The Journal of Neuroscience, January 15, 1996, 16(2):553–562

Kinetics of Stimulus-Coupled Secretion in Dialyzed Bovine Chromaffin Cells in Response to Trains of Depolarizing Pulses

Elizabeth P. Seward and Martha C. Nowycky

Department of Anatomy and Neurobiology, Medical College of Pennsylvania and Hahnemann University, Philadelphia, Pennsylvania 19129

Stimulus-secretion coupling in bovine chromaffin cells was investigated with whole-cell patch-clamp recordings and capacitance detection techniques to monitor exocytosis in response to trains of depolarizing pulses. Two kinetically discrete modes of exocytotic responses were observed. In one mode, the first depolarization of a train elicited a large increase in membrane capacitance (C_m; mean ~70 fF). This secretory mode was characterized by small Ca^{2+} requirements, relative insensitivity to the pipette Ca^{2+} chelator concentration, and rapid depletion of the secretory response. This mode of stimulus-secretion coupling was labile and was seen only in response to the first and, occasionally, the second stimulus train of whole-cell recordings.

The second type of exocytotic response persisted for the remainder of the whole-cell recordings and consisted of two distinct phases. During the earliest pulses of a stimulus train, Ca^{2+} entry did not evoke C_m increases. Instead, C_m responses were elicited by later pulses, despite diminished Ca^{2+} entry per pulse caused by Ca^{2+} channel inactivation. The secretory phase was initiated after a specific "threshold" amount of Ca^{2+} had entered the cell, which was determined by the concentration, but not the binding kinetics, of the Ca^{2+} chelator in the pipette. In both the early and the secretory phases, the response of the cell was proportional to cumulative Ca^{2+} entry, regardless of current amplitude, pulse duration, or number of pulses.

Threshold-type secretory kinetics has been described previously in peptide-secreting neurohypophysial (NHP) nerve terminals (Seward et al., 1995). Secretory kinetics with minimal Ca^{2+} requirements has not been observed in that preparation. Chromaffin cells appear to possess a broader repertoire of stimulus-secretion coupling modes than NHP terminals.

Key words: calcium-secretion coupling; membrane-capacitance detection; exocytosis; large dense core-vesicles (LDCV); calcium chelators (EGTA, BAPTA); chromaffin cells

Ca^{2+}-secretion coupling in adrenal chromaffin cells is a complex cellular process that involves Ca^{2+}-dependent preparatory and fusion steps and an extensive array of modulatory phenomena involving both second-messenger systems (Burgoyne and Morgan, 1993) and cytoskeletal elements (Cheek and Barry, 1993; Trifaro et al., 1993). The physiological stimulus for catecholamine secretion is acetylcholine release from the splanchnic nerve, which triggers bursts of action potential (AP) activity (Brandt et al., 1976; Ozawa and Sand, 1986; Zhou and Misler, 1995). As voltage-gated Ca^{2+} channels are activated by each AP within a burst, bursts cause complex pulsatile Ca^{2+} elevations near the membrane as well as a slower global summation (Thayer and Miller, 1990; Yamada and Zucker, 1992; Nowycky and Pinter, 1993; Peng and Zucker, 1993; Stuenkel, 1994).

To avoid the complexities and uncertainties inherent in pulsatile Ca^{2+} entry, two groups have studied the kinetics of Ca^{2+}-stimulated exocytosis of large dense-cored vesicles (LDCVs) in response to flash photolysis of caged Ca^{2+} compounds (Heinemann et al., 1993, 1994; Neher and Zucker, 1993; Thomas et al., 1993). In both chromaffin cells and melanotrophs, flash photolysis of demeclocycline (DM)-nitrophen abruptly elevates intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) into the micromolar to hundreds of micromolar range and elicits membrane capacitance (C_m) changes (ΔC_m) that differ in speed and amplitude. Based on the distinct kinetic components of the C_m response, both groups have produced models of Ca^{2+} secretion-coupling that are similar enough to be summarized together. The following are the essential features. (1) At rest, LDCVs exist in sets of functionally distinct "pools" or "states" in a cell. (2) Movement between pools is modeled as a series of simple chemical reactions. (3) The pools are arranged sequentially, leading from least prepared to most prepared and ready for exocytosis. (4) Movement between pools is reversible with the exception of the final exocytotic step. (5) The step(s) leading from the final resting state to final fusion has a third- or fourth-order dependence on $[Ca^{2+}]_i$.

Although the models are attractive in their simplicity, the flash photolysis experiments on which they are based have certain limitations. Among the most important of these limitations are the nonphysiologically high and prolonged Ca^{2+} elevations that are produced as well as cell dialysis in the absence of Mg-ATP. Two studies of rat chromaffin cells using capacitance (Horrigan and Bookman, 1994) and amperometric techniques (Zhou and Misler, 1995) that examined the effect of voltage-gated Ca^{2+} entry have reported exocytotic components that are not described by the above models.

In a previous report, we used stimulus-train protocols that mimic AP bursts to examine the coupling of LDCV release to

Received July 14, 1995; revised Sept. 15, 1995; accepted Oct. 2, 1995.

This work was supported by Grant NS 22281 from the National Institute of Neurological and Communicative Disorders and Stroke (M.C.N.) and a Medical Research Council Fellowship (E.P.S.). We thank Drs. Natalya Chernevskaya, Kathrin Engisch, Alla Fomina, and Patrice Mollard for critical comments on this manuscript and for many discussions throughout the project.

Correspondence should be addressed to Dr. Martha C. Nowycky, Department of Anatomy and Neurobiology, Medical College of Pennsylvania, 3200 Henry Avenue, Philadelphia, PA 19129.

Dr. Seward's present address: Glaxo Institute for Molecular Biology, 14 Champs des Aulx, 1228 Plan-les-Ouates, Geneva, Switzerland.

pulsatile, voltage-gated Ca^{2+} entry in peptide-secreting neurohypophysial (NHP) terminals. We found that exocytosis exhibits a kinetic pattern, which we called "threshold" secretion (Seward et al., 1995). In this secretory mode, Ca^{2+} entry during initial depolarizations does not elicit secretion until a certain critical amount of summed Ca^{2+} entry occurs. Subsequent Ca^{2+} entry is responsible for the final steps leading to fusion and exocytosis. In this study, we perform similar experimental manipulations in bovine chromaffin cells. We find that chromaffin cells also respond with threshold-type secretion but have an additional kinetically distinct response that differs in its Ca^{2+} dependence.

Some of this work has been presented previously in abstract form (Seward and Nowycky, 1995).

MATERIALS AND METHODS

Chromaffin cell culture

Adult bovine chromaffin cells were prepared as described in Vitale et al. (1991). After digestion and purification on a Percoll gradient, cells were plated in 10% fetal calf serum and 90% Dulbecco's modified Eagle's medium on collagen-coated coverslips at a density of 1×10^5 cells/35 mm Petri dish. Most of the recordings were made between 36 and 72 hr after plating. The exceptions are recordings portrayed in Figure 6, which were carried out between 3 and 7 d after plating.

Recording conditions

Solutions. Intracellular solution was as follows (in mM): Cs-glutamate 145, Cs-[4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid] (HEPES) 10, NaCl 9.5, bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetra-acetic acid (BAPTA) 0.3, and Mg-ATP 2, pH 7.2, unless otherwise stated. External solution was as follows (in mM): NaCl 130, KCl 2, glucose 10, HEPES 10, $MgCl_2$ 1, $CaCl_2$ 5, and tetrodotoxin 0.002, pH 7.2.

Capacitance detection. Whole-cell Ca^{2+} channel currents and capacitance measurements were performed with a List EPC-7 as described previously (Lim et al., 1990) using a computer-based, phase-tracking method (Neher and Marty, 1982; Joshi and Fernandez, 1988; Fidler and Fernandez, 1989). Details are described in Mollard et al. (1995) and Seward et al. (1995). To check for possible contamination of C_m traces by Na^+ channel-gating charges (Horrigan and Bookman, 1995), control experiments were performed in 0 extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) or in the presence of 100 μM cadmium to block Ca^{2+} entry. Bovine chromaffin cells have much smaller Na^+ currents than rat chromaffin cells, and gating charge movements do not contribute to the C_m responses. Experiments were performed at room temperature (24–27°C).

$[Ca^{2+}]_i$ measurements. Fura-red (0.3 mM, Molecular Probes, Eugene, OR) was used to monitor changes in cytosolic Ca^{2+} as described in Mollard et al. (1995).

RESULTS

Ca^{2+}-stimulated exocytosis was monitored with the C_m detection technique in whole-cell recordings of cultured bovine chromaffin cells. Cells were stimulated with trains of short depolarizing pulses that mimic physiological bursts of APs (Brandt et al., 1976; Ozawa and Sand, 1986; Zhou and Misler, 1995). Two kinetically distinct patterns of C_m responses were observed.

Two kinetically distinct patterns of ΔC_m in single dialyzed cells

Figure 1*A* illustrates a typical response to the first stimulus train after establishing the whole-cell configuration ("break-in"), and Figure 1*B* illustrates the response to the third stimulus train. The timing of individual pulses is indicated by gaps in the C_m and conductance (G) traces. In Figure 1*Ai*, a large C_m jump (~92 fF) occurs during the first depolarizing pulse. This is followed by five progressively diminishing large jumps (Fig. 1*Aiii*), for a total ΔC_m of ~310 fF. The responses to the remaining 14 depolarizing pulses consist of smaller, uniform steps for which the average amplitude is 12 fF. The C_m trace elicited by the third train (Fig. 1*Bi*) does not have large jumps at the beginning of the train; instead, the average amplitude of the small steps for the first 6 pulses actually is lower than that for the last 14 pulses (7.7 vs 14.9 fF; Fig. 1*Biii*).

Individual jump amplitudes per pulse are plotted in Figure 1, *Aiii* and *Biii*, as a function of cumulative Ca^{2+} entry. This value is obtained by integrating the Ca^{2+} current evoked by each depolarizing pulse and then summing with previous Ca^{2+} entry during the stimulus train. The large C_m jumps during the first train (Fig. 1*Aiii*) occur below 10×10^7 total Ca^{2+} ions in this example. During the third train (Fig. 1*Biii*), the larger average jumps begin after 10×10^7 Ca^{2+} ions have entered the cell.

We will refer to the large C_m jumps evoked by small amounts of Ca^{2+} entry as "docked" secretion, analogous with fast synapses and rapid responses in both melanotrophs and chromaffin cells (Neher and Zucker, 1993; Thomas et al., 1993); we will refer to the second type of exocytotic response as threshold secretion, analogous with similar responses in NHP nerve terminals (Seward et al., 1995). The remainder of the paper describes differences between the two secretory modes in terms of time-dependent run-down, Ca^{2+} requirement, and effect of Ca^{2+} chelators.

Disappearance of docked secretion and run-down of threshold secretion during cell dialysis

In dialyzed bovine chromaffin cells, the characteristic pattern of large C_m jumps during the beginning of the train (first 6 pulses of Fig. 1*A*) is seen only during the first and sometimes second stimulus trains after break-in, whereas the pattern illustrated in Figure 1*B* is obtained repeatedly during the whole-cell recording. Typically, the first stimulus train was delivered within ~3 min of establishing the whole-cell configuration, i.e., after break-in and initiation of cell dialysis. Exchange of small molecules such as ethylene glycol bis(β-aminoethyl)ether-*N,N,N′,N′*-tetra-acetic acid (EGTA) and BAPTA is calculated and measured to be complete within 20 sec (Pusch and Neher, 1988; Neher and Augustine, 1992; Thomas et al., 1993). Thus, the presence of large C_m jumps in response to small amounts of Ca^{2+} influx is not caused by insufficient diffusion of the exogenous Ca^{2+} chelators. This is supported further by the presence of both docked and "not-docked" kinetic responses within a single trace (Fig. 1*A*).

In several experiments, the first depolarizing train was delivered as much as 10 min after break-in; large-amplitude C_m jumps still were obtained. In Figure 2, the ΔC_m response per pulse is plotted for the first and second stimulus trains, which were given at 10 min (Fig. 2*A*) and 13 min (Fig. 2*B*) after establishing whole-cell recording. Large-amplitude jumps in response to small amounts of Ca^{2+} entry still are seen during the first train, but not in response to the second train. It appears that within the time frame of ~10 min of whole-cell dialysis, docked vesicles do not "undock." However, the ability to replenish this secretory mode or vesicular pool is lost within a few minutes of whole-cell dialysis.

In contrast to the rapid disappearance of the docked secretory pattern in whole-cell recordings, the second kinetically distinct secretory pattern was relatively stable for prolonged recording and perfusion periods. Figure 3 illustrates a method for estimating the decline of secretory robustness over time. The amount of total $\Delta C_m/\Sigma Ca^{2+}$ for each train was measured in five cells that were stimulated every 3 min for up to 1 hr. These values were normalized to either the second or the third stimulating train, whichever train was the first that did not exhibit docked secretion. Secretory robustness declined monoexponentially with a time constant of 26 min.

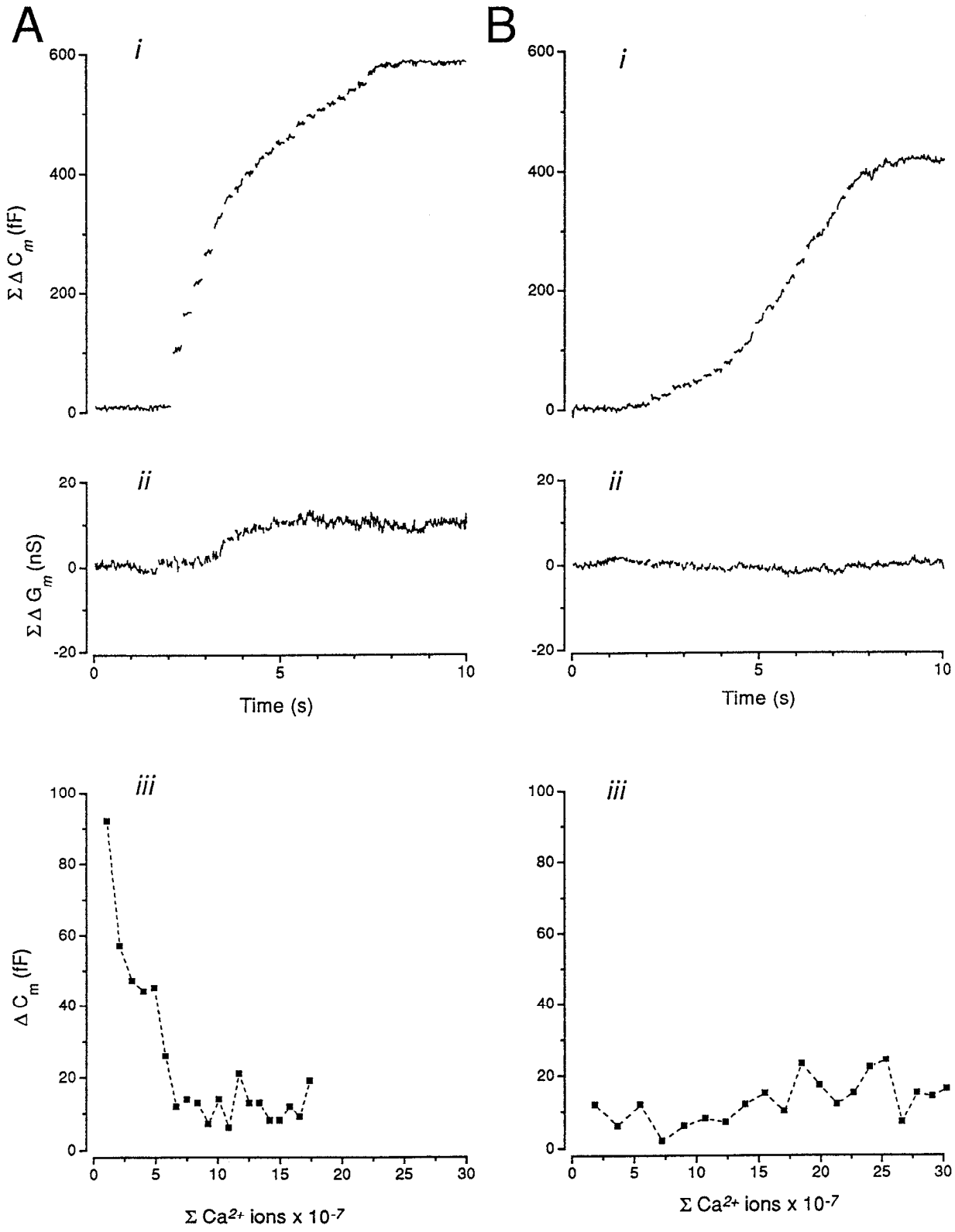

Figure 1. Two kinetically distinct secretory patterns are observed in dialyzed chromaffin cells. *A*, *B*, Cumulative changes in whole-cell C_m (*i*) and *G* (*ii*). Data from a single chromaffin cell illustrating typical patterns of C_m increases evoked by the first (*A*) and third (*B*) train of depolarizing pulses after break-in. Stimulus trains consisted of 20 depolarizations to +10 mV at 4 Hz. Gaps in the traces indicate timing of depolarizing pulses during which capacitance detection is interrupted. The first stimulus train (*A*) was given 2 min after establishing the whole-cell recording; pulse duration was 10 msec. The third stimulus train (*B*) was given 6 min later; pulse duration was 6 msec. Pulse duration was decreased to compensate for the increase in Ca^{2+} current amplitude (run-up) that occurred in most cells. Plots of C_m jump amplitudes during each pulse as a function of cumulative Ca^{2+} influx (*iii*). Cumulative Ca^{2+} influx is determined by integrating Ca^{2+} current and summing it to previous Ca^{2+} entry. Cell B0124; 0.1 mM $[BAPTA]_i$.

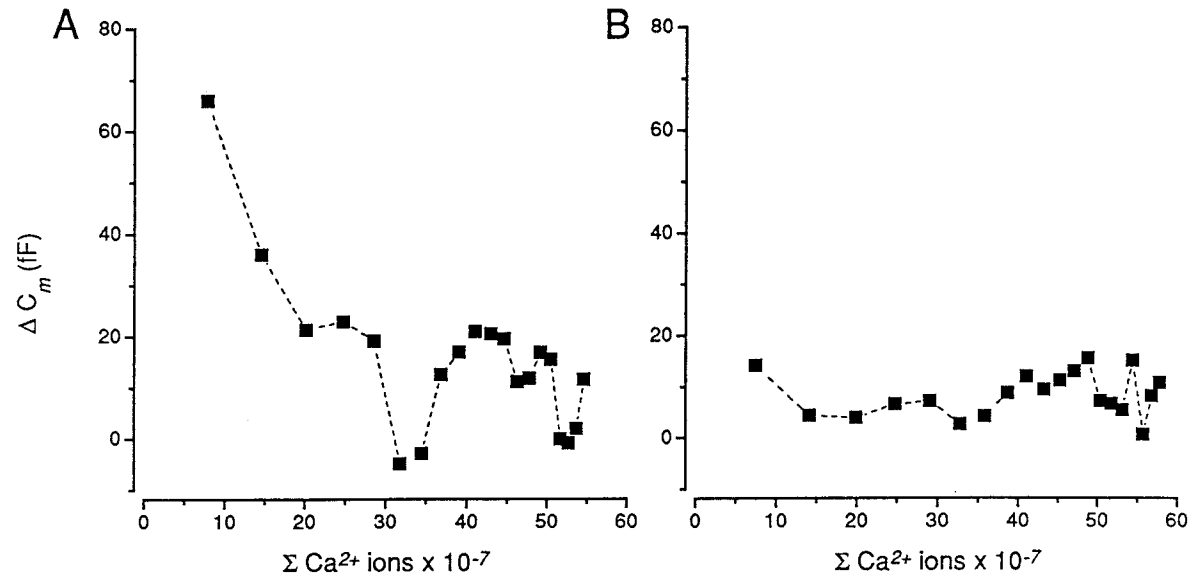

Figure 2. Docked secretory kinetics is observed during the first stimulus train even after prolonged cell dialysis. *A*, *B*, Plots of individual C_m jump amplitudes versus cumulative Ca^{2+} entry as in Figure 1. *A*, The first stimulus train was given ~10 min after establishing the whole-cell configuration. Prominent, large C_m jumps are elicited by the first five depolarizations. *B*, The second stimulus train was given 3 min later, and all small C_m responses are <20 fF. Cell B0585; 0.5 mM $[BAPTA]_i$.

Ca^{2+} dependence of docked versus threshold secretion

Docked secretion

Because the large-amplitude C_m jumps elicited at the beginning of a train were seen only during the first and/or second stimulus train of whole-cell recording experiments, we compared the Ca^{2+} requirements of the C_m response to the first depolarizing pulse (C_{m1}) of the first and third trains of a population of cells. Amplitude histograms for C_{m1} jumps elicited by the first (Fig. 4*A*) and third (Fig. 4*B*) trains are shown for 37 cells dialyzed with 0.3 mM BAPTA. The average C_{m1} of the first train was 69 ± 9 fF (mean ± SEM), with 61% of responses >50 fF. The average C_{m1} of the third train for the same cells is 23 ± 3 fF, with only 8% of responses >50 fF.

The relationship between C_{m1} jump amplitude and amount of Ca^{2+} entry during the first depolarizing pulse is illustrated in Figure 4*C*. C_{m1} amplitudes for the first (*solid squares*) and third (*open circles*) trains are plotted against the summed Ca^{2+} entry for each first pulse. Data are binned as indicated in the figure legend. During the first stimulating train, large C_{m1} jumps averaging ~70 fF are elicited by $<5 \times 10^7$ Ca^{2+} ions and pulse durations as short as 6 msec. Pulses with larger amounts of Ca^{2+} entry produced similar C_{m1} amplitudes, suggesting that very small amounts of Ca^{2+} are sufficient to elicit maximal C_{m1} responses for a single depolarizing pulse. During the third train, the same number of Ca^{2+} ions elicited maximal C_{m1} responses of ~20 fF amplitude. The much larger vertical error bars for C_{m1} responses during the first train reflect the presence of both small and large amplitude jumps as seen in Figure 4*A*, whereas the tighter error bars of the C_{m1} responses during the third train are attributable to the absence of large jumps (Fig. 4*B*).

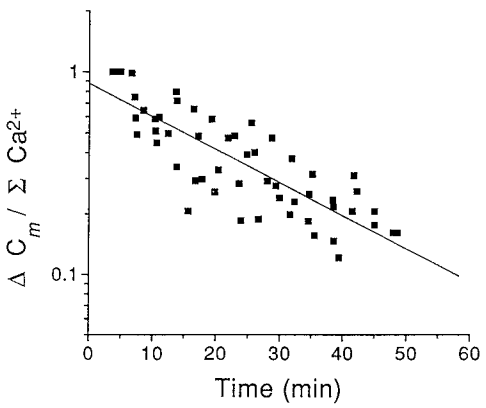

Figure 3. Threshold secretion runs down slowly during whole-cell dialysis. Pooled data from five whole-cell recordings normalized to the second or third stimulus train of the recording; total of 56 depolarizing trains. *Line* is drawn through the data and fit by linear regression ($r = 0.84$) for $\tau_{run\text{-}down}$ = 26.45 min. All cells recorded with 0.3 mM $[BAPTA]_i$.

A characteristic feature of the docked secretory mode is that the first pulse of a train almost always elicited the largest C_m jump. The next several pulses of a stimulus train elicited progressively smaller responses, as seen in Figures 1*A* and 2*A*, giving the appearance of a limited vesicular pool that could be released by this secretory mode. The total amount of docked secretion in a single cell usually consisted of 200–500 fF; small C_m jumps seen later in the train (Figs. 1*Ai*, 2*A*) probably reflect the activation of threshold secretion.

Threshold secretion

During threshold secretion, the earliest pulses of a train elicit little or no C_m increases and, if pulse durations are brief, C_m jumps late in the train are small and relatively uniform. Typical examples are seen in Figures 1*B* and 2*B*.

The relationship between ΔC_m changes and Ca^{2+} entry during threshold secretory kinetics is illustrated in the experiment shown in Figure 5. The cell was stimulated with three stimulus trains that were identical except for pulse durations. The first and last Ca^{2+} current records for each stimulus train are shown superimposed in Figure 5*i* for pulse durations of 10, 20, and 40 msec (*A*, *B*, and *C*, respectively). Corresponding C_m traces are illustrated in Figure 5*Aii–Cii*. The train with the shortest pulse durations elicited the

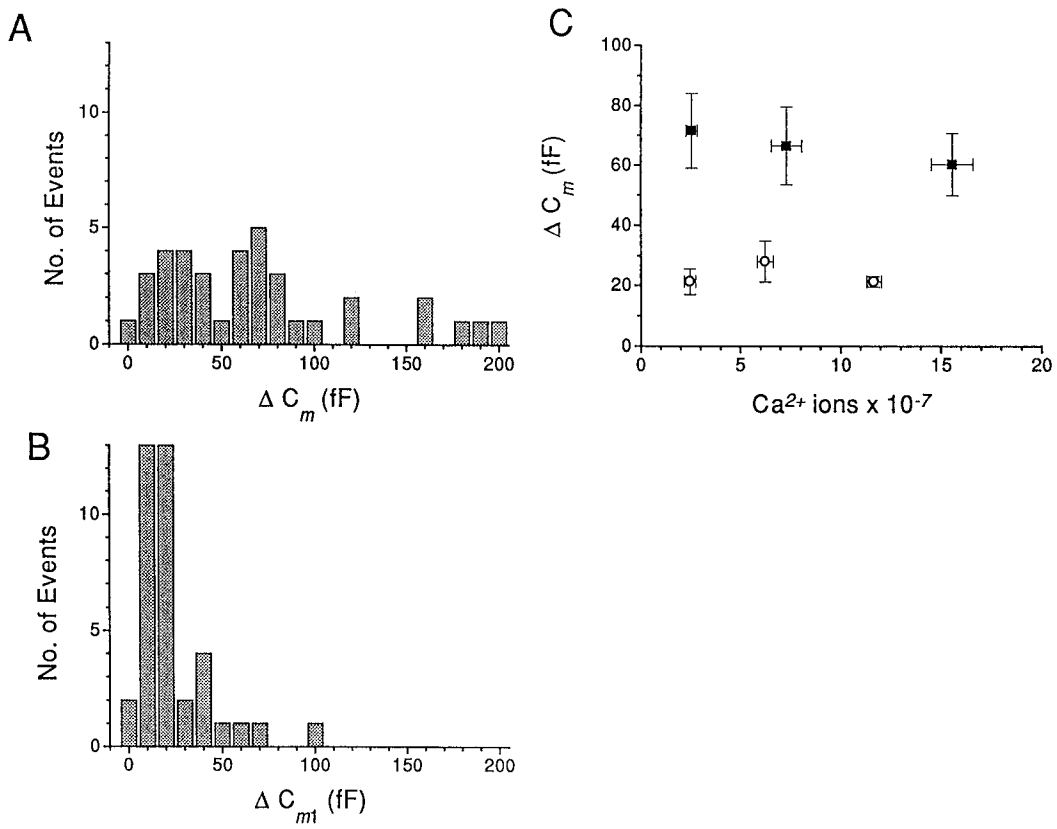

Figure 4. Docked secretory kinetics is lost very quickly during whole-cell dialysis. *A*, *B*, Frequency histogram of the amplitude of the first C_m jump (C_{m1}) evoked by the first stimulus of the first (*A*) and third (*B*) depolarizing train after breaking into the cell. Data are from 37 chromaffin cells stimulated with depolarizations to +10 mV, with a mean duration of 19 ± 1 msec (range 6–40 msec). In *A*, C_m jump amplitudes are distributed fairly evenly, with more than half >50 fF. In *B*, only three cells had C_m jumps >50 fF. *C*, Integrated Ca^{2+} entry for the depolarizing pulses shown in *A* (*solid squares*) and *B* (*open circles*). Data are binned for Ca^{2+} values of 0–5 × 10^7 ions, 5.5–10 × 10^7 ions, and >10.5 × 10^7 ions. All cells recorded with 0.3 mM $[BAPTA]_i$.

least total secretion (Fig. 5*Aii*), whereas the trains of 20 and 40 msec durations gave similar total C_m responses. It is evident in Figure 5*Aii–Cii* that C_m increases begin at different times in the train (*second arrow*). Shorter pulse durations result in a greater number of pulses that fail to elicit detectable C_m increases. Thus, for the 10 msec pulse train there is no response until about the 14th or 15th stimulus pulse, or ~3.5 sec after the first depolarizing stimulus, whereas for the 40 msec pulse train the response begins on the 5th or 6th stimulus pulse, or at ~1.5 sec.

C_m changes elicited by trains with different stimulus parameters are consistently related to cumulative Ca^{2+} entry as shown in Figure 5*iii*. It is apparent by comparing the three plots that the effect of Ca^{2+} entry during a stimulus train can be divided into two discrete phases. During each train, C_m increases do not begin until after a critical amount of summed Ca^{2+} entry, which we call threshold. The lines drawn through the cumulative C_m points during the second secretory phase are fit by linear regression, and the threshold value is taken as the intersection with the ordinate. In this experiment with 0.3 mM BAPTA, the threshold value is ~20 × 10^7 Ca^{2+} ions.

Threshold secretory kinetics consists of two Ca^{2+}-sensitive phases even when the amount of Ca^{2+} entry during a single pulse is very large. Figure 6*A* is a plot of cumulative C_m increases versus cumulative Ca^{2+} entry from an experiment with 0.5 mM EGTA in the pipette in which the duration of depolarizing pulses ranged between 46 and 393 msec. During each train, C_m increases did not begin until ~55 × 10^7 Ca^{2+} ions had entered, even though the number of ions during the first pulse varied from ~6.7 to 40.2 × 10^7 for the shortest and longest pulse durations, respectively. Also, during the secretory phase C_m increases are related to the amount of cumulative Ca^{2+} entry. For longer pulse durations, e.g., >72 msec, each depolarization elicits a relatively large amplitude C_m jump that is proportional to the amount of Ca^{2+} entry during that pulse.

The relationship between cumulative C_m increase and cumulative Ca^{2+} entry during threshold secretory kinetics also is observed when Ca^{2+} influx per pulse is varied by protocols other than changing pulse durations. Figure 6*B* contains data from a cell in which $[Ca^{2+}]_o$ was increased from 5 to 10 mM. The pulse protocols were identical except for the number of pulses (10 pulses in 10 mM $[Ca^{2+}]_o$, 20 pulses in 5 mM $[Ca^{2+}]_o$) that were required to reach approximately the same amount of total Ca^{2+} entry. Under these conditions, the Ca^{2+} flux per pulse doubles at the higher $[Ca^{2+}]_o$. Despite the differences in train duration (2.5 vs 5 sec) and Ca^{2+} flux per pulse, the Ca^{2+}-ΔC_m dependence of the two traces coincides almost exactly. Thus, Ca^{2+}-secretion coupling during threshold secretion is a function of cumulative Ca^{2+} entry rather than of any specific parameter of Ca^{2+} current amplitude, current duration, or the time or number of pulses required to reach a certain value of total Ca^{2+}.

Effects of Ca^{2+} chelators on docked versus threshold secretion

In NHP terminals, low concentrations of Ca^{2+} chelators strongly influenced the secretory response by controlling the amount of Ca^{2+} entry necessary to reach threshold. We tested the effect of two chelators, EGTA and BAPTA, on the threshold secretory response, and we tested two concentrations of BAPTA on the docked secretory response.

Thresholds were estimated from the *x*-axis intercept of a line fitted by linear regression to the secretory phase in plots of cumulative C_m changes versus cumulative Ca^{2+}, as illustrated in Figure 5. The effect on the threshold values for three concentrations of EGTA (0.2, 0.5, and 1.0 mM) and BAPTA (0.1, 0.3, and

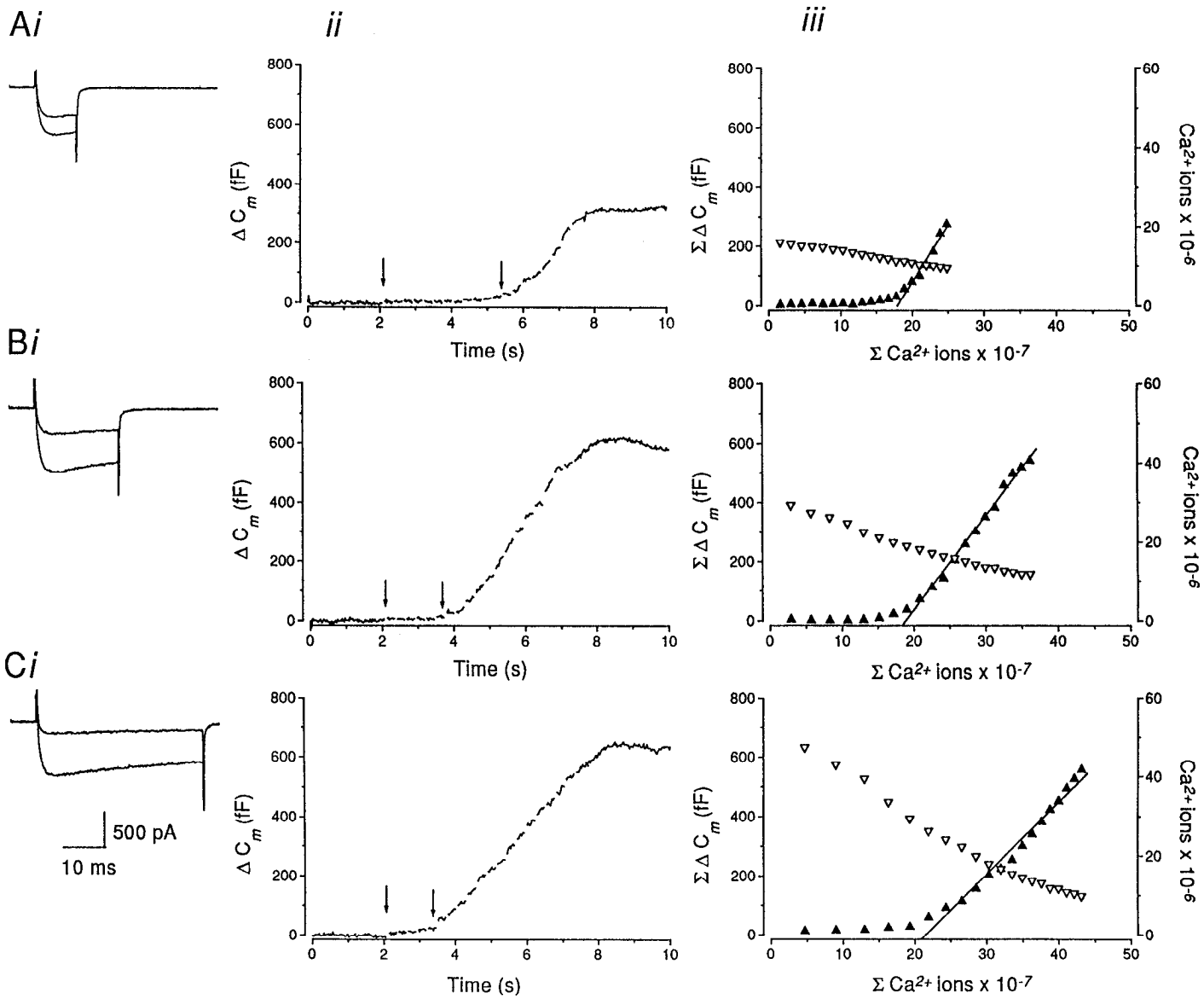

Figure 5. Stimulus trains with different pulse durations trigger C_m increases after similar amounts of cumulative entry. *A–C*, Data from a single chromaffin cell stimulated with three trains of 20 pulses with pulse durations of 10 msec (*A*), 20 msec (*B*), and 40 msec (*C*) (+20 mV, 4 Hz). *i*, The 1st and 20th Ca^{2+} current traces of each train are superimposed to illustrate the amount of channel inactivation. *ii*, Capacitance traces. The *first arrow* (from *left*) marks the first depolarizing pulse as a reference point. The *second arrow* indicates the transition between the threshold and secretory phases. *iii*, Plots of cumulative C_m increases (*solid triangles*) and integrated Ca^{2+} entry per pulse (*open inverted triangles*) versus cumulative Ca^{2+} entry. During the secretory phase, data are fit by linear regression (*line*), and the threshold Ca^{2+} influx required to initiate exocytosis is determined by extrapolation of the fitted line to the *x*-axis. For the *top trace*, the fitted line has a slope of 3.8 fF/10^6 Ca^{2+} ions and a threshold of 18 × 10^7 Ca^{2+} ions (r = 0.98). For 20 msec pulse durations, the slope is 3.2 fF/10^6 Ca^{2+} ions and the threshold is 18 × 10^7 Ca^{2+} ions (r = 1); for 40 msec pulse durations, the slope is 2.4 fF/10^6 Ca^{2+} ions and the threshold is 21 × 10^7 Ca^{2+} ions (r = 0.99). Cell B0313; 0.3 mM $[BAPTA]_i$.

0.5 mM) is summarized in Figure 7*A*. The results presented in the graph were obtained over a broad range of stimulus paradigms, including various pulse durations and current amplitudes. As in NHP terminals, low concentrations of buffers increased the threshold value for Ca^{2+} entry. EGTA, a chelator with slow binding kinetics, and BAPTA, a chelator with much faster binding kinetics, had similar effects on threshold.

We compared the effects of two concentrations of BAPTA on docked secretion. The data in Figure 7*B* represent the average jump amplitude for C_{m1} of the first stimulus train in two groups of cells dialyzed with 0.1 or 0.3 mM BAPTA. Data for 0.3 mM BAPTA are from the population of cells shown in Figure 4*A*. Lowering the BAPTA concentration by one-third had no effect on the average C_{m1} jump amplitude. The inset in Figure 7*A* illustrates the differences between Ca^{2+} requirements for eliciting 70 fF of docked secretion versus initiating the secretory phase in threshold secretion. The values for docked secretion represent the average Ca^{2+} ions required to elicit C_{m1} in 0.1 mM BAPTA (n = 15) as well as the average of the first binned subset from Figure 4*C* (n = 25). Clearly, large jumps during docked secretion occur in response to much less Ca^{2+} entry than is required for reaching threshold values. The relatively weak sensitivity of docked vesicles to low concentrations of BAPTA is reminiscent of the insensitivity of fast synaptic transmission to Ca^{2+} chelators and indicates that the vesicles are in relatively close vicinity to Ca^{2+} channels.

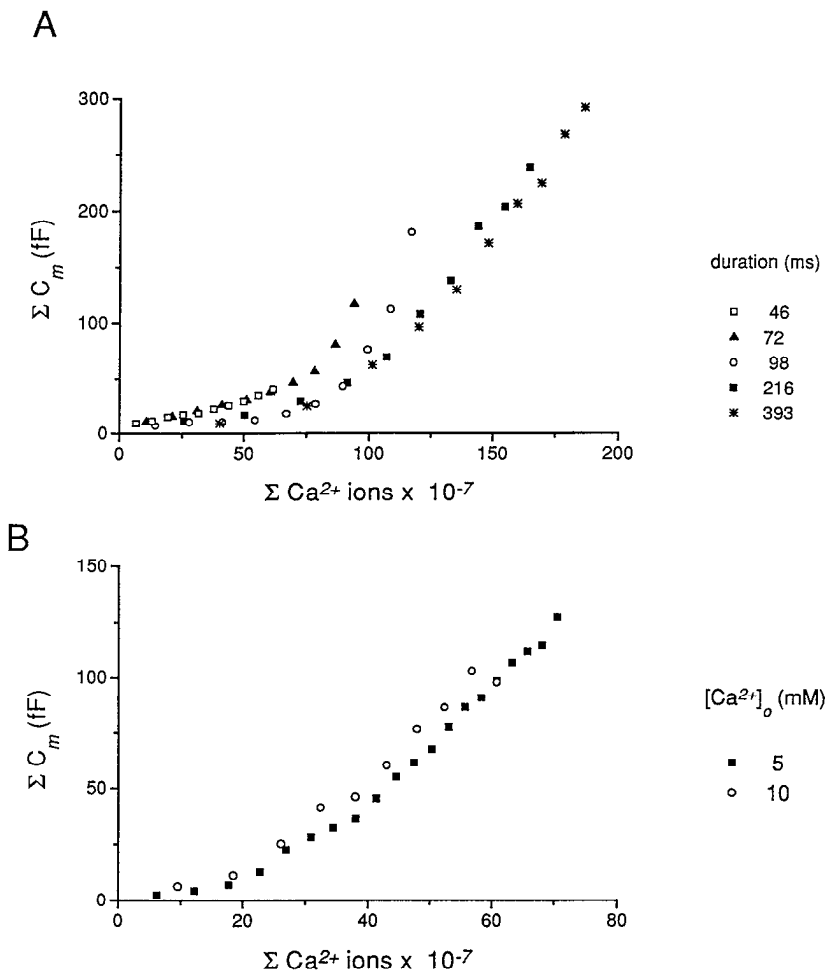

Figure 6. Threshold secretory kinetics is observed when Ca^{2+} entry is varied by pulse duration or changes in Ca^{2+} flux. Plots of cumulative ΔC_m versus cumulative Ca^{2+} influx. *A*, Pulse duration is varied between 46 and 393 msec. At the shortest duration (46 msec) (*open triangles*), total Ca^{2+} entry does not reach threshold. At the longest pulse duration (393 msec) (*asterisks*), the first two depolarizations produce almost no secretion. Once threshold is exceeded (estimated as $\sim 55 \times 10^7$ ions), ΔC_m increase is nearly a linear function of further Ca^{2+} entry. Cell A05G; 0.5 mM $[EGTA]_i$. *B*, In another cell, the rate of Ca^{2+} entry is doubled by increasing $[Ca^{2+}]_o$ from 5 to 10 mM. Ten depolarizations in 10 mM $[Ca^{2+}]_o$ elicit nearly identical total C_m changes to 20 depolarizations in 5 mM $[Ca^{2+}]_o$. The cumulative C_m versus cumulative Ca^{2+} plots superimpose with a threshold value of $\sim 20 \times 10^7$ Ca^{2+} ions. Cell A09C; 0.2 mM $[EGTA]_i$.

To determine whether the threshold was the point at which the Ca^{2+} chelator was saturated, we monitored averaged cytosolic Ca^{2+} levels by including 0.2 mM Fura-red in the presence of 10 mM EGTA. Fura-red is chemically derived from BAPTA and has faster binding kinetics than EGTA but similar Ca^{2+} affinity as EGTA. During trains of pulses lasting several seconds, Ca^{2+} first binds to Fura-red but then quickly equilibrates with the much larger capacity provided by the EGTA. A recording of the C_m trace (Fig. 8*A*) is compared with the fluorescent signal of the Ca^{2+} dye (Fig. 8*B*). C_m increases begin well below the maximum Ca^{2+} signal, indicating that exogenous Ca^{2+} chelators are not saturated at the threshold level.

DISCUSSION

Chromaffin cells secrete catecholamines in response to bursts of APs that elicit complex pulsatile patterns of Ca^{2+} entry and diffusion. We have used trains of depolarizing pulses to examine the secretory response of dialyzed bovine chromaffin cells. In this study, we describe two kinetically distinct modes of Ca^{2+}-secretion coupling. The modes differ in Ca^{2+} dependence and in their sensitivity to dialysis in the whole-cell patch-clamp configuration.

Secretory run-down in chromaffin cells

Secretory responsiveness decays rapidly in permeabilized chromaffin cells compared with intact controls. The rate of decay, as well as the properties of the remaining secretory response, depends on the diameter of holes produced by the permeabilizing agent and the composition of the permeabilization medium (for review, see Sontag et al., 1991). The whole-cell patch-clamp recording mode can be viewed as another variant of permeabilization techniques in which the cell is dialyzed with a specific pipette solution through a single 0.5- to 2-μm-diameter hole.

Threshold secretion declined slowly with an average time constant of 26 min. The slow, but still significant, run-down of threshold secretion may reflect the disappearance of or decline in critical secretory components during whole-cell recordings. The run-down described here differs from that in a previous study in which the secretory response was reported to "wash out" with a time constant of 1–4 min (see Fig. 8 in Augustine and Neher, 1992). Because the pipette solutions are similar in the two studies, the differences may arise from stimulus paradigms. In the previous study, long depolarizations (200–500 msec) were administered every 100 sec. With similar protocols, we also observed diminished secretory responses and less reproducibility (data not shown). In the present study, trains consisted of brief depolarizing pulses (5–100 msec) separated by 3–5 min. The longest pulse durations were used only at high Ca^{2+} chelator concentrations. Frequent and prolonged elevation of $[Ca^{2+}]_i$ to high levels may damage the secretory capability of chromaffin cells and obscure the time course of actual secretory run-down.

In contrast to threshold secretion, the second type of secretory kinetics is extremely labile and is observed only in response to the first and/or second stimulus train during whole-cell dialysis. The

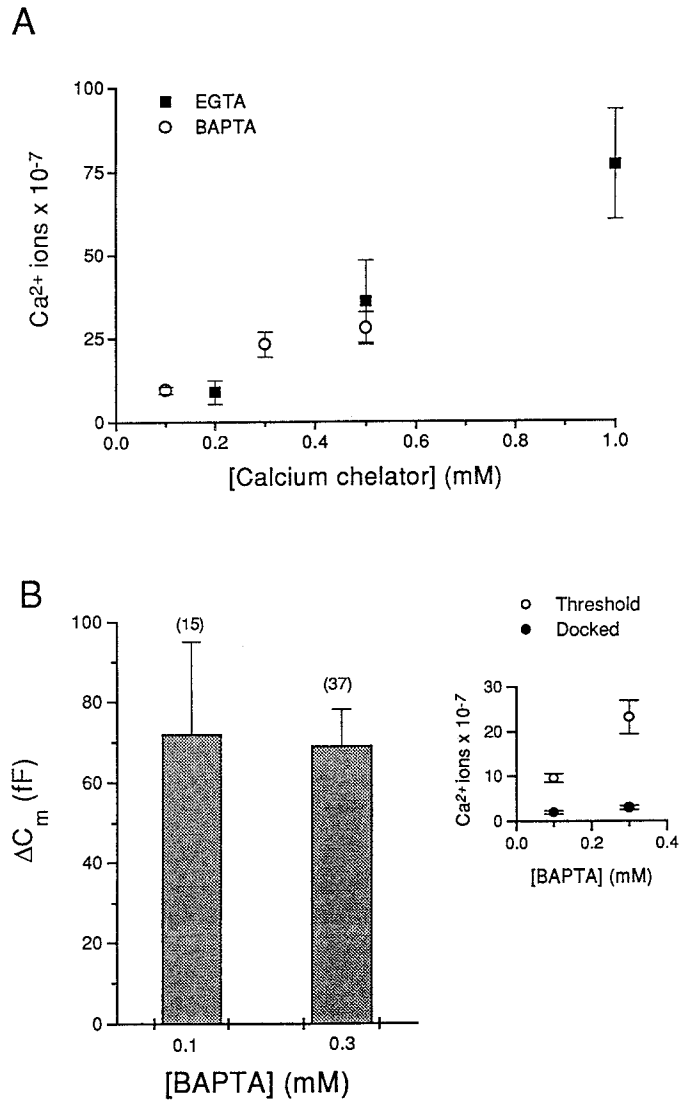

Figure 7. The concentration of the Ca^{2+} chelator regulates the value of the threshold amount of Ca^{2+} entry required for secretion. *A*, Estimated threshold values for cells with 0.2, 0.5, and 1.0 mM $[EGTA]_i$ (*solid squares*) and 0.1, 0.3, and 0.5 mM $[BAPTA]_i$ (*open circles*). *B*, Effect of Ca^{2+} chelator concentration on the amplitude of the response to the first depolarizing pulse of the first train. The number of cells is indicated in *parentheses above each bar*. Pulse protocols in 0.1 or 0.3 mM BAPTA consist of depolarizations to +10 mV for 6–40 msec. Data were collected between 2 and 6 min after break-in. *Inset*, Same data as in *A* for 0.1 and 0.3 mM BAPTA (*open circles*); average amount of Ca^{2+} entry for all experiments in 0.1 mM BAPTA (n = 15) and for the first bin of Figure 4*C* (n = 25; 0.3 mM BAPTA) (*solid circles*). The average for all cells in 0.3 mM BAPTA was $5.97 \times 10^7 \pm 9.14$ (SEM).

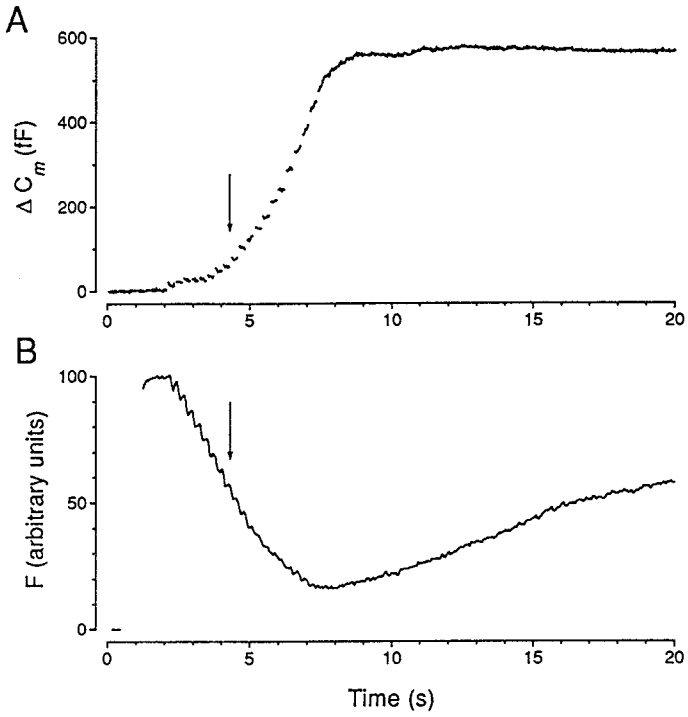

Figure 8. C_m increases begin before saturation of Ca^{2+} chelators. Recording from a cell with 10 mM EGTA and 0.2 mM Fura-red in the pipette. *A*, Capacitance trace in response to a train of 20 depolarizations (+10 mV, 100 msec, 4 Hz). *B*, Fluorescence intensity (*F*) expressed in arbitrary units.

rapid loss of this kinetic component may reflect a requirement for some small metabolite or protein to promote this state. However, possible alternative explanations are discussed below.

Characteristics of threshold secretion

The properties of threshold secretion in chromaffin cells are similar to those observed in peptide-secreting NHP terminals (Lim et al., 1990; Seward et al., 1995). In both preparations, threshold secretion consists of two Ca^{2+}-sensitive phases, the first of which is preparatory in nature and does not cause secretion. Exocytosis begins only after a critical threshold amount of Ca^{2+} entry that is governed by the concentration, but not species, of Ca^{2+} chelator. In the second phase, the final exocytotic step(s) appears to require elevated submembrane Ca^{2+}, because most C_m increases occur only when Ca^{2+} channels are open. The species of Ca^{2+} chelator, i.e., EGTA versus BAPTA, affects the secretory phase to a greater extent than the threshold phase (Seward et al., 1995). Thus, the two phases of threshold secretion appear to reflect two discrete Ca^{2+}-dependent steps within a single secretory mode. Finally, the critical parameter regulating both phases is cumulative Ca^{2+} entry during a stimulus train, rather than specific details of Ca^{2+} entry such as current amplitude or single-channel flux rates, which would produce different spatial and temporal gradients of submembrane Ca^{2+} concentration (Sala and Hernandez-Cruz, 1990; Nowycky and Pinter, 1993; Seward et al., 1995).

Secretory responses for which kinetics resembles threshold secretion appear in all studies of bovine chromaffin cells stimulated with trains of depolarizing pulses (Augustine and Neher, 1992; von Ruden and Neher, 1993; Artalejo et al., 1994). Threshold-like secretion also is observed in melanotrophs (Thomas et al., 1990) and pancreatic β cells (Ammala et al., 1993). In pancreatic β cells, the Ca^{2+}-dependent threshold occurs when average cytosolic $[Ca^{2+}]_i$ is 0.5 μM. As in NHP terminals and chromaffin cells, exocytosis is related to cumulative Ca^{2+} entry regardless of pulse protocol (Ammala et al., 1993). Threshold-like secretory behavior has been described in chicken II luteinizing hormone–releasing hormone (LHRH)-secreting sympathetic preganglionic fibers (Peng and Zucker, 1993) and may be a universal mechanism for secretion of LDCV.

Characteristics of docked secretion

In chromaffin cells, an additional form of Ca^{2+}-secretion coupling is observed with strikingly different Ca^{2+} dependence from threshold secretion. In this secretory mode, vesicles are released in response to amounts of Ca^{2+} entry well below threshold and are relatively insensitive to different concentrations of Ca^{2+} chelators. We use the term "docked" as both analogous with fast synapses where, presumably because of the close proximity of vesicle fusion sites to Ca^{2+} channels, Ca^{2+} chelators have only weak effects on stimulus-induced secretion (Adler et al., 1991; von Gersdorff and Matthews, 1994), and following the nomenclature of Thomas et al. (1993) to describe the most rapid phase of release in melanotrophs.

Because of its labile nature, docked secretion is missed easily if the cell is depolarized before initiating capacitance measurements, and it has not been reported previously in studies of bovine chromaffin cells stimulated with depolarizing pulses. A kinetic response that resembles docked secretion was reported in pancreatic β cells (see Figs. 5, 6, 8 in Ammala et al., 1993), but it was not studied further.

Two types of secretory patterns have been reported in rat adrenal chromaffin cells (Horrigan and Bookman, 1994). These patterns have been ascribed to the existence of two vesicular pools: an immediately releasable pool (IRP) of ~17 secretory vesicles of 35 fF, and a readily releasable pool (RRP), which is distinct and larger, consisting of ~170 vesicles. The kinetics of exocytosis of IRP in some respects resembles docked secretion in that it proceeds at lower amounts of Ca^{2+} entry and is less sensitive to buffers than the release of RRP. However, the correspondence between the IRP release in rat cells and the docked secretion in bovine chromaffin cells is not exact. Differences include (1) the persistence of IRP exocytosis during whole-cell recordings in rat, (2) the smaller size of the IRP pool in rat cells, and (3) the tight correlation between pulse duration and C_m responses during IRP secretion.

In experiments with flash photolysis of caged Ca^{2+} compounds, a series of kinetic responses is reported for both melanotrophs and bovine chromaffin cells (Neher and Zucker, 1993; Thomas et al., 1993; Heinemann et al., 1994). The earliest component is called "docked secretion" (Thomas et al., 1993) or secretion of a RRP of vesicles (also called "ultrafast"; Neher and Zucker, 1993; Heinemann et al., 1994). This component may be the same as the docked secretion described here. However, it is difficult to be certain, because the initial flow of caged Ca^{2+} compound into the cell produces a Ca^{2+} transient as intracellular Mg^{2+} displaces Ca^{2+} from the DM-nitrophen. It is likely that the large C_m responses evoked by the Ca^{2+} transient (>1000 fF in bovine chromaffin cells) (Neher and Zucker, 1993; Heinemann et al., 1994) include a majority of the labile docked pool.

Physiological implications

A major question raised by these observations is whether docked secretion represents the final step of the threshold pathway or whether docked and threshold secretion are parallel pathways involving different pools of vesicles or different release sites. We favor the interpretation of parallel pathways, because docked secretion never has been observed by us in NHP terminals and because we have not observed docking during a stimulus train. However, it is possible that docking is an optional step in a single secretory pathway that is lost once a key factor is dialyzed away and that this component is not present in NHP terminals. Alternatively, docking always may occur during threshold secretion, and perhaps we have not detected it because docked vesicles are released immediately.

The complexity of kinetic components presented here implies that a single adrenal chromaffin cell does not respond identically to electrical activity at all times. In the docked mode, a relatively rapid and reliable response might be elicited by a single or a few APs. In threshold mode, on the other hand, secretion would occur only during bursts of APs after a critical number of APs triggers this mode. The rules that govern the presence of one or the other secretory mode remain to be unraveled.

REFERENCES

Adler EM, Duffy SN, Augustine GJ, Charlton MP (1991) Alien intracellular calcium chelators attenuate neurotransmitter release at the squid giant synapse. J Neurosci 11:1496–1507.

Ammala C, Eliasson L, Bokvist K, Larsson O, Ashcroft FM, Rorsman P (1993) Exocytosis elicited by action potentials and voltage-clamp calcium currents in individual mouse pancreatic β-cells. J Physiol (Lond) 472:665–688.

Artalejo CR, Adams ME, Fox AP (1994) Three types of Ca^{2+} channel trigger exocytosis with different efficacies in chromaffin cells. Nature 367:72–76.

Augustine GJ, Neher E (1992) Calcium requirements for secretion in bovine chromaffin cells. J Physiol (Lond) 450:247–271.

Brandt BL, Hagiwara S, Kidokoro Y, Miyazaki S (1976) Action potentials in the rat chromaffin cell and effects of acetylcholine. J Physiol (Lond) 263:417–439.

Burgoyne RD, Morgan A (1993) Regulated exocytosis. Biochem J 293:305–316.

Cheek TR, Barry VA (1993) Stimulus-secretion coupling in excitable cells: a central role for calcium. J Exp Biol 184:183–196.

Fidler N, Fernandez JM (1989) Phase tracking: an improved phase detection technique for cell membrane capacitance measurements. Biophys J 56:1153–1162.

Heinemann C, Chow RH, Neher E, Zucker RS (1994) Kinetics of the secretory response in bovine chromaffin cells following flash photolysis of caged Ca^{2+}. Biophys J 67:2546–2557.

Heinemann C, von Ruden L, Chow RH, Neher E (1993) A two-step model of secretion control in neuroendocrine cells. Pflügers Arch 424:105–112.

Horrigan FT, Bookman RJ (1994) Releasable pools and the kinetics of exocytosis in adrenal chromaffin cells. Neuron 13:1119–1129.

Joshi C, Fernandez JM (1988) Capacitance measurements. An analysis of the phase detection technique used to study exocytosis and endocytosis. Biophys J 53:885–892.

Lim NF, Nowycky MC, Bookman RD (1990) Direct measurement of exocytosis and calcium currents in single vertebrate nerve terminals. Nature 344:449–451.

Mollard P, Seward EP, Nowycky MC (1995) Activation of nicotinic receptors triggers exocytosis from bovine chromaffin cells in the absence of membrane depolarization. Proc Natl Acad Sci USA 92:3065–3069.

Neher E, Augustine GJ (1992) Calcium gradients and buffers in bovine chromaffin cells. J Physiol (Lond) 450:273–301.

Neher E, Marty A (1982) Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine chromaffin cells. Proc Natl Acad Sci USA 79:6712–6716.

Neher E, Zucker RS (1993) Multiple calcium-dependent processes related to secretion in bovine chromaffin cells. Neuron 10:21–30.

Nowycky MC, Pinter MP (1993) Time courses of calcium and calcium-bound buffers following calcium influx in a model cell. Biophys J 64:77–91.

Ozawa S, Sand O (1986) Electrophysiology of excitable endocrine cells. Physiol Rev 66:887–952.

Peng Y-Y, Zucker RS (1993) Release of LHRH is linearly related to the time integral of presynaptic Ca^{2+} elevation above a threshold level in bullfrog sympathetic ganglia. Neuron 10:465–473.

Pusch M, Neher E (1988) Rates of diffusional exchange between small cells and a measuring patch pipette. Pflügers Arch 411:204–211.

Sala F, Hernandez-Cruz A (1990) Calcium diffusion modeling in a spherical neuron. Relevance of buffering properties. Biophys J 57:313–324.

Seward EP, Chernevskaya NI, Nowycky MC (1995) Exocytosis in peptidergic nerve terminals exhibits two calcium-sensitive phases during pulsatile calcium entry. J Neurosci 15:3390–3399.

Seward EP, Nowycky MC (1995) Stimulus-secretion coupling patterns in dialyzed chromaffin cells: effects of Ca buffers (Abstr). Biophys J 68:117A.

Sontag J-M, Sarafian T, Bader M-F, Aunis D (1991) Characterization of cytoplasmic proteins essential for exocytosis in chromaffin cells. Biochem Soc Trans 19:1090–1094.

Stuenkel EL (1994) Regulation of intracellular calcium and calcium buffering properties of rat isolated neurohypophysial nerve endings. J Physiol (Lond) 481:251–271.

Thayer SA, Miller RJ (1990) Regulation of intracellular free calcium concentration in single rat dorsal root ganglion neurones in vitro. J Physiol (Lond) 425:85–115.

Thomas P, Surprenant A, Almers W (1990) Cytosolic Ca^{2+}, exocytosis, and endocytosis in single melanotrophs of the rat pituitary. Neuron 5:723–733.

Thomas P, Wong JG, Lee AK, Almers W (1993) A low affinity Ca^{2+} receptor controls the final steps in peptide secretion in pituitary melanotrophs. Neuron 11:93–104.

Trifaro J-M, del Castillo AR, Vitalc ML (1993) Dynamic changes in chromaffin cell cytoskeleton as a prelude to exocytosis. Mol Neurobiol 6:339–358.

Vitale ML, del Castillo AR, Tchakarov L, Trifaro J-M (1991) Cortical filamentous actin disassembly and scinderin redistribution during chromaffin cell stimulation precede exocytosis: a phenomenon not exhibited by gelsolin. J Cell Biol 113:1057–1067.

von Gersdorff H, Matthews G (1994) Dynamics of synaptic vesicle fusion and membrane retrieval in synaptic terminals. Nature 367:735–749.

von Ruden L, Neher E (1993) A Ca-dependent early step in the release of catecholamines from adrenal chromaffin cells. Science 262:1061–1065.

Yamada WM, Zucker RS (1992) Time course of transmitter release calculated from simulations of a calcium diffusion model. Biophys J 61:671–682.

Zhou Z, Misler S (1995) Action potential-induced quantal secretion of catecholamines from rat adrenal chromaffin cells. J Biol Chem 270: 3498–3505.