Rapid Fluctuations in Transmitter Release from Single Vesicles in Bovine Adrenal Chromaffin Cells

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ABSTRACT Single-vesicle release of catecholamines from chromaffin cells can be detected in real time as current spikes by the electrochemical method of amperometry. About 70% of spikes are preceded by a small "foot," the trickle of transmitter out of the early fusion pore. In addition, 20–50% of foot signals exhibit rapid fluctuations that we interpret as flickering of the fusion pore. There are also "stand-alone" foot signals, which may reflect transient fusions, in which the vesicles do not collapse completely into the plasma membrane. The number and frequency of the foot flickering are affected by intracellular Ca^{2+} concentration.

INTRODUCTION

Recently, increasing effort has been directed toward unraveling the mechanisms of exocytotic release of transmitters and hormones from secretory cells. Several highly conserved proteins have been identified as members of a putative "fusion complex" that is thought to be involved in the final steps of exocytosis (Südhof, 1995; Scheller, 1995). Defining the function of these proteins requires rapid assays of membrane fusion and transmitter release, as the final steps of exocytosis occur on the time scale of a millisecond. For nonneuronal cells (i.e., cells that lack a postsynaptic membrane that would serve as a fast and sensitive bioassay of release) patch-clamp capacitance measurements and the electrochemical method of amperometry provide fast and exquisitely sensitive tools for real-time, in situ monitoring of cell membrane addition or transmitter release, respectively (Gillis, 1995; Chow and von Rüden, 1995).

Amperometry has been applied previously to the study of secretion in chromaffin cells (Wightman et al., 1991; Chow et al., 1992) and mast cells (Alvarez de Toledo et al., 1993). In adrenal chromaffin cells, secretion leads to spiking current transients in amperometric recordings. Each oxidative current spike was shown to be due to the transmitter released from a single dense-core vesicle. Most of the spikes were preceded by a slower trickle or "foot" of release that was attributed (Chow et al., 1992) to the leak of transmitter out of the early fusion pore—the initially narrow, aqueous channel that connects the fused vesicle lumen with the cell exterior (Almers, 1990). This idea was difficult to confirm in chromaffin cells, where the single granules are so small that the capacitance changes due to exocytosis are too small to be resolved in standard whole-cell capacitance measurements (Neher and Marty, 1982). However, in mast cells from *beige* mice, the secretory granules are giant (1–2.5 μm diameter). Thus, granule fusion can be visualized directly with the light microscope (Zimmerberg et al., 1987; Breckenridge and Almers, 1987), and it is possible with whole-cell capacitance measurements to resolve the time course of single vesicle fusion. The initially narrow fusion pore has a lifetime of up to several seconds and may rapidly fluctuate about a small mean diameter ("flicker"), or even close transiently, before expanding irreversibly (Fernandez et al., 1984; Almers and Tse, 1990; Monck and Fernandez, 1994). Furthermore, simultaneous capacitance and amperometric measurements have confirmed that transmitter starts to trickle out during capacitance flickering—that is, while the pore is still narrow and before the vesicle has completely collapsed into the plasma membrane (Alvarez de Toledo et al., 1993). Interestingly, fluctuations in the conductance of the fusion pore were highly correlated with fluctuations in the amperometric signal, showing that the early fusion pore may restrict the rate of transmitter efflux from the vesicle.

We have reexamined the morphology of amperometric events recorded from patch-clamped bovine chromaffin cells, taking advantage of the exceptionally low noise and high temporal resolution of carbon fiber electrodes insulated with polyethylene. We report that 20–50% of amperometric foot signals have rapid but readily resolvable and quantifiable fluctuations or "flickers"; these may reflect flickering of the fusion pore. Although the capacitance flickers reported in mast cells were slow (<40 Hz), the flickers resolved here are >5 times faster. Under some conditions, the average flicker frequency is dependent on the intracellular Ca^{2+} concentration. In addition, we provide evidence for the existence of "stand-alone foot" signals, which may represent transient vesicle fusions in which the vesicles do not collapse completely into the plasma membrane.

These data provide insight into the kinetics of exocytotic transmitter release from chromaffin granules, often taken as a model system for the study of synaptic vesicle exocytosis.

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Furthermore, they illustrate that the resolution of the amperometric approach, at least in chromaffin cells, far exceeds that obtained by capacitance measurement. Part of this work has appeared in abstract form (Zhou et al., 1995).

MATERIALS AND METHODS

Cell preparation and solutions

Bovine chromaffin cells were enzymatically dispersed and cultured (Zhou and Neher, 1993) and were used within 1 to 4 days of plating. Experiments were performed at room temperature (23°C). The external solution consisted of (in mM): 140 NaCl, 2.8 KCl, 2 MgCl2, 10 glucose, 10 HEPES (pH set to 7.2 with NaOH). The standard internal solution consisted of (in mM) 130 K glutamate, 8 NaCl, 1 $MgCl_2$, 2 MgATP, 20 diglycolic acid, 10 HEPES (pH 7.2 with CsOH). The calcium buffer was diglycolic acid, included at 20 mM concentration for all solutions. Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was set by adding $CaCl_2$ to obtain a final total concentration of 450.5 μM or 2.07 mM, which gave an estimated free $[Ca^{2+}]_i$ of 10 and 50 μM, respectively. The Ca^{2+} buffer capacity of the diglycolic acid solutions were 40 (Zhou and Neher, 1993). The free calcium concentrations were checked with a calcium electrode (Orion Research, Cambridge, MA) and found to be within 10% of the desired values.

Electrophysiology

Stimulation of secretion was achieved by dialysis of single cells against a patch pipette solution of high $[Ca^{2+}]$ (10 or 50 μM). Single cells were voltage clamped at −70 mV with access resistance in range of 3–7 MΩ (4.8 ± 1.0 SD). Recordings in which the access resistance exceeded 10 MΩ were rejected. In most cells, secretion started within a few seconds after patch rupture to attain the whole-cell recording configuration, and it continued for 10 to 20 min.

$[Ca^{2+}]_i$ was assumed to be set by the internal solution as long as the patch pipette seal was "tight" (>200 MΩ). This was supported by earlier experiments in which $[Ca^{2+}]_i$ was monitored with fura-2 (Zhou and Neher, 1993). In these experiments, it was consistently observed that $[Ca^{2+}]$ was reasonably well controlled when seal resistance (R_{seal}) > 200 MΩ, but that external Ca^{2+} entered the cell through a leaky seal and increased $[Ca^{2+}]_i$ irreversibly to very high levels (Zhou, unpublished observation). In leaky cells with R_{seal} < 200 MΩ, we assumed that $[Ca^{2+}]_i$ was ≫ 50 μM, because the extracellular Ca^{2+} concentration was 2 mM—far higher than that of any of our internal solutions. Whole-cell currents were acquired together with membrane capacitance and access resistance by using a computer-controlled patch-clamp amplifier EPC-9 (HEKA, Lambrecht, Germany).

Amperometry

Carbon fiber electrodes (CFEs) were used for electrochemical monitoring of quantal release of CA from single cells as described previously (Chow and von Rüden, 1995). Polyethylene-insulated CFEs (pCFEs) (Chow et al., 1992) were modified and used in such a way as to ensure high detection sensitivity (Zhou and Misler, 1995a). Briefly, to ensure intact insulation of the carbon fiber before the tip-cutting process, the polyethylene at the pCFE tip was heated by a soldering iron to form a thinly insulated region of ~100 μm in length. Because the electrode noise depends significantly on the electrode capacitance, which is nearly proportional to the length of the thinly insulated tip (Zhou and Kang, 1990; Neher and Chow, 1995), the tip was cut to a final length of 10–50 μm. The pCFE was mounted on a manipulator, allowing precise positioning of the pCFE tip at the crux of spring-loaded metal iris 8-mm scissors (Stolz Instrument Co., St. Louis, MO) attached to the recessed surface of a magnetic stand base. It was often possible to reuse the same pCFE by recutting it multiple times. Recutting the tip had the additional important function of ensuring that the sensor surface was free of any cellular debris that would act as a diffusion barrier. Immediately before an experiment, the tip was inserted into methanol for 2–5 s. Instead of 3 M KCl, we used mercury as the back-filling solution to make the contact between the CFE and the Ag wire input to the amplifier, because, after several hours of exposure of the carbon fiber to KCl solution, the noise increases significantly, for unknown reasons. Total RMS noise of our amperometric recordings was 0.8–3 pA with a low-pass filter of 3 kHz, which is about 5–10 times larger than the internal noise of an EPC-7 patch-clamp amplifier. Records were made in the amperometric mode with a patch-clamp amplifier, which held the pCFE at + 800 mV.

Data collection and analysis

Amperometric signals were low-pass filtered at 3 kHz bandwidth, then sampled at 2 to 5 kHz by an ITC-16 computer interface (Instrutech Corp., Elmont, NY) driven by a Macintosh computer operating IGOR (WaveMetrics, Lake Oswego, OR) and the Pulse Control XOPs (Herrington and Bookman, 1993). Cell membrane capacitance, access conductance, and whole-cell currents were routinely recorded at 2 Hz by an Atari computer-controlled system. For each individual amperometric spike, the spike amplitude, rise time, half-height duration ($t_{1/2}$), foot duration, spike and foot charges, number of foot flickers, and the frequency of foot flickers (see below) were analyzed on Macintosh computers with macro programs in IGOR written by Chow and Zhou.

Analysis of foot flickers

To quantify the size of foot fluctuations, the amperometric current trace was smoothed with a binomial smoothing operation provided in IGOR, with a "smooth factor" of 10. The processed trace was then differentiated. Within a foot signal in the differentiated trace, we identified a "foot flicker" as a spike whose absolute amplitude exceeds a threshold of 3 (or 5) times the standard deviation in the background noise. For each particular single-vesicle release event, the background noise was evaluated 30 ms before the foot signal (see Fig. 3 *A*). The foot duration is defined according to the method of Chow and von Ruden (1995): a line is drawn through points in the ascending limb of an event at 35% and 60% of the peak amplitude. Another line is drawn through the baseline. The end of the event is the intercept of the two derived lines. To identify the beginning of an event the unprocessed trace is scanned from the peak of the event backward in time to the point at which the signal first drops below the baseline plus 2 standard deviations of the baseline noise.

The number of flickers (N_f; see Fig. 3 for definition) detected in a given "foot" divided by the foot duration (D_{ft}) defines the frequency of flickers (F_f) in that "foot":

$$F_f = \frac{N_f}{D_{ft}} \tag{1}$$

N_f and F_f are parameters describing the fluctuations of each foot signal. Note that each upward (fusion pore expanding) or downward (fusion pore closing) deflection in the amperometric trace generates a positive or negative "spike" in the differentiated trace. Both upward and downward spikes were summed to obtain the flicker numbers, because, for the present analysis, we do not assume that they are necessarily coupled (fusion pore opening may occur independently of closing, and vice versa). Thus, a single fast "foot spike" may generate two foot flickers (N_f = 2).

To calculate the number of flickers versus percentage of total foot events from the histogram of foot flickers (Fig. 3 *B*), the cumulative distribution functions (PN) is used:

$$PN(x) = \sum_{i>0}^{x} N_f(i) \Big/ \sum_{i>0}^{\infty} N_f(i), \tag{2}$$

where i and x are flicker number; $N_f(x)$ is the histogram function of number of foot flickers.

To calculate frequency of foot flickers versus percentage of total foot events from the histogram of foot flickers (Fig. 3 *C*), the cumulative distribution functions (PF) are used:

$$PF(x) = \sum_{i>0}^{x} F_f(i) \Big/ \sum_{i>0}^{\infty} F_f(i), \quad (3)$$

where i and x are flicker frequency; $F_f(x)$ is the histogram function of frequency of foot flickers. Note that according to Eqs. 2 and 3, events without flickers (first bars in Fig. 4, *B* and *C*) were not included in the cumulative functions PN(x) and PF(x).

RESULTS

Secretion evoked by intracellular Ca^{2+} dialysis

Experiments combining whole-cell patch-clamp recording and carbon fiber amperometry were made on single bovine chromaffin cells. When a chromaffin cell was dialyzed against a high-[Ca^{2+}] (10 or 50 μM) pipette solution in the whole-cell voltage-clamp configuration, we observed both increases in membrane capacitance, representing an increase in plasma membrane surface area, and intermittent amperometric spikes, representing quantized release of catecholamines (CAs). Both signals were well correlated and presumably reflected the exocytosis of CA-containing chromaffin granules.

Fig. 1 shows traces typical of those recorded from the 24 cells whose secretory events were analyzed for presentation here. In this figure, amperometric current, whole-cell current, access resistance, and membrane capacitance were monitored in parallel. In the rest of this work, only the amperometric current spikes were analyzed in detail. With each cell, we recorded continuously until secretion appeared to cease (usually after about 5–10 min), probably because of depletion of releasable vesicles (Burgoyne, 1991; Augustine and Neher, 1992) or loss into the pipette of cytosolic factors critical for continued exocytosis (Penner et al., 1987). In the cell shown in Fig. 1, the membrane capacitance increased 1.5-fold from an initial value of 6 pF over 200 s of whole-cell dialysis against 50 μM [Ca^{2+}]. This corresponds to the release of about 3200 granules, each of 280 nm diameter (assuming 2.5 fF per vesicle; Neher and Marty, 1982). For 24 cells, the average value of C_m increase during prolonged Ca^{2+} dialysis was 9.1 ($\pm$6.9 SD) pF, corresponding to the exocytosis of about 3600 vesicles.

Flickering during the "foot"

Chow et al. (1992) reported that 50–70% of the large amplitude (>50 pA) and rapid rise time (<3 ms) amperometric signals had a small "foot" immediately preceding the major spike. This "foot" was interpreted as representing the trickle of catecholamine through the early and narrow fusion pore, before the completed fusion of the secretory granule with the plasma membrane. Here we show that

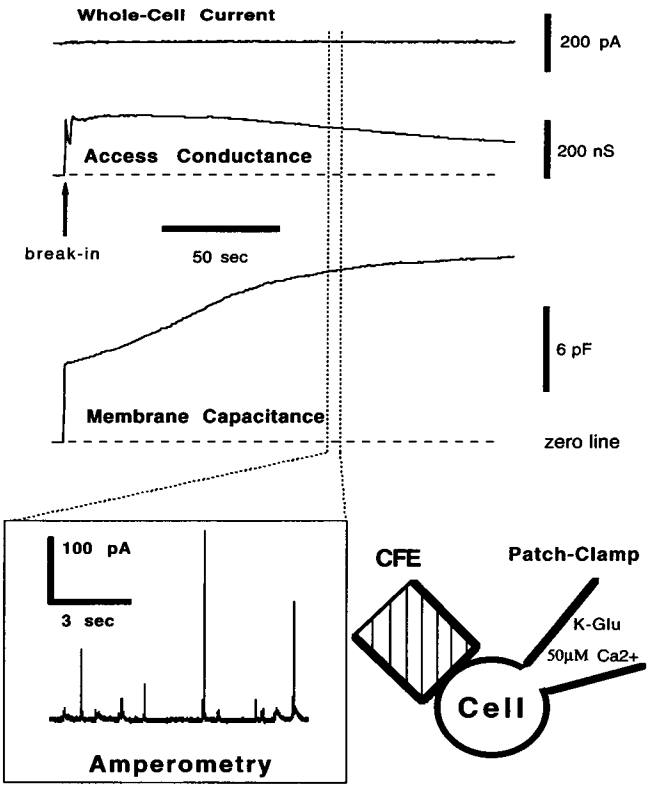

FIGURE 1 Stimulation and recording configuration. This cell was dialyzed against a pipette solution containing 50 μM Ca^{2+} to promote secretion. Whole-cell current, access resistance, and membrane capacitance were recorded by the patch pipette. The amperometric signals were recorded by a carbon fiber electrode. In this particular cell the membrane capacitance increased 1.5-fold during 200 s of whole-cell dialysis.

significant fluctuations occur during many of the foot signals (see Fig. 2). These fluctuations far exceed the background noise and often have small (5–50 pA) "subspikes" or "flickers" with a half-width or half-height duration of $t_{1/2} \leq$ 0.5–2 ms.

Analysis of these flickers revealed the following properties. First, flickers occur during the foot signal, suggesting that flickers and foot signals reflect aspects of the same underlying process. Second, the half-width or half-height duration ($t_{1/2}$) of a flicker is roughly equal to or less than that of the major spike (0.5–4 ms), suggesting that release sites that give rise to both the major spikes and foot flickers are $\leq$ 0.5 μm from the CFE sensor (see diffusion models of Schroeder et al., 1994; Chow and Ruden, 1995). Third, the charge transfer during one flicker is <1% of the charge of the major "spike." Fourth, flickers often sit on a plateau of slower fluctuations (significantly exceeding background noise). Based on these properties, we suggest that i) the flickers are part of the foot signal and that ii) the entire foot signal represents the escape of CAs through the fusion pore, which transits through several low-conductance states before it ultimately dilates irreversibly and permits the major spike of CA release.

To quantitate the features of the fluctuations occurring in a foot, we counted and tabulated the number of flickers

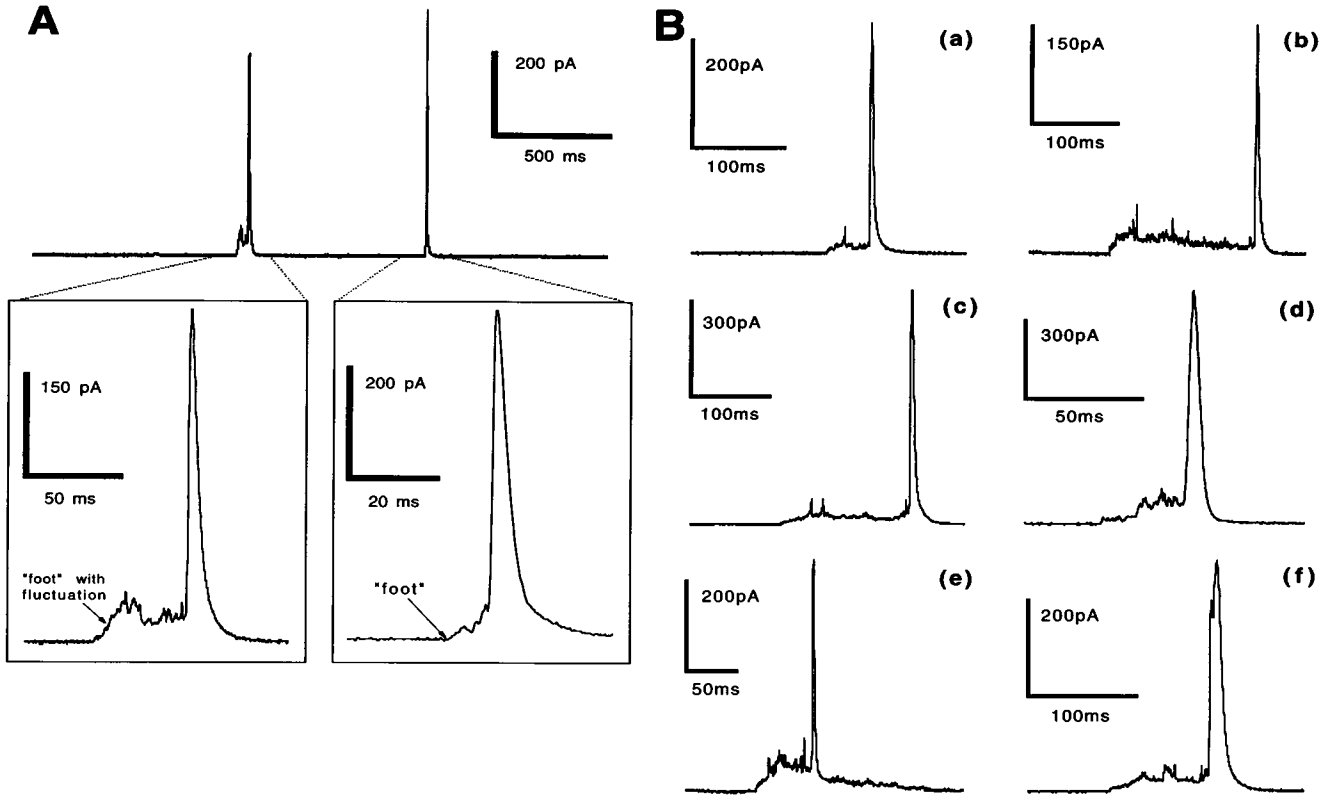

FIGURE 2 Examples of "feet" of amperometric signals with obvious "flickers." (*A*) Two well-separated spike events with significant fluctuations on the small feet preceding the major spikes. (*B*) More examples of events with foot flickers.

("flicker number," N_f) and then calculated frequency of flickers in each foot ("flicker frequency," F_f) using a method involving differentiation of the foot signal and then searching the differentiated record for deflections (individual flickers) having amplitudes exceeding the baseline noise by a certain threshold (defined in terms of standard deviations of the baseline noise; see Fig. 3 *A* and Methods section). Fig. 3, *B* and *C*, presents the statistical analysis of ~1500 large (>100 pA) and rapid (rise time ≤ 1 ms) events recorded from 24 cells, in which 5 cells had a $[Ca^{2+}]_i = 10$ μM, 12 cells had a $[Ca^{2+}]_i = 50$ μM, and 7 cells had a $[Ca^{2+}]_i \gg 50$ μM. For a threshold of 3 SD ($p < 0.01$) and a rise time <1 ms, about 50% of the foot events had at least one flicker, and the average flicker number was 1.4 for all fast events analyzed. When we considered only those events with at least one flicker, the average flicker number was 2.7 and the average flicker frequency was 170 Hz. For a threshold of 5 SD ($p < 0.001$), about 35% of the foot events had at least one flicker. The average flicker number was 1.1 for all fast events analyzed. When we considered only events with at least one flicker, the average flicker number was 2.1 and the average flicker frequency was 140 Hz.

Dependence of foot flicker kinetics on intracellular $[Ca^{2+}]$

Occasionally, during a recording the seal resistance suddenly dropped from > 1 GΩ to < 200 MΩ. This was followed consistently by an increase in the slope of the membrane capacitance trace and in the frequency of amperometric spikes. Both of these changes probably reflected a rise in intracellular $[Ca^{2+}]$ to levels far exceeding that of the pipette solution, leading to an increased rate of granule fusion. Because the rate of secretion increased even when the pipette solution contained 50 μM free Ca^{2+}, the intracellular $[Ca^{2+}]$ undoubtedly attained levels far exceeding 50 μM. Of course, concentrations of other intracellular ions may be altered in leaky cells, but it is the change in [Ca2+] that is most likely to be responsible for the enhanced rate of secretion (Knight and Baker, 1982).

Elevation of $[Ca^{2+}]_i$ above basal level stimulates secretion in chromaffin cells (Heinemann et al., 1994). Thus, it was of interest to investigate whether differing $[Ca^{2+}]_i$ might affect the dynamics of exocytosis of individual granules. Fig. 4 shows the cumulative distribution function (see Materials and Methods) of foot flickers at $[Ca^{2+}]_i = 10$ μM ($n = 3$), $[Ca^{2+}]_i = 50$ μM ($n = 8$), and $[Ca^{2+}]_i \gg 50$ μM ($n = 7$), respectively. These data show that the foot flicker parameters are affected by $[Ca^{2+}]_i$. At $[Ca^{2+}]_i \gg 50$ μM, the average flicker number (N_{fa}) and average flicker frequency (F_{fa}) are $N_{fa} = 2.45$ and $F_{fa} = 200$ Hz. In contrast, at $[Ca^{2+}]_i = 10$ μM, N_{fa} and F_{fa} are 35% and 104% smaller. Larger N_{fa} and F_{fa} at higher $[Ca^{2+}]_i$ suggest that persistently elevated $[Ca^{2+}]_i$ can affect the fusion pore even after the vesicle is fused with the plasma membrane.

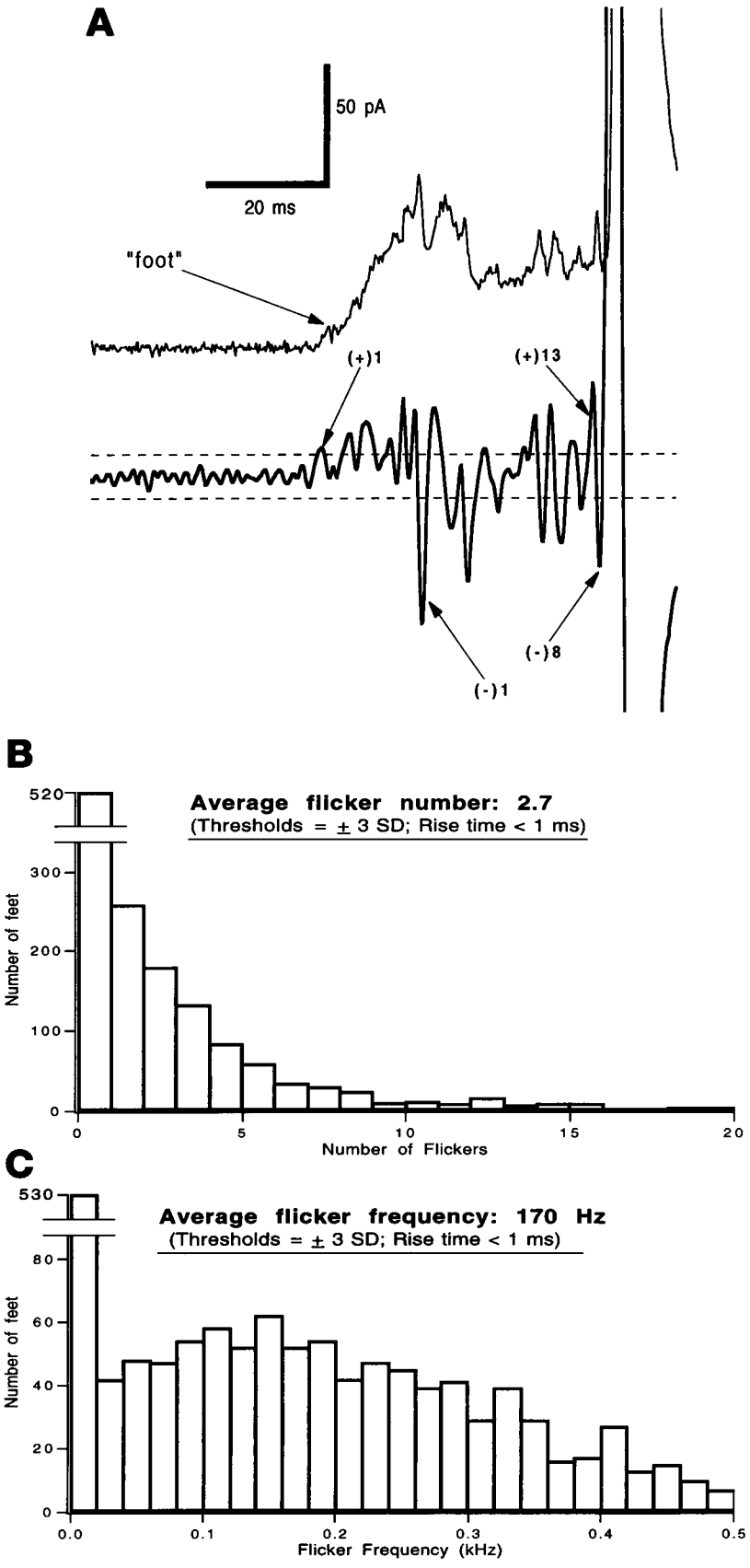

FIGURE 3 Quantitative analysis of the "flickery foot." (*A*) Raw amperometric current trace consisting of i) an *event* (a "spike" preceded by a "foot") and ii) the 30 ms of "background" preceding the start of the "event." Immediately below the raw trace is a differentiated trace, the dotted line that represents a threshold criterion of 5 SD, based on the noise in the background segment. In the differentiated trace each positive or negative excursion beyond the threshold was counted; those preceding the "spike" were counted as "flickers." The upper continuous trace is the data trace, and the lower continuous trace is the differentiated record. The dotted line is the threshold defined as the mean of the baseline ± 5 SD. Note that there are more upward deflections exceeding the threshold than downward deflections. In *B* and *C*, this analysis was applied to ~1500 well-isolated, large (≥100 pA) and fast rise time (≤1 ms) single release events using a threshold criterion of ±3 SD.

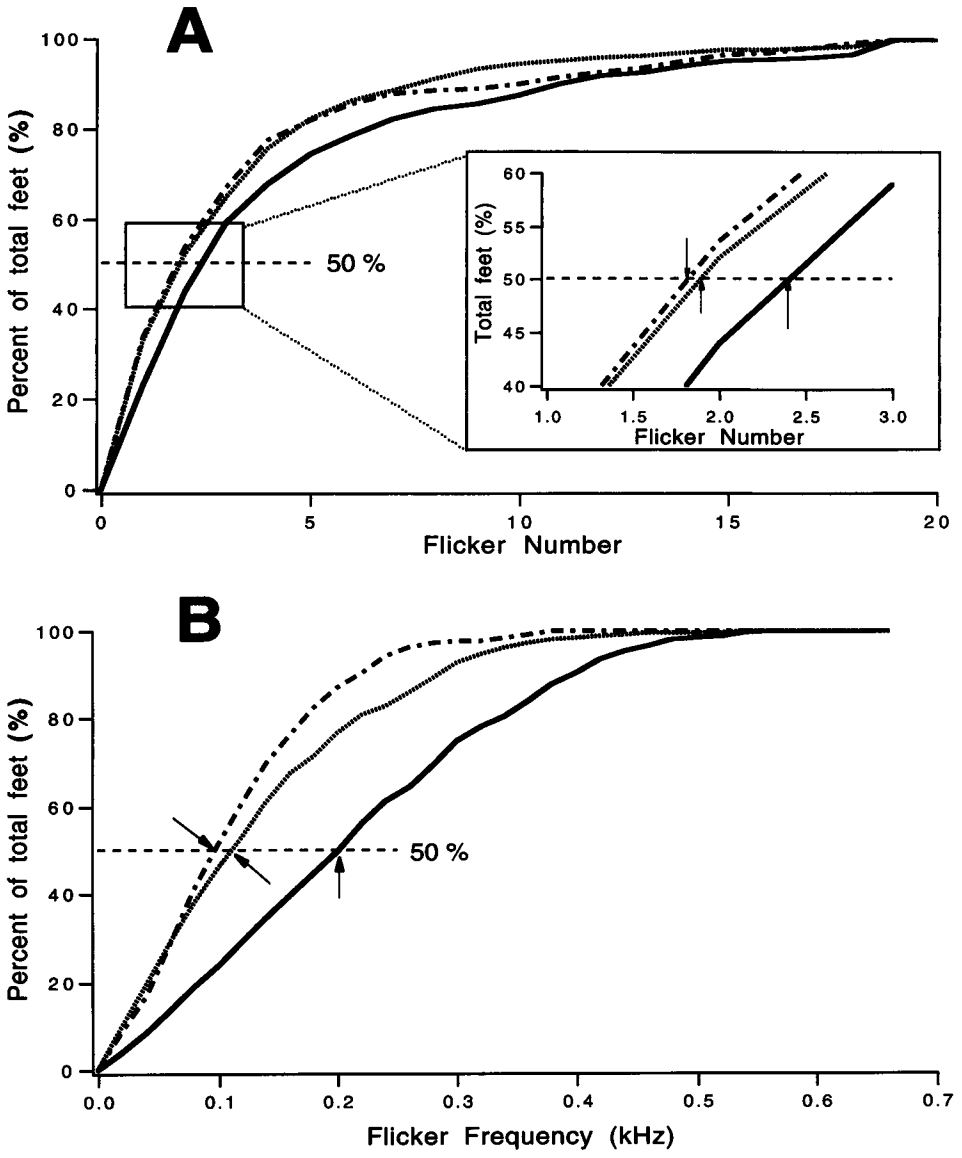

FIGURE 4 $[Ca^{2+}]_i$ dependence of foot flickers. (*A*) $[Ca^{2+}]_i$ dependence of the number of flickers per foot. The cumulative distribution functions (see Materials and Methods) showing the percentage of total feet with at most x flickers, PN(x). PN(2) = 40% means that 40% of feet have ≤2 flickers. (*B*) $[Ca^{2+}]_i$ dependence of the frequency of foot flickers.

Stand-alone foot signals

Although each foot signal examined above was followed by a large rapid spike (the "foot-spike" pattern), we also observed many foot-like events that were not followed by large spikes. In Fig. 5 *A*, such a "stand-alone" foot event is seen in the same record as a foot-spike event. That the flickers ($t_{1/2}$ = 0.5–2 ms) in the stand-alone foot were as fast as those in the foot-spike event suggests that the sites of transmitter release underlying both types of events were comparably close to the sensor surface. Fig. 5 *B* shows more examples of stand-alone feet.

Stand-alone feet were found in most cells. For quantitation, we counted as stand-alone foot events only those events with $N_f \geq 3$ (i.e., more than one flickery spike, because one spike may have N_f = 1 or 2; see Materials and Methods). This criterion was used because only when two or more fast flickery spikes are superimposed on a broad, low-amplitude signal can one distinguish that signal from an event due to vesicle fusion far from the carbon fiber sensor.

From 10 cells we collected 76 stand-alone feet and 253 foot-spike events. For the purpose of comparison, the latter events were selected with the same criterion (the foot signals had $N_f >= 3$), and in addition, the spike events had to have rise times of <5 ms and an amplitude of >100 pA. In total there were 784 foot-spike events with a rise time of <5 ms and an amplitude of >100 pA, of which about a third (253) had $N_f \geq 3$ (for the remaining 531 events, $N_f \leq 2$). In addition, for analysis we defined the foot of a stand-alone foot event as ending at the instant its last flicker began (this last flicker being considered as an aborted spike).

Fig. 6 compares characteristic features of the stand-alone feet (see histograms *A–D, left*) with those of foot-spike events (see histograms *right*), for events selected based on the criteria of a rise time of <5 ms and an amplitude of >3

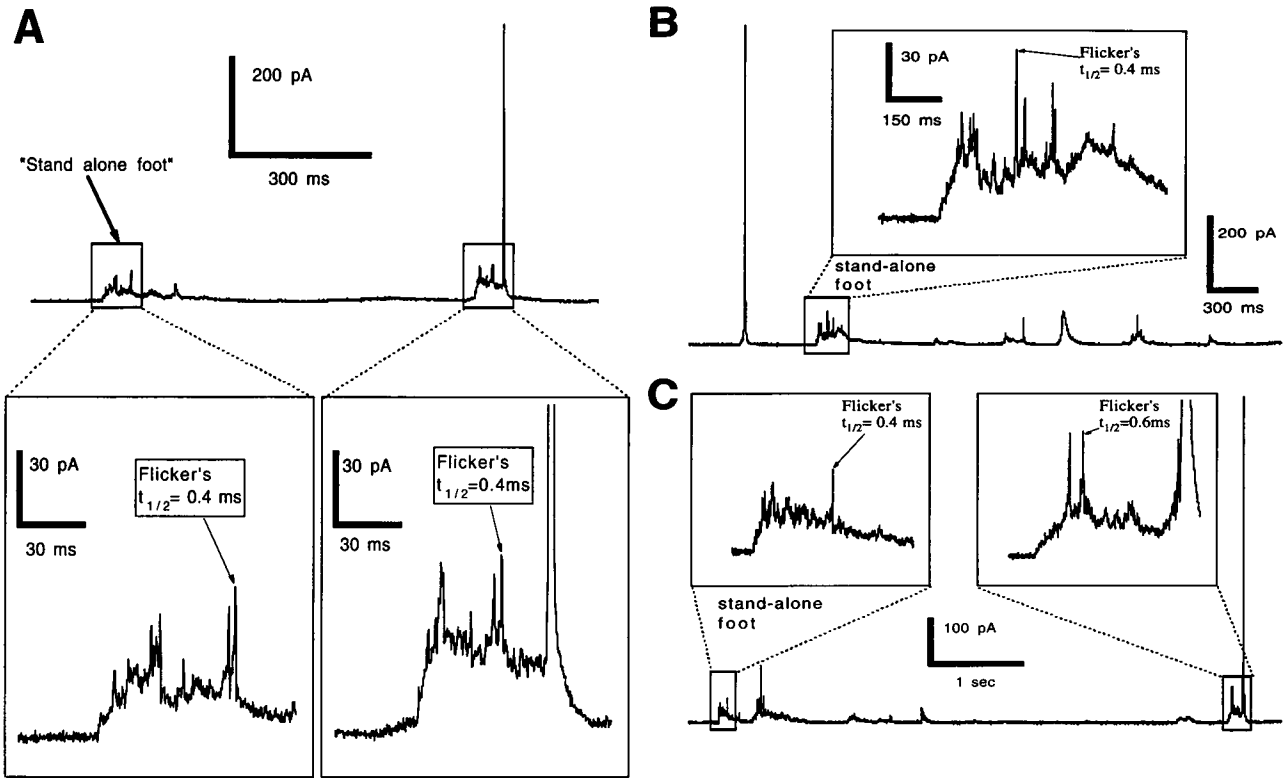

FIGURE 5 Evidence for stand-alone feet. (*A*) Typical stand-alone foot preceding the more usual foot-spike event. Comparison of both feet reveals that their kinetics (slow fluctuations and fast flickering) are similar but not identical. The rapid upstroke and brief duration of these flickers (half-height duration = 0.4 ms) suggests that the sites of CA release giving rise to them are close to the pCFE sensor (distance < 1 μm, according to diffusion calculations). (*B* and *C*) Other examples of stand-alone feet.

SD threshold. These histograms show that i) the average charge (1.51 pC) of stand-alone foot signals is 77% of the average charge (1.95 pC) of foot-spike signals; ii) the average foot duration for the stand-alone foot signals (97 ms) is nearly double that for foot-spike signals (52 ms); and iii) the average flicker frequency of the foot signals in stand-alone foot events (96 Hz) is about half that of the foot-spike signals (190 Hz). The significance of these observations will be discussed below.

DISCUSSION

Carbon fiber microelectrodes can detect quantal release of catecholamines from individual chromaffin cells (Wightman et al., 1991; Chow et al., 1992; Zhou and Misler, 1995a). In the present work, we have demonstrated that the dynamics of quantal release are more complex than previously observed.

Exquisite sensitivity of pCFE amperometry

As we have reported here, fast flickers can be resolved in the early exocytotic release time course for large (280 nm) dense-core vesicles in bovine adrenal chromaffin cells. The improved sensitivity and temporal resolution may be due to 1) enhanced precision of pCFE tip insulation and cutting (Zhou and Misler, 1995a); 2) use of a freshly cut pCFE tip for each new cell—which ensures a clean detecting surface (free of adherent secretory products that would create a diffusion barrier for transmitter) at the beginning of a recording; 3) touching the tip of the electrode to the cell membrane to minimize the diffusion distance from release site to detection surface; 4) using the amperometric mode, which allows measurements at the diffusion-limited rate (unlike fast cyclic voltammetry, which is limited by the frequency with which the waveform is applied); and 5) the 3 to 5 times lower capacitance of pCFE as compared to glass-insulated CFEs, which is reflected in lower noise (Chow et al., unpublished data; Neher and Chow, 1995). In fact, with this approach quantal release of much smaller, synaptic vesicles (50-nm diameter) can be detected in nerve terminals (Zhou and Misler, 1995b).

Flickers during single-vesicle transmitter release

The dynamic features of chromaffin cell exocytosis as seen in amperometric recordings may be related to the time course of formation and dilation of the fusion pore linking the chromaffin granule and the plasma membrane. The early fusion pore may resemble an ion channel in being partially

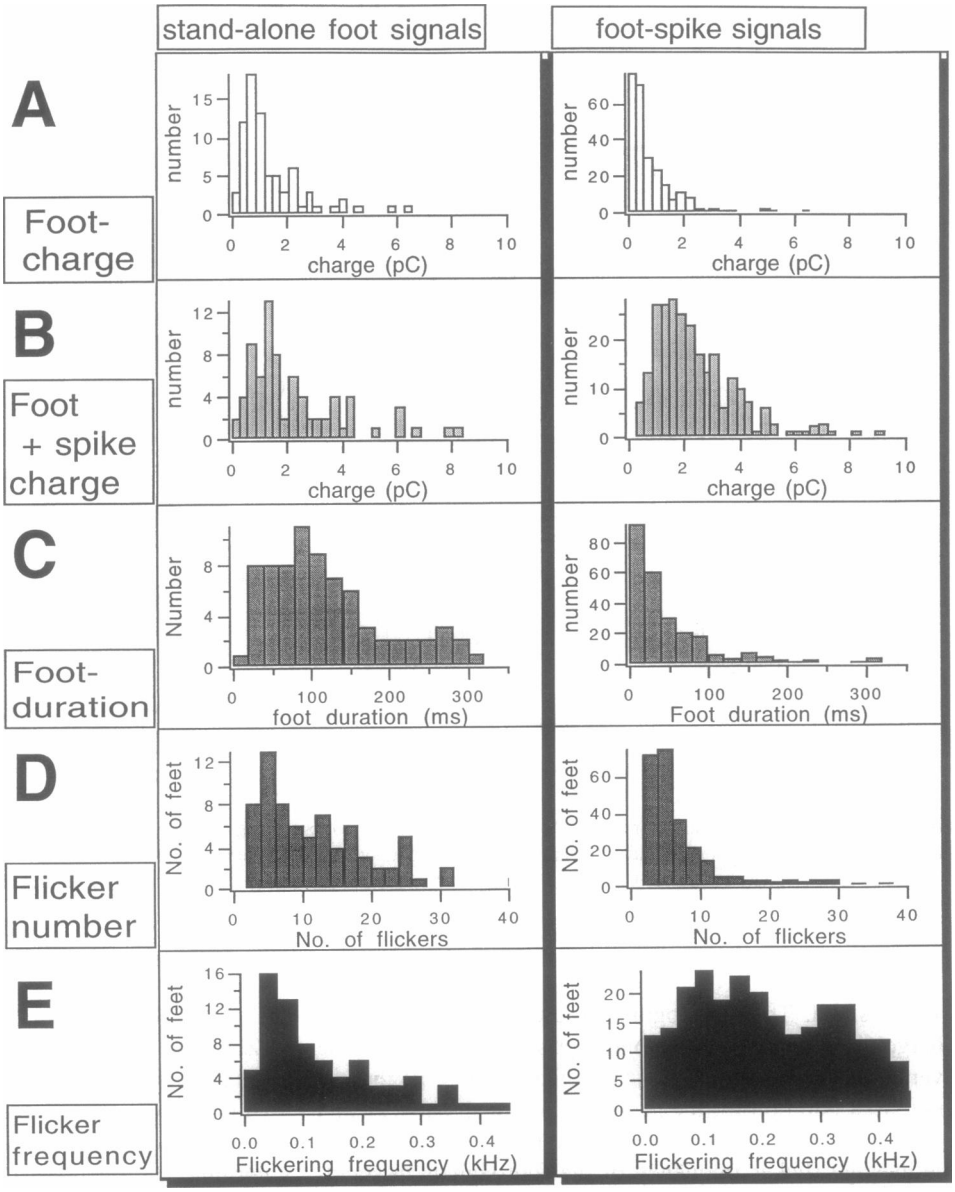

FIGURE 6 Statistics for stand-alone feet. The characteristics of stand-alone foot signals and the more usual foot-spike signals were compared by analyzing 76 stand-alone feet and 253 foot-spike events (each containing at least three flickers). All events were recorded from the same 10 cells (see *A–E*). See text for explanation.

lined by polypeptide chains (Almers and Tse, 1990), or it may be entirely lined with lipid but bracketed by a protein scaffold (Monck and Fernandez, 1994). Like an ion channel, the pore may "flicker" open and shut several times before it dilates and the vesicle membrane collapses into the plasma membrane.

In amperometric recordings, between 20% and 50% of the large spiking release events are preceded by a pedestal or foot, during which a significant fraction of the transmitter may be released. These feet often show complex fluctuations or flickers. Analyzed with a simple spike-detector algorithm, the flickers appear to occur with an average frequency of about 200 Hz and often last for less than 0.4 ms.

The individual amperometric current events represent release of free catecholamines into the extracellular space during the fusion of a granule containing a dense-core matrix with the plasma membrane. Fluctuations in the foot seen at the start of these amperometric events represent rapid changes in the release rate and probably reflect rate-limiting steps in the process of fusion pore dilation and/or granule matrix decondensation (Wightman et al., 1995). If release of CA from the matrix into a diffusable pool is very rapid, but fusion pore dilation is rate limiting, fluctuations in the conductance of the fusion pore would limit access of CA to the extracellular space. In this case, the flicker would be due to fluctuations in the diameter of the fusion pore and the subsequent spike due to final irreversible expansion of the fusion pore. The small amplitude of the foot would be a consequence of the limited conductance of the early fusion pore. At the other extreme, if stabilization of the nascent fusion pore were very rapid, but decondensation of the internal proteoglycan matrix of the chromaffin granule that

binds and/or traps CAs were the rate-limiting factor for release, onset of bursts of release from regions of the decondensing matrix in closest contact with extracellular solution could irregularly augment slower background release to produce a fluctuating foot signal. Subsequent rapid, autocatalytic dissociation of the rest of the matrix would produce the major spike of release.

It is very probable that both mechanisms contribute to rate-limiting aspects of exocytosis/degranulation. However, given the extremely short durations of individual flickers ($t_{1/2}$ = 0.4–2 ms), the high temporal correlation for mast cell granules between flickers in amperometric foot signals and in the fusion pore conductance (Alvarez de Toledo et al., 1993), and the lack of other data specifically addressing the detailed time course of release of neurotransmitters from a granule matrix, we favor the hypothesis that fluctuations in the structure of the fusion pore are the major determinant of the fast flickers during amperometric foot events.

The sensitivity of the foot flickers to alterations in ionic conditions or pharmacological agents may serve as a clue to the nature of the process underlying it. The observation that increasing $[Ca^{2+}]_i$ from 10 μM to >50 μM increases both the average number of flickers in a foot as well as their repetition frequency suggests that some features of the fusion process after the initiation of fusion are $[Ca^{2+}]_i$ dependent. In the simplest case, the Ca^{2+} site affecting the evolution of the fusion pore may be the same as that which triggers fusion. However, there are other possibilities. Persistently elevated Ca^{2+} is likely to have effects on a number of second messenger systems, any one of which may lead to phosphorylation or other covalent modification of relevant proteins, thereby changing the energetics by which conformational states are achieved and/or maintained. In addition, the continuous presence of elevated Ca^{2+} may influence the composition of the plasma and/or vesicle membranes, e.g., by activation of phospholipases or phospholipid transfer proteins. The membrane lipid composition in many cases has strong effects on membrane curvature and thereby on the energetics of fusion process (Zimmerberg et al., 1991).

Stand-alone foot signals

Up to 20% of release events appear to consist entirely of a "stand-alone foot." Because these events are broad and low in amplitude, they could be mistaken for the diffusionally smeared signals due to events arising far from the carbon fiber surface. However, one important characteristic of these events strongly argues against this interpretation and suggests instead that they are stand-alone foot events, arising by the same mechanism that leads to the normal foot signals: superimposed on the broad pedestal of current there are often small, fast spikes of current, with half-widths as short as 0.5 ms. Such fast signals must originate near the detector (Chow and von Rüden, 1995). Furthermore, as discussed above, similar rapid fluctuations are seen in the release pattern during the foot signals of the more typical foot-spike events.

One discernible difference between the stand-alone foot events and the foot signals that precede a large spike is that the stand-alone feet are significantly longer and have a lower flicker frequency. Assuming that the flickers represent transitions in pore conductance, the early fusion pore underlying the stand-alone foot might be more rigid (i.e., more stable and less able to dilate). The absence of a terminal release spike could be due either to premature and stable closure of the fusion pore ("kiss-and-run" exocytosis; Fesce et al., 1994) or to complete escape of the contents of the vesicle before full dilation of the fusion pore. If premature fusion pore closure were the explanation, one might expect the amount of transmitter released in a stand-alone foot to be less than that in a foot-spike event. In fact, the amount released is on average only about 20% less than that released during a foot-spike event. Further experiments will be necessary to distinguish between these possibilities.

Comparison with mast cells and neurons

Transmitter substances are packaged in secretory vesicles ranging from >1000-nm-diameter vesicles in mast cells to ~280-nm-diameter vesicles in chromaffin cells to ~50-nm-diameter vesicles in neurons. To date, it is not known how closely related the process of exocytosis in these three systems really is, in part because it has not been easy to obtain high-resolution measurements of single-vesicle fusion events with similar techniques. For example, in non-excitable mast cells, time-resolved dynamic features of single-vesicle fusion, observed repeatedly using capacitance measurements, have allowed modeling of the formation of a fusion pore. However, in excitable chromaffin cells and neurons, capacitance changes attributable to single vesicle release are too small to be resolved in standard whole-cell recording, although they can be seen in cell-attached patches (Neher and Marty, 1982).

In principle it should be possible to perform amperometry for vesicles across the range of sizes. Our improved resolution of catecholamine release from chromaffin cells permits some comparison with previously published data on serotonin release from mast cells. 1) In chromaffin cells, 20–50% of quantal release events are preceded by feet with complex fluctuations or flickers, as compared with <1% in mast cells (Spruce et al., 1990). 2) The average frequency of foot flickers in chromaffin cells is about 200 Hz (with the duration of some flickers lasting ≤0.4 ms), as compared with <40 Hz in mast cells (Alvarez de Toledo et al., 1993). 3) In chromaffin cells, more than 10% of the total charge transferred during an amperometric event occurs during a foot, and release during stand-alone feet is 77% that occurring during a conventional foot-spike amperometric event. In mast cells, release during a foot is <2% that of a total event (Alvarez de Toledo et al., 1993). These data show that the release of transmitter during the foot plays a more important role in catecholamine release from chromaffin cells than in serotonin release from mast cells. As ampero-

metric recordings are now possible from neurons (Zhou and Misler, 1995b), it will be possible to analyze the kinetics of synaptic vesicle fusion as well.

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