Real-time dynamics of the F-actin cytoskeleton during secretion from chromaffin cells

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Summary

Transmitted light images showed an intricate and dynamic cytoplasmic structural network in cultured bovine chromaffin cells observed under high magnification. These structures were sensitive to chemicals altering F-actin-myosin and colocalised with peripheral F-actin, β-actin and myosin II. Interestingly, secretagogues induced a Ca2+-dependent, rapid (>10 second) and transitory (60-second cycle) disassembling of these cortical structures. The simultaneous formation of channel-like structures perpendicular to the plasmalemma conducting vesicles to the cell limits and open spaces devoid of F-actin in the cytoplasm were also observed. Vesicles moved using F-actin pathways and avoided diffusion in open, empty zones. These reorganisations representing F-actin transfer from the cortical barrier to the adjacent cytoplasmic area have been also confirmed by studying fluorescence changes in cells expressing GFP-β-actin. Thus, these data support the function of F-actin-myosin II network acting simultaneously as a barrier and carrier system during secretion, and that transmitted light images could be used as an alternative to fluorescence in the study of cytoskeleton dynamics in neuroendocrine cells.

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Key words: Exocytosis, Confocal microscopy, Adrenomedullary cells, Cytoskeleton, Vesicle transport

Introduction

Understanding the role of the cytoskeleton during exocytosis has been a central aspect for research in the neuroscience field in recent years. In fact there is controversy whether cytoskeletal elements play similar or different barrier, transport or scaffolding functions in neuronal and neuroendocrine cellular systems (Halpain, 2003). Recent work using fluorescent tagging of actin, has suggested that in nerve terminals F-actin acts as a structure maintaining the presence of essential regulatory factors (Sankaranarayanan et al., 2003). This is contrary to previous studies as it promotes a more active role of cytoskeletal elements during neurosecretion. For example in the exocytotic model of adrenomedullary chromaffin cells, it has been described that a dense mesh of filamentous actin (F-actin) excludes secretory vesicles from the subplasmalemmal region (Lee and Trifaró, 1981; Aunis and Bader, 1988; Plattner et al., 1997). Upon stimulation, only a small population of docked granules (1-3% of total vesicles) are rapidly released and constitute the ready releasable vesicle pool (Neher and Zucker, 1993; Horrigan and Bookman, 1994), and the rest of the chromaffin vesicles released during continuous or repetitive stimulation are recruited from a reserve pool located behind the F-actin barrier (Vitale et al., 1995; Gil et al., 2000). These vesicles gain access to the plasma membrane to release the stored catecholamines because a local and transient disruption of the F-actin cortical network occurs (Perrin and Aunis, 1985; Cheek and Burgoyne, 1986; Vitale et al., 1995), a process suggested to be controlled by actin-severing proteins such as scinderin (Rodriguez del Castillo et al., 1990) or alternatively by network reorganisation based in the interaction between F-actin and molecular motors such myosin (Nakanishi et al., 1989; Gutiérrez et al., 1989; Ñeco et al., 2002; Ñeco et al., 2003; Ñeco et al., 2004). More recently, this model of the F-actin network as a passive barrier has been redefined by a number of contributions using the technique of total internal reflection fluorescence microscopy (TIRFM) allowing direct visualisation of vesicles within 300 nm of the cellular limits. These studies performed in living PC12 (Lang et al., 2000) and chromaffin cells (Steyer and Almers, 1999; Oheim and Stühmer, 2000; Johns et al., 2001), have suggested an active role for an actin-myosin transport system in propelling secretory vesicles through the subplasmalemmal zone.

In this study we address cytoskeleton dynamics during secretion using both transmitted light scanning microscopy and confocal fluorescence. The former is used as a new tool allowing the direct visualisation of complex cytoplasmic structures inside cell cytosol. These structures, with differential density to the passage of visible light, are strictly sensitive to actin and myosin inhibitors and present a distribution characteristic of the F-actin network. More interestingly, there is a clear association between the dynamics of both chromaffin granule movements and these cytoplasmic structures, which appear to suffer complex reorganisations in the subcortical and internal cytoplasmic regions during the secretory cycle of chromaffin cells. Taken together, these studies and the data gathered using fluorescent-tagged β-actin support an active
dual role of F-actin as a barrier and carrier system during secretion of neuroendocrine cells.

Materials and Methods
Isolation and culture of bovine chromaffin cells
Chromaffin cells were prepared from bovine adrenal glands by collagenase digestion and further separated from debris and erythrocytes by centrifugation on Percoll gradients as described (Almazán et al., 1984; Gil et al., 1998). Cells were maintained in monolayer cultures using 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 and were harvested in 35 mm Petri dishes (500,000 cells/dish, Corning, NY, USA). Cells were used between the second and fourth day after plating. All experiments were performed at room temperature (21-22°C). Chemicals were from Sigma (Madrid, Spain).

Dynamic confocal microscopy
Cells were loaded with quinacrine using a 4 µM concentration in culture medium for 10 minutes as described (Neco et al., 2002; Neco et al., 2003; Neco et al., 2004). Fluorescent emission from quinacrine trapped in the acidic vesicles was investigated using an Olympus Fluoview FV300 confocal laser system mounted in a BX-50 WI upright microscope incorporating a 100× LUMPlan FI water-immersion objective. Excitation was performed using Ar and HeNe visible light lasers. This system allows for Z-axis reconstruction (0.5-0.55 µm theoretical Z slice) and time-lapse dynamic studies with time resolutions ranging from 0.1 second for 200×150 pixels image acquisition (adequate for region studies) to about 0.6 second for images of 400×300 pixels (for visualisation of the entire cell). Simultaneous acquisition of transmitted light images was obtained using the channel implemented in the confocal microscope and using bright field optics (theoretical depth of field is 0.55-0.65 µm). The intensity of this channel was adjusted to avoid saturation of the subcortical region and visualise the lighter cytoplasmic structures. Distribution of the F-actin network was studied using rhodamine coupled to phalloidin in intact or permeabilised cells. As this chemical presents low permeability throughout the plasmalemma, intact cells were labelled enabling Z-axis confocal reconstructions. Microtubules were visualised in intact cells using 1 µM taxol conjugated with Bodipy 564/570 (Molecular Probes, Eugene, OR). Incubation of this compound for 15-20 minutes at 37°C allowed efficient labelling of the total population of cultured chromaffin cells. Other actin and myosin affecting chemicals were used as described (Gil et al., 2000; Neco et al., 2002; Neco et al., 2003; Neco et al., 2004).

Production of GFP-actin and mutant and wild-type forms of RLC-GFP constructs
A GFP-actin construct was obtained by Nhel/MluI digestion of pEGFP-actin (Clontech, Palo Alto, CA) and inserted into an XbaI/EcoRI-digested pHSVpuc amplicon, upstream the IE 4/5 promoter. Packaging and amplification of this vector were performed using standard procedures (Lim et al., 1996). A plasmid encoding chicken gizzard smooth muscle myosin II regulatory light chain (RLC; a gift of Dr Kendrick-Jones, MRC, Cambridge, UK) was used for site-directed PCR cassette mutagenesis. The modifications were confirmed by sequencing in both directions. pRLC-wt-GFP and pRLC-T18A/S19A-GFP were generated using pEGFP-N1. The final construct was inserted in pHSVpuc and amplicons produced as indicated above. Primary cultures of chromaffin cells were infected with Herpes simplex virus (HSV-1) amplicon containing the constructs described above as described elsewhere (Gil et al., 2002). Efficiency of virus infection was determined by fluorescent microscopy, using serial dilutions of purified virus. The dilution chosen for further experiments (20-30 µl virus per 1 ml of medium) produced 10-15% infection efficiency.

Image analysis and particle tracking
Analysis of frames was performed using the program of public domain ImageJ with plug-ins for ROI measurement, image average and comparison of multiple channel images and 3D reconstruction (obtained from http://rsb.info.nih.gov/ij/). A multi-tracker plug-in was used to determine particle centroid in time-lapse studies as described earlier (Neco et al., 2003; Neco et al., 2004). The x-y coordinates determined for vesicles or threshold visible images (structure particles obtained after threshold of the transmitted light channel images) were transferred to Igor Pro, where specialised macros were used to calculate total lateral displacement and the mean square displacement (MSD) for any given time interval based in the equations defined by Qian et al. (Qian et al., 1991).

\[
\text{MSD}(n \delta t) = \frac{1}{(N-n)} \sum_{j=1}^{N-n} [(x(j \delta t + n \delta t) - x(j \delta t))^2 + (y(j \delta t + n \delta t) - y(j \delta t))^2],
\]

where images were taken at intervals \( \delta t \) and the coordinates of a granule in one image were \( x(j \delta t) \) and \( y(j \delta t) \) and in other image \( x(j \delta t + n \delta t) \) and \( y(j \delta t + n \delta t) \). In each temporal sequence the displacement during the interval \( n \delta t \) can be measured for \( (N-n) \) intervals, where \( N \) is the total number of images acquired.

This equation is a good description of vesicle movement when subjected to a single coefficient of diffusion; the downward curvature of the experimental data indicated the diffusion of vesicles in a cage, which can be described by the approximate equation (Saxton and Jacobson, 1997),

\[
\text{MSD}(\Delta t) = r_c^2 \left[ 1 - A_1 \exp (-4 A_2 D \Delta t / r_c^2) \right].
\]

This equation represents the first two terms in an infinite series, where \( A_1 \) and \( A_2 \) are constants (0.99 and 0.85, respectively). \( D \) is the coefficient of confined diffusion, and \( r_c \) is the radius of the theoretical circular cage. Therefore the MSD could be used to calculate \( r_c \) when \( \Delta t \rightarrow \infty \) and the data reach an asymptotic value.

The Student’s t-test for paired samples or the two-way ANOVA test was used to establish statistical significance among the different experimental data (samples were considered significantly different when \( P<0.05 \)). All data were expressed as mean±s.e.m. of experiments performed in a number (\( n \)) of individual cells. The data presented represents experiments performed with cells from at least three different cultures.

Results
Transmitted light scanning microscopy allows observation of dynamic cytoplasmic structures in bovine chromaffin cells
Recently, confocal microscopy analysis of cells labelled with 4 µM quinacrine has allowed us to make direct observation of vesicle movement throughout the entire cytoplasm of cultured bovine chromaffin cells (Neco et al., 2002; Neco et al., 2003; Neco et al., 2004). During these experiments, we were somewhat surprised by the complex images obtained by scanning microscopy of the transmitted light channel implemented in addition to fluorescence detection. These visible light images showed an intricate network of
cytoplasmic structures forming polygonal cages as shown by the different optical density of its walls in relation to internal spaces. Furthermore, and apart from its non-confocal nature, it was also observed that the visible information changed notably in adjacent planes separated by 1 μm (Fig. 1). The density of these structures increased in the cytoplasmic region beneath the plasma membrane, forming a peripheral ring that was clearly shown when we focussed the planes adjacent to the equatorial cell section (Fig. 1A,C). A density profile along the cell (Fig. 1C, line) shows that the majority of the vesicles appeared to be located within 1-2 μm of the internal limits of this peripheral ring (Fig. 1E). Furthermore, the visualisation of these structures in the y-z and x-z planes showed cytoskeletal cavities with vesicles located in their borders (Fig. 1F). The complexity of these cavities is best observed in 3D reconstructions (Fig. 1G) showing the continuity of these cages along the z-axis. Interestingly, the cytoplasmic structures are dynamic, as observed by time-lapse microscopy, and a close inspection of changes in a peripheral area showed variations in the density of the cortical structure with the infrequent formation of disruptions allowing vesicle penetration in the subplasmalemmal area as can be observed in for the structures (Fig. 2A) and vesicles (Fig. 2B) indicated by arrows. This dynamic behaviour of the structures visualised with transmitted light is obvious in the sample video obtained of a 1-minute sequence acquiring images at 1 Hz (Movie 1 in supplementary material). The dynamics could be analysed by measuring the time-dependent variations of averaged intensity in a region of interest (ROI), such as that indicated in Fig. 2. In these measurements, vesicles tend to stay for periods of 2-7 seconds in the confocal plane presenting short cycle oscillations in fluorescence, whereas visible light intensity changed in cycles of time ranging from 10 to 20 seconds (Fig. 2C), indicating the slower dynamics of these structures when viewed by transmitted light scanning microscopy. Further assessment of this difference in movement can be made by tracking the x-y positions of the vesicles and particle structures obtained after the threshold of the transmitted light images (Fig. 2D). In this way it was possible to observe an average displacement of 0.79±0.09 μm over 20 seconds (for 60 threshold particles in 16 cells) for the transmitted light structures and 3.75±0.50 μm/20 seconds (n=135 vesicles corresponding to 11 cells) for the vesicle displacement in the confocal plane. These data and the corresponding distributions (Fig. 2D) indicated that on average transmitted light structures moved at a speed that was four- to fivefold lower than chromaffin granules in the cytoplasm of these cells. Indeed, using this type of data we obtained the mean square displacement (MSD) at different time intervals (Equation 1) and these values were averaged to generate the MSD versus time plot (Fig. 2E). Assuming that movement is governed by a single coefficient of diffusion, its value can be derived from the slope (slope = 4D/πD) (Qian et al., 1991). The diffusion coefficient was calculated to be 5.3±0.4×10^{-3} μm²/second for moving granules (n=27) and 2.11±0.06×10^{-3} μm²/second for structure movement (n=18). Both the movement of granules and structures were strictly calcium dependent, as incubation of chromaffin cells in a media lacking additional calcium and in the presence of 1 mM EGTA for 20 minutes drastically affected the diffusion coefficients calculated under these conditions which were at least 100 times lower than control values (3.8±0.6×10^{-5} μm²/second for moving granules, and 4.5±0.3×10^{-5} μm²/second for structure movement, n=19 in both cases), as can be observed in the accelerated video (Movie 2 in supplementary material).

Fig. 1. Simultaneous visualisation of granules and cytoplasmic structures by confocal scanning microscopy. Granules were detected in quinacrine-treated chromaffin cells by epifluorescence whereas cytoplasmic structures were evident in the transmitted light channel implemented in an Olympus Fluoview FV300 microscope incorporating a 100× LUMPlan Fl water-immersion objective. (A-D) Images of four planes separated by 1.0 μm along the Z axis. Structure density increased in the region beneath the plasmalemma forming a peripheral ring and extending into the cytoplasm with the appearance of polygonal cages. The nucleus presents a less defined blurry organisation. (E) Vesicle fluorescence and optical density of the transmitted light image across the line depicted in C. Vesicles accumulate in the proximity of the peripheral ring visualised by transmitted light scanning microscopy. (F) Cross-sectional images of transmitted light structures in a chromaffin cell in the conventional X-Y plane and two X-Z and Y-Z cytoplasmic planes (indicated by coloured lines in the X-Y section). (G) 3D reconstruction of a portion of the cytoplasmic structures showing an intricate network. Black surfaces represent solid structure. Bar, 10 μm (A-D); 2 μm (F); 1 μm (G).
with transmitted light scanning microscopy could be limiting such vesicle movements, we measured the wall to wall maximal X and Y distances for an high number of such cages \((n=272\) measurements in five cells) and obtained an average value of \(1.5\pm0.7\ \mu m\) (mean±s.d.), that closely matches the diameter of \(1.72\ \mu m\) for the theoretical circular cage imprisoning vesicle movement.

Chemicals affecting F-actin alter the structure and dynamics of the cytoplasmic structures visualised with transmitted light

What is the molecular nature of these visible structures? Based on its distribution accumulating in the cell periphery, our first guess would be the cytoskeletal network of F-actin forming a subplasmalemmal barrier in chromaffin cells (Trifaró et al., 1984; Perrin and Aunis, 1985). In consequence, we treated chromaffin cultures with specific agents affecting such filamentous protein. Treatment of cultured cells with \(1\ \mu M\) latrunculin A, a chemical stabilizing monomeric actin (Spector et al., 1983), for 15 minutes resulted in drastic changes in the characteristics of these visible structures. First, there was an obvious alteration of the cell shape that was accompanied by a reduction of the intensity of the peripheral cortical network (Fig. 3). Quantification by measurement of the density of the cell cortex showed a 60% decrease in the average optical intensity of this cortical structure in measurements performed in nine cells before and after latrunculin treatment (Fig. 3C).

Moreover, the incubation of chromaffin cells with latrunculin A also affected the internal network of polygonal cages (Fig. 3D), which continuously decreased its optical density during incubation with this chemical (Fig. 3E).

Furthermore, the F-actin stabiliser phalloidin coupled to the fluorophore rhodamine could be used to study the colocalisation of this filamentous protein with the structures observed by transmitted light microscopy. Using a \(1\ \mu M\) concentration of this chemical incubated for 30 minutes (Gil et al., 2000), we observed a spatial coincidence of both the peripheral transmitted light structures (Fig. 4A) and the cortical F-actin labelling (Fig. 4B), that presented significant overlap (Fig. 4C). In some cells intracellular phalloidin-rhodamine labelling of cytoplasmic structures can be observed colocalising extensively with that observed using transmitted light (Fig. S1 in supplementary material). Interestingly, treatment of the cells with this F-actin stabiliser drastically affected both the dynamics of vesicle mobility and transmitted light structures, as under these conditions vesicles were immobilised in contact with the visible cytoplasmic structures that become static upon stabilisation (Fig. 4D,E). These experiments also proved that the transmitted light image was enriched with the visual information coming from the plane studied, otherwise, there was little probability of coincidence between vesicle and structure location. Taken together, these experiments showed that F-actin activity governs the structure and motility of the network observed by transmitted light scanning microscopy.
GFP-β-actin and the regulatory light chain of myosin II colocalise with the transmitted light network within the cortical region

We have shown in a recent publication that myosin II is associated with the F-actin network in the cytoplasm and periphery of chromaffin cells (Ñeco et al., 2002; Neco et al., 2004). Based on this, we conceived experiments expressing both the β-actin and regulatory light chain (RLC) of myosin II linked to enhanced green fluorescence protein (GFP) by their N-terminal and C-terminal domains respectively, using an amplicon-based strategy that has proven successful for the terminal and C-terminal domains respectively, using an amplicon-based strategy that has proven successful for the expression of other proteins in chromaffin cells in culture (Gil et al., 2002; Neco et al., 2004). GFP-β-actin was heavily expressed in the cytoplasm and the cortical region of chromaffin cells (Fig. 4F), and colocalised with the cortical density visualised with transmitted light (Fig. 4G,H), whereas in the cytoplasm there was a partial alignment. In addition, when we expressed RLC-GFP and specially an inactive unphosphorylatable form, T18A/S19A RLC-GFP (Fig. 4K), in chromaffin cells, it could be observed in the cytoplasm forming a network that matched that visualised with transmitted light (Fig. 4L,K). In addition, if granules were labelled with quinacrine, its brilliant fluorescence could be observed in association with both the transmitted light structures and the GFP fluorescence associated with T18A/S19A RLC-GFP (Fig. 4K).

Interestingly, the expression of this inactive form of RLC yielded a static network of transmitted light structures whereas the wild-type form of RLC sustained both vesicle (Ñeco et al., 2004) and structure mobility (D.G., unpublished observation).

Secretagogues induce changes in the density and distribution of the visualised transmitted light structures

The cellular distribution, colocalisation with phalloidin, GFP-β-actin and RLC-GFP and its sensitivity to chemicals affecting actin-myosin strongly suggest that the structure visualised by transmitted light scanning microscopy might be composed of the chromaffin cell cytoskeleton complex of F-actin modulated by the activity of structural myosin II (Kumakura et al., 1994; Neco et al., 2002; Neco et al., 2004). Thus, transmitted light scanning microscopy could be used to study the cytoskeletal dynamics that accompanies secretion, obtaining a dynamic picture of the complex rearrangements of cytoskeletal elements occurring during the secretory cycle. The stimulation of the cells with 10 μM acetylcholine resulted in a 2-4% increase in the cell perimeter as a consequence of exocytotic membrane incorporation and the simultaneous formation of evident discontinuities in the subcortical structures (Fig. 5A and Movie 3 in supplementary material). Measurements of optical density in a ROI located over one of the detected disruptions, such as those depicted in Fig. 5B at 2-second intervals, showed that the formation of these subcortical patches within a few seconds was fast enough to encompass secretion (Fig. 5D). In contrast with these rapid changes occurring in the cell periphery, we...
also observed the reorganisation of intracellular structures forming polygons, increasing the open space in their interior (Fig. 5C). This process, however, takes tens of seconds to fully develop as shown in the images of this panel taken at 10-second intervals. These changes were observed in dozens of cells stimulated with either physiological agonist or by sustained superfusion with high potassium stimulatory solution and they were strictly dependent of the presence of calcium in the stimulatory medium. In similar experiments, stimulation for 1 minute with acetylcholine in a medium lacking Ca²⁺ (and in the presence of 1 mM EGTA) produced no apparent changes in the cytoskeletal peripheral integrity compared with resting conditions (Fig. S3 in supplementary material), whereas the posterior perfusion of this cell with this secretagogue in a medium containing 2 mM CaCl₂ induced rapid and drastic changes in the cytoskeletal structure.

In another series of experiments, cells were depolarised briefly with a 10-second superfusion with 59 mM KCl. Under these conditions cells secrete catecholamines for 10-12 seconds as measured using single cell amperometry (Gil et al., 1998; Ñeco et al., 2003), and the cortical cytoskeleton disassembles in parallel (Fig. 6A,B and compressed Movie 4 in supplementary material), after that, the cortical barrier...
subcortical area to the adjacent cytoplasmic regions, as ROI measurements within this zone showed that, with a few seconds delay, increases were detected in the optical density encompassing the parallel decrease of the peripheral barrier (Fig. 6A,B, comparison of the optical density in cortical and interior ROIs). The concept of cortical F-actin transfer rather than its destruction was also supported when the average transmitted light intensity of the overall region studied in Fig. 6 did not change significantly during secretion (Fig. 6B). We observed in many stimulated cells, that the reorganisation of F-actin implied the formation of channel-like structures perpendicular to the membrane plane (see second and third frame of Fig. 6A). A detailed observation of the granule positions during secretion showed that quinacrine-loaded vesicles accessed the cortical region through the newly opened disruptions and they were frequently found in the narrow space left by the channel-forming structures. Finally, they increased their presence in the subplasmalemmal regions 10-15 seconds after disruption of the cortical barrier as detected by measuring quinacrine fluorescence within a ROI located in the exterior of the cortical structure (Fig. 6C).

Vesicles moved along F-actin during stimulus-dependent cytoskeletal reorganisations

During cell stimulation, F-actin reorganised and formed disruptions and the open cytoplasmic spaces as described above, but could the vesicles use such spaces to diffuse and access the subplasmalemmal area? A simple, yet accurate way to observe vesicle positions is to obtain the cumulative image of vesicles in relation to cytoskeletal structures during secretion or even under resting conditions. Cumulative images obtained over 15 seconds in non-stimulated cells show that vesicles moved in the internal face of the cytoskeletal barrier and appeared to avoid the dark area located in the centre of polygons formed by the F-actin network (Fig. 6D, arrows). After stimulation, most of the vesicles either were released or moved to other cell areas but the vesicles observed moved in contact with the cytoskeletal network and again avoided the use of open spaces (arrows in Fig. 6E). These observations were made in dozens of stimulated cells and indicated that during rest conditions or stimulatory activity, chromaffin granules moved preferentially along F-actin structures avoiding access to bare cytoplasmic zones.

Changes in the fluorescence of GFP-β-actin-expressing cells support the F-actin dynamics observed using transmitted light scanning microscopy

In order to confirm the observations made with transmitted light scanning microscopy, we studied the cytoskeletal dynamics in chromaffin cells expressing GFP-β-actin construct, where fluorescence is distributed in the cell periphery and also in cytoplasmic patches produced by overexpression (Fig. 7A). These patches corresponded to F-actin aggregates as they were labelled by phalloidin (Fig. 7B,C), which also stained the peripheral ring corresponding to the cortical F-actin barrier. Additional proof of the accumulation of GFP-β-actin in the F-actin cortical network, was obtained by treatment with 1 μM latrunculin A. This substance affected the integrity of the peripheral fluorescence...
that decreased in the cortical area and continuously increased in parallel in the internal cytoplasm as shown in the ROI determinations (Fig. 7D,E), thus validating the use of this fluorescent construct to study of cortical F-actin dynamics in chromaffin cells.

Interestingly, cells overexpressing GFP-β-actin manifested fluorescent changes encompassing those occurring in the transmitted light channel upon cell stimulation by depolarisation. In these experiments a temporal decrease of fluorescence integrated in the cortical zone and the parallel increase in the fluorescence of the cytoplasmic subcortical area was observed and similar results were obtained by analysing the transmitted light images. Furthermore, the formation of cortical disruptions during stimulation and F-actin transfer has also been observed in cells expressing the GFP-β-actin construct (Fig. 8) for a depolarised cell. These experiments proved simultaneous detection of these disruptions in the confocal images of GFP-β-actin fluorescence (Fig. 8A) and in the transmitted light channel images (Fig. 8B). Determination of fluorescence intensity at different distances from the cell limits demonstrated GFP-β-actin transfer from the external to the internal zones during the formation of such disruptions (Fig. 8C). Taking these observations from both transmitted light and fluorescence GFP-β-actin images together, it is clear that during stimulation F-actin redistributed to leave open space in the peripheral barrier and to form the trails for vesicle movement.

Discussion

Transmitted light scanning microscopy provides a new alternative to studying dynamic changes in the F-actin cytoskeleton

Although confocal fluorescence microscopy has found widespread use in cell biology, the use of transmitted light scanning microscopy implemented in confocal microscopes is not as widespread. In this work we have shown, using a variety of pharmacological and molecular biology tools in chromaffin cells, that the complex images obtained by transmitted scanning microscopy probably representing variations in cytoplasmic viscosity, are closely associated with the activity of the network of F-actin regulated by interaction with myosin II. Moreover, the study of fluorescence changes after
A model to understand the double role of F-actin as both a barrier and a support for vesicle access to the subplasmalemmal area in chromaffin cells

Even if the combination of pharmacological tools and the observed distribution and colocalisation of the transmitted light structures with phalloidin, GFP-β-actin, and myosin II RLC labelling may constitute convincing evidence of its F-actin identity, the complexity of the changes occurring in these structures during cell stimulation is additional and important proof of its nature. As shown in Figs 5-8, using both transmitted light and confocal observation of GFP-β-actin, three types of major change were characterised in the cytoplasmic F-actin distribution after secretory stimulation: fast and discrete disruptions in the cortical peripheral barrier, formation of channel-like structures perpendicular to the membrane plane, and slower changes in the interior of the cytoplasm with the appearance of the formation of wider open spaces devoid of F-actin. The formation of cortical disruptions in response to different secretagogues was the key element supporting the role of peripheral F-actin as an effective barrier avoiding vesicle access to secretory active sites (Aunis and Bader, 1988; Vitale et al., 1995). Our experiments are the first to show the real-time dynamics of this process, in resting conditions, vesicles tend to locate within 200-400 nm of the membrane, following secretagogue superfusion and within 5-10 seconds, F-actin reorganises itself forming cortical disruptions of about 0.5-2 μm and the vesicles penetrate to previously forbidden areas in the cell limits. After 10-20 seconds of stimulation the general disposition of F-actin, that was parallel to the membrane, changed to a perpendicular alignment increasing its concentration in deeper subcortical regions, thus forming frequently channel-like open spaces of about 300-400 nm used by granules to access the membrane. A static view of these structures was previously shown when the cortical region of rhodamine-phalloidin-labelled chromaffin cells was studied by z-reconstruction of confocal images (Ñeco et al., 2003). Vesicles using these channel-like structures will be accessing docking sites 10-20 seconds after initiation of secretion and therefore will refill the pools of docked vesicles released during sustained stimulation. Finally, after 60-100 seconds, F-actin reorganisation in response to transient stimuli recovered the original distribution of parallel to the plasma membrane and again vesicles were restricted in their access to active sites. This reorganisation of F-actin is of extreme importance as it provides the basis to understanding the double nature of the cytoskeletal network of F-actin as a dense barrier that does not allow vesicle access to the docking sites and also, after stimulation and reorganisation, as providing the system to transport vesicles from the cytoplasmic pool to specific points of the peripheral area. In contrast to these rapid changes, continuous cell stimulation also induced the slow formation of intracellular spaces lacking F-actin with the aspect of empty polygons. We do not know the functional relevance of this process as vesicles do not seem to access the interior of these zones as they only move only along the F-actin walls. The formation of these polygons during secretion is supported by electron microscopy studies in fixed chromaffin cells (Tchakarov et al., 1998).

Taking these observations together, an active role as both a barrier and a carrier for F-actin-myosin is suggested in this neuroendocrine model, but our studies do not exclude another scaffold function as has been proposed in presynaptic terminals (Sankaranarayanan et al., 2003). We observed that these transmitted light structures are also easily visible and dynamic in the cell body of motoneurons and hippocampus neurons, therefore the possible application of transmitted light scanning microscopy to the study of secretion in neuronal and other cells could open new alternatives for the study of transport and secretion processes in living cells.

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