SYMPOSIUM REPORT

Activity-dependent control of bulk endocytosis by protein dephosphorylation in central nerve terminals

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Bulk endocytosis is the process by which nerve terminals retrieve large amounts of synaptic vesicle membrane during periods of strong stimulation intensity. The process is rapidly activated and is most probably calcium dependent in a similar manner to synaptic vesicle exocytosis. This article briefly summarizes the current knowledge of bulk endocytosis with respect to its activation, kinetics and molecular mechanism. It also presents recent data from our laboratory showing that the dephosphorylation of a group of endocytosis proteins called the dephosphins by the Ca^{2+}-dependent protein phosphatase calcineurin is key to the activity-dependent stimulation of the process. Possible downstream effectors of calcineurin are discussed such as the large GTPase dynamin I and its phosphorylation-dependent interaction partner syndapin I.

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Multiple synaptic vesicle retrieval pathways in central nerve terminals

The retrieval of synaptic vesicle (SV) membrane after exocytosis is essential for the maintenance of synaptic transmission in central nervous system synapses. Multiple routes of membrane retrieval have been described (Fig. 1), the best characterized being clathrin-dependent endocytosis (Murthy & De Camilli, 2003; Royle & Lagnado, 2003; Conner & Schmid, 2003), the most controversial being 'kiss-and-run' (Harata *et al.* 2006) and the most enigmatic being 'bulk' endocytosis (Royle & Lagnado, 2003). Bulk endocytosis differs from the other two modes of membrane retrieval in that it retrieves membrane for greater than one vesicle (the other two retrieve single SVs). It does so by invaginating large areas of presynaptic membrane from which SVs can be generated over time. These large endosomes can remain attached to the plasma membrane for a considerable length of time, while constantly allowing SVs to bud from them (Takei *et al.* 1996; Gad *et al.* 1998).

Bulk endocytosis has been reported in various different neuronal systems, including classical small central nerve terminals (Takei *et al.* 1996; Marxen *et al.* 1999; Leenders *et al.* 2002), atypical large central nerve terminals such as retinal biopolar neurones (Holt *et al.* 2003; Paillart *et al.* 2003) and the calyx of Held (de Lange *et al.* 2003), frog and snake neuromuscular junctions (Richards *et al.* 2000, 2004; Teng & Wilkinson, 2000; Teng *et al.* 2007) and lamprey reticulospinal synapses (Gad *et al.* 1998). In almost all instances bulk endocytosis is activated by strong stimulation protocols, which lead to the suggestion that it is purely an emergency mechanism that controls nerve terminal surface area during times of intense activity (Royle & Lagnado, 2003). However it is of key importance to understand this recycling pathway, since bulk endocytosis will also be activated during physiological processes such as long-term potentiation and synaptic integration and also during pathological processes such as epiliptogenic burst firing. Thus bulk endocytosis will contribute to the ability of the nerve terminal to respond to intense stimuli and it is vital that this mechanism is understood.

Properties of bulk endocytosis

Bulk endocytosis was initially thought to be a slow process, since endosomal intermediates have been shown to remain attached to the plasma membrane for long periods after stimulation (Takei *et al.* 1996; Gad *et al.* 1998). However the formation of these endocytic vacuoles is very fast, with bulk endosomes observed within 1–2 s of strong nerve terminal stimulation (Marxen *et al.* 1999; Leenders *et al.* 2002; Teng *et al.* 2007). This suggests that bulk endocytosis

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is a triggered event, most likely to be activated by the same Ca^{2+} stimulus that evokes SV exocytosis. Bulk endocytosis has been previously suggested to be activated by the accumulation of SV membrane in the plasma membrane; however, the speed of bulk endosome formation argues against this. Thus bulk endocytosis is rapidly triggered and possibly activated by Ca^{2+} influx, suggesting a requirement for a Ca^{2+} sensor in the process.

Bulk endocytosis was first demonstrated in central nerve terminals using electron microscopy (Takei *et al.* 1996), and this has remained an important tool in observing the process. However, these studies are labour intensive and do not provide real time information on the process. Since the turn of the decade, bulk endocytosis has also been able to be visualized using fluorescent methods, opening up new possibilities to investigate its activation and molecular mechanism. The first studies were performed on the frog neuromuscular junction, where it was shown that the lipid-binding styryl dye FM1-43 could selectively label bulk endocytosis when compared with its more hydrophilic counterpart FM2-10 (Richards *et al.* 2000). The proposed reason for this disproportionate labelling was that FM2-10 was washed out of the bulk endosomes that were still connected to the plasma membrane, whereas the more hydrophobic FM1-43 could not be removed. The selective labelling of bulk endosomes was confirmed by photoconversion of the dyes and examination of nerve terminals with electron microscopy (Richards *et al.* 2000). In addition to styryl dyes, large molecular weight dextrans tagged with fluorescent molecules have also been used to demonstrate the presence and kinetics of bulk endocytosis in motor nerve terminals (Holt *et al.* 2003; Teng *et al.* 2007). These fluid phase markers are too large to be accumulated inside single SVs, and therefore they selectively label the bulk endocytosis pathway.

These flourescent studies have allowed the traffic of SVs generated by bulk endocytosis to be followed in real time. It transpires that the primary route for these SVs is to replenish the reserve pool of SVs. This conclusion was first drawn in the frog neuromuscular junction, since SVs labelled by FM1-43 during strong stimulation could not immediately undergo exocytosis, whereas those SVs labelled with FM2-10 could (Richards *et al.* 2000). The FM1-43-labelled SVs could eventually be released, but only after a delay of approximately 10–15 min. The most obvious explanation for this time lag is that this is the time required to generate new SVs from bulk endosomes. Recently our group has shown that SVs derived from bulk endocytosis also replenish the reserve SV pool in central nerve terminals (Evans & Cousin, 2007). In these studies, a sustained component of exocytosis was observed that was attributable to the reserve pool when FM1-43 was loaded into SVs using a strong stimulus, but was absent when FM2-10 was loaded under identical conditions. Thus SVs generated by bulk endocytosis are unable to be immediately used and rejoin the SV recycling pool in the reserve pool.

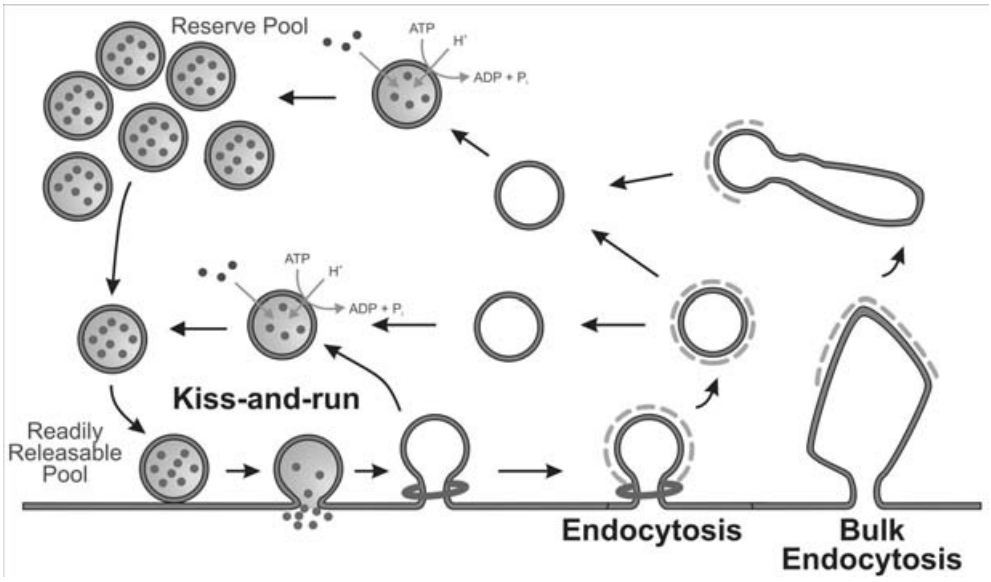

Figure 1. Multiple synaptic vesicle retrieval pathways in central nerve terminals
Three different mechanisms are propsed to retrieve synaptic vesicle (SV) membrane after exocytosis in nerve terminals. Kiss-and-run is a mechanism where the SV never fully fuses with the plasma membrane and retrieves intact. Classical clathrin-dependent endocytosis involves the invagination of a single clathrin-coated bud from the plasma membrane before its fission and uncoating. Bulk endocytosis is the process where large areas of nerve terminal membrane are invaginated to produce endosomes from which SVs can bud.

Since the kinetics of bulk endocytosis and fate of SVs derived from this pathway are now known, it is perhaps surprising that very little is understood about the molecular mechanism of the process itself. While it is thought that clathrin-dependent endocytosis is responsible for SV budding from bulk endosomes (Takei *et al.* 1996), the molecules that activate and mediate the invagination and fission of the membrane are relatively unknown. Bulk endocytosis has been linked to the process of macropinocytosis in non-neuronal cells where membrane protrusions gather large amounts of the fluid phase in a 'cell drinking' mechanism (Holt *et al.* 2003; Teng *et al.* 2007). Macropinocytosis is dependent on the activation of Rho family GTPases which stimulate the actin-driven formation of these protrusions (Conner & Schmid, 2003). Interestingly in both the frog neuromuscular junction and in retinal bipolar neurones, disruption of actin function with pharmacological agents resulted in an inhibition of bulk endocytosis (Holt *et al.* 2003; Richards *et al.* 2004). In addition, bulk endocytosis was also retarded by inhibition of phosphatidylinositol 3-kinase activity (Holt *et al.* 2003; Richards *et al.* 2004), suggesting a link between this signalling cascade and the actin dynamics required for the process. Thus a requirement for some molecules in bulk endocytosis has been identified, with actin dynamics and rearrangement a major factor in the process.

The dephosphins are activity-dependent triggers for bulk endocytosis

Bulk endocytosis is activated by strong stimulation and is likely to be immediately triggered by the same Ca^{2+} stimulus that activates the exocytosis machinery. Thus the Ca^{2+} sensor for bulk endocytosis must be a Ca^{2+}-binding protein which activates certain endocytosis proteins during strong, but not mild, nerve terminal stimulation. One protein that fulfils all of these criteria is the Ca^{2+}-dependent protein phosphatase calcineurin. Calcineurin is activated by Ca^{2+} influx in nerve terminals and dephosphorylates the set of endocytosis proteins called the dephosphins (Cousin & Robinson, 2001). The dephosphins are grouped together by their ability to be dephosphorylated by calcineurin on stimulation, and by the fact that they are all essential for SV endocytosis. The dephosphins are; the large GTPase dynamin I, the adaptor protein AP180, and the accessory proteins amphiphysin I/II, synaptojanin, epsin, esp15 and phosphatidylinositol phosphate kinase type Iγ (PIPKIγ). After their stimulus-dependent dephosphorylation, the dephosphins are rephosphorylated by their respective protein kinases, such as cyclin-dependent kinase 5 (cdk5), which rephosphorylates dynamin I, synaptojanin and PIPKIγ *in vivo* (Tan *et al.* 2003; Lee *et al.* 2005).

We have identified both the dephosphorylation of the dephosphins and their subsequent rephosphorylation as essential events in bulk endocytosis (Evans & Cousin, 2007). The evidence for this is as follows: (i) inhibition of either calcineurin or cdk5 by either pharmacological antagonists or overexpression of dominant negative constructs in primary neuronal culture arrested the uptake of FM1-43 but not FM2-10 during strong stimulation; (ii) inhibition of cdk5 blocked the uptake of the fluid-phase marker horseradish peroxidase (HRP) into plasma-membrane-generated endosomes, but not single SVs, during strong stimulation; (iii) inhibition of cdk5 had no effect on either FM1-43 loading or HRP labelling if nerve terminals were challenged with a mild stimulus; and finally (iv) the sustained component of exocytosis observed when SVs were labelled with FM1-43 was abolished when either calcineurin or cdk5 activity was inhibited during the dye-loading phase (Evans & Cousin, 2007).

These findings place some or all of the dephosphins as the key mediators of bulk endocytosis, with their dephosphorylation by calcineurin as the activity-dependent trigger for the process. There is ample evidence that calcineurin can fulfil the temporal requirements of this role, since the dephosphorylation by calcineurin of the dephosphins is synchronous and rapid (<1 s; Robinson *et al.* 1994). Furthermore the intracellular free Ca^{2+} increase required to maximally dephosphorylate the dephosphins in nerve terminals (approximately 1 μM; Sihra *et al.* 1992) correlates well with the predicted increase in intracelluar free Ca^{2+} during strong stimulation. However, the key test as to whether calcineurin is the activity-dependent Ca^{2+} sensor for bulk endocytosis is whether it is able to dephosphorylate its substrates only at stimulation frequencies at which bulk endocytosis is observed. Unpublished experiments recently performed in our laboratory confirm that this is the case. In these experiments no dephosphorylation of the calcineurin substrate dynamin I was observed at mild stimulation frequencies, but a robust dephosphorylation was seen with increasing stimulation frequency in our neuronal cultures. Thus we propose that the activity-dependent dephosphorylation of the dephosphins is the trigger for bulk endocytosis, and that some if not all of the dephosphins are involved in the process.

Perspectives

The identification of calcineurin as the activity-dependent trigger for bulk endocytosis raises some provocative concepts. For example, it suggests that some if not all of the dephosphins perform roles in both clathrin-dependent and bulk endocytosis. This should not be too surprising since a molecule such as dynamin I will

be required for membrane fission in both processes. Dynamin I already has an identified phosphorylation-dependent binding partner, which is the endocytosis protein syndapin (Anggono *et al.* 2006). Dynamin I also has a phosphorylation-independent interaction with amphiphysin (Tan *et al.* 2003; Anggono *et al.* 2006) and this interaction is essential for clathrin-dependent endocytosis (Grabs *et al.* 1997; Wigge *et al.* 1997). Thus dynamin I has two major interactions: one which is phosphorylation independent (i.e. will occur regardless of stimulation intensity) and is essential for clathrin-dependent endocytosis (amphiphysin), and the other which is phosphorylation dependent (i.e. will occur only during strong stimulation) and is therefore implicated in bulk endocytosis (syndapin). Syndapin is an excellent candidate for a bulk endocytosis effector since is it heavily linked to the control of actin polymerization through its well characterized interaction with nucleating protein N-WASP (Kessels & Qualmann, 2002). Since actin dynamics are essential for bulk endocytosis, this places the dynamin–syndapin interaction as the key event in the process. In addition, the central region of syndapin has a series of NPF amino acid repeats that interact with a family of proteins that mediate budding from endosomes (Braun *et al.* 2005), possibly indicating a role in SV budding from bulk endosomes. Finally syndapin has an F-BAR domain at its N terminus (Itoh *et al.* 2005; Tsujita *et al.* 2006), and this is similar to the N-BAR domain found on both amphiphysin and endophilin (Peter *et al.* 2004). This domain can bind and tubulate lipid both *in vitro* and *in vivo* and may facilitate the invagination and possibly fission of the bulk endosome. Therefore, the phosphorylation-dependent recruitment of syndapin by the activity-dependent dephosphorylation of dynamin I may be the key molecular event in bulk endocytosis, a process which is central to nerve terminal function.

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