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VESICLE MOVEMENTS ARE GOVERNED BY THE SIZE AND DYNAMICS OF F-ACTIN CYTOSKELETAL STRUCTURES IN BOVINE CHROMAFFIN CELLS

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Abstract—Dense vesicles can be observed in live bovine chromaffin cells using fluorescent reflection confocal microscopy. These vesicles display a similar distribution, cytoplasmic density and average size as the chromaffin granules visualized by electron microscopy. In addition, the acidic vesicles labeled with Lysotracker Red comprised a subpopulation of the vesicles that are visualized by reflection fluorescence. A combination of fluorescence reflection and transmitted light images permitted the movements of vesicles in relation to the cortical cytoskeleton to be studied. The movement of vesicles located on the outside of this structure was restricted, with an apparent diffusion coefficient of $1.0\pm0.4\times10^{-4}$ $\mu m^2/s$. In contrast, vesicles located in the interior moved much more freely and escaped from the visual confocal plane. Lysotracker labeling was more appropriate to study the movement of the faster moving vesicles, whose diffusion coefficient was five times higher. Using this type of labeling we confirmed the restriction on cortical movement and showed a clear relationship between vesicle mobility and the kinetics of cytoskeletal movement on both sides of the cortical cytoskeleton. This relationship was further emphasized by studying cytoskeletal organization and kinetics. Indeed, an estimate of the size of the cytoskeletal polygonal cages present in the cortical region and in the cell interior agreed well with the calculation of the theoretical radius of the cages imprisoning vesicle movement. Therefore, these data suggest that the structure and kinetics of the cytoskeleton governs vesicle movements in different regions of chromaffin cells.

Key words: exocytosis, confocal microscopy, adrenomedullary cells, cytoskeleton, vesicle transport.

Chromaffin granules that contain catecholamines are synthesized in the internal membrane biogenesis pathway of the ER and Golgi systems, and they must be transported to the cell periphery where the stored materials can be released by exocytosis (Winkler, 1977). Therefore, the kinetics of vesicle displacement exerts a fundamental influence on how neuroendocrine cells accomplish their function. Indeed, the existence of large reservoirs and the capacity to replenish the released vesicles is a remarkably feature that differentiates these cells from other excitable cells such as neurons. Vesicle transport studies have been based on the visualization of such organelles using fluorescent markers, which include dyes which accumulate in these acidic vesicles like FM-1-43, Acridine Orange or Lysotracker Red (Smith and Betz, 1996; Steyer et al., 1997; Becherer et al., 2003), or through the expression of green fluorescent protein (GFP) coupled to vesicular proteins (Lang et al., 1997). Such fluorescent markers were initially analyzed by dynamic conventional epifluorescence or confocal microscopy. More recently, the introduction of evanescent wave microscopy has facilitated a more in depth characterization of vesicle mobility in the immediate proximity of the plasma membrane, demonstrating an increasing restriction in the movements of chromaffin granules as they reach their secretory sites (Steyer and Almers, 1999; Oheim and Stuhmer, 2000; Johns et al., 2001). Theoretically, this limitation in vesicle mobility could be attributed to the presence of tethering or docking elements that interact with vesicles in the proximity of the plasmalemma (Waters and Hughson, 2000; Toonen et al., 2006). However, they could also be the product of a major change in the density of cytoskeletal structures in the cortical region which would limit granule movement by reducing the physical space in which vesicles could be displaced (Steyer and Almers, 1999; Oheim and Stuhmer, 2000). In this sense, the role played by cytoskeletal elements in these processes remains under debate since the use of chemical agents that affect F-actin or myosin have been reported to cause variable impact on vesicle mobility (Oheim and Stuhmer, 2000; Neco et al., 2004; Allersma et al., 2006). However, the relative lack of specificity of such chemicals may be a key factor when interpreting these conflicting results. In the interior of the cytosol, the coordinated activity of cytoskeletal structures such F-actin filaments and microtubules is likely to be involved in vesicle transport, and it will be important to determine how these events are controlled in order to fully understand the early stages of these secretory cycles (Neco et al., 2003).

We recently reported that dynamic changes in the structure and function of the F-actin-myosin II cytoskeleton during secretion could be studied using transmitted light in confocal microscopes (Giner et al., 2005). In this work, optical changes in the gel/sol properties of the cytoplasm allowed us to visualize the polygonal cages that constitute the cytoskeletal network in the cytoplasm of chromaffin cells. We now use this technique in combination with vesicle tracking by fluorescence reflection or epifluorescence confocal microscopy to analyze the dynamics of vesicle

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; MSD, mean square displacement; PBS, phosphate-buffered saline; ROI, region of interest; TIRFM, total internal reflection fluorescence microscopy.

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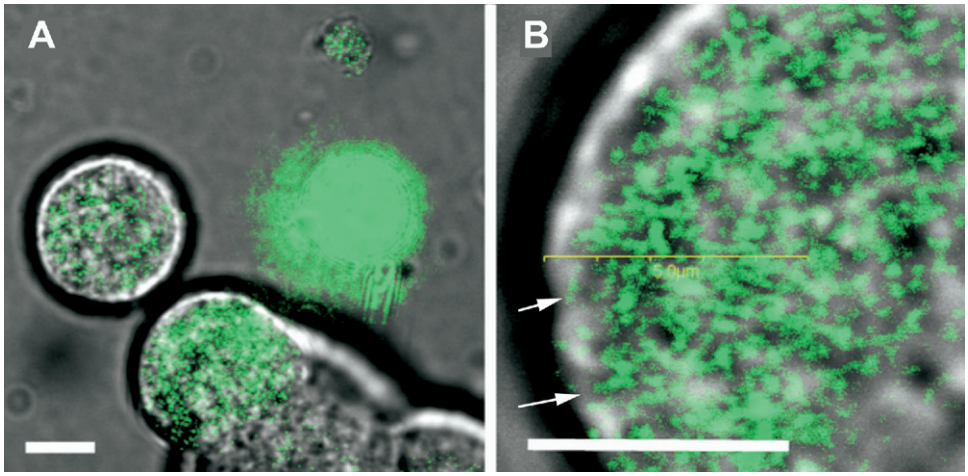

Fig. 1. Chromaffin cells visualized by reflection of fluorescence and transmitted light using a confocal scanning microscope. Images of chromaffin cells were obtained on an Olympus IX-70 microscope using a confocal Fluoview FV300 system incorporating a 100× Plan Apo oil objective. (A) Transmitted light images showing the F-actin network forming a cortical structure of greater density and extending into the cytoplasm. Fluorescence reflection is visualized in green and forms a typical circular pattern in the center of the reflecting dichroic mirror. (B) Detail of the image A, showing a magnification of the cortical region, in which the polygonal cages forming the cytoskeletal structure in the interior of the cell can be seen. Vesicular structures reflecting fluorescence are seen in different areas of the cytosol. Arrows indicate areas of the cortical barrier with lower density. Scale bar=10 μm.

transport in different regions of chromaffin cell cytosol, regions characterized by their distinct cytoskeletal organization. Our results, support the notion that the organization of the cell cytoskeleton, and specifically the dynamics and size of the cytoskeletal cages, are major factors that affect chromaffin granule dynamics on both sides of the cortical F-actin barrier.

EXPERIMENTAL PROCEDURES

Isolation and culture of bovine chromaffin cells

Chromaffin cells were isolated from bovine adrenal glands following collagenase digestion and they were separated from the debris and erythrocytes by centrifugation on Percoll gradients as described elsewhere (Almazan et al., 1984; Gil et al., 1998). Cells were maintained in 35 mm Petri dishes as monolayer cultures with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 10 μM cytosine arabinoside, 10 μM 5-fluoro-2′-deoxyuridine, 50 IU/ml penicillin and 50 μg/ml streptomycin (500,000 cells/dish, Corning Inc., Corning, NY, USA). The cells were used between the second and fourth day after plating and all experiments were performed at room temperature (21–22 °C). Chemicals were obtained from Sigma (Sigma Co., Madrid, Spain).

Confocal dynamic images of chromaffin granules and the F-actin cytoskeleton

Fluorescent reflection and epifluorescence was investigated using an Olympus Fluoview FV300 confocal laser system mounted on a IX-71 inverted microscope incorporating a 100× Plan Apo oil-immersion objective with 1.40 n.a. Excitation was achieved using Ar and HeNe visible light lasers. This system permits *z* axis reconstruction (0.5–0.55 μm theoretical z slice) and dynamic time lapse studies, with a time resolution ranging from 0.1 s and with 200×150 pixel image acquisition (adequate for region studies) to about 0.6 s for images of 400×300 pixels (for visualization of the entire cell). Simultaneous acquisition of transmitted light images provided information of the distribution of the F-actin cytoskeleton and they were obtained using the channel implemented in the confocal microscope and using bright field optics (the theoretical depth of field is 0.55–0.60 μm, see Giner et al., 2005). The intensity of this channel was adjusted to avoid saturation of the sub-cortical region and to visualize the lighter cytoplasmic structures. Chromaffin granules are visualized by fluorescent reflection using 488 nm Ar laser illumination and a fluorescein filter set without neutral filters limiting the past of the reflection of fluorescence in the dichroic mirror. It is easily obtained in a confocal microscope by varying a bit the angle of the dichroic mirror and eliminating neutral filters in one of the emission channels. When it is correctly achieved we visualize an annulus of reflection in the center of the image (see Fig. 1). To study chromaffin granules using epifluorescence the cells were incubated with the acidic Lysotracker Red dye (1 μM, Invitrogen Corporation, Carlsbad, CA, USA) for 15 min at room temperature in DMEM. After extensive washing, the cells were visualized at a laser excitation of 543 nm and using rhodamine filters in the emission pathway.

In other experiments, the distribution of F-actin and the granules was studied after fixation. Briefly, cells were fixed using 4% paraformaldehyde (PFA) in phosphate-buffered saline solution (PBS, of composition in mM; NaCl 140, KCl 3.35, Na_2HPO_4 1, KH_2PO_4 1.84 and pH 7.2) for 20 min and then permeabilized with 0.2% Triton X-100 in 3.6% formaldehyde for 10 min. The cells were incubated overnight with a rabbit antiserum against bovine dopamine β-hydroxylase (Chemicon International Inc., Temecula, CA, USA) diluted 1:200 in PBS 1% BSA and after extensive washing, this antiserum was detected over 2 h with a goat anti-rabbit Cy2-coupled antibody diluted 1:200 (Amersham Inc., Madrid, Spain). Control staining was performed by detecting pre-immune serum with the secondary antibody mentioned above. After extensive rinsing with PBS, cells were incubated for 15 min with a solution of 0.5 μg/ml of rhodamine–phalloidin in PBS to stain F-actin. Other chemicals that were employed to modify actin and myosin were used as described previously (Neco et al., 2003, 2004; Giner et al., 2005).

Electron microscopy of chromaffin cells

Electron microscopy of chromaffin cells was performed as described elsewhere (Gutierrez et al., 1997). Briefly cells (2×10^6 cells/well) were collected by low speed centrifugation (1000×*g* for 2 min) and fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.05 M cacodylate buffer at pH 7.3 for 2 h. The pellets