Compensatory and excess retrieval: two types of endocytosis following single step depolarizations in bovine adrenal chromaffin cells

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1. Endocytosis following exocytosis evoked by single step depolarizations was examined in bovine adrenal chromaffin cells using high resolution capacitance measurements in perforated-patch voltage clamp recordings.

2. Endocytosis was detected as a smooth exponential decline in membrane capacitance to either the pre-stimulus level (‘compensatory retrieval’) or far below the pre-stimulus level (‘excess retrieval’). During excess retrieval, >10% of the cell surface could be internalized in under 5 s.

3. Compensatory retrieval was equal in magnitude to stimulus-evoked exocytosis for membrane additions >100 fF (about fifty large dense-cored vesicles). In contrast, excess retrieval surpassed both the stimulus-evoked exocytosis, and the initial capacitance level recorded at the onset of phase-tracking measurements. Cell capacitance was not maintained at the level achieved by excess retrieval but slowly returned to pre-stimulus levels, even in the absence of stimulation.

4. A large percentage of capacitance increases < 100 fF, usually evoked by 40 ms depolarizations, were not accompanied by membrane retrieval.

5. Compensatory retrieval could occur with any amount of Ca$^{2+}$ entry, but excess retrieval was never triggered below a threshold Ca$^{2+}$ current integral of 70 pC.

6. The kinetics of compensatory and excess retrieval differed by an order of magnitude. Compensatory retrieval was usually fitted with a single exponential function that had a median time constant of 5.7 s. Excess retrieval usually occurred with double exponential kinetics that had an extremely fast first time constant (median, 670 ms) and a second time constant indistinguishable from that of compensatory retrieval.

7. The speed of compensatory retrieval was Ca$^{2+}$ dependent: the largest mono-exponential time constants occurred for the smallest amounts of Ca$^{2+}$ entry and decreased with increasing Ca$^{2+}$ entry. The Ca$^{2+}$ dependence of mono-exponential time constants was disrupted by cyclosporin A (CsA), an inhibitor of the Ca$^{2+}$- and calmodulin-dependent phosphatase calcineurin.

8. CsA also reduced the proportion of responses with excess retrieval, but this action was caused by a shift in Ca$^{2+}$ entry values below the threshold for activation. The lower total Ca$^{2+}$ entry in the presence of CsA was due to an increase in the rate of Ca$^{2+}$ current inactivation rather than a reduction in peak amplitude.

9. Our data suggest that compensatory and excess retrieval represent two independent, Ca$^{2+}$-regulated mechanisms of rapid membrane internalization in bovine adrenal chromaffin cells. Alternatively, there is a single membrane internalization mechanism that can switch between two distinct modes of behaviour.
Endocytosis of plasma membrane occurs via several morphologically distinguishable membrane invaginations, including clathrin-coated pits, caveolae or other uncoated vesicles, and large vacuoles. These structures exist in most cells (for review see Liu & Robinson, 1995). Neurosecretory cells that exocytose transmitter/hormone in response to electrical activity may possess a unique means to maintain cell integrity in the face of intensive stimulation, or alternatively may simply use one of the above mechanisms to reuptake recently added membrane. For example, clathrin is highly enriched in neural tissue (De Camilli & Takei, 1996, and references therein). However, internalization via clathrin cages is a relatively slow process that takes minutes to complete (reviewed in Henkel & Almers, 1996), leading to the suggestion that additional, more rapid mechanisms must exist. One hypothesis suggests vesicles do not completely fuse during stimulation but only transiently join the plasma membrane via a fusion pore that rapidly recloses after transmitter is released (‘kiss and run’; Fesce, Grohovaz, Valtorta & Meldolesi, 1994; Henkel & Betz, 1995). On the other hand, there is morphological evidence from freeze fracture and transmission electron microscopy studies that rapid internalization can occur at the active zones of stimulated nerve terminals without the formation of clathrin-coated pits (Miller & Heuser, 1984; Koenig & Ikeda, 1996).

In contrast to fusion-mediated transmitter release, which can be monitored using postsynaptic receptor responses, it has been difficult to study potentially rapid mechanisms of membrane uptake in real time. Recently, a method for detecting small changes in the amount of surface membrane using high resolution capacitance measurements has been developed for whole-cell patch clamp recording (Neher & Marty, 1982). With this technique, rapid endocytic responses (detected as decreases in membrane capacitance) have been observed in a number of cell types: melanotrophs, gonadotrophs, goldfish bipolar neurons, hair cells, pancreatic β-cells, calf and adult bovine adrenal chromaffin cells (reviewed in Henkel & Almers, 1996), posterior pituitary nerve terminals (Hsu & Jackson, 1996), PC12 cells (Kasai et al., 1996), salamander rods (Rieke & Schwarz, 1996) and dorsal root ganglion cell bodies (Huang & Neher, 1996).

In all of the preparations mentioned above, capacitance decreases due to endocytosis are smoothly exponential. The measured rates of endocytosis vary 100-fold in different preparations, with the slowest time constants in the tens of seconds (Huang & Neher, 1996) and the fastest <100 ms (Heinemann, Chow, Neher & Zucker, 1994). This wide range of rates can occur in the same preparation under different experimental conditions (Heinemann et al., 1994; Burgoyne, 1995) or even during a single round of endocytosis following a train of depolarizations (Artalejo, Henley, McNiven & Palfrey, 1995).

The extent of membrane retrieval also varies widely. In some experiments, endocytosis is incomplete, retrieving only a portion of the stimulus-evoked increase in membrane surface area (Thomas, Suprenant & Almers, 1990; Hsu & Jackson, 1996). Retrieval can also be far greater than the amount of exocytosis (‘excess retrieval’), even in those preparations that also show incomplete responses (Neher & Zucker, 1993; Thomas, Lee, Wong & Almers, 1994; Artalejo et al., 1995; Artalejo, Elhandani & Palfrey, 1996; Hsu & Jackson, 1996; Kasai et al. 1996). In melanotrophs, excess retrieval is rapid (τ, <500 ms; Thomas et al., 1994), but in nerve terminals of the posterior pituitary, excess retrieval occurs with a slower time constant (2 s; Hsu & Jackson, 1996). The variability in endocytic parameters, even within a single cell type/preparation, could be due to modifications of a single endocytotic process under different experimental conditions. Alternatively, the differences may reflect multiple mechanisms of endocytosis under distinct regulatory controls, which have unique capacities and kinetics.

Both exocytosis and endocytosis run down with time during whole-cell recording, although the run-down of endocytosis is faster (Åmmålå, Eliasson, Bokvist, Larsson, Ashcroft & Rorsmann, 1993; Parsons, Lenzi, Almers & Roberts, 1994; Burgoyne, 1995; Eliasson et al., 1996). This may reflect a differential dependence on lower molecular weight cytoplasmic constituents for endocytosis vs exocytosis. In support of this conclusion, Artalejo et al. (1995) reported that run-down of endocytosis during whole-cell recording is prevented when GTP is included in the recording solution. However, even in the presence of GTP, endocytosis can be lost during whole-cell recording (Burgoyne, 1995). Some of the variability in endocytic parameters observed during capacitance measurements may reflect the differential loss of one or more endocytotic mechanisms during whole-cell perfusion.

The intracellular milieu can be preserved by using a variant of the whole-cell patch clamp technique, perforated-patch recording (Horn & Marty, 1988). Several brief reports suggest that endocytosis is maintained during perforated-patch recording (Åmmålå et al., 1993; Parsons et al., 1994; Proks & Ashcroft, 1995), and in a more detailed study, Eliasson et al. (1996) reported that endocytosis was observed in only 25% of whole-cell recordings, but occurred in over 70% of perforated-patch recordings.

We have previously used the perforated-patch technique in bovine adrenal chromaffin cells to examine the Ca2+ dependence of endocytosis of large dense-cored vesicles in response to depolarization-evoked Ca2+ entry (Engisch & Nowycky, 1996). Here we report that in perforated-patch recordings of individual bovine adrenal chromaffin cells there are two distinct types of retrieval events following depolarization-evoked Ca2+ entry. ‘Compensatory retrieval’ quantitatively recovers membrane added during stimulation. ‘Excess retrieval’ is an extremely large internalization event that appears to be unrelated to stimulus-evoked exocytosis. We show that compensatory and excess retrieval can be further distinguished by maintenance during voltage clamp recording, kinetics, and Ca2+ requirements.
METHODS

Cell preparation
Chromaffin cells were prepared from adult bovine adrenal glands (obtained from a local abattoir) by collagenase digestion and cultured on collagen-coated glass coverslips as described in Vitale, del Castillo, Tchakarov & Trifaro (1991). Culture media consisted of Dulbecco’s modified Eagle’s medium supplemented with 25 mM Hepes, and fetal bovine serum, anti-mitotic agents and antibiotics were added immediately prior to plating. Cells were used from day 3 to day 7 after plating, culture media were partially replaced on day 3 and day 6.

Electrophysiological solutions
Standard external recording solution consisted of (mM): 130 NaCl, 2 KCl, 10 glucose, 10 Hepes-Na salt, 1 MgCl₂, 5 N-methyl-D-glucamine, and 5 CaCl₂ (pH 7.2, 295 mosmol l⁻¹). Perforated-patch (internal) solution contained (mM): 145 cesium glutamate, 10 Hepes free acid, 9.5 NaCl, 0.5 Na₂BAPTA (pH 7.2, 305–310 mosmol l⁻¹). A stock solution of amphotericin B (Calbiochem; 125 mg ml⁻¹ in DMSO) was prepared every 2 h by ultrasonication, and kept protected from light at room temperature (25–28 °C); amphotericin B was added to the internal solution (final concentration, 0.5 mg ml⁻¹) and the solution was homogenized using a Pro-250 homogenizer (Pro Scientific, Monroe, CT, USA) for 7–10 s immediately prior to use. Pipettes were briefly dipped in amphotericin B-free internal solution and backfilled with amphotericin B-containing solution. Cells were continuously perfused with external saline at a rate of 1–2 ml min⁻¹ and all experiments were performed at room temperature (25–28 °C).

Cyclosporin A (CsA, gift of Dr R. Nichols, Allegheny University of the Health Sciences, PA, USA) was kept as a 1 mM stock solution in 100 % ethanol and stored in aliquots at −20 °C. CsA was diluted in external recording solution (1 mM) and applied to cells via the DAD-12 computerized microperfusion system (Adams List, Westbury, NY, USA), via 100 μm diameter quartz tubing.

Capacitance measurements and stimulus protocols
Individual bovine chromaffin cells were voltage clamped using a List EPC-7 patch clamp amplifier. Capacitance detection was performed using a computer-based phase-tracking algorithm (Fidler & Fernandez, 1989) as previously described (Engisch & Nowycky, 1996). Briefly, a 15 mV root mean square (r.m.s.), 1.4 kHz sine wave was added to the holding potential of −90 mV through the voltage-command input of the amplifier, and the resulting current output analysed at two orthogonal phase angles. Each data point represents the average of ten sinusoidal cycles and was collected with a temporal resolution of 18 ms. Data acquisition was initiated when the access conductance after patching became > 70 nS; access conductance usually stabilized at ~100 nS. Cells were held at −90 mV, and single depolarizations to +20 mV for varying durations were applied at an interval of 90–120 s. During trains of depolarizations, pulses were applied at 200 ms intervals.

Amperometry
Amperometric electrodes were manufactured according to Kawagoe, Zimmerman & Wightman (1993). Briefly, a single 8 μm carbon fibre was inserted into a glass capillary and pulled on a two-stage microelectrode puller (Narishige). The fibre extending from the pulled end of the glass was cut with iridectomy scissors before dipping the tip in freshly prepared liquid epoxy resin. The epoxy resin was allowed to dry overnight before curing at 150 °C for 2–24 h. Electrodes were used within 1–3 days of manufacture; the carbon fibre tip was cut with a scalpel blade immediately prior to use.

Figure 1. Decays in capacitance following single depolarizations can be fitted with either a single exponential function or the sum of two exponentials

Cₘ traces from 2 cells stimulated by 320 ms depolarizations from −90 to +20 mV (gap in capacitance recording occurs during depolarization). Exponential fits of capacitance decays were carried out in Microcal Origin (see Methods). In A and B, single (continuous curve) and double (dashed curve) exponential fits of the same capacitance decay are shown superimposed for the two endocytotic responses. A, a single exponential fit deviated substantially from the observed decay in capacitance, but the capacitance decrease was well fitted with a double exponential function. B, a double exponential fit of the capacitance decay was not visually distinguishable from a single exponential fit; the single τ and τ₁ from the double exponential function differed <2-fold. Ca²⁺ current integrals were 94.4 pC and 121 pC for cells in A and B, respectively. (Cell L060401 and L092403.)
recording. Carbon fibre electrodes were backfilled with a 1 M KCl solution, and held at +700 mV using a modified PC-501 amplifier (Warner Instruments Corp., New Haven, CT, USA); oxidative currents due to catecholamine release were measured under voltage clamp conditions. After obtaining a seal and during perforation, a carbon fibre microelectrode was manipulated onto the top of a chromaffin cell so that the two were touching. Amperometric events evoked by depolarization were acquired at 1 kHz in Axobasic by triggering a second computer via the stimulus template put out by the capacitance sampling software.

Data analysis
Calcium entry, in picocoulombs, was calculated from integration of calcium currents (using limits that excluded the majority of Na⁺ current). All currents were digitally leak-subtracted prior to calculating total Ca²⁺ entry. Cells with leak currents > 25 pA were discarded from analysis.

Membrane capacitance (Cm) decays during endocytosis were fitted using a non-linear least squares fitting algorithm in Origin (Microcal, Version 3.5) to the functions:

\[ Y = Y_0 + A_1 e^{-\frac{(X-X_0)}{\tau_1}} \]  

(mono-exponential fit), or

\[ Y = Y_0 + A_1 e^{-\frac{(X-X_0)}{\tau_1}} + A_2 e^{-\frac{(X-X_0)}{\tau_2}} \]  

(double exponential fit), where \(X_0\) is the initial time value, \(Y_0\) is the asymptotic value of the \(Y\) variable for large \(X\) values, \(A_1\) and \(A_2\) are the amplitudes of the first and second components, respectively, and \(\tau_1\) and \(\tau_2\) are the decay constants.

Limits for fitting exponential decays were set within single capacitance traces at the peak of the post-stimulus value subsequent to the depolarization, and at the end of the trace (total duration ~20 s). Mono-exponential fits were attempted initially, but responses were re-analysed using a double exponential fit if the single exponential fit deviated substantially from the actual response by visual inspection (Fig. 1A). In addition, a double exponential fit was not accepted if the fit was visually indistinguishable from a mono-exponential fit (Fig. 1B). To analyse responses with time constants > 10 s, three sequential capacitance traces were used (total duration ~60 s).

Several values obtained from the exponential fit of a \(C_m\) decay were used as quantitative estimates of endocytotic parameters. The total amount of endocytosis, in femtofarads, was estimated from the coefficient \(A_1 + A_2\) for double exponential fits. A plot of the amount of endocytosis estimated by eye vs. \(A_1\) and \(A_2\) showed good agreement. The undershoot, in femtofarads, was estimated from \(Y_0\), which agreed well with the visual estimation of minimum post-stimulus level.

Contiguous capacitance traces (Figs 3 and 6) were aligned manually by eye by adding offset femtofarad values to the 990 points in each trace.
subsequent trace using Origin software. This is necessary because a new phase angle is calculated at the start of each capacitance trace and the absolute capacitance values of each trace do not match those of neighbouring traces. The real time value \( t, 0 \) at seal formation was acquired for each capacitance point and stored in a separate data file. Between each trace is a gap of \( \sim 1 \) s due to the time required for resetting the phase angle; longer time gaps occurred when phase detection was suspended to manually adjust capacitance compensation or input new parameters for the stimulus protocol.

Amperometric traces were imported into Origin, digitally filtered with a Fourier algorithm, and subjected to a peak detection algorithm. Baseline noise identified as peaks by the computer program, was manually de-selected.

Statistical comparisons were performed using Student's \( t \) test for normally distributed data (\( C_m \) jumps), or the non-parametric Mann–Whitney \( U \) test for non-normally distributed data (time constants). Data are plotted as means ± s.e.m. unless otherwise noted.

RESULTS

In perforated-patch recording of bovine adrenal chromaffin cells, single step depolarizations evoke increases in membrane capacitance (\( C_m \) jumps) corresponding to exocytosis of large dense-cored vesicles. We previously found that the amount of exocytosis increases with increasing total \( Ca^{2+} \) entry according to the function:

\[
\Delta C_m = g \left( \sum \left( Ca^{2+} \text{ ions} \right) \right)^{1.5},
\]

where \( \Delta C_m \) is the change in \( C_m \), \( \sum \left( Ca^{2+} \text{ ions} \right) \) is obtained from the integral of the \( Ca^{2+} \) current, and \( g \) is a proportionality constant (Engisch & Nowycky, 1996). In the course of these experiments we observed that \( C_m \) jumps were usually followed by smooth, exponential decays in cell capacitance, which we interpret as endocytosis.

Endocytosis can be measured separately from exocytosis

Capacitance recording detects the net sum of membrane addition and subtraction. Endocytosis, measured as a decrease in \( C_m \), often occurred immediately following the return to capacitance measurement. Occasionally there was a slow, upward drift in capacitance subsequent to the depolarization, and endocytosis appeared to begin after a delay (Fig. 2A, arrow). It is possible that for most single pulses, exocytosis continues long after the depolarization but is not detected due to overlapping endocytosis. If true, the capacitance decrease observed would be slower than the actual underlying endocytosis.

Figure 3. Excess retrieval and compensatory retrieval: two types of endocytosis that can occur in an individual bovine adrenal chromaffin cell

Plot of cell capacitance throughout a 25 min recording period. Stimulation protocols are indicated with symbols, above is the total \( Ca^{2+} \) entry (\( Q_{Ca} \)) for each protocol. The cell was stimulated with 3 single long duration depolarizations (320, 320 and 640 ms), and each evoked large \( C_m \) jumps that were followed by decreases in capacitance. Endocytosis, after the first two long depolarizations, rapidly undershot the pre-stimulus \( C_m \) level by \( >100 \) fF (Excess retrieval); the third long depolarization evoked exocytosis followed by compensatory retrieval back to the pre-stimulus level. Individual capacitance traces (duration \( \sim 20 \) s) were aligned manually in Micrcal Origin (see Methods); calibration pulses have been eliminated for clarity. (Cell L070908)
To address this issue directly, we used carbon fibre amperometric detection of catecholamine release (Wightman et al. 1991). The amperometric current recording is shown below the capacitance trace ($C_m$) for the same stimulation in Fig. 2A and on an expanded time scale in Fig. 2B. The release of catecholamine-containing vesicles directly beneath the carbon fibre is detected as a series of spikes, superimposed on an increase in baseline current that is probably due to catecholamine release further away from the carbon fibre electrode. Although the spikes are relatively few in number (the 8 μm diameter carbon fibre samples ~10% of the chromaffin cell surface; Chow, von Rüden & Neher, 1992), they cluster during the beginning of the depolarization; one large spike occurs ~150 ms after the depolarization is over. Only one or two tiny spikes, barely above baseline noise, are detectable for the next 15 s of amperometric recording (Fig. 2A).

The release of catecholamine-containing vesicles directly beneath the carbon fibre is detected as a series of spikes, superimposed on an increase in baseline current that is probably due to catecholamine release further away from the carbon fibre electrode. Although the spikes are relatively few in number (the 8 μm diameter carbon fibre samples ~10% of the chromaffin cell surface; Chow, von Rüden & Neher, 1992), they cluster during the beginning of the depolarization; one large spike occurs ~150 ms after the depolarization is over. Only one or two tiny spikes, barely above baseline noise, are detectable for the next 15 s of amperometric recording (Fig. 2A).

The timing of spikes elicited during six 320 ms and two 640 ms depolarizations is illustrated in Fig. 2C. The bulk of release occurred during the depolarization for both protocols (91 and 87% for 320 and 640 ms duration pulses, respectively). These data demonstrate that the amount of vesicle fusion that occurs after a prolonged depolarizing pulse is only ~10% of that occurring during the depolarization. Furthermore, catecholamine release is virtually undetectable by amperometry within 300 ms of the depolarization. Thus, endocytotic processes that occur in the seconds following a depolarization are not substantially affected by on-going exocytosis. However, the speed of endocytotic events measured just after the depolarization may be slightly underestimated.

Compensatory and excess retrieval: two types of endocytosis in bovine adrenal chromaffin cells

Figure 3 shows a continuous plot of membrane capacitance obtained over a 26 min recording in perforated-patch mode. The record was constructed by aligning the beginning of each capacitance trace to the end of the previous trace (one trace is ~20 s in duration). During this recording, the cell...
was depolarized fourteen times with various protocols and the amount of Ca\(^{2+}\) entry during each stimulus is indicated by the height of a bar above the trace.

The continuous record portrays the various dynamic changes in surface area that occur during a perforated-patch experiment. Three long duration pulses evoke large increases in surface area, reflecting exocytotic \(C_m\) jumps that are easily visible even on this compressed scale (320 ms, ○; 640 ms, ◊). The most dramatic change in surface area occurs after the first long depolarizing pulse: \(> 400 \mu F\) or \(-10\%\) of surface membrane is rapidly endocytosed. Because the minimum reached is considerably below the \(C_m\) level just prior to the stimulus, we term this type of response 'excess' retrieval, as was done previously by Almers and coworkers (Thomas et al. 1990, 1994) and others (Neher & Zucker, 1993; Proks & Ashcroft, 1995; Artalejo et al. 1996; Hsu & Jackson, 1996). The cell surface area did not remain at the minimum level but slowly, over several minutes, recovered to approximately the pre-stimulus level. The second long depolarizing pulse also evoked an endocytotic event that surpassed the pre-stimulus level and then slowly recovered, but which was much smaller than the response to the first 320 ms depolarization. The third long pulse (640 ms) evoked a \(C_m\) jump that was followed by membrane retrieval to the pre-stimulus level, a process we term 'compensatory' retrieval.

In addition, shorter duration pulses (40 ms, □) evoked small amounts of exocytosis that are difficult to see on the compressed scale and were followed by variable amounts of endocytosis.

**Figure 5.** Compensatory retrieval is accurate and reliable for large \(C_m\) jumps

(A) The magnitude of endocytosis vs. the \(C_m\) jump for single depolarizations (40—640 ms duration, symbol for each duration as indicated). Total endocytosis in femtofarads was determined from the amplitude of the best exponential fit to the \(C_m\) decay (\(A_1 + A_2\), see Methods) whilst the \(C_m\) jump was calculated from the average across the first 10 capacitance points subsequent to the depolarization. Therefore some compensatory endocytotic responses are significantly larger than the \(C_m\) jump, because membrane added after the time used for \(C_m\) jump calculation was retrieved accurately (i.e. ○, 550 F at exocytosis, 780 F at endocytosis). Excess retrieval events (undershoot \(> 100 F\)) were excluded from analysis. Inset, expansion of the region 0—100 F. Many \(C_m\) jumps in this region were not accompanied by endocytosis (decay undetectable based on inability to be fitted by an exponential function).

(B) Stimulus-evoked responses were sorted independently by (a) exocytosis (i.e. size of \(C_m\) jump) or (b) \(Ca^{2+}\) entry, and the percentage of responses accompanied by endocytosis was calculated for each bin.

**Compensatory retrieval accurately recovers membrane added during large \(C_m\) jumps**

We first examined the reliability and accuracy of membrane retrieval for those stimuli that did not have excess retrieval: that is, by excluding all responses that undershot the pre-stimulus level by \(> 100 F\). We found that compensatory retrieval was preferentially associated with longer depolarizations that evoked larger amounts of exocytosis. Small increases in \(C_m\) evoked by short depolarizations (<100 F, equivalent to the release of fewer than fifty large dense-cored vesicles) were retrieved slowly, or not at all, as shown on an expanded time scale for two cells in Fig. 4 (40 ms pulses). In the same cells, longer depolarizations evoked exocytosis that was followed by compensatory retrieval, i.e. a decline in \(C_m\) back to the pre-stimulus level. The decay in cell capacitance during compensatory retrieval was smoothly exponential, and could usually be fitted with a single exponential (Cell 2, 160 ms pulse) or occasionally the sum of two exponentials (Cell 1, 320 ms pulse; see also Fig. 1). Compensatory retrieval is a relatively rapid endocytotic
mechanism; after large exocytotic jumps, $C_m$ usually returned to baseline within a single capacitance trace (~20 s).
The relationship between exocytosis and compensatory retrieval is summarized for 142 responses in Fig. 5A. The amount of endocytosis is plotted as a function of the $C_m$ jump (Exocytosis) for all stimulus durations. For large $C_m$ jumps the data cluster around the line of identity, suggesting that displacements over a certain magnitude are quantitatively matched by endocytosis. It is important to note that the accuracy of compensatory retrieval was obscured when the entire population of endocytotic events, including those classified as excess retrieval, was considered. The simplest explanation for the accuracy is that membrane from recently exocytosed vesicles is retrieved by compensatory endocytosis.

As can be seen in the inset to Fig. 5A (an expansion of the region 0–100 fF), the situation is different for small $C_m$ jumps. A number of $C_m$ jumps below 100 fF are not accompanied by endocytosis (0 fF endocytosis). The probability of eliciting endocytosis increases with the size of the $C_m$ jump. Only 20% of $C_m$ jumps $\leq 20$ fF were followed by endocytosis, whilst 100% of the $C_m$ jumps

![Figure 6. Regulation of cell surface area during prolonged perforated-patch recordings](image)

Continuous capacitance records, obtained by aligning single sequential traces as described in Methods. Capacitance acquisition was initiated when access conductance became $>70$ nS. On a slow time scale, gradual stimulus-independent changes in cell capacitance become apparent. Cell 1 and Cell 3 both show slow upward drift in $C_m$ at the beginning of the record that sums with $C_m$ jumps evoked by 40 ms pulses, whilst Cell 2 has a slow $C_m$ decline. The first 320 ms pulse depolarization often triggered rapid and large amplitude excess retrieval that could truncate (Cells 1 and 2) or even obliterate (Cell 3) the $C_m$ jump. After excess retrieval, $C_m$ did not normally remain at the new level but instead increased slowly, appearing to approach the initial or pre-stimulus level. This post-excess retrieval increase in capacitance could occur in the absence of depolarization-induced Ca$^{2+}$ entry (Cell 1). Only 1 cell out of 8 that exhibited excess retrieval (11 continuous plots were assembled) remained more or less at the level reached by the excess retrieval event (Cell 2). Excess retrieval tended to become smaller with repeated stimulations (compare responses to 320 or 640 ms depolarization, early vs. late in the recordings). Stimulation protocols: $\Box$, single 40 ms pulse; $\Delta$, single 160 ms pulse; $\bigcirc$, single 320 ms pulse; $\bullet$, single 640 ms pulse, $\ast$, train of 5 ms pulses (35); $\times$, train of 40 ms pulses (20). (Cells L060204, L091101, L081902.)
above 80 fF were retrieved (Fig. 5Bb). In terms of amount of Ca\(^{2+}\) entry, 30% of depolarizations that allowed ≤40 pC had endocytosis; an ~100% success rate of endocytosis was reached above 60 pC (Fig. 5Ba). Under our experimental conditions, small \(C_m\) jumps were usually elicited by 40 ms pulses (Δ, Fig. 5A), although a few 160 ms (Δ) and 320 ms (O) duration pulses were followed by little or no endocytosis. We compared the distributions of Ca\(^{2+}\) current integrals for the 40 ms pulses with and without endocytosis, but the distributions overlapped completely (not shown). Indeed, exocytotic responses evoked by 40 ms pulses within a single cell can be followed by no endocytosis or be completely retrieved (e.g. first vs. final 40 ms pulse in Fig. 3).

**Excess retrieval is not proportional to stimulated exocytosis**

Our data suggest that membrane may accumulate on the cell surface following short depolarizations. Excess retrieval may be a mechanism to preserve cell size that is triggered when a sufficient Ca\(^{2+}\) entry signal occurs. To examine if bouts of excess retrieval were related to previously exocytosed membrane, we followed the total \(C_m\) of the cell as membrane was added and retrieved after depolarizations.

Figure 6 shows continuous plots of capacitance recordings from three cells, which range from 25 to 36 min in duration. Different symbols directly above each trace indicate the pulse protocol of stimulations (see legend). Usually single pulses were used, but Cell 2 was stimulated three times with trains of depolarizing pulses. In each of these examples the first prolonged depolarization triggered a large, extremely rapid excess retrieval that decreased the cell surface by >5%. Prior to the excess retrieval event, \(C_m\) either increased slowly (Cell 1 and Cell 3, including the addition of membrane during brief pulses), decreased slowly (Cell 2) or remained stable (e.g. Fig. 3). The slow increases and decreases in cell capacitance accumulated over minutes and only became apparent on the compressed time scale shown.

Two observations suggest that excess retrieval is not simply a mechanism for endocytosing membrane left by previous stimulations. First, excess retrieval always internalized more membrane than was added during the entire duration of recording preceding the stimulation. Second, whilst it is possible that excess retrieval also recovered membrane added prior to, or during patch clamping, \(C_m\) usually did not remain at the new level after excess retrieval but instead recovered to near the pre-stimulus level. In Cell 1, following the excess retrieval event, membrane capacitance increased at a rate of ~1–2 fF s\(^{-1}\) for at least 5 min in the complete absence of stimulation. On the other hand, stimulation-evoked exocytosis during the return to \(C_m\) baseline levels could sum with the recovery mechanism (e.g. a 40 ms pulse after the first excess retrieval event in Cell 3 and after the second excess retrieval event in Fig. 3) or be retrieved by the

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**Figure 7.** Excess retrieval events following single step depolarizations

A, excess retrieval after a single 320 ms depolarization to +20 mV (from a holding potential of −90 mV; see inset for inward current trace) that undershoots the pre-stimulus level (dotted line) by 213 fF. The decay was well fitted by a single exponential (dashed curve). Total Ca\(^{2+}\) entry was 120 pC (Cell N020302). B, excess retrieval could be extremely rapid, reaching a maximum undershoot hundreds of femtofarads below the pre-stimulus level in under 5 s (undershoot, 440 fF). This large undershoot represented a decrease in total cell capacitance as it required a readjustment of the capacitance compensation circuitry. The endocytotic response was fitted with the sum of two exponentials (dashed curve), as were most rapid excess retrieval events. Inset: total Ca\(^{2+}\) entry, 320 ms depolarization, 143 pC (Cell N120502). The accompanying conductance traces (G) do not show parallel equal (or opposite) changes.
compensatory mechanism (e.g. Cell 3, Δ; Fig. 3, ※). Cell 2 is
the only example of a cell that maintained the $C_m$ level
reached by excess retrieval, out of eight recordings
containing at least one excess retrieval event.

Excess retrieval was not reproducibly evoked by long
duration pulses within a given cell, either because excess
retrieval was inaccurate, or because the mechanism was lost
during voltage clamp recording (e.g. Fig. 6; Cell 3, second ○
and ◊). The latter explanation appears to be the case, since
excess retrieval did not vary randomly — there was a
consistent trend towards smaller excess retrieval events
with repeated stimulations, or 'run-down.' The loss of excess
retrieval responses during a recording cannot be attributed
to wash-out of a key cytoplasmic constituent because
experiments were performed in perforated-patch mode. It is
possible that the molecular components required for excess
retrieval cannot be reassembled on the time scale of a
typical perforated-patch clamp recording (10—40 min), at
room temperature.

Figure 7 illustrates excess retrieval events evoked by the
first long depolarizing pulse from an additional two cells on
an expanded time scale. Similar to compensatory retrieval,
the $C_m$ decay during excess retrieval was smoothly
exponential, occurring with either mono-exponential kinetics
(Fig. 7A) or, more frequently, double exponential kinetics
(Fig. 7B). In these examples, the undershoot after excess
retrieval was ~150 fF and > 400 fF for Fig. 7A and 7B,
respectively. The large undershoots that occur during excess
retrieval reflect reductions in cell capacitance and are not
paralleled by comparable changes in the conductance, or $G$
trace (Fig. 7A and B).

As illustrated in Fig. 2, exocytosis occurs throughout a
320 ms depolarization, and in some cells for ~300 ms after
the depolarization is over. Therefore the ~200 ms first time
constant for the double exponential fit in Fig. 7B may
slightly underestimate how fast membrane can be endo-
cytosed during excess retrieval. Actually, a number of
responses with large excess retrieval appear to begin during
the depolarizing pulse, effectively truncating or even
obliterating the exocytotic jumps. Therefore, in a previous
study concerning the Ca$^{2+}$ dependence of exocytosis
following single depolarizations (Engisch & Nowycky, 1996)
cells with excess retrieval were stimulated a second or third
time so that the $C_m$ jump could be measured in the absence
of contaminating endocytosis.

Excess retrieval requires a threshold amount of Ca$^{2+}$
entry

Because of the decrement in excess retrieval with multiple
stimulations (Figs 3 and 6), we examined the Ca$^{2+}$
dependence of the type of endocytotic response evoked by
the first long stimulus in individual cells. The maximal
undershoot after the first long pulse was plotted as a
function of integrated Ca$^{2+}$ entry for sixty-nine individual
cells (Fig. 8A; stimulus durations indicated with symbols as
shown). To compare post-stimulus changes for a range of
smaller amounts of Ca$^{2+}$ entry , we also included the response
to the 40 ms stimulation closest in time (prior to the long
pulse) for each cell. Excess retrieval, defined as an
undershoot of > 100 fF, occurred only if the amount of
Ca$^{2+}$ entry during the depolarization was >70 pC (arrow):
an amount never achieved by a 40 ms pulse under our
recording conditions (5 mM extracellular Ca$^{2+}$). Above this

![Figure 8](image-url)

**Figure 8. Excess retrieval requires a threshold amount of Ca$^{2+}$ entry for activation**

A, plot of the undershoot (in femtofarads, relative to pre-stimulus baseline) as a function of total Ca$^{2+}$ entry, for the first long duration (> 160 ms) stimulation of an experiment. A point for the 40 ms stimulation immediately prior to the long duration stimulation is also included for each cell. The shaded region covers undershoots ≤ 100 fF that are classified as compensatory retrieval. No excess retrieval occurred if the Ca$^{2+}$ current integral was ≤ 70 pC (arrow). B, endocytotic responses to 320 ms pulses were sorted by amount of Ca$^{2+}$ entry and classified as either compensatory or excess retrieval. The percentage of responses with excess retrieval increased with increasing amount of Ca$^{2+}$ entry, to a maximum of ~70%. 
value, the amount of excess retrieval was highly variable and did not show a strict dependence on Ca\(^{2+}\) entry. On the other hand, the percentage of responses with excess retrieval for the first stimulus pulse increased dramatically with increasing Ca\(^{2+}\) entry (Fig. 8B), although there were still a number of responses categorized as compensatory (undershoot, 0–100 fF; Fig. 8A, shaded region) across the entire range of integrated Ca\(^{2+}\) entry. The maximum percentage of responses with excess retrieval reached a plateau at 70% (Fig. 8B). Thus, excess retrieval requires a threshold amount of Ca\(^{2+}\), but is not always triggered even by the first long stimulus in individual cells.

The kinetics of compensatory retrieval and excess retrieval differ by an order of magnitude
As illustrated in Figs 1, 4 and 7, endocytosis following \(C_m\) jumps was a smooth mono- or double exponential function that was fitted with remarkably little error. Histograms of time constants obtained from fits of endocytosis following single depolarizations (40–640 ms) are shown in Fig. 9. The first time constant (\(\tau_1\)) for double exponential fits is very fast (median, 670 ms) (Fig. 9Aa). The second time constant (\(\tau_2\)) is approximately 10-fold slower, with a median of 6.3 s, and a number of responses with time constants > 10 s (Fig. 9Ab). For endocytosis fitted with a mono-exponential function, the distribution of time constants resembles that of \(\tau_2\) rather than \(\tau_1\) (median 5.7 s; Fig. 9Ac).

When endocytotic responses for 320 ms pulses were separated according to whether excess retrieval occurred (undershoot > 100 fF below the pre-stimulus baseline), the majority of excess retrieval events were fitted with a double exponential function (85%; Fig. 9B). In contrast, compensatory retrieval responses were usually fitted with a mono-exponential function (75%; Fig. 9B) that has a 10-fold slower time constant. Since the median first time constant for double exponential events is 670 ms, this result indicates that excess retrieval is an extremely rapid endocytotic mechanism. These data also suggest that mono-exponential endocytosis is the manifestation of a single mechanism, which we have termed compensatory, whilst double exponential responses are a mixture of a very rapid excess retrieval mechanism and compensatory retrieval.

![Figure 9](image-url)
The time constants for single exponential endocytotic responses are Ca\(^{2+}\) dependent

As illustrated in Fig. 9A, the time constants from mono-exponential fits were not normally distributed, but had a tail of responses with a wide range of high values. The slowest time constants were associated with the smallest amounts of Ca\(^{2+}\) entry. Figure 9C shows the means of time constants from single exponential fits, binned by amount of Ca\(^{2+}\) entry (●). For Ca\(^{2+}\) entry below 25 pC the mean is >15 s (n = 17); this value decreases to less than half (6.1 s) at 100–125 pC (n = 23). A few large values greatly affect the mean, so the median values for each Ca\(^{2+}\) entry bin are also plotted (○). The median decreases from 12 to 4.5 s between ~15 and ~110 pC. The difference in time constant values between the smallest Ca\(^{2+}\) entry range and those at 110 pC did not reach statistical significance (P > 0.1, Mann–Whitney U test) but many of the slowest decays could not be fitted with an exponential function (i.e. thirty-five responses in the lowest entry bin). Therefore the shortening of time constants with increasing Ca\(^{2+}\) entry is greatly underestimated here.

Figure 10. CsA decreases total Ca\(^{2+}\) entry and inhibits exocytosis

A, inward current traces before and after perfusion with 1 μM CsA. A, CsA had little effect on peak current, but increased the rate of inactivation during the pulse. The change in inactivation rate developed slowly during continued perfusion with CsA, as illustrated by currents evoked by 40 ms pulses given prior to (−4 min), and 8 and 15 min after the perfusion started. B, in the same cell, the effect on inactivation was more apparent for currents evoked by 320 ms pulses, which were given once before (−7 min), and once after (17 min CsA) CsA had been applied for at least 15 min. In control, untreated cells there was no change in rate of inactivation for currents evoked by 320 ms depolarizations given >15 min apart (not shown), although in some cells the peak current amplitude declined slightly during the course of an experiment.

C, comparison of total integrated Ca\(^{2+}\) entry for 320 ms depolarizations in the absence of CsA (n = 28) vs. integrated Ca\(^{2+}\) entry for 13 responses after treatment with CsA for at least 15 min (*, P < 0.05, Student’s t test). B, plot of C\(_{\text{m}}\) jumps, binned by amount of Ca\(^{2+}\) entry, for control responses and responses elicited in the presence of CsA. A standard curve obtained from the average of 27 Ca\(^{2+}\)-secretion relationships obtained in a previous study (Engisch & Nowycky, 1996) is superimposed on the data (dashed curve). Control responses (□) lie close to the standard curve. However, C\(_{\text{m}}\) jumps after CsA treatment (▲) are significantly less than control responses at the highest Ca\(^{2+}\) entry range attained in the presence of CsA (P < 0.05, Student’s t test).
Does cyclosporin A stimulate excess retrieval in perforated-patch recordings?

Cyclosporin A (CsA) is an inhibitor of the Ca²⁺- and calmodulin-dependent phosphatase calcineurin (Liu, Farmer, Lane, Friedman, Weissman & Schreiber, 1991; Schreiber & Crabtree, 1992). Calcineurin is thought to modulate endocytosis by dephosphorylating dynamin (Liu, Sim & Robinson, 1994; Nichols, Suplick & Brown, 1994). Recently, in a study of whole-cell capacitance measurements in calf adrenal chromaffin cells it was shown that external application of CsA induced extremely large amplitude excess retrieval following trains of depolarizations (Artalejo et al. 1996).

We examined whether CsA treatment affected excess retrieval in perforated-patch recordings of bovine chromaffin cells. We applied CsA (1 μM) for at least 15 min prior to giving a test depolarization. CsA is membrane permeant and was applied by one of three protocols: cells were pre-incubated in CsA (n = 3), CsA was applied after patching under voltage clamp conditions (access conductance, > 70 nS).

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**Figure 11.** Compensatory retrieval is slower and less accurate after CsA application

A, four examples of relatively large C_m jumps (close to or >100 fF) elicited in the presence of CsA. Three sequential C_m traces were aligned for each response shown. After CsA treatment, C_m decay were slow and often incomplete. a and b, (cell L073002) response to 160 ms depolarization (integrated Ca²⁺ entry, 77 pC) and 320 ms depolarization (110 pC), respectively; c, (cell L081102) response elicited by 320 ms depolarization (75 pC); d, (cell L080102) response to a 320 ms depolarization (72 pC). B, after treatment with CsA, the time constants for mono-exponential decay no longer displayed any Ca²⁺ dependence. Time constants were binned by amount of Ca²⁺ entry; only the three lowest ranges were observed in the presence of CsA. Values for time constants in these range for control responses were replotted from Fig. 9 for comparison. At the highest range, the time constant was significantly different from control (P < 0.05, Mann-Whitney U test). C, effect of CsA treatment on excess retrieval. The undershoot (relative to pre-stimulus baseline) was plotted as a function of integrated Ca²⁺ entry for responses in the presence of CsA (▲) and control responses in the same experiments or from cells in sister cultures ( ○, data are a subset of the data presented in Fig. 8A). Shaded region cover undershoots ≤100 fF that are classified as compensatory retrieval. CsA treatment did not alter the threshold Ca²⁺ requirement of excess retrieval, but due to the increase in rate of inactivation, a higher proportion of 320 ms pulses induced Ca²⁺ entry that fell at or below the threshold amount (arrow).
before any long duration stimulations \((n=7)\), or control responses were obtained and then the cell was perfused with CsA for \(>15\) min \((n=3)\).

**CsA inhibits Ca\(^{2+}\) entry and exocytosis**

In the presence of CsA, the calcium current integral was significantly decreased, due to an increase in the rate of inactivation rather than a reduction in the peak of the Ca\(^{2+}\) current (Fig. 10Aa and b). This action significantly reduced total Ca\(^{2+}\) entry, integrated over a 320 ms pulse (Fig. 10Ac; \(P<0.01\), Student's \(t\) test). In addition, exocytosis in the presence of CsA was reduced more than expected from the decrease in Ca\(^{2+}\) entry, based on the relationship between Ca\(^{2+}\) entry and exocytosis obtained in control cells (Engisch & Nowycky, 1996). Figure 10B compares \(C_m\) jumps in control cells (\(\square\)) and cells treated with CsA (\(\Delta\)) for the same amount of Ca\(^{2+}\) entry. The dotted curve represents a standard transfer function:

\[
\Delta C_m = 0.147(\sum(C^{2+}_{\text{entry}}))^{\frac{3}{5}},
\]

obtained by averaging input–output relationships \((C_m\) jumps vs. total Ca\(^{2+}\) entry, in pC) from twenty-seven control cells in a previous study (Engisch & Nowycky, 1996). In the highest Ca\(^{2+}\) entry range achieved in the presence of CsA, the mean \(C_m\) jump was significantly smaller than the mean \(C_m\) jump for control cells in the same range \((P<0.05, \text{Student's } t \text{ test})\). This is in contrast to other methods for decreasing Ca\(^{2+}\) entry, such as changing test potential, decreasing pulse duration, or applying the Ca\(^{2+}\) channel antagonists \(\omega\)-conotoxin GVIA or \(\omega\)-agatoxin IVA, all of which reduce exocytosis proportionally to their effects on total Ca\(^{2+}\) entry in bovine chromaffin cells (Engisch & Nowycky, 1996). The reason for this additional effect on exocytosis by CsA treatment is not understood; inhibition of calcineurin may induce depression of Ca\(^{2+}\)-secretion coupling or, alternatively, prevent release of a pool of vesicles usually accessed only by large amounts of Ca\(^{2+}\) entry.

**Effects of CsA on compensatory retrieval**

In addition to its effect on Ca\(^{2+}\) currents and exocytosis, CsA modulated the process of compensatory retrieval. Mono-exponential endocytosis (which we equate with the compensatory retrieval mechanism) was either slower than control responses (Fig. 11Aa), or if similar in time course, did not return \(C_m\) completely to pre-stimulus levels (Fig. 11Ab and c). Occasionally a completely normal endocytotic response occurred, rapidly returning \(C_m\) to baseline (Fig. 11Ad). The Ca\(^{2+}\)-dependent shortening of time constants with increasing Ca\(^{2+}\) entry observed in control, untreated cells appeared to be inhibited by CsA. In Fig. 11B, time constants from mono-exponential fits of responses from CsA-treated cells are shown as a function of Ca\(^{2+}\) entry. There is no apparent decrease in the time constant with increasing Ca\(^{2+}\) entry, and at the highest range \((n=9)\) the mean time constant was significantly different from that of untreated controls \((n=23; P<0.05, \text{Mann–Whitney } U \text{ test})\).

We considered the possibility that endocytosis was slowed in the presence of CsA due to the additive effects of lower Ca\(^{2+}\) entry and reduced exocytosis. To determine whether smaller \(C_m\) jumps were associated with slower time constants, we sorted control responses in the range 70–90 pC from the smallest to the largest \(C_m\) jump and took the lower half of the values. The mean \(C_m\) jump for this subset of data \((58.6 \pm 3.8 \text{ fF}, n=12)\) was similar to the mean for CsA-treated cells \((50.5 \pm 9.1 \text{ fF})\), but the time constant was not significantly different from that of the bin as a whole \((P>0.25, \text{Mann–Whitney } U \text{ test})\). Thus, slow mono-exponential time constants in the presence of CsA cannot be explained solely by the smaller amount of exocytosis.

**Excess retrieval occurs less often after CsA treatment, due to a decrease in Ca\(^{2+}\) entry**

Excess retrieval runs down with repeated stimulations, so a cell could not be used as its own control to compare responses before and after CsA treatment. Therefore, cells were treated with CsA for at least 15 min prior to application of the first 320 ms depolarizing pulse and responses in this group of cells were compared with those of control (untreated) cells from the same cultures. In the presence of CsA, only two out of ten cells had excess retrieval (under-shoot >100 fF below the pre-stimulus level) after the first 320 ms pulse, whereas 64% of the first 320 ms pulses in control cells triggered excess retrieval. This is only an apparent inhibition of excess retrieval by CsA as it can be entirely attributed to the reduction in integrated Ca\(^{2+}\) entry after CsA treatment. In Fig. 11C, the amount of excess retrieval (i.e. the under-shoot) for responses in CsA-treated cells is plotted as a function of integrated Ca\(^{2+}\) entry (\(\Delta\)), as described in Fig. 8. Integrated Ca\(^{2+}\) entry fell at or below the threshold required to trigger excess retrieval for six out of ten cells incubated in 1 μM CsA; excess retrieval occurred in two out of four of the remaining cells. Although after treatment with CsA the number of cells with Ca\(^{2+}\) entry values above the trigger level was very low, there is no reason to conclude that the proportion of responses with excess retrieval has been altered. The responses after CsA treatment further confirm the existence of a threshold requirement for induction of excess retrieval, and clearly show that CsA does not lower or eliminate this Ca\(^{2+}\) requirement.

**DISCUSSION**

We have used a computer-based capacitance detection technique in bovine adrenal chromaffin cells to record membrane retrieval following exocytosis evoked by single depolarizations. Long depolarizations (180–640 ms) activated two different types of endocytotic response: 'compensatory retrieval', endocytosis that returned \(C_m\) to the pre-stimulus level, or 'excess retrieval', endocytosis that caused \(C_m\) to fall >100 fF below the pre-stimulus level. The absolute magnitude of membrane internalization during excess retrieval was extremely large: >10% of the total surface area in
many cases (400 fF for a 4 pF cell), whereas compensatory retrieval responses were comparable to exocytotic responses, so only a few were as large as 400 fF. The two types of response had different kinetics. During compensatory retrieval, capacitance usually decayed mono-exponentially with a median time constant of \(\sim 6\) s. The decay in \(C_m\) during excess retrieval was extremely rapid and was fitted with two time constants (\(r_1, \sim 600\) ms; \(r_2, \sim 6\) s). In addition to differing in the extent and speed of membrane internalization, compensatory and excess retrieval had different requirements for Ca\(^{2+}\) entry: compensatory retrieval could occur after small amounts of entry during brief pulses, but excess retrieval was only triggered by Ca\(^{3+}\) entry \(\geq 70\) pC. Finally, compensatory and excess retrieval were differentially sensitive to inhibition of the Ca\(^{3+}\)-dependent phosphatase calcineurin by CsA. Compensatory retrieval was slowed several-fold, independently of a reduction in Ca\(^{2+}\) entry by CsA, whereas in the presence of CsA excess retrieval was less likely, but this was completely explained by a reduction in Ca\(^{2+}\) entry below the threshold for activation of excess retrieval. While not absolute proof, these results, taken together, strongly suggest that compensatory and excess retrieval represent two distinct endocytic mechanisms, although we cannot rule out that there is a single mechanism that takes on two distinct sets of properties.

Do exocytosis and endocytosis overlap?

We confirmed with carbon fibre amperometry that endocytic responses occurring in the seconds following depolarization were not contaminated by exocytosis because catecholamine release essentially ceased within 300 ms of the depolarization. However, the first phase of double exponential endocytotic decays (time constant, \(\sim 600\) ms) would be affected by post-stimulus exocytosis. It is therefore possible that both compensatory and excess retrieval have two kinetic components, but in some cases the first phase of endocytosis was obscured by exocytosis. The first kinetic component is fast enough to sometimes obliterate the \(C_m\) jump, but the rate of release subsequent to the depolarization is slow compared with the rate during the pulse. If the first component of endocytosis is obscured by post-stimulus exocytosis, it is unlikely to be a major contributor to compensatory retrieval.

Excess retrieval is unrelated to large dense-cored vesicle exocytosis

We established that excess retrieval was not related to previously exocytosed membrane that had accumulated on the surface during patching, or during brief depolarizations unaccompanied by endocytosis. If excess retrieval represented a restorative mechanism, the cell should have maintained the new \(C_m\) level achieved by excess retrieval. Instead, after a large amplitude excess retrieval event the cell capacitance usually (seven out of eight cells) increased as it returned to a level that could be maintained for the duration of the recording. A similar return to resting levels after excess retrieval was reported by Artalejo et al. (1996) using capacitance measurements recorded in whole-cell voltage clamp of calf adrenal chromaffin cells, although the return appeared to be at least an order of magnitude faster than observed here. After a large inaccurate endocytotic event, bovine and calf adrenal chromaffin cells appear to possess an exocytotic mechanism, presumably Ca\(^{2+}\) independent, that can return the cell surface area to its desired set point.

Excess retrieval has also been observed following large increases in intracellular Ca\(^{2+}\) induced by flash photolysis of DM-nitrophen (Thomas et al. 1990, 1994; Neher & Zucker, 1993; Heinemann et al. 1994). It has been suggested that excess retrieval triggered by photoreleased Ca\(^{2+}\) is retrieving membrane deposited during a Ca\(^{2+}\) transient that occurs with DM-nitrophen-loading of the cell. However, when examined quantitatively, the correlation between the amount of membrane added during the loading transient and the amount of excess retrieval was relatively poor (\(r = 0.43\); Thomas et al. 1994). Furthermore, membrane capacitance can also recover following excess retrieval induced by photoreleased Ca\(^{2+}\) (see Fig. 3 in Neher & Zucker, 1993).

Is endocytosis Ca\(^{2+}\)-dependent?

To couple endocytosis to exocytosis, a cell may have a mechanism to ‘sense’ increases in surface area. Alternatively, the same signal that evokes vesicle fusion may be used to stimulate endocytosis — i.e. Ca\(^{2+}\) ions. There is some evidence that a distinct divalent cation requirement exists for endocytosis. In calf chromaffin cells, endocytosis is inhibited when Ba\(^{2+}\) is substituted for Ca\(^{2+}\), but Ba\(^{2+}\) ions can support exocytosis (Artalejo et al. 1996). On the other hand, in pancreatic \(\beta\)-cells rapid endocytosis with properties resembling excess retrieval can still be evoked in the presence of high extracellular concentrations of Ba\(^{2+}\) (Proks & Ashcroft, 1995). These differences could be due to the existence of multiple Ca\(^{2+}\) sensors corresponding to multiple endocytic mechanisms, although the displacement of Ca\(^{2+}\) from intracellular stores has not been ruled out in the latter case.

Our results suggest that excess retrieval responds directly to Ca\(^{2+}\), rather than being regulated by exocytosis. An alternative possibility, that a cell responds to net displacement away from a resting level, is unlikely since a brief pulse should have occasionally raised \(C_m\) above the triggering level. Extremely fast endocytosis (time constant, 62 ms) that only occurred above a threshold level of Ca\(^{2+}\) (\(\sim 40\) pM) has been previously observed in bovine chromaffin cells, using flash photolysis of DM-nitrophen to increase intracellular Ca\(^{2+}\) (Heinemann et al. 1994). In pancreatic \(\beta\)-cells, endocytosis required a much lower threshold of \([Ca^{2+}]_i\) (2 pM; Eliasson et al. 1996).

The match between the amount of exocytosis and endocytosis (Fig. 5.A) suggests that compensatory retrieval is regulated by the amount of exocytosis rather than Ca\(^{2+}\) entry. However Ca\(^{2+}\) did influence compensatory retrieval: increasing Ca\(^{2+}\) entry shortened the time constant for mono-exponential decays, which were usually associated with
compensatory retrieval. Time constants decreased to a plateau value of \(-6\,s\) that did not shorten further for the highest range of \(Ca^{2+}\) entry. Large \(Ca^{2+}\) loads may inhibit endocytosis, as has been reported for rod bipolar neurons (von Gersdorff & Matthews, 1994). Alternatively, there may be some limit to the capacity of the endocytic mechanism (Wu & Betz, 1996), which is exceeded by the large \(C_m\) jumps evoked in this range.

Our data suggest it is the \(Ca^{2+}\) activation of calcineurin that increases the speed of compensatory retrieval, since this is prevented by application of CsA. Dephosphorylation of dynamin by calcineurin is thought to activate dynamin by reducing its GTPase activity and thereby promoting the GTP-bound state of the protein (Liu et al., 1994; Robinson, Liu, Powell, Fykse & Sudhoff, 1994). Our results support this hypothesis by providing evidence that endocytosis is slowed when dephosphorylation of dynamin is likely to be prevented (in the presence of a calcineurin inhibitor). However, we have not directly demonstrated that the effects we observed are due to preventing the dephosphorylation of dynamin. There are several other known substrates of calcineurin, including DARP-32, (phosphatase) inhibitor 1, and GAP-43/neuromodulin (King et al., 1984; Liu & Storm, 1989), although unlike dynamin their roles in endocytosis have yet to be established.

CsA does not stimulate excess retrieval

CsA reduced the probability of eliciting excess retrieval, primarily because after CsA treatment \(Ca^{2+}\) current integrals tended to fall below the threshold for activation of excess retrieval. Our results appear to directly contradict a previous report that CsA treatment greatly increases the extent of excess retrieval and prevents the return of membrane capacitance to resting levels (Artalejo et al., 1996). There are two major differences between our report and the previous study: (1) the absence of the facilitation \(Ca^{2+}\) channel in adult bovine adrenal chromaffin cells (Engisch & Nowycky, 1996) and (2) in the present study large amplitude excess retrieval events were elicited routinely; in Artalejo et al. (1996) such responses were seen only in the presence of CsA (see Table 1, Artalejo et al., 1996). The activation of the facilitation channel can double the average amplitude of \(Ca^{2+}\) currents (Artalejo, Ariano, Perlm an & Fox, 1990). Moreover, the facilitation channel may be unaffected by CsA treatment. It is possible that CsA potentiates excess retrieval, if there is sufficient \(Ca^{2+}\) entry to trigger the retrieval mechanism. The difference in the probability of triggering excess retrieval could also be due to recording conditions (perforated-patch vs. whole-cell recording mode; high external Na\(^+\) vs. no external Na\(^+\)), age (adult bovine chromaffin cells vs. calf cells) or stimulation method (single depolarizations vs. trains of depolarizations). We do not think our results are due to an inability to inhibit calcineurin under our conditions, since CsA treatment had multiple effects on bovine chromaffin cells, including acceleration of \(Ca^{2+}\) current inactivation, inhibition of exocytosis and prevention of a \(Ca^{2+}\)-dependent shortening of mono-exponential time constants.

Conclusion

It is clear that multiple membrane retrieval mechanisms exist, on the basis of morphological evidence, participation of distinct proteins and recent physiological studies using high resolution capacitance detection (including the present work). Each mechanism may be tailored to serve a particular cellular function, depending on whether rapid recovery is more important than accuracy, whether a trigger event occurs (e.g. \(Ca^{2+}\) entry or sudden addition of membrane) and whether the cell is carrying out housekeeping functions to maintain a stable shape or size, or instead suddenly requires a change in shape or direction. Our data suggest that some of the different physiological properties of endocytosis observed in bovine adrenal chromaffin cells are attributable to two distinct endocytotic pathways, one that offsets membrane added during exocytosis (compensatory retrieval) and another that is triggered by high levels of \(Ca^{2+}\) and is not related to exocytosis (excess retrieval). The role of this latter mechanism is unknown, but it may be invoked under conditions in which the cell needs to alter cell volume rapidly. Furthermore, we found that the rate of compensatory retrieval can be regulated by \(Ca^{2+}\) entry, in a manner that is sensitive to inhibition of calcineurin by cyclosporin A.

Note added in proof

A recent paper reports that in addition to dynamin, two other proteins implicated in synaptic vesicle endocytosis, amphiphysin I and synaptojanin, undergo depolarization-dependent dephosphorylation that is blocked by cyclosporin A and FK506, but not okadaic acid (R. Bauerfeind, K. Takei & P. De Camilli. ‘Amphiphysin I is associated with coated endocytic intermediates and undergoes stimulation-dependent dephosphorylation in nerve terminals’, Journal of Biological Chemistry (in the Press)). This suggests that calcineurin is a key \(Ca^{2+}\)-dependent regulator of a group of target proteins that may act in concert to control endocytosis.


Two types of endocytosis in bovine chromaffin cells


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