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A pre-embedding immunogold approach for detection of synaptic endocytic proteins in situ

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Abstract

During the past decade, many molecular components of clathrin-mediated endocytosis have been identified and proposed to play various hypothetical roles in the process [Nat. Rev. Neurosci. 1 (2000) 161; Nature 422 (2003) 37]. One limitation to the evaluation of these hypotheses is the efficiency and resolution of immunolocalization protocols currently in use. In order to facilitate the evaluation of these hypotheses and to understand more fully the molecular mechanisms of clathrin-mediated endocytosis, we have developed a protocol allowing enhanced and reliable subcellular immunolocalization of proteins in synaptic endocytic zones in situ. Synapses established by giant reticulospinal axons in lamprey are used as a model system for these experiments. These axons are unbranched and reach up to 80–100 μm in diameter. Synaptic active zones and surrounding endocytic zones are established on the surface of the axonal cylinder. To provide access for antibodies to the sites of synaptic vesicle recycling, axons are lightly fixed and cut along their longitudinal axis. To preserve the ultrastructure of the synaptic endocytic zone, antibodies are applied without the addition of detergents. Opened axons are incubated with primary antibodies, which are detected with secondary antibodies conjugated to gold particles. Specimens are then post-fixed and processed for electron microscopy. This approach allows preservation of the ultrastructure of the endocytic sites during immunolabeling procedures, while simultaneously achieving reliable immunogold detection of proteins on endocytic intermediates. To explore the utility of this approach, we have investigated the localization of a GTPase, dynamin, on clathrin-coated intermediates in the endocytic zone of the lamprey giant synapse. Using the present immunogold protocol, we confirm the presence of dynamin on late stage coated pits [Nature 422 (2003) 37] and also demonstrate that dynamin is recruited to the coat of endocytic intermediates from the very early stages of the clathrin coat formation. Thus, our experiments show that the current pre-embedding immunogold method is a useful experimental tool to study the molecular mechanisms of synaptic vesicle recycling.

Keywords: Endocytosis; Synapse; Immunocytochemistry; Electron microscopy; Dynamin; Synapsin

1. Introduction

In neuronal synapses, a pool of neurotransmitter-filled vesicles is aggregated at the synaptic active zone, where membrane fusion takes place. During synaptic activity, clathrin-mediated endocytosis of synaptic vesicles has been shown to be critical for sustaining neurotransmitter release (Slepnev and De Camilli, 2000). The reuptake of vesicle membrane via clathrin-mediated endocytosis occurs lateral to the active zone. Clathrin molecules are recruited on the invaginating membrane where they form polygonal cages. The budding vesicle is later pinched off from the membrane in a dynamin-dependent process. In addition to clathrin and dynamin, a number of accessory proteins have been implicated in clathrin-coat formation including epsin, amphiphysin, endophilin and intersectin (Conner and Schmid, 2003). At the light microscopic level, it has been shown that accessory proteins are accumulated at the endocytic zone during synaptic activity (Estes et al., 1996; Roos and Kelly, 1999; Guichet et al., 2002). However, the precise contribution of these components to membrane invagination, coat formation, and scission is unknown. To fully understand

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the molecular mechanism of the clathrin-dependant vesicle recycling, it would be valuable to know the precise location of each of these proteins during endocytosis.

It is generally difficult to localize endocytic proteins in identified intact synapses at high resolution. These structures are usually small, about 1–2 μm in diameter, and it is rather complicated to access their interiors in situ (Slepnev and De Camilli, 2000). So far, the most highly efficient immunogold labeling of synaptic endocytic zones has been achieved using in vitro preparations of ruptured synaptosomes (Takei et al., 1995; Ringstad et al., 1999). In this paper, we describe a novel procedure that allows efficient ultrastructural localization of proteins in synaptic endocytic zones in situ in combination with excellent ultrastructural preservation of the tissue.

2. Methods

2.1. Animals, dissection and stimulation procedures

Adult lampreys (*Lampetra fluviatilis*) were kept at 4 °C in aerated fresh water aquaria. Animals were anesthetized by immersion in 100 mg/l tricaine metasulphonate (MS-222, Sigma). After decapitation trunk segments of spinal cord on notochord were dissected out in oxygenated ice-cold Ringer's solution (138 mM NaCl, 2.1 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 4 mM glucose, 2 mM HEPES, 0.5 mM L-glutamine; pH 7.4) and cut into pieces about 5 cm long. Axons were stimulated by incubation of the specimens in Ringer's solution containing 30 mM KCl for 20 min at 4 °C. As a control, pieces of spinal cord were incubated in low Ca^{2+} (0.1 mM $CaCl_2$, 4.0 mM $MgCl_2$), Ringer's solution for the same period of time. Preparations immunostained with the proposed protocol (see below) were fixed for 4 h in 3% para-formaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4) and then processed for electron microscopy.

2.2. Immunogold labeling

Spinal cords were cut into two parts from the ventral side of spinal cords in a vibratome (Fig. 1). Sections were blocked for 30 min in 1% human serum albumin in TPBS (Tris-phosphate buffered saline; pH 7.4) at 4 °C and

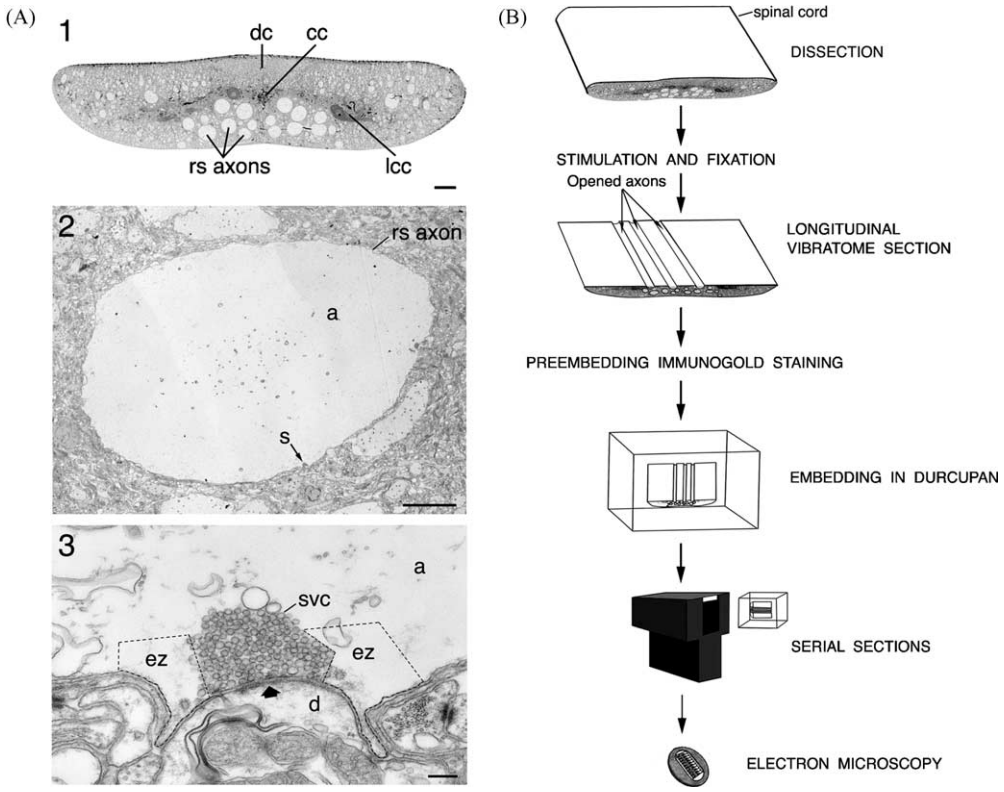

Fig. 1. Schematic representation of the model system and labeling procedure. (A1) Light microscopic image of a transverse section of the lamprey spinal cord and the position of ventral giant reticulospinal axons (rs axons). (A2) Low power electron micrograph of a transverse section of a reticulospinal axon with synapses on its surface. One synapse (s) is shown in A3 at higher magnification. (A3) Electron micrograph of a reticulospinal synapse stimulated by action potentials to induce synaptic vesicle recycling. Endocytic zones are located lateral to the active zone (thick arrow). Most of the endocytic events occur in the range of 0.5 μm from the edge of the active zone. The endocytic zone area investigated in the present study is designated with dashed lines. a: axoplasm, s: synapse, svc: synaptic vesicle cluster, d: dendrite, dc: dorsal columns, cc: central canal, lcc: lateral cell column. Bars: A1, 100 μm; A2: 10 μm; A3: 200 nm. (B) Schematic representation of the labeling procedure. The lamprey spinal cord is dissected, stimulated, fixed and cut longitudinally in a vibratome into two parts to open axons. The preparation is then labeled using a pre-embedding immunogold technique, post-fixed, embedded in resin, and processed for electron microscopy.

incubated overnight with primary antibodies on a shaker. They were washed in TPBS for 6–8 h, and then incubated with secondary antibodies conjugated to 5 nm gold particles (diluted 1:50; in TPBS) overnight. After washing for 6–8 h in TPBS, sections were post-fixed in 3% glutaraldehyde in 0.1 M PB for 1 h followed by 1% osmium tetraoxide in the same buffer for 1 h, dehydrated in ethanol, and embedded in Durcupan ACM (Fluka, UK). Control specimens were embedded in LR Gold at low temperature (−25 °C) directly after stimulation and stained with antibodies using a standard post-embedding immunogold protocol (Bloom et al., 2003).

Semithin and ultrathin sections were cut in an ultrotome (Leica UCT) with a diamond knife (Diatome). Semithin sections were stained with 0.5% toluidine blue and photographed in a Nikon microscope. Ultrathin sections were mounted on grids and counterstained with uranyl acetate and lead citrate. Sections were examined and photographed in a Tecnai 12 electron microscope.

Density of gold particles in endocytic zones was obtained from an area 0.5 μm form the border of active zone and 0.5 μm from the presynaptic membrane (Fig. 1A3). Excel™ software was used for statistical evaluation of the data.

2.3. Antibodies

Anti-dynamin rabbit polyclonal antibodies (LD-1) against the GTP-binding domain of the lamprey dynamin I (aa 1-320), were affinity purified and used at a dilution of 1:1000. The DG-1 antibodies were directed against aa 1–750 of rat dynamin I (Grabs et al., 1997). They were used at 2 μg/ml. The DG-1 antisera recognized a single band corresponding to the molecular weight of lamprey dynamin I in lamprey spinal cord extract (data not shown). Rabbit polyclonal antibodies against lamprey anti-synapsin D domain were used as described previously (Bloom et al., 2003). Immunogold reagents were purchased from Amersham Bioscience, Sweden.

3. Results

Giant lamprey synapses and their endocytic zones are present on the inner surface of unbranched axons of reticulospinal neurons (Fig. 1A). These synapses can be easily identified at the electron microscopic (EM) level. Furthermore, they can be stimulated electrically at different frequencies or by high $[K^+]_e$ to induce synaptic vesicle recycling (Shupliakov and Brodin, 2000).

In order to access the endocytic zone with reagents, the lamprey spinal cord was cut in a vibratome to open axons along the longitudinal axis (referred in the text to as “opened axons,” see Fig. 1B). We first tested if the cutting procedure damaged the organization of axoplasm and synapses. Axons were stimulated to induce synaptic vesicle recycling (see Section 2) and fixed in 3% para-formaldehyde/0.1% glutaraldehyde to minimize the damage. Specimens were embedded in Durcupan and transverse semithin and ultrathin sections were cut from the opened axons (Figs. 1 and 2). Sections were counterstained and examined in light and electron microscopes. Large areas of the axoplasm remained in the giant axons after cutting along the longitudinal axis (Fig. 2A). At the ultrastructural level, no changes were detected compared to synapses from intact axons of the same specimen (Fig. 2B and C). Synapses from opened

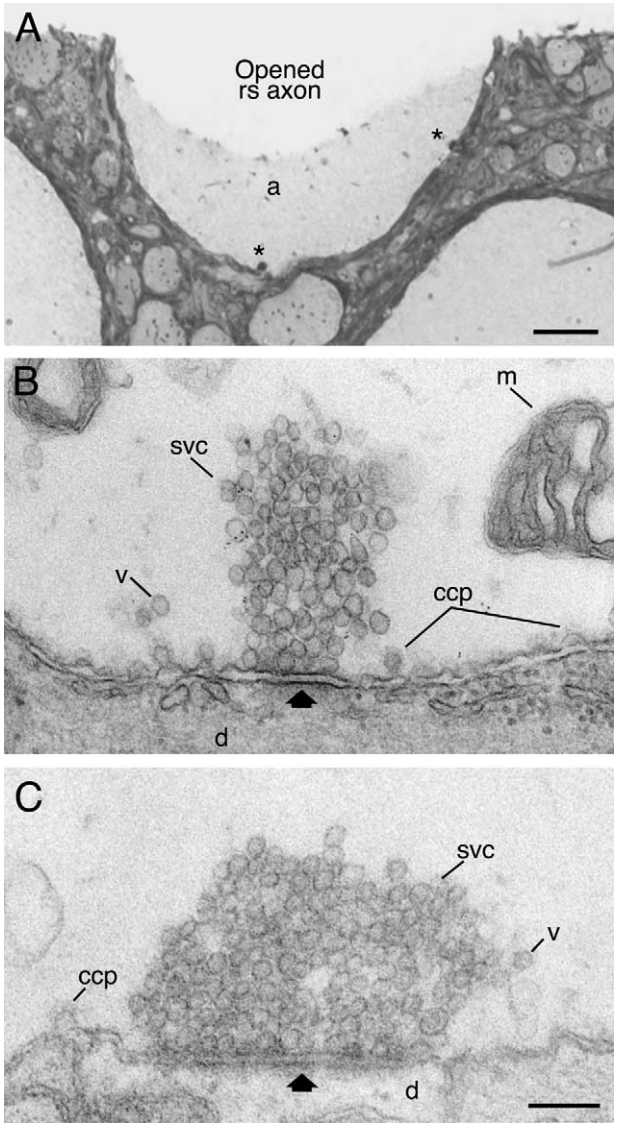

Fig. 2. Morphology of “opened” giant axons. (A) Light micrograph of a semithin section from a Durcupan embedded preparation showing an opened axon. Note the presence of axoplasm (a) and vesicle clusters (asterisks) in the axon. (B) Electron micrograph of a synapse in an opened axon stimulated by high $[K^+]_e$. Numerous clathrin coated pits (ccp) are present at endocytic zones. Thick arrow indicates active zone. Note synaptic vesicles (v) in transit from the endocytic zone to the vesicle cluster. (C) Electron micrograph of a control synapse in a neighboring intact axon. m: mitochondrion, ccp: clathrin-coated pits. Other designations as in Fig. 1. Scale bars: (A) 10 μm; (B–C) 200 nm.

axons appeared to have a normal ultrastructure with synaptic vesicle clusters accumulated at active zones, clathrin-coated intermediates, and vesicles migrating back to vesicle clusters in the cytomatrix of endocytic zones (Fig 2B). Also, their mitochondria and axoplasmic matrix had a normal appearance.

Having established good ultrastructural preservation using this technique, we then investigated if endocytic zones in opened axons were accessible to antibodies without the addition of detergents. Pre-embedding immunogold procedures were employed. Vibratome sections of opened axons were incubated with primary antibodies, followed by incubation in gold-conjugated secondary antibodies (Fig. 1B). After the staining procedure, specimens were post-fixed and processed for electron microscopy. Antibodies against dynamin and synapsin, two different proteins with established functions at the synapse, were used (Slepnev and De Camilli, 2000; Bloom et al., 2003). In both cases, a strong accumulation of gold particles was observed in synaptic endocytic zones (Figs. 3 and 4). Ultrastructure of synapses as compared to control intact synapses was not affected. In control specimens incubated without primary antibodies, no labeling was detected in opened axons. The antibodies did not label endocytic zones in unstimulated axons.

Since the current protocol allowed us to combine selective immunolabeling with a good structural preservation of endocytic intermediates, we investigated how early dynamin associates with the intermediates in synapses during clathrin coat formation. It has been shown in studies in cell cultures of non-neuronal cells that dynamin could be localized to the plasma membrane during endocytosis (Damke et al., 1994; Iversen et al., 2003). In synapses, however, the involvement of dynamin at early stages of membrane retrieval remained unclear (Takei et al., 1995). With our enhanced efficiency of labeling, we were able to show the colocalization of dynamin with the coated membranes starting from the initial stages of the clathrin pit formation in the synapse (Fig. 3A, B and C). Dynamin was also clearly associated with the late stages of clathrin-coated intermediates (Fig. 3 A, D and E). All clathrin-coated pits ($n = 300$) observed in opened axons in these experiments displayed immunogold labeling. Both tested antibodies produced similar labeling patterns.

We also tested the efficiency of the post-embedding technique in dynamin labeling experiments. It was not

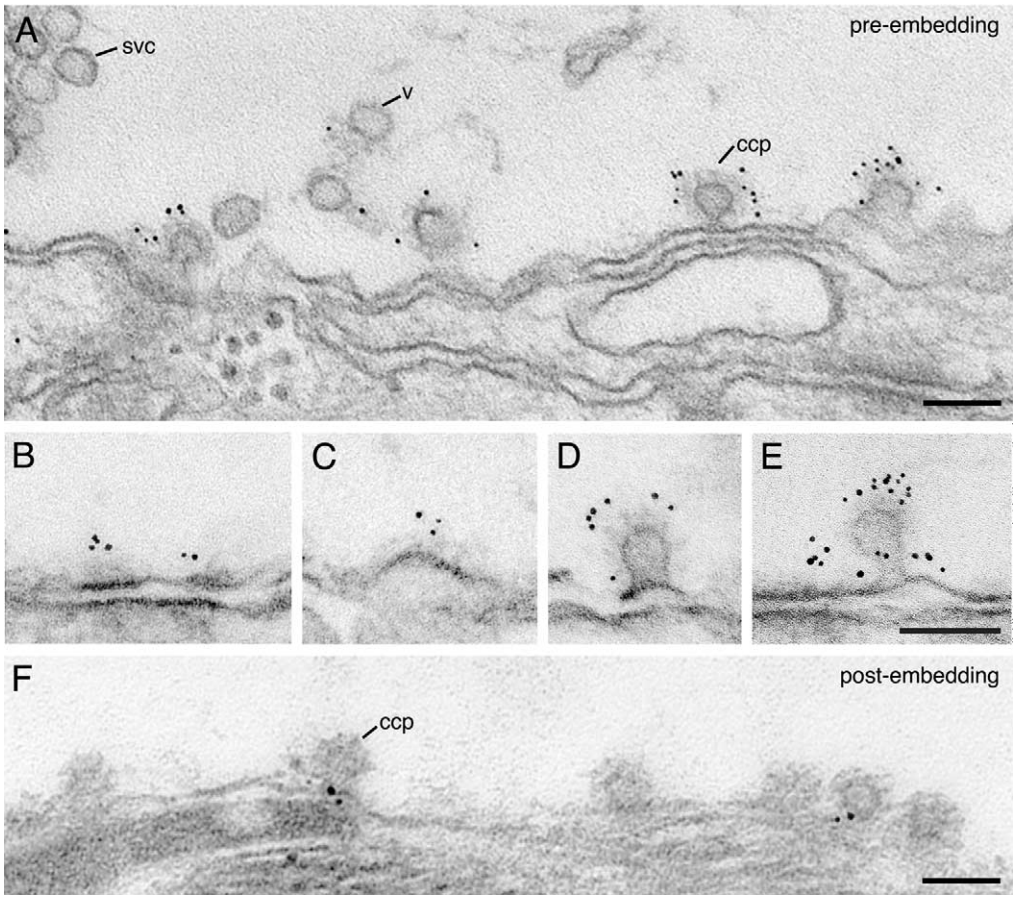

Fig. 3. Localization of dynamin on coated intermediates in the lamprey giant synapse. (A) Electron micrograph of the endocytic zone of a reticulospinal synapse labeled with LD-1 antibodies using our pre-embedding method. Note selective accumulation of gold particles over endocytic intermediates. (B–E) Endocytic intermediates at different stages labeled with the current method. Note immunogold particles associated with the coat starting from early stages of endocytosis. Gold particles are also associated with coats and necks of coated pits at later stages. (F) Clathrin-coated pits labeled with dynamin antibodies (DG-1) using a standard post-embedding protocol. Designations as in Figs. 1 and 2. Scale bars: (A) and (F), 200 nm; (B–E), 200 nm.

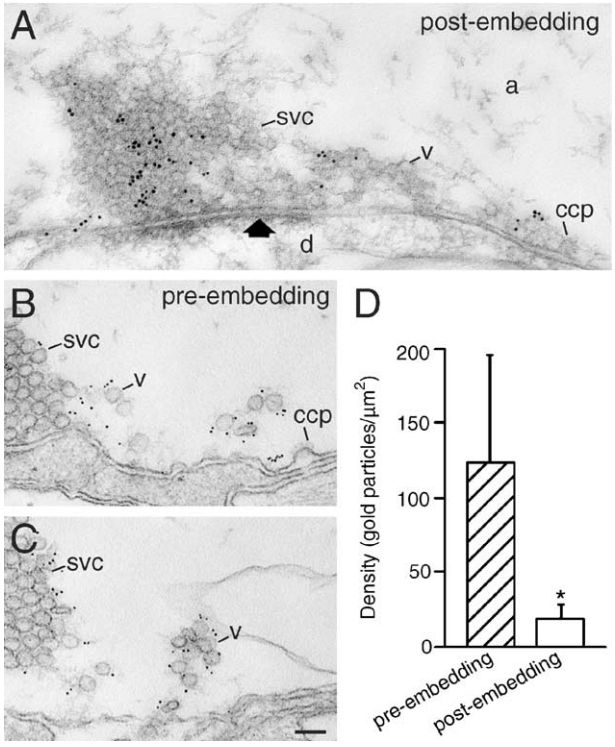

Fig. 4. Synapsin labeling in synaptic endocytic zones. (A) Electron micrograph of a synapse labeled for synapsin using a standard post-embedding protocol. (B, C) Electron micrographs of endocytic zones from two stimulated giant synapses labeled for synapsin using pre-embedding technique. Note the high density of immunogold labeling on synaptic vesicles in the endocytic zone and the lack of gold particles on the presynaptic membrane and coated intermediates. (D) Histogram showing densities of gold particles in endocytic zones of synapses stained using the present pre-embedding method and the standard post-embedding technique. Designations as in Figs. 1 and 2. Scale bar: (A–C), 100 nm.

possible, however, to show the association of dynamin labeling with coated pits at early stages reliably (Fig. 3F). Since coated intermediates are about 80 nm and electron microscope sections are a similar thickness, most of the antigens on the surface of endocytic intermediates will be hidden within the section and are therefore not accessible to antibodies. Indeed, in our experiments labeling for dynamin using post-embedding immmunogold methods, only 32% ($n = 142$) of the coated intermediates had gold particles recognizing dynamin antibodies on their surface.

To confirm the specificity of dynamin labeling using the current protocol, we labeled synapses in open axons for synapsin, a synaptic vesicle protein well-characterized to be present on synaptic vesicles and excluded from endocytic intermediates (Bloom et al., 2003). In agreement with our earlier results obtained using post-embedding immunogold techniques (Fig. 4A, see also Bloom et al., 2003), synaptic vesicle clusters were decorated with gold particles detecting synapsin antibodies. In addition, synapsin immunoreactivity was also found in the filamentous cytomatrix of the endocytic zone, which contained synaptic vesicles being transported back to the vesicle cluster (Fig. 4B; Bloom et al., 2003). Significantly higher efficiency (seven-fold increase) of immunogold labeling in the endocytic zone area was achieved as compared to the post-embedding protocol (Fig. 4; $P > 0.001$; $n = 9$; Student's t-test). No labeling was found in association with clathrin-coated endocytic intermediates (Fig. 4B).

4. Discussion

The proposed model system and approach for detection of endocytic proteins in this paper employ the pre-embedding immunogold method. The main advantage of our approach is that the membrane can be removed from one side of the axon, thus providing access for reagents to the sites of endocytosis without detectable ultrastructural damage. As a large number of endocytic zones within the same axon are simultaneously exposed to reagents, the reproducibility of immunolabeling can be easily verified. A similar pre-embedding technology has been used earlier in in vitro experiments with ruptured synaptosomes and synaptic membranes after addition of cytosol (Takei et al., 1995; Ringstad et al., 1999). However, these experiments are limited by the potential extraction of cytoplasmic components and addition of extrasynaptic components during formation of endocytic intermediates. The opened-axon-model allows for good preservation of an intact endocytic intermediates and minimal loss of components normally present in the cytomatrix of the endocytic zone.

Commonly, detergents are added to antibodies to increase penetration into tissues. This is not required to label endocytic zones in opened axons, and as a result, membrane structures remained well-preserved. Although the proposed procedure has a number of unique features, several limitations should be noted. As with all pre-embedding techniques, interaction of proteins with binding partners can prevent detection of certain epitopes hidden by protein–protein interactions.

In the present study, two different proteins, dynamin and synapsin, were localized at synaptic endocytic zones. In agreement with earlier studies, dynamin was localized to the neck of constricted coated pits, where it has been proposed to be involved in the scission reaction (see review Hinshaw, 2000). In addition, we found gold particles associated with the clathrin coat. Thus, our data support the finding that dynamin is also present on the coat surface during clathrin assembly (Damke et al., 1994; Iversen et al., 2003), an observation which was not fully supported by in vitro studies (Takei et al., 1995). Moreover, the current protocol allowed us to show that dynamin becomes associated with the clathrin coat complex from the very early stages of its formation in the synapse.

Dynamin has not been shown to bind clathrin directly (see review Hinshaw, 2000). Therefore, this suggests that other molecules, which interact with clathrin during the initial stages of the clathrin-coat formation could recruit dynamin to the endocytic membrane. One molecule which

could potentially play such role is amphiphysin I, which possesses both the clathrin-AP2 binding sequence and an SH3-domain which selectively binds dynamin (Slepnev and De Camilli, 2000).

Using a post-embedding technique, synapsin has been recently identified in the endocytic zones of active synapses (Bloom et al., 2003). Synapsin has been proposed to be involved in organization of the actin-rich cytomatrix in the endocytic zone. In agreement with these findings, we detected synapsin in the endocytic zone in association with vesicles migrating to the vesicle cluster and in the filamentous matrix. As in post-embedding experiments, our current pre-embedding protocol did not reveal the presence of synapsin on the plasma membrane or on clathrin-coated intermediates, despite the much higher efficiency of the labeling achieved. This further confirms the view that synapsin dissociates from synaptic vesicles during the synaptic vesicle cycle (Bloom et al., 2003) and supports the use of this technique for the subsynaptic localization of proteins.

When antibodies are applied to opened axons they can access the entire cytoplasmic surface of coated intermediates, which results in 3D labeling pattern of an endocytic intermediate. It is possible that future studies performed with this immunolabeling approach will allow the study of 3D ultrastructural distribution of molecules using intermediate-voltage microscopy in combination with electron tomography (Gustafsson et al., 2002; Lenzi et al., 2002). If electron tomography is applied, reagents conjugated with smaller colloidal gold particles should be most probably used (e.g. 1 nm). This will reduce the steric hindrance during the labeling procedure and allow an even higher resolution of detection of molecular components of the endocytic machinery.

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