Morphological evidence for tracer uptake at the active zones of stimulated frog neuromuscular junction

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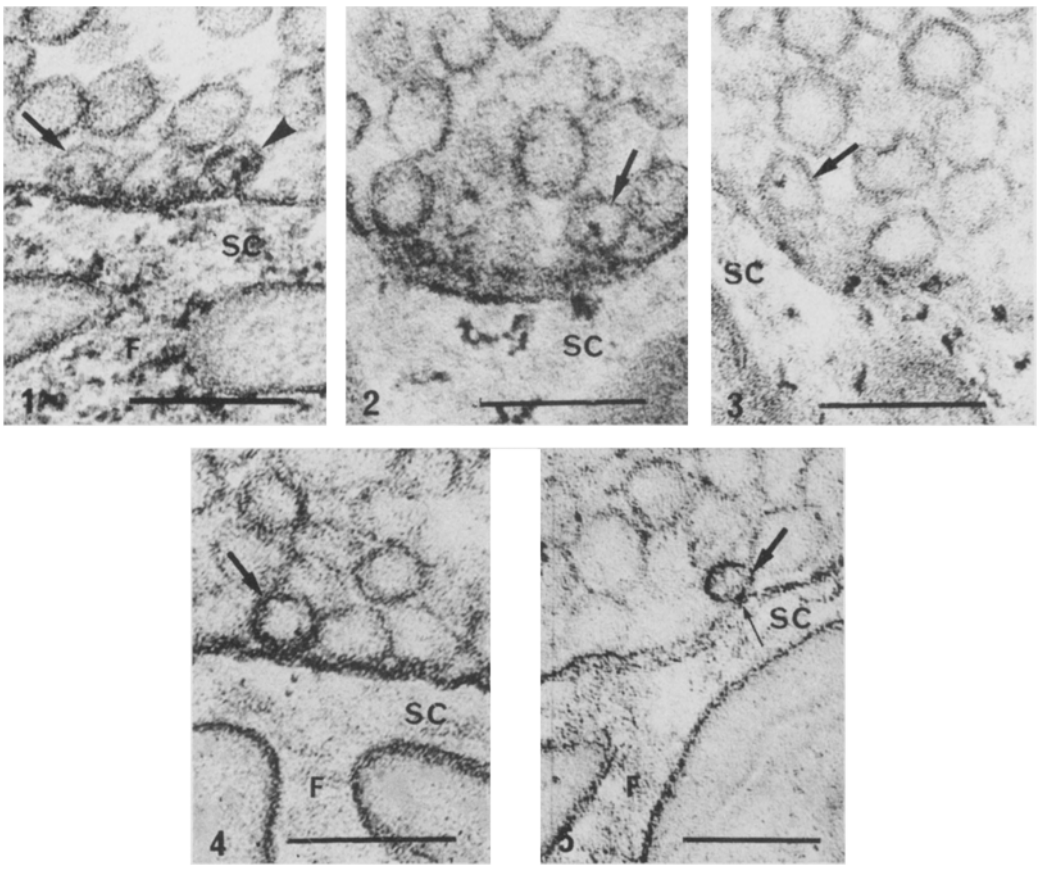
Summary. Lanthanum or ferritin added to the fixative were found in small and non-coated vesicles located at active zones in nerve-muscle preparations stimulated by potassium during cold aldehyde fixation. The presence of labeled vesicles at active zones supports the hypothesis that a double process of exo-endocytosis might occur under moderate stimulation conditions.

Neuromuscular preparations continuously stimulated over a long period of time are able to release several times more quanta than the number of synaptic vesicles they originally contained[2]. This implies a rapid and local turnover of synaptic vesicles after exocytosis. Evidence for membrane recovery from axolemma in frog neuromuscular junction was obtained in experiments showing that stimulation increases the uptake of extracellular tracers into synaptic vesicles. Some investigators interpreted their pictures of tracer uptake as illustrating synaptic vesicles pinching off directly from the presynaptic membrane after exocytosis[3,4]. Others showed that synaptic vesicles collapse flat after exocytosis and that recovery of membrane occurs from coated vesicles formed in regions distant from active zones[5-7]. However, several mechanisms of recycling can occur, depending on the rate of stimulation. Indeed, no apparent increase in the number of coated vesicles was observed in chronically stimulated terminals; only the frequency of coated pits was higher in the terminals stimulated at a high rate[2]. Moreover, it has recently been confirmed that vesicle openings are of different shapes when transmitter release occurs during cold chemical fixation[8,9]. Vesicles which are open into the synaptic cleft appear as pockets the membrane of which is fused with the presynaptic membrane. Some of the pockets with a large aperture have omega-like profiles of the same size and curvature as synaptic vesicles. Others are smaller and almost closed. The presence of small-sized vesicles at some active zones was interpreted as resulting from the closing of such small pockets. This view is supported by the present findings showing that extracellular tracer uptake from the synaptic cleft occurs in small non-coated vesicles located at active zones when spontaneous release is highly accelerated by K^+ during cold chemical fixation.



Figures 1-5. Frog neuromuscular junctions stimulated with 20 mM K^+ during cold chemical fixation. A tracer (La^{3+} in figs 1-3; ferritin in figs 4 and 5) was added to the fixative. In figure 1, electron-dense granules of La^{3+} are observed in the synaptic cleft (SC) and folds (F) and in a small pocket (arrow head) located at an active zone. In figures 1-5, small vesicles (large arrows) not connected to the presynaptic membrane, contain tracers. In figure 5, the membrane of the labeled small vesicle displays a distinct point of labeling (thin arrow) contiguous to the presynaptic membrane (bars = 0.1 µm; figs 1-4: ×214,000; fig. 5: ×176,000).

Experiments were performed on isolated cutaneous pectoris muscles of the frog *Rana esculenta*. The muscles were bathed in standard Ringer solution containing (in mM) NaCl, 111; KCl, 2; $CaCl_2$, 2; $NaHCO_3$, 2; (pH 7.2) and kept at 0–1 °C by cooling device. In experiments with lanthanum, normal Ringer's was replaced 3 min before fixation by $NaHCO_3$ free-Ringer solution. The chemical fixation was done at the same temperature by replacing Ringer's by a cold solution of 2.5% glutaraldehyde in 0.1 M cacodylate buffer which contained 2 mM Ca^{2+} and a tracer which was either ferritin (0.8%) (Sigma, with less than 0.1% Cd) or 2 mM $LaCl_3$.

The spontaneous acetylcholine (ACh) release was accelerated during fixation by adding 20 mM KCl to the fixative. The 1st solution of fixative was replaced 30 min latter by 2.5% glutaraldehyde in 0.1 M cacodylate buffer for preparations soaked in ferritin, or by 2.5% glutaraldehyde in 0.1 M phosphate buffer for preparations soaked in La^{3+}. Phosphate buffer was used for precipitating La^{3+} and obtaining fine electron-dense crystals[10]. Blocks of muscles containing endplate regions were postfixed for 1 h in 2% (w/v) osmium tetroxide in veronal-acetate buffer (pH 7.4). They were stained with 0.5% (w/v) uranyl acetate in the same buffer for 20 min, dehydrated in graded ethyl alcohols and embedded in Spurr's resin. Thin longitudinal sections (about 60 nm), either unstained or slightly stained with lead citrate, were examined in a 301 Philips electron-microscope. The area of labeled and unlabeled synaptic vesicle cross-sectional profiles was calculated from electron micrographs enlarged at a magnification of $\times 150{,}000$ and mounted on a digital table analyser (MOP-Kontron). Comparison of areas was done by using the Student paired t-test. Each pair of values was made up of the area of a labeled vesicle and of the mean area of 6 unlabeled synaptic vesicles located around the labeled one on the same active zone profile.

In sections from preparations fixed at rest (without added K^+), ferritin and La^{3+} filled only the synaptic cleft and folds. In stimulated preparations, some of the pockets located at active zones[9] were filled with both markers. Figure 1 shows an example of vesicle opening into the junctional cleft which has been penetrated by the tracer. At other active zones, vesicles containing a marker, and without an apparent connection with the presynaptic membrane were observed (figs 1–4). Since the thickness of sections was of the same order of magnitude as the equatorial diameter of the synaptic vesicles, it was logical to suppose that if these small vesicles were open, the opening would be noticed. In figures 4 and 5, the marker (ferritin) which covers both the presynaptic membrane and the inner side of the membrane of small vesicles gives evidence of their endocytotic origin. In addition, figure 5 shows a distinct labeled point at the base of the small vesicle which may indicate the 'scar' of the former point of attachment of the vesicle to the presynaptic membrane. Most of the observed labeled vesicles appeared smaller than the surrounding ones. In order to substantiate this view, measurements of the area of labeled and unlabeled synaptic vesicles were performed. The values obtained in 42 active zone profiles show that the mean area of labeled vesicles was 797.78 ± 27.11 nm^2 while it was of 1203.30 ± 23.10 nm^2 for the unlabeled vesicles. The paired t-test performed on the data shows that the difference between the 2 means is significant at the 0.001 level of significance (mean difference: 405.52 ± 1.96; $t = 16.2$ for 41 degrees of freedom).

Finally, a longer treatment of muscle blocks with uranyl acetate (4 h) followed by an additional uranyl grid stain did not result in the appearance of a coat on the small vesicles located at the active zones.

Hence, the present results show that extracellular tracers penetrate into non-coated vesicles located at active zones of frog neuromuscular junctions when transmitter release is accelerated by K^+ during cold chemical fixation. The findings suggest that a local recycling of vesicles could be involved in the rapid recovery of the synaptic vesicle store in the nerve terminal. Whether the reformed vesicles at the active zones are refilled with ACh or are destined for another role remains in question. However, they might explain why the newly synthesized ACh is preferentially released in numerous cholinergic terminals[11–15]. Indeed, it is possible that the newly synthesized ACh may be preferentially loaded into these empty or partially empty vesicles located at active zones, and may therefore be immediately released. A new population of synaptic vesicles loaded with freshly synthesized ACh has also been shown to appear with stimulation in the perfused electric organ[16,17]. The present results provide a view supplementary to the widely-admitted opinion that vesicle openings are related only to ACh release[7]. They support the hypothesis, already suggested[18], that a double process of exo-endocytosis might occur at the active zones of the frog neuromuscular junction under moderate stimulation conditions. Recent findings that recycling of synaptic vesicles occurs by specific retrieval of vesicle membrane also support this view[19]. Although the previous and present results favor the hypothesis of a local exo-endocytosis at the active zone, it is not yet possible to discard completely the idea that retrieved vesicles may be due to the action of fixative. To rule out this possibility, further work with combined quick-freezing and cryo-substitution techniques needs to be done. These experiments are now in progress in our laboratory.

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