ELSEVIER

Journal of Neuroscience Methods 153 (2006) 27–42

JOURNAL OF NEUROSCIENCE METHODS

www.elsevier.com/locate/jneumeth

Vesicular roundness and compound release in PC-12 cells

D. Germain [a], D. Maysinger [b], M.I. Glavinović [c,*]

[a] *Department of Computer Engineering, McGill University, Montreal, Canada*
[b] *Department of Pharmacology and Therapeutics, McGill University, Montreal, Canada*
[c] *Department of Physiology, McGill University, 3655 Sir William Osler Promenade, Montreal P.Q., Canada H3G 1Y6*

Received 17 May 2005; received in revised form 22 September 2005; accepted 3 October 2005

Abstract

The principal goals of this study were to establish a quantitative morphological analysis of spatial and regional properties of dense core vesicles, and to use this analysis to assess whether homotypic fusion is prominent in chronically treated PC-12 cells at elevated release levels. Simple computerized image processing of electron-micrographs provided the binary images of vesicular dense cores, whilst the artificial intelligence methods were needed to determine the vesicular membranes. As in the past, the presence of large, highly irregular vesicles, provided the morphological evidence of fused vesicles, but the irregularity of vesicular shape was assessed quantitatively—from its roundness. Free space of each vesicle was determined from the distance to its nearest-neighbor, or from the size of its Voronoi polygon. Within a Voronoi polygon, each point is closer to that vesicle than to any other vesicle. Large vesicles were not less round and did not have larger free space, as expected if they result from fusion of several smaller vesicles.

In conclusion, we present a novel and rigorous morphological analysis of spatial and regional properties of dense core vesicles. The results demonstrate that the homotypic fusion is not prominent in PC-12 cells, before or following a chronic treatment that enhances release.

Keywords: Homotypic fusion; Voronoi polygon; Neural network; Vesicular clustering; Pheochromocytoma

1. Introduction

One of the most fundamental hypotheses of neurobiology – quantal hypothesis – is based on the assumption that the quantal size is constant (Del Castillo and Katz, 1954). There is now a growing body of experimental evidence demonstrating that the quantal size of dense core vesicles can change, sometimes greatly with stimulation (Bokvist et al., 2000; Glavinović and Trifaro, 2002; Pothos et al., 2002), but there is no unanimity as to what the origin of such changes is. In this study, we evaluate using morphometry in PC-12 (pheochromocytoma) cells, whether quantal size rises because several vesicles fuse and subsequently release their content through a single fusion pore (often referred to as compound secretion).

The formation of secretory granules is initiated in the trans-Golgi network. The exocytosis of these granules occurs at low rate with no stimulation, but external stimuli amplify it. Immature granules can fuse with each other and with mature granules (homotypic fusion). The fusion of immature granules is part of the vesicular maturation process, and both the mature and immature granules can fuse with the plasma membrane (heterotypic fusion; Tooze et al., 1991; Urbe et al., 1998) but after maturation the granule–granule fusion machinery becomes inactivated (Urbe et al., 1998). Several lines of evidence—electrophysiological for mast cells (Alvarez de Toledo and Fernandez, 1990), biochemical for mast cells (Guo et al., 1998) and endocrine cells (Tooze et al., 1991), optical for pituitary lactotrophs (Cochilla et al., 2000), but also ultrastructural for eosinophils and mast cells (McLaren et al., 1997; Lawson et al., 1978), pancreatic β-cells (Orci and Malaisse, 1980), acinar cells (Senda et al., 1989) and pituitary somatotrophs (Nakagawa et al., 1995) support the presence of compound exocytosis (Urbe et al., 1997). The number of smaller vesicles fusing to form a larger vesicle appears to be different in different cell types. In unstimulated horse eosinophils most granules are formed by the fusion of 7–15 smaller granules (Henderson and Chi, 1985), but in human cells they consist of one to two smaller granules (Hartmann et al., 1995). More importantly, it is unclear how long the 'fused multi-vesicular complexes' exist prior to secretion, how spatially distributed

* Corresponding author. Tel.: +1 514 398 6002; fax: +1 514 398 7452.
E-mail address: mladen.glavinovic@mcgill.ca (M.I. Glavinović).

0165-0270/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneumeth.2005.10.003

they are and how much more prominent they become with acute or chronic treatments that enhance the release. In neutrophils, the compound exocytosis accounts for ∼22% of total exocytosis (Lollike et al., 2002) and guanosine 5′-γ-thiotriphosphate (GTPγS; Scepek and Lindau, 1993) and cyclic adenosine-3′,5′-monophosphate (cAMP) enhance homotypic fusion but not heterotypic fusion (Cochilla et al., 2000; Machado et al., 2001).

Given the ability of both mature and immature granules for heterotypic fusion, any change in homotypic fusion should contribute to change of quantal size. However, greater quantal size may also be due to preferential release of large vesicles, which are better barriers to Ca^{2+} diffusion (Glavinović and Rabie, 2001; Shahrezaei and Delaney, 2004). The release of transmitter is triggered by highly localized Ca^{2+} whose concentration may be >100 μM (Llinas et al., 1992; Mennerick and Matthews, 1996; Jeremic et al., 2004). In addition, greater quantal size may be due to a greater fractional release of vesicular content (Burgoyne and Barclay, 2002). Finally, the changes of the catecholamine synthesis and availability of neurotransmitter in the cytosol, changes in the expression of the vesicular monoamine transporters and changes in the vesicular pH may also alter the quantal size though slowly (Pothos et al., 1996, 2002; Sulzer and Pothos, 2000). To evaluate how prominent the homotypic fusion is, and thus to distinguish among possible explanations for the changes of quantal size, we have used well-developed mathematical methods of spatial analysis (Voronoi, 1908; Delaunay, 1932; Aurenhammer, 1991) and determined the vesicular regional (Voronoi polygons and vesicle–plasma membrane and vesicle–vesicle nearest-neighbor distances) and spatial parameters (radius and roundness). Our results argue that the homotypic fusion is not prominent in PC-12, and thus that the compound secretion does not contribute greatly to increase the presynaptic quantal size.

2. Materials and methods

2.1. Cell cultures

PC-12 cells used in this study, clone number 13088, were obtained from the American Type Culture Collection and were used for no more than 10–12 passages. Growth medium consisted of RPMI 1640 (Sigma) supplemented with 10% horse serum (Gibco) and 5% fetal bovine serum (Gibco), as well as antibiotics (penicillin and streptomycin; Gibco). One day prior to an experiment, a flask of confluent cells was passaged and plated onto a poly-L-lysine-coated 24-well tissue culture plate at a density of 50,000 cells per well. The serum contained (5%) RPMI 1640 medium. In some cases, a specific tyrosine phosphatase inhibitor potassium bisperoxo(1,10-phenantroline)-oxovanadate (bpv[phen]; Posner et al., 1994) was added to the cells (1 μM; 24 h). After starvation (4 h), cells were washed with phosphate buffer (0.1 M; pH 7.4) and fixed in glutaraldehyde (2.5% in phosphate buffer; 1 h). Then, cells were post-fixed with OsO_4 (1%) and KFe (1.5%) in phosphate buffer (0.1 M, for 1 h). This post-fixation protocol is frequently used for a better preservation of membranes. We have also tested the mixtures of paraformaldehyde (2.5%) with glutaraldehyde (0.5–1%), but they did not result in better vesicle preservation. A standard procedure was used for the EM of ultrathin sections (Phillips 410 LS).

2.2. Determination of vesicular dense cores in PC-12 cells

Fig. 1A shows the image of a PC-12 cell. The images have to be processed to evaluate the vesicular spatial and regional properties. The processing of the images proceeded in several stages. First, because the digitized images of the secretory cells produced very large files whose processing was very slow, the images were pre-filtered using the bilinear transformation, which is a widely used method. This reduced the size of the files to be analyzed and speeded up the analysis, but 'by visual impression' did not reduce the resolution greatly. Second, the images were thresholded, and the 'right' threshold value was arrived at iteratively. Several threshold values were typically tried and the one judged to be the 'best' – 'by eye' – was taken. The thresholding was primarily needed to decide where the limits of dense cores lie, and initially we did not use it for vesicle selection, which was decided entirely 'by eye'. However, the thresholding was found to be a very useful tool for assisting in the selection of vesicles. When the threshold was well chosen typically less then 1% (and never more then 2%) of vesicles and 'small blobs' needed to be reclassified. Thresholding made white all points below a chosen level of darkness, and black all those above, i.e. it creates a binary image. The values of the points give the optical density and range between 0.0 and 1.0, where 0.0 was an indication of a completely white dot and 1.0 of a completely

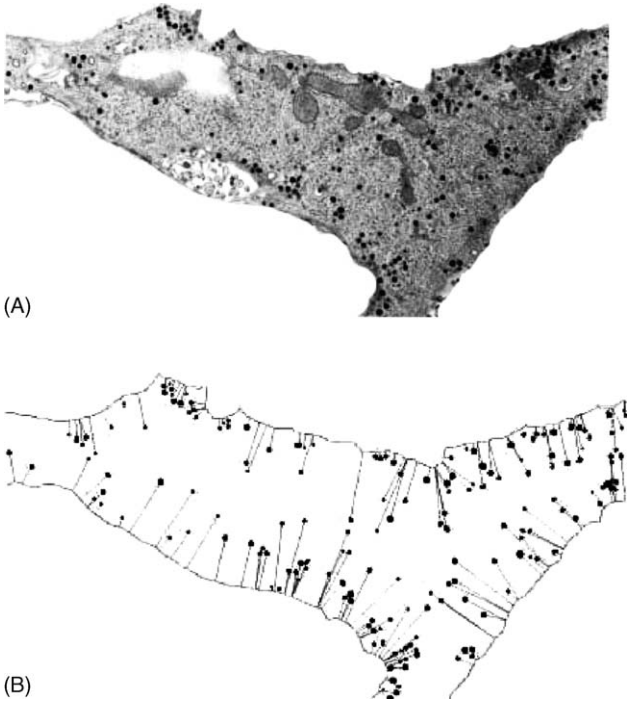

Fig. 1. (A) Electron-micrograph of a PC-12 cell. (B) Contour of the same PC-12 cell and the vesicular dark cores. The dark cores were determined following pre-filtering of the whole cell, thresholding, removal of the 'small blobs' and determination of the false-positives and false-negatives (see text). Lines indicate the shortest distance between a vesicle and the plasma membrane.