Ba²⁺ Ions Evoke Two Kinetically Distinct Patterns of Exocytosis in Chromaffin Cells, but Not in Neurohypophysial Nerve Terminals

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The coupling between divalent cations and exocytosis of large dense-cored vesicles (LDCV) was studied with capacitance-detection techniques in nerve terminals of the rat neurohypophysis (NHP) and bovine chromaffin cells. Ba²⁺ substitution for Ca²⁺ produced kinetically distinct responses in the two preparations.

In NHP terminals, Ba²⁺ ions behave as weak substitutes for Ca²⁺. Exocytotic events occur principally during depolarizing pulses, i.e., events are “stimulus-coupled” to Ba²⁺ entry through voltage-gated Ca²⁺ channels. Stimulus-coupled exocytosis apparently requires elevated submembrane cation concentrations that dissipate rapidly on hyperpolarization-induced Ca²⁺-channel closure. Intracellular dialysis of NHP terminals with Ba²⁺ does not evoke exocytosis, nor does it interfere with depolarization-evoked Ca²⁺ influx and exocytosis.

In chromaffin cells, Ba²⁺ ions evoke a small quantity of stimulus-coupled secretion, but the dominant response is an additional pronounced poststimulus capacitance increase that

outlasts channel closures by 20–50 sec. “Stimulus-decoupled” exocytosis is slow (~25–40 fF/sec) compared with Ca²⁺-evoked stimulus-coupled exocytosis (~1000 fF/sec). Decoupled secretion is not attributable to Ba²⁺ displacement of intracellular Ca²⁺ ions, because it is insensitive to 10 mM EGTA or thapsigargin. Slow exocytosis is initiated by inclusion of Ba²⁺ ions in the recording pipette and continues steadily for 5–12 min, producing a total increase of several thousand fF, which ultimately doubles or triples the original cell-surface area.

We propose that two pathways of regulated exocytosis with distinct kinetics and divalent cation sensitivity exist in chromaffin cells. Only a single kinetic pattern is detected in NHP terminals, suggesting that mechanisms for secretion are not universally distributed in excitable cells.

Key words: calcium-secretion coupling; membrane-capacitance detection; exocytosis; chromaffin cells; neurohypophysis; asynchronous release; large dense-cored vesicles

Neurohypophysial (NHP) nerve endings and chromaffin cells are two classic systems for the investigation of Ca²⁺-regulated secretion of large dense-cored vesicles (LDCV) (Douglas and Poisner, 1964; Douglas and Rubin, 1964). The two systems share many properties: (1) secretion is triggered by bursts of action potentials (APs) rather than single APs (Ozawa and Sand, 1986); (2) both respond to low concentrations of intracellular Ca²⁺ in permeabilized preparations (~1–10 μ M) (Knight and Baker, 1982; Cazalis et al., 1987); and (3) each shows relatively rapid decay of the maximal secretory response despite maintained Ca²⁺ levels (Baker and Rink, 1975; Stuenkel and Nordmann, 1993). However, differences in Ca²⁺-secretion coupling have also been noted: (1) in whole-cell recordings, secretion in NHP terminals is abolished within ~15 sec at elevated Ca²⁺ concentrations (Lindau et al., 1992), but in chromaffin cells continues for minutes, although at submaximal rates (Augustine and Neher, 1992); and (2) NHP terminals respond to trains of depolarizing pulses with a single kinetic mode termed “threshold,” whereas chromaffin cells con-

tain an additional response termed “docked” secretion (Seward et al., 1995; Seward and Nowycky, 1996).

In many excitable cells including both NHP terminals (Ishida, 1968; Cazalis et al., 1987) and chromaffin cells (Douglas and Rubin, 1964; Forsberg and Pollard, 1988; Heldman et al., 1989; TerBush and Holz, 1992), Ba²⁺ ions are potent secretagogues. Previous studies on Ba²⁺-induced secretion in NHP terminals concluded that Ba²⁺ ions evoke vasopressin release, although they are only approximately one-third as effective as equimolar concentrations of Ca²⁺ ions (Cazalis et al., 1987). Studies on intact and permeabilized chromaffin cells are much more extensive. Although both Ca²⁺ and Ba²⁺ ions evoke catecholamine secretion, there are kinetic and other differences between the release produced by the two ions. Ba²⁺-induced secretion is always slower than that evoked by Ca²⁺, yet Ba²⁺ elicits much more total catecholamine release (Izumi et al., 1986; Heldman et al., 1989; Castillo et al., 1992; Michelena et al., 1993). Ba²⁺-induced secretion does not self-limit as does Ca²⁺-induced secretion (Heldman et al., 1989; Castillo et al., 1992; Michelena et al., 1993; Jankowski et al., 1994), which accounts for the difference in total release. Also, Ca²⁺-induced secretion is blocked by trifluoperazine and imipramine, whereas Ba²⁺-induced secretion is insensitive to both agents (Heldman et al., 1989).

Here we examine Ba²⁺-induced secretion in single NHP terminals and chromaffin cells using the capacitance-detection technique, which has excellent temporal resolution and sensitivity. Although the technique only reports changes in surface membrane area, there is extensive earlier evidence that Ba²⁺ does

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Figure 1. Ba^{2+} substitutes weakly for Ca^{2+} in triggering exocytosis from NHP terminals. *A*, Superimposed C_m traces from two terminals with typical patterns of exocytosis observed with external Ca^{2+} and Ba^{2+} . In extracellular Ca^{2+} , the stimulus train consisted of 5 pulses, 46 msec in duration, and in extracellular Ba^{2+} of 20 pulses, 72 msec in duration. Depolarizing pulses are indicated by vertical bars below the C_m traces. ΔC_m jumps evoked by Ca^{2+} pulses were non-uniform (4–17 fF), whereas those triggered by Ba^{2+} influx were fairly uniform and small (0–4 fF). *B*, Data from *A* plotted as cumulative Ca^{2+} (open triangles) and Ba^{2+} (filled triangles) influx versus cumulative C_m increases. In 30 terminals, the mean Ca^{2+} influx required to evoke a C_m increase of 50 ± 1 fF was $154 \pm 14 \times 10^6$ ions, whereas in Ba^{2+} an equivalent C_m increase (49 ± 6 fF) required $535 \pm 119 \times 10^6$ ions. Thus, Ba^{2+} ions are approximately one-fifth as potent as Ca^{2+} ions in evoking stimulus-coupled secretion. *C*, Ba^{2+} -evoked secretion is proportional to the amount of Ba^{2+} influx and persists in the presence of 10 mM EGTA. Superimposed C_m traces are from a single terminal stimulated with 3 trains of 30 pulses. The first Ba^{2+} current evoked by each train is shown below. In *a*, pulses were of 200 msec duration, TP = -10 mV; in *b*, 200 msec, $+20$ mV; and in *c*, 400 msec, $+20$ mV. In 10 mM EGTA, a 49 ± 6 fF C_m increase was achieved by $4600 \pm 500 \times 10^6$ Ba^{2+} ions ($n = 3$), or ~ 8 –9 times as many as needed in the standard solution with 0.5 mM EGTA.

release peptide hormones from NHP and catecholamines from chromaffin cells (see above). We report that, in both preparations, Ba^{2+} ions trigger exocytosis by substituting weakly for Ca^{2+} through a conventional, stimulus-coupled pathway. However, in chromaffin cells, but not in NHP terminals, the bulk of Ba^{2+} activated secretion takes place through a kinetically distinct, slow, stimulus-decoupled pathway.

Some of these results have been presented in abstract form (Seward and Nowycky, 1991; Chernevskaya et al., 1995).

MATERIALS AND METHODS

Preparations. NHP terminals were prepared as described previously (Lim et al., 1990; Seward et al., 1995). The pipette solution consisted of (in mM): Cs-D-glutamate 115, HEPES 40, MgCl_2 1, CsCl 10, Mg-ATP 2.5, and EGTA 0.5, adjusted to pH 7.2 with CsOH. Terminals were continuously superfused with external solution consisting of (in mM): NaCl 145, KCl 5, MgCl_2 1, CaCl_2 or BaCl_2 10, HEPES 10, glucose 10, and 1 μM tetrodotoxin, adjusted to pH 7.2 with NaOH.

Adult bovine chromaffin cells were cultured for 2–4 d as described in Vitale et al. (1991). The internal solution consisted of (in mM): Cs-D-glutamate 145, HEPES 10, NaCl 9.5, Mg-ATP 2, and bis(2-aminophenoxy)ethane- N,N,N',N' -tetra-acetic acid (BAPTA) 0.3, adjusted to pH 7.2 with CsOH. The external solution consisted of (in mM): NaCl 130, KCl 2, MgCl_2 1, CaCl_2 or BaCl_2 5, HEPES 10, glucose 10, and 1 μM tetrodotoxin, adjusted to pH 7.2 with NaOH. For the experiments shown in Figure 6, thapsigargin (1 μM) was added to the external solution after establishing whole-cell recording.

Calculation of free intracellular Ba^{2+} concentrations. In some experiments, 1 mM Ba^{2+} was added to the pipette solution. To chelate Ca^{2+} present in distilled water and to control cytosolic Ca^{2+} that might be released from intracellular stores or displaced from proteins, 1 mM EGTA was used instead of BAPTA because it is much more selective for Ca^{2+} than Ba^{2+}

(Fabiato and Fabiato, 1979). Free Ba^{2+} concentration was calculated using the program Maxchelator (version 6.72, C. Patton, Hopkins Marine Station, Pacific Grove, CA), using binding values estimated by Fabiato and Fabiato (1979). The final concentration of free Ba^{2+} concentration in the intracellular solution was estimated as 0.17 mM for NHP terminals and 0.15 mM for chromaffin cells. It is important to note that these are calculations based on available information on Ba^{2+} –EGTA interactions that may not be exact for the specific solutions used.

Recording conditions. Whole-cell Ca^{2+} -channel currents and capacitance measurements were performed 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). All voltage-pulse stimulations are from a holding potential of -90 mV to a test potential (TP) of $+20$ mV. Capacitance data are sampled at 18–28 msec intervals. C_m “jumps” are calculated by averaging four to five points before and after a depolarizing pulse. “Slow” C_m ($C_{m\text{-slow}}$) increases were calculated by subtracting the sum of C_m jumps from the total C_m increase for a given trace. Mean trace duration is 18 sec.

Pipette diameters. Patch pipettes used for experiments on NHP terminals had ~ 1 μm diameters and resistances of 3–5 M Ω . Pipettes for chromaffin cell experiments had ~ 1.5 –2 μm diameters and resistances of 1.5–3 M Ω .

Statistics. In Figure 6C, statistical comparisons were made using unpaired Student's *t* test for groups in which the SDs were equal and Mann–Whitney test for unequal SDs.

RESULTS

NHP terminals

Ba^{2+} weakly elicits C_m responses coupled to depolarizing pulses

The capacitance-detection technique was used to monitor exocytotic events in response to trains of depolarizing pulses. Figure 1A contains traces from two NHP terminals that are representative of

Figure 2. C_m jump amplitudes elicited by trains of pulses recorded in Ca^{2+} and Ba^{2+} . Histogram of individual C_m jump amplitudes obtained in Ca^{2+} and Ba^{2+} (250 events each). Individual ΔC_m events in Ca^{2+} ranged up to 79 fF, with 27% of the events >10 fF. In Ba^{2+} , maximal events were 13 fF, and only 2% of the events were >10 fF.

those elicited in extracellular solutions containing Ca^{2+} or Ba^{2+} and that are selected to illustrate similar total amounts of evoked ΔC_m . With Ca^{2+} as the divalent cation, C_m increases occur mostly as jumps during periods of Ca^{2+} influx (Seward et al., 1995). The C_m jump size is often larger on the second or third depolarizing pulse (Fig. 1A), as described previously (Lim et al., 1990; Seward et al., 1995). Ba^{2+} acts as a weak substitute for Ca^{2+} , because all Ba^{2+} -induced jumps are smaller and much more Ba^{2+} entry is required to elicit equivalent total C_m increases (note 5 vs 20 pulses indicated below traces and longer pulse durations in Ba^{2+}). A plot of cumulative ΔC_m versus cumulative divalent cation influx for these traces (Fig. 1B) indicates that approximately fivefold greater Ba^{2+} than Ca^{2+} entry is needed to induce similar total C_m changes.

The magnitude of the total C_m response is proportional to the amount of Ba^{2+} -ion influx during a train. In Figure 1C, three capacitance traces are illustrated along with the Ba^{2+} current evoked by the first pulse of each stimulus train. Minimal Ba^{2+} entry has no effect on C_m , whereas progressively larger currents elicit proportionally larger C_m changes. The C_m traces have the typical appearance of threshold secretion in which the earliest pulses of the train do not evoke C_m increases, whereas later pulses trigger C_m jumps once a threshold amount of divalent cation entry is achieved (Seward et al., 1995). Even when Ba^{2+} influx per pulse is very high as in this terminal with large Ca^{2+} -channel currents (~ 1 nA), all C_m jumps are small and C_m increases remain tightly coupled to depolarizations.

Individual C_m jump amplitudes recorded from 35 terminals in extracellular Ba^{2+} versus extracellular Ca^{2+} are plotted in Figure 2. There is a prominent difference in C_m jump amplitude distributions for the two ions. Both Ba^{2+} and Ca^{2+} evoke many small events (<10 fF). However, the large jumps seen in response to Ca^{2+} are not evoked by Ba^{2+} , even though the Ba^{2+} influx per pulse was usually greater than for Ca^{2+} .

Ba^{2+} acts independently of Ca^{2+}

To determine whether Ba^{2+} causes nonspecific damage, we tested mixtures of Ca^{2+}/Ba^{2+} (5:5 mM), which is predicted to cause significant influx of Ba^{2+} (Hess and Tsien, 1984). Responses to depolarizing pulses resemble those elicited by Ca^{2+} alone (i.e.,

larger-amplitude C_m jumps; $n = 3$; data not shown), indicating that there is no damage and that Ba^{2+} ions do not block access to critical release sites. To determine whether Ba^{2+} acts directly or through release of Ca^{2+} from internal stores, we used a high concentration of intracellular EGTA, a chelator with much greater affinity for Ca^{2+} versus Ba^{2+} (Fabiato and Fabiato, 1979). Ten millimolar EGTA abolished Ca^{2+} -evoked response in most terminals (Lim et al., 1990; Seward et al., 1995), even when cumulative Ca^{2+} influx was 20-fold greater than required with 0.5 mM EGTA. In contrast, Ba^{2+} still elicited C_m increases with 10 mM EGTA, although the total amount of Ba^{2+} entry had to be increased by approximately eight- to ninefold (compare legends of Fig. 1B,C).

Because Ca^{2+} - and Ba^{2+} -evoked secretion occurs mostly during depolarizations, that is, periods of active cation entry, it appears that the fusion machinery of NHP terminals responds only to elevated submembrane divalent cation concentrations, i.e., exocytosis is stimulus-coupled. To test this further, we dialyzed NHP terminals with Ba^{2+} (1 mM total, estimated at 0.17 mM free). Figure 3 illustrates that intracellular dialysis with Ba^{2+} has no significant effect on membrane capacitance, as expected if vesicle fusion requires elevated divalent cation concentrations near the membrane. A depolarizing train elicits normal exocytotic response if the terminal is simultaneously bathed in extracellular Ca^{2+} , providing further evidence that the weak responsiveness to Ba^{2+} is not attributable to nonspecific damage or occlusion of critical receptors.

Bovine chromaffin cells

Ba^{2+} weakly elicits stimulus-coupled secretion and stimulates an additional slow response

Although Ca^{2+} -evoked secretion in chromaffin cells shares many similarities with the response in NHP terminals (Seward and Nowicky, 1996), Ba^{2+} -evoked secretion is markedly different in the two preparations. Typical chromaffin-cell responses to trains of depolarizing pulses in Ca^{2+} and Ba^{2+} are illustrated by two superimposed traces in Figure 4. In Ca^{2+} (top trace), most of the C_m increase occurs as jumps during depolarizing pulses when Ca^{2+} concentration is at its highest in the vicinity of the plasma membrane. After the last pulse, C_m increases terminate almost immediately. This secretory pattern is similar to what is observed in NHP terminals and is termed stimulus-coupled secretion. When Ba^{2+} is substituted for extracellular Ca^{2+} , C_m increases begin later in the train and jumps are much smaller, although divalent cation influx is increased approximately fivefold per pulse (Fig. 4, bottom trace; compare current traces). During the train, C_m increases occur both as slow drifts between pulses and as C_m jumps. However, the bulk of the C_m response occurs as a prolonged slow rise that outlasts the end of the depolarizing train (arrow) by tens of seconds. This response is termed stimulus-decoupled secretion to emphasize the temporal dissociation between the stimulus train and most of the C_m increase.

Table 1 contains a summary of some of the properties of Ba^{2+} induced stimulus-decoupled secretion. The total amount of C_m increase (both coupled and decoupled) reflects the fusion of several hundred vesicles. The highest secretory rate for decoupled secretion is obtained immediately after the end of a stimulus train and averages 32 ± 9 fF sec $^{-1}$ ($\pm$ SD; $n = 9$). In most cells, slow C_m increases continue for 20–50 sec after the train. The rate of decay of poststimulus secretion could be fit by a single exponential that falls with a time constant of 8.6 ± 1.9 sec.

Stimulus-decoupled secretion is not attributable to toxic effects

Figure 3. Intracellular dialysis with 1 mM Ba^{2+} does not elicit C_m increases in NHP terminals and does not block depolarization-induced Ca^{2+} -stimulated secretion. The standard intracellular solution was modified by addition of 1 mM Ba^{2+} . The chelator was increased to 1 mM EGTA, yielding an estimated free EGTA concentration of ~ 0.17 mM (see Materials and Methods). During the time indicated by the solid bar, the terminal was stimulated by a train of 20 depolarizing pulses, 40 msec in duration, +20 mV, 5 Hz. The first depolarization exceeded the threshold and induced a relatively large C_m jump. Each C_m increase (indicated by breaks in the trace) was followed by very rapid C_m decreases, which are observed occasionally in these terminals and may reflect a rapid endocytotic process.

of Ba^{2+} because prominent stimulus-coupled events are observed after reintroduction of Ca^{2+} . Figure 5A illustrates Ca^{2+} -induced C_m increases before (i) and after (iii) stimulation in Ba^{2+} (ii). The second response to Ca^{2+} is smaller because of secretory rundown (Seward and Nowycky, 1996), but it terminates within 2 sec of the last pulse. Slow C_m increases could be evoked several times by repeated stimulation with Ba^{2+} . When three pulse trains are given in rapid succession (Fig. 5B; note continuous time scale), there is little depression of the secretory response to the third train and, instead, C_m increases to the second and third stimulus begin after a shorter delay than is evident in the first trace of the series.

The magnitude of ΔC_m is correlated with the absolute amount of Ba^{2+} entry and is saturable. The two traces in Figure 5C are from a single cell stimulated with two pulse durations. In the trace with short pulses (i), there is less total ΔC_m and it proceeds at a slower rate than for greater Ba^{2+} entry (ii), but is similarly

prolonged. The relationship between total amount of Ba^{2+} entry and C_m increases for both stimulus-coupled (open squares) and stimulus-decoupled (solid squares) responses for all recordings with 0.3 mM intracellular BAPTA is plotted in Figure 5D. When the total Ba^{2+} entry is $< 0.5 \times 10^9$ ions, no stimulus-decoupled secretion is observed (data not shown). The rate of stimulus-decoupled secretion never exceeded 50 fF/sec, even at the largest values of Ba^{2+} entry.

Ba^{2+} evokes stimulus-decoupled secretion independently of Ca^{2+}

Chromaffin cells contain large-capacity, active intracellular Ca^{2+} stores that can support exocytosis in the absence of APs and voltage-gated Ca^{2+} entry (Cheek and Thastrup, 1989; O'Sullivan et al., 1989; Mollard et al., 1995) and might contribute to the Ba^{2+} -elicited C_m increases. To test this, we used a combination of thapsigargin, a Ca^{2+} -ATPase inhibitor that depletes intracellular stores, and high concentrations of EGTA. A representative experiment is illustrated in Figure 6A. The recording was performed

Figure 4. Ca^{2+} and Ba^{2+} ions elicit exocytotic responses with different kinetics in chromaffin cells. Two superimposed traces from one cell. Stimulus trains consisted of 20 depolarizing pulses, 20 msec in duration for Ca^{2+} and 100 msec for Ba^{2+} . The current traces evoked by the first and last depolarizing pulse of each train are plotted below. The timing of the last depolarizing stimulus is indicated by arrows. Other pulses are indicated as breaks in the C_m trace. Asterisk marks 100 fF calibration deflections.

Table 1. Rates of slow, Ba^{2+} -induced C_m increases in single cells

Experimental conditions	ΣBa^{2+} influx ($\times 10^{-9}$ ions)	ΣC_m increase (fF)	Initial poststimulus rate (fF sec $^{-1}$)	τ_{decay} (sec)
0.3 mM BAPTA $_i$	2.3	1083	28	9
	4.2	724	17	9
0.3 mM BAPTA $_i$	3.1	1037	36	10
	1.5	991	25	11
1 μM Thp $_o$	4.1	869	45	6
10 mM EGTA $_i$	5.1	446	36	7
	7.1	485	41	8
	6.5	472	41	6
10 mM EGTA $_i$	10.4	640	25	11
1 μM Thp $_o$

Figure 5. Ba^{2+} -evoked stimulus-decoupled secretion is reversible, can be evoked multiple times, and depends on amount of Ba^{2+} entry. *A*, Ba^{2+} -evoked slow secretion is reversed in Ca^{2+} . Three C_m traces from a single chromaffin cell (0.3 mM BAPTA_i): *i*, recorded 3.5 min after break in with 5 mM extracellular Ca^{2+} and stimulated with 20 pulses, 20 msec in duration (C_m calibration of 100 fF); *ii*, 8 min later with 5 mM extracellular Ba^{2+} , stimulated with 20 pulses, 100 msec in duration; *iii*, 14 min later, after reintroduction of 5 mM extracellular Ca^{2+} . Note appearance of slow endocytosis and a 100 fF poststimulus drift, but which terminates within 2 sec, in contrast to Ba^{2+} -evoked secretion. *B*, Multiple Ba^{2+} -evoked slow C_m increases may be evoked in a single cell. Consecutive C_m traces in response to three stimuli each consisting of 20 pulses, 100 msec in duration (0.3 mM BAPTA_i, 5 mM Ba^{2+} , and 1 μ M thapsigargin). Previous exposure to Ba^{2+} decreases the delay in the onset of exocytosis in the second and third stimuli. *C*, Ba^{2+} dependence of C_m increase. Two traces from a cell stimulated with (*i*) 20 pulses of 20 msec duration, with a cumulative Ba^{2+} influx of 9×10^8 ions and C_m increase of 174 fF, (*ii*) 20 pulses of 100 msec duration, with a cumulative Ba^{2+} influx of 25×10^8 ions and 566 fF C_m increase (0.3 mM BAPTA_i, 5 mM Ba^{2+} , and 1 μ M thapsigargin). *D*, Mean $\pm$ SEM C_m increases evoked by trains of depolarizations plotted against cumulative Ba^{2+} influx/train: filled symbols, poststimulus C_m increases; open symbols, C_m jumps coupled to depolarizations. Results are from 13 cells with 0.3 mM BAPTA_i.

Figure 6. Ba²⁺ influx triggers slow, stimulus-decoupled exocytosis via a Ca²⁺-independent mechanism. *A*, Three C_m traces from a single chromaffin cell recorded with 10 mM EGTA in the internal solution. *a*, C_m increases evoked by 20 depolarizations, 100 msec duration in Ca²⁺. High concentrations of EGTA greatly attenuated the secretory response of cells to Ca²⁺ influx (Seward and Nowycky, 1996). *b*, Addition of 1 μ M thapsigargin to the superfusate had no effect on resting C_m and minimal effect on Ca²⁺-evoked C_m increases in the presence of 10 mM EGTA. *c*, C_m response to 20 depolarizations of 200 msec duration after substitution of external Ca²⁺ with Ba²⁺ and in the continued presence of thapsigargin. The kinetics of the slow poststimulus C_m increase is indistinguishable from recordings without high EGTA or thapsigargin pretreatment. *B*, *C*, Stimulus-coupled and -decoupled secretions were estimated by dividing C_m traces into changes occurring during depolarizations (see Materials and Methods) and those occurring during the interpulse interval and after the last depolarizing pulse. The fraction of stimulus-decoupled secretion is given directly in *C*. In *B*, the remaining stimulus-coupled secretion is expressed in terms of ΔC_m /divalent cation, which allows direct comparisons between the efficacy of Ca²⁺ versus Ba²⁺. *B*, Ba²⁺ (hatched bars) is less efficient than Ca²⁺ (solid bars) in evoking stimulus-coupled secretion. The mean C_m increase during jumps per Ca²⁺ or Ba²⁺ ion is plotted in control conditions (Con; 0.3 mM intracellular BAPTA), after application of 1 μ M thapsigargin (Thp; 0.3 mM intracellular BAPTA), and with 10 mM extracellular EGTA, for the number of experiments shown in parentheses. This normalization makes no assumptions about the relationship between Ca²⁺ influx and amount of C_m increase. *C*, The Ba²⁺-induced slow secretion is insensitive to thapsigargin treatment and 10 mM EGTA. Same data set as in *B*, but measuring only C_m increases that occurred between pulses or after stimulus termination. Ten micromolar EGTA completely abolished stimulus-decoupled secretion evoked by Ca²⁺. Both thapsigargin (Thp) and high EGTA had significant effects ($p < 0.01$) on Ca²⁺-induced slow secretion (solid bars), whereas effects on Ba²⁺-induced slow secretion (hatched bars) are not significantly different ($p > 0.05$). Con, Control.

with 10 mM intracellular EGTA in the pipette. The first two traces are elicited in external Ca²⁺ before (*a*) and 4 min after (*b*) application of 1 μ M thapsigargin. Because of the high internal chelator concentration, there is a <50 fF C_m increase for a stimulus protocol that typically would elicit >300 fF ΔC_m at lower chelator concentrations (see Fig. 6*B* in Seward and Nowycky,

1996). The extracellular solution was then exchanged for one containing Ba²⁺ and thapsigargin. In the continued presence of 10 mM EGTA and 1 μ M thapsigargin, Ba²⁺ entry induces the characteristic slow, prolonged C_m increase [Fig. 6*A*(*c*)]. Thus, Ba²⁺-evoked secretion is not caused by displacement of Ca²⁺ ions from intracellular stores or other sources.

Figure 7. Intracellular dialysis with Ba^{2+} elicits massive C_m increases in chromaffin cells. The slow capacitance rise began ~ 10 – 20 sec after establishing whole-cell recording mode. Each line represents an ~ 18 sec capacitance recording interval. Gaps between traces represent interruptions during which the C_{slow} compensation circuitry of the EPC-7 patch clamp was adjusted because of the magnitude of the exocytotic response. To estimate the amount of exocytosis that occurred during the interruptions, secretory rates were calculated at the end of one trace and the beginning of the next available capacitance trace, and the estimated exocytosis is represented by the position of the second trace. The small upward deflections on several of the traces are 100 fF calibration pulses. Pipette solution contained 1 mM Ba^{2+} and 1 mM EGTA, instead of the usual 0.3 mM BAPTA, because EGTA is more selective for Ca^{2+} over Ba^{2+} than is BAPTA and, therefore, was included at higher concentration to control cytosolic Ca^{2+} . The initial cell capacitance was ~ 5 pF. *Inset*, Immediately after the last trace shown, the cell was stimulated with a train of 20 depolarizations, 40 msec in duration, 4 Hz.

In the experiment illustrated in Figure 6A, a combination of thapsigargin and high EGTA is used to eliminate any participation of Ca^{2+} ions. Thapsigargin or high EGTA alone were also used as tools to study the divalent cation dependency of stimulus-coupled and -decoupled secretion. For this, we divided the changes in C_m into the fraction of the response occurring during the depolarizing pulses (i.e., stimulus-coupled secretion; Fig. 6B) and the fraction that occurred between pulses and after the last stimulus of the train (stimulus-decoupled secretion, $C_{m\text{-slow}}$; Fig. 6C). Stimulus-coupled secretion is expressed as ΔC_m per amount of divalent cation entry (Fig. 6B). Ba^{2+} ions appear to substitute ~ 14 -fold less potently than Ca^{2+} ions in stimulus-coupled secretion (Fig. 6B). Thapsigargin treatment does not influence the coupled response to either divalent cation significantly. Ten millimolar EGTA dramatically reduces the efficacy of Ca^{2+} (9-fold) and has a significant, although smaller, effect on the efficacy of Ba^{2+} (3.4-fold), as expected from the lower affinity of the chelator for Ba^{2+} (Fabiato and Fabiato, 1979). Thus, the efficacy of both Ca^{2+} and Ba^{2+} in evoking stimulus-coupled secretion is reduced by Ca^{2+} chelators (Seward and Nowycky, 1996), but is insensitive to block of divalent cation uptake into intracellular stores.

Thapsigargin and 10 mM EGTA have qualitatively different effects on slow secretion. In control Ca^{2+} conditions, $\sim 16\%$ of total changes in C_m occur as slow interpulse drifts and post-train increases ($C_{m\text{-slow}}$). Thapsigargin increases the amount of Ca^{2+} -induced stimulus-decoupled drift approximately twofold, whereas 10 mM EGTA completely abolishes it. These results are consistent with a model in which residual Ca^{2+} promotes a modest additional amount of secretion between and after periods of active Ca^{2+} entry. The amount of residual Ca^{2+} is prolonged by preventing Ca^{2+} uptake into intracellular stores and is completely eliminated by higher concentrations of Ca^{2+} chelator.

In Ba^{2+} , the majority of the secretory response occurs as $C_{m\text{-slow}}$ (65%; Fig. 6C). Thapsigargin has no significant effect on the fraction (Fig. 6C) or the total amount of $C_{m\text{-slow}}$ (Table 1). Ten millimolar EGTA slightly diminishes the average amount of $C_{m\text{-slow}}$ and increases the number of Ba^{2+} ions necessary to elicit stimulus-decoupled secretion. For cells in which total Ba^{2+} influx exceeded 5×10^9 ions, the mean $C_{m\text{-slow}}$ was 176 ± 36 fF ($n = 13$),

compared with 312 ± 59 fF in 0.3 mM BAPTA ($n = 17$). High EGTA had no significant effect on the kinetics of the poststimulus response (Table 1), nor on the relative contribution of $C_{m\text{-slow}}$ to the overall secretory response (Fig. 6C). The results with thapsigargin are expected and are consistent with the inability of native Ca^{2+} -ATPases to remove Ba^{2+} (Kwan and Putney, 1990). The relatively weak effect of 10 mM EGTA on the decoupled response is the opposite of what is expected for a residual Ca^{2+} model.

Intracellular dialysis with Ba^{2+} elicits slow secretion in chromaffin cells

von Ruden et al. (1993) reported that intracellular dialysis with Ba^{2+} induced slow C_m increases in bovine chromaffin that did not exceed an average maximum of 30 fF/sec over the range of 0.1–1 mM Ba^{2+} (recordings with 0.1 mM Fura-2). We performed similar experiments with an internal solution that contained 1 mM Ba^{2+} , with 1 mM EGTA substituted for BAPTA to control for Ca^{2+} ions in the cytosol (see Materials and Methods). A representative result is illustrated in Figure 7. Within seconds of establishing the whole-cell dialysis mode, the cell capacitance begins to increase at a rate that never exceeds 50 fF/sec and is typically ~ 20 fF/sec or slower. The slow C_m increase continues for 6–12 min ($n = 13$); during this time, the original cell-surface area doubles or triples. This massive exocytosis is visible under the microscope as an increase in cell diameter and transition to a bumpy and irregular surface (data not shown).

The massive exocytosis induced by intracellular Ba^{2+} does not interfere with Ca^{2+} -stimulated coupled exocytosis. The example illustrated in Figure 7 (*inset*) shows, on an expanded time and capacitance scale, Ca^{2+} -coupled exocytosis triggered ~ 10 min after the initiation of dialysis with Ba^{2+} . Once the cell is stimulated with a train of 20 pulses (timing indicated by bar), discrete C_m jumps are observed. The secretory response has threshold-like kinetics, because later pulses in the train are more effective than early ones in eliciting C_m jumps (Seward and Nowycky, 1996). The presence of a qualitatively typical secretory response to Ca^{2+} entry through voltage-gated channels indicates that massive Ba^{2+} -induced exocytosis does not obliterate or damage the stimulus-

coupled mechanism and that there is no particular priming of vesicles by Ba^{2+} .

Robust coupled exocytosis is seen when cells are stimulated with depolarizing trains both within 1–2 min of whole-cell dialysis with Ba^{2+} ($n = 5/5$) and as much as 8–10 min later ($n = 4/4$). Secretory responsiveness eventually fails: in two chromaffin cells first stimulated after prolonged dialysis (15 and 24 min), there was no stimulus-coupled secretion; however, Ca^{2+} currents were markedly reduced at these late times. At earlier time points, Ca^{2+} -induced, stimulus-coupled secretion could be elicited up to three or four times before the secretory responsiveness was exhausted. Thus, as for NHP terminals, dialysis of chromaffin cells with Ba^{2+} does not interfere with the stimulus-coupled exocytosis induced by elevated submembrane Ca^{2+} ions. In addition, in chromaffin cells the massive exocytosis induced by the stimulus-decoupled pathway does not obliterate or damage sites for stimulus-coupled exocytosis. Finally, dialysis with Ba^{2+} ions in both cell types does not potentiate or otherwise influence the stimulus-coupled response.

DISCUSSION

Ba^{2+} is a weak agonist for stimulus-coupled secretion

Ba^{2+} ions appear to be an adequate although weak substitute for Ca^{2+} in conventional stimulus-coupled secretion in both NHP terminals and chromaffin cells. In NHP terminals, the kinetics of Ba^{2+} - and Ca^{2+} -induced exocytosis are similar, in that capacitance increases do not begin until after several depolarizing pulses (i.e., after achieving threshold; Seward et al., 1995), then occur mostly during depolarizations and terminate rapidly after the last pulse of a stimulus train. The observation that stimulus-coupled secretion occurs mostly during periods of active cation influx implies that the final step(s) of threshold secretion requires elevated levels of divalent cation concentration in submembrane sites and that the concentration falls below critical levels within milliseconds of channel closure (Sala and Hernandez-Cruz, 1990; Nowycky and Pinter, 1993).

Ba^{2+} ions are weak agonists for conventional stimulus-coupled exocytosis in a number of different preparations. A small amount of stimulus-coupled secretion is observed in chromaffin cells (this paper) and in pancreatic β cells (Proks and Ashcroft, 1995). Similarly, at fast synapses Ba^{2+} ions either weakly evoke AP-coupled EPSPs of low quantal content (Blioch et al., 1968; Silinsky, 1978; Zengel and Magleby, 1981; Augustine and Eckert, 1984) or are completely ineffective (McLachlan, 1977; Stanley, 1993). Proteins such as synaptotagmins with different affinities for both Ca^{2+} and Ba^{2+} may participate in stimulus-coupled secretion in a wide variety of neuronal and endocrine cells (Davletov and Sudhof, 1993; Li et al., 1995).

Ba^{2+} triggers stimulus-decoupled secretion in chromaffin cells

In chromaffin cells, the major response to Ba^{2+} entry consists of slow exocytosis that outlasts a stimulus train by tens of seconds. Slow exocytosis begins only after a large amount of Ba^{2+} entry and then proceeds at characteristic rates. Remarkably, the rates are similar under different experimental conditions, including in the presence of high concentrations of divalent cation chelators (10 mM EGTA) or when triggered by continuous Ba^{2+} dialysis. von Ruden et al. (1993) demonstrated that over a 10-fold range of Ba^{2+} concentrations (0.1–1 mM), the secretory rate averaged 25 fF/sec. This suggests that both in dialysis experiments and in

experiments with depolarization-induced Ba^{2+} entry, the slow secretory rate may be an intrinsic property of the stimulus-decoupled mechanism.

Intracellular dialysis with Ba^{2+} and the resulting exocytosis neither interfere with nor potentiate stimulus-coupled secretion. As in NHP terminals, Ba^{2+} ions apparently do not occlude sites that are critical for stimulus-coupled exocytosis. Perhaps surprisingly, massive addition of vesicular membrane to the plasma membrane also does not destroy or block the stimulus-coupled secretory machinery. Finally, dialysis with Ba^{2+} does not potentiate stimulus-coupled secretion and, therefore, does not appear to participate in any divalent cation preparatory steps that have been postulated in recent models of stimulus-coupled LDCV secretion (Heinemann et al., 1994; Seward et al., 1995).

The possibility that prolonged exocytosis elicited by Ba^{2+} entry through voltage-gated channels is attributable to retention of Ba^{2+} ions within restricted spaces or specialized Ba^{2+} -buffering mechanisms found in chromaffin cells, but not in NHP terminals, cannot be excluded absolutely. It seems unlikely because few Ca^{2+} -binding proteins are able to bind Ba^{2+} (Chao et al., 1984) and because measurements of Ba^{2+} diffusion in snail neurons showed slightly faster rates than for Ca^{2+} diffusion (Nasi and Tillotson, 1985). Nevertheless, further proof will require the discovery of a tool that selectively eliminates one but not the other kinetic mode.

The relationship between stimulus-coupled and stimulus-decoupled secretion

Ca^{2+} -ion entry through voltage-gated channels induces a small amount of slower capacitance increases between pulses and after the final depolarization (see Fig. 5C). The amount of slow capacitance increase is approximately doubled by blocking Ca^{2+} uptake into stores with thapsigargin and is abolished by 10 mM EGTA. Although there is no absolute way to distinguish whether these capacitance changes are attributable to induction of the decoupled mechanism or residual Ca^{2+} acting via coupled release, the observations are consistent with a residual Ca^{2+} mechanism.

Why does Ca^{2+} influx during depolarizations not elicit much stimulus-decoupled secretion? We can only speculate that because Ca^{2+} ions activate many proteins that do not bind Ba^{2+} , including proteins with the E-F hand motif such as calmodulin (Chao et al., 1984), or with C2 domains, such as protein kinase C (PKC), activation of a Ba^{2+} -insensitive protein somehow actively inhibits the stimulus-decoupled pathway. For example, phosphorylation of annexins I and II by PKC abolishes their ability to induce chromaffin granule aggregation (Johnstone et al., 1993). Alternatively, because synaptotagmin isoforms have different affinities for Ca^{2+} and Ba^{2+} (Davletov and Sudhof, 1993; Li et al., 1995), and because multiple isoforms are present in chromaffin cells (Mizuta et al., 1994; Roth and Burgoyne, 1994), the two ions may be selectively activating distinct molecular entities.

Stimulus-decoupled secretion is observed in other cell types

In a paper that appeared during the review process, slow Ba^{2+} -evoked secretion that persists long after stimulus trains was reported to occur in most pancreatic β cells recorded in perforated-patch mode (Proks and Ashcroft, 1995). The authors tentatively conclude that the slow second phase of secretion may result from retention of Ba^{2+} in the cytoplasm of essentially intact cells. However, the changes in capacitance appear to have a similar time course as in our whole-cell recordings (compare their Fig. 6 to our

Table 1). Extremely prolonged secretory responses to Ba^{2+} were seen when the ion was puffed onto intact bovine chromaffin cells that were not voltage-clamped (Jankowski et al., 1994). In that study, catecholamine release, measured with amperometric techniques, continued for several minutes after a 15 sec puff of Ba^{2+} . This extended response appears to involve a combination of Ca^{2+} release from intracellular stores and possibly subsequent Ca^{2+} influx through the plasma membrane. Thus, although the physiological significance of the decoupled pathway is unknown at present, one possibility is that it may be activated by G-protein-coupled receptor mechanisms that produce lower intracellular Ca^{2+} concentrations than those apparently achieved during stimulus-coupled secretion (Cheek and Thastrup, 1989; O'Sullivan et al., 1989).

Ba^{2+} ions also evoke slow, stimulus-decoupled secretion at fast synapses. Despite their weak activation of AP-coupled EPSPs, Ba^{2+} ions are severalfold more potent than Ca^{2+} ions in eliciting an increased frequency of miniature end-plate potentials (MEPPs) between individual stimuli and after stimulus trains, a form of release also known as "tonic" or "asynchronous" release (Blioch et al., 1968; McLachlan, 1977; Silinsky, 1978; Zengel and Magleby, 1981; Guan et al., 1988; Quastel and Saint, 1988). The increased MEPP frequency builds up slowly over several seconds during a train and then decays slowly as a prominent tail after the train, lasting as long as 30 sec (McLachlan, 1977; Silinsky, 1978; Zengel and Magleby, 1981; Guan et al., 1988), kinetics that resembles that observed in chromaffin cells and pancreatic β cells (this paper) (see also Jankowski et al., 1994; Proks and Ashcroft, 1995).

Recently, Geppert et al. (1994) reported that in mice carrying a mutation of synaptotagmin I, AP-coupled synchronous or phasic neurotransmitter release is impaired, whereas at least some asynchronous release processes are unaffected. Similarly, mutation of *Drosophila* synaptotagmin strongly decreases evoked transmitter release, but has no effect on spontaneous release (Littleton et al., 1993). Both groups suggest that the coexistence of multiple release mechanisms accounts for these results. Further work should establish whether synchronous and asynchronous release at fast synapses corresponds to the stimulus-coupled and -decoupled mechanisms described here.

In this study, we describe a secretory pathway for release of LDCVs that is present in chromaffin cells, but not in NHP terminals. The lack of a stimulus-decoupled mechanism in NHP terminals is thus far unique and supports the hypothesis that stimulus-coupled and -decoupled pathways differ in at least one critical divalent cation-sensitive step, rather than reflect different kinetics of Ba^{2+} versus Ca^{2+} binding to a single class of receptors. Although the similarity between secretory pathways has been stressed during recent years, there may also be diversity within a single cell type. In light of the many forms of endocytosis that utilize distinct proteins, it is not surprising to find diversity in exocytotic mechanisms as well.

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