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Isolation of mouse chromaffin secretory vesicles and their division into 12 fractions

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ABSTRACT

The study of chromaffin secretory vesicles (SVs) has contributed immensely to our understanding of exocytosis. These organelles, also called chromaffin granules, are a specific type of large dense secretory vesicle found in many endocrine cells and neurons. Traditionally, they have been isolated from bovine adrenal glands due to the large number of SVs that can be obtained from this tissue. However, technical advances now make it possible to obtain very pure preparations of SVs from mice, which is particular interesting for functional studies given the availability of different genetically modified strains of mice. Despite the small size of the mouse adrenal medulla (400–500 μm and less than 2 mg in weight), we have successfully carried out functional studies on SVs isolated from WT and knockout mice. As such, we present here our method to purify crude vesicles and to fractionate mouse chromaffin SVs, along with examples of their functional characterization.

Introduction

Exocytosis is a key means of communication, particularly among neurons, and it entails the fusion of secretory vesicles (SVs) with the cell membrane to release part or all of their content into the extracellular space. Large numbers of SVs can be obtained from bovine adrenal glands and as such, this tissue has served as an important source of SVs for many decades, allowing biochemists to characterize many of their features, such as their vesicular content, lipid and protein composition, and other dynamic characteristics [1–3]. Chromaffin granules are a special type of large dense core vesicles (LDCVs) [4,5] that have also been isolated from human pheochromocytoma cells [6]. The study of these granules has allowed us to define their vesicular pH [7,8], the concentrations of amines [9–11], ATP [12,13] and calcium [14–16], and how these solutes are regulated. However, bovine tissue has serious limitations due to its heterogeneous origin, the difficulty of treating animals with specific agents or the impossibility of using genetically modified animals.

The development of micro-techniques (flow cytometry, fluorescence microscopy, proteomics, electrochemistry, etc.) enables minute amounts of animal tissue to be studied in distinct ways. When employed in conjunction with the genetically modified mice currently available, this advance makes the use of isolated SVs [17] or LDCVs from mouse adrenal glands a very attractive approach to perform functional studies. Thus, we describe here how to obtain purified LDCVs from mouse adrenal glands.

To the best to our knowledge, this is the first full description of a method to fractionate SVs from mouse adrenal glands capable of producing sufficient amounts of highly purified LDCVs to allow the analyses of solutes and membrane-bound proteins. These vesicles retain most of their ability to acidify their lumen, and for amine and ATP uptake.

Materials and methods

Reagents

The salts used to prepare the buffers should be reagent grade. All reagents are available from Sigma-Aldrich unless otherwise stated (Cat. No.): DNase I (D4527), ethylenediaminetetraacetic acid EDTA

Abbreviations used: CgA, Chromogranin A; CgB, Chromogranin B; DLS, Dynamic light scattering; LDCVs, Large dense core vesicles; SgII, Secretogranin II; SVs, Secretory vesicles.

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(E6758), HEPES (H3375), oligomycin (O5001), Trizma® base (T6791), Triton™ X-100 (T9284), Iodixanol (OptiPrep™ (D1556), cOmplete® protease inhibitors (Roche: Cat. No. 04693116001), and sucrose (Merck: Cat. No. 1.07651).

Animals

We use 6–8 week old C57BL/6J mice of 25–30 g body weight, the adrenal gland from which weigh approximately 2 mg. The use of animals conforms to all institutional and governmental regulations, and the animals were sacrificed by cervical dislocation.

At least 16 adrenal medullas (8 mice) are required to prepare the crude LDCV purification. However, if LDCVs are to be separated by continuous gradient centrifugation at least 20 mice are needed.

Equipment for vesicle isolation

The equipment required includes: a glass homogenizer, 0.1 mL volume (Jencons Scientific Ltd: Cat. No. 361.047); a degassing ultrasound bath (Branson, 2510 MT: Cat. No. Z244910); a stereo binocular-microscope coupled to a cold light source; a gradient station (BioComp Instruments, Inc, New Brunswick, Canada: Cat. No. 153–002); open-top 5 mL centrifuge tubes (SETON Scientific Petaluma, CA: Cat No. 7022); 150 mm glass Pasteur pipettes (Normax, Marinha Grande, Leiria, Portugal. Cat No. 5426015); a refrigerated microfuge and 2 mL microcentrifuge tubes suitable for centrifugation at 25,000 × g (Eppendorf: Cat. No. 0030.120.094); and an ultracentrifuge (e.g., Optima™ L-100 XP: Beckman Coulter, Inc.) with a SW 55 Ti swinging bucket rotor.

Reagent setup

All solutions should be stored at 4 °C.

Locke's buffer

Passed through a 0.22 μm filter (in mM): NaCl (154), KCl (5.6), $NaHCO_3$ (3.6), HEPES (5) and glucose (5.6) [pH 7.3].

Homogenization buffer

Passed through a 0.22 μm filter (in mM): sucrose (250), EDTA (1), $MgSO_4$ (1), KCl (10), HEPES (10), the cOmplete protease inhibitor mixture (1x), oligomycin 1 μM, DNase I 10 μg/mL and [pH 7.0] (KOH). Measured osmolarity is ≈310 mOsm.

Working solution

5 vol of commercial OptiPrep™ (60% Iodixanol) diluted in 1 volume of a filtered solution containing (in mM): sucrose (250), EDTA (6), $MgSO_4$ (6), HEPES (60), KCl (60) and protease inhibitors (1x) [pH 7] (KOH).

TENT buffer

A solution containing (in mM): Tris-Cl (50) [pH 8.0], EDTA (2), NaCl (150), protease inhibitors (1x) and Triton X-100 (final concentration, 1%).

Procedure

Isolation of the crude LDCV fraction

Once the abdomen has been opened and the adrenal glands located (Fig. 1A), these organs are removed and placed immediately in sterile ice-cold Locke's buffer. The white-yellow mouse adrenal gland is about the size and shape of a pinhead, and this color allows it to be easily distinguished from the kidney and the surrounding fat. The adrenal medullary tissue is yellow-orange while the cortex is darker (Fig. 1B). The glands are placed on a piece of Whatman®

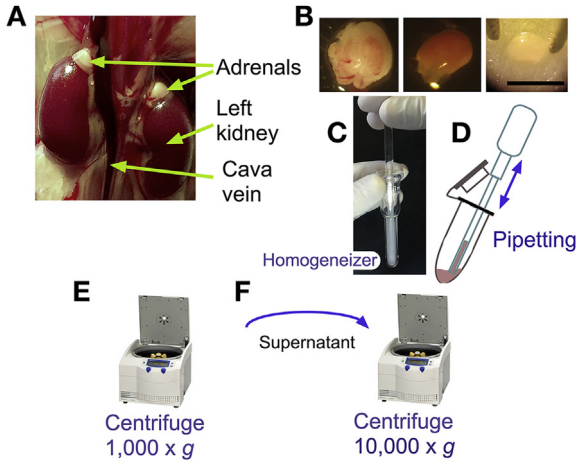

Fig. 1. General procedure to isolate LDCVs from the mouse adrenal medulla. Once both adrenal glands have been located (**A**), they are removed and (**B**) the adrenal medulla is exposed by peeling off the cortex. Note the different aspect of (from left to right) the fat, the whole gland and the adrenal medulla. Scale bar = 1 mm. (**C**) The medullas are trimmed and pooled, homogenized in a glass homogenizer (**D**) and further dispersed by repeated pipetting. Two sequential centrifugations **E** and **F** are employed to obtain a crude LDCV fraction (see text).

filter paper soaked in Locke's buffer and they are cleaned from the surrounding fat using a scalpel under a surgical microscope coupled to a cold light source. Subsequently, the medulla is carefully isolated from the cortex and placed into the glass homogenizer with 50 μL of homogenization buffer (Fig. 1 C, see below). Care is observed to keep the tissue moist and cold throughout the isolation procedure.

The adrenal medullas are triturated using 25 strokes to ensure that most of the cells (>90%) are broken. The glass homogenizer is washed using a Pasteur pipette in successive steps of 100 μL with ice-cold homogenization buffer, filling a 2 mL microcentrifuge tube (Fig. 1D). The tube is centrifuged for 10 min at 1000 × g and 4 °C to sediment the nuclei and incompletely broken cells (Fig. 1 E).

The supernatant is then centrifuged at 10,000 × *g* for 20 min and 4 °C to obtain an enriched pellet of crude LDCVs (Fig. 1F). The resulting pellet should be highly enriched in LDCVs with little mitochondrial and lysosomal contamination. This enriched but heterogeneous fraction contains sufficient vesicles for transmitter quantification by HPLC, for sizing by dynamic light scattering (DLS) or for flow cytometry. This crude preparation can also be used for further purification and separation (see below).

LDCV fractionation

We prepare the 5, 8 and 26% (w/v) iodixanol (Optiprep®) solutions by diluting a working solution (50% iodixanol) in homogenization buffer. The solution must be degassed and kept in ice-cold water. To form the continuous gradient, we add the 8% iodixanol solution to centrifuge tubes (open-top centrifuge tubes polyclear Seton Ref. 7022) to the level marked in the Gradient Station manual for short caps. We then inject the 26% iodixanol solution into the bottom of each tube until it reaches the same mark, taking special care not to disturb the interface between the two solutions when removing the cannula and avoiding the formation of air bubbles. Each tube is completely filled with 5% solution. We next plug the tube with the short cap (Cat No. 105-413-6, BioComp Ins.) and rotate them for 55 s at an angle of 87° and 28 rpm in the Gradient Station. The crude pellet is re-suspended in 115 μL of 5% iodixanol solution and 100 μL of this solution is used to fill the centrifuge tubes to the very top, adding only the 5% solution to one tube as a control to measure the density of the gradient (Fig. 2) [18]. After a