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Multicore Vesicles: Hyperosmolarity and L-DOPA Induce Homotypic Fusion of Dense Core Vesicles

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Abstract

Aim— A fraction of vesicles in cells treated with hypertonic solution exhibit multiple dense cores and this is enhanced by treatment with L-3,4-dihydroxyphenylalanine (L-DOPA). These cells were examined to determine if the multicore vesicles are the product of endocytosis or homotypic fusion.

Methods— Electron microscopy was used to determine the number of multicore vesicles and amperometry was used to examine if the multicore vesicles are a competent fraction of the readily releasable pool.

Results— In this study, we observed that a substantial portion (15.3%) of large dense core vesicles in PC12 cells contained multiple cores in hypertonic saline loaded with L-DOPA, and amperometric measurements show a bimodal distribution of quantal sizes in treated cells.

Conclusions— The results suggest that the multicore vesicles are formed from homotypic fusion of LCDVs prior to exocytosis.

Keywords

Exocytosis; Secretory granules; Electron microscopy; PC12; Cationized ferritin; Quantal size; Amperometry; Catecholamines

Introduction

The volume of secretory vesicles in both PC12 cells and cultured neurons can be enlarged by treatment with L-3,4-dihydroxyphenylalanine (L-DOPA) (Colliver et al. 2000; Pothos 2002), and L-DOPA treatment leads to larger quantal release per vesicle fusion event (Pothos et al. 1996). Quantal size can also be manipulated by other means, such as activation of second messenger systems, autoreceptors, or the use of VMAT inhibitors (Pothos 2002). These manipulations have the potential to influence synaptic plasticity (Choi et al. 2003).

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Electron microscopy (EM) images of secretory cells show that vesicles usually contain exactly one dense core per vesicle. However, we show that hyperosmotic conditions appear to increase the frequency that large dense-core vesicles (LDCVs) in rat pheochromocytoma (PC12) cells fuse with one another prior to exocytosis (homotypic fusion). Incubation with the dopamine precursor L-DOPA augments this effect. Amperometric measurements suggest these multicore vesicles remain a competent fraction of the readily releasable pool. These treatments, although not physiological, provide evidence that mature vesicles can interact with each other.

Methods

PC12 cells were maintained as described previously (Kozminski et al. 1998). Cells were treated with low-or high-salt salines for 10 min (5 mM KCl, 10 mM HEPES, 5 mM glucose, 2 mM CaCl₂, 1.2 mM MgCl₂, and 150/332/460 mM NaCl, pH 7.4). Subsequently, cells were incubated for 60 min in isotonic saline with or without L-DOPA (100 μ M), cationized ferritin (5–500 mg/ml; Sigma, St. Louis, MO), or the combination (Danon et al. 1972; Farquhar 1978). Cells were rinsed for 10 min in saline with osmolarity corresponding to the first incubation period, pelleted, fixed (Karnovsky 1965), and prepared for electron microscopy by conventional methods (Colliver et al. 2000). Vesicle diameters were corrected for sampling bias inherent in random transections of vesicles (Aherne and Dunnill 1982).

Amperometry (5- μ m carbon fiber beveled at 45°, + 650 mV holding potential) data were filtered at 2 kHz (Axopatch 200B, Molecular Devices Corp.) and digitized at 5 kHz. Peaks were detected using MiniAnalysis (www.Synaptosoft.com) with a threshold of 5 times the RMS noise. Quantal size distributions were analyzed for the presence of multiple distributions using PeakFit (SPSS, Inc.) All comparisons employed a t-test unless stated otherwise. Results were considered significant if $P < 0.05$. Values are mean $\pm$ SEM.

Results

A substantial portion of vesicles contained multiple dense cores when the osmolarity was increased (Table 1). No multicore vesicles were found in control cells incubated in isotonic (335 mOsm) saline (data not shown), but 3.5% and 8.6% of vesicles had multiple cores at 700 mOsm and 965 mOsm, respectively (Fig. 1a). Addition of 100 μ M L-DOPA to the 700 mOsm hypertonic saline increased the percentage of multicore vesicles from 3.5% to 15.3% ($P < 0.05$; Fig. 1b), whereas L-DOPA increased the percentage of multicore vesicles under isotonic conditions to a modest 0.3%.

Vesicular volume was 6.0 ± 2.0 aL for multicore vesicles in cells incubated in 700 mOsm saline compared to 2.1 ± 0.2 aL for single-cored vesicles in the same cells ($P < 0.05$), signifying a 292 % increase. In cells treated with 700 mOsm saline and L-DOPA, vesicular volume was 14.0 ± 2 aL for the multicore vesicles versus 5.9 ± 0.9 aL for single-cored vesicles ($P < 0.05$), a 237% increase.

To ascertain whether multicore vesicles reflected a population of recycled protein cores captured during endocytosis or vesicles fusing with each other independent of exocytosis, we employed a marker for endocytosis. Incubation with 500 μ g/ml cationized ferritin produced optimal endocytic labeling of LDCVs in isotonic control cells (Fig. 1c). However, in hypertonic saline, endocytosed particles were only observed when the concentration of cationized ferritin was reduced to 50 μ g/ml (Fig. 1d; unsuccessful experiments employed a range of 5–500 μ g/ml). At the low-ferritin concentrations, too few vesicles were labeled to enable quantitative analysis, and at higher concentrations the ferritin label usually formed large extracellular clumps away from the plasma membrane and was not trapped by endocytosing vesicles. Moreover, incubating cells with 500 μ g/ml ferritin in isotonic saline (335 mOsm) caused a

slight increase in the percentage of multicore vesicles observed from 0% to 1.4%. Assuming the few multicore vesicles observed following incubation in isotonic saline containing 100 μ M L-DOPA were generated in the same manner as those observed in hyperosmotic solutions, we examined, whether any of these multicore vesicles contained the endocytosis tracer when 500 μ g/ml ferritin was used.

After examining several hundred cells that had been incubated in isotonic saline containing ferritin and L-DOPA, eleven cells were identified that contained at least one multicore vesicle. In these cells, 11 ± 3 % of the 684 single-core vesicles contained the ferritin label; however, no ferritin localized with any of the 58 multicore vesicles identified. In all, ferritin was detected in 385 subcellular compartments resembling vesicles or small endosomes, but only 59 of these vesicle-like compartments contained both ferritin and a dense core. If the majority of these 385 ferritin-containing compartments are indeed recaptured vesicles, then the dense core is not retained during endocytosis in PC12 cells in up to 85% of events. If this assumption is correct, then 15% of vesicle fusion events are of the kiss-and-run variety, leading to dense core retention. As EM analysis of sectioned vesicles underestimates the number of with a dense core, the fraction of events that are kiss-and-run is probably higher.

Amperometry was performed on cells before and after hypertonic and L-DOPA treatment to deduce whether the presence of multicore vesicles affected the quantal size of stimulated secretion. When the distribution of quantal sizes from cells before treatment was compared to quantal sizes from the same cells after treatment, the post-treatment distribution was more positively skewed (3.78 ± 0.10) than the pre-treatment distribution (2.20 ± 0.09). The best fit of two curves to the hypertonic- L-DOPA-treated distribution sorted 84 % and 16 % of quanta into the smaller and larger portions of the bimodal distribution (Fig. 2), respectively, similar to the portion of single core (84.7%) and multicore (15.3%) vesicles observed with EM. Similar analyses of the quantal size distribution before and after L-DOPA treatment in isotonic saline produced nearly identical skew measurements (2.54 ± 0.10 before compared to 2.53 ± 0.12 after L-DOPA) without bimodal distributions. Thus the treatment with hypertonic salines and L-DOPA was not cytotoxic and both single core and multicore vesicles were capable of exocytosis.

Discussion

Homotypic fusion has been observed between immature secretory granules during vesicle maturation (Tooze et al. 1991; Wendler et al. 2001) and evidence for homotypic fusion has been provided in adrenal chromaffin cells (Amatore et al. 2005). Multicore vesicles have also been previously observed (Farquhar 1978; Tooze and Huttner 1990). However, methods to reproducibly generate multicore vesicles have not been previously available. The electron microscopy results presented here suggest that multicore vesicles are formed from homotypic fusion of LCDVs prior to exocytosis. Amperometric measurements support this conclusion as the distribution of quantal sizes includes a bimodal distribution, likely arising from exocytosis of dopamine from two pools of vesicles (single and multicore LCDVs). Thus it appears that mature, competent secretory vesicles are not inhibited from exchanging contents during maturation and that some fuse in the cytosol, at least under hyperosmotic conditions. The synergy of both treatments may be the result of swollen, unstable vesicles migrating in an osmotically reduced cytosolic volume that increases the probability of vesicular collisions. In a cell-free system osmotic swelling has been shown to drive the fusion of vesicles to planar membranes (Cohen et al. 1982).

Although competent vesicles do not appear to fuse together under normal conditions, their tendency to fuse in hyperosmotic solutions reveals that the mechanisms preventing homotypic fusion are not robust. Even L-DOPA treatment or the presence of cationized-ferritin in solution

induced the formation of a few (~ 1%) multicore vesicles under isotonic conditions, further suggesting that barriers to homotypic fusion are relatively weak. Therefore, it is possible that vesicles have the ability to exchange phospholipids and possibly proteins under normal conditions. Such possibilities are worthy of further exploration, as exchange of lipid and proteins between vesicles could serve a regulatory purpose. For instance, such a membrane exchange capacity could help explain why vesicles swell and shrink in volume in response to L-DOPA and reserpine treatment, respectively (Colliver et al. 2000), rather than altering the concentration of dopamine packaged in each vesicle. This phenomenon likely results from an ability of vesicles to shed or absorb phospholipids more easily than altering the driving force for neurotransmitter uptake or the neurotransmitter binding capacity of the dense core. Furthermore, a recent report suggests at least one vesicular protein, the vesicular ATPase, is tightly regulated (Takamori et al. 2006). The copy number of the vesicular ATPase varies between 1 and 2 copies per vesicle. It is possible that at least one copy of this essential transporter is actively maintained by regulated exchange between vesicles, similar to how vesicles accrue multiple cores under the conditions examined in this study. These observations demonstrate the plastic nature of secretory vesicle pools and open up the possibility that treatments could affect neuronal communication by altering the homotypic fusibility of secretory vesicles.

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Abbreviations

PC12	Rat Pheochromocytoma
TEM	Transmission Electron Microscopy
L-DOPA	L-3,4-dihydroxyphenylalanine
VMAT	Vesicular Monoamine Transporter
AL	Attoliters (10^{-18} l)

Fig. 1.

A portion of dense core vesicles in PC12 cells exhibits multiple cores. Two examples of each treatment are provided. (a) Cells bathed in hyperosmotic (700 mOsm) saline. (b) Cells bathed in hyperosmotic saline and loaded with L-DOPA. (c) Control cells labeled with ferritin. (d) Hyperosmotic, L-DOPA-treated cells labeled with ferritin. Arrows with stems indicate examples of multicore vesicles. Arrowheads indicate vesicles that incorporated the ferritin label (about 11% of cells). Images were collected from 10 to 12 cells per condition, with similar results

Fig. 2.

Two populations of vesicles undergoing exocytosis. Histogram of amperometric peak area (fC) for events ($n = 587$) collected from PC12 cells ($n = 5$) pre-treated with $100 \mu\text{M}$ L-DOPA in isotonic (335 mOsm) saline, then rinsed and stimulated in hypertonic (700 mOsm) saline. Smoothed traces reflect a double distribution in the data, generated using PeakFit software ($r^2 = 0.93$). The smaller distribution of larger area peaks encompasses 16% of the total detected peaks

Table 1

Percentage of vesicles having multicores increases with high osmolarity and L-DOPA treatment

Osmolarity (mOsm)	Multicore vesicles (%)	
	No L-DOPA	L-DOPA
335	0.0	0.3
700	3.5	15.3
955	8.6	