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Exocytosis of Catecholamine (CA)-containing and CA-free Granules in Chromaffin Cells*

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Recent evidence suggests that endocytosis in neuroendocrine cells and neurons can be tightly coupled to exocytosis, allowing rapid retrieval from the plasma membrane of fused vesicles for future use. This can be a much faster mechanism for membrane recycling than classical clathrin-mediated endocytosis. During a fast exo-endocytotic cycle, the vesicle membrane does not fully collapse into the plasma membrane; nevertheless, it releases the vesicular contents through the fusion pore. Once the vesicle is depleted of transmitter, its membrane is recovered without renouncing its identity. In this report, we show that chromaffin cells contain catecholamine-free granules that retain their ability to fuse with the plasma membrane. These catecholamine-free granules represent 7% of the total population of fused vesicles, but they contributed to 47% of the fusion events when the cells were treated with reserpine for several hours. We propose that rat chromaffin granules that transiently fuse with the plasma membrane preserve their exocytotic machinery, allowing another round of exocytosis.

Exocytosis is an ubiquitous cellular function used to discharge nonpermeable compounds across the plasma membrane. After exocytosis, the membrane excess at the cell surface is internalized by endocytosis, a function required to maintain a constant cell membrane area and allow the reutilization of vesicle components. Traditionally, endocytosis has been considered to be clathrin-mediated, and it occurs with a much slower time course than exocytosis (1). In clathrin-mediated endocytosis, the internalized membrane is thought to mix with endosomal compartments from which new vesicles emerge to maintain a functional pool of secretory vesicles (2, 3). This cycle is well established, and many details have been elucidated in the last few years (4–8). However, several studies indicate that endocytosis may also be more tightly coupled to exocytosis, giving rise to a faster exo-endocytosis sequence. Patch clamp capacitance measurements directly measure changes in the membrane area associated with exo- and endocytosis. With this method, a rapid form of endocytosis was observed in which the same amount of previously exocytosed membrane is retrieved rapidly in response to physiological stimuli (9). In synaptic vesicles, using FM1–43 fluorescent membrane marker, it has been shown that the amount of membrane taken up by endocytosis equals the amount of membrane added by exocytosis. This result suggested that at least some internalized vesicles directly enter the pool of releasable vesicles, bypassing intermediate endosomal compartments during the recycling process (10). In chromaffin cells, measurements of single fusion events and the simultaneous recording of CA[1] release by patch amperometry have demonstrated that a granule may reversibly fuse with the plasma membrane and release its CA content with a time course indistinguishable from that of irreversible fusion events. The frequency of reversible events (also termed "kiss-and-run" events) increases gradually as the Ca^{2+} concentration is elevated (11). We are now able to show that chromaffin granules that have lost their CA content are able to undergo exocytosis. The percentage of these CA-free granules increased in reserpine-treated cells. The CA-free granules are slightly smaller in size and show a delayed fusion pore expansion as compared with CA-containing granules. Our results show the existence of CA-free granules in chromaffin cells and the ability of such granules to fuse with the plasma membrane. These results point to the possibility that part of the vesicle pool is rapidly recovered, allowing a faster availability of vesicles for future use.

EXPERIMENTAL PROCEDURES

Preparation of Chromaffin Cells—Chromaffin cells were obtained from Sprague-Dawley rats (200–400 g) and cultured as described previously (12). Recordings were made on days 1–3 in culture. The bath solution contained 140 mM NaCl, 2.7 mM KCl, 10 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES/NaOH, and 5–10 mM glucose. The pH was adjusted to 7.3, and the osmolarity was, on average, around 300 mmol/kg. The pipette solution contained either (*a*) 5 mM $CaCl_2$, 100 mM TEA-Cl, 50 mM NaCl, 1 mM $MgCl_2$ and 10 mM HEPES/NaOH, (*b*) 90 mM $CaCl_2$, 40 mM TEA-Cl, and 10 mM HEPES/NaOH, or (*c*) 90 mM $BaCl_2$, 40 mM TEA-Cl, and 10 mM HEPES/NaOH. All experiments were done at room temperature. For reserpine experiments, cells were incubated for 1–6 h in 1 μM reserpine and subsequently washed in reserpine-free medium for at least 20 min before recording.

Measurement of Single Fusion Events and CA Release—Changes in membrane capacitance and catecholamine release were recorded simultaneously by patch amperometry (11, 13). Briefly, cell-attached patch clamp was achieved with a carbon fiber 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 fibers as described previously (14). The CFE was positioned at a distance 1–5 μm from the tip opening under the microscope. The pipette electrode was connected to ground, whereas the CFE was connected to the I/V converter of a homemade amperometric amplifier via a 3 M KCl solution. The CFE was continuously held at +800 mV. Amperometric currents were filtered with an 8-pole Bessel filter set at 3 KHz. Patch pipettes were pulled in three stages with a programmable puller (P-97; Sutter Instruments) and coated with a silicone compound (Sylgard 184; Dow

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[1] The abbreviations used are: CA, catecholamine; CFE, carbon fiber electrode; F, farad; S, siemens; ACh, acetylcholine; tetraethylammonium.

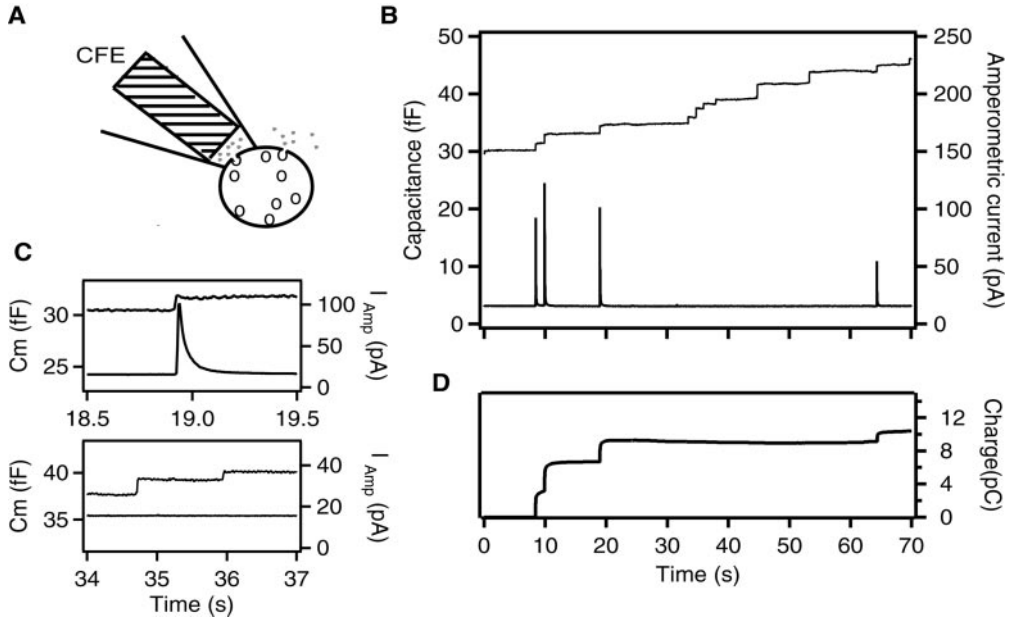

FIG. 1. **Patch amperometry recording showing CA-containing and CA-free granules.** *A,* patch amperometry configuration illustrating that only fusion events occurring in the membrane patch are detected electrochemically. *B,* simultaneous recording of membrane capacitance (*top trace*) and amperometric current (*bottom trace*). *C,* the two events from *B* are shown in an expanded time scale. *D,* time integral of the amperometric signal shown in *B, bottom trace*.

Corning). Pipettes were fire-polished and had a typical resistance in the bath of around 2 megohms. Pipette resistance typically increased up to 3–4 megohms when the carbon fiber approached the tip opening. To calculate the time integral of the amperometric signal, we subtracted the constant direct current level in the CFE.

For cell-attached capacitance measurements, we used a patch clamp amplifier (EPC-7; HEKA-Elektronik). Command voltage was applied to the bath. Changes of patch admittance were measured as described previously (15, 16) 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 a 1- or 3-ms time constant, 24 db. Data acquisition and analysis were done with a 15 A/D converter (IDA 15125; Indec Systems Inc.) with locally written software. Fusion pore openings were analyzed as described previously (16). 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 previously (17, 18), assuming a cylindrical pore 15-nm long and a conductivity of the pipette solution of 15 mS/cm^{-1} (see Eq. 11.1 in Ref. 19).

RESULTS

Patch amperometry allows resolution of exocytosis of single secretory vesicles approaching the size of synaptic vesicles and, in addition to this, detects the associated release of oxidizable vesicle contents (11, 13). Measurements are performed by combining high-resolution cell-attached membrane capacitance (15, 16) and electrochemical detection of CA inside the patch pipette. One advantage of patch amperometry is that it only detects fusion and the release of CA within the membrane patch under the pipette (~1% of the cell surface) (Fig. 1*A*). Cell-attached capacitance measurements have an excellent signal:noise ratio for chromaffin granule exocytosis, and the amperometric transients recorded under these conditions are detected with minimal loss of molecules due to the restricted space at the pipette tip (11, 13). Fig. 1*B* shows a patch amperometry recording from a chromaffin cell immediately after seal formation. The *top trace* illustrates step increments in patch membrane capacitance due to the irreversible fusion of single chromaffin granules with the plasma membrane. The *bottom trace* shows amperometric spikes representing CA detection with a CFE placed inside the patch pipette. The first three capacitance steps were accompanied by amperometric spikes. However, all but one of the remaining irreversible fusion events showed no detectable release of CA (blank events). In this patch, we recorded 19 capacitance steps but only 6 amperometric spikes, *i.e.* only 32% of the fused granules released CA. Fig. 1*C* shows a step increment in capacitance followed by release (*top panel*) and two steps not accompanied by amperometric transients (*bottom panel*) in more detail. This suggests that the properties of the step increases in capacitance were similar in both types of events. To better check whether there is release of any oxidizable compound during the fusion of blank events, we assessed the time integral of the amperometric signal (Fig. 1*D*). It is clear that an increase in the charge (2.5 ± 1.0 picocoulomb/fusion event in this cell, mean ± S.D.; $n = 4$) only occurred when there was an amperometric spike.

The fusion of blank events occurred in 7% (28 of 400) of the membrane patches recorded, 64% (18 patches) of which showed at least one fusion event accompanied by CA release. Within the recording from one particular patch, the time courses of the amperometric transients were similar regardless of the presence of blank events. Fig. 2*A* shows a superposition of 10 amperometric spikes from a patch in which 6 blank events were recorded. Because all events in patch amperometry are detected from the same very limited area on the cell surface (the membrane patch), and the distance between the release sites and the electrochemical detector is similar for all events, all amperometric transients displayed similar kinetics. Fig. 2*B,* which shows normalized amperometric spike amplitudes, better illustrates this point. Only a slight increase in the spike half-height width of 4 ms (from 26 to 30 ms) was observed during the recording in this cell (Fig. 2*C,* ●). The reason for the broadening of this spike was not determined, but it was typically observed in all recordings regardless of the presence of blanks. The observations that most amperometric spikes have similar half-height widths and that blank events are detected in between spikes (Fig. 2*C,* ○) are good indications that blank events are not due to a change in the sensitivity of the carbon fiber electrode.

Size Distribution of Capacitance Blank Events—To determine whether the size of the CA-free granules was within the range of chromaffin granules, we compared blank events with

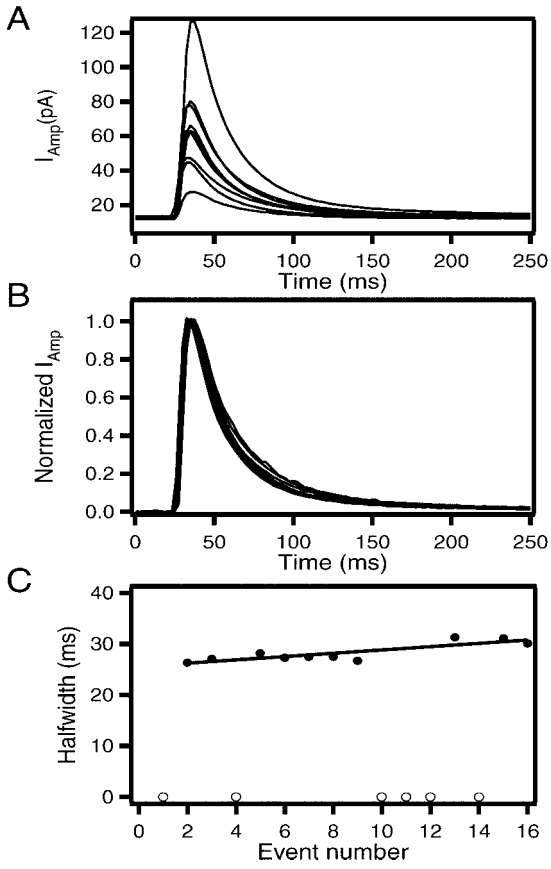

FIG. 2. **Comparison of amperometric spike events from a cell with blanks.** Spikes have been superimposed (*A*) and normalized (*B*). *C,* half-height width of the spikes during the recording. *Open circles* represent events without CA release; *filled circles* represent events with CA release.

those accompanied by amperometric spikes. Fig. 3, *A* and *D,* shows frequency histograms of capacitance step sizes. Distributions for both types of events were similar, although the mean capacitance value for blank events was 0.86 ± 0.46 fF (±S.D.; n = 53; 18 cells), which was 31% less than that of CA-containing granules (1.25 ± 0.7 fF; ±S.D.; n = 142; 8 cells). The transformation of granule capacitance to granule radius predicts an average (±S.D.) radius of 96 ± 25 nm for CA-containing granules (Fig. 3*B*) and 80 ± 21 nm for non-CA-containing granules (Fig. 3*E*). The average volume difference between these two populations of granules is about 58%. Fig. 3*C* shows a frequency histogram of the charge under the amperometric spike (assuming 2 charge electrons/CA molecule) for CA-containing granules. The mean charge ± S.E. was 1.56 ± 0.2 picocoulombs, a value similar to that obtained in cells in which no blank events were recorded.

To further explore the origin of CA-free granules, we did experiments in cells incubated with reserpine, which blocks the vesicular CA transporter. With reserpine, 47% of the fusion events recorded were CA-free (25 of 53 steps recorded; 12 cells). The step size distribution of fusion events in reserpine-treated cells had an average value ± S.D. of 0.94 ± 0.6 fF (n = 53) (Fig. 3*G*), corresponding to a mean ± S.D. granule radius of 84 ± 22 nm (Fig. 3*H*). These values are in between the granule radius measured for CA-containing and CA-free granules. In reserpine-treated cells, the amount of CA in CA-containing granules was greatly reduced, giving an average ± S.E. value of 0.38 ± 0.07 picocoulomb (28 spikes). These results indicate that the granule size distribution in reserpine-treated cells is similar to the size distribution of CA-free granules.

CA Concentration of Chromaffin Granules in Cells with Blanks—Regardless of the presence or absence of blank events in the recordings, the amount of CA released from individual chromaffin granules was usually proportional to granule size in nontreated cells. Granule volumes were calculated from the size of the capacitance steps, assuming that the granules are perfect spheres and that the membrane has a specific membrane capacitance of 10 fF/μm^2. Mean granule CA concentration was estimated from the slope of the line correlating moles of CA (estimated from the integral of the spike amperometric currents) and granule volumes. Fig. 4 shows this relationship in four cells. Although the estimated mean CA concentration varied greatly from cell to cell, granules of one particular cell generally had either a homogenous CA concentration or did not release any detectable CA at all. Granules containing a very small amount of CA were recorded only as exceptions (Fig. 4, *arrows*). The average ± S.E. of granular CA concentration measured in reserpine-treated cells was 0.64 ± 0.07 M (28 events), a significantly smaller amount of CA than that contained in typical chromaffin granules (range, 1–4 M).

Time Dependence of Blank Fusion Events—Fig. 5 shows the time sequence of fusion for CA-free and CA-containing granules in two representative recordings. Blank events are illustrated as points with CA concentration = 0. There appears to be no preferred time distribution of blank events along the recordings. Blank events seem to occur randomly between the exocytosis of CA-containing vesicles, indicating that both CA-free and CA-containing granules were ready for fusion. These data suggest that these two populations of granules are within the same pool of releasable vesicles.

Pore Characteristics in CA-free Granules—To study the effect of CA content on fusion pore opening, the time courses of fusion pore conductance were compared in CA-containing and CA-free vesicles. Pore conductance was calculated for those events in which the increase in the imaginary part of the admittance (capacitance trace) was accompanied by a detectable transient increase in the real part of the admittance (conductance trace). Fig. 6 compares, in the same cell, the time course of two fusion pores in which an amperometric spike was present (Fig. 6*B*) or absent (Fig. 6*A*). The panels show the imaginary part of the admittance (proportional to the patch membrane capacitance, trace labeled *Im*/Ω), the real part of the admittance (proportional to the patch conductance, trace labeled *Re*), the calculated pore conductance (*Gp*) (16), and the amperometric current (*Am*). In Fig. 6*A,* the pore conductance developed in at least two phases. A phase of small fusion pore conductance (circa 100 pS) lasting 300 ms was followed by a fast rise of fusion pore conductance to values of >1 nS. In Fig. 6*B,* only the fast phase is observed. In this membrane patch, no current projection onto the real part of the admittance could be detected during the fusion of all of the CA-containing granules (n = 5), indicating that a possible phase of low fusion pore conductance was shorter than our time resolution (sampling rate, 2.5 ms/point). On the contrary, in all CA-free granules, the time course of the pore expansion could be measured. This trend was observed in four different cells. The average duration ± S.E. for low fusion pore conductance was almost five times shorter in CA-containing granules (12 ± 1.8 ms; n = 50) than in CA-free granules (58 ± 21 ms; n = 20). Fig. 6, *C* and *D,* shows normalized time distributions of fusion pore conductance values for CA-free and CA-containing chromaffin granules, respectively. CA-free granules spent >80% of their expansion time with a conductance of <200 pS (Fig. 6*C*), and the average pore conductance was 195 pS (n = 234). On the contrary, CA-containing granules had an average conductance of 560 pS (n = 86), spending <20% of their expansion time with a con-

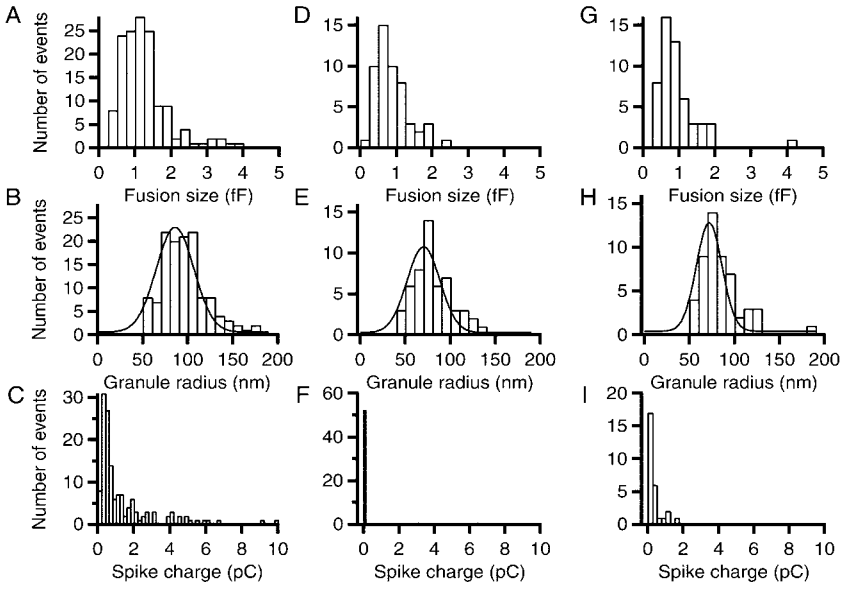

FIG. 3. **Comparison of CA-containing (*A–C*) and CA-free (*D–F*) granules, and events recorded in cells incubated with reserpine (*G–I*).** *A, D,* and *G,* capacitance step size distribution. *B, E,* and *H,* granule radius distribution. *C, F,* and *I,* spike charge distribution of amperometric spikes.

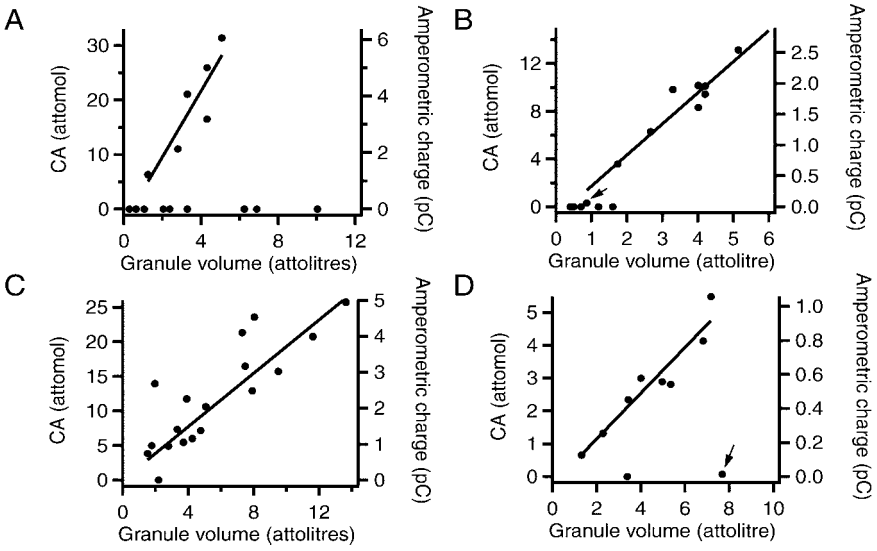

FIG. 4. **Relationship between CA content and granule volume in four cells (*A–D*).** Note that CA-free granules are represented as a *dot* at the *bottom axis*. The *arrows* in *B* and *D* point to granules partially filled with CA.

ductance of <200 pS (Fig. 6*D*). In both cases, events in which the fusion pore could not be measured were considered fusion events with pore duration < 2.5 ms. These results indicate that although chromaffin granules fuse with the membrane regardless of the CA contents in the granule, CA-free granules tend to enlarge their fusion pores slowly, continuing at smaller conductance values for longer time periods.

DISCUSSION

This work describes the exocytosis of CA-containing and CA-free granules in chromaffin cells in rats. Our results are consistent with the hypothesis that CA-free granules result from chromaffin granules that have previously fused with the plasma membrane, released their CA contents, and once emptied, internalized. However, there are other possibilities that could explain the existence of stepwise capacitance blank events in chromaffin cells. One possibility is that these blank events represent the fusion of acetylcholine (ACh)-containing vesicles. Such vesicles have been described in chromaffin cells (20, 21) and would appear as blank events in our amperometric recordings because ACh cannot be measured electrochemically by a carbon fiber electrode as used here. This possibility is unlikely for two reasons: (*a*) ACh is stored in synaptic-like microvesicles with diameters ranging from 30–90 nm (22), corresponding to step changes in capacitance between 0.02 and 0.25 fF, and these values do not overlap with the step size distribution obtained for the capacitance blank events measured in our experiments (Fig. 3, *D* and *E*); and (*b*) during blank fusion events, we do not observe a transient conductance increase, as expected if the released ACh activates the nicotinic receptors of chromaffin cells. Another possibility is that blank events are indeed chromaffin granules that release CA, but the released molecules escape electrochemical detection. A possible source of detection failure of CA in patch amperometry could be that fused granules release their contents into a compartmentalized space within the patch pipette. This could happen in situations where part of the membrane patch is tightly attached to the pipette wall, forming a pocket of membrane that could slow down diffusion of molecules toward the carbon fiber detector. For example, if this pocket has a volume 100 times larger than the volume of a chromaffin granule, and it is connected to the pipette interior through a leak pathway with a conductance similar to a fusion pore, we should expect that when a chromaffin granule releases its CA content, CA would accumulate at a 100 times lower concentration into this pocket and would leak to the carbon fiber detector 100 times more slowly. This will give rise to a very small but very prolonged amperometric signal that should appear in the time integral of the amperometric recording. As shown in Fig. 1*D,* we did not

observed any increase in the amperometric charge during and many seconds after the fusion of blank events.

To further explore the origin of blank events, we performed experiments in cells incubated with reserpine (1 μM). This treatment depletes chromaffin granules of CA without affecting the contents of other granule components such as dopamine β-hydroxylase (23). In our experiments, the amount of blank events recorded increased from 7% in nontreated cells to 47% in cells incubated with reserpine. In reserpine-treated cells, the CA content in the remaining granules was significantly smaller than that in non-reserpine-treated CA-containing granules, suggesting that reserpine effectively depleted chromaffin granules of CA before fusing with the plasma membrane. The step size distribution of reserpine-treated cells was more like that observed for blank events than that observed for CA-containing granules. This strongly suggests that CA-free granules in fact arise from depleted chromaffin granules and not from a different type of granule or through constitutive exocytosis.

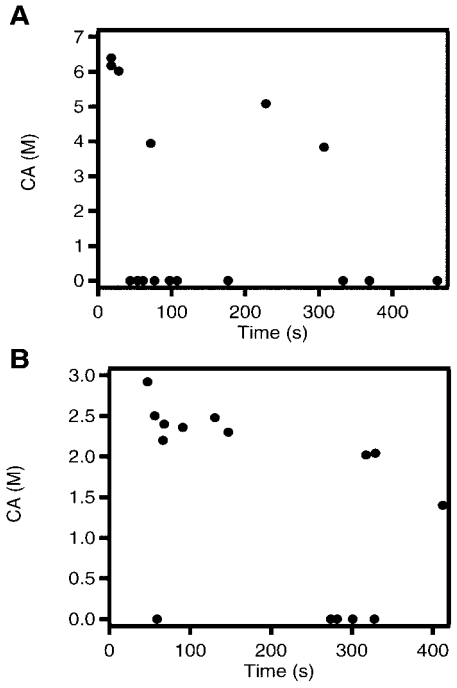

FIG. 5. *A* and *B,* time distribution of CA concentration of fusing granules during the course of two experiments. CA-free events are scattered throughout the recording, indicating that CA-free and CA-containing granules intermixed within the same pool of granules.

The CA-free granules apparently have a smaller mean diameter than CA-containing ones. This could be due to the inclusion of some constitutive exocytotic events on the CA-free granule size distribution, which the great majority is < 0.4 fF (24). Alternatively, smaller-sized granules could result from the loss of some membrane during the preceding fusion event in which catecholamine was released. Membrane transfer from the plasma membrane to secretory granules during reversible fusion events has been reported in mast cells (25). In the electric organ of the marine ray *Torpedo,* two populations of vesicles can be distinguished after stimulation (26, 27). One has the same size as synaptic vesicles found at rest, whereas the other has a smaller diameter (25%). These two populations were attributed to preformed and recycling vesicles, respectively (28, 29). Large-sized vesicles were full of ACh, whereas smaller-sized vesicles were partially filled with ACh. We do not usually find partially filled granules in cells non-treated with reserpine; we usually find granules with high or no CA content. Partially loaded granules were recorded only occasionally (Fig. 4, *arrows*). The absence of partially filled granules in our recordings may be due to several possibilities. CA granules might not be refilled because the reloading machinery in the granule membrane was not ready, either because the proton gradient necessary for the transport of CA was not reestablished or because the CA transporter was no longer operative. Another possibility is that depleted granules did not have enough time to reload. The CA refilling time for a single chromaffin granule is currently unknown.

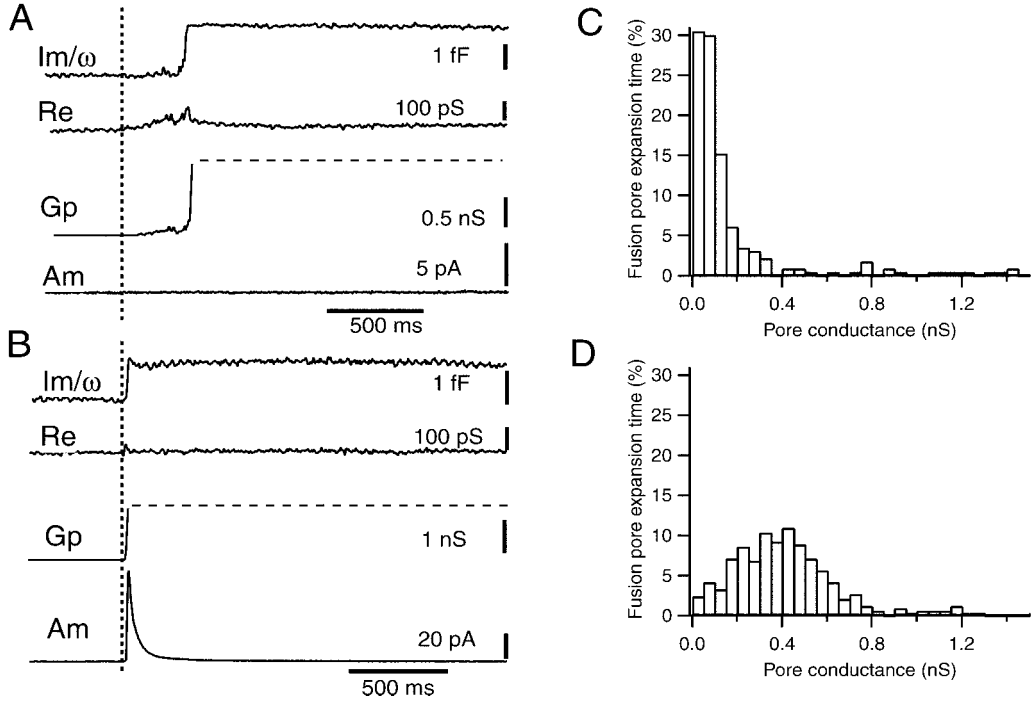

FIG. 6. **Kinetics of the fusion pore opening in CA-containing and CA-free granules.** *A* and *B,* recordings of capacitance (*Im*/Ω), conductance (*Re*), pore conductance (*Gp*), and release (*Am*) from the same cell. *A,* CA-free granule; *B,* CA-containing granule. *C* and *D,* pore conductance distribution during the expansion of the exocytotic fusion pore in events without CA release (*C*) and events accompanied by CA release (*D*).

In our experiments, irreversible blank events appeared to be randomly intermixed with CA-releasing events along the recording, without a clear distribution preference (Fig. 5). Similar results have been reported in the electric organ of the *Torpedo* (30). This result suggests that CA-free and CA-full granules in chromaffin cells are within the same pool of releasable vesicles, at the same stage in the sequence of events leading to fusion-competent vesicles. It should be noted that during our recordings, no external stimulus was applied, and most fusion events occur spontaneously or after spontaneous action potential firing.

The expansion of exocytotic fusion pores has been studied previously in mast cells (31–33) and horse eosinophils (34). It was found that fusion pore expansion is accelerated by increased cytosolic calcium concentrations (33, 34) and by protein kinase C activation with phorbol ester (35). Accelerated fusion pore expansion leads to a more rapid release; in particular, it leads to a shortened time between the initial opening of the fusion pore and the initiation of rapid release (33). In the experiments described here, we find a marked increase in the delay between fusion pore formation and rapid expansion for CA-free vesicles. The initial fusion pore formation, however, does not appear to be markedly affected, indicating that fusion pores formed independently of the granule contents. However, fusion pore expansion was delayed. CA-free granules remain in a low conductance state for longer periods than do CA-containing granules, although CA-free granules could eventually fully expand the fusion pore. This suggests that the release of CA or the process associated with it, *i.e.* expansion of the granule matrix during exocytosis, could have an important role in hastening fusion pore expansion. This result is in contrast to that reported previously in beige mouse peritoneal mast cells (32), in which inhibition of the granule matrix swelling apparently did not affect the kinetics of fusion pore expansion during exocytosis. However, it should be mentioned that in those experiments, only 90% of granule matrix swelling could be inhibited by a high concentration of histamine chloride (140 mM) in the bath. Additional experiments with complete inhibition of matrix swelling in beige mouse mast cells will be required to clarify the importance of matrix swelling in the kinetics of fusion pore expansion.

Cell membrane capacitance increments not accompanied by a detectable amperometric signal have been described in bovine chromaffin cells (36) and in mast cells (37). These increases in capacitance have been attributed either to the fusion of intracellular vesicles (or organelles) distinct from the specific chromaffin granules or to the fusion of mast cell granules with the plasma membrane. Supporting the hypothesis of nonspecific membrane fusion, changes in capacitance have also been described in cells whose main function is thought to be nonsecretory, such as fibroblasts or Chinese hamster ovary cells (38). However, stepwise membrane capacitance increments unaccompanied by serotonin release could be resolved and unambiguously attributed to the fusion of single secretory granules in peritoneal mast cells from ruby-eye mice (39). These fusion events in mast cells appeared at the beginning of the secretory response. They were attributed to the fusion of granules that had previously discharged their contents into the extracellular medium during transient fusion events (39). This is consistent with the high incidence of capacitance flickers in mast cells from ruby-eye mice.

Flickering of the fusion pore between an open and closed state has been demonstrated with electrophysiological techniques in mast cells (31, 40, 41) and chromaffin cells (11, 13). Such transient fusion events may also occur in synaptic vesicles. Using optical methods, it has been proven that exocytosed vesicles can be directly reformed from the plasma membrane without passing through the early endosome (10). During transient fusion events, rapid and complete CA release can occur in rat chromaffin cells (11). The existence of exocytosis-coupled endocytosis with full discharge of the granule contents implies the presence of neurotransmitter-depleted vesicles inside the cell. One intriguing possibility is that CA-free granule exocytosis arises from granules that have been depleted of CA through a preceding transient fusion with the plasma membrane and lacked the time or capability to reload the transmitter.

In summary, we have found a population of CA-free granules, with a size that is compatible with that of chromaffin granules, that is competent for fusion. These CA-free granules presumably arise from chromaffin granules that have previously released their contents via a kiss-and-run event and directly return into the pool of releasable vesicles. The fusion pore expansion in these vesicles is significantly slower than that in CA-containing vesicles, thus suggesting that the ion exchange processes with the granular matrix may play a role in the mechanism of fusion pore expansion in chromaffin granules.

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