Red, yellow, green go! – a novel tool for microscopic segregation of secretory vesicle pools according to their age


Abstract

Large dense-core vesicles (LDCVs) were labelled in cultured bovine adrenal chromaffin cells expressing fluorescent chimaeric ‘cargo’ proteins that were targeted to these secretory vesicles. When the cells were stimulated with nicotine 48 h after transduction, the fractional loss of fluorescent LDCVs was much greater than the fractional catecholamine secretion, implying selective release of newly assembled vesicles. This was confirmed using a fluorescent ‘timer’ construct that changes its fluorescence emission from green to red over several hours, and by measurement of the location and mobility of LDCVs in live cells by confocal fluorescence microscopy. Newly assembled (green) LDCVs were located mostly in peripheral regions of the cells, were virtually immobile and could be released by nicotine, but not by Ca2+; in contrast, older (red) LDCVs were centrally located and relatively mobile, and their exocytotic release was triggered by Ca2+, but not by nicotine. We describe the image restoration procedure that is necessary in order to analyse the behaviour of LDCVs labelled with this construct.

Introduction

Neuroendocrine cells contain numerous large dense-core vesicles (LDCVs) that are filled with cargo proteins destined for secretion. However, only a small fraction of these vesicles is membrane-docked and able to be released rapidly in response to elevated cytosolic Ca2+ concentrations at any time [readily releasable pool (RRP)]. The larger, remaining pool is assumed to be the cytosolic reserve pool (RP), from which more LDCVs can be recruited to the RRP. Little is known about the segregation of these vesicle pools or the temporal sequence in which vesicles enter these pools (see Figure 1): LDCVs could be released after random selection from the RP (Figure 1b); alternatively, either the oldest (Figure 1a) or most recently assembled (Figure 1c) vesicles could be released preferentially.

Here we present a new fluorescent probe for specifically labelling LDCVs, atrial natriuretic factor (ANF)-timer, which changes its emission spectrum with time. ANF, a cargo protein that is targeted to the vesicle lumen of endocrine cells, was fused to the fluorescent protein DsRed-E5. DsRed-E5 is a mutant of the red fluorescent coral protein DsRed, and is characterized by unusually slow maturation, during which the initially green emission changes to red over approx. 16 h [1]. Thus newly assembled LDCVs appear green, those of intermediate age are yellow and older vesicles (>16 h) are red. Expression of this fusion protein in bovine adrenal chromaffin cells (BCCs) reveals segregation of LDCVs, on the basis of age, into distinct vesicle pools with distinct properties. We will demonstrate the use of this novel fusion protein in microscopic studies analysing the localization, mobility and release behaviour of LDCVs from different pools [2].

Labelling of LDCVs with a fluorescent ‘cargo’ protein

We used Semliki Forest Virus (SFV)-based transduction to target a fluorescent fusion protein, ANF fused to enhanced green fluorescent protein (EGFP), to the lumen of LDCVs in BCCs [2,3]. Cells were fixed 48 h after infection and imaged using confocal laser scanning microscopy (CLSM) in three dimensions at Nyquist sampling rate. Raw data were deconvolved using the Huygens 2 (SVI) software package on an Octane workstation (SGI), and three-dimensional images were reconstructed. Restored images showed 157 ± 17 (mean ± S.E.M.) fluorescent, punctate objects per cell (n = 12), which were concentrated towards the footplate of the cells. To confirm that the objects labelled with ANF–EGFP were LDCVs, cells were co-stained with Lysotracker DND-99, a red fluorescent dye, which accumulates in acidic compartments of the cell. Three-dimensional images from these cells were subjected to a co-localization analysis using Colocalization 2.0 (Bitplane). The co-localization data extracted confirmed that 97% ± 0.2% of the green voxels co-localized with red voxels, whereas only 1 ± 0.002% of red voxels co-localized with green. As Lysotracker Red...
Figure 1 | Three models suggested for the sequence of LDCV release with regard to vesicle age
(a) 'Oldest first'. LDCVs in chromaffin cells bud from the Golgi and translocate sequentially in an orderly queue to the RP, where they mature, and eventually move to the plasma membrane, from which they are released upon stimulation and fuse with the plasma membrane. (b) Random release. A second model suggests a sequential release of LDCVs as described in (a), except that LDCVs of different ages are recruited randomly to the plasma membrane, from which they are released. (c) 'Youths jump the queue'. Newly synthesized LDCVs translocate swiftly to the plasma membrane, and a fraction is released upon stimulation. LDCVs that are not released translocate to the RP, from which they can either be re-recruited to the plasma membrane or move to the vesicle pool, which is not releasable by nicotine stimulation.

DND-99 stains all acidic compartments in the cell and not only LDVCs, the upper estimate for LDCVs in a BCC is approx. 15 000.

Nicotinic stimulation selectively releases newly assembled LDCVs
Upon maximal stimulation with 20 µM nicotine for 15 min, the cells lost 60 ± 7% of their ANF–EGFP-labelled vesicles. Given that these vesicles cannot be older than 48 h, this suggests that an unexpectedly high proportion of newly synthesized LDCVs was released by nicotine. This does not agree with the sequential model shown in Figure 1(a), given that the lifetime of LDCVs in these cells is approx. 18 days, and most of the old LDCVs would not be labelled during this relatively short experimentation period. These results also do not agree with the random release of LDCVs (see Figure 1b), which would predict approx. 15% release of labelled vesicles, consistent with the catecholamine release measured in population assays (see below).

‘Time-stamping’ LDCVs with a fluorescent timer protein
To generate a fluorescent labelling tool that could be used to provide more refined information about the age of single vesicles, ANF was fused to a mutant red fluorescent protein, DsRed-E5. This mutant fluorophore shows an unusually slow maturation of its fluorescence from green to red emission over a time course of approx. 16 h [1]. The resulting fluorescent fusion protein, ANF–DsRed-E5 or ANF-timer, was introduced into BCCs and imaged as described above (see [2]). To establish that ANF-timer was targeted to post-Golgi LDCVs in BCCs, cells were fixed, immunolabelled with either an antibody against chromogranin A (CgA; a major cargo protein of LDCVs in BCCs) or an antibody against a Golgi protein [4]. A total of 54 confocal sections (2 µm thick) were imaged as described above, and three-dimensional images were restored after deconvolution of the raw data. Figures 2(a) and 2(b) show the punctate distribution of CgA (blue) and ANF-timer (green, yellow and red) throughout the cell. Co-localization analysis revealed that the majority of ANF-timer (84.2 ± 7.2% of green, 87.6 ± 0.9% of yellow and 91.1 ± 3.7% of red voxels) co-localized with blue voxels representing CgA. Extracted voxels showing co-localization events are depicted in yellow in Figure 2(c).

Since preparations of BCCs can potentially contain a proportion of contaminating adhering cells, such as fibroblasts, the co-localization of CgA with the ANF-timer label also confirmed that the cells to be analysed were BCCs, because CgA represents a specific LDCV marker. This was corroborated by immunolabelling BCCs with antibodies directed against two other LDCV markers: ANF and the vesicle membrane protein dopamine β-mono-oxygenase (results not shown). In all cases, these vesicle markers co-localized with ANF-timer label in punctate objects, showing unambiguously that the cells studied were BCCs. Furthermore, as shown below, exocytosis of LDCVs could be evoked by specific exocytotic stimuli, another exclusive property of secretory cells.

In contrast with CgA co-labelling, there was no significant co-localization of ANF-timer label with an immunolabel
detecting a protein of the Golgi network (Figure 2d) (only 1.9 ± 0.001% and 1.75 ± 0.002% of green and red voxels respectively co-localized with blue voxels), which indicates that the majority of ANF-timer-labelled objects were post-Golgi LDCVs, developing their green fluorescence with a delay after vesicles budded from the Golgi network.

All BCCs analysed in these studies were well-adhering, flattened out cells, for the following reasons. First, a significant proportion of round, semi-detached cells, which are sometimes used for electrophysiological experiments, showed a strong nuclear stain upon labelling with Acridine Orange (AO), which suggests that these cells were entering apoptosis. Flat cells did not show any nuclear AO stain. Secondly, the flat morphology of cells significantly reduces the number of scans in the Z-plane during imaging, and thus decreases the effects of photobleaching. For these reasons we chose to analyse adhering rather than rounded cells.

Image restoration is essential for the analysis of ANF-timer-transduced cells

Raw confocal image stacks from BCCs transduced with ANF-timer are difficult to analyse because of common optical aberrations, such as lack of resolution in the Z-axis and chromatic aberration. This makes image restoration an essential step in the data analysis in order to distinguish LDCVs by their colour. Deconvolved images were restored in three dimensions and then subjected to an object-independent extraction of LDCV colours using Colocalization 2.0 (Bitplane) software. For each optical level, two-dimensional plots of fluorescence intensity (green channel versus red channel) were generated, and gating was used to extract pixels that were exclusively green or red respectively (see Figures 3a and 3b). A third region of interest included all pixels showing co-localization events and therefore defined as yellow pixels (Figure 3c). This approach provides an object-independent, computer-based method for the extraction of vesicle colours, in order to avoid errors introduced by a purely visual analysis of image data.

The subcellular location and mobility of LDCVs depend upon their age

ANF-timer-transduced BCCs contain LDCV subpopulations of distinct colour and distribution. Figure 3(e) shows a projection of 48 confocal sections (0.2 µm thickness) of a representative BCC, 48 h after infection with SFV–ANF-timer. Characteristically, green LDCVs were located in peripheral areas and towards the footplate of the cell (see also Figures 3b and 3c, which show LDCV localization in a YZ section), commonly assumed to be preferred areas of vesicle release. In contrast, yellow and red LDCVs were situated in more central areas of the cell. Mean numbers of ANF-timer-labelled LDCVs per cell were 27 ± 5 green, 83 ± 13 yellow and 14 ± 5 red (n = 12).

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Figure 3 | Computer-based, object-independent colour extraction facilitates the analysis of ANF-timer-labelled vesicles

(a)-(c) One- and two-dimensional fluorescence intensity plots for the green and red channels, which were used to define regions of interest to extract voxels that are exclusively green (a) or red (b), or show co-localization of green and red and thus appear yellow (c). The three panels on the left-hand side contain 2-D plots (green vs red fluorescence intensities) with the appropriate regions of interest that were used for gating. The three panels on the right-hand side depict the extracted voxels for each region of interest. Newly synthesized LDCVs, which are located predominantly in the cell periphery in an immobile state, are released preferentially upon nicotine stimulation. Panels (d)-(f) show projections of 48 optical sections (0.2 µm thick) of a representative ANF-timer-transduced BCC in the XY plane (d, scale bar = 8 µm) and the YZ plane (e and f). All images were scanned by CLSM and deconvolved as described in [2], which significantly improves the resolution in the Z plane (see e and f). (g) Projection of 48 confocal sections of an ANF-timer-transduced BCC after maximal stimulation.
If newly synthesized, green LDCVs are translocated rapidly to the plasma membrane, where they become morphologically docked, their mobility should be significantly different from that of yellow and red vesicles. Therefore the mobility of LDCVs was determined from their trajectories, and the mean square displacement \((d^2)\) was calculated and plotted against time for the three different vesicle populations. Data can be fitted by the equation \(d^2 = D't\) [5–7]. Green LDCVs were virtually immobile, with an apparent diffusion coefficient of \(D' = 7 \times 10^{-12} \text{cm}^2 \cdot \text{s}^{-1}\), whereas yellow and red LDCVs were significantly more mobile, with apparent diffusion coefficients of \(D' = 3.7 \times 10^{-15} \text{cm}^2 \cdot \text{s}^{-1}\) and \(D' = 1.4 \times 10^{-9} \text{cm}^2 \cdot \text{s}^{-1}\) respectively. In addition, a proportion of red LDCVs showed saltatory movements, partially in a retrograde direction, which could be an indication that these vesicles were moving from peripheral to central areas of the cell.

To analyse vesicle release behaviour further, ANF-timer-transduced BCCs were imaged and analysed after maximal stimulation with nicotine and compared with unstimulated cells. As in BCCs expressing ANF–EGFP, loss of 63 ± 15% of all labelled LDCVs was recorded. However, the three sub-populations showed dramatic differences in release according to their age: 99 ± 1% of the green, newly synthesized LDCVs were released, in comparison with release of 63 ± 5% of yellow LDCVs and no loss of exclusively red LDCVs. This result clearly indicates the preferential release of newly synthesized LDCVs upon nicotine stimulation in BCCs.

**Biochemical assays of secretion are consistent with single-vesicle assays**

To corroborate these results from imaging data, we carried out population assays to compare catecholamine and ANF secretion from cells transduced with ANF–EGFP with secretion from non-transduced cells (for methods, see [2]). The total catecholamine content of non-transduced BCCs was 73 ± 8 fmol/cell \((n = 51)\), compared with 52 ± 7 fmol/cell \((n = 22)\) in ANF-timer-transduced cells. Upon maximal nicotine stimulation at 48–72 h after infection, 15.5 ± 2.7% \((n = 31)\) and 14.4 ± 2.4% \((n = 31)\) of the catecholamine was released from transduced and non-transduced cells respectively. This suggests that SFV transduction of BCCs does not disturb their normal secretion behaviour, and corroborates findings showing that SFV infection of BCCs does not alter the expression of proteins involved in regulated exocytosis [3]. These results also agree with reports demonstrating that neurons from SFV-infected rat hippocampal slice cultures do not show differences in excitable membrane properties compared with non-transduced neurons [8].

The cellular content of ANF in non-transduced BCCs was 99 ± 12 fmol/10^6 cells, and that in ANF–EGFP-transduced cells was 335 ± 45 fmol/10^6 cells, a 3-fold overexpression. However, a large proportion of ANF was secreted from ANF–EGFP-expressing cells, i.e. 85.4 ± 18.5% \((n = 12)\) compared with only 18.8 ± 6.1% \((n = 15)\) from non-transduced BCCs. This large fractional release from ANF–EGFP-transduced BCCs is in agreement with previously published data and the imaging results, which showed loss of 60% of ANF–EGFP-labelled LDCVs and 63.4% of total LDCVs from ANF-timer-transduced BCCs, supporting the hypothesis that newly synthesized secretory vesicles are released preferentially.

**Total internal reflection fluorescence (TIRF) microscopy confirms that LDCV cargo is released by exocytosis**

To establish that the loss of LDCVs as seen in the confocal microscopic analysis was indeed due to vesicle fusion, we applied TIRF microscopy to visualize the stimulated release of fluorescent vesicle contents. ANF–EGFP-transduced BCCs were imaged as described [9]. Upon nicotine stimulation during real-time imaging, the fluorescent content of a subpopulation of LDCVs dissipated from vesicles that had been immobile for more than 2 s (a movie is available for viewing at URL: http://www.biochemsoctrans.org/bst/031/bst0310851add.htm). The same results were obtained for AO-loaded LDCVs, of which 27 ± 18% of docked vesicles were released. Control experiments carried out with no stimulus did not show any exocytosis of LDCVs above basal levels \((2.2 ± 0.2\%)\).

**Different secretagogues act on different pools of LDCVs**

Since it has been reported that Ba^{2+}, unlike nicotine, acts on the RP rather than on the RRP in BCCs and neurons [10,11], we used ANF-timer-transduced BCCs to study the release of different vesicle pools by these two secretagogues.

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with 20 µM nicotine for 15 min. Most green (newly synthesized) and a fraction of the yellow LDCVs are lost (scale bar = 10 µm). (b) Projected image of 24 confocal sections (0.2 µm thick) of a BCC after stimulation with 5 mM Ba^{2+}, showing a decrease in the number of yellow LDCVs, whereas green vesicles remain in peripheral areas (scale bar = 10 µm). Supplementary data/a multimedia adjunct (Movie 1) is available at URL: http://www.biochemsoctrans.org/bst/031/bst0310851add.htm. An ANF–EGFP-transduced, live BCC was imaged using a TIRF microscope (for methods, see [9]) at 4.5 Hz. Nicotine (20 µM) was added at t = 15 s. Two ‘flashes’, which indicate the dissipation of the AO into the extracellular space due to vesicle fusion with the plasma membrane, are visible from immobile LDCVs.
In imaging experiments, the total fraction of released LDVCs was similar with both, i.e. 63 ± 15% upon nicotine and 58 ± 9% upon Ba2+ stimulation. However, very strikingly, Ba2+ failed to release any green LDVCs, whereas the loss of yellow LDVCs was increased to 83 ± 20% (compared with 64 ± 5% with nicotine). Cell population assays revealed that a larger fraction of catecholamine was secreted using a 5 mM Ba2+ stimulus (60 ± 9% in non-transduced and 53 ± 3% in ANF-timer-transduced cells), compared with only approx. 15% upon nicotine stimulation, as has been reported previously [12]. An explanation for this larger fraction of catecholamine that is released by Ba2+ compared with nicotine could be that Ba2+ acts only on older vesicles, which are assembled before transduction and include a large proportion of unlabelled vesicles.

To confirm that Ba2+ cannot trigger the release of immobile, docked LDVCs, which are the youngest vesicles, we analysed AO-loaded BCCs by TIRF microscopy. AO labels all LDVCs, including those that are older than 48 h and therefore not fluorescent in ANF-timer-transduced cells. In contrast with nicotine, which triggered the release of 27 ± 18% of docked LDVCs ($n = 230$ vesicles from six cells), Ba2+ did not release any docked vesicles ($n = 165$ vesicles from five cells) over comparable time periods (4–10 min). Although AO does not give any information about the vesicle age, we assume that immobile, docked vesicles are part of the RRP, as established above. However, stimulation with Ba2+, unlike that with nicotine, did not affect the already docked LDVCs.

This is in agreement with previous results [7], which suggested release of mobile vesicles upon Ba2+ stimulation of PC12 cells. In addition, we carried out time-lapse imaging of central sections of AO-loaded BCCs using CLSM (results not shown). We observed significant depletion of vesicles from central areas of cells upon stimulation with 5 mM Ba2+, which was not observed in experiments with nicotine stimulation or without stimulation. Taken together, these results indicate that Ba2+ does not affect newly synthesized, docked LDVCs. However, cell population assays, imaging data from PC12 cells [7], imaging of ANF-timer-transduced BCCs and confocal time lapse movies all suggest that Ba2+ triggers the release of older vesicles of the RP, possibly acting through a mechanism different from that operating in nicotine stimulation.

**Discussion**

With the introduction of ANF-timer, we have a powerful, novel tool that allows us not only to specifically label LDVCs but also to obtain refined information about their age. This has made it possible to segregate LDVCs into different subpopulations according to their age. We have shown that newly synthesized LDVCs are located in peripheral areas, from which they are preferentially released by nicotine, which explains previous reports that newly synthesized peptides are secreted quickly [13,14]. The swift translocation of newly synthesized LDVCs from the Golgi to the cell membrane agrees with a recent analysis of vesicle dynamics in PC12 cells [15].

LDVCs that are not released within 16 h of assembly retreat from the cell membrane and enter the RP (see Figure 1c). Release of these older vesicles can be triggered by Ba2+, suggesting that different secretagogues act on vesicles of different ages. The underlying mechanisms need further investigation.

The preferential release of newly synthesized LDVCs offers at least two advantages for endocrine cells. First, changes in the expression of vesicle proteins or biosynthetic enzymes can be rapidly ‘translated’ into changes in the composition of the membranes or cargo of LDVCs. This reduces the time between changes in expression and their possible effectiveness in the RRP from many days down to a few hours. Secondly, the only way to ensure that more unstable vesicle components, which may have half-lives in the order of hours rather than days, are available for release is again the preferential recruitment, rapid translocation and release of the youngest LDVCs.

This work was funded by Wellcome Trust grants.

**References**


Received 23 March 2003