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Clathrin and synaptic vesicle endocytosis: studies at the squid giant synapse

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Abstract

The role of clathrin-mediated endocytosis in SV (synaptic vesicle) recycling has been studied by combining molecular biology, physiology and electron microscopy at the squid giant synapse. Procedures that prevent clathrin from assembling into membrane coats, such as impairment of binding of the AP180 and AP-2 adaptor proteins, completely prevent membrane budding during endocytosis. These procedures also reduce exocytosis, presumably an indirect effect of a reduction in the number of SVs following block of endocytosis. Disrupting the binding of auxilin to Hsc70 (heat-shock cognate 70) prevents clathrin-coated vesicles from uncoating and also disrupts SV recycling. Taken together, these results indicate that a clathrin-dependent pathway is the primary means of SV recycling at this synapse under physiological conditions.

Keywords

adaptor protein; clathrin; plasma membrane; squid giant synapse; synaptic vesicle; vesicle recycling

Introduction

The role of CCVs (clathrin-coated vesicles) in SV (synaptic vesicle) endocytosis has been debated for more than 30 years. Early observations of coated vesicles in nerve terminals led to the suggestion that SVs might be recycled locally via these structures [1,2]. Heuser [3,4] and Heuser and Reese [5] were the first to demonstrate this by using electron microscopy to track an extracellularly applied tracer, HRP (horseradish peroxidase), over time following synaptic stimulation. The HRP was found first in coated vesicles, then in larger endosomal-like cisternae and finally in SVs. This led to the formal proposal that following exocytosis

SVs are retrieved from the plasma membrane via the formation of coated vesicles [3–5]. Ceccarelli et al. [6,7] questioned the physiological relevance of the Heuser and Reese model, noting that high and possibly unphysiological levels of synaptic activity were used. They proposed an alternative model suggesting that SV endocytosis proceeds rapidly by simply closing the exocytotic fusion pore rather than a specific membrane retrieval mechanism. Over the years, evidence has accumulated in support of both models [8,9].

We now know that clathrin is the major protein component of coated vesicles, and the debate continues over whether CCV formation is the predominant mechanism for recycling SVs. One major line of evidence supporting a clathrin-independent pathway has come from membrane capacitance measurements, which allows for real-time detection of endocytosis [8,9]. While endocytosis in nerve terminals has been observed to require tens of seconds [10–13], a more rapid, and possibly clathrin-independent, endocytosis has also been observed to occur within milliseconds [14] to a few seconds [15–20] in some synaptic terminals and neurosecretory cells. Some argue that this rapid endocytosis is far too fast for the multiple budding steps of the classical CCV pathway to occur [8]. In support of this, rapid endocytosis in chromaffin cells is not blocked by antibodies against clathrin [21]. However, rapid endocytosis is blocked by antibodies against dynamin, a major player in the clathrin pathway. Therefore a role for CCVs cannot be entirely ruled out.

When two kinetically distinct rates of membrane retrieval were found at hippocampal synapses, it was hypothesized that the slower process is clathrin-dependent and that the faster process is clathrin-independent. However, in a more recent version of the Heuser and Reese model, SVs are retrieved via CCV formation in a single budding step from the plasma membrane without requiring an endosomal intermediate [22–25]. Furthermore, it has been observed that SV recycling occurs on a continuum with time constants ranging from 4 to 90 s and with a maximum capacity of one SV per second [19]. Thus it is possible that clathrin-mediated endocytosis could occur on the fast timescale that has been observed for rapid endocytosis.

The clathrin model is well supported by genetic and acute perturbation studies. When clathrin binding or accessory proteins are genetically perturbed, defects in synaptic transmission are consistently observed. For example, genetic disruptions of dynamin [26,27], the clathrin assembly adaptor proteins AP-2 and AP180 [28–30], synaptojanin [31], amphiphysin [32] and Eps15 [33] all cause defects in SV endocytosis. However, these studies do not address whether the clathrin pathway is essential for SV recycling, since these proteins are often involved in other processes. For example, dynamin also plays a role in ‘rapid endocytosis’, which may not be clathrin-dependent [21]. Furthermore, since AP-2 plays a general role in receptor-mediated endocytosis, loss of AP-2 α -adaptin subunit may disrupt a large number of essential membrane trafficking events [34]. That many of these accessory proteins interact with non-clathrin-binding partners further complicates the matter. Therefore acute perturbation studies, in which reagents that directly target clathrin binding or specific protein–protein interactions were utilized, have provided an important complementary approach for assessing the role of the clathrin pathway in synaptic endocytosis.

The two major systems that have been used are the lamprey synapse and the squid giant synapse. In the lamprey system, a number of protein–protein interactions between clathrin accessory proteins, including dynamin, amphiphysin and endophilin, have been implicated in SV endocytosis [35–38]. In the squid system, we have shown that reagents that inhibit clathrin assembly [39–41] or uncoating [42] perturb SV endocytosis. Because the work at the squid giant synapse utilizes very low rates of stimulation (0.03 Hz), this further allowed us to evaluate the roles of the clathrin pathway under physiological conditions.

Role of clathrin assembly in SV endocytosis

To probe the function of the clathrin pathway in SV endocytosis, we perturbed several protein–protein interactions that are involved in this pathway. For this purpose, we developed several novel reagents that could be microinjected into the squid giant synapse and asked whether these reagents affected SV endocytosis.

We began by considering the role of clathrin assembly into membrane coats. Alignment of the sequences of numerous clathrin-binding proteins has revealed the presence of a conserved clathrin-binding motif that includes a DLL (Asp-Leu-Leu) consensus sequence [40]. Taking advantage of this information, we designed binding site peptides that mimic the DLL-containing regions of the clathrin assembly proteins, AP180 and AP-2 (AP2 pep). Each of these peptides was capable of competitively inhibiting both AP180 and AP-2 from assembling clathrin into cages. AP2 pep produced a concentration-dependent inhibition of clathrin assembly by AP-2 (Figure 1A) and AP180 (results not shown). A mutant peptide in which the clathrin-binding DLL motif was mutated to AAA (AP2 pep DLL) did not inhibit clathrin assembly (Figure 1A, open symbols), thus allowing this peptide to serve as a control.

To probe the physiological function of clathrin-binding reactions *in vivo*, the DLL-motif peptides were microinjected into the squid giant pre-synaptic terminal as they were being stimulated every 30 s (0.03 Hz) [43]. We first monitored neurotransmitter release, which can be assayed by measuring the slope of PSPs (post-synaptic potentials) elicited in response to single pre-synaptic action potentials. Because neurotransmitter release represents the net sum of all exocytotic and endocytotic reactions within the terminal, it is very sensitive to disruption of endocytosis. Indeed, we found that AP2 pep when microinjected into pre-synaptic terminals inhibited neurotransmitter release (Figure 1B). This effect was concentration-dependent and was specifically related to inhibition of clathrin-binding reactions, because it was not observed with the control peptide, AP2 pep DLL [40]. There was an excellent correlation between the ability of several different clathrin-binding-site peptides to inhibit neurotransmitter release and their ability to inhibit clathrin assembly by AP-2, indicating that the inhibition of neurotransmitter release is likely to arise from impairment of clathrin binding (Figure 1C).

To identify further the site of action of AP2 pep within the SV trafficking cycle, we used electron microscopy to examine the ultrastructure of squid terminals following injection of this peptide during 0.03 Hz stimulation. AP2 pep caused a dramatic change in the structural phenotype in comparison with terminals injected with the control AP2 pep DLL (Figure

2A). Most striking was a severe reduction in the number of SVs (Figure 2A, right panel). Quantification of the number of SVs in hundreds of images like those in Figure 2(A) revealed that the vesicle number was reduced by nearly 70% in the presence of AP2 pep. This was likely to be due to an inhibition of endocytosis, because the number of CCVs was reduced even more severely. Better evidence for this conclusion comes from analysis of all membrane-bound compartments involved in SV trafficking, including SVs and CCVs, as well as endosomal structures and the pre-synaptic plasma membrane. The total amount of membrane associated with the sum of these compartments was relatively constant, though there was a shift in this membrane from the SVs to the plasma membrane (Figure 2B). Thus inhibition of clathrin assembly by AP2 pep blocks SV trafficking at a step that is required for the formation of CCVs and the subsequent reformation of SVs.

Measurements of membrane capacitance changes associated with endocytosis recently confirmed this conclusion. The capacitance of the plasma membrane is directly related to the membrane surface area, allowing membrane capacitance measurements to serve as a time-resolved assay for both exocytosis and endocytosis [14,21,44]. Stimulation of squid pre-synaptic terminals causes an increase in the capacitance associated with exocytosis, and the subsequent recovery of capacitance, is attributable to endocytosis [45]. Endocytosis proceeds with a time constant of a few hundred seconds at the squid pre-synaptic terminal (Figure 3A), with the rate of endocytosis inversely related to the amount of membrane added during exocytosis [46]. AP2 pep initially has little effect on the stimulus-induced rise in membrane capacitance, but completely blocks the endocytosis-associated recovery of capacitance (Figure 3B). This indicates that clathrin-mediated endocytosis is responsible for the recovery of the capacitance signal and that this form of endocytosis is the sole detectable means of membrane retrieval under our experimental conditions.

Role of Eps15 in SV endocytosis

We also have used the squid giant synapse to examine the function of Eps15, an accessory protein that has been implicated in clathrin-mediated endocytosis in many systems [33,34]. Eps15 is found at the edge of clathrin-coated pits, suggesting that it is involved in the formation of CCVs. We therefore evaluated the role of Eps15 in clathrin assembly and SV trafficking [39].

Eps15 alone had no ability to assemble clathrin *in vitro*. However, it was able to stimulate clathrin assembly by the adaptor protein AP180. This action is due to Eps15 binding to AP180 at two sites that contain an NPF motif, because peptides derived from these sites inhibit the ability of Eps15 to stimulate clathrin assembly. These peptides, termed NPF1 and NPF2, thus can serve as probes of the physiological role of the ability of Eps15 to stimulate clathrin assembly.

When microinjected into squid giant pre-synaptic nerve terminals, both NPF1 and NPF2 inhibited neurotransmitter release [39]. This inhibitory activity was concentration-dependent, with NPF1 acting at somewhat lower concentrations than NPF2. These effects were due to inhibition of the binding of Eps15 to AP180, because a mutant NPF1 peptide that did not inhibit binding also did not inhibit neurotransmitter release. While the NPF1

peptide had little effect on the number of SVs in squid pre-synaptic terminals, it caused a significant reduction in the number of clathrin-coated pits and CCVs. This is consistent with a role for Eps15 in regulation of clathrin coat assembly and indicates that Eps15 participates in CCV formation during SV endocytosis, providing further evidence that the clathrin pathway is involved in this process.

Role of auxilin in uncoating of endocytic vesicles

The results described above indicate that clathrin-mediated endocytosis is the predominant form of membrane retrieval in the squid pre-synaptic terminal. We next asked whether the uncoating of CCVs during endocytosis employs auxilin, a protein that serves as a cofactor for Hsc70 (heat-shock cognate 70)-dependent uncoating of vesicles [47,48]. For this purpose, we considered the physiological actions of a form of auxilin with mutations in the HPD motif within the domain that is required for binding to Hsc70. This mutant protein, termed auxilin HPD, is incapable of binding to Hsc70 *in vitro* [42]. Further, auxilin HPD has no ability to uncoat CCVs but does prevent wild-type auxilin from promoting uncoating. Thus this mutant form of auxilin acts as a dominant-negative inhibitor of the auxilin-catalysed uncoating of CCVs, allowing its use as a probe of auxilin function during SV trafficking.

When microinjected into the squid giant pre-synaptic terminal, auxilin HPD produced several alterations consistent with impairment of uncoating of CCVs [42]. First, auxilin HPD, but not wild-type auxilin, inhibited neurotransmitter release evoked by pre-synaptic action potentials. This effect was concentration-dependent and was similar to that observed when clathrin assembly was inhibited by clathrin binding-site peptides (Figure 1A), consistent with impairment of the endocytic pathway. However, auxilin HPD had very distinct effects on pre-synaptic ultrastructure. In particular, this inhibitor produced a 6-fold increase in the number of CCVs within the pre-synaptic terminal, as would be expected from blockade of vesicle uncoating. We therefore conclude that auxilin-dependent uncoating of CCVs is also important for SV endocytosis.

Conclusions

A major goal of our work at the squid giant synapse was to determine the significance of the clathrin pathway for endocytosis during physiological rates of synaptic activity. In order to address this question, we developed a collection of reagents that perturb different steps of the clathrin pathway and studied their effects on synaptic transmission at the squid giant synapse. We found that reagents that disrupt clathrin assembly *in vitro* also disrupt synaptic transmission and SV endocytosis *in vivo*, even under conditions of 'low synaptic activity' (0.03 Hz) [40,41]. Analysis of the disrupted terminals revealed a massive depletion of SVs and CCVs, and a concomitant increase in plasma membrane area, indicating that endocytosis was inhibited at the step of CCV formation at the plasma membrane [40,41]. We also found that reagents that perturb CCV uncoating *in vitro* disrupt synaptic transmission and SV recycling under conditions of low-frequency stimulation [42]. Analysis of these disrupted terminals revealed a 6-fold increase in the number of CCVs, indicating

that SV endocytosis was inhibited at the uncoating step. We conclude that the clathrin pathway is important for SV recycling even when demand for SVs is low.

These studies also allowed us to learn more about the molecular mechanisms of the clathrin coating and uncoating reactions. We discovered that all of the clathrin assembly proteins contain multiple copies of a degenerate clathrin-binding element and that these proteins promote clathrin assembly by a multivalent cross-linking mechanism [40]. We also determined that interactions between a well-conserved HPD motif in the J domain of auxilin and Hsc70 are critical for recruiting Hsc70 to CCVs and promoting the uncoating reaction [42]. Therefore this approach of acutely perturbing specific protein–protein interactions in a physiological preparation has been very fruitful both for evaluating the physiological significance of a particular protein–protein interaction as well as elucidating the precise molecular mechanisms underlying endocytotic reactions in the nerve terminal. Taken together, the case is now very strong that clathrin-mediated endocytosis is a major pathway for SV endocytosis under physiological conditions.

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Abbreviations used

AP	adaptor protein
AP2 pep	binding site peptide that mimics the DLL-containing region of AP-2
CCV	clathrin-coated vesicle
HRP	horseradish peroxidase
Hsc70	heat-shock cognate 70
PSP	post-synaptic potential
SV	synaptic vesicle

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Figure 1. Role of clathrin neurotransmitter release

(A) AP2 pep inhibits assembly of clathrin by AP-2 *in vitro*. A mutant form of this peptide, AP2 pep Δ DLL, is much less effective. (B) Microinjection of AP2 pep (time indicated by the bar) into the squid giant pre-synaptic terminal reversibly inhibits neurotransmitter release. Transmitter release was assessed as the slope of PSPs evoked by pre-synaptic action potentials. (C) Correlation between the ability of several peptides to inhibit clathrin assembly *in vitro* (abscissa) and neurotransmitter release at the squid synapse (ordinate). Taken from Morgan et al. [40] with permission. © 2000 Society for Neuroscience.

Figure 2. AP2 pep inhibits SV endocytosis

(A) Ultrastructure of squid giant pre-synaptic terminals microinjected with AP2 pep (right panel) and the control, mutant AP2 pep DLL (left panel). (B) The total amount of pre-synaptic membrane was conserved following microinjection of AP2 pep, with the decrease in membrane in SVs offset by an increase in the surface area of the pre-synaptic plasma membrane (PM). These changes indicate that AP2 pep inhibits endocytosis. Taken from Morgan et al. [40] with permission. © 2000 Society for Neuroscience.

Figure 3. AP2 pep inhibits membrane fission during endocytosis

(A) Under control conditions, a brief train of pre-synaptic action potentials (20 Hz, 15 s, represented by the bar beneath the x -axis) elicits a rise in membrane capacitance (C_m) due to exocytosis. This is followed by recovery of the capacitance as endocytosis retrieves vesicular membrane from the plasma membrane. (B) Following microinjection of AP2 pep, recovery of the capacitance signal is eliminated, indicating a block of membrane fission during endocytosis.