Actin polymerization regulates clathrin coat maturation during early stages of synaptic vesicle recycling at lamprey synapses

ARTICLE *in* THE JOURNAL OF COMPARATIVE NEUROLOGY · AUGUST 2006

Impact Factor: 3.23 · DOI: 10.1002/cne.21006 · Source: PubMed

3 AUTHORS, INCLUDING:

Jennifer Bourne
University of Colorado
16 PUBLICATIONS **1,066** CITATIONS
SEE PROFILE

Vincent Pieribone
Yale University
102 PUBLICATIONS **6,098** CITATIONS
SEE PROFILE

THE JOURNAL OF COMPARATIVE NEUROLOGY 497:600–609 (2006)

Actin Polymerization Regulates Clathrin Coat Maturation during Early Stages of Synaptic Vesicle Recycling at Lamprey Synapses

JENNIFER BOURNE,[1] JENNIFER R. MORGAN,[2] AND VINCENT A. PIERIBONE[1]*
[1]John B. Pierce Laboratory, Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06519
[2]Department of Cell Biology, Yale University School of Medicine, Howard Hughes Medical Institute, New Haven, Connecticut 06510

ABSTRACT

Although it is established that presynaptic actin participates in synaptic vesicle recycling at several synapses, the earliest stages at which actin polymerization is employed during this process are still unclear. To address this, we prevented actin polymerization at lamprey synapses by applying latrunculin B or swinholide A. Latrunculin and swinholide depolymerize actin by sequestering actin monomers and, in addition, swinholide can sever existing actin filaments. When injected into individual presynaptic axons of the intact spinal cord, fluorescently labeled monomeric actin rapidly incorporated in a calcium-dependent manner into a stable, filamentous actin network concentrated at endocytic zones. This pool of actin was disrupted completely by latrunculin. At stimulated synapses, specific disruption of actin polymerization with latrunculin and swinholide induced a selective increase in unconstricted clathrin-coated pits and, in the case of swinholide, an additional increase in the size of plasma membrane evaginations. These results indicate that actin polymerization participates initially in the maturation of clathrin-coated pits during early stages of synaptic vesicle recycling. J. Comp. Neurol. 497:600–609, 2006.

Indexing terms: clathrin; endocytosis; latrunculin; swinholide

Following neurotransmitter release, synaptic vesicles within the presynaptic terminal must be rapidly and efficiently recycled via endocytosis. Often, clathrin-mediated endocytosis is the predominant mechanism for selectively retrieving the vesicular membrane and protein contents from the plasma membrane (Heuser and Reese, 1973; Brodin et al., 2000). Functional links between clathrin-mediated endocytosis and the actin cytoskeleton have been identified (Lee and De Camilli, 2002; Merrifield et al., 2002, 2005; Shupliakov et al., 2002; Bloom et al., 2003; Kaksonen et al., 2003, 2005; Carreno et al., 2004). Accordingly, many proteins have the potential to link actin to the clathrin coat (reviewed in Qualmann and Kessels, 2002; Schafer, 2002; Engqvist-Goldstein and Drubin, 2003). In neurons, actin polymerization may be involved in several steps of synaptic vesicle trafficking, including vesicle mobilization, exocytosis, and endocytosis (Cole et al., 2000; Morales et al., 2000; Shupliakov et al., 2002; Sakaba and Neher, 2003; Richards et al., 2004). In contrast, other studies report minimal roles for actin in synaptic vesicle trafficking and instead suggest that actin scaffolds synaptic proteins or regulates bulk membrane endocytosis (Job and Lagnado, 1998; Holt et al., 2003; Sankaranarayanan

Grant sponsor: National Institutes of Health; Grant number: F31 NS043866 (to J.B.); Grant number: F32 MH067385 (to J.R.M.); Grant number: R01 NS037823 (to V.A.P.); Grant sponsor: John B. Pierce Laboratory.

The first two authors contributed equally to this work.

Current address for J.R. Morgan: Department of Biology, Bowdoin College, Brunswick, ME 04011.

Current address for J. Bourne: Synapses and Cognitive Neuroscience Center, Medical College of Georgia, Augusta, GA 30912.

*Correspondence to: Vincent A. Pieribone, The John B. Pierce Laboratory, 290 Congress Ave., New Haven, CT 06519.
E-mail: vpieribo@jbpierce.org

Received 14 July 2005; Revised 9 September 2005; Accepted 9 February 2006

DOI 10.1002/cne.21006

Published online in Wiley InterScience (www.interscience.wiley.com).

et al., 2003). Therefore, the roles of actin at synapses remain unclear, and thus it is important to explore further the dynamic regulation and precise functions of presynaptic actin polymerization.

The highly dynamic nature of actin filament formation has been studied in a variety of cultured cell systems including neurons (Theriot and Mitchison, 1991; Belmont and Drubin, 1998; Bernstein et al., 1998; Colicos et al., 2001; Merrifield et al., 2002; Star et al., 2002; Sankaranarayanan et al., 2003). However, our understanding of neuronal actin dynamics is largely limited to developing synapses that are still undergoing maturation, where rapidly turning-over actin participates in axon outgrowth, de novo synapse formation, molecular scaffolding, and synaptic plasticity (Lin et al., 1994; Fischer et al., 1998; Colicos et al., 2001; Star et al., 2002; Sankaranarayanan et al., 2003). In contrast, much less is known about the mechanisms that regulate actin dynamics at the synapses of intact, adult nervous tissues, such as those in the lamprey spinal cord and neuromuscular junctions, and at specialized synapses, such as retinal bipolar neurons and the Calyx of Held, where actin is participating primarily in vesicle trafficking events (Cole et al., 2000; Shupliakov et al., 2002; Bloom et al., 2003; Holt et al., 2003; Sakaba and Neher, 2003; Morgan et al., 2004; Richards et al., 2004). Thus, identifying the precise roles for actin in presynaptic function has been difficult because its contribution—perhaps not unexpectedly—varies according to developmental stage, history of synaptic activity, and synapse type.

The giant synapses of the adult lamprey reticulospinal axons afford an ideal model system in which to address the functions of presynaptic actin polymerization at synapses because of a prominent ring of filamentous actin (F-actin) surrounding the large vesicle clusters at endocytic, or "periactive" zones (Pieribone et al., 1995; Roos and Kelly, 1999; Dunaevsky and Connor, 2000; Shupliakov et al., 2002; Bloom et al., 2003; Morgan et al., 2004). Furthermore, because these synapses can be examined within axons of mature animals and because these synapses are quiescent unless stimulated with synaptic activity, one can analyze in isolation the function of presynaptic actin in membrane trafficking without the complications of ongoing developmental processes or unevoked synaptic activity. Previous studies of actin function at lamprey giant synapses showed that this network of F-actin at periactive zones plays a predominant role in late stages of synaptic vesicle recycling (Shupliakov et al., 2002; Bloom et al., 2003). For example, actin was implicated in transporting vesicles back to the cluster following endocytosis in a manner that depends on prior actin filament formation and the synaptic-vesicle associated protein, synapsin. This was assessed by ultrastructural observations of stimulated lamprey synapses in the presence of F-actin stabilizing reagents, phalloidin, and an N-ethylmaleimide-inhibited subfragment of myosin I (NEM-S1) (Shupliakov et al., 2002; Bloom et al., 2003). Furthermore, actin has been implicated in earlier stages of vesicle recycling at lamprey synapses, as assessed by an accumulation of clathrin-coated pits at synapses treated either with phalloidin, *Clostridium botulinum* C2 toxin, or with reagents that inhibit the interaction between phosphatidylinositol phosphate kinase type 1γ and the actin-binding protein talin (Shupliakov et al., 2002; Morgan et al., 2004). Therefore, actin has many functions during vesicle recycling at lamprey synapses.

To discriminate these functions, we set out to determine the initial step at which actin polymerization is required for synaptic vesicle recycling. To do so, we utilized two actin-disrupting toxins, latrunculin B and swinholide A, which have not been applied previously to this model system. Unlike phalloidin and NEM-S1, which bind to polymerized, filamentous actin, latrunculin and swinholide prevent the initial polymerization of actin by binding monomeric actin and preventing its incorporation into filaments. Therefore, latrunculin and swinholide are expected to perturb presynaptic actin function at the earliest step at which it is required. Although the C2 toxin also prevents actin polymerization, the lack of a comprehensive and quantitative morphometric analysis precluded a full understanding of the function of actin polymerization during these early stages of synaptic vesicle recycling (Shupliakov et al., 2002).

Here, using fluorescence light microscopy, we show that monomeric globular actin (G-actin) is rapidly incorporated into the F-actin rings at periactive zones in a calcium-dependent manner. Although the incorporation of G-actin is rapid, the F-actin structure can remain stable for several hours. When latrunculin B is bath-applied, these stable actin structures completely disappear. Using electron microscopy, we demonstrate that both latrunculin and swinholide induce a dramatic build-up of clathrin-coated pits at stimulated synapses. Quantitative, morphometric analysis of the clathrin-coated pits reveals a selective increase in unconstricted, coated pits. Additionally, swinholide causes a remarkable build-up of plasma membrane adjacent to active zones. Thus, actin polymerization is first required for clathrin-coated pit maturation during an earlier stage of synaptic vesicle recycling than has been previously demonstrated.

MATERIALS AND METHODS

Microinjection experiments

Adult lampreys (*Ichthyomyzon unicuspis*) were anesthetized with Tricaine methane sulfonic acid (0.1 g/L tank water; Finquel; Argus Chemicals, Vernio, Italy). Following anesthetization, a segment (4–5 cm) of the spinal cord was dissected out as previously described (Pieribone et al., 1995) and transferred to a cooled (7–10°C) bath containing oxygenated lamprey Ringer's (in mM): 91 NaCl, 2.1 KCl, 2.6 $CaCl_2$, 1.8 $MgCl_2$, 4 glucose, 2 HEPES, 0.5 L-glutamine (pH 7.4). The spinal cord was pinned to a glass-bottomed chamber, ventral side up for easy identification and access to the reticulospinal axons. Alexa Fluor 488-conjugated actin from rabbit muscle (Molecular Probes, Eugene, OR) was diluted to 2.5 mg/ml (180 μM) in G buffer, containing 2 mM Tris, 0.2 mM Mg^{2+} ATP, and 0.2 mM $CaCl_2$ (pH 8.0). Rhodamine phalloidin (Molecular Probes) was diluted to 100 μM in lamprey internal solution (180 mM KCl; 10 mM HEPES-KOH, pH 7.4). In some cases, lamprey spinal cords were pretreated with either Latrunculin B (2.5 μM; 0–60 minutes) or with 0 Ca^{2+} lamprey Ringer's (where Ca^{2+} was replaced by Mg^{2+}; 15 minutes) before injection of actin. All fluorescent reagents were pressure-injected directly into reticulospinal axons via borosilicate glass microelectrodes using a Picospritzer II (General Valve, Marietta, GA). Intracellular recordings

of axons were performed via an Axoclamp 2B amplifier (Axon Instruments, Burlingame, CA), and electrophysiological parameters of axons were monitored throughout injections. Standard fluorescence microscopy was performed on an Olympus BHX-50 upright microscope with a 40× (0.8 NA) water-immersion objective, and images were collected with a MicroMax cooled CCD camera (Roper Scientific, Tucson, AZ).

Electron microscopy

Electron microscopy was performed as previously described (Pieribone et al., 1995). Briefly, latrunculin B (2.5 μM; Molecular Probes) or swinholide A (50 nM; Kamiya Biomedical, Thousand Oaks, CA) was bath-applied to a segment of spinal cord for 1 hour or overnight at 10°C. After treatment with the toxins, single axons were impaled with 3M KCl-filled electrodes and then stimulated with action potentials elicited by injecting current pulses (1 ms; 30–100 nA; 20 Hz, 5 minutes). Following the stimulation period, fixative (3% gluteraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.4) was gently added to the chamber while continuing to elicit action potentials. Fixation occurred within the first minute, as assessed by the disappearance of action potentials. The entire spinal cord segment was fixed first in the chamber for 1 hour. Then a smaller piece of spinal cord including the injected axon was dissected out and left in fresh fixative overnight at room temperature. Following fixation, preparations were washed in 0.1 M Na cacodylate buffer, postfixed for 1 hour in 2% osmium tetroxide (EM Sciences, Fort Washington, PA), dehydrated in a graded acetone series, and embedded in Durcapan ACM (Fluka, Buchs, Switzerland). Serial ultrathin sections were cut on a Leica Ultracut UTC ultramicrotome and mounted onto formvar-coated copper slot grids. Sections were counterstained with saturated uranyl acetate and 0.5% lead citrate and then examined using a Zeiss 109 microscope at 3,000 or 15,000× magnification.

3D reconstructions

Three-dimensional reconstructions of serial sectioned control and toxin-treated synapses were made by tracing vesicles and membranes from electron micrographs into a 3D-CAD program (Ashlar-Velum Xenon) and then lofting a surface between the membrane "ribs" using a skin function. Photomicrographs were aligned on a graphics tablet (Wacom) using fiduciary structures in individual sections prior to the input. Multiple fiduciary structures were used for each section pair to avoid straight-line biasing. Synaptic vesicles (yellow) and coated structures (red) were added to the scenes separately. Final scenes were then oriented and rendered using Xenon.

Morphometric analysis

The number of vesicles was counted at each synapse from images taken near the center of the active zone. From these same images the plasma membrane (PM) curvature ratio was determined by measuring a straight-line distance of 0.5 μm from the edge of the active zone to the plasma membrane divided into the curved distance along the membrane between the same two points. This value is called the "PM curvature ratio" (Fig. 6). The total number of coated structures associated with the active zone was counted and staged according to the following criteria: Stage 1 (early coat, little invagination), Stage 2 (fully coated invagination, no constriction of the neck), Stage 3 (fully invaginated pit with constricted neck, with or without protein collar), and Stage 4 (apparently free coated vesicle). Stage 3 vesicles can be mistaken for Stage 4 if the neck of the coated pits is outside the image plane.

RESULTS

Presynaptic actin is dynamic at resting synapses

When injected into living axons of the intact lamprey spinal cord, fluorescently labeled phalloidin, an F-actin binding toxin, accumulates into rings surrounding synaptic vesicle clusters at the periactive zone (Fig. 1C) (Shupliakov et al., 2002; Morgan et al., 2004). Because phalloidin prevents actin filaments from disassembling, its presence prevents examination of actin dynamics to any great detail. Therefore, in order to examine actin dynamics at synapses Alexa488-labeled monomeric G-actin was injected into unstimulated lamprey axons (Fig. 1). The fluorescent actin was rapidly incorporated into distinct ring-like structures at synapses, indicating actin turnover at these quiescent synapses (Fig. 1A). When subsequently injected into the same axon, rhodamine-phalloidin tightly colocalized with actin (Fig. 1B,C), indicating that the actin rings are composed predominately of polymerized F-actin. When coinjected with phalloidin, the Alexa488 fluorescence associated with synaptic actin was doubled (because phalloidin stabilizes F-actin and thereby effectively promotes polymerization) (Fig. 1D; n = 72 actin synapses, five phalloidin synapses; $P < 0.005$; Student's *t*-test). Pretreatment of spinal cords with the actin-depolymerizing toxin latrunculin B prevented the accumulation of actin at synapses (Fig. 1E). Similarly, removal of extracellular calcium prevented the de novo incorporation of Alexa488-actin into perisynaptic rings (Fig. 1F; n = 11 of 12 axons). In contrast, although the fluorescence was somewhat less bright than in controls (Fig. 1G), removal of extracellular calcium did not prevent Alexa488-phalloidin from labeling preexisting actin structures, indicating that the overall synaptic architecture was preserved (Fig. 1H; n = 5 of 5 axons).

We next examined the time course of Alexa488-actin (~43,000 MW) diffusion and compared it to that of an inert, freely diffusible molecule of similar size, Alexa488-dextran (40,000 MW). Dextran fluorescence reached a peak within 5 minutes following injection and then continually decreased over time. In contrast, both the axonal and synaptic actin fluorescence remained high for up to 5 hours (Fig. 1I). These results indicate that there is a rapidly turning over, yet stable, F-actin network at resting lamprey synapses.

To determine the time course over which latrunculin B depolymerizes actin at lamprey synapses, the synaptic actin was first labeled by microinjecting Alexa488-actin into axons. Latrunculin B (2.5 μM) was then bath-applied and the fluorescence associated with actin was monitored over time. In the absence of latrunculin, there was relatively no change in the synaptic actin fluorescence over the time course of an hour (Fig. 2A,C). However, when latrunculin was added to the bath, the synaptic actin fluorescence completely disappeared (Fig. 2B,C). The time constant of the effect of latrunculin is 7.7 minutes (n = 9 synapses), providing another indication that this stable F-actin network is turning over rapidly.

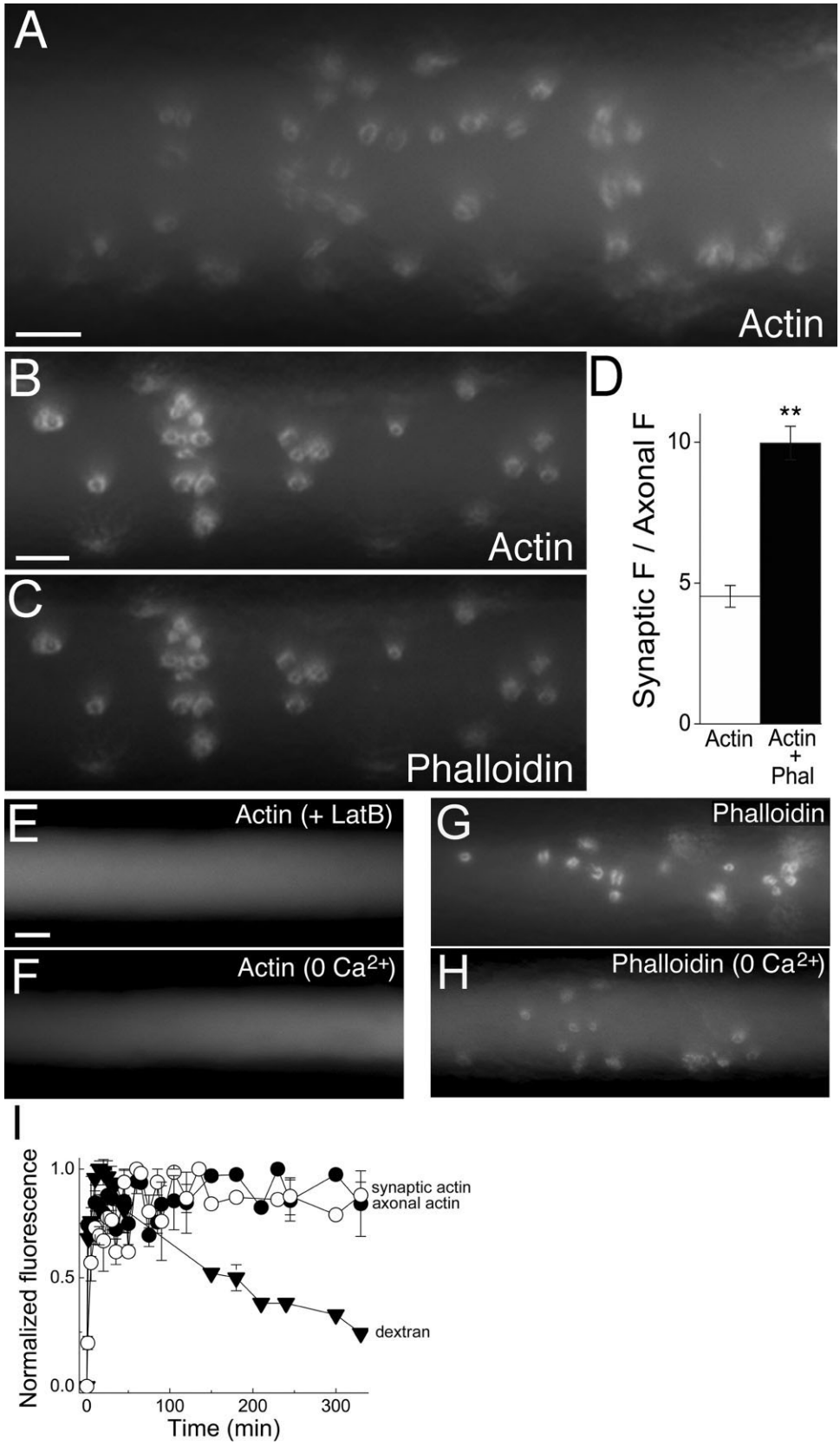

Fig. 1. A dynamic ring of F-actin exists at endocytic zones of synapses. **A:** Fluorescence images of a living axon injected with Alexa 488-actin reveals de novo synaptic accumulations of actin. **B,C:** Images of an axon injected first with Alexa 488-actin (B) and then with rhodamine-phalloidin (C) reveal a complete overlap in the distribution patterns and indicate that the actin structure is filamentous. **D:** Quantification of the average actin fluorescence at synapses in the absence or presence of phalloidin. Error bars indicate ± SEM. **E,F:** Axons preincubated either with latrunculin B (E) or zero calcium lamprey Ringer (F) and then injected with Alexa 488-actin. Note that actin fails to accumulate at synapses. **G,H:** Axons in normal lamprey Ringer (G) or preincubated with zero calcium lamprey Ringer (H) prior to injection of Alexa 488-phalloidin. In both cases, Alexa 488 recognizes preformed synaptic F-actin structures. **I:** Time course of axonal actin (dark circles), synaptic actin (open circles), and dextran (dark triangles) diffusion reveals that synaptic actin accumulations are stable for several hours. Scale bars = 10 μm.

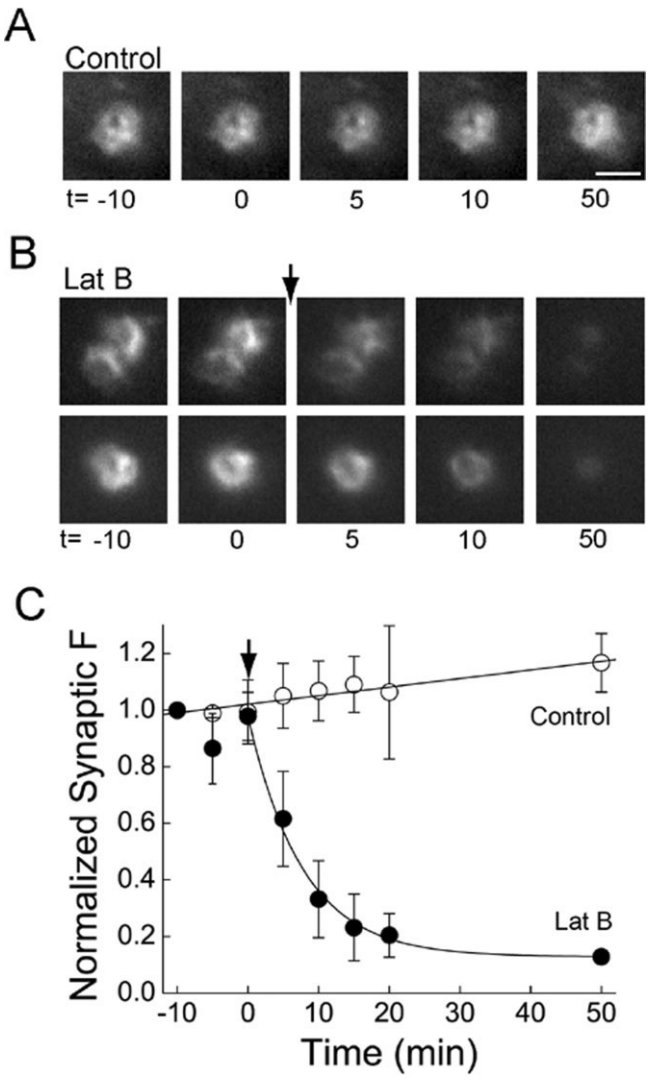

Fig. 2. Time course showing effect of latrunculin B on actin at synapses. **A:** Fluorescence images of a synapse labeled with Alexa-488 actin shows very little change in synaptic actin fluorescence over a 1-hour time period. **B:** Images showing time-dependent depolymerization of synaptic actin. Latrunculin (2.5 μM) was bath-applied at t = 0 minutes (arrow in B,C). **C:** Time course of the effect of latrunculin B on synaptic actin fluorescence. Data represent mean synaptic actin fluorescence ± SEM. Scale bar = 3 μm.

Actin polymerization participates in the maturation of clathrin-coated vesicles during synaptic vesicle recycling

Evidence from previous studies indicates that actin turnover participates in many steps during synaptic vesicle recycling at lamprey synapses (Shupliakov et al., 2002; Bloom et al., 2003; Morgan et al., 2004). However, we wanted to determine at which step actin polymerization is first required for synaptic vesicle recycling. To do so, we examined the ultrastructure of stimulated and unstimulated synapses both with latrunculin and with swinholide, another toxin designed to specifically prevent the formation of actin filaments. Under control conditions at the ultrastructural level, resting lamprey reticulospinal synapses have discrete vesicle clusters, a smooth presynaptic plasma membrane, and few clathrin-coated structures (Fig. 3A,B) (Wickelgren et al., 1985; Pieribone et al., 1995). When stimulated to induce synaptic vesicle trafficking (20 Hz; 5 minutes), small plasma membrane evaginations filled with a dense actin cytomatrix form around the active zones (Bloom et al., 2003) and the number of clathrin-coated structures slightly increases (Fig. 3C,D). Following a 1-hour pretreatment of spinal cords with either latrunculin B (2.5 μM) or swinholide A (50 nM), there was no effect on the organization of unstimulated synapses (Figs. 4A, 5A,B). In contrast, stimulated synapses treated with latrunculin or with swinholide exhibited an increase in the number of clathrin-coated profiles (Figs. 4B–F, 5C–G). Three-dimensional reconstructions of stimulated latrunculin- and swinholide-treated synapses revealed clathrin-coated structures that appeared at distances farther from the synapse than observed in controls (Figs. 4F, 5F,G). In addition, stimulated swinholide-treated synapses also exhibited large, protruding evaginations that were largely devoid of actin cytomatrix (Fig. 5C–E,G).

Quantification of these ultrastructural effects revealed that latrunculin and swinholide induced a 2.5-fold increase in the total number of clathrin-coated pits at stimulated synapses (Fig. 6A; n = 17 synapses for each condition; $P < 0.0001$, Student's *t*-test). When the coated pits were classified into morphologically discrete stages (see Materials and Methods) (Brodin et al., 2000; Gad et al., 2000), it became apparent that latrunculin and swinholide increased the number of unconstricted (Stage 2) and constricted (Stage 3) coated pits (Fig. 6B). When normalized to the total number of coated pits, a selective increase in the percent of unconstricted coated pits was revealed (Fig. 6C), indicating an arrest or delay of clathrin-coated pit maturation. Measurement of the plasma membrane evaginations revealed that swinholide also induced a significant 29% increase in the size of these structures over controls, further implicating actin polymerization in the earliest stages of synaptic vesicle recycling (Fig. 6D; $P < 0.05$). Latrunculin B, a less aggressive actin toxin, decreased the size of membrane evaginations by 37%. Taken together, these results indicate that actin polymerization is initially required during early phases of clathrin coat maturation during synaptic vesicle recycling.

DISCUSSION

Presynaptic actin at mature, central synapses is highly dynamic yet stable

Actin turnover is critically important during the development of the nervous system (Lin et al., 1994; Gungabissoon and Bamburg, 2003). In developing synapses of hippocampal neurons cultured for 1–3 weeks where maturation processes are likely still ongoing, dynamic turnover of actin has been observed both presynaptically and postsynaptically (Colicos et al., 2001; Star et al., 2002; Sankaranarayanan et al., 2003). However, very little is known about the dynamics of actin and the mechanisms that regulate its polymerization at intact, mature synapses, such as those of the giant lamprey reticulospinal axons, where actin is likely serving very different roles in membrane trafficking. Here, we show that monomeric

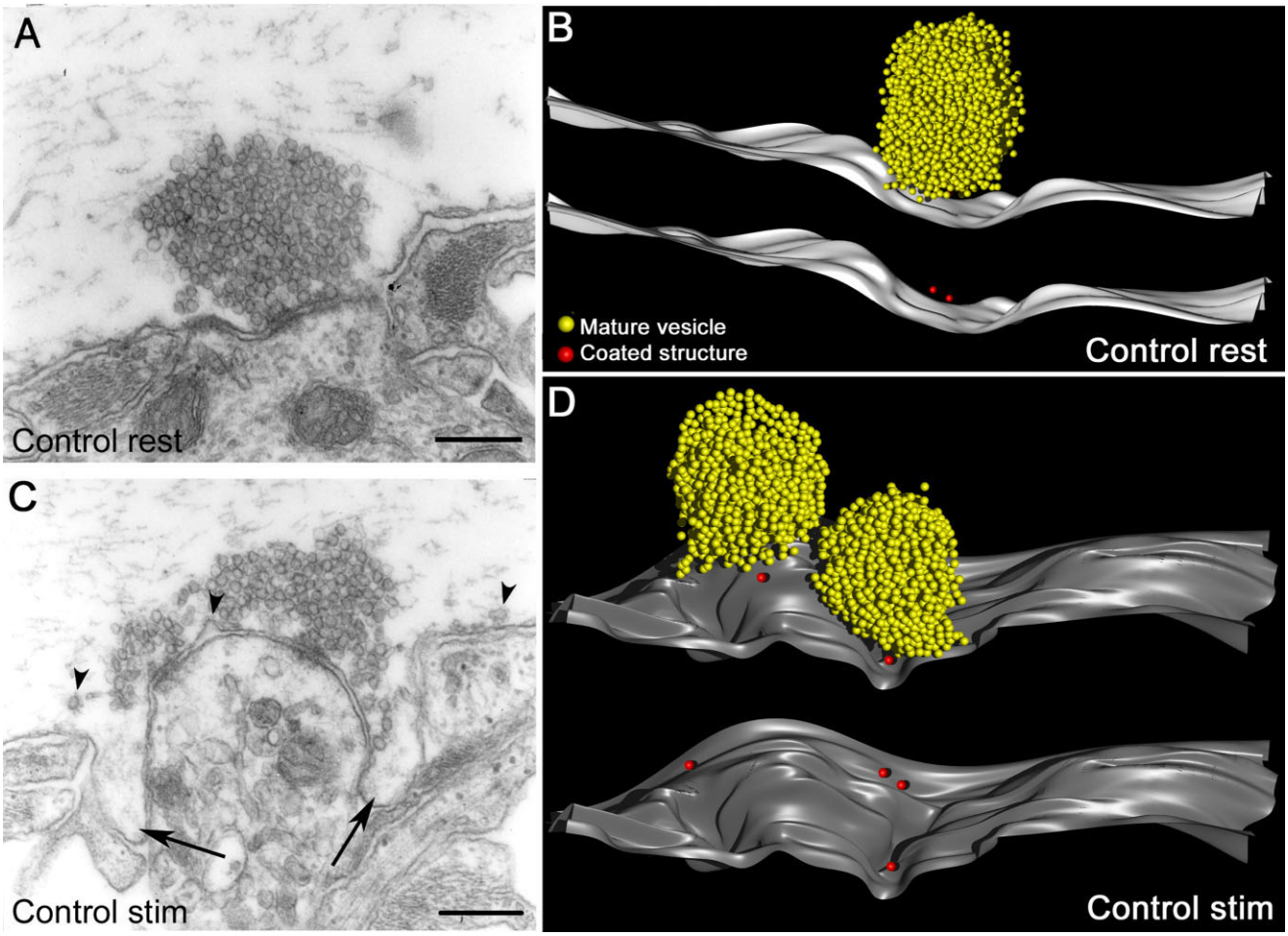

Fig. 3. Ultrastructure of control synapses. **A:** Electron micrograph of an unstimulated synapse showing a large, tight synaptic vesicle cluster. **B:** Computer reconstruction of an unstimulated synapse made from 10 serial sections. The membrane is depicted with (top) and without (bottom) the synaptic vesicle cluster. **C:** Image of a synapse stimulated at 20 Hz for 5 minutes from an adjacent axon. Note the appearances of small plasma membrane evaginations at the periactive zone (arrows) and a few coated pits (arrowheads). **D:** Computer reconstruction of a stimulated synapse made from 15 serial sections. Scale bars = 0.5 μm.

G-actin is rapidly incorporated via polymerization into the ringlike F-actin network, which exists at endocytic, "periactive" zones of lamprey synapses (Fig. 1) (Shupliakov et al., 2002; Bloom et al., 2003; Morgan et al., 2004). This actin network is a stable structure, as indicated by the constant actin fluorescence that is retained at synapses for many hours. Although stable, the synaptic actin network is also dynamically turning over, as indicated by the prevention of actin incorporation upon pretreatment of synapses with a low concentration (2.5 μM) of the actin-depolymerizing toxin latrunculin B. Furthermore, latrunculin causes a complete dispersion of synaptic actin within 8 minutes of the toxin application (Fig. 2). This is in agreement with a study of presynaptic actin dynamics in cultured hippocampal neurons, where latrunculin caused a rapid dispersion (τ = 20 seconds) of actin from presynaptic terminals and an arrest of activity-dependent actin dynamics (Sankaranarayanan et al., 2003). In hippocampal neurons, actin turnover at synapses occurs within a minute (Star et al., 2002; Sankaranaryanan et al., 2003). The slower time course for the action of latrunculin at lamprey synapses is likely due to the much larger pool of actin that has to be disrupted at these giant synapses. It may also reflect an intrinsic difference in the turnover rates for actin filaments, which would not be surprising given the distinct roles of actin polymerization at hippocampal and lamprey synapses. Additionally, latrunculin may also be slower at penetrating axons within an intact spinal cord preparation when compared to axons of cultured neurons. Whatever the case, our results show that the actin network at lamprey synapses is a stable structure in which the actin is constantly being turned over. These results additionally suggest the presence of a mechanism for sequestering monomeric actin at synapses that could serve to support the dynamic turnover of actin filaments.

In addition, the integration of G-actin into the perisynaptic F-actin ring is dependent on the presence of extracellular calcium. When the spinal cord was bathed in zero calcium Ringer, Alexa488-actin failed to accumulate at synapses following its injection. Although we do not yet completely understand either the mechanisms by which

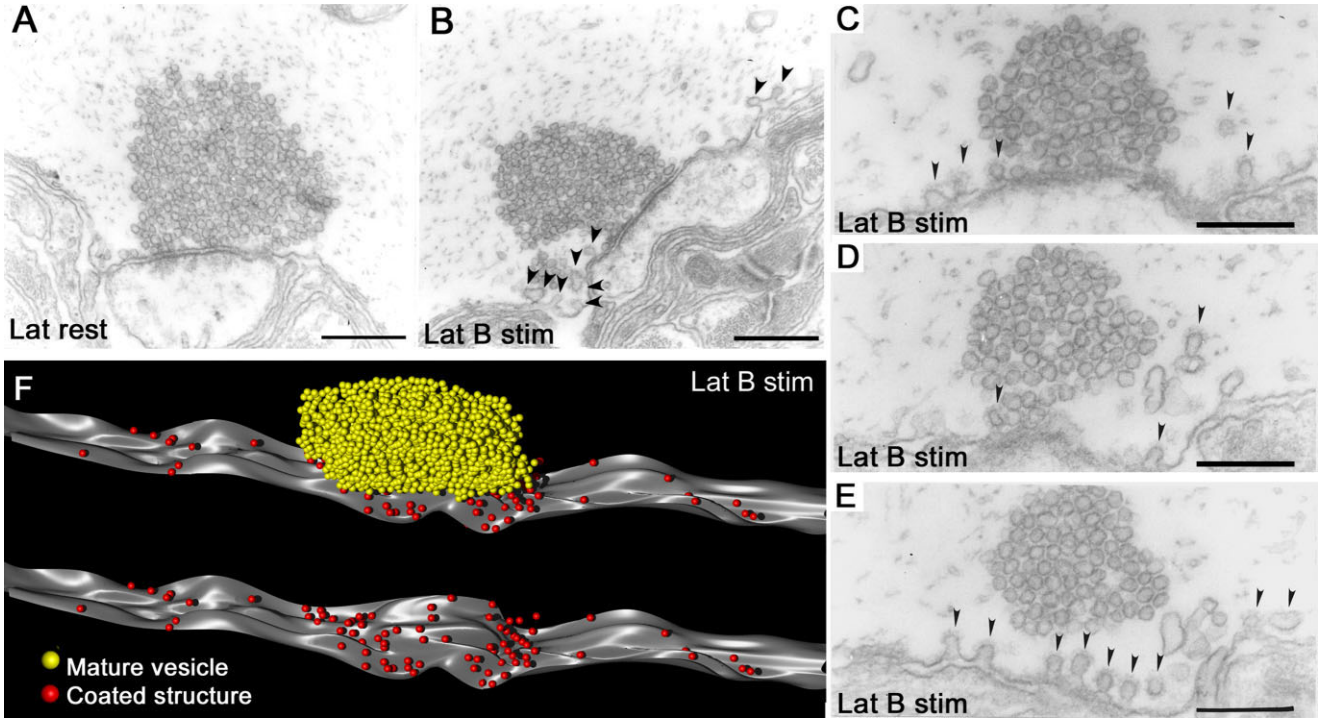

Fig. 4. Ultrastructure of synapses within latrunculin-treated axons. **A:** Electron micrograph of an unstimulated synapse treated with latrunculin B shows no obvious effect on the synaptic morphology (compare to Fig. 2A). **B:** Image of a stimulated synapse treated with latrunculin B. Note the dramatic increase in the number of coated pits (arrowheads). **C–E:** Serial sections through another stimulated synapse treated with latrunculin B. **F:** Computer reconstruction of another stimulated synapse treated with latrunculin made from 14 serial sections. The membrane is depicted with (top) and without (bottom) the synaptic vesicle cluster. Note that the coated pits extend well beyond the immediate vicinity of the active zone. Scale bars = 0.5 μm.

calcium enters the presynaptic compartment from the extracellular space in these resting axons or the means by which calcium affects actin-related accessory molecules, this result indicates that the presynaptic actin network is positively regulated by calcium. Similarly, the de novo appearance of presynaptic actin puncta in varicosities of cultured hippocampal neurons requires calcium, indicating a positive role for calcium in regulating presynaptic actin during synapse formation (Colicos et al., 2001). These new results suggest that presynaptic actin could be positively regulated by synaptic activity, which causes an elevation of intracellular calcium within the presynaptic compartment via opening of voltage-gated calcium channels. In support of this, synaptic stimulation increases the amount of actin at synapses and causes an elaboration of the presynaptic actin network (our unpubl. results).

Actin polymerization is initially required for clathrin coat maturation during early phases of synaptic vesicle recycling

The present study, as well as previous studies, show a close physical and functional relationship between actin and clathrin-coated vesicles during synaptic vesicle recycling. In both lamprey and mammalian synapses, clathrin-coated vesicles have been observed in close proximity to the actin cytoskeleton (Cremona et al., 1999; Gad et al., 2000; Wenk et al., 2001; Shupliakov et al., 2002; Bloom et al., 2003). A dramatic demonstration of this in lamprey synapses occurs following disruptions of synaptojanin, a phosphatidylinositol phosphatase that regulates the levels of phosphatidylinositol-(4,5)-bisphosphate, which is a positive regulator both for actin nucleation and clathrin assembly at synapses (Cremona et al., 1999; Gad et al., 2000). When synaptojanin is perturbed, there is a build-up of clathrin-coated pits with constricted necks as well as free clathrin-coated vesicles. The coated vesicles are surrounded by a hypertrophied actin cytoskeleton, which suggests a role for actin in late phases of synaptic vesicle recycling. Such a role for actin in propelling the endocytic vesicle away from the plasma membrane following vesicle fission has been observed in nonneuronal cells (Merrifield et al., 1999, 2002). In support of this, previous studies at lamprey synapses showed endocytic vesicles at the ends of thick bundles of polymerized actin filaments located within the periactive zone of stimulated synapses (Shupliakov et al., 2002; Bloom et al., 2003). Most often, these endocytic vesicles appeared to be in transport back to the vesicle cluster. Thus, actin participates in late stages of vesicle recycling at lamprey synapses. However, until now the potential roles of actin in earlier stages of synaptic vesicle recycling were unclear.

Here we show that actin also has a crucial role in the initial phases of clathrin-mediated endocytosis during synaptic vesicle recycling. When specific actin-depolymerizing toxins, latrunculin and swinholide, were applied to lamprey spinal cords, there was a significant build-up of clathrin-coated pits at stimulated synapses (Figs. 3–5). These clathrin-coated pits were unconstricted

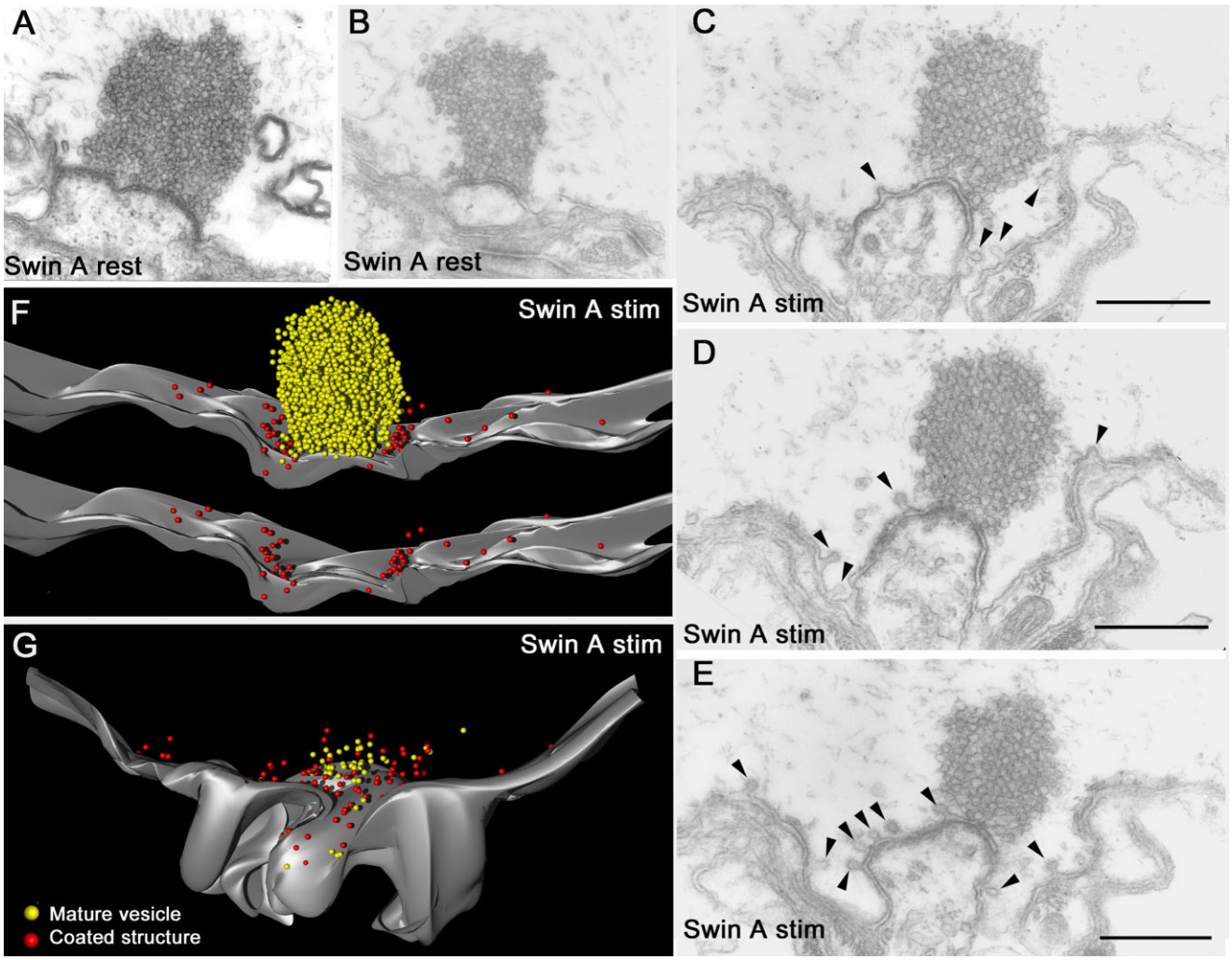

Fig. 5. Ultrastructure of synapses within swinholide-treated axons. **A,B:** Electron micrographs of unstimulated synapses treated either for 1 hour (A) or 24 hours (B) with swinholide show no obvious effect on the synaptic morphology. **C–E:** Three sample sections from a series taken of a stimulated synapse treated with swinholide. Note the dramatic increase in the number of coated pits (arrowheads) and appearance of large membrane evaginations. **F:** Computer reconstruction of a stimulated synapse treated with swinholide made from 10 serial sections. The membrane is depicted with (top) and without (bottom) the synaptic vesicle cluster. **G:** Computer reconstruction of another stimulated synapse treated with swinholide made from 16 serial sections. Note the elaborate membrane evaginations and the accumulation of coated pits. Scale bars = 0.5 μm.

and lacked the obvious necks or collars that are associated with function of the GTPase dynamin in vesicle constriction and fission from the plasma membrane (Takei et al., 1996; Schmid et al., 1998; Brodin et al., 2000). Interestingly, dynamin is thought to function as an actin regulatory protein (Lee and De Camilli, 2002; Krueger et al., 2003; Schafer, 2004). A similar phenotype was observed at lamprey synapses treated with the actin-depolymerizing C2 toxin (Shupliakov et al., 2002). However, the present comprehensive morphometric analysis of many synapses treated with latrunculin and swinholide unequivocally shows for the first time that actin polymerization is necessary for the maturation of clathrin-coated vesicles from an unconstricted to a constricted stage at lamprey synapses (Figs. 4–6). Interestingly, when these synapses are treated with the F-actin stabilizing toxin phalloidin, there is also an accumulation of unconstricted clathrin-coated pits (Shupliakov et al., 2002). However, unlike synapses treated with swinholide and latrunculin, the coated pits observed in the presence of phalloidin often have long necks extending from the plasma membrane, indicating a perturbation of a slightly later stage of clathrin-mediated synaptic vesicle recycling. Because latrunculin and swinholide prevent altogether the polymerization of G-actin into F-actin, any effect on these later stages of clathrin-mediated vesicle recycling would be effectively masked.

In the case of swinholide, a more aggressive toxin that both sequesters actin monomers and severs existing filaments, the increase in coated pits was accompanied by an increase in the size of the plasma membrane evaginations (Figs. 5, 6). These perisynaptic evaginations are structurally similar to those that have been observed in a number of different types of synapses, including those in the sympathetic ganglia of the cat (Pysh and Wiley, 1972), the

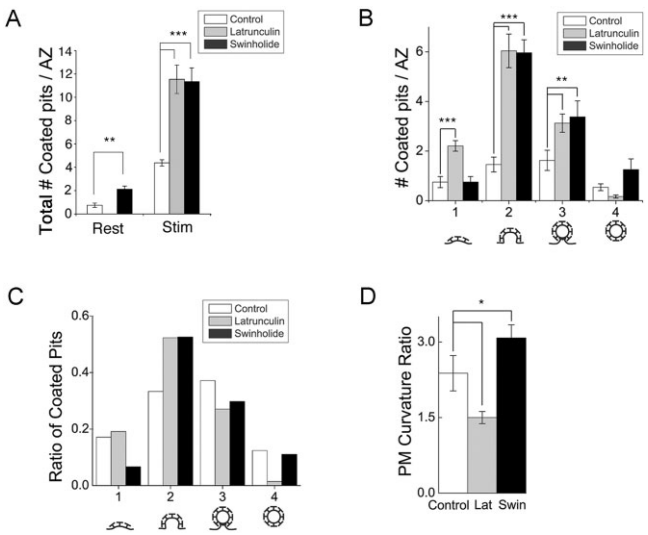

Fig. 6. Latrunculin and swinholide inhibit the progression of clathrin-mediated endocytosis at synapses. **A:** Quantification of the total number of clathrin-coated pits (CCPs) at stimulated control, latrunculin B-, and swinholide-treated synapses. **B:** Quantification of the number of CCPs according to their stage of maturation. Note the selective increase in unconstricted and constricted (Stages 2 and 3) CCPs. **C:** Quantification of the ratio of CCPs at each stage reveals a specific increase in the unconstricted CCPs (Stage 2) and indicates a perturbation in the progression of maturation. **D:** Quantification of the plasma membrane (PM) curvature ratio for control and toxin-treated synapses. Data derived from 17 each of control, latrunculin-, and swinholide-treated synapses from four axons. All data represent means and ± SEM for each condition. Statistical significance was established using Student's *t*-test (*$P < 0.05$; **$P < 0.005$, ***$P < 0.0005$).

retina of the turtle (Schaeffer et al., 1982), and the sense cells of the electrosensory organs of the thornback ray (Fields and Ellisman, 1988). Similar to our findings, latrunculin A causes an increase in complex membrane folds that probably originate from the plasma membrane at stimulated frog neuromuscular junctions (Richards et al., 2004). Furthermore, latrunculin B perturbs bulk endocytosis in retinal bipolar neurons (Holt et al., 2003). However, such bulk endocytosis is not involved in replenishing the pools of synaptic vesicles (Holt et al., 2003). In contrast, at lamprey synapses these plasma membrane evaginations are the source for replenishing synaptic vesicles via clathrin-mediated endocytosis following stimulation (Fig. 5) (Gad et al., 1998, 2000; Morgan et al., 2004). Therefore, we favor the interpretation that the enlarged plasma membrane evaginations observed in the presence of swinholide are due to a perturbation of the very earliest stages of clathrin-mediated endocytosis, for example, during the initiation of clathrin assembly by AP180 and AP-2 (Morgan et al., 2000).

The precise molecular mechanisms that link actin and clathrin at synapses remain to be determined. Recently, we showed that phosphatidylinositol-(4,5)-bisphosphate [$PIP(4,5)P_2$] is one molecule that links actin and the clathrin coat at lamprey synapses (Morgan et al., 2004). When $PIP(4,5)P_2$ synthesis was perturbed, there was both a decrease in the levels of synaptic actin and a concomitant increase in the same type of unconstricted clathrin-coated pits that we observe here with latrunculin and swinholide treatment (Morgan et al., 2004). This is likely due to the known dual roles of $PIP(4,5)P_2$ in actin nucleation via its interaction with N-WASP and in clathrin coat formation via its interactions with the clathrin adaptors (Qualmann and Kessels, 2002). Other potential molecular links between actin and the clathrin coat include N-WASP, cortactin, PACSIN/syndapin, Hip1R, and intersectin, which may also contribute to the observed phenotype (Qualmann and Kessels, 2002; Engqvist-Goldstein and Drubin, 2003, Merrifield et al., 2004, 2005). However, these molecular links remain to be explored in future studies.

In agreement with our findings, it has been observed that actin participates in endocytosis and receptor internalization during early stages of clathrin coat maturation in yeast and in nonneuronal mammalian cells (Kaksonen et al., 2003, 2005; Yarar et al., 2005). Furthermore, several studies directly implicate actin as part of the fission machinery for both clathrin-coated vesicles as well as mitochondria (De Vos et al., 2005; Zhu et al., 2005). Thus, the participation of actin in early steps of endocytosis is a conserved feature between various cell types and complex cellular processes.

ACKNOWLEDGMENTS

The authors thank Pietro De Camilli for support and discussion.

LITERATURE CITED

Belmont LD, Drubin DG. 1998. The yeast V159N actin mutant reveals roles for actin dynamics in vivo. J Cell Biol 142:1289–1299.

Bernstein BW, DeWit M, Bamburg JR. 1998. Actin disassembles reversibly during electrically induced recycling of synaptic vesicles in cultured neurons. Brain Res Mol Brain Res 53:236–251.

Bloom O, Evergren E, Tomilin N, Kjaerulff O, Löw P, Brodin L, Pieribone VA, Greengard P, Shupliakov O. 2003. Co-localization of synapsin and actin during synaptic vesicle recycling. J Cell Biol 161:737–747.

Brodin L, Löw P, Shupliakov O. 2000. Sequential steps in clathrin-mediated synaptic vesicle endocytosis. Curr Opin Neurobiol 10:312–320.

Carreno S, Engqvist-Goldstein AE, Zhang ZX, McDonald KL, Drubin DG. 2004. Actin dynamics coupled to clathrin-coated vesicle formation at the trans-Golgi network. J Cell Biol 165:781–788.

Cole JC, Villa B, Wilkinson RS. 2000. Disruption of actin impedes transmitter release in snake motor terminals. J Physiol 523:579–586.

Colicos MA, Collins BE, Sailor MJ, Goda Y. 2001. Remodeling of synaptic actin induced by photoconductive stimulation. Cell 107:605–616.

Cremona O, Di Paolo G, Wenk MR, Luthi A, Kim WT, Takei K, Daniell L, Nemoto Y, Shears SB, Flavell RA, McCormick DA, De Camilli P. 1999. Essential role of phosphoinositide metabolism in synaptic vesicle recycling. Cell 99:179–188.

De Vos KJ, Allan VJ, Grierson AJ, Sheetz MP. 2005. Mitochondrial function and actin regulate dynamin-related protein 1-dependent mitochondrial fission. Curr Biol 15:678–683.

Dunaevsky A, Connor EA. 2000. F-actin is concentrated in nonrelease domains at frog neuromuscular junctions. J Neurosci 20:6007–6012.

Engqvist-Goldstein EF, Drubin DG. 2003. Actin assembly and endocytosis: from yeast to mammals. Annu Rev Cell Dev Biol 19:287–332.

Fields RD, Ellisman MH. 1988. Functionally significant plasticity of synaptic morphology: studies on the ribbon synapse of the ampullae of Lorenzini. Neuroscience 25:705–720.

Fischer M, Kaech S, Knutti D, Matus A. 1998. Rapid actin-based plasticity in dendritic spines. Neuron 20:847–854.

Gad H, Low P, Zotova E, Brodin L, Shupliakov O. 1998. Dissociation between Ca2+-triggered synaptic vesicle exocytosis and clathrin-mediated endocytosis at a central synapse. Neuron 21:607–616.

Gad H, Ringstad N, Löw P, Kjaerulff O, Gustafsson J, Wenk M, Di Paolo G, Nemoto Y, Crun J, Ellisman MH, Di Camilli P, Shupliakov O, Brodin

L. 2000. Fission and uncoating of synaptic clathrin-coated vesicles are perturbed by disruption of interactions with the SH3 domain of endophilin. Neuron 27:301–312.

Gungabissoon RA, Bamburg JR. 2003. Regulation of growth cone actin dynamics by ADF/cofilin. J Histochem Cytochem 51:411–420.

Heuser JE, Reese TS. 1973. Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J Cell Biol 57:315–344.

Holt M, Cooke A, Wu MM, Lagnado L. 2003. Bulk membrane retrieval in the synaptic terminal of retinal bipolar cells. J Neurosci 23:1329–1339.

Job C, Lagnado L. 1998. Calcium and protein kinase C regulate the actin cytoskeleton in the synaptic terminal of retinal bipolar cells. J Cell Biol 143:1661–1672.

Kaksonen M, Sun Y, Drubin DG. 2003. A pathway for association of receptors, adaptors, and actin during endocytic internalization. Cell 115:475–487.

Kaksonen M, Toret CP, Drubin DG. 2005. A modular design for the clathrin- and actin-mediated endocytosis machinery. Cell 123:305–320.

Krueger EW, Orth JD, Cao H, McNiven MA. 2003. A dynamin-cortactin-Arp2/3 complex mediates actin reorganization in growth factor-stimulated cells. Mol Biol Cell 14:1085–1096.

Lee E, De Camilli P. 2002. Dynamin at actin tails. Proc Natl Acad Sci U S A 99:161–166.

Lin CH, Thompson CA, Forscher P. 1994. Cytoskeletal reorganization underlying growth cone motility. Curr Opin Neurobiol 4:640–647.

Merrifield CJ, Moss SE, Ballestrem C, Imhof BA, Giese G, Wunderlich I, Almers W. 1999. Endocytic vesicles move at the tips of actin tails in cultured mast cells. Nat Cell Biol 1:72–74.

Merrifield CJ, Feldman ME, Wan L, Almers W. 2002. Imaging actin and dynamin recruitment during invagination of single clathrin-coated pits. Nat Cell Biol 4:691–698.

Merrifield CJ, Qualmann B, Kessels MM, Almers W. 2004. Neural Wiskott Aldrich syndrome protein N-WASP and Arp 2/3 complex are recruited to sites of clathrin-mediated endocytosis in cultured fibroblasts. Eur J Cell Biol 83:13–18.

Merrifield CJ, Perrais D, Zenisek D. 2005. Coupling between clathrin-coated-pit invagination, cortactin recruitment, and membrane scission observed in live cells. Cell 121:593–606.

Morales M, Colicos MA, Goda Y. 2000. Actin-dependent regulation of neurotransmitter release at central synapses. Neuron 27:539–550.

Morgan JR, Prasad K, Hao W, Augustine GJ, Lafer EM. 2000. A conserved clathrin assembly motif essential for synaptic vesicle endocytosis. J Neurosci 20:8667–8676.

Morgan JR, Di Paolo G, Werner H, Shchedrina VA, Pypaert M, Pieribone VA, De Camilli P. 2004. A role for talin in presynaptic function. J Cell Biol 167:43–50.

Pieribone V A, Shupliakov O, Brodin L, Hilfiker-Rothenfluh S, Czernik AJ, Greengard, P. 1995. Distinct pools of synaptic vesicles in neurotransmitter release. Nature 375:493–497.

Pysh JJ, Wiley RG. 1972. Morphologic alterations of synapses in electrically stimulated superior cervical ganglia of the cat. 176:191–193.

Qualmann B, Kessels MM. 2002. Endocytosis and the cytoskeleton. Int Rev Cytol 220:93–144.

Richards DA, Rizzoli SO, Betz WJ. 2004. Effects of wortmannin and latrunculin A on slow endocytosis at the frog neuromuscular junction. J Physiol 15:77–91.

Roos J, Kelly RB. 1999. The endocytic machinery in nerve terminals surrounds sites of exocytosis. Curr Biol 9:1411–1414.

Sakaba T, Neher E. 2003. Involvement of actin polymerization in vesicle recruitment at the Calyx of Held synapse. J Neurosci 23:837–846.

Sankaranarayanan S, Atluri PP, Ryan TA. 2003. Actin has a molecular scaffolding, not propulsive, role in pre-synaptic function. Nat Neurosci 6:127–135.

Schaeffer SF, Raviola E, Heuser JE. 1982. Membrane specializations in the outer plexiform layer of the turtle retina. J Comp Neurol 204:253–267.

Schafer DA. 2002. Coupling actin dynamics and membrane dynamics during endocytosis. Curr Opin Cell Biol 14:76–81.

Schafer DA. 2004. Regulating actin dynamics at membranes: a focus on dynamin. Traffic 5:463–469.

Schmid SL, McNiven MA, De Camilli P. 1998. Dynamin and its partners: a progress report. Curr Opin Cell Biol 10:504–512.

Shupliakov O, Bloom O, Gustafsson JS, Kjaerulff O, Löw P, Tomilin N, Pieribone V A, Greengard P, Brodin L. 2002. Impaired recycling of synaptic vesicles after acute perturbation of the pre-synaptic actin cytoskeleton. Proc Natl Acad Sci U S A 99:14476–14481.

Star EN, Kwiatkowski DJ, Murthy VN. 2002. Rapid turnover of actin in dendritic spines and its regulation by activity. Nat Neurosci 5:239–246.

Takei K, Mundigl O, Daniell L, De Camilli P. 1996. The synaptic vesicle cycle: a single vesicle budding step involving clathrin and dynamin. J Cell Biol 133:1237–1250.

Theriot JA, Mitchison TJ. 1991. Actin microfilament dynamics in locomoting cells. Nature 352:126–131.

Wenk MR, Pellegrini L, Klenchin VA, Di Paolo G, Chang S, Daniell L, Arioka M, Martin TF, De Camilli P. 2001. PIP kinase Igamma is the major PI(4,5)P(2) synthesizing enzyme at the synapse. Neuron 32:79–88.

Wickelgren WO, Leonard JP, Grimes MJ, Clark RD. 1985. Ultrastructural correlates of transmitter release in pre-synaptic areas of lamprey reticulospinal axons. J Neurosci 5:1188–1201.

Yarar D, Waterman-Storer CM, Schmid SL. 2005. A dynamic actin cytoskeleton functions at multiple stages of clathrin-mediated endocytosis. Mol Biol Cell 16:964–975.

Zhu J, Zhou K, Hao JJ, Liu J, Smith N, Zhan X. 2005. Regulation of cortactin/dynamin interaction by actin polymerization during the fission of clathrin-coated pits. J Cell Sci 118:807–817.