Calcium triggers calcineurin-dependent synaptic vesicle recycling in mammalian nerve terminals

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Background: Following exocytosis at the synapse, synaptic vesicle components are recovered by endocytosis. Morphological analysis has suggested that this occurs by a clathrin-mediated pathway, and the GTPase dynamin is thought to be involved in 'pinching off' endocytosing vesicles. The finding that the calcium-dependent phosphatase calcineurin can dephosphorylate dynamin and two other proteins implicated in endocytosis (amphiphysin and synaptojanin) has suggested a potential role for calcium and dephosphorylation in regulating synaptic vesicle endocytosis.

Results: We tested this hypothesis with an endocytosis assay in isolated nerve terminals (synaptosomes) that relies on the use of the fluorescent dye FM2-10. In synaptosomes, vesicle recycling occurs predominantly via a pathway dependent on both dynamin and amphiphysin. We found that endocytosis could be stimulated maximally at calcium concentrations that yielded only low levels of exocytosis, suggesting that the two processes had different calcium sensitivities and hence independent calcium sensors. Using the specific inhibitors cyclosporin A and FK506, we identified calcineurin as a calcium sensor for endocytosis and showed that its activity is essential for synaptic vesicle endocytosis in synaptosomes.

Conclusions: Our results suggest that dynamin-dependent synaptic vesicle endocytosis is triggered by calcium influx occurring upon nerve-terminal depolarisation. An essential mediator of calcium's effect is calcineurin, the activation of which leads to dephosphorylation of at least four proteins implicated in endocytosis – dynamin, amphiphysin 1, amphiphysin 2 and synaptojanin. Our findings also imply that endocytosis and exocytosis may occur in tandem *in vivo* simply because they share a responsiveness to calcium influx, rather than because they are mechanistically coupled.

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Background

Synaptic vesicle endocytosis is thought to occur by a clathrin-mediated mechanism [1]. Support for this hypothesis is given by morphological observations of clathrin-coated vesicle intermediates after nerve-terminal stimulation and also by the marked enrichment in nerve terminals of the molecular machinery for the clathrin-mediated pathway (including clathrin, adaptors and dynamin) [2,3]. It is likely that there are other synaptic vesicle endocytosis pathways in addition to the clathrin-mediated one, but their roles and relative significance are unclear [1].

The importance of endocytosis for the sustained activity of neurons is highlighted by the phenotype of *shibire*, a temperature-sensitive mutant of the GTPase dynamin in *Drosophila* [4,5]. In *Drosophila* bearing this mutation, there is an accumulation of invaginated vesicle intermediates that have not 'pinched off' from the plasma membrane and a rapid onset of paralysis at the non-permissive temperature. The electron-dense collars around the necks of these endocytosing vesicle intermediates resemble both dynamin rings formed *in vitro* [6] and the dynamin rings observed in isolated nerve terminals (synaptosomes) that have been lysed and treated with the non-hydrolysable GTP analogue GTPγS [7]. Thus, dynamin is likely to be a key protein in the fission stage of synaptic vesicle endocytosis. Amphiphysin, a major dynamin binding partner is proposed to be involved in dynamin recruitment to the necks of coated vesicles [8–11]. Amphiphysin is concentrated in nerve terminals and is present as a heterodimer of two isoforms, both of which bind to dynamin [12]. Disruption of the interaction between amphiphysin and dynamin in either the lamprey giant reticulospinal synapse [10] or in fibroblasts [11] leads to an inhibition of clathrin-mediated endocytosis, probably through a blockade of dynamin recruitment. Synaptojanin and endophilin (or SH3p4) have also been implicated in synaptic vesicle endocytosis because they are concentrated in nerve terminals and interact with

components of the endocytosis machinery [13–15]. Synaptojanin additionally has a phosphatidylinositol 5′ phosphatase activity that might modify lipids during endocytosis [13,16].

In response to nerve-terminal depolarisation, dynamin is dephosphorylated by the calcium–calmodulin-activated protein phosphatase calcineurin (also known as protein phosphatase 2B) [17]. This has led to the speculation that a phosphorylation cycle might regulate synaptic vesicle endocytosis [18]. In support of a role for calcineurin, this phosphatase is known to be enriched in nerve terminals where it colocalises with amphiphysin and indeed dephosphorylates amphiphysin and synaptojanin at the same time as it dephosphorylates dynamin [19]. On a broader scale, calcineurin activity in the brain is also implicated in synaptic plasticity, nerve regeneration and brain disease [20–22].

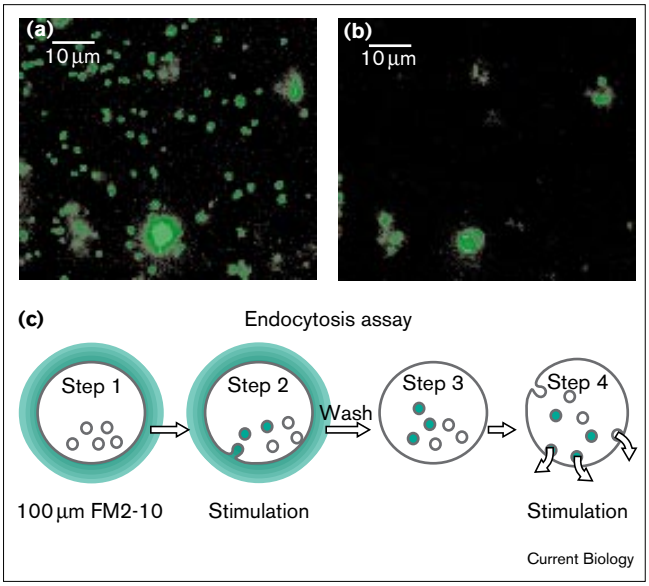
Exocytosis in the nerve terminal is stimulated by calcium entry through voltage-dependent calcium channels and involves synaptotagmin as a calcium sensor [23–25]. Is there also a calcium sensor for endocytosis in mammalian nerve terminals or is endocytosis merely driven by a preceding exocytosis event? If dephosphorylation by calcineurin is important for endocytosis, a dependence on calcium influx would be expected. Reports of calcium sensitivity are conflicting, however; they have suggested that endocytosis can either be activated or inhibited by calcium, or that endocytosis is calcium-independent [26–30]. This might be due to a variation in the calcium sensitivity of endocytosis in different cell types or preparations, reflecting differences in their endocytosis pathways. We set out to investigate synaptic vesicle endocytosis using a preparation of synaptosomes from rat brain cortices. In this preparation both exocytosis and endocytosis can be followed in parallel and we show that endocytosis occurs predominantly by a pathway that requires amphiphysin and dynamin interaction and that it is dependent on calcium and calcineurin.

Results

Using FM2-10 to follow endocytosis in synaptosomes from rat brain

FM dyes are highly fluorescent when inserted into lipid membranes but their fluorescence is negligible in aqueous solution (for review, see [31]). When synaptosomes were incubated with the dye FM2-10, it partitioned into the membrane. A depolarising stimulus that causes synaptic vesicle endocytosis resulted in dye-loaded vesicles being internalised (‘loading’). Subsequent exocytosis caused by a second depolarising stimulus released internalised dye and a decrease in fluorescence was observed (‘unloading’). Figure 1a,b shows confocal microscopy images of a synaptosome preparation after loading with FM2-10 and stimulation of vesicle cycling. Active synaptosomes fluoresced, due to the labelled vesicles that they contained, and could be visualised clearly after washing away the excess dye (Figure 1a). Exocytosis caused by the second depolarising stimulus resulted in the recycled synaptic vesicles releasing the trapped dye (Figure 1b), and consequently the fluorescence was largely lost as the dye rapidly diluted into the aqueous phase. The field shown in Figure 1a is a selected one that demonstrates the phenomenon of destaining most effectively. In practice it was hard to obtain such images consistently and reproducibly. Synaptosomes adhere poorly to coverslips; additionally, due to their small size, it was usually difficult to find the same plane of focus on the same synaptosomes before and after any particular treatment. Also, varying degrees of myelin contamination presented a problem. For these reasons, quantitative measurements of fluorescence changes were made using a large population of synaptosomes in a spectrofluorimeter as shown by the diagram in Figure 1c. The fluorescence decrease, ΔF, resulting from the second depolarising stimulus was recorded at step 4 and is a measure of the amount of dye that was taken up into vesicles that are recycled. By changing the conditions under which dye was taken up and subsequently measuring ΔF, we were able to determine the effects of those conditions on synaptic vesicle recycling.

Figure 1



The endocytosis assay for synaptosomes. **(a,b)** Confocal microscopy images of synaptosomes that have been loaded with and have subsequently released FM2-10. **(c)** Schematic illustration of the endocytosis assay; (a) and (b) correspond to steps 3 and 4 of (c), respectively. Synaptosomes were incubated with FM2-10 at 37°C (step 1) and vesicle endocytosis was stimulated as described in Materials and methods (step 2). The dye-loaded synaptosomes were then washed to repolarise them and to remove the external dye (step 3). Vesicle cycling was measured by a further stimulation (step 4) that caused dye-labelled vesicles to be released (see Materials and methods for details).

Synaptic vesicle recycling in synaptosomes is by a pathway dependent on dynamin and amphiphysin

We have presented evidence previously to suggest that the interaction between dynamin and amphiphysin 1 plays an essential role in receptor-mediated endocytosis [11]. Dynamin binds to the Src homology 3 (SH3) domain of amphiphysin 1 via the PSRPNR (in single-letter amino-acid code) sequence in the dynamin polyproline domain, and a peptide corresponding to this region of dynamin blocks binding to amphiphysin 1 and 2 *in vitro* [12,32]. We myristoylated the peptide containing this sequence (Myr-P4; QVPSRPNRAP) in order to make it membrane permeable and added it to our synaptosome preparation. Figure 2a shows that at 50 μM, Myr-P4 effectively inhibited vesicle recycling, whereas a myristoylated peptide containing the same amino acids as Myr-P4 but in a scrambled order (QPPASNPRVR) had no effect. Exocytosis of glutamate was not affected by Myr-P4 indicating that the inhibition was occurring in the endocytosis part of the synaptic vesicle cycle (Figure 2b).

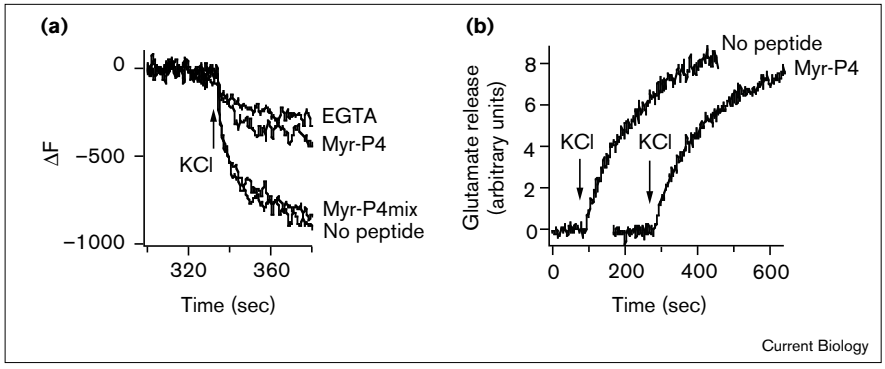
From this finding we conclude that in our synaptosome preparation, synaptic vesicle endocytosis is occurring predominantly via a dynamin-dependent pathway and probably via an amphiphysin-dependent pathway (although it is possible that other SH3 domain proteins are also involved). This conclusion agrees with recent studies demonstrating that endocytosis is dynamin-dependent in the lamprey giant reticulospinal synapse [10].

Characteristics of the synaptic vesicle recycling assay

In our synaptosome preparation, 30 mM KCl and an extracellular calcium concentration ($[Ca^{2+}]_o$) of 1.3 mM was used for depolarisation, which achieved maximal exocytosis of glutamate (these conditions were used in previous work [33]). Depolarisation with 30 mM KCl in the presence of 2 mM EGTA was used to give a value for ΔF or glutamate release in the absence of a calcium stimulus. A signal was observed with EGTA in both dye-loading and glutamate-release assays. Although this signal might represent a small calcium-independent component to both exocytosis and endocytosis, there are other explanations for its occurrence. In the glutamate-release assay, the signal seen upon depolarisation in the presence of EGTA has been previously attributed to a reversal in the direction of action of a glutamate transporter occurring as a result of the change in the plasma membrane electro-chemical potential upon depolarisation [33]. The fluorescence of FM dyes has been shown to be voltage-dependent [31], thus depolarisation of the membrane at step 4 in our synaptic vesicle recycling assay could cause a small change in fluorescence of the residual dye in the plasma membrane. In support for this explanation, we always observed a small response to stimulation in the presence of EGTA, of approximately the same magnitude, from any preparation of synaptosomes, even if the synaptosomes were not very active by other measurements.

In all of our experiments we defined the maximum stimulated exocytosis or endocytosis as the increase in the signal obtained with 1.3 mM $[Ca^{2+}]_o$ and 30 mM KCl (henceforth referred to as standard conditions) over that obtained with 2 mM EGTA and 30 mM KCl. When characterising our assay we observed that stimulation of exocytosis to a level above that achieved with our standard conditions was not possible even when using KCl concentrations of up to 60 mM (data not shown). This suggests that our standard conditions caused exocytosis of the whole of the readily releasable vesicle pool in synaptosomes. We also observed that if the synaptosomes were allowed to repolarise between each standard stimulation, at least three such stimulations on the same synaptosome sample could elicit maximum glutamate release with little decrease between the amount released on the first stimulation and that released on the second or third (data not shown). This observation implies to us that the amount of synaptic

Figure 2



The dependence of endocytosis on amphiphysin and dynamin. **(a)** The ΔF was measured when the dye had been loaded using standard conditions with or without 50 μM Myr-P4 peptide. Myr-P4 inhibited recycling of FM2-10 by 85 ± 10% ($n = 3$). A peptide comprising a scrambled Myr-P4 sequence (Myr-P4mix; 50 μM) had no inhibitory effect on synaptic vesicle recycling. A ΔF was always observed upon depolarisation in the presence of EGTA; we believe that this signal is an artefact most likely caused by a slight change in the fluorescent properties of the dye when the membrane potential is changed [31]. **(b)** Glutamate-release traces showing that 50 μM Myr-P4 had no inhibitory effect on exocytosis during the second (unloading) stimulus. There was also no effect of the peptide on glutamate release during the first (loading) stimulus (data not shown).

vesicle recycling compensates for the amount of exocytosis stimulated under our standard conditions. If this were not the case, an inhibition of exocytosis upon stimulations subsequent to the first would most likely have been observed.

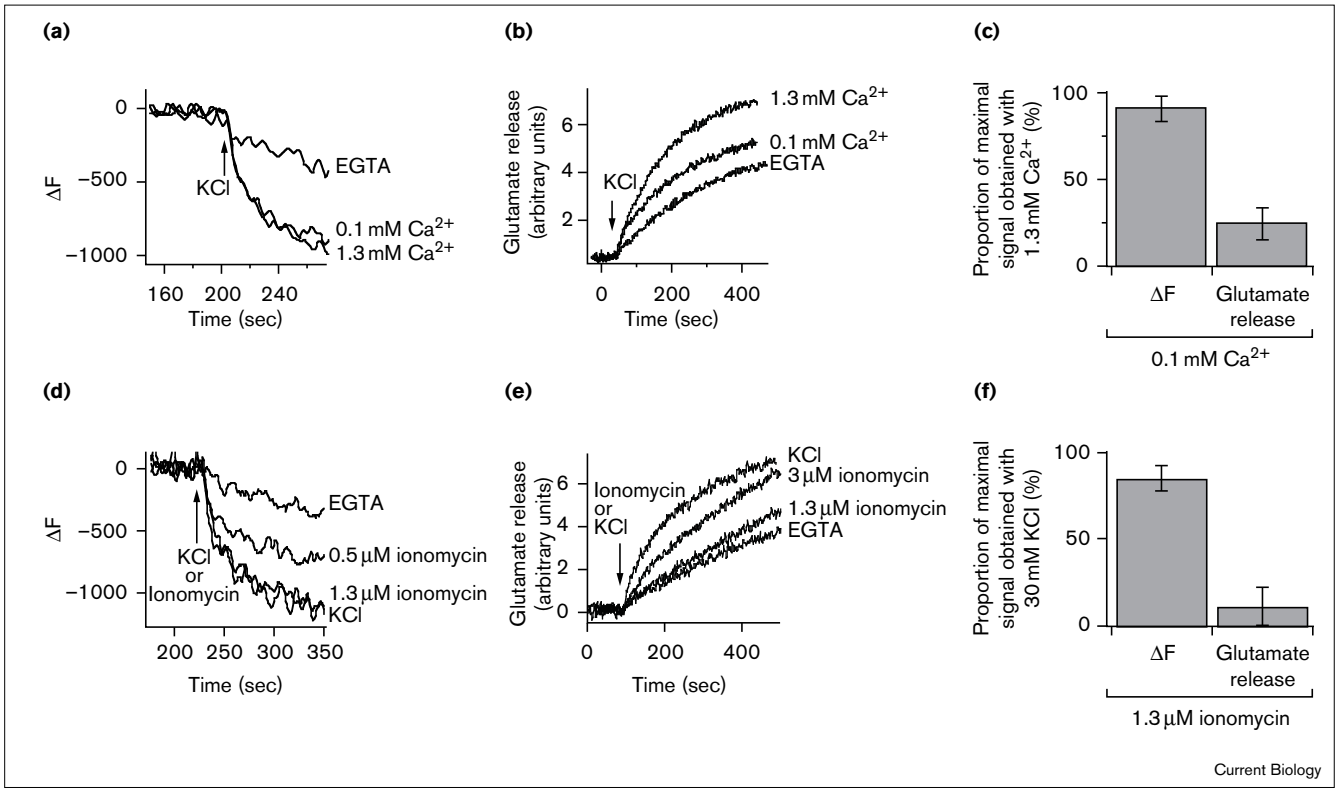
Exocytosis and endocytosis of synaptic vesicles have different calcium sensitivities

Exocytosis is a calcium-dependent process and it has become the accepted view that endocytosis follows exocytosis because of a 'coupling' between the two processes. In testing the calcium-dependence of both exocytosis and endocytosis it was observed that lowering the $[Ca^{2+}]_o$ to 0.1 mM limited the release of glutamate to $25 \pm 9\%$ of maximal levels. Endocytosis, however, still occurred at $92 \pm 8\%$ of its maximal extent with 0.1 mM $[Ca^{2+}]_o$ (Figure 3a–c). That exocytosis and endocytosis can be stimulated to occur simultaneously but to differing extents suggests that they might not be tightly mechanistically coupled. It is possible that vesicle release and recycling occur in tandem *in vivo* as a result of the calcium signal generated by an action potential at the presynaptic terminal that is sufficient to stimulate both processes.

Ionomycin-induced calcium influx alone is sufficient to trigger synaptic vesicle endocytosis

To further characterise the calcium-dependence of endocytosis we used the calcium ionophore ionomycin to allow calcium entry into synaptosomes in the absence of depolarisation. We observed that ΔF was almost maximal ($85 \pm 5\%$) with 1.3 μM ionomycin as the dye-loading stimulus followed by unloading under standard conditions (Figure 3d,f). This concentration of ionomycin was,

Figure 3



The calcium-dependence of endocytosis. **(a)** Measurement of ΔF after dye was loaded for 45 sec with 30 mM KCl and a $[Ca^{2+}]_o$ of either 1.3 mM or 0.1 mM. From multiple observations we found that both of these dye-loading stimuli elicited approximately the same ΔF. **(b)** Glutamate-release measurements showed that 0.1 mM $[Ca^{2+}]_o$ could elicit only a small fraction of the maximal exocytotic release. Release in the presence of EGTA was due to the reversal of the plasma membrane glutamate transporter, discharging glutamate from the cytoplasm [33]. The additional release in the presence of calcium was the exocytotic component. **(c)** The average of multiple traces from (a) and (b) showing the proportion of the maximal dye loading (represented by ΔF) and glutamate release obtained when the $[Ca^{2+}]_o$ was lowered from 1.3 mM to 0.1 mM during dye loading. The average ΔF was $92 \pm 8\%$ of maximal ($n = 7$). The average glutamate release was $25 \pm 9\%$ of maximal ($n = 3$). **(d)** Ionomycin (1.3 μM) stimulated maximal endocytosis of dye. The $[Ca^{2+}]_o$ was 1.3 mM for both ionomycin and KCl stimulations. **(e)** Ionomycin at 1.3 μM was insufficient to cause significant glutamate exocytosis. Ionomycin at 3 μM caused moderate glutamate release but was still not equivalent to KCl depolarisation. The $[Ca^{2+}]_o$ was 1.3 mM for both ionomycin and KCl stimulations. **(f)** The average of multiple traces from (d) and (e). Comparison of the ability of 1.3 μM ionomycin to replace 30 mM KCl in stimulating dye loading or glutamate release. The average dye loading (ΔF) was $85 \pm 5\%$ of maximal ($n = 5$) and glutamate release was $< 15\%$ of maximal ($n = 3$).

however, unable to elicit comparable exocytosis of glutamate (Figure 3e,f). These results indicate that a raised intracellular calcium concentration ($[Ca^{2+}]_i$) alone, at a concentration lower than that required to cause exocytosis, triggers endocytosis. The results also confirm that substantial endocytosis can occur with only minimal preceding exocytosis (although this might not be a physiological event — see Discussion). From our experiments we also noted that lower ionomycin concentrations could be used to stimulate sub-maximal endocytosis. At low ionomycin concentrations the exact level of stimulation of endocytosis was fairly variable (particularly between batches of ionomycin and different synaptosome preparations), but approximately 50% of the maximal level of endocytosis could be stimulated by 0.5 μM ionomycin in conjunction with 1.3 mM $[Ca^{2+}]_o$.

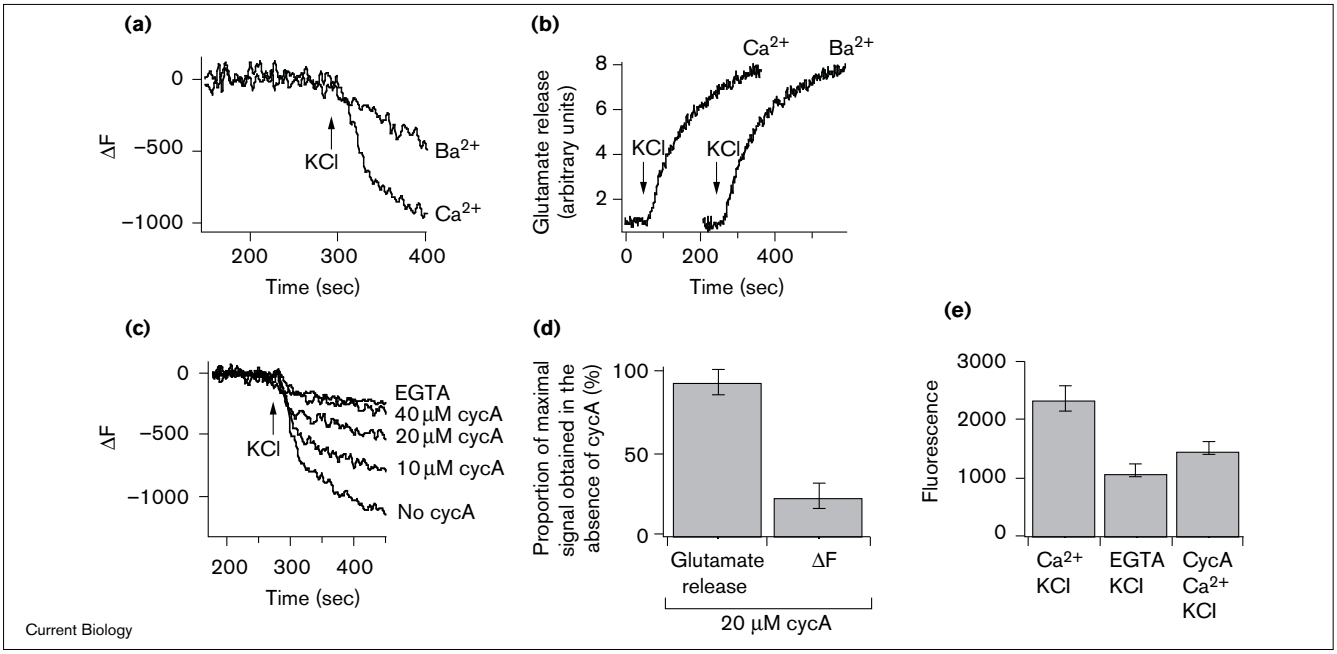
Barium does not substitute effectively for calcium in supporting synaptic vesicle recycling

Barium is capable of replacing calcium in supporting exocytosis [34], and thus when barium was substituted for calcium, there was no effect on glutamate release; an inhibition of dye recycling was observed, however, (Figure 4a,b). As barium does not form an active complex with calmodulin [35], this finding indicates a possible requirement for calcium and calmodulin in synaptic vesicle recycling. Studies using chromaffin cells have also suggested that calcium and calmodulin are essential for stimulated endocytosis [36].

Inhibitors of calcineurin potently block synaptic vesicle recycling

Calcineurin has been shown previously to dephosphorylate dynamin, synaptojanin and amphiphysin 1 [17,19]. Cyclosporin A, in a complex with cyclophilin, binds to the regulatory subunit of calcineurin, causing calcineurin to be potently and selectively inhibited [37]. Application of 20 μM cyclosporin A to synaptosomes 3 minutes before dye loading using standard conditions was found to decrease ΔF to 24.5 ± 8% of the control value (Figure 4c,d). Increasing the cyclosporin A concentration to 40 μM or more caused nearly complete inhibition of

Figure 4



Calcineurin activity is required for synaptic vesicle recycling. **(a)** Barium did not substitute effectively for 1.3 mM $[Ca^{2+}]_o$ in supporting FM2-10 dye recycling. $[Ba^{2+}]_o$ at 1.3 mM was substituted for calcium only on the first stimulation. The ΔF after dye loading with barium was much lower than the control, in which dye unloading was stimulated as normal. **(b)** Glutamate release after stimulation with KCl and 1.3 mM $[Ba^{2+}]_o$ was comparable to the release stimulated with KCl and 1.3 mM $[Ca^{2+}]_o$. **(c)** Dye-unloading traces showing the effects on FM2-10 recycling of three different concentrations of cyclosporin A (cycA) added 3 min before dye loading. **(d)** A comparison of the effect of 20 μM cyclosporin A on FM2-10 recycling and on glutamate release. FM2-10 recycling occurred at only 24.5 ± 8% of maximal ($n = 3$) whereas glutamate release was essentially unaffected (96 ± 9% of maximal; $n = 3$) in the presence of 20 μM cyclosporin A. **(e)** Results from the assay for endocytosis of newly formed synaptic vesicles (see Materials and methods) confirmed that endocytosis was calcium-dependent and was inhibited by 20 μM cyclosporin A. The fluorescence levels shown are FM2-10 fluorescence (plus background) of the synaptic vesicle fractions of synaptosomes that were depolarised using the stimuli indicated. The fluorescence level of the synaptic vesicle fraction from synaptosomes stimulated in the presence of 2 mM EGTA represents the background fluorescence, not the stimulated dye uptake, because unstimulated or inactive synaptosomes incubated with dye and then fractionated gave a similar signal.

dye recycling (Figure 4c). Cyclosporin A was not cytotoxic at the concentrations used and had no inhibitory effect on glutamate release at 20 μM (Figure 4d). A similar inhibition of vesicle cycling was obtained using another selective calcineurin inhibitor, FK506 (maximum inhibition at 4 μM; data not shown). These results demonstrate an essential role *in vivo* for calcineurin in synaptic vesicle recycling, contrary to published findings for endocytosis in chromaffin cells ([36], but see also [30]).

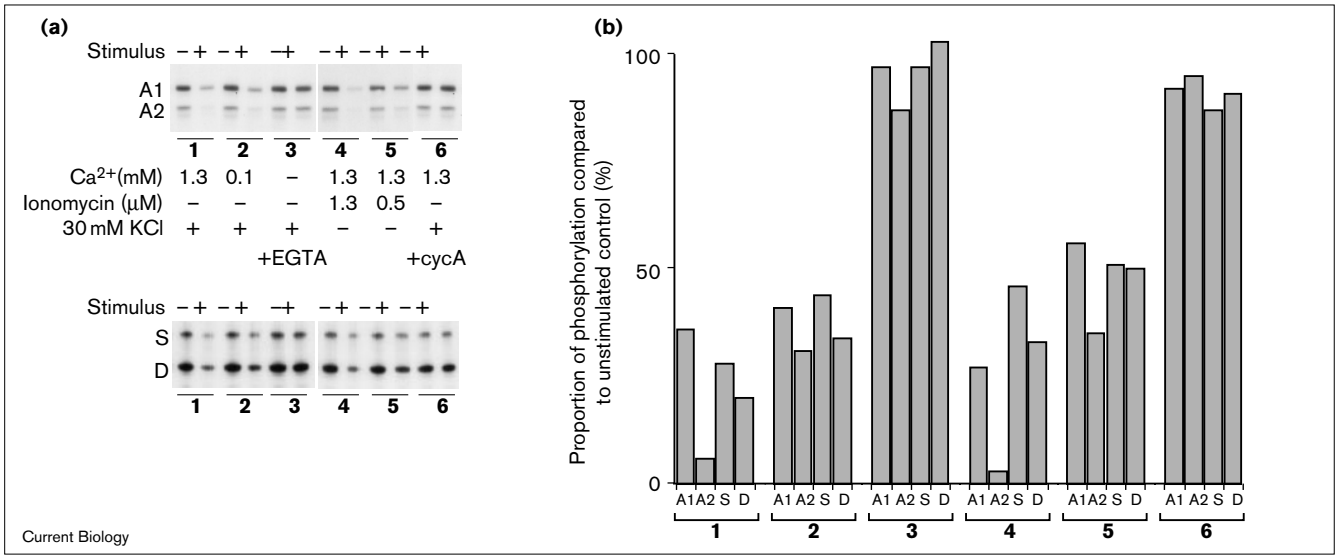
Identification of the step at which calcineurin is involved

The assay described so far in this paper can demonstrate only that calcineurin activity is required at some point in the synaptic vesicle recycling pathway. This point could be either when endocytosed synaptic vesicles are formed or when these vesicles are conveyed back to sites of exocytosis in a functional state. We have recently perfected a stage-specific assay for synaptic vesicle endocytosis in synaptosomes that directly measures the amount of dye uptake into small vesicles that has occurred after a single stimulus. This assay involves a stimulation protocol for dye uptake exactly as used in the assay described previously (Figure 1c and Materials and methods), except that after stimulation and washing away extraneous dye, the synaptosomes are hypotonically lysed and centrifuged to separate large membrane fragments from synaptic vesicles. The FM2-10 fluorescence from the synaptic vesicle fraction is then measured. Each experimental condition for stimulating dye uptake is compared with a positive and negative control (the positive control being stimulation with standard conditions and the negative control stimulation with 2 mM EGTA and 30 mM KCl). When 20 μM cyclosporin A was added 3 minutes before stimulation of dye uptake using standard conditions, fluorescence from the resulting synaptic vesicle fraction decreased to 31.5 ± 10% of control (Figure 4e). We interpret these results to indicate that cyclosporin A is inhibiting the endocytic formation of new synaptic vesicles that normally occurs when the synaptosomes are depolarised.

Cyclosporin A inhibits dephosphorylation of several proteins that have been implicated in endocytosis

Using ^{32}P-labelled synaptosomes, we showed that calcimeurin reversibly dephosphorylated amphiphysin 2 upon stimulation of synaptosomes, in addition to dephosphorylating amphiphysin 1, dynamin and synaptojanin (Figure 5a,b). Amphiphysin 2 phosphorylation was reduced by almost 95% whereas dephosphorylation of the other proteins was reduced by approximately 70% of their original phosphorylation levels (see also [12]). The differing extents of dephosphorylation might indicate that calcineurin has differing affinities for these substrates, or might indicate that there are multiple phosphorylation sites in some of the proteins but not others.

Figure 5



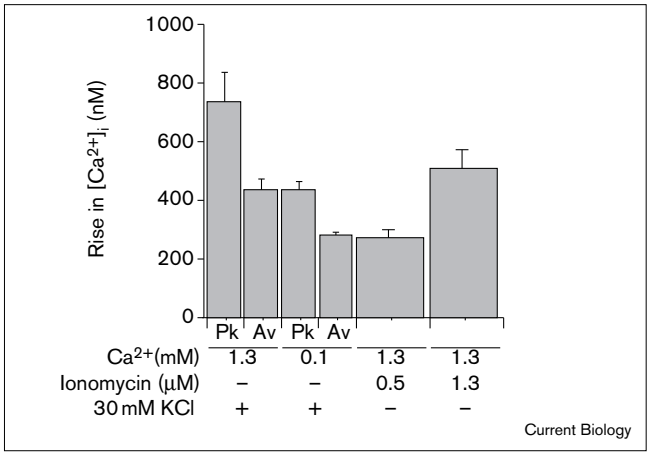
Endocytosis proteins are dephosphorylated by calcineurin in response to synaptosome depolarisation. The ^{32}P-labelled proteins from synaptosomes depolarised by a range of stimulus conditions (labelled 1–6 in the figure) were isolated and visualised by autoradiography as described in Materials and methods. EGTA was at 2 mM and cyclosporin A (cycA) at 20 μM. **(a)** Autoradiographs showing ^{32}P-labelled amphiphysin 1 (A1), amphiphysin 2 (A2), synaptojanin (S) and dynamin (D). The level of phosphorylated protein present after the depolarising stimulus (+) can be compared with the level of phosphorylated protein before the stimulus (–) shown adjacently. **(b)** Graph of the amount of phosphorylated protein present after each depolarising stimulation – using the conditions 1–6 described in (a) – as a percentage of the level of phosphorylated protein before stimulation. The phosphorylated bands were quantitated on a phosphorimager.

Cyclosporin A was shown to inhibit effectively dephosphorylation of all four proteins, indicating that the stimulated dephosphorylation was calcineurin-dependent (Figure 5a,b). Also shown in this figure is the extent of dephosphorylation elicited by all the other depolarising stimuli that we have discussed in the paper. Depolarisation using only 0.1 mM $[Ca^{2+}]_o$ but a standard KCl concentration still elicited a dephosphorylation of between 59% and 69%. Ionomycin and 1.3 mM $[Ca^{2+}]_o$ as the depolarising stimulus also caused dephosphorylation in a manner dependent on the concentration of ionomycin. At 1.3 μM ionomycin, dephosphorylation of amphiphysin 1, synaptojanin and dynamin was about 70% (97% for amphiphysin 2), whereas at 0.5 μM, these proteins dephosphorylated by about 50%. In our endocytosis assay we found that 1.3 μM ionomycin produced near maximum vesicle recycling (represented by ΔF), whereas 0.5 μM ionomycin elicited approximately 50% of the maximum, so there is a correlation between dephosphorylation and synaptic vesicle recycling. As we expected, 2 mM EGTA and 30 mM KCl did not cause dephosphorylation; these conditions had not caused synaptic vesicle recycling either.

$[Ca^{2+}]_i$ measurements confirm a correlation between calcium influx and synaptic vesicle recycling

Calcineurin is a calcium–calmodulin-dependent enzyme, therefore it is consistent that the calcium influx that occurs upon nerve-terminal depolarisation should be essential for calcineurin activation. We used the membrane-permeable calcium-sensing dye Fura-2 AM to show that the $[Ca^{2+}]_i$ elicited by the different depolarising stimuli correlated with the amount of synaptic vesicle recycling that they caused. Our measurements showed that our standard depolarising stimulus of 1.3 mM $[Ca^{2+}]_o$, in conjunction with either 1.3 μM ionomycin or 30 mM KCl caused an increase in the average $[Ca^{2+}]_i$ of about 500 nM, from a resting level of approximately 100 nM, during the minute following the stimulus (Figure 6). These stimuli produced maximal synaptic vesicle recycling. The calcium influx caused by 0.5 μM ionomycin and 1.3 mM $[Ca^{2+}]_o$ was 269 ± 24 nM. This value seems to correlate well with a stimulation of about 50% maximal synaptic vesicle recycling. However, 0.1 mM $[Ca^{2+}]_o$ with standard KCl concentrations, though able to maximally stimulate synaptic vesicle recycling, only caused 286 ± 8 nM increase in $[Ca^{2+}]_i$. The explanation for this seemingly anomalous finding might be that in contrast to stimulation with ionomycin, stimulation using KCl produced an initial peak increase in $[Ca^{2+}]_i$ (lasting for approximately 10 seconds) above the subsequent average level, to which it then fell back. We found that this peak level with 0.1 mM $[Ca^{2+}]_o$ reached 440 ± 26 nM. This could explain how depolarisation in the presence of this level of calcium could still trigger endocytosis maximally.

Figure 6



Increases in $[Ca^{2+}]_i$ as a result of a range of depolarising stimuli. Graph showing $[Ca^{2+}]_i$ after stimulation of synaptosomes by the conditions shown. The rise in calcium shown is that in excess of the baseline level (the baseline ranged between 50 and 150 nM). All the calcium rises were measured as the average increase that had occurred in the period 15–60 sec after stimulation. For stimulations with KCl, however, an initial peak of calcium influx (Pk) lasting for approximately 5–10 sec was observed before a lower steady level of increased calcium (Av) was attained. Note that 1.3 μM ionomycin and 30 mM KCl treatments in the presence of 1.3 mM Ca^{2+} generated approximately the same average $[Ca^{2+}]_i$ of about 500 nM (509 ± 68 nM and 433 ± 35 nM for ionomycin and KCl, respectively). This is interesting in the light of Figure 3e,f, which showed that only the KCl treatment stimulated significant glutamate release (see Results and Discussion).

Discussion

Studies in a variety of systems, ranging from chromaffin cells to hippocampal neurons to goldfish retinal bipolar cells, have produced diverse findings with respect to the role of calcium in endocytosis [26–30,38]. In this paper we have shown that in mammalian nerve terminals, calcium triggers endocytosis. In some previous reports (notably from studies on the *Drosophila* mutant *shibire* and in hippocampal cells) it has been concluded that endocytosis is inextricably coupled to exocytosis and that calcium stimulates endocytosis only because it stimulates exocytosis. In the case of the *shibire* mutant, the block of endocytosis and the accumulation of budding vesicles that occurs at the non-permissive temperature can be relieved at the permissive temperature in the absence of calcium [5]. This has been interpreted previously to suggest that endocytosis could be calcium-independent. In hippocampal cells, endocytosis is able to proceed in the presence of EGTA once exocytosis has been stimulated by depolarisation in the presence of calcium [28,29].

In contrast to these observations, our experiments with ionomycin demonstrate that calcium alone stimulates endocytosis, even when there is little preceding exocytosis (Figure 3d–f). We propose that in the light of our results,

the correct interpretation of the *shibire* observation is that the calcium-dependent step of endocytosis occurs before the stage of the cycle at which *shibire* becomes blocked. Thus, invaginating vesicle intermediates formed by stimulation in the presence of calcium at the non-permissive temperature would be able to endocytose independently of calcium when returned to the permissive temperature. Interpreting the experimental evidence in this way leads us to suggest that the calcium-dependent step of endocytosis is probably not the fission or budding-off of a newly formed vesicle, but a previous stage such as the functional recruitment of components such as dynamin to coated pits. With respect to the hippocampal cell studies, the simple explanation to fit both these findings and our own is that the calcium influx used to stimulate exocytosis in the hippocampal studies was sufficient to stimulate endocytosis as well, so that, following the initial calcium-dependent stages, endocytosis could be completed in the presence of EGTA.

Calcineurin has a low dissociation constant (K_d) for calcium (in the order of hundreds of nM) that corresponds to the average intracellular levels that we have shown to stimulate synaptic vesicle recycling *in vivo* [35] (Figure 6); more significantly, we have shown that inhibition of calcineurin activity effectively inhibits synaptic vesicle endocytosis (Figure 4c–e). As calcineurin is a protein phosphatase, it must follow that if calcineurin is essential for vesicle recycling then protein dephosphorylation must also be essential. Several calcineurin substrates that are likely to be involved in endocytosis have now been identified: dynamin [17], synaptojanin, amphiphysin 1 [19] and amphiphysin 2 (identified as a substrate in this paper). All these proteins are dephosphorylated in unison in response to nerve-terminal depolarisation [12,19]. The exact consequences that dephosphorylation and rephosphorylation of these proteins have for endocytosis are not yet known, although the effects of phosphorylation state on the characteristics of some of these proteins *in vitro* are being elucidated [17,39].

It is possible that calcium is directly involved in the regulation of endocytosis in addition to its effects mediated through calcineurin activation. It has been reported that the association of dynamin with membranes can be stimulated by calcium *in vitro* [40] and that the GTPase activity of dynamin is inhibited by calcium [41], although the high concentrations of calcium required for these effects are much greater than those reported to occur *in vivo* (hundreds of μM).

One might expect endocytosis to be a tightly regulated process in the nerve terminal so that the surface area of the synaptic plasma membrane does not fluctuate wildly. This assertion has led to a widely accepted view that endocytosis occurs only at membrane sites that are 'labelled' by synaptic vesicle components. Indeed there is evidence suggesting that synaptotagmin acts as a receptor for the endocytosis machinery [42]. If this hypothesis is correct it is difficult to explain why we have found it possible to differentially stimulate endocytosis and exocytosis to the extent that we could observe endocytosis occurring near maximally after very sub-maximal levels of exocytosis. It might be that we are observing a peculiarity of synaptosomes, for example their plasma membranes may have incorporated synaptic vesicles at some stage during preparation. Alternatively, our observations may reflect a flexibility in the system: there might always be a pool of recoverable membrane at the synapse, exocytosis adding to this pool and endocytosis drawing from it.

One interesting final point concerns the implications of our findings on the spatial relationship between the sites of exocytosis and endocytosis. Figure 6 shows that both 1.3 μM ionomycin and 30 mM KCl in conjunction with 1.3 mM $[Ca^{2+}]_o$ elicited a $[Ca^{2+}]_i$ of roughly the same magnitude (approximately 500 nM); it was shown in Figure 3e,f, however, that 1.3 mM ionomycin was insufficient to cause substantial glutamate release, whereas 30 mM KCl stimulated maximal release. The apparent anomaly between these two findings may be explained by the fact that KCl depolarisation stimulates calcium entry at specific sites identified by calcium channels whereas ionomycin allows calcium to leak in across any region of membrane into which it inserts. Thus, with KCl depolarisation, localised high concentrations of calcium far in excess of 400 nM could be generated around calcium channels (estimations of the calcium concentration in this micro-domain necessary to drive secretion are in the order of tens to hundreds of μM [26,43]) whereas with ionomycin-induced calcium influx such calcium concentrations would not occur. Synaptotagmin, the proposed calcium sensor for exocytosis, has a K_d in the micromolar range [23–25] and so would require the higher $[Ca^{2+}]_i$ proposed to exist in micro-domains around calcium channels in order to be activated. Calcineurin, on the other hand, with a K_d in the order of hundreds of nM [35], could be activated by bulk cytoplasmic calcium levels after nerve-terminal depolarisation. Thus, it would be possible for sites of endocytosis to be distant from calcium channels, whereas sites of exocytosis would have to be close to the channels.

Conclusions

Our results strongly support the hypothesis that calcium influx, occurring when nerve terminals are depolarised, triggers synaptic vesicle endocytosis via activation of calcineurin. The activated calcineurin dephosphorylates several of the proteins involved in endocytosis and this is proposed to set the molecular machinery of endocytosis in motion.

Materials and methods

Synaptosome preparation

Rat brain synaptosomes were prepared essentially as described [44] but incorporating the following points. A rat brain was dissected on ice to remove the mid-brain and hind-brain, meninges and as much myelin as possible. This is particularly important as myelin in the synaptosome preparation traps large amounts of FM2-10 resulting in a high background fluorescence and thus a greatly decreased signal to noise ratio. The brain was then homogenised in approximately 20 ml 0.32 M sucrose, 20 mM Hepes pH 7.3 using six strokes with a potter homogeniser at around 800 rpm. The brain homogenate was then centrifuged in a Sorvall SS-34 rotor for 2 min at 5000 rpm and the pellet discarded. The supernatant was centrifuged again in the same rotor for a further 12 min at 11,500 rpm. The pellet was resuspended in approximately 8 ml 0.32 M sucrose, 20 mM Hepes and this was loaded onto percoll gradients (4%, 10% and 23%) and centrifuged at 18,000 rpm for 11 min in a Beckman SW40Ti rotor. The synaptosomes were removed from the interface between the 10% and 23% percoll leaving any remaining myelin behind in the 4% percoll layer. The synaptosomes were diluted into Hepes-buffered medium (HBM; 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM Na_2HPO_4, 1 mM $MgCl_2$, 20 mM Hepes, 10 mM glucose, pH 7.3), centrifuged for a final time for 14 min at 11,500 rpm in the Sorvall SS-34 rotor and the pellet resuspended in approximately 4 ml HBM.

Synaptic vesicle recycling assay in rat brain cortical synaptosomes

Synaptosomes (500 µl, prepared as described above) were diluted to 1 ml in HBM in a stirred cuvette and incubated with FM2-10 (100 µM) and calcium (normally 1.3 mM) for 3 min at 37°C. Vesicle cycling was stimulated with 30 mM KCl for 45 sec. The sample was washed with two short 10 sec spins, to remove externally bound dye, and resuspended in 1 ml fresh HBM to allow repolarisation. The synaptosomes were gently stirred at 37°C for approximately 10 min to further remove dye from the plasma membrane. Labelled vesicles were released during a second round of vesicle cycling, stimulated with 30 mM KCl, 1.3 mM $[Ca^{2+}]_o$. We measured the decrease in fluorescence (ΔF) when the dye was exocytosed in a Fluoromax-2 spectrofluorimeter, exciting at 467 nM and collecting at 550 nM. The dye FM-143 has been used previously to measure exocytosis in synaptosomes [45], but FM2-10 is more easily washed from external membranes giving a lower background signal, which is why it was used in our study.

Synaptic vesicle endocytosis assay in rat brain cortical synaptosomes.

Synaptosomes were prepared as for the synaptic vesicle recycling assay. They were loaded with dye in the same way as with the previous assay excepting that double the concentration of synaptosomes was used. The extraneous dye was washed away from the loaded synaptosomes by the same protocol as described for the previous assay. Following two washes, the synaptosomes were spun briefly a third time and resuspended in lysis buffer (20 mM Hepes). The synaptosomes were left to stand in this hypertonic medium for approximately 10 min, then were briefly vortexed and returned to 150 mM ionic strength by the addition of KCl. The lysed synaptosomes were centrifuged for 10 min at 28K in a Beckman TLA 100.4 rotor. The supernatants were removed and measured for FM2-10 fluorescence in the spectrofluorimeter.

Confocal microscopy

Synaptosomes were prepared as described above and plated on polylysine-coated coverslips for 30 min. A coverslip was transferred to a perfusion chamber containing an optical window and the synaptosomes loaded with FM2-10 following the same procedure as for the endocytosis assay. The labelled synaptosomes were visualised using an MRC 1024 confocal microscope with a ×100 oil immersion objective.

Glutamate-release assay in rat brain cortical synaptosomes

This was performed as described [33]. Briefly, an enzyme-coupled assay was used in which production of NADPH by glutamate dehydrogenase is monitored in a spectrofluorimeter as a measure of glutamate production.

In vivo *labelling of endocytosis proteins with ^{32}P*

Synaptosomes isolated from one brain were resuspended in 5 ml HBM and ^{32}P-orthophosphate was added to 1 mCi/ml. The synaptosomes were incubated for approximately 30 min at 37°C to allow phosphoprotein labelling to occur. The synaptosomes were then spun at low speed for 4 min in a bench-top centrifuge and the supernatant containing excess ^{32}P discarded. The labelled synaptosomes were resuspended in HBM, this amount of material being sufficient for at least 20 immunoprecipitation/pull-down experiments. Aliquots of synaptosomes were left unstimulated or stimulated for 45 sec by various conditions, pelleted and lysed by addition of 20 mM Hepes containing 0.1% Triton X-100, 10 µM cyclosporin A, 500 nM staurosporine and a protease inhibitor cocktail [12]. The lysed material was then returned to 150 mM ionic strength by addition of NaCl and centrifuged for 15 min in a microfuge to pellet debris. Dynamin and synaptojanin were affinity isolated from the supernatants using glutathione-S-transferase (GST)-tagged recombinant amphiphysin 1 SH3 domain immobilised on glutathione agarose beads [12]. Amphiphysin 1 and 2 were immunoprecipitated with a polyclonal rabbit antibody (Ra1.2) [12]. Labelled proteins were separated on denaturing polyacrylamide gels and autoradiographed for detection. When comparisons of the extent of protein phosphorylation between two samples were made, the gels were also immunoblotted to confirm that comparisons were being made between equivalent amounts of protein.

Calcium measurements using Fura-2 AM

Synaptosomes were prepared in the same way and used at the same concentration as the synaptic vesicle recycling assay and were incubated with 5 µM Fura-2 AM for 30 min at 37°C. The sample was spun for 10 sec in a microfuge and the pellet resuspended in 1 ml fresh HBM (this removes excess Fura-2 AM). The levels of $[Ca^{2+}]_i$ elicited by various depolarising stimuli were measured by monitoring emission from Fura-2 excited at 340 nm and 380 nm. The Fura-2 was calibrated by lysing the synaptosomes with Triton X-100 (to give the maximum signal from the dye when exposed to the high calcium concentration of the external medium) followed by addition of excess EGTA (to obtain the minimal dye signal with effectively no calcium). The $[Ca^{2+}]_i$ were calculated from these data as described [46].

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