channel opening, the change in the real part of the admittance (Re) is equal to the conductance change (Re=G). For full incorporation of a vesicle, the change in the imaginary part (Im) is equal to the vesicle capacitance (Cv) multiplied by the angular frequency of the sine wave (Im=$2\pi f$Cv, where f is the sine-wave frequency)[9]. When a narrow fusion pore forms, connecting the vesicular lumen to the extracellular space, then we get changes in both in-phase current (Re) and out-of-phase current (Im)[15–17]. Vesicle capacitance and fusion-pore conductance (Gp) can then be calculated from the admittance change, as Cv=(Re^2+Im^2)/Im and Gp=(Re^2+Im^2)/Re (ref. 15). Traces in Fig. 3 show the fusion-pore conductance during two kiss-and-run events, one accompanied by release of catecholamines in a spike-like form (fast kiss-and-run; Fig. 3a) and the other accompanied by slow catecholamine release (stand-alone foot; Fig. 3c).

In the example shown in Fig. 3a, the conductance of the pore first attained a plateau value of ~450 pS, where it remained fluctuating for about 40 ms. During this period the pore leaked some catecholamines, producing the observed foot in the amperometric trace (Fig. 3a, fourth trace). A frequency histogram of pore conductance during the occurrence of the amperometric foot, in 20 different events like those in Fig. 3a, is shown in Fig. 3b. The average pore conductance during the foot obtained from all events with a foot of sufficient duration was 400 pS. This value is very similar to the pore conductance estimated during the foot in mast-cell granules[10] and in bovine chromaffin granules using patch amperometry[11]. After about 60 ms, the pore conductance briefly exceeded 1 nS, and then the pore closed completely. Pore expansion coincided with the onset of the amperometric spike. The conductance of expanded pores could not be accurately determined because of the small pro-

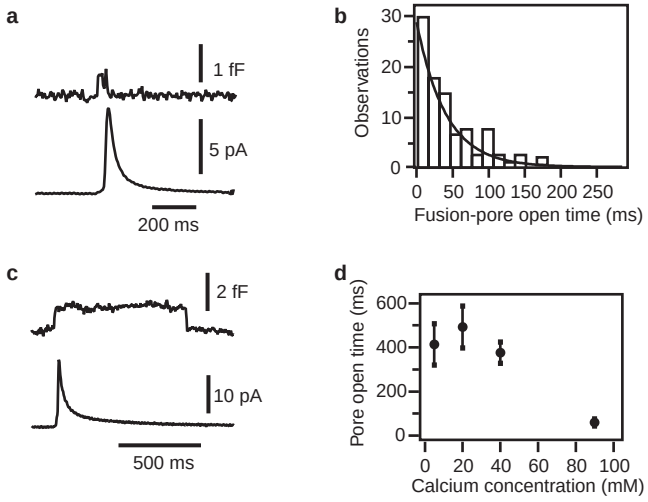

Figure 4 **Ca^{2+} dependence of the closure of the exocytotic fusion pore. a**, A fast kiss-and-run fusion event measured in the presence of 90 mM Ca^{2+}. **b**, Frequency histogram of fusion-pore open times measured in the presence of 90 mM Ca^{2+}. **c**, Event detected with a pipette containing 40 mM Ca^{2+}. The amperometric spike occurred quickly after a brief expansion of the fusion pore; closure of the pore was delayed for 770 ms. **d**, Duration of fast, transient capacitance events at different pipette Ca^{2+} concentrations (mean ±s.e.m.; *n*=8, *n*=16, *n*=26, *n*=90, from left to right).

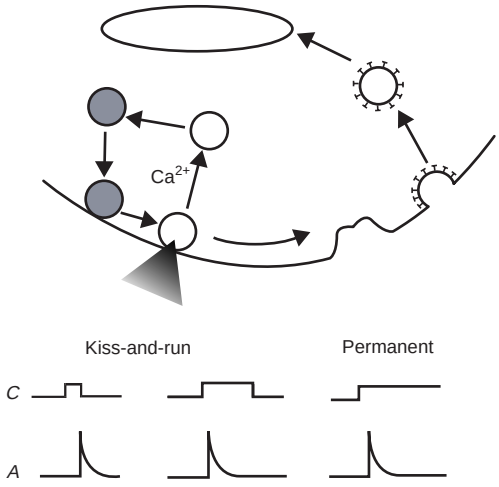

Figure 5 **Model for coupled exocytosis and endocytosis, incorporating our experimental results.** The kiss-and-run mechanism (left) preserves vesicle identity and allows for fast reloading of neurotransmitter. Permanent fusion (right) leads to full merging of vesicle and plasma membranes, which are internalized by protein-coated pits and recycled through endosomes. Darkly shaded circles represent neurotransmitter-full vesicles; white circles represent empty vesicles. The dark triangle represents neurotransmitter release. The lower part on the figure shows schematic capacitance (*C*) and amperometric (*A*) traces.

jection in the Re trace. In 12 events, a value for the fusion-pore conductance at the onset of the spike could be determined; the average fusion-pore conductance was 1.43±0.23 nS (±s.e.m.). This is an underestimate of the real value, because fusion-pore conductances larger than this cannot be detected for the vesicle size and sine-wave frequency used here. Furthermore, the lifetime of the expanded state is comparable to our time resolution of 5 ms and the pore conductance spike is thus low-pass-filtered. We conclude that fast kiss-and-run events are characterized by brief expansion of the fusion pore beyond a conductance of 1.4 nS (and probably larger than this), allowing for rapid and complete transmitter release from the vesicle as indicated by rapid amperometric spikes.

A typical stand-alone foot event is analysed in Fig. 3c. This event lasted 445 ms and analysis of fusion-pore conductance revealed that the fusion pore rapidly attained a maximum conductance of 600 pS; this later decreased steadily to 160 pS before the pore closed (Fig. 3c, Gp trace). The amperometric signal developed slowly, like in a regular foot, without spike, probably indicating that the pore size limits release.

The fusion pore closes more quickly at high Ca^{2+} concentrations. The duration of fast kiss-and-run events, as measured by the duration of capacitance flickers, was variable (Fig. 4). Examples of characteristic events obtained in the presence of 90 and 40 mM Ca^{2+} in the pipette are shown in Fig. 4a and c, respectively. The frequency distributions of the duration of capacitance flicker were well fitted by a single exponential. With 90 mM Ca^{2+}, a time constant of 41 ms was obtained, indicating the mean pore open time for this experimental condition (Fig. 4b). At lower pipette Ca^{2+} concentrations, the flicker duration or mean pore open time was markedly prolonged (Fig. 4d). Interestingly, the pore remained open for several hundred milliseconds after complete neurotransmitter release had occurred (Fig. 4c).

Discussion

Our results indicate that Ca^{2+} may modulate the mode of exocytosis–endocytosis by regulating the rate at which fusion pores re-close following fusion (Fig. 5). At low Ca^{2+} concentrations, re-closure of the fusion pore is unlikely and vesicles preferentially incorporate completely into the plasma membrane, probably to be internalized later by a conventional mechanism that passes through endosomal structures. With increasing Ca^{2+} concentration, the probability and rate of re-closure of the fusion pore increase, leading preferentially to kiss-and run events.

The role of calcium in endocytosis following exocytosis has been controversial. An inhibition of endocytosis by high intracellular Ca^{2+} concentrations has been reported in retinal bipolar cells by using whole-cell capacitance methods[22]. However, a rapid form of endocytosis, with time constants ranging from 300 ms to 1 s, has been observed only in the presence of high intracellular Ca^{2+} concentrations[23–26] and occurs only if the Ca^{2+} load exceeds a certain threshold value[27]. The rapid sequence of exocytosis and endocytosis of single vesicles that we have described here may be the mechanism by which this rapid form of endocytosis occurs.

Although the pipette Ca^{2+} concentrations used here are not physiological for rat chromaffin cells, the concentration attained at the intracellular membrane surface may be close to those attained in nerve terminals under physiological conditions. We may therefore be mimicking, in a chromaffin cell, the conditions found in the active zone of a synapse. Recently, the kinetics and regulation of exocytosis–endocytosis in hippocampal synapses were studied using FM dyes[7]. Klingauf *et al.* interpreted their destaining kinetics using the kiss-and-run model for exocytosis. In the model in ref. 7, the lifetime of the fused state decreased with increasing Ca^{2+} concentration, in agreement with our direct measurements. Assuming that full fusion followed by slow internalization did not occur, Klingauf *et al.*[7] calculated an upper limit of 2 s for the time constant of rapid endocytosis in the presence of an extracellular Ca2+ concentration of 8 mM. Klingauf *et al.* suggest that this time constant may be an overestimate, if slow endocytotic processes following permanent fusion occur in parallel with the fast endocytotic events; thus the average time constant of ~0.5 s that we obtained (with pipette Ca^{2+} concentrations in the range 5–20 mM) agrees well with their results.

In addition to the effect of Ca^{2+} on the internal side of the membrane, the high Ca^{2+} concentration in the external solution may prevent swelling of chromaffin granule matrices, because Ca^{2+} efficiently generates condensation of granule matrices in goblets[28]

and beige mouse mast cells[29,30].

Our results indicate that rat chromaffin granules can release their catecholamine content rapidly and in a spike-like form through their transient connection to the plasma membrane, and that this is a Ca^{2+}-dependent process. What might be the function of the Ca^{2+}-mediated modulation of endocytosis? During phases of high electrical activity, when vesicles undergo exocytosis at a high rate, kiss-and-run is an excellent mechanism by which the pool of releasable vesicles can be rapidly refilled. This probably occurs through a direct pathway that bypasses the endosomal compartment, as suggested[8]. What then is the advantage of the alternative recycling mechanism, involving full fusion and conventional endocytosis? This mechanism could operate during phases of low electrical activity. When release rates are low, vesicles could fuse fully with the plasma membrane, be internalized through protein-coated pits, and be processed through the endosomal compartment. This would allow the reassembly of the synaptic vesicles, eliminating defective membrane proteins and other components. This would be the time for housekeeping activities and quality control. The Ca^{2+} dependence of the kiss-and-run method may thus ensure both rapid recycling during periods of high activity and the replacement of used vesicles during periods of low activity. □

Methods

Cells and solutions.

Chromaffin cells were obtained from Sprague–Dawley rats (200–400 g) and cultured as described[31]. Recordings were made on days 1–3 in culture. The bath solution contained (in mM): 140 NaCl, 2.7 KCl, 10 $CaCl_2$, 1 $MgCl_2$, 10 HEPES/NaOH and 5–10 glucose, pH 7.3. The pipette solution contained (in mM): 5 $CaCl_2$, 100 tetraethyl ammonium chloride, 50 NaCl, 1 $MgCl_2$ and 10 HEPES/NaOH, or 20 $CaCl_2$, 150 tetraethyl ammonium chloride and 10 HEPES/NaOH, or 40 $CaCl_2$, 120 tetraethyl ammonium chloride and 10 HEPES/NaOH, or 90 $CaCl_2$, 40 tetraethyl ammonium chloride and 10 HEPES/NaOH. All experiments were done at room temperature.

Measurements of membrane capacitance and catecholamine release.

Changes in membrane capacitance and catecholamine release were recorded simultaneously by patch amperometry[11]. Briefly, cell-attached patch clamp was achieved with a carbon-fibre electrode (CFE) introduced into the patch pipette. A special holder with two Ag/AgCl electrodes was used. The CFE was prepared from 5-μm-diameter carbon fibres as described[32]. The CFE was positioned at distances 1–5 μm from the patch-pipette-tip opening under the microscope. The pipette electrode was connected to ground and the CFE was connected to the *I–V* converter of a home-made amperometric amplifier by a 3 M KCl solution. The CFE was held continuously at +800 mV. Amperometric currents were filtered with a 8-pole Bessel filter set at 3 kHz. Patch pipettes were pulled in three stages with a programmable puller (P-97, Sutter Instruments).

For measurements of cell-attached capacitance, we used a patch-clamp amplifier (EPC7, HEKA-Elektronik). Command voltage was applied to the bath. Changes in patch admittance were measured as described[9,15] with a lock-in amplifier (SR830, Stanford Research Systems) using a sine-wave amplitude of 25 mV (root mean square) at a frequency of 20 kHz. The output filter was set to time constant 1 or 3 ms, 24 dB. Capacitance and conductance traces in Fig. 1a were digitally filtered to remove a periodic oscillation of 0.05 Hz that was probably the result of fluctuations at the level of the bath solution. To convert capacitance changes to granule volumes, we used a proportionality constant of 10 $fF\,\mu m^{-2}$ for the specific membrane capacitance. Data acquisition and analysis were done with a 15 bit analog-to-digital converter (IDA 15125, Indec Systems Inc.) with locally written software. Fusion-pore openings were analysed as described[15]. Pore conductance, Gp, was calculated from the real (Re) and imaginary (Im) parts of the admittance after baseline subtraction: $Gp=(Re^2+Im^2)/Re$. Pore diameter was estimated from Gp as described[16,33], assuming a cylindrical pore 15 nm long and a conductivity of the pipette solution of 15 mS cm^{-1} (see equation 11.1 in ref. 34). Six amperometric events observed without a change in capacitance or conductance (that is, the fusion pore opened and closed faster than our time resolution (2.5 or 5 ms) for cell-attached capacitance measurements) were included in the first bin of the histogram shown in Fig. 4b.

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