FEBS Letters 505 (2001) 414–418 FEBS 25260

Direct observation of membrane retrieval in chromaffin cells by capacitance measurements

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Received 9 July 2001; accepted 13 August 2001

First published online 6 September 2001

Edited by Maurice Montal

Abstract This study was focussed on the identification of the endocytic organelles in chromaffin cells which retrieve large, dense core vesicle (LDCV)-membrane components from the plasma membrane. For this purpose, 'on-cell' capacitance measurements and electron microscopy were employed. We found capacitance steps and capacitance flickers, corresponding to single exo- and endocytic events. The analysis revealed that the total membrane surface of completely fused LDCVs is recycled by large endocytic vesicles and smaller, most likely clathrin-coated vesicles, at approximately the same ratio. These results were confirmed by rapid-freeze immuno-electron microscopy, where an extracellular marker was rapidly internalized into endocytic vesicles that morphologically resembled LDCVs.

Key words: Endocytosis; Chromaffin cell; Clathrin-coated vesicle; Capacitance measurement; Immuno-electron microscopy

1. Introduction

Catecholamines are stored in large, dense core vesicles (LDCVs) and released from chromaffin cells during cholinergic stimulation. Vesicles fuse either completely with the plasma membrane, a process termed 'exocytosis', or the contents are liberated by the 'kiss-and-run' mechanism [1–4].

The membrane of completely fused LDCVs becomes recycled quickly by endocytosis, preventing continued growth of the plasma membrane surface [5]. Obviously, the recovery of LDCV-specific lipids and proteins is essential for proper functions, and this process involves the accumulation of LDCV components in specialized locations for endocytosis. There are only a few studies that focus on the characterization of the endocytic organelles in chromaffin cells. First described in context with receptor-mediated endocytosis, clathrin-coated vesicles are the standard endocytic organelle for membrane internalization. This universal concept was proposed as one of the major pathways for synaptic vesicle recycling at the frog neuromuscular junction [6], and the idea was supported by several groups, presenting biochemical [7,8] and morphological evidence [9].

It was proposed that the LDCV membrane does not completely intermix with the plasma membrane, since the membrane of LDCVs contains high amounts of lysolecithin and the plasma membrane does not [10]. The problem of separating vesicle from plasma membrane components could be solved, if the LDCV membrane stays together like a raft and becomes recovered in one piece [11–13]. So far there is little evidence for this pathway in neuroendocrine cells, thus, we used a time-resolved 'on-cell' capacitance measurement technique to determine the size of the endocytic organelles.

In this study we provide direct evidence that chromaffin cells utilize both molecular-independent pathways for endocytic membrane retrieval, namely endocytosis, by clathrin-coated vesicles and by large endocytic vesicles, corresponding to LDCVs.

2. Materials and methods

2.1. Cells and buffers

Bovine chromaffin cells were prepared from adrenal medulla glands, cultured as described [14], and used within 2–10 days after preparation. The bath and pipet stimulation buffer to trigger exocytosis of LDCVs contained (in mM) 120 NaCl, 10 $CaCl_2$, 2 KCl, 2 $MgCl_2$, 20 tetraethylammonium and 20 HEPES/NaOH (pH 7.25). The stimulation buffer was used in all stimulation experiments in bath and pipet. Additionally, the pipet contained 0.1 mM carbachol, and experiments were performed at room temperature (23°C) or physiological temperature (35°C) on a heatable stage (LN-PCT-2, Luigs and Neumann).

2.2. Electron microscopy

Chromaffin cells were grown for 10 days on sapphire coverslips (Rudolf Bruegger SA, CH 6648 Minusio) to a density of 20 000 cells ml^{-1}. The cells were incubated in stimulation buffer with 0.1 mM carbachol and 25 mg ml^{-1} horseradish peroxidase (HRP) (Serva, P31941) for 1 min at 37°C. The coverslips were blotted onto Whatman filter-paper and quickly frozen in liquid ethane. They were subsequently processed for freeze substitution and embedded in Lowicryl HM-20, as described previously [15], with slight modifications. Modifications included a 90° tilt angle and a specially designed coverslip holder (FH-Cryotec, Singapore) for carrying out the freeze substitution. Ultrastructural localization of HRP was performed by the immuno-gold technique with a HRP antibody (Sigma, P7899) in a concentration of 0.04 mg ml^{-1} linked to protein A conjugated with 9 nm gold particles. Gold samples for multiple-labeling cytochemistry were prepared as described elsewhere [16].

2.3. On-cell capacitance measurements

The patch pipets (borosilicate glass, wall thickness 0.38 mm, outer diameter 1.5 mm, World Precision Instruments) were coated with dental wax. After fire-polishing, the pipets had resistances between 1.8 and 2.5 MΩ. After sealing, the pipet potential was set to +20

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Abbreviations: LDCVs, large, dense core vesicles; HRP, horseradish peroxidase

PII: S0014-5793(01)02861-7

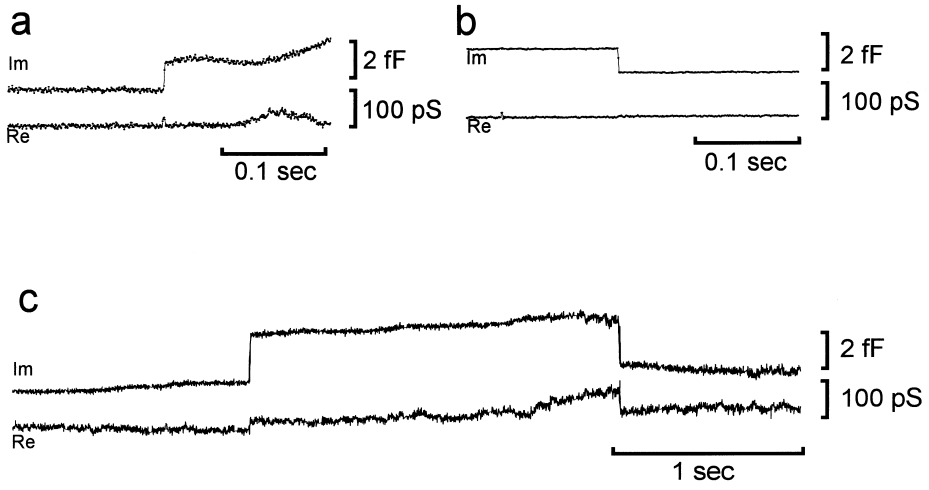

Fig. 1. Stimulation dependent capacitance steps. *Im* and *Re* traces recorded from chromaffin cells. a: Upward capacitance step indicates the fusion of a LDCV. b: Downward capacitance step shows the internalization of a large endocytic vesicle. c: An upward and a subsequent downward step represent kiss-and-run type fusion events. The persistent conductance between the two steps in the *Re* trace indicates the opening of a transient pore.

mV, keeping the patch potential 20 mV more negative than the cells resting potential.

The capacitance of the membrane patch was continuously monitored for 10–20 min. An EPC-7 patch-clamp amplifier was connected to a SRS 830DSP lock-in amplifier, which overlaid an 8 kHz sine wave onto the holding potential. The lock-in amplifier analyzed the resulting current that is phase-shifted with respect to the command voltage. This relation can be expressed as the cells complex admittance, which has a real part (*Re*) that corresponds to the conductance and an imaginary part (*Im*) 90° out of phase, which is equivalent to the capacitance of the patch [17]. The data were recorded at 100 Hz or 350 Hz via an A/D converter (Labmaster TL-1 DMA, Axon Instruments) into a PC-computer under Windows95. The measuring setup was described in detail earlier [3]. The software is available on the internet at www.synosoft.de.

2.4. Capacitance-step analysis

Analysis of large (>0.5 fF) capacitance steps were done semi-automated by several computer routines. The traces were at first scanned manually for upward or downward capacitance steps. Step amplitudes were measured by fitting regression lines to the *Im* (capacitance) and the *Re* (conductance) traces over 150 ms before and after the step, and determining their vertical distance at the position of the step. Steps were accepted if they passed the following criteria:

1. The *Im*-step amplitude was at least three times larger than the corresponding *Re*-step amplitude
2. The pre- and post-step rms-noise was less the 250 aF
3. The step amplitude was between 0.5 and 5 fF

Steps between 0.15 and 0.5 fF were analyzed by an automatic computer algorithm, and the step frequency was corrected for detection errors, as described earlier [3].

Capacitance flicker steps were excluded from this analysis. This subset of steps was defined as: One step follows another step of approximately the same amplitude and the opposite direction; the two steps are not separated by more than 5 s.

A specific capacitance of 5 fF μm^{-2} was used for calculation of vesicle diameter and surface from the capacitance data [18,19].

3. Results

On-cell capacitance measurements have been shown to be very useful to study interactions of small single vesicles and LDCVs with the plasma membrane [1–3,20–22]. This technique enables the quantification of exo- and endocytosis and the determination of individual vesicle subpopulations, responsible for retrieval of exocytosed LDCV membrane during stimulation.

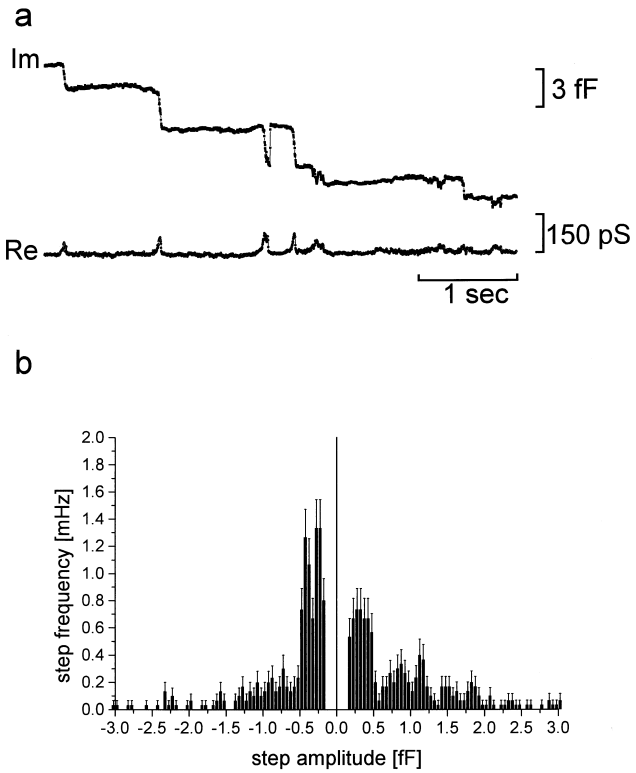

Fig. 2. Membrane recycling by large and small vesicles. a: *Im* and *Re* traces recorded from a chromaffin cell in stimulation buffer at 35°C. b: Histogram of capacitance-step amplitudes between 0.15 and 3 fF from 118 cells. Negative values indicate endocytic and positive exocytic steps. Error bars give the square root of the step count in each bin, scaled to unit of frequency. Steps smaller than 0.5 fF were detected by a fully automated step-detection algorithm.

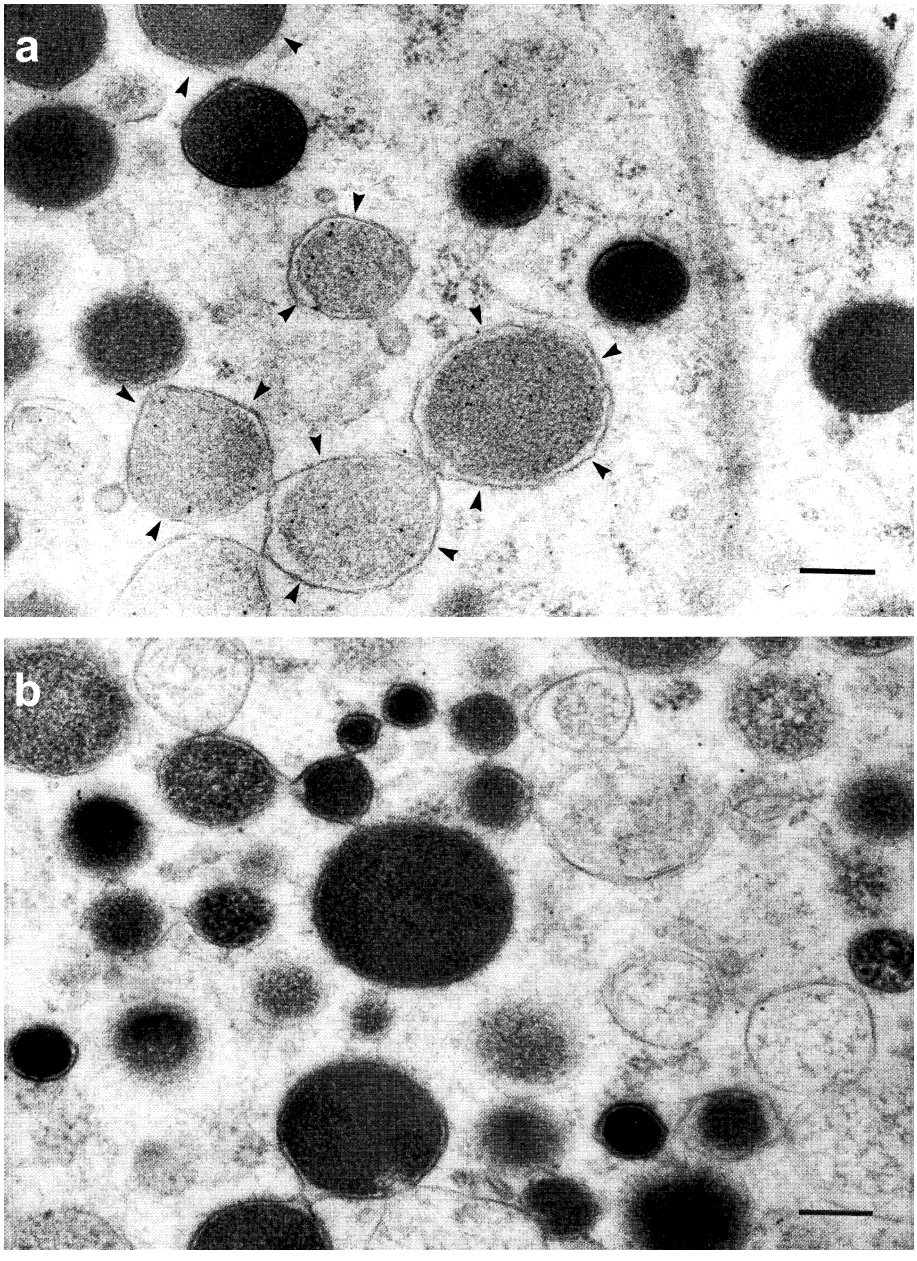

Fig. 3. Endocytosis of LDCV membrane by large vesicles. a: Electron micrograph of large endocytic vesicles containing the extracellular marker HRP. HRP is labeled with anti-HRP antibodies conjugated with 9 nm gold particles. Arrowheads indicate vesicles, labeled with gold particles. The cells were stimulated with carbachol in stimulation buffer for 1 min at 37°C in the presence of extracellular HRP. b: Control: incubated under the same conditions without HRP. Scale bars are 200 μm on both images.

3.1. Solitary steps and capacitance flickers

Unstimulated bovine chromaffin cells have only very low frequency (one step in 111.1 min in a patch) of endocytosis of LDCV [3]. Fig. 1 shows capacitance recordings from stimulated cells. The solitary upward and downward steps represent the fusion (Fig. 1a) or the fission (Fig. 1b) of a single vesicle. Occasionally, there was an upward step (Fig. 1c), rapidly succeeded by a downward step of approximately the same size. A continuous electrical conductance was registered between the pair of steps, indicating the existence of an aqueous channel. These events were interpreted as 'transient fusions' or kiss-and-run events [1–3,21]. Since in these cases the LDCV membrane stays intact without fusing into the plasma membrane, we excluded this type of events from our analysis and focussed on the recovery of completely collapsed LDCV-membrane components.

3.2. Membrane retrieval by large and small vesicles

The membrane of completely fused LDCVs was internalized by large (>0.5 fF) and small (<0.5 fF) endocytic vesicles. 0.5 fF corresponds to 0.1 μm^2 surface of a spherical vesicle with a diameter of 180 nm. Fig. 2a shows a capacitance trace from a chromaffin cell, where four large endocytic steps occurred within 5 s. The mean amplitude of downward steps (1.41 fF $\pm$ 0.16 S.E.M.) was about identical to that of upward steps (1.39 fF $\pm$ 0.09 S.E.M.). These capacitance values correspond to diameters of 299 nm for endocytic vesicles, respectively, 297 nm for exocytic LDCVs. The frequencies of large

Table 1
Membrane traffic balance

	Membrane turnover (μm^2 $cell^{-1}$ min^{-1})	Endocytosis (%)
Exocytic vesicles	+1.37	
Large endocytic vesicles	−0.73	55.7
Small endocytic vesicles	−0.58	44.2

Cells ($n = 118$) were stimulated by carbachol in the pipet at 35°C; exocytic vesicles: 0.15–5 fF; large endocytic vesicles: 0.5–5 fF; small endocytic vesicles: 0.15–0.5 fF.

endocytic steps were lower than the corresponding frequencies of exocytic steps. The difference between exo- and endocytosis was compensated by a higher frequency of small endocytic vesicles. In our analysis we used the frequency-unit mHz, meaning that 1 mHz corresponds to one step per 1000 s. The histogram in Fig. 2b shows that cells had a higher frequency (7.2 mHz) of small endocytic steps compared to small exocytic steps (4.5 mHz). In contrast, the same cells had more large exocytic steps (6.7 mHz) than large endocytic steps (4.4 mHz). The frequencies of all exocytic (11.3 mHz) and all endocytic (11.6 mHz) steps were almost identical. Table 1 presents the calculated balance of the membrane traffic in cells, stimulated with the cholinergic agonist carbachol. Exocytosis and endocytosis were in balance at large. The number of small vesicles of less than 0.5 fF that were determined by the automatic computer algorithm, was corrected for detection errors [3]. These small vesicles endocytosed a slightly smaller amount of membrane than vesicles larger than 0.5 fF.

3.3. HRP uptake by large vesicles

HRP, an endocytosis marker, was applied to identify endocytic organelles. The electron micrograph in Fig. 3a shows that quick-frozen carbachol-stimulated cells internalized HRP at 37°C within 1 min by large vesicles, comparable to the size of LDCVs, while unstimulated cells did not pick up the endocytosis marker (Fig. 3b). In general, the dense core of HRP-labeled vesicles was lighter compared to most LDCVs. Small clathrin-coated vesicles were very rare in all of our electron micrographs and no HRP was found inside.

4. Discussion

We included all single endocytic vesicles between 0.15 fF (∼100 nm) and 5 fF (∼560 nm) for the qualitative and quantitative analysis of endocytosed LDCV membrane. Most of these vesicles had a capacitance between 0.15 and 0.4 fF (∼130 nm), corresponding to the size of clathrin-coated vesicles [7,23]. It appears very likely that clathrin-coated vesicles are involved in endocytosis of LDCVs [24,25]. However, this subpopulation accounted only for about half of total internalized membrane, leaving the rest to be recaptured by vesicles larger than 0.5 fF (∼180 nm). This ratio between large and small vesicles was independent of temperature (data not shown). Our results show that LDCVs can be recycled rapidly and directly after exocytosis. This finding confirms an earlier hypothesis that found evidence against the exclusive recycling of LDCV membrane by clathrin-coated vesicles, because injected anti-clathrin antibodies did not inhibit rapid endocytosis in chromaffin cells [26]. Additionally it has been shown that the membrane of fused granules stays together in a raft and can be captured as a whole entity [27,28]. Very similar observations were made on sea urchin eggs, where granular membrane becomes recycled directly after exocytosis [13].

In order to identify the organelles for endocytic recycling of LDCV membrane after complete fusion with the plasma membrane, we labeled endocytic compartments with HRP as endocytosis marker for electron-microscopy analysis. In these experiments, the cells were stimulated with carbachol at physiological temperature and immediately fixed by the rapid-freeze technique [29]. We found immuno-gold-labeled HRP in large vesicles of about the size of LDCVs. These findings confirm former observations by Winkler's group [28], who showed very similar results on a much more extended time scale. However, we did not find higher levels of HRP-containing clathrin-coated vesicles or other labeled structures [30]. We can not exclude that the large vesicles originated from fused clathrin-coated vesicles, but it is hard to imagine that this pathway can be accomplished within 1 min, because it was shown that the maturation of LDCVs from clathrin-coated vesicles involves an intermediate endocytic compartment and takes at least 30 min [28]. Thus, it is most likely that the endocytic granules were directly recycled from formerly fused LDCVs or originated from kiss-and-run LDCVs, because all large labeled endocytic vesicles contained an intact dense core.

In summary, our real-time capacitance measurements provide direct evidence that more than 50% of all completely fused chromaffin granules become directly recycled without the participation of small clathrin-coated vesicles.

Acknowledgements: The authors and this work were supported by the Max-Planck-Gesellschaft.

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