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A confocal study on the visualization of chromaffin cell secretory vesicles with fluorescent targeted probes and acidic dyes

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ABSTRACT

Secretory vesicles have low pH and have been classically identified as those labelled by a series of acidic fluorescent dyes such as acridine orange or neutral red, which accumulate into the vesicles according to the pH gradient. More recently, several fusion proteins containing enhanced green fluorescent protein (EGFP) and targeted to the secretory vesicles have been engineered. Both targeted fluorescent proteins and acidic dyes have been used, separately or combined, to monitor the dynamics of secretory vesicle movements and their fusion with the plasma membrane. We have now investigated in detail the degree of colocalization of both types of probes using several fusion proteins targeted to the vesicles (synaptobrevin2-EGFP, Cromogranin A-EGFP and neuropeptide Y-EGFP) and several acidic dyes (acridine orange, neutral red and lysotracker red) in chromaffin cells, PC12 cells and GH_3 cells. We find that all the acidic dyes labelled the same population of vesicles. However, that population was largely different from the one labelled by the targeted proteins, with very little colocalization among them, in all the cell types studied. Our data show that the vesicles containing the proteins more characteristic of the secretory vesicles are not labelled by the acidic dyes, and vice versa. Peptide glycyl-L-phenylalanine 2-naphthylamide (GPN) produced a rapid and selective disruption of the vesicles labelled by acidic dyes, suggesting that they could be mainly lysosomes. Therefore, these labelling techniques distinguish two clearly different sets of acidic vesicles in neuroendocrine cells. This finding should be taken into account whenever vesicle dynamics is studied using these techniques.

1. Introduction

Secretory vesicles have low pH, around 5.5, a property that share with other vesicular organelles such as the lysosomes. The low pH of all these vesicles should allow them to accumulate lipophilic compounds of acidic nature and, in fact, compounds with these characteristics such as acridine orange or neutral red label a vesicular population in neurons and neuroendocrine cells that has been classically assumed to correspond largely to the secretory vesicles (Kuijpers et al., 1989; Steyer et al., 1997; Steyer and Almers, 1999; Straub et al., 2000; Oheim and Stühmer, 2000). Thus, the dynamics of the vesicles labelled with acridine orange has been extensively investigated to monitor vesicle motion, fusion with the plasma membrane and other characteristics of the latter steps before fusion (Steyer et al., 1997; Steyer and Almers, 1999; Oheim and Stühmer, 2000; Li et al., 2004).

More recently, several chimeric proteins targeted to the secretory vesicles and containing EGFP have been engineered and expressed in different cells (Lang et al., 1997; Tsuboi et al., 2000; Ohara-Imaizumi et al. 2002; Bezzi et al., 2004; Allersma et al., 2004, 2006), and used also to investigate the dynamics of the secretory vesicles. In some cases, cells expressing one of these constructs were also labelled with acridine orange to monitor at the same time the dynamics of the vesicles, using the specifically targeted EGFP marker, and the event of fusion, by following the disappearance of the loaded dye (Tsuboi et al., 2000; Bezzi et al., 2004). These papers showed an extensive colocalization among the two types of probe. However, it has been reported more recently that acridine orange metachromasie, that results in the concomitant emission of green and red fluorescence from acridine orange, generates systematic colocalization errors between acridine orange and EGFP in vesicular organelles (Nadrigny et al., 2007). According to this work, the green emission from acridine orange overlaps with that of EGFP and produces a false apparent colocalization on dual-color images.

Abbreviations: EGFP, enhanced green fluorescent protein; NPY, neuropeptide Y; VAMP, vesicle-associated membrane protein; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; GPN, glycyl-L-phenylalanine 2-naphthylamide.

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We have now made a detailed study of the colocalization of several EGFP-probes targeted to the secretory vesicles and several acidic dyes. Our results show that both kinds of labelling methods produce a clear vesicular pattern, but surprisingly there was little coincidence among the vesicular patterns generated using EGFP-probes and those obtained with acidic dyes.

2. Materials and methods

2.1. Preparation and culture of chromaffin cells, PC12 cells and GH_3 cells

Ethical approval for this study was granted from the investigation committee and the animal experimentation committee of the Faculty of Medicine, University of Valladolid. Cow adrenal glands were kindly supplied by the veterinaries of the slaughterhouse Justino Gutiérrez of Laguna de Duero (Valladolid). Bovine adrenal medulla chromaffin cells were isolated as described previously (Moro et al., 1990), plated on 12 mm glass polilysine-coated coverslips (0.25×10^6 cells per 1 ml medium) and cultured in high-glucose (4.5 g/l) Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 50iu ml^{-1} penicillin and 50iu ml^{-1} streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2. PC12 rat pheochromocytoma cells were grown in high-glucose (4.5 g/l) Dulbecco's modified Eagle's medium supplemented with 7.5% fetal calf serum, 7.5% horse serum and 2 mM glutamine. GH_3 adenohypophyseal cells were grown in RPMI 1640 culture medium supplemented with 2.5% fetal bovine serum, 15% horse serum, 2 mM glutamine, 100 iu ml^{-1} penicillin and 100 iu ml^{-1} streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Cells were seeded over glass bottom Petry dishes coated with poly-L-lisine (0.01 mg/ml).

2.2. Preparation and expression of the EGFP-targeted probes

The VAMP-enhanced green fluorescent protein (EGFP) construct has been described previously (SantoDomingo et al., 2008). For construction of adenoviral vectors, full-length cDNA encoding these constructs was subcloned into the pShuttle vector and then used for construction of the corresponding adenoviral vector by using an AdenoX adenovirus construction kit (Clontech). Cells were infected with an adenovirus for expression of this construct. Infection was carried out the day after cell isolation and Ca^{2+} measurements were performed 48–72 h after infection. Efficiency of infection of chromaffin cells with the adenovirus carrying the VAMP-EGFP chimera was estimated to be about 60%.

The chromogranin A-EGFP and neuropeptide Y-EGFP constructs were kindly provided by Dr. J.D. Machado, University of La Laguna, Spain. Transfections of these constructs were carried out using Metafectene (Biontex, Germany).

2.3. Confocal studies

Cells were imaged at room temperature on a Leica TCS SP2 confocal spectrophotometer using a 63× oil immersion objective. EGFP-containing constructs and acridine orange were excited with the 488 nm line of the Argon laser, and the fluorescence emitted between 500 and 530 nm was collected. Fluorescence from lysotracker red or neutral red dyes was excited with the 543 nm line of the green He-Ne laser and the fluorescence emitted between 600 and 700 nm was collected. The above settings were carefully chosen to assure that there was no interference from the green fluorochrome in the red channel, or viceversa. Lack of bleed-through between the two channels can be clearly appreciated in many of the figures. Images for each fluorochrome at every confocal plane were recorded sequentially frame by frame at a rate of 0.8 frames per second. No significant movement of the granules was observed when consecutive images of the same fluorophore were taken at this rate. For loading with the acidic dyes, cells were incubated for 1–5 min with either 100 nM acridine orange, 50 nM lysotracker red or 1 μM neutral red, added directly to the cell chamber in the stage of the microscope.

For colocalization analysis we have used the toolbox JACoP (Bolte and Cordelières, 2006) under ImageJ software (public domain image processing program developed by Wayne Rasband at the National Institutes of Health, Bethesda, U.S.A.) to obtain the Pearson's correlation coefficients (Manders et al., 1992) from deconvolved images of each channel. When this coefficient that can vary between −1 and +1 is applied to image colocalization, values close to +1 indicate colocalization, while values close to zero indicate lack of correlation. The values obtained in each case are given in the Figure Legends. In Fig. 1A, the composite images showing the colocalized pixels were obtained with the Colocalization Finder plugin from the ImageJ software.

2.4. Fluorescence microscopy measurements

Cells expressing VAMP-EGFP were mounted in a cell chamber in the stage of a Zeiss Axiovert 200 microscope under continuous perfusion. Single cell fluorescence was excited at 480 nm using a Cairn monochromator (200 ms excitation every 2 s, 10 nm bandwidth) and images of the emitted fluorescence obtained with a 40× Fluar objective were collected using a 495DCLP dichroic mirror and a E515LPV2 emission filter (both from Chroma Technology) and recorded by a Hamamatsu ORCA-ER camera. Single cell fluorescence records were analyzed using the Metafluor program (Universal Imaging). Experiments were performed at room temperature.

3. Results

3.1. Subcellular dual-color localization of VAMP-EGFP and acidic dyes: lysotracker red and neutral red

Given that VAMP-EGFP and acridine orange fluorescences cannot be well distinguished, we have used other two acidic dyes having a fluorescence spectrum that can be easily separated from that of EGFP by choosing the appropriate emission windows, as described in Methods: lysotracker red and neutral red. Fig. 1A and B show a series of confocal images of two chromaffin cells expressing VAMP-EGFP and then stained with lysotracker red. It can be observed that both VAMP-EGFP and lysotracker red generated a vesicular pattern. In addition, VAMP-EGFP also labelled the plasma membrane. This was expected, as it is an integral protein of the vesicle membrane and remains in the plasma membrane after fusion. However, the vesicular patterns observed with both probes were clearly different and mostly non-coincident. Because yellow pixels are sometimes difficult to see over the red and green background, a series of images showing in bright white the few coincident pixels has been included in Fig. 1A to make clear that the coincidence is marginal. Accordingly, Pearsońs correlation coefficients were also close to zero (see legend). In addition to the lack of colocalization, vesicle distribution and size was very different in both groups: vesicles stained with lysotracker red were less in number and generally bigger than those labelled by VAMP-EGFP.

Similar findings were observed in PC12 and GH_3 cells. Fig. 2 shows confocal planes of each of these cells expressing VAMP-EGFP and then stained with lysotracker red. Although it is difficult to exclude some small degree of colocalization, in part due to the large density of vesicles labelled by VAMP-EGFP, it is clear that the vesicular patterns in both cases are completely different, and