

Intravesicular Calcium Release Mediates the Motion and Exocytosis of Secretory Organelles

A STUDY WITH ADRENAL CHROMAFFIN CELLS^{*[5]}

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Secretory vesicles of sympathetic neurons and chromaffin granules maintain a pH gradient toward the cytosol (pH 5.5 versus 7.2) promoted by the V-ATPase activity. This gradient of pH is also responsible for the accumulation of amines and Ca²⁺ because their transporters use H⁺ as the counter ion. We have recently shown that alkalinization of secretory vesicles slowed down exocytosis, whereas acidification caused the opposite effect. In this paper, we measure the alkalinization of vesicular pH, caused by the V-ATPase inhibitor bafilomycin A1, by total internal reflection fluorescence microscopy in cells overexpressing the enhanced green fluorescent protein-labeled synaptobrevin (VAMP2-EGFP) protein. The disruption of the vesicular gradient of pH caused the leak of Ca²⁺, measured with fura-2. Fluorimetric measurements, using the dye Oregon green BAPTA-2, showed that bafilomycin directly released Ca²⁺ from freshly isolated vesicles. The Ca²⁺ released from vesicles to the cytosol dramatically increased the granule motion of chromaffin- or PC12-derived granules and triggered exocytosis (measured by amperometry). We conclude that the gradient of pH of secretory vesicles might be involved in the homeostatic regulation of cytosolic Ca²⁺ and in two of the major functions of secretory cells, vesicle motion and exocytosis.

Secretory granules from chromaffin cells are large dense core vesicles similar to vesicles present in many other neuroendocrine cells and in sympathetic neurons (1). Chromaffin granules are extremely efficient concentrating solutes, so that catecholamines reach 500–1000 mM (2, 3) and ATP reaches 125–300 mM (4). The mechanisms used to obtain these large concentrations of solutes have intrigued researchers for three decades. Intravesicular aggregation has been proposed as the mechanism for reducing osmotic forces resulting from the large amount of solutes concentrated in vesicles.

Chromaffin granules maintain a pH gradient across their membranes of ~2 orders of magnitude, ~5.5 inside and ~7.3 in the cytosol. This gradient is held stable by the activity of a specific H⁺-ATPase (V-ATPase). Vesicular H⁺ are used as antiporters to accumulate catecholamines by the vesicular monoamine transporter VMAT-2 (5) or Ca²⁺ (6). The presence of a vesicular matrix composed of solutes and chromogranins has been proposed as the chelating method to reduce the osmotic forces (7) to allow the accumulation of catecholamines at high concentrations. Most of the intravesicular solutes are not free but associated to the matrix, where the main proteic components are chromogranins, whose pK_a is ~5.5 (7, 8). Therefore, intravesicular pH can regulate the ability of chromogranin A to form aggregates (9). It is plausible that the regulation of vesicular pH could play an important role in the accumulation of Ca²⁺ and catechols (10, 11). We have recently demonstrated that the vesicular pH is closely associated with the modulation of the kinetics and quantal characteristics of the exocytosis of catecholamines (12).

However, the importance of vesicular Ca²⁺ is frequently ignored. ~30% of the total chromaffin cell volume is occupied by ~20,000 granules (13). The estimated free Ca²⁺ concentration in the granules has been reported to be 40–80 μM in PC12 (14) and 50–100 μM in chromaffin cells (15).³ Moreover, the granules accumulate 20–40 mM of total Ca²⁺, more than 99% in bounded form (11), which is in striking contrast to the ~100 nM Ca²⁺ concentration found in the cytosol, thus creating a Ca²⁺ concentration gradient of up to 10⁵-fold across the granule membrane. Therefore, the chromaffin granules contain far more Ca²⁺ than any other organelle, accounting for ~60% of the total calcium in the chromaffin cell (16). However, despite the fact that the Ca²⁺ released from secretory vesicles is probably the closest to its sensors for triggering motion and/or exocytosis, its real participation on cell physiology is still under debate. However, it is not easy to design an experimental approach in bovine chromaffin cells to demonstrate the role of intravesicular Ca²⁺ in these functions under physiological stimuli, such as acetylcholine, because they also promote massive Ca²⁺ entry and intracellular mobilization of Ca²⁺ from other sources. Bafilomycin A1 (Baf),⁴ a potent and highly specific inhibitor of the H⁺-ATPase (V-ATPase) (17, 18), is a powerful tool for studying the role of pH in vesicular Ca²⁺ turnover

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³ J. Alvarez, unpublished data.

⁴ The abbreviations used are: Baf, bafilomycin A1; TIRFM, total internal reflection fluorescence microscopy; EGFP, enhanced green fluorescent protein.

pH Gradient and Chromaffin Vesicles

because it directly alkalinizes chromaffin granules with few effects on other structures, at least in the nanomolar range (19).

We have found that bafilomycin A1-mediated alkalinization causes the release of Ca^{2+} from granules to cytosol, and this effect is able to increase the lateral motion of chromaffin granules and to trigger exocytosis, even in Ca^{2+} -free medium. Therefore, intravesicular Ca^{2+} may represent a novel source of Ca^{2+} capable of creating a local microdomain able to control both granule motion and exocytosis.

EXPERIMENTAL PROCEDURES

Materials—Urografin[®] was obtained from Schering España (Madrid, Spain). Culture plates were purchased from Nunc (Roskilde, Denmark). Lipofectamine[™] 2000, Oregon Green[®] 488 BAPTA-2, and trypsin-versene were from Invitrogen. OptiPrep[™] was from Axis-Shield Plc. All other drugs were obtained from Sigma. Salts used for buffer preparation were reagent grade.

DNA Constructs—Human pro-neuropeptide Y construct fused with EGFP (NPY-EGFP) was kindly provided by Dr. W Almers (Vollum Institute, Oregon Health & Science University) (20). Synaptobrevin 2, fused on the C terminus (luminal) to EGFP (VAMP2-EGFP) was the kind gift from Dr. R. W. Holz (University of Michigan, Ann Arbor, MI) (21).

Cell Culture and Transfection—Bovine chromaffin cells were isolated by adrenal medulla digestion with collagenase IA and further Urografin[®] centrifugation, as described (22). The cells were suspended in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 50 IU ml^{-1} penicillin, and 50 $\mu\text{g ml}^{-1}$ gentamicin and incubated at 37 °C and 5% CO_2 . For amperometry experiments, 5×10^4 cells were plated on 12-mm diameter glass coverslips. The cells were used at room temperature between 1 and 5 days of culture. To transfect bovine chromaffin cells, the Ca^{2+} phosphate precipitation method was used, as described (23). The cells were cultured on 6-well culture plates at 1.2×10^6 cells/ cm^2 and transfected with 0.5–1 $\mu\text{g ml}^{-1}$ plasmid DNA (VAMP2-EGFP). After transfection, for TIRFM experiments, the cells were removed from these dishes using a rubber policeman and replated on 25-mm \varnothing coverslips coated with calfskin collagen to promote cell adhesion. The experiments were performed 4–8 days after the preparation of cultures.

PC12 cells were maintained in 75-ml flasks at 37 °C/5% CO_2 in RPMI 1640 supplemented with 10% fetal calf serum and 5% horse serum. For transfection, PC12 cells were grown up to 60% of confluence for 2 days, then washed with phosphate-buffered saline, and detached with trypsin-versene. The cells were then replated on poly-L-lysine-coated coverslips ($n = 1.518$; Warner Instruments, Hamdem, CT), placed in 6-well plates and transfected with 2 μg of plasmid DNA (NPY-EGFP) using Lipofectamine[™] 2000 in accordance with the manufacturer's instructions.

Amperometry—Carbon fiber microelectrodes of 5- μm radius (Thornel P-55; Amoco Corp., Greenville SC) were prepared as described (24). Electrodes were calibrated in a flow cell and accepted for cell studies when the application of noradrenaline (50 μM) resulted in an oxidation current of 300–400 pA, which is reduced by 80–100 pA under stop flow conditions.

Amperometric measurements were performed with the carbon fiber microelectrode gently touching the cell membrane using an Axopatch 200B (Axon Instruments, Foster City, CA) (22). Signals were low pass filtered at 1 kHz and collected at 4 kHz using locally written software (LabView for Macintosh, National Instruments, Austin, TX). Data analysis was conducted using locally written macros for IGOR (Wavemetrics, Lake Oswego, OR) (25).

Cytosolic Ca^{2+} Measurement—Chromaffin cells plated onto 12-mm coverslips were loaded in standard Krebs-HEPES buffer containing 2 μM fura-2 AM for 45 min at room temperature. The cells were then washed for 45 min at room temperature and placed in a cell chamber mounted on the stage of a Zeiss Axiovert 200 microscope under continuous perfusion. Single cell fluorescence was excited at 340 and 380 nm (100 ms of excitation at each wavelength every 2 s, 10-nm bandwidth) using a monochromator (Cairn Res. Faversham, UK). Images of the emitted fluorescence, obtained with a 40 \times Fluor objective, were collected using a 400DCLP dichroic mirror and a D510/80 emission filter (Chroma Technology, Rockingham, VT) and then recorded by a ORCA-ER camera (Hamamatsu Photonics, Hamamatsu, Japan). Single cell fluorescence records were ratioed and calibrated into $[\text{Ca}^{2+}]_c$ values off-line, by using the Metafluor program (Universal Imaging Corporation, PA), as described (26). The experiments were performed at 37 °C using an on-line heater.

Evanescent Field Microscopy—TIRFM images were obtained by directing an argon ion laser (488 line; Melles Griot (Carlsbad, CA) model 35-LAP-431-208 or 3W Lexel (Fremont, CA) model 95) through a custom side port to a side facing dichroic mirror Q495LPw/AR, and a HQ500 LP emission filter (Chroma Technology) on an Olympus IX70 microscope (Olympus, Melville, NY). The beam was focused on the periphery of the back focal plane of a 100 \times 1.65 N.A. oil-immersion objective (Olympus); thus the laser beam was incident on the coverslip at 58.4–64.2° from the normal. Digital images were captured by a cooled CCD camera (SensiCam; Cooke, Romulus, MI) and acquired at 2 Hz. Cell perfusion was performed by using a computer-controlled perfusion apparatus (model DAD-6VM; ALA Scientific Instruments, Westbury, NY).

Image Analysis—Fluorescence intensity profiles, from the whole cell stacks, were plotted *versus* time. Local background, defined as the average fluorescence outside the region of interest, was subtracted in each frame. For individual granules, a circle of 0.9- μm diameter was drawn around the center, and the average fluorescence intensity therein was calculated. The local background was the average fluorescence outside the region. To avoid misinterpretation caused by z axis motion, changes in vesicular pH were conducted only on still labeled granules that did not collide with neighboring granules. These were tracked through time sequence stacks, as described (21, 27). A data set was generated containing the x - y coordinates of the granule positions from the stacks of images. The trajectory in the plane of the membrane was plotted for each granule. We used the distance of the median (x, y) of the data set to the furthest point to define the radius of a circle that would encompass every data point to calculate the maximum range of a given trajectory. The

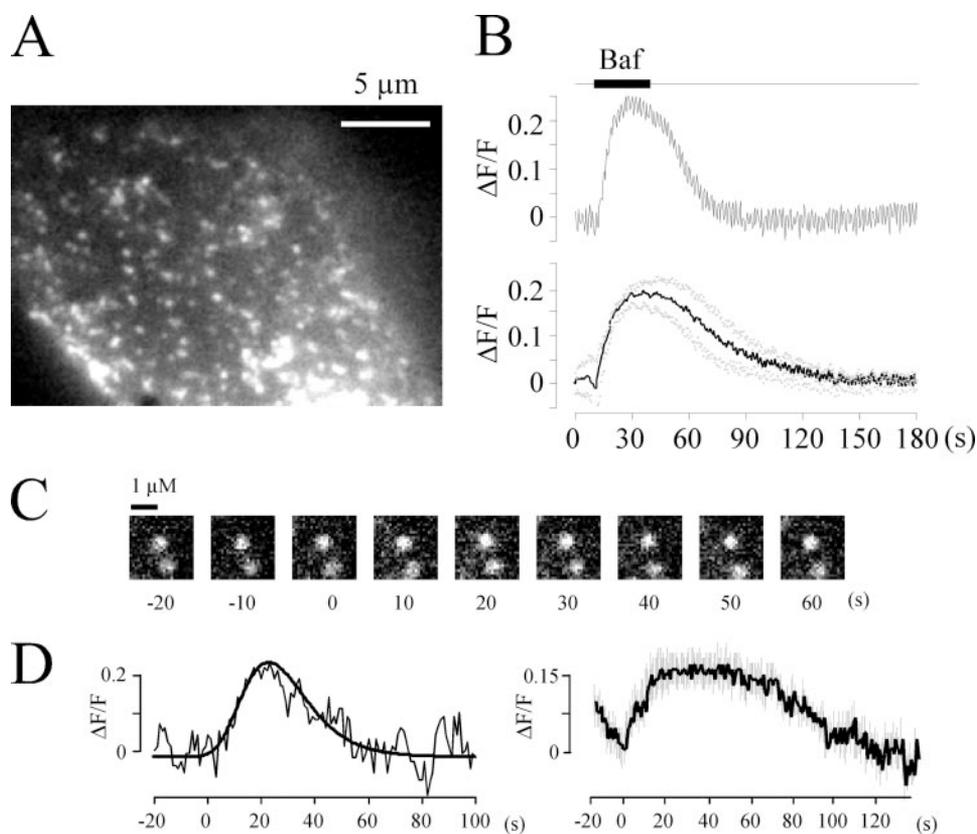


FIGURE 1. Bafilomycin A₁ alkalinizes chromaffin granules. *A*, a TIRFM image of a chromaffin cell transfected with VAMP2-EGFP. *B*, *upper trace* shows an original trace of fluorescence intensity of a movie acquired at 2 Hz during 3 min, and the *bottom traces* illustrate the average trace of the intensity \pm S.E. of fluorescence from four cells. *C*, time lapse for alkalinization of the intravesicular lumen in response to bafilomycin (100 nM) of a typical chromaffin granule expressing the intravesicular pH probe VAMP2-EGFP. Only vesicles situated in the bottom plane of the cell (150 nm deep) were visible. *D*, original trace of fluorescence intensity from the time-lapse in *C*, and average trace of the intensity of fluorescence \pm S.E. of fluorescence from 19 granules.

analysis of all the images was performed using MetaMorph (Molecular Devices, Sunnyvale, CA).

Purification of Bovine Chromaffin Granules—Chromaffin granules were obtained from homogenates of bovine adrenal medulla by differential centrifugation, as previously described (28). The chromaffin granules were separated from mitochondria on a discontinuous isosmotic density gradient of iodixanol (OptiPrep™) in water 60% (w/v) (29). The gradient consisted of two solutions of 8 and 16% (w/v) of OptiPrep in a solution of 0.3 M sucrose, containing 6 mM EDTA, 6 mM MgSO₄, and 60 mM HEPES, pH 7.0. The ultracentrifugation was conducted at 100,000 \times *g* for 1 h at 4 °C in a Beckman model L8–70 ultracentrifuge, with a type 50Ti fixed angle rotor. After centrifugation, the chromaffin granules were taken from the sediment, and resuspended in the intracellular solution containing 20 mM HEPES, 155 mM potassium glutamate, 0.1 mM MgCl₂, 10 mM glucose, 0.01 mM ascorbic acid, 2 mM ATP-Mg, 2 mM ATP-Na, pH 7 (KOH).

Calcium Release from Isolated Granules—Chromaffin granules were kept on ice before starting the experiments. Fluorescent of Oregon green BAPTA-2 on the solutions was monitored in a 3-ml polystyrene cuvette at 523-nm emission with 494-nm excitation wavelength, using a Cary Eclipse spectrofluorometer (Varian, Palo Alto, CA). The temperature was kept at 37 °C, and a magnetic stirrer ensured continuous mixing. An aliquot of 50

μ l of chromaffin granules at the concentration of 3 mg protein ml⁻¹, determined by the modified Folin-Lowry procedure (30), was added to the cuvette containing intracellular solution buffer and 1 μ M of the non-permeant dye Oregon green BAPTA-2. Different drugs were applied by micropipette in the Ca²⁺-free solution chromaffin granules suspension. The maximum of Oregon green BAPTA-2 fluorescence intensity was determined by adding 1% Triton X-100, and the minimum of fluorescence was determined by the addition of 5 mM EGTA.

RESULTS

Bafilomycin Transiently Alkalinizes Secretory Granules—Chromaffin cells expressing the protein VAMP2-EGFP were used to test the direct effect of the H⁺-ATPase inhibitor bafilomycin A1 on the pH of single granules. This protein is then sorted into secretory granules (21) exhibiting a pH-dependent fluorescence emission. Fig. 1*A* shows TIRFM images from cells exhibiting fluorescence spots close to the plasma membrane, which were identified as single large dense-core

granules. This approach was successfully used to monitor the intravesicular changes of pH. The application of Baf (100 nM) for 30 s in the vicinity of a cell produced a 2-fold increase in the cell fluorescence, indicating an alkalinization process (Fig. 1*B*, *upper trace*). This transient increase of the fluorescent emission was observed for ~50 s and then falling to the basal level. The averaged result for four cells is illustrated in the *bottom trace* of Fig. 1*B*.

We selected single granules as described under “Experimental Procedures” to analyze this phenomenon more extensively. Fig. 1*C* shows a series of time frames of a typical chromaffin granule expressing VAMP2-EGFP and the transient alkalinization caused by Baf. Thus, the transient fluorescence increase induced by Baf was also observed in individual granules. Fig. 1*D* shows the intensity profile of fluorescence from a single granule *versus* time (*left panel*), and the averaged fluorescence (background subtracted) obtained from 19 granules (four cells, *right panel*), which remained still for at least 120 s.

Bafilomycin Increases the Granule Motion—An unexpected observation from TIRFM experiments was that bafilomycin increased the lateral motion of chromaffin granules (see supplemental Movie S1 and Fig. 2*A*). In the presence of bafilomycin 80% of the vesicles were more mobile, and 36% increased their motility 2-fold. To explore the relation between the alkalinization and granule motion, we analyzed the mobility in the lateral

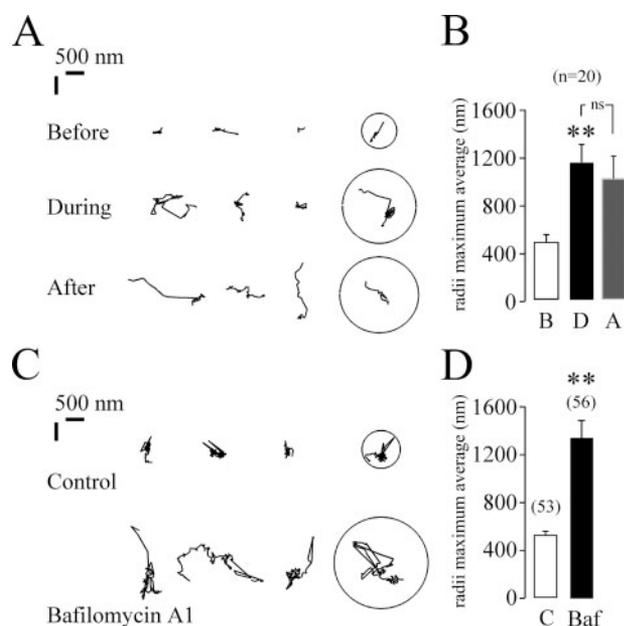


FIGURE 2. Alkalinization increases the motion of secretory vesicles. *A*, chromaffin cells expressing VAMP2-EGFP as the intravesicular probe were perfused with Baf. Labeled granules were identified and their position tracked via the sequence of images. The plot shows representative trajectories of chromaffin granules before, during, and after the application of 100 nM Baf. The circles represent the area with the maximum radius that includes the trajectory of the given granule. *B*, bar diagrams show the averages \pm S.E., of the radii of 20 granule trajectories before (*B*), during (*D*), and after (*A*) the application of Baf. *C*, examples of trajectories from PC12 granules labeled with NPY-EGFP. *D*, bar diagrams from averaged radii \pm S.E. Tracking come from 53 granules of seven control cells and from 56 granules of eight cells incubated with Baf. Calibration bars, 500 nm. **, $p < 0.01$, Kolmogorov-Smirnov test.

plane (parallel to the membrane) of single secretory granules. We used TIRFM stacks of chromaffin granules to track the position of single labeled chromaffin granules before, during and after the application of Baf. We only analyzed granules that did not collide with the neighboring granules, during the recording period. The mobility of each granule was analyzed by determining its x - y coordinate positions and then calculating the radius of the circle containing the trajectories. The plots from Fig. 2*A* show examples of four representative tracks of chromaffin granules before, during, and after the application of Baf. Fig. 2*B* shows a 43% increase in granule motion, observed on the addition of Baf in chromaffin cells. However, we could not conclude that changes in the fluorescence of given granules were associated with an increase in their motion because mobile granules also increased their z displacement that exponentially affected the light they emitted.

PC12 cells contain fewer granules than chromaffin cells. For this reason, it is fitting to follow their trajectories in the x - y plane. We transiently expressed the NPY-EGFP protein in PC12 cells that sorted into secretory granules, as previously described (20). TIRFM stacks were recorded at 2 Hz for 5 min in PC12 cells preincubated with or without Baf (100 nM). Fig. 2*C* shows examples of granule trajectories (supplemental Movie S2), and the averaged data are shown in Fig. 2*D*.

Bafilomycin A1 Releases Ca^{2+} from Intracellular Stores—Chromaffin cells loaded with fura-2 were used to test whether the alkalinization of secretory vesicles by Baf (50 nM) caused the release of Ca^{2+} . Fig. 3*A* shows a typical control cell (from 14

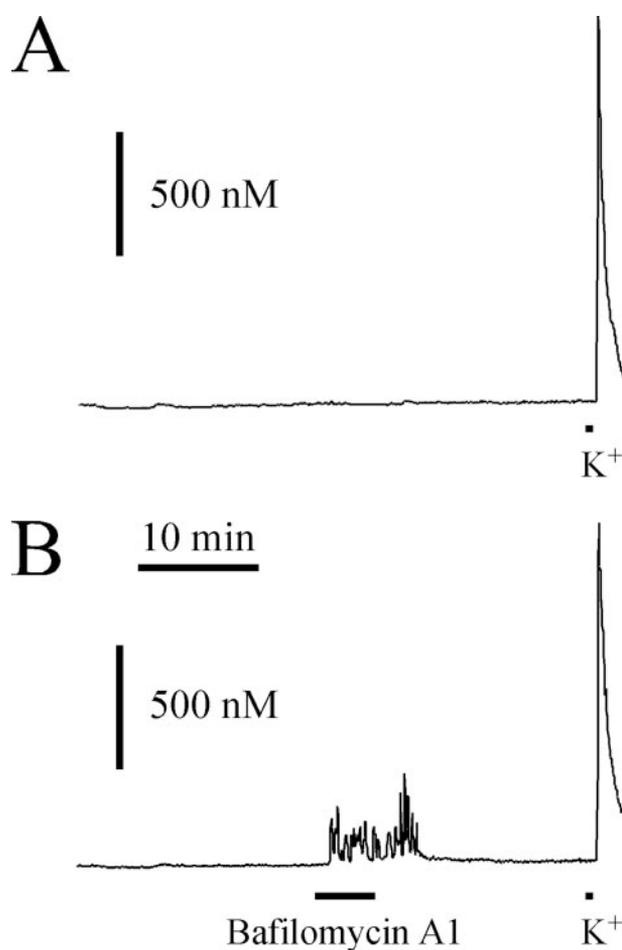


FIGURE 3. Bafilomycin A1 causes a transient elevation in the $[Ca^{2+}]_c$. Bovine chromaffin cells were loaded with fura-2, as described under "Experimental Procedures". *A*, typical fluorescent trace showing the stable base line of $[Ca^{2+}]_c$ and the effect of stimulation with K^+ (70 mM) by a single pulse of 30 s after 40 min of recording. *B*, effect of 5 min perfusion of bafilomycin A1 (50 nM), indicated by the solid horizontal line before the 30-s pulse of 70 mM K^+ .

cells) that had a stable base line for 40 min before receiving 30 s stimulation with K^+ (70 mM). Fig. 3*B* shows a typical recording of a cell (from 35) after the application of Baf for 5 min. Although the increase of cytosolic free $[Ca^{2+}]_c$ was observed in all the cells, the pattern of Ca^{2+} elevations varied from cell to cell and was oscillatory, as in Fig. 3*B*, or exhibited a stable plateau (not shown). In all the cases, the effects of Baf were reversible. The magnitude of the change was much lower than that evoked by K^+ , but the total amount of the Baf-induced released Ca^{2+} , throughout the stimulus (the integral of the effect of Baf), could be enough to cause biological effects. We observed a delayed onset of 95 ± 11 s between drug application and Ca^{2+} signals.

Alkalinization of Chromaffin Granules Causes the Release of Ca^{2+} —The experiments shown in Fig. 4 were conducted using fluorescence time lapse to address the question of whether Baf, by reducing the pH gradient across granule membrane, may trigger the release of intravesicular Ca^{2+} . Chromaffin-isolated granules were maintained in suspension in the cuvette of a spectrofluorometer at 37 °C, in a Ca^{2+} -free buffer containing 4 mM ATP (see "Experimental Procedures"). We used the non-

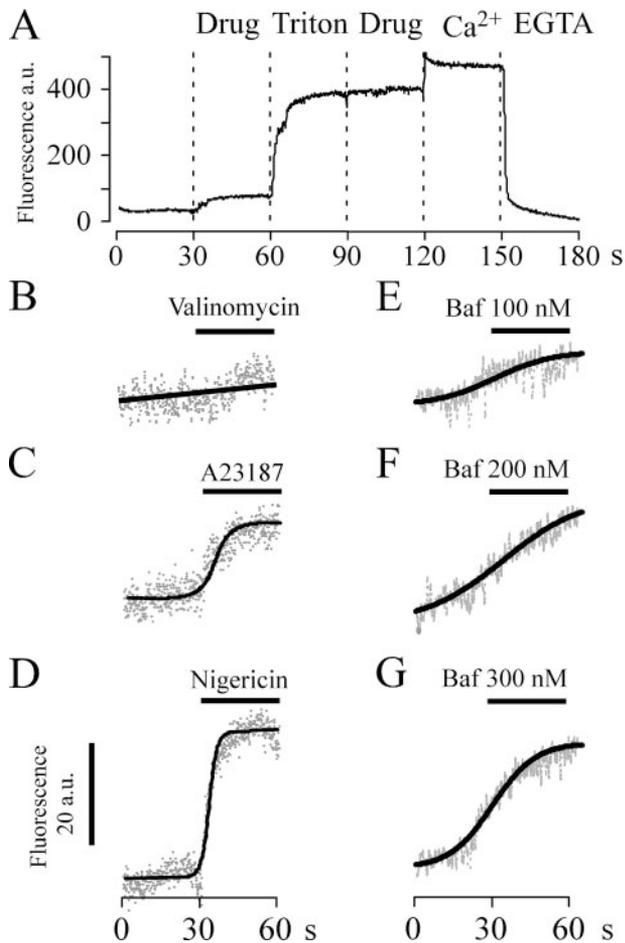


FIGURE 4. Release of vesicular Ca^{2+} *in vitro* elicited by various agents. A, diagram of the experimental protocol. Chromaffin granules were suspended in 2 ml of intracellular solution containing the nonpermeant dye Oregon Green[®] 488 BAPTA-2 (1 μM). B–E, the fluorescence was measured in a spectrofluorometer using excitation light at 494 nm, and the emission was set at 523 nm. 50 μl of granule suspension was added to a buffer containing 1 μM valinomycin (B), 25 μM of A23187 (C), 2 μM nigericin (D), or 100–300 nM bafilomycin A1 (E–G).

permeant dye Oregon Green[®] 488 BAPTA-2 to measure free Ca^{2+} . Basal fluorescence of the Ca^{2+} probe was monitored for 30 s. Subsequently, to assess the release of Ca^{2+} , a first round of drugs was added to the final concentration indicated in the legend of the Fig. 4. Then chromaffin granule membranes were ruptured by adding Triton X-100 (final concentration, 1%) to release all the intravesicular Ca^{2+} . No further effects were observed, if any of the drugs were added after Triton. Finally, to standardize the responses, 1 mM Ca^{2+} and 5 mM EGTA (final concentration) were successively added. This protocol resulted in a signal as illustrated in Fig. 4A, and the typical effects of the drugs are presented in Fig. 4 (B–G).

These results indicate that Ca^{2+} ionophore A23187 (25 μM) caused the leak of Ca^{2+} from the secretory vesicles. The release was monitored as a slow increase in the fluorescence that fits a sigmoid profile (Fig. 4C). Conversely, nigericin (2 μM), a H^+/K^+ exchanger ionophore that disrupts the pH gradient, caused the fastest and biggest Ca^{2+} displacement, out of granules (Fig. 4D). Then we observed that 100 nM Baf, in the same concentration used in intact chromaffin cells, promoted the release of Ca^{2+} from chromaffin granules (Fig. 4E) and Baf (300 nM) released

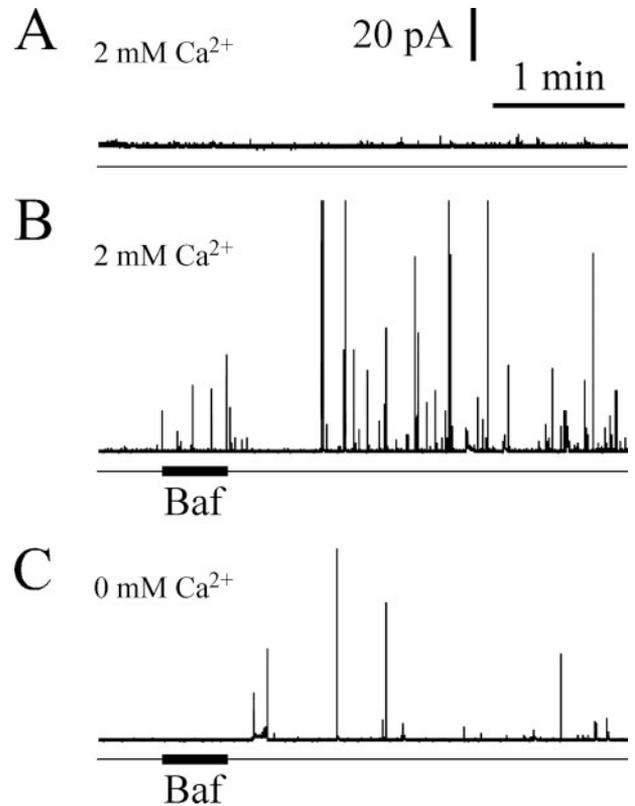


FIGURE 5. Bafilomycin produces exocytosis even in the absence of extracellular Ca^{2+} . Amperometrical record in response to: pressure ejection of Krebs-HEPES (A); bafilomycin A1 (100 nM) in the presence of physiological external Ca^{2+} (2 mM) (B); and bafilomycin A₁ (100 nM) in the absence of Ca^{2+} (5 mM EGTA added) (C).

Ca^{2+} to a similar extent as A23187 did (Fig. 4G), thus indicating the crucial role of the pH in the maintenance of the vesicular calcium. Then we observed that 100–300 nM Baf promoted the release of Ca^{2+} from chromaffin granules (Fig. 4, E–G). The Baf effects were concentration- and time-dependent, although a concentration of 300 nM was required to observe rapid and substantial effects (Fig. 4, E–G). However, valinomycin at 1 μM , an ionophore that does not modify the gradient of pH, did not significantly release Ca^{2+} from isolated vesicles (Fig. 4A).

Bafilomycin Triggers Exocytosis—To test whether the efflux of calcium from secretory vesicles has functional implications on the exocytosis, we performed amperometry experiments. Although TIRFM allows the monitoring of exocytotic events, they were seldom observed (see supplemental Fig. S1). Nevertheless, amperometry with carbon fiber microelectrodes recorded, at the level of single exocytotic events, the effects of bafilomycin on the release of catecholamines from bovine chromaffin cells.

A brief application of the Krebs-HEPES buffer did not elicited secretion, thus ruling out the possibility that secretion could be triggered by mechanical stimulation (Fig. 5A). However, a 30-s application of Baf (100 nM) triggered exocytosis both in the presence (Fig. 5B) and in the absence of external Ca^{2+} (5 mM EGTA; Fig. 5C). Although the frequency of exocytotic events, recorded along 4 min, was smaller in the absence of external Ca^{2+} (0.118 *versus* 0.350 Hz), their quantal characteristics were similar (data not shown).

DISCUSSION

The idea that intravesicular Ca^{2+} could be involved in the exocytotic process was first postulated by Borowitz in 1967 (31). Nevertheless, the physiological role of cell organelles in the homeostasis of Ca^{2+} is still currently under debate. Endoplasmic reticulum was considered as the main source of Ca^{2+} , and the mobilization of Ca^{2+} stores by IP_3 was first discovered in this organelle. However, the involvement of other organelles like mitochondria, nucleus, and Golgi in the uptake, release, and cytosolic redistribution of Ca^{2+} have also been recently proven (32). Even considering that secretory vesicles contain large amounts of Ca^{2+} (15) and that this cation is crucial for processes that take place “just across their membrane,” like vesicle movement or exocytosis, they have received little attention in terms of Ca^{2+} homeostasis. The main argument has been that vesicular Ca^{2+} is sequestered into the vesicular matrix and has little turnover. Intravesicular Ca^{2+} kinetics seems to follow a bi-compartmental model where the total amount of chelated Ca^{2+} is estimated to be $\sim 40 \text{ mM}$ (15), whereas free Ca^{2+} is ~ 3 orders of magnitude lower. This probably accounts for the rapid recovery of free Ca^{2+} after its depletion of the free compartment with caffeine or pH-disrupting agents like carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (14). The recent development of targeted aequorins inside secretory vesicles has directly confirmed that the free Ca^{2+} fraction inside these organelles is $\sim 20\text{--}40 \mu\text{M}$ (15) and can be mobilized by caffeine, ATP, or depolarizing stimuli (14, 15).

Secretory vesicles accumulate Ca^{2+} and catecholamines by specific antiporters that use H^+ as a counter ion. Several protonophores (33) or weak bases (12, 34, 35) induce both the alkalization of granules and the release of Ca^{2+} toward the cytosol. We have demonstrated that some drugs like hydralazine, which are accumulated in chromaffin granules, displace catecholamines and Ca^{2+} toward the cytosol (36). In addition, we have recently shown that the reduction of the pH gradient across the granule membrane produces a reduction in the catecholamine content of chromaffin granules (12). This paper has been aimed to test whether the modifications in the vesicular pH could promote the release of Ca^{2+} and to check whether the elevation of Ca^{2+} in the vicinity of granules also plays a role in the vesicle motion and exocytosis.

Bafilomycin is a highly selective blocker (in the nanomolar range) of the V-ATPase, which maintains the pH value near 5.5 in the inner vesicle (37). Indeed, it is a powerful tool to directly study the effects of vesicle alkalization in a reversible way. Although other cell organelles like lysosomes, endocytic vesicles, and early endosomes acidify their lumen by means of the V-ATPase, the cell volume occupied by chromaffin granules is large enough ($\sim 30\%$) to account for the main part of the observed effects of bafilomycin.

In this paper we have demonstrated that transient alkalization of granule lumen (Fig. 1) releases Ca^{2+} from granules (Fig. 4) toward the cytosol (Fig. 3) that drastically increases the movement of granules (Fig. 2) and promotes exocytosis (Fig. 5). There is increasing evidence about the ability of vesicles to trigger their own exocytosis by releasing their internal Ca^{2+} (35). In addition, the tight relation observed between the raise of vesic-

ular pH and the release of Ca^{2+} (33) and catecholamines (12) suggests that bafilomycin A1 causes a significant vesicular dissociation of Ca^{2+} from the matrix. Hence, the resultant elevated $[\text{Ca}^{2+}]_c$ triggers exocytosis. Therefore, these data provide strong evidence that intravesicular Ca^{2+} may represent a novel source of Ca^{2+} able to create a specific microdomain of Ca^{2+} in the exact location to control both granule motion and exocytosis.

Most of the vesicular Ca^{2+} seems to be closely associated with other soluble components (ATP, ascorbate, catecholamines, and chromogranins) inside the vesicle to form the matrix and to reduce the osmotic forces caused by these large amounts of osmotically active substances. However, the free fraction of Ca^{2+} reaches $40 \mu\text{M}$ (14), which is in equilibrium with the Ca^{2+} bound ($\sim 40 \text{ mM}$) (15), allowing a rapid recovery after an acute depletion like that caused by Ca^{2+} -induced Ca^{2+} release (14) or the alkalization mediated by the activation of several second messenger routes (12).

The main problem to demonstrate whether the intravesicular Ca^{2+} is actively participating in granule motion and exocytosis, under physiological conditions, is the difficulty in differentiating this Ca^{2+} from the Ca^{2+} arriving from other sources. All known secretagogues increase free cellular Ca^{2+} by activating its entry from external media and/or promoting its release from internal stores. We have shown here that vesicular alkalization induces Ca^{2+} release from the granules. Given that many second messenger routes activated by neurotransmitters and drugs have been reported to modify the vesicular pH, it seems plausible that the pH gradient across the vesicular membrane could be a necessary link between physiological stimuli and the regulation of Ca^{2+} release from the secretory vesicles.

Although bafilomycin is not a physiological stimulus, the results presented in this paper reveal a novel mechanism for the release of Ca^{2+} from secretory vesicles, which is controlled by vesicular pH. Cell stimulation by different mechanisms, mediated by either inositol triphosphate receptors, ryanodine receptors, or plasma membrane Ca^{2+} channels, has been reported to induce transient vesicular Ca^{2+} release (14). Other stimuli that activate guanylate cyclase or adenylate cyclase that alkalize the vesicular lumen might also mimic these mechanisms. The physiological relevance of the Ca^{2+} release from secretory vesicles will require further investigation.

In this paper, we demonstrate that transient changes in the pH gradient, through the vesicular membrane, cause a substantial release of Ca^{2+} . Taking into account the poor diffusion of Ca^{2+} through the cytosol (38), we consider it highly plausible that vesicular Ca^{2+} could be playing a relevant physiological role in the approach of the granule to the membrane (39, 40) and in its own exocytosis.

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