Developmental change in the calcium sensor for synaptic vesicle endocytosis in central nerve terminals

Karen J. Smillie, Gareth J. O. Evans and Michael A. Cousin

Membrane Biology Group, Division of Biomedical and Clinical Laboratory Sciences, University of Edinburgh, Edinburgh, UK

Abstract
Synaptic vesicle endocytosis is stimulated by calcium influx in mature central nerve terminals via activation of the calcium-dependent protein phosphatase, calcineurin. However, in different neuronal preparations calcineurin activity is either inhibitory, stimulatory or irrelevant to the process. We addressed this inconsistency by investigating the requirement for calcineurin activity in synaptic vesicle endocytosis during development, using vesicle recycling assays in isolated nerve terminals. We show that endocytosis occurs independently of calcineurin activity in immature nerve terminals, and that a calcineurin requirement develops 2–4 weeks after birth. Calcineurin-independent endocytosis is not due to the absence of calcineurin activity, since calcineurin is present in immature nerve terminals and its substrate, dynamin I, is dephosphorylated on depolarization. Calcineurin-independent endocytosis is calcium-dependent, since substitution of the divalent cation, barium, inhibits the process. Finally, we demonstrated that in primary neuronal cultures derived from neonatal rats, endocytosis that was initially calcineurin-independent developed a calcineurin requirement on maturation in culture. Our data account for the apparent inconsistencies regarding the role of calcineurin in synaptic vesicle endocytosis, and we propose that an unidentified calcium sensor exists to couple calcium influx to endocytosis in immature nerve terminals.

Keywords: calcineurin, calcium, endocytosis, FM2-10, nerve terminal, synaptic vesicle.


Endocytosis of synaptic vesicles (SVs) is essential for the maintenance of neurotransmission in central nerve terminals. This is because endocytosis retrieves SVs from the neuronal plasma membrane, allowing them to become available for exocytosis after their subsequent recycling and refilling with neurotransmitter. To ensure the efficiency of this retrieval process, SV exocytosis and endocytosis are closely coupled both temporally and spatially in nerve terminals. This high degree of coupling originates from the influx of extracellular calcium (Ca\(^{2+}\)) into nerve terminals on their depolarization (Cousin 2000). The vast majority of this Ca\(^{2+}\) influx occurs at active zones. Active zones have a high density of voltage-dependent Ca\(^{2+}\) channels and on nerve terminal depolarization, intracellular free Ca\(^{2+}\) levels can reach as high as 1 mM (Lin and Scheller 2000). Such high levels are required since the proposed Ca\(^{2+}\) sensor for exocytosis, synaptotagmin, has a very low affinity for Ca\(^{2+}\) (Yoshihara et al. 2003). After SV fusion with the plasma membrane, the same Ca\(^{2+}\) influx stimulates SV endocytosis. SV endocytosis occurs in regions surrounding the active zone called ‘endocytosis zones’. However, the levels of Ca\(^{2+}\) required to stimulate the process are approximately 100-fold lower (Cousin 2000). The Ca\(^{2+}\) sensor for SV endocytosis in mature central nerve terminals is the Ca\(^{2+}\)-dependent protein phosphatase calcineurin (CaN) (Marks and McMahon 1998; Cousin and Robinson 2001; Cousin et al. 2001).

CaN was identified as the Ca\(^{2+}\) sensor in isolated central nerve terminals (synaptosomes) using fluorescent assays of SV recycling (Marks and McMahon 1998; Cousin et al. 2001). However, in other neuronal preparations, the CaN dependence of SV endocytosis differs dramatically. For example, in primary neuronal cultures no requirement for CaN in SV endocytosis has ever been demonstrated, even though the process is Ca\(^{2+}\)-dependent (Sankaranarayanan and Ryan 2001). Furthermore, at neuromuscular junctions in Drosophila larvae, CaN inhibits rather than stimulates SV endocytosis (Kuromi et al. 1997; Kuromi and Kidokoro

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Address correspondence and reprint requests to Michael A. Cousin, Membrane Biology Group, Division of Biomedical and Clinical Laboratory Sciences, George Square, University of Edinburgh, Edinburgh, UK, EH8 9XD. E-mail: M.Cousin@ed.ac.uk

Abbreviations used: Ba\(^{2+}\), barium; Ca\(^{2+}\), calcium; CaM, calmodulin; CaN, calcineurin; CGN, cerebellar granule neurone; CsA, cyclosporin A; SV, synaptic vesicle.
Ca2+ influx couples to a different Ca2+ sensor to stimulate SV endocytosis between neuronal preparations could result from a change in the coupling of Ca2+ influx to the endocytosis sensor during development. We used a system that has an essential requirement for CaN activity (synaptosomes) and tested whether this was present throughout development, using fluorescent assays of SV recycling. We show that CaN activity only becomes essential for SV endocytosis between the ages of 2 and 4 weeks after birth. The same developmental pattern was also observed in primary neuronal culture. Our study highlights the fact that Ca2+ influx couples to a different Ca2+ sensor to stimulate SV endocytosis in immature and adult animals.

Materials and methods

Materials
Cyclosporin A and cypermethrin were from CN Biosciences (Nottingham, UK). FM2-10 was from Invitrogen (Paisley, UK). Dynamin I antibody (C-16) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Calcineurin antibody was from BD Biosciences (Oxford, UK). Coverslips were from Raymond Lamb (Eastbourne, UK). All tissue culture plastics were obtained from either Falcon (Plymouth, UK) or Greiner (Dursley, UK). Penicillin/streptomycin, phosphate-buffered salts, foetal calf serum and Minimal Essential Medium were obtained from Invitrogen (Paisley, UK). Dynamin I antibody (C-16) was from Santa Cruz Biotechnology (Nottingham, UK). FM2-10 was from Invitrogen (Paisley, UK). Cyclosporin A and cypermethrin were from CN Biosciences (Oxford, UK).

Synaptosomes
FM2-10 exocytosis and endocytosis assays
Synaptosomes were prepared from rat cerebral cortex by centrifugation on discontinuous Percoll gradients (Dunkley et al. 1986). SV exocytosis was measured using the loading and unloading of FM2-10, as previously described (Cousin and Robinson 2000). Briefly, synaptosomes (0.6 mg protein in 2 mL) were incubated for 5 min at 37°C in plus Ca2+ Krebs-like solution (118.5 mM NaCl, 4.7 mM KCl, 1.18 mM MgCl2, 0.1 mM KH2PO4, 20 mM HEPES (N-tris(hydroxy-methyl)-methyl-2-aminoethane-sulfonic acid), 5 mM NaHCO3, 5 mM glucose, 1.2 mM Na2SO4, 1.2 mM MgCl2, 1.3 mM CaCl2, pH 7.4). The total recycling pool of SVs was loaded with FM2-10 (100 μM) by evoking SV recycling with 50 mM KCl (50 mM NaCl removed to maintain osmolarity) for 2 min. This corresponds to S1 loading (Fig. 5a). CGNs were washed and after 15 min, FM2-10 was unloaded by two sequential 30 s KCl challenges at 20 s and 120 s (S1 unloading, Fig. 5a). This S1 protocol provides an estimate of the total number of SVs turned over during KCl stimulation. This was repeated for CGNs pre-incubated with 40 μM CsA for 15 min before and during S2 loading (for SV endocytosis) or S2 unloading (for SV exocytosis). FM2-10 unloading was quantified by monitoring the total decrease in fluorescence after normalizing fluorescence in individual nerve terminals to an arbitrary value. FM2-10 unloading was visualized using a Nikon (Kingston-Upon-Thames, UK) epifluorescence microscope (Diaphot-TMD) and ×20 objective at 480 nm excitation and > 510 nm emission. Fluorescent images were visualized using a Hamamatsu Orca-ER CCD digital camera (Welwyn Garden City, UK) and offline imaging software (Compix Imaging Systems, PA, USA). Data from at least three independent experiments, each containing at least 70 nerve terminals, were collated and presented as the ratio of the total unloading of FM2-10 at S2 and S1, respectively (S2/S1).

Western blotting
The amount of dynamin I, calcineurin or phosphorylated dynamin I in synaptosomes was monitored by western blotting using antibodies
directed against either dynamin I, calcineurin, or phospho-serine-774 and phospho-serine-778 [both on dynamin I (Tan et al. 2003)]. Blots were scanned and analysed using GeneSnap and GeneTools ( SynGene, Cambridge, UK) software.

Results

SV endocytosis is CaN-dependent in adult, but not young animals

SV endocytosis in mature central nerve terminals is dependent upon CaN activity (Marks and McMahon 1998; Cousin et al. 2001). To determine whether CaN activity is also essential for SV endocytosis in immature nerve terminals, we performed exocytosis and endocytosis assays using the styryl dye FM2-10 (Cousin and Robinson 2000). Figure 1a displays the protocol for monitoring the effect of the CaN antagonist, CsA, on both SV exocytosis and endocytosis. When CsA was applied to adult synaptosomes that received a standard load of FM2-10, it had no significant effect on KCl-evoked unloading and thus, exocytosis (Fig. 1b). This confirms previous studies where CsA had no effect on KCl-evoked Ca\(^{2+}\)-dependent glutamate release in adult nerve terminals (Nichols et al. 1994; Marks and McMahon 1998; Cousin et al. 2001; Baldwin et al. 2003). When CsA or a different CaN antagonist, cypermethrin, was applied prior to FM2-10 loading, a significant inhibition of KCl-evoked unloading was observed (Fig. 1c and data not shown). Previous studies have confirmed that this inhibition is a direct result of a block in SV endocytosis and not subsequent recycling (Marks and McMahon 1998). Thus, CsA inhibits the accumulation of FM2-10 by endocytosis, since exocytosis is unaffected by the drug.

To determine whether CaN was essential for SV endocytosis through development, we repeated these assays using synaptosomes derived from 2-week-old animals. CsA produced a small, equivalent and significant inhibition of both FM2-10 unloading and loading in these immature nerve terminals (Figs 1d and 1e). Cypermethrin also had a similar effect on FM2-10 loading in immature nerve terminals (data not shown). This small inhibition of FM2-10 loading in the endocytosis assay was a direct result of an upstream inhibition of SV exocytosis and not SV endocytosis, since the CsA inhibited FM2-10 unloading (SV exocytosis) to the same extent. CsA had similar effects on Ca\(^{2+}\)-dependent glutamate release from immature synaptosomes (data not shown). Since SV endocytosis is dependent on the prior amount of exocytosis, the decrease in the number of SVs loaded with FM2-10 was due to a decrease in availability of SVs to retrieve. Thus, at 2 weeks, SV endocytosis is independent of CaN activity.

To investigate when a requirement for CaN activity develops, we examined the effect of CsA on SV exocytosis and endocytosis in a range of animal ages (Fig. 2a). To obtain an accurate estimate of SV endocytosis, we used the retrieval efficiency calculation (endocytosis/exocytosis; Cousin and Robinson 1998), since the amount of SV endocytosis is dependent on the prior amount of exocytosis. We...
determined that SV endocytosis becomes CaN-dependent 4 weeks after birth (Fig. 2b). By 16 weeks, the requirement for CaN starts to plateau until maturity is reached, where almost half of all SV endocytosis is dependent on CaN activity. Thus, immature nerve terminals do not require CaN activity for SV endocytosis, and this requirement develops with age.

**SV endocytosis in young animals is Ca\(^{2+}\)-dependent**

One reason for the lack of CaN dependence of endocytosis in immature nerve terminals could be that the enzyme is either not present or not active. To test this possibility we probed lysates from both immature and mature nerve terminals for the presence of CaN. Figure 3 shows that there is no difference in the amount of CaN present between immature and mature nerve terminals. To test whether the CaN present in immature nerve terminals is active, we examined the phosphorylation status of one of its substrates, dynamin I. Dynamin I is co-ordinately dephosphorylated by CaN in mature nerve terminals at two sites on nerve terminal depolarization (Tan et al. 2003; Larsen et al. 2004). When probed with phospho-specific antibodies directed against these sites, dynamin I was dephosphorylated on both during depolarization of immature nerve terminals, and this dephosphorylation was abolished by CsA and cypermethrin (Fig. 3, data not shown). Thus, CaN is present and active in immature nerve terminals.

SV endocytosis may not require CaN activity in immature nerve terminals because the process may not be Ca\(^{2+}\)-dependent at this stage of development. To test this possibility we stimulated SV recycling with the divalent cation, barium (Ba\(^{2+}\)). Ba\(^{2+}\) inhibits a presynaptic voltage-sensitive K\(^+\) channel, inducing transient plasma membrane depolarizations similar to action potentials (Cousin and Robinson 1998; Marks and McMahon 1998). It also stimulates SV exocytosis by entering nerve terminals through voltage-dependent Ca\(^{2+}\) channels and substituting for Ca\(^{2+}\). However, Ba\(^{2+}\) does not support SV endocytosis in mature nerve terminals, due to its inability to activate calmodulin (CaM)/CaN (Cousin and Robinson 1998; Marks and McMahon 1998). Ca\(^{2+}\) cannot inhibit K\(^+\) channels and is therefore unable to evoke SV exocytosis independently. We assayed Ba\(^{2+}\)-evoked release of endogenous glutamate.
Ba\(^{2+}\) has complex effects on FM2-10 unloading (data not shown). Ba\(^{2+}\) efficiently stimulated glutamate release (Fig. 4a) but could not evoke SV endocytosis in immature nerve terminals (Fig. 4b). Thus, SV endocytosis in immature nerve terminals is robustly Ca\(^{2+}\)-dependent, suggesting that a different Ca\(^{2+}\) sensor must activate the process.

SV endocytosis develops CaN-dependency with age in primary neuronal cultures

To demonstrate the developing requirement for CaN in SV endocytosis, we used primary cultures of CGNs. Primary neuronal cultures are usually derived from embryonic or neonatal tissue, possibly explaining why a requirement for CaN in SV endocytosis has never been reported. We would predict that SV endocytosis would be initially CaN-independent and then develop a requirement for CaN on maturation in culture. We devised a protocol to test this hypothesis (Fig. 5a). CGNs are loaded with FM2-10 using KCl stimulation and the accumulated dye is then unloaded with two KCl challenges (S1). The same nerve terminals are loaded with CsA present either before (endocytosis) or after (exocytosis) the S2 loading stimulus. Inhibition of exocytosis or endocytosis by CsA is estimated as the ratio of total FM2-10 unloaded at S2 to that at S1.

Figure 5(b–d) shows a CGN field after S1 loading (Fig. 5b) and S2 loading (Fig. 5c) and unloading data from a typical control experiment (Fig. 5d). Note that the total amount of unloading for both S1 and S2 are equal, reflecting the reproducibility of loading and unloading. CsA had no effect on the S2/S1 ratio for either SV exocytosis or endocytosis in CGNs after 10 days in vitro (Fig. 5e). Thus, in immature CGN cultures both SV exocytosis and endocytosis is CaN-independent. To determine if a requirement for CaN in SV endocytosis develops with age, we performed the same protocol using CGNs older than 30 days in vitro. A robust and reproducible inhibition of SV endocytosis by CsA was observed, with no effect on SV exocytosis (Fig. 5e). Thus, in both primary neuronal cultures and acutely isolated nerve terminals a requirement for CaN activity in SV endocytosis develops on nerve terminal maturation.

Discussion

Ca\(^{2+}\) influx stimulates both SV exocytosis and endocytosis in mature nerve terminals (Cousin and Robinson 1998; Marks and McMahon 1998; Cousin 2000). The protein phosphatase CaN is activated by this influx and the subsequent dephosphorylation of a group of proteins,
called the dephosphins, is essential for SV endocytosis (Cousin and Robinson 2001). We demonstrate that the requirement for CaN activity develops 2–4 weeks after birth using nerve terminals prepared from both immature and adult animals. The lack of CaN dependence of SV endocytosis in immature nerve terminals is not explained by an absence or inactivation of CaN, since dynamin I is still dephosphorylated on nerve terminal depolarization. Likewise, it is not because of a lack of Ca\(^{2+}\) dependency, since SV endocytosis is abolished by substituting the divalent cation Ba\(^{2+}\) for Ca\(^{2+}\) (Chan and Robinson 1998; Marks and McMahon 1998). Finally, we have shown that SV endocytosis is CaN-dependent in mature, but not immature primary neuronal cultures.

**A developmental change in the CaN dependence of SV endocytosis**

A developmental requirement for CaN activity in SV endocytosis explains the apparent contradictions in the published literature. The only other experimental system to demonstrate an obligatory requirement for CaN activity is the bovine chromaffin cell (Chan and Smith 2001; Chan et al. 2003). Two forms of endocytosis were observed in cells derived from adult animals during stimulation with action potential waveforms: a CaN-independent form that predominated at basal frequencies (termed type I endocytosis) and a CaN-dependent form that was activated by higher firing frequencies (termed type II endocytosis) (Chan and Smith 2001). In a different study using adult chromaffin cells, CaN antagonists abolished the Ca\(^{2+}\)-dependency of endocytosis evoked by square waveform depolarizations (termed compensatory retrieval) (Engisch and Nowycky 1996). This loss of Ca\(^{2+}\)-dependency was explained by a selective inhibition of type II endocytosis, since compensatory retrieval is likely to be a mixture of both type I and type II retrieval (Chan and Smith 2001). In contrast, chromaffin cells derived from immature animals have no requirement for CaN. Various approaches to disrupt CaN function all resulted in an acceleration of a rapid and excess retrieval of membrane that was stimulated by high intensity square waveform pulses (Artalejo et al. 1996). This suggests that CaN activity may inhibit endocytosis in younger animals. A similar inhibitory role for CaN is apparent at the neuromuscular junction of *Drosophila* larvae, where CaN antagonists greatly increase FM1-43 uptake into nerve terminals (Kuromi et al. 1997; Kuromi and Kidokoro 1999). This form of SV endocytosis was strictly Ca\(^{2+}\)-dependent, as was the excess retrieval pathway reported in calf chromaffin cells (Artalejo et al. 1996). When these results are considered with our data it appears that SV endocytosis is Ca\(^{2+}\)-dependent at all ages of animal, from pre to postnatal. However, during development the requirement for CaN changes from a putative inhibitory role in immature nerve terminals to a stimulatory role in mature nerve terminals.

**An unidentified Ca\(^{2+}\) sensor for SV endocytosis in immature nerve terminals**

We suggest that the lack of CaN dependency in immature nerve terminals is due to a switch in the Ca\(^{2+}\) coupling of SV endocytosis during development. In other words, Ca\(^{2+}\) influx is still required to stimulate SV endocytosis, but the sensor that transduces its effect is different. A requirement for this unidentified sensor may continue into adulthood, since CaN antagonists do not always abolish SV endocytosis (Cousin et al. 2001, but see Marks and McMahon 1998). One possibility is that Ca\(^{2+}\) influx controls essential interactions between endocytosis proteins, such as that observed for dynamin I with both endophilin and syntrophin (Daly and Ziff 2002; Chen et al. 2003). Another is that the Ca\(^{2+}\) binding protein CaM, is the Ca\(^{2+}\) sensor, since excess membrane retrieval in calf adrenal chromaffin cells has an absolute requirement for its activity (Artalejo et al. 1996). Possible downstream targets are still unclear. However, CaM may act via the release of a Ca\(^{2+}\)-dependent binding partner such as GAP-43, rather than the activation of a Ca\(^{2+}\)-dependent target. In cortical neurones overexpression of GAP-43 mutants that constitutively bind CaM disrupted SV endocytosis, whereas GAP-43 mutants unable to bind CaM enhanced the process (Neve et al. 1998). Thus, the release of a Ca\(^{2+}\)-independent CaM-interacting protein may be a possible mechanism to control SV endocytosis. This has previously been observed in yeast, where the release of the Ca\(^{2+}\)-independent binding partner, unconventional myosin I (Geli et al. 1998), and the Ca\(^{2+}\)-dependent activation of the actin binding protein, Arp2/3, are both essential for endocytosis (Kubler et al. 1994; Schaefer-Brodbeck and Riezman 2000). Thus, CaM may play a dual role in the control of SV endocytosis by activating CaN and releasing a Ca\(^{2+}\)-independent binding partner.

We have uncovered a developmental switch in the Ca\(^{2+}\) coupling of SV endocytosis in central nerve terminals. In immature animals, SV endocytosis has no requirement for CaN activity. However, this requirement develops from 4 weeks after birth through to adulthood. Our data explain previous inconsistencies in the published data and indicate that a different Ca\(^{2+}\) sensor must stimulate the process in immature nerve terminals.

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References


