

Recycling of intact dense core vesicles in neurites of NGF-treated PC12 cells

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Abstract Exocytic fusion in neuroendocrine cells does not always result in complete release of the peptide contents from dense core vesicles (DCVs). In this study, we use fluorescence imaging and immunoelectron microscopy to examine the retention, endocytosis and recycling of chromogranin B in DCVs of NGF-treated PC12 cells. Our results indicate that DCVs retained and retrieved an intact core that was available for subsequent exocytic release. The endocytic process was inhibited by cyclosporine A or by substitution of extracellular Ca^{2+} with Ba^{2+} and the total recycling time was less than 5 min.

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1. Introduction

Regulated secretion from neurons and neuroendocrine cells is a result of the exocytic fusion of either synaptic vesicles or dense core vesicles (DCV). Synaptic vesicles store non-peptide transmitters such as glutamate or acetylcholine, whereas DCVs are responsible for secretion of peptide hormones and neuropeptides as well as amines.

While there are similarities with respect to the mechanisms for Ca^{2+} -dependent exocytosis for both types of vesicles, functional recycling may be fundamentally different processes. The synaptic vesicle cycle includes membrane recycling and import of cargo molecules directly from the cytosol allowing multiple rounds of exocytosis and cargo loading to occur locally in the synaptic terminal [1–3]. For DCVs whose main secretory cargo is a peptide hormone, functional recycling following exocytic release of cargo would likely include traffic to biosynthetic compartments of the Golgi to reload with cargo [4]. However, DCVs such as chromaffin granules that release monoamines could possibly undergo recycling processes more similar to that of synaptic vesicles [5]. Monoam-

ines transporters reside in the membranes of DCVs [3,6], indicating that these cargo components can be locally imported. For the DCVs of this type to retain functionality, they would presumably have to be endocytosed intact and retain their peptide cores that help store the soluble transmitters [7,8].

In the present study, we used NGF-treated PC12 cells with fluorescence imaging and electron microscopy to test the hypothesis that intact dense cores of catecholamine containing vesicles are internalized following exocytosis and recycle locally for use during subsequent rounds of exocytosis in the neurite tips.

2. Materials and methods

2.1. Cell culture and reagents

PC12 cells (ATCC, Manassas, VA) were cultured in F12K media with 2.5% FBS and 15% donor horse serum (Mediatech, Inc., Herndon, VA). NGF-treated cells were prepared by plating cells in poly-L-lysine coated Permanox chamber slides (Nalge Nunc, Naperville, IL) containing culture media supplemented with 2.5 ng/ml 2.5S murine NGF (Invitrogen, Carlsbad, CA). Cells were cultured 4–6 days before experiments.

The standard external solution contained 140 mM NaCl, 2 mM KCl, 1 mM MgCl_2 , 5 mM CaCl_2 , 10 mM D-glucose and 10 mM HEPES, pH 7.2. High K^+ external solution was identical except that it contained 100 mM KCl and 42 mM NaCl. When indicated, the 5 mM CaCl_2 was replaced with 10 mM BaCl_2 . Cyclosporine A (Calbiochem) was added at 10 μM to cells in standard external solution for 10 min prior to stimulation with high K^+ external solution containing 10 μM cyclosporine A.

Secondary antibodies (Jackson Immunoresearch Labs, West Grove, PA), mouse anti-chromogranin B (Transduction Labs/BD Pharmingen, San Diego, CA) and rabbit anti-synaptophysin (Synaptic Systems, Goettingen, Germany) were used.

2.2. Stimulus dependent labeling of dense cores with anti-chromogranin B

To label chromogranin B (CgB) exposed to the cell surface in response to exocytic stimulation [9], cells were incubated in high K^+ external solution containing 1:100 anti-CgB and 1% BSA for 5 min at 37 °C. Cells were then rinsed in ice cold PBS and fixed in ice cold 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.3% Triton-X100 before labeling with 1:400 Cy2-labeled donkey anti-mouse secondary antibody and mounted with Vectashield containing DAPI (Vector Labs, Burlingame, CA). Control experiments were routinely conducted in which anti-CgB was included in standard external solution instead of high K^+ external solution and showed no staining, indicating that staining was dependent upon exocytic stimulation. Only cells from passage 3–10 after thaw of the ATTC stock were used, since cells maintained in culture for more than 10 passages showed an increase in stimulus-independent uptake of anti-CgB.

To test for internalization of anti-CgB, cells were stimulated as above and then a brief (~60 s) rinse with 0.5 M NaCl in 0.5% acetic acid adjusted to pH 2.0 was performed prior to fixation. This acid

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Abbreviations: DCV, dense core vesicle; CgB, chromogranin B; HRP, horseradish peroxidase

wash procedure was sufficient to remove all anti-CgB bound and exposed to the cell exterior as determined by a lack of staining when fluorescent secondary antibodies were applied after acid wash without permeabilization with TritonX-100. Thus, any staining observed in acid-washed cells after permeabilization was due to anti-CgB that had been internalized.

To measure the amount of anti-CgB internalized during stimulation, parallel experiments were conducted in which cells were processed with or without acid wash. Cells were selected at random and all images were acquired under identical conditions. The integrated fluorescence of the neurite tips of all tips was measured. Exocytosis was expressed as the mean fluorescence of tips in the non-acid wash conditions (i.e., total exocytic labeling with anti-CgB). The amount of anti-CgB internalized was determined by taking the mean of fluorescence from tips in acid washed cells divided by the mean fluorescence from tips in non-acid washed cells (i.e., the ratio of internalized anti-CgB over total exocytic labeling with anti-CgB). This ratio, representing the amount of endocytosis, varied between different cultures and ranged from 40% to 60% of exposed CgB being internalized during 5 min. To determine the effect of Ba^{2+} or cyclosporine A on endocytosis, acid and non-acid wash experiments for both standard control conditions and test conditions were conducted in parallel. Exocytosis and endocytosis for test conditions were then normalized to the values from the corresponding standard control conditions to give the percent of control. Values are given as means \pm S.E.M.

To test for recycling of internalized anti-CgB, cells were stimulated as above in high K^+ external solution containing anti-CgB, rinsed with acid solution, then rinsed rapidly in standard external solution and then immediately restimulated a second time in high K^+ external solution containing Cy2 donkey anti-mouse secondary antibody. The wash procedures required 60–90 s. At the end of the second stimulation, cells were fixed and permeabilized and also stained for synaptophysin immunoreactivity to aid visualization of neurite tips.

2.3. Fluorescence imaging and analysis

Image acquisition and analysis was performed with a system running Slidebook software (Intelligent Imaging Innovations, Denver, CO) comprising a Zeiss Axiovert S100 inverted microscope, 63X oil immersion objective (1.4 na, PlanApochromat, Zeiss) and Cooke Sensicam CCD camera.

2.4. Electron microscopy

Cells were stimulated for 5 min in high K^+ external solution containing anti-CgB, fixed in ice cold 4.0% paraformaldehyde, 0.05% glutaraldehyde in 100 mM phosphate buffer, treated with 0.1% $NaBH_4$ and then 0.05% TritonX-100 for 30 min at room temperature. Cells were blocked with 0.2% BSA-c prior to incubation in 6 nm gold-labeled Protein G (Aurion, Electron Microscopy Sciences) in 0.2% BSA-c for 18 h at 4 °C. Cells were postfixed in 2.5% glutaraldehyde for 30 min and then 1.0% OsO_4 for 15 min and then dehydrated in ethanol and embedded in Epon. Thin sections were post-stained with 5% uranyl acetate and Reynolds lead citrate, viewed and photographed with a Hitachi 7000 transmission electron microscope. Control experiments were processed identically except that anti-CgB was omitted from the high K^+ external solution.

3. Results

3.1. Stimulus-dependent retrieval of dense core contents

Exocytic anti-CgB labeling was abundant throughout the cell body, neurite extension and neurite tips (Fig. 1A). These results are in agreement with the previously reported exocytic labeling with anti-CgB in undifferentiated PC12 cells [9].

The labeling observed in Fig. 1A could be due to either anti-CgB still exposed to the extracellular solution or anti-CgB that had been internalized during stimulation. To address this, cells were stimulated for 5 min with high K^+ solution containing anti-CgB, then rinsed rapidly in ice cold acid wash solution to remove surface exposed antibodies prior to fixation, permeabilization and detection with fluorescent secondary antibodies.

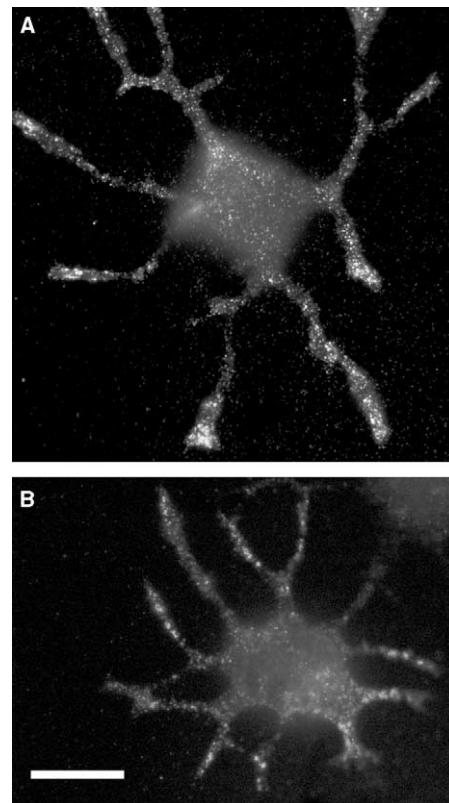


Fig. 1. Retention of CgB. (A) Cells were stimulated in the presence of anti-CgB antibodies prior to fixation, permeabilization and detection with secondary antibody. (B) Cells in which an acid wash was added prior to fixation to remove anti-CgB still exposed to the surface. Images for (A) and (B) were acquired with the same settings and are displayed on the same intensity scale. Scale bar is 10 μ m for both images.

The detected anti-CgB following the acid wash was due to structures that had internalized the antibody during the high K^+ treatment (Fig. 1B).

3.2. Inhibition of endocytosis by cyclosporine A and substitution of Ca^{2+} by Ba^{2+}

We used quantitative image analysis to estimate the amount of granule retrieval in the neurite tips. The integrated fluorescence of tips in cells that were not treated with the acid wash revealed the total amount of labeling that occurred during stimulated exocytosis. This value provides a measure of exocytosis. The ratio of fluorescence in acid washed samples to non-acid washed samples is a measure of the fraction of the anti-CgB that was internalized during the 5 min high K^+ stimulation.

When extracellular Ca^{2+} was replaced by Ba^{2+} , the fraction of anti-CgB that was internalized was reduced while total exocytosis was slightly increased compared with the control (fraction internalized control = 0.40 ± 0.02 ; fraction internalized Ba^{2+} treated = 0.12 ± 0.01 , normalized endocytosis = 30%; Fig. 2). Treatment with the calcineurin inhibitor cyclosporine A resulted in inhibition of total exocytosis (Fig. 2) consistent with the effect of this reagent on exocytosis in chromaffin cells [10]. The fraction of exocytosed CgB that was internalized was also reduced (fraction internalized control = 0.60 ± 0.07 ; fraction internalized cyclosporine treated = 0.16 ± 0.02 , normalized endocytosis = 27%; Fig. 2).

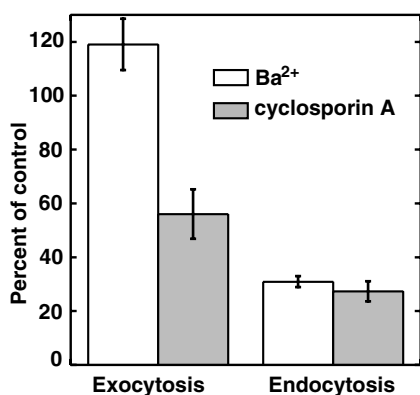


Fig. 2. Endocytosis of anti-CgB is inhibited by Ba^{2+} or by cyclosporine A. Exocytosis and endocytosis under test conditions are normalized to values from standard conditions. (see Section 2 for details).

3.3. Internalized anti-CgB was contained in dense cores

We used immunogold labeling to determine the ultrastructural characteristics of the structures containing endocytosed anti-CgB. The internalized anti-CgB was clearly associated with dense core containing organelles in the neurite tips (Fig. 3). Over 70% of cores within the neurite tips had gold

associated with them. There was also gold associated with patches of electron dense material at the cell surface (Fig. 3C). The majority of internalized label was associated with cores that appeared spherical and similar in size to unlabeled cores (Fig. 3D). No protein G-gold labeling of cores was observed in control experiments in which anti-CgB was omitted (Fig. 3E).

3.4. Local recycling of endocytosed secretory granules

Cells that internalized anti-CgB were washed with acid solution and then were immediately stimulated a second time for 5 min with high K^+ solution containing Cy2-labeled donkey anti-mouse secondary antibody (Fig. 4). Labeling is due to exocytosis of granules that had internalized anti-CgB following the first stimulation and was largely restricted to neurite tips with labeling at the cell body being observed infrequently and with less intensity. The recycling anti-CgB in the tips did not co-localize with anti-synaptophysin (Fig. 4C), a marker of small clear vesicles.

4. Discussion

We provide evidence for retrieval and recycling of intact dense core granules in the neurites of NGF-treated PC12 cells. Our results are consistent with morphological studies of

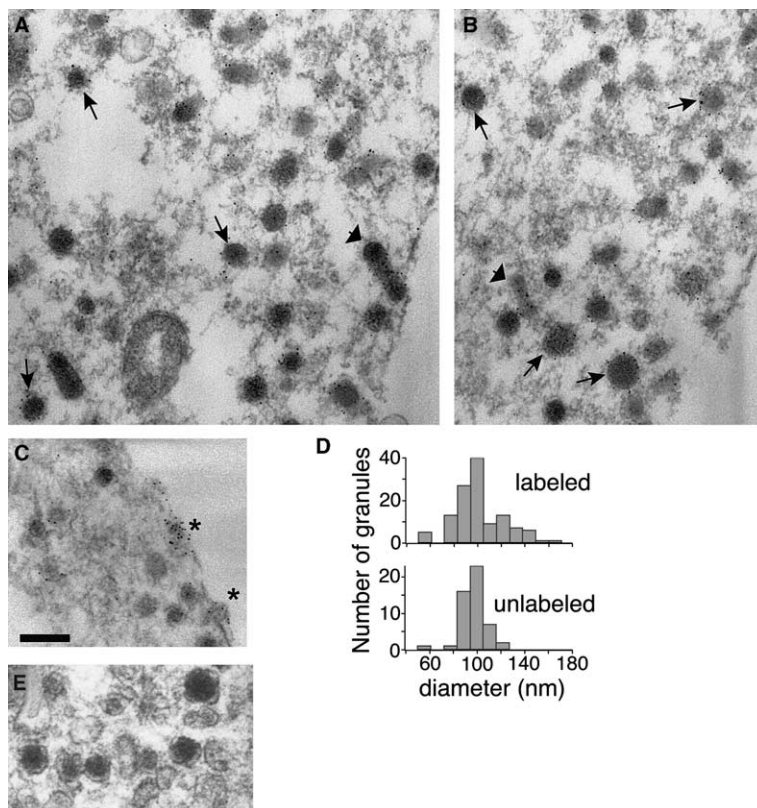


Fig. 3. Internalized anti-CgB is associated with dense cores. Cells were labeled with 6 nm protein G gold following stimulation in the presence of anti-CgB. All images are from the tips of neurites. (A, B) Gold was mostly associated with spherical cores (arrows) but was rarely observed associated with cores in elongated structures (arrow heads). (C) Surface-bound anti-CgB was also associated with electron dense material or cores (*). (D) The diameter of spherical gold-labeled cores (top; mean 99.3 ± 16.0 nm) was not different from the diameter of unlabeled cores (bottom, mean 94.5 ± 8.4 nm). (E) Control cells were treated identically except that anti-CgB was omitted from the stimulation solution. Scale bar is 200 nm for all images.

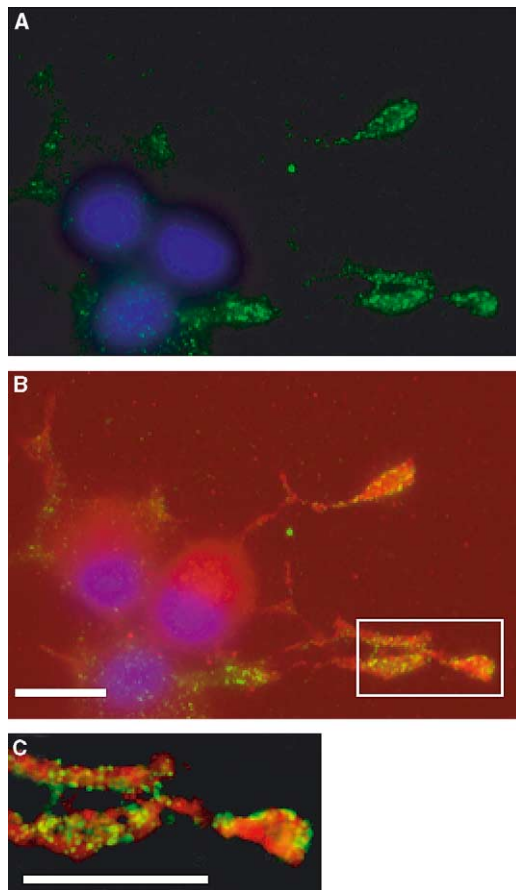


Fig. 4. Recycling of anti-CgB in neurite tips. (A) Cells that had internalized anti-CgB during a first round of stimulated exocytosis were stimulated a second time in the presence of labeled secondary antibody (green). (B) The same cells were also processed for immunostaining of total synaptophysin (red) to indicate the location of both the neurites and cell body. The nuclei are stained with dapi (blue). (C) Higher magnification of the region boxed in (B) demonstrates that recycling of anti-CgB did not co-localize with synaptophysin. Scale bar is 10 μ m in all images.

chromaffin cells in which no detectable loss of dense cores was observed regardless of the amount of secretion [11,12]. Slow and/or partial release of peptide content has also been observed in a variety of endocrine cells [13–16].

Biophysical studies demonstrating rapid endocytic processes coupled to DCV fusion reviewed in [17,18] and the partial release of amine coupled to the flickering behavior of fusion pores [8,19] are consistent with transient fusion events that may allow for retention of dense cores. Studies of this type have provided tremendous insight into kinetic coupling between exocytosis and endocytosis. However, they do not provide morphological correlates of these processes nor do they directly address retention of the cores. Retention and endocytosis of the cores could be controlled by the extent of fusion pore expansion, the duration of opening of the pore as well as the physical attachment of the proteins in the core to the membrane. Full pore dilation and granule collapse may contribute to the surface bound anti-CgB observed in our electron microscopy analysis that was not rapidly internalized.

Stimulus-dependent uptake of antibodies against DCV membrane proteins has been used to investigate recycling of

vesicle membrane in chromaffin cells [20–22] and neurons [23]. These studies focused on longer time scales and found recycling back to the cells surface after prolonged times (typically hours) [20,21]. When stimulus-dependent uptake of anti-CgB was monitored in undifferentiated PC12 much of the antibody was degraded within 1 h, the fate of the remaining anti-CgB was not determined [9]. The non-specific fluid phase marker HRP has been used to demonstrate a recycling process in chromaffin cells detectable 5 min after an exocytic stimulus [24]. Recent studies demonstrated that HRP was found in dense core granules at the end of a 2 min high K^+ stimulation of PC12 cells [14] and chromaffin cells [25]. Together, these results are consistent with rapid local recycling of DCVs, although the possibility of HRP traffic through other endosomal systems was not ruled out.

Capacitance measurements from chromaffin cells have begun to unravel the mechanisms controlling rapid DCV membrane retrieval. Endocytosis is dependent on different isoforms of dynamin and upon clathrin, depending on stimulation conditions [26,27]. Substitution of Ba^{2+} for Ca^{2+} did not support rapid endocytosis in chromaffin cells under certain stimulation conditions [26,28], suggesting that distinct mechanisms are used under different conditions. The capacitance approach allows for fine control of stimulation and direct comparisons with our results using high K^+ treatment are difficult. However, it is worth noting that endocytosis of anti-CgB was inhibited by replacement of extracellular Ca^{2+} by Ba^{2+} and by the calcineurin inhibitor cyclosporine A. These results are consistent with the effects of this drug on rapid endocytosis in chromaffin cells [10,29] and suggest a role for dephosphins [30].

Block of dynamin function has been found to cause rundown of catecholamine secretion from chromaffin cells, suggesting that recycling of DCVs was required to maintain catecholamine secretion [31]. We found that the internalized granules in the neurite tips were available for a second exocytic stimulation within minutes of the first exocytic stimulation. These results provide direct support for the hypothesis that DCVs responsible for secretion of monoamines remain relatively intact and are capable of local recycling processes more similar to that of synaptic vesicles. The number of times a DCV can recycle is likely related to the factors governing attachment of CgB [9,32,33] and other matrix proteins to the membranes. Regulation of dense-core retention [13,33,34] could clearly impact DCV recycling and sustained secretion.

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