Cell Tissue Res (2001) 304:159–164
DOI 10.1007/s004410000320

SHORT COMMUNICATION

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Morphological and functional characterization of beige mouse adrenomedullary secretory vesicles

Received: 14 April 2000 / Accepted: 30 October 2000 / Published online: 1 March 2001

Abstract We tested whether the giant secretory granules observed in the mast cells of the naturally occurring mutant beige mouse (BM) (C57BL/6N-*bg*) were also present in the adrenal chromaffin cells. The presence of large chromaffin granules (CG) would be a valuable tool for the study of exocytosis in neuronal tissues. Conversely, the observation of large vesicles within chromaffin cells that are different from CG could indicate that CG are of a different origin than granules of mast cells. Ultrastructural analysis demonstrated the presence of large lysosomal-like vesicles in the BM, and also a discrete increase in the number of CG with diameters larger than 240 nm but not of giant CG. In addition, amperometric measurements of single-event exocytosis, using carbon fiber microelectrodes, showed no differences between the quantal size of secretory events from BM and wild-type or bovine chromaffin cells. Minor but significant differences were found between the kinetics of exocytosis in BM cells andwild-type mouse cells. We conclude that CG, but not the abnormal-sized vesicles found in BM chromaffin cells contribute to the catecholamine secretion and that abnormal secretory granules are not present in adrenergic cell lineage.

Keywords Amperometry · Exocytosis · Mast cells · Chediak-Higashi syndrome · Microelectrodes · Mouse, beige (C57BL/6N-bg)

This work was supported in part by CICYT (PB97-1483) and FEDER (1FD97-1065-C03-01). JFG is recipient of a fellowship from the Gobierno de Canarias

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Introduction

Adrenal medullary chromaffin cells release catecholamines (CA) into the bloodstream in response to stress situations. These cells have served as a biological model for the study of the stimulus-secretion coupling process of neural tissues because the mechanisms involved in secretion, the released products and their neuroectodermic origin are similar to those of adrenergic neurons (Winkler 1988; Ungar and Phillips 1983; Borges 1997).

Another popular model for the study of exocytosis is provided by mast cells, which release serotonin, histamine and other substances as part of anaphylactic reactions. The discovery of giant secretory vesicles within mast cells of beige mouse (BM) (Chi and Lagunoff 1975) prompted a series of elegant studies which, combining optical and electrophysiological techniques, have contributed a great deal to our knowledge of the exocytotic phenomenon (Oberhauser et al. 1996; Zimmember et al. 1987; Alvarez de Toledo et al. 1993).

The BM has been used as a model for the study of Chediak-Higashi syndrome (Penner and Prieur 1987). The features of this disease are partial albinism, photophobia, nystagmus, large eosinophilic, peroxidase-positive inclusion bodies in the myeloblasts and promyelocytes of the bone marrow, neutropenia, abnormal susceptibility to infection, and peculiarly malignant lymphoma. The disease results in defective membrane targeting of the proteins present in secretory lysosomes, and causes the presence of giant organelles within the cytoplasm of many cell types (Perou et al. 1996). This alteration is linked to the *TCRG* gene on BM chromosome 13 (Holcombe et al. 1987).

Mast cell granules exhibit some analogies with chromaffin granules (CG): they store high amounts of amines; the secretory products are complexed with intragranular matrix (heparan sulfates vs chromogranins); and their exocytotic kinetics vary in a similar way to changes in temperature or ionic environment (Pihel et al. 1996). However, mast cells are not closely related to neuronal lineage, and their very slow degranulation process does

not mimic that occurring in neuroendocrine tissues (Knight et al. 1989). It is possible that the genesis, sorting and maturation of both organelles follow different routes. If this were true, it could be that the presence of organelles, corresponding to mast cell granules, might share the cytosolic space with CG in the chromaffin cell.

There are a few unsolved questions regarding the exocytotic phenomenon; the solutions are limited by the smallness of CG (Moser and Neher 1997). These questions relate to the nature of the fusion pore (Oberhauser et al. 1996), the functional role of the intragranular matrix (Borges et al. 2000) and to whether partial fusion is the result of complete or partial CA release (Albillos et al. 1997). If giant CG were found in BM chromaffin cells, they would be a very valuable model for the study of the exocytotic phenomenon.

The present study had two aims: (1) to investigate whether the mutation that causes the presence of giant granules in some cell types of BM (Blume et al. 1969; Oliver and Essner 1973; Jensen et al. 1997) may also affect the secretory vesicles of neuroendocrine cells and (2) to test whether this mutation affects the sorting of a different organelle, indicating that mast cell granules and CG have different origins (Arvan and Castle 1998). To address these questions we have conducted morphometric and amperometric studies on cultured chromaffin cells from BM and compared the results with those obtained in murine cells from wild-type mouse and in bovine cells.

Materials and methods

Animals

Homozygous beige mice (C57BL/6N-*bg*) were obtained from Jackson Laboratories (Bar Harbor, Me., USA) and maintained in a local animal house. Control mice (Swiss, OF1) were purchased from IFFACREDO (Barcelona, Spain). All animal procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of La Laguna University.

Chromaffin cell culture

Bovine adrenal chromaffin cells enriched in adrenaline through a single-step Urografin gradient were prepared as described elsewhere (Moro et al. 1990). Cells were plated onto 12-mm-diameter glass coverslips contained in 24-well culture plates at an approximate density of 5×10^5 cells/well. Murine adrenal chromaffin cells were isolated and cultured following the procedure described previously for the rat (Gandía et al. 1995). Cells were maintained at 37°C in a water-saturated, 5% CO_2 environment and were used at room temperature within 1–5 days of isolation.

Microscopy studies

Mast cells were obtained from peritoneal washing and from ear skin. Specimens were fixed in 4% formaldehyde solution and stained by the Papanicolaou method. Adrenal medullae were excised from both beige and control mice and incubated in Locke's solution containing 4% formaldehyde. Tissues were immersed in paraffin and stained with hematoxylin-eosin.

Murine adrenal glands were fixed in 1% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.3 for 2 h. After fixation, tissues were postfixed in 1% osmium tetraoxide, dehydrated in alcohol and embedded in Epon. Thin sections were stained with lead and uranyl acetate and examined in an electron microscope. Chromaffin granule and clear vesicle sizes were quantified by direct measurement of diameters from calibrated micrographs (×25,500). Measurements of about 20 granules/photograph were done in duplicate and performed double blind. The total number of clear vesicles with a diameter larger than 200 nm was determined by counting.

Amperometric detection of exocytosis

Carbon fiber microelectrodes were prepared as described by Kawagoe et al. (1993). Carbon fibers of 5 µm radius (Thornel P-55; Amoco Corp., Greenville, S.C., USA) were kindly donated by Prof. R.M. Wightman, University of North Carolina at Chapel Hill, USA. Electrochemical recordings were performed using an EI-400 potentiostat (Ensman Inst., Bloomington, Ind., USA) The potential of the carbon fiber electrode vs a Ag/AgCl pellet reference electrode was +650 mV. Electrodes were tested using a flow injection system.

Glass coverslips with adhering cells were placed in a perfusion chamber mounted on the stage of an inverted microscope (DM-IRB Leica, Weltzlar, Germany). Cells were incubated in a Krebs-HEPES solution containing (mM): NaCl (140); KCl (5); $MgCl_2$ (1.2); $CaCl_2$ (2), HEPES (10), and glucose (11), pH 7.35 (NaOH). Carbon electrodes were gently touched against the cell membrane. Cell secretion was stimulated by pressure ejection of 5 mM $BaCl_2$, 5 s duration pulses, from a glass micropipette situated 40 µm from the cell.

Amperometry data analysis

Amperometric signals were low pass filtered at 400 Hz, sampled at 4 KHz using a PCI-1200 card, and collected using software written locally for Labview (National Inst., Austin, Tex., USA) on a G3 Apple-Macintosh computer. Data analysis was carried out using locally written macros for IGOR (Wavemetrics, Lake Oswego, Ore., USA). These macros allow automatic digital filtering and secretory spike identification and construct histograms of the kinetic parameters used for spike classification (Segura et al. 2000). The macros can be obtained at no charge from the web site (http://webpages.ull.es/users/rborges).

Several kinetic parameters were measured from each secretory spike (Jankowski et al. 1993): (1) the maximum amplitude (I_{max}) of the oxidation current, expressed in pA; (2) the granule charge (Q), expressed in pC, was obtained as the area under the curve. Q can be normalized as the cubic root ($Q^{1/3}$) because it reflects the vesicle size better, the sphere volume being proportional to the third power of its radius. (3) The width of the spike at the half of its height is calculated and expressed in ms ($t_{1/2}$).

Materials

Cultured media, fetal calf serum and collagenase type IA were purchased from Sigma (Madrid, Spain). Urografin was purchased from Schering (Spain). All salts used for buffer preparation and chemicals employed for microscopy studies were reagent grade.

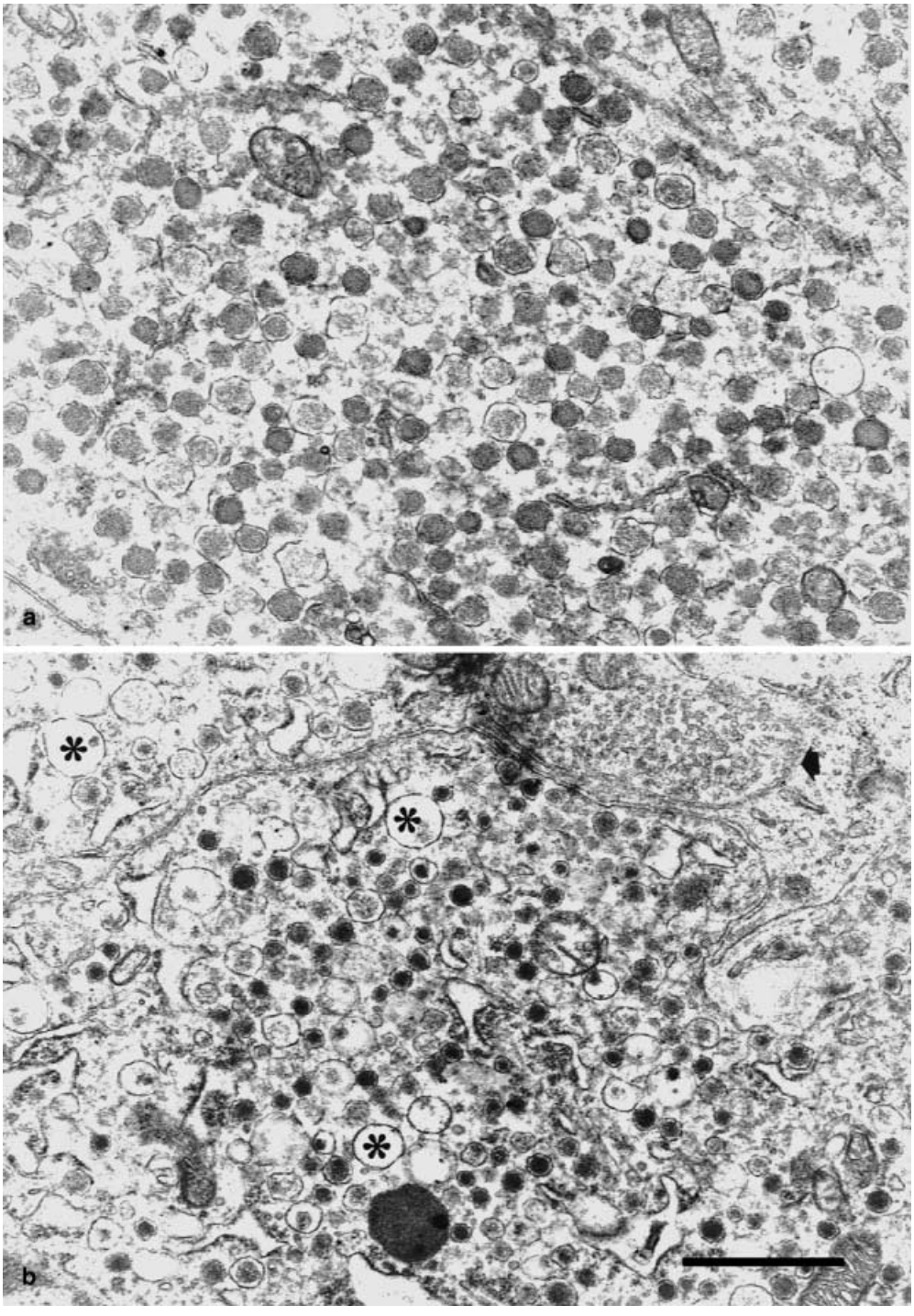

Fig. 1a, b Electron micrographs from adrenal medullary tissues. **a** Control cells from a Swiss mouse. **b** Chromaffin tissue from a beige mouse. A cholinergic terminal is indicated by an *arrow*. *Asterisks* indicate lysosomal-like vacuoles. *Bar* 500 nm

Results and discussion

Microscopy observations on BM tissues showed abnormal granule size in mast cells but not in chromaffin cells

The presence of abnormal granule size within BM mast cells was confirmed by light microscopy. Mast cells, whether obtained from ear skin or by peritoneal washing, had a reduced number of granules, 8–20/cell (n=25), and they were 2.0±0.3 μm (mean±SEM) in size. In contrast, mast cells from control mice contained a larger number of granules, which were 0.6±0.2 μm in size. The number of granules present in single sections of normal mice cells could not be precisely counted, but estimated to be around 80–100.

In the adrenal gland, light-microscopy examination revealed no abnormal granules in cells from either control mice or BM, indicating that they were too small to be distinguished from the background.

Examples of electron-microscopy images from adrenomedullary tissues are shown in Fig. 1. No appreciable differences were observed in the cell sizes or in their relations with other structures, such as endothelial cells or nerve terminals. Chromaffin granule distribution was similar, and no consistent differences between granule matrix or their relation with membrane were found.

Morphometric analysis was conducted to evaluate the diameter of CG. Data were obtained from chromaffin cells from five different adrenals of BM and five of control mice. Figure 2a shows the histogram of normalized CG size distribution. There was a clear displacement to the right on the histograms, corresponding to the population of CG larger than 240 nm in BM compared with control. However, CG number and size differences were within the range considered normal, and not as different as observed in the case of mast cells. Granules measured from control mice and BM, diameter sizes were (means± SEM) 151±1.9 nm (control, *n*=455) vs 173±2.6 nm (beige, *n*=832). This observation was consistent in all the preparations studied and is not easy to explain, but it is possible that some of the mechanisms involved in the granule package could be involved. Also, it is plausible that there were intrinsic differences derived from the natural origin of both mouse strains. Although both animal types belong to the *Mus musculus domesticus* subspecies, they have been separate for several decades of laboratory breeding.

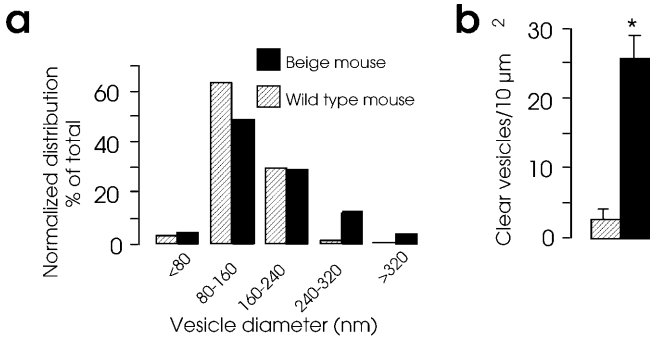

Fig. 2a, b Morphometric studies on chromaffin cell organelles. **a** Normalized CG size distribution in BM and control adrenal chromaffin cells. *Abscissa* shows the granule distribution at the ranges indicated. Diameters were measured from EM micrographsy. **b** Clear vesicles >200 nm in diameter (means±SEM) counted from EM photographs (5 sections/gland; 4 mice of each type), obtained from adrenomedullary sections. **P*>0.01 Student's *t*-test

Large vacuoles were present in BM but not in wild-type mouse chromaffin cells

Large, clear vesicles are seldom observed in normal mice. However, abundant large, lysosomal-like vacuolar organelles were observed within BM cells. They did not contain any intragranular dense matrix and were not closely associated with the cell membrane (Fig. 1b, asterisks). Figure 2b shows the different distributions of large vacuolar structures (diameter over 200 nm) within control and BM chromaffin cells.

The presence of normal CG and abnormally large clear vesicles (Figs. 1, 2) suggests that the sorting process of vesicles in the BM is also altered in chromaffin cells. It could indicate that clear vesicles, probably lysosomes, but not CG, are the counterpart of mast cell granules in chromaffin cells. These large vacuolar structures do not contribute to the amperometric signal, or the amount of CA released is too small to be detected by the electrode. Amperometric data discounted the idea that clear vesicles, even if they are similar to mast cell granules, can accumulate CA. This may be due to the lack of uptake mechanisms and granular matrix capable of concentrating neurotransmitters.

Amperometric analysis of exocytosis revealed no differences in the quantal release of adrenaline

When chromaffin cells were stimulated with a brief pulse of Ba^{2+}, secretion was observed as secretory spikes corresponding to single exocytotic events. Owing to the large variations observed within spikes, even from the same cell, it becomes necessary to compute several hundred events to perform statistical analysis of the kinetic parameters. As shown in Table 1, each of the three animal cells studied exhibited a similar spike-firing frequency throughout the 6 min of recording time. In addition, the onset of secretions observed in the three species occurred within 2–3 s upon the end of Ba^{2+} application. These data suggest that the number of readily releasable CG present in the BM was similar to the numbers in wild-type mouse and cow.

To ensure correct determination of the kinetic characteristics of secretory events, overlapping or very noisy

Table 1 Kinetic characteristics of secretory spikes. Data (means±SEM) were obtained from Fig. 3

*, # Significant differences from values in cells from *cows and # control mice (*P*<0.01, U-Mann-Whitney test)

	Spikes/6 min	I_{max} (pA)	$t_{1/2}$ (ms)	Q (pC)	$Q^{1/3}$
Bovine *n*=549	161±15	31.8±1.8	27.0±0.9	1.01±0.06	0.91±0.02
Control *n*=291	173±12	24.5±3.7*	39.4±2.1*	1.09±0.12	0.94±0.01
Beige *n*=286	158±8	38.8±2.3#	23.0±1.0#	1.12±0.06	0.96±0.02

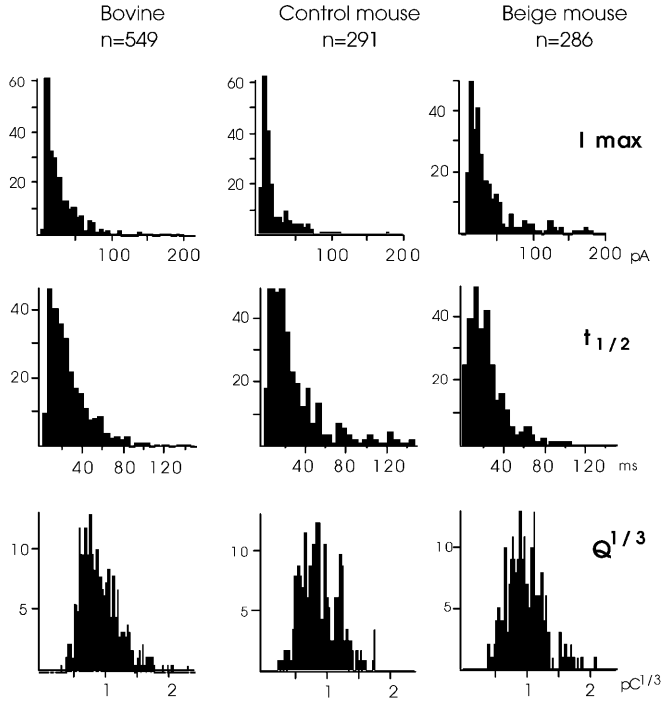

Fig. 3 Secretory spike characteristics. Histograms from secretory spikes were obtained under the same conditions from adrenal chromaffin cells in cow, control (Swiss) mice and beige mice. Data are from 18, 12 and 20 different cells, respectively. The number of spikes counted in each group is indicated at the *top*; overlapped, very noisy or distorted spikes were not analyzed. *Columns* at the *right* of each graph indicate an increase in the number of higher (I_{max}), wider ($t_{1/2}$) or larger ($Q^{1/3}$) spikes. $Q^{1/3}$ results from the cubic root of Q data (see Materials and methods). Averaged data are summarized in Table 1

spikes were discarded. This explains the apparent discrepancies found between the number of spikes counted within 6 min of recording and those used for the measurements of kinetic parameters (Table 1).

Histograms corresponding to the kinetic characteristics of secretory spikes are shown in Fig. 3. Values for the maximum oxidative current (I_{max}), the spike width at the half height ($t_{1/2}$) and the net charge taken from the area under the spike trace (Q) were measured. These parameters roughly indicate the concentration of CA at the electrode tip (I_{max}), the releasing speed of quantal packets ($t_{1/2}$) and the apparent CG adrenaline content (Q). Assuming that the intragranular concentration of CA is homogeneous throughout a granule population, Q should be proportional to the granule size. For this reason, because size is proportional to the third order of vesicle radii, a normalization of Q data can be achieved by taking the cubic root of the charge, so that this normalization is represented as $Q^{1/3}$.

Values reported for $Q^{1/3}$ in the literature have varied, depending on the laboratory of origin and the experimental conditions. For instance, in a previous study we found larger Q values than reported here, but the values were similar for rats and cows: 1.14 and 1.09 $pC^{1/3}$, respectively (Finnegan et al. 1996). A similar size was reported by Alés et al. (1999) for the rat: 1.15 $pC^{1/3}$. Conversely, other authors have found smaller values for mice: 0.54 $pC^{1/3}$ (Moser and Neher 1997). Secretory spikes from BM occurred with a faster kinetic than that observed in control animals and, although statistically different, they can be considered normal values.

Since two different vesicles were observed by electron microscopy in the BM, the idea that both of them store and release CA seems plausible. If this were so, histograms of Q would exhibit a bimodal distribution showing that amperometric signals from giant CG produce spikes with large charges. Conversely, spikes resulting from large clear vesicles would have a small charge and a short $t_{1/2}$ provided that they did not have an intravesicular matrix (chromogranin A) capable of concentrating CA to the submolar levels observed in CG. Although a second group of bigger spikes seems to appear in the $Q^{1/3}$ histogram, no statistical differences were observed between any two of the three types of cells studied (Fig. 3). The comparison between bovine cells and cells from wild-type mice does not support the existence of two different secretory vesicles or the presence of a significant population of larger functional CG.

Spike characteristics are summarized in Table 1. On average, secretory spikes from BM had a larger I_{max} and a shorter $t_{1/2}$ than normal mice. Comparisons of mice with bovine chromaffin cells were pertinent because this species is the most widely used for secretory studies. Although there were slight differences among the three types of cells studied, these can be attributed to variability in the experimental conditions, such as electrode sensitivity or cell responsiveness.

BM chromaffin cells do not have any major value as a model for the study of exocytosis, even though these cells could be of interest for the study of the intracellular vesicle sorting routes. This paper is one of the few available reporting on an investigation on mouse adrenal secretion. Because work on single chromaffin cells has overruled the need for large numbers of cells, mouse adrenals offer an interestingattractive model for the study of exocytosis. In addition, the increasing number of available transgenic mice offer an interesting field of research on a wide range of aspects related to catecholaminergic systems, such as exocytotic machinery, receptors, ion channels and amine carriers.

Recent technological improvements have allowed partial visualization of CG fusion (Terakawa et al. 1993) and single-event analysis by cell-attached capacitance combined with amperometry (Albillos et al. 1997). However, the smallness of CG and sympathetic dense core vesicles does not allow characterization of the exocytotic process with the fine resolution carried out in BM granules (Marszalek et al. 1997). For this it would be necessary to find a biological model with secretory vesicles large enough to conduct kinetic analysis combined with direct observation of granule fusion. Unfortunately, the lysosomal-like vesicles observed in the BM were not CG, and they lacked an intravesicular matrix. In addition, true CG observed in these cells were indistinguish-

able from CG of control mice. Moreover, in contrast to that observed in mast cells, the total number of CG present in both animal types was similar, suggesting that chromaffin cells did not show differences in their CG biogenesis.

A cell contains from 15,000 to 30,000 CG. The intragranular CA concentration has been estimated at 0.5–1 M (Schroeder et al. 1996; Albillos et al. 1997). In addition, other soluble components, such as ATP, ascorbate and peptides, are co-stored and released together with CA. The intriguing nature of the mechanism used by CG, complexing these components to maintain the intragranular environment isotonic with the cytoplasm and the functional role of the intragranular matrix in the exocytotic process have received considerable attention from scientists. However, these problems, together with the elusive characterization of the nature of the fusion pore in neuroendocrine cells, arise partly from the small size of CG. For these reasons, an animal model which possesses large CG will bring about a great advance in our knowledge of the exocytosis process.

This study suggests that mast and chromaffin granules come from a different sorting route and that only the former are altered in BM and, probably, in patients with Chediak-Higashi syndrome.

Acknowledgments We thank Dr. F. Valladares for his help with the initial part of the work and Drs. A.D. Powel and M.A. Brioso for their critical reading of the manuscript.

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