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Myosin-Va proteolysis by Ca^{2+}/calpain in depolarized nerve endings from rat brain

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Abstract

Myosin-Va is a molecular motor that may participate in synaptic vesicle cycling. Calpain cleaves myosin-Va in vitro at methionine 1141 in the tail domain. We show that intracellular proteolysis of myosin-Va occurs in rat cortical synaptosomes depolarized in the presence of calcium, evidenced by the formation of an 80 k polypeptide that co-migrates in SDS–PAGE with the 80 k fragment produced by the in vitro proteolysis of myosin-Va by calpain. Anti-myosin-Va antibody recognized this polypeptide in Western blots and immunoprecipitated it from synaptosome extracts. Calpastatin, a calpain-specific inhibitor, or leupeptin, a general cysteine protease inhibitor, suppressed or blocked formation of the 80 k polypeptide depending on membrane permeability. We conclude that myosin-Va undergoes intracellular proteolysis by endogenous calpain, when synaptosomes are depolarized in the presence of calcium, at the same cleavage site previously identified in vitro, thus, making it a target for calcium signaling during synaptic activation.

Keywords: Myosin-Va; Calpain; Synaptosome; Depolarization; Calcium; Proteolysis

The influx of ionic calcium at nerve endings via specific ion channels leads to the activation of several events related to synaptic vesicle cycling, including exocytosis and neurotransmitter release, phosphorylation of synaptic vesicle-associated proteins such as synapsin and calmodulin-dependent protein kinase type II (CaMKII), and depolymerization of actin filaments. Recent studies have implicated myosin motors in essential events related to exocytosis and synaptic function [1–5]. Myosin-Va (M-Va), an actin-based molecular motor, exquisitely regulated by calcium [6–8], has been localized to the synaptic region, binding to and interacting with several synaptic vesicle associated proteins, including CaMKII [4], syntaxin [4], and VAMP/synaptobrevin [2,9]. The exact role of M-Va at the synapse has not been determined, but suggestions have been made that this molecular motor may be involved in transport of synaptic vesicles from reserve pools towards the active zone where membrane fusion and neurotransmitter release occur [4,5,10].

M-Va is a substrate for calpain proteolysis in in vitro assays [7,11,12]. The major site of cleavage on the M-Va molecule has been determined to be one amino acid downstream of a PEST site in the tail region of M-Va [7]. Since PEST sites have been implicated as recognition sites for fast intracellular proteolysis by calpain and other proteases [13], the presence of a PEST site in M-Va and its susceptibility to proteolysis in vitro suggest that proteolytic processing may have a role in the cellular function of M-Va. In this paper, we demonstrate that limited proteolytic degradation of M-Va can be induced in isolated, intact nerve terminals by depolarization in the presence of extracellular calcium, resulting in the production of a relatively stable 80 k polypeptide that co-migrates with the 80 k fragment of biochemically purified M-Va cleaved in vitro by exogenous calpain. The size and immunoreactivity of the fragment formed, the requirement for calcium, and the effect of calpain-specific

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inhibitors suggest that endogenous calpain is responsible for site-specific cleavage of M-Va in depolarized nerve terminals.

Materials and methods

Materials. Reagents and chemicals were purchased from Sigma (St. Louis, MI). Percoll and anti-rabbit IgG conjugated to HRP were from Amersham Biosciences (Piscataway, NJ). Triton X-100 was from Pierce (Rockford, IL). Pansorbin cells, calpastatin (human), calpastatin peptide, leupeptin, and aprotinin were from Calbiochem (La Jolla, CA). Electron microscopy material and reagents were purchased from Electron Microscopy Sciences (Fort Washington, PA). Polyclonal antibody against the medial tail domain of chicken M-Va, referred to here as "anti-M-Va," was produced and affinity purified as previously described by Costa et al. [4].

Synaptosomes. Synaptosomes were prepared from rat cerebral cortex and purified over a Percoll gradient as described by Nagy and Delgado-Escueta [14]. All experiments were performed on synaptosomes immediately after preparation.

Electron microscopy. For morphological determinations, synaptosomes were fixed with 2% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, for 18 h at 4 °C, washed, and post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate, pH 7.3, for 2 h at 4 °C. Synaptosomes were gradually dehydrated in ethanol and embedded in araldite resin. Ultra-thin sections were observed under a Phillips electron microscopy (model EM 208). For immunoelectron microscopy, synaptosomes were rapidly frozen by impact onto a liquid nitrogen-cooled sapphire block using a modified Life Cell CF-100 slamming machine (Life Cell Corporation, The Woodlands, TX) as previously described by Moreira et al. [15]. Immunolocalization of M-Va within synaptosomes was performed by the protein A/colloidal gold method slightly modified from Moreira et al. [15] using anti-M-Va as the primary antibody. Negative controls of the reaction were done by incubation of grids following the same procedures but omitting the primary antibody.

Synaptosome incubations. Freshly prepared synaptosomes were incubated at 25 °C in a water bath and then depolarized with the addition of 50 mM KCl in the presence of 2.5 mM $CaCl_2$ or 2.5 mM EGTA. At the times 0, 5, 10, 15, 20, 30, 45, and 60 min, 100 μl was drawn from the reaction, interrupted by the addition of cold TCA (10%, final concentration), and kept on ice for 20 min. Each aliquot was centrifuged at 10,000*g* for 5 min at 4 °C. The resulting pellets were washed with cold ethanol, dried, and prepared for SDS–PAGE. In experiments with protease inhibitors, synaptosomes were pre-incubated for 10 min with 5 μM of the following calpain inhibitors: calpastatin, whole molecule (mw. 14,000), calpastatin, active peptide (mw. 3178), and leupeptin (mw. 475). As a negative control, the serine protease inhibitor, aprotinin, was used at the same concentration. Similar assays were also performed in the presence of 0.05% Triton X-100, added to the synaptosomes 10 min before incubation with protease inhibitors. Depolarization for 15 min and further treatment were as described above.

Immunoprecipitation. Depolarized synaptosomes were homogenized in Hepes buffer (40 mM Hepes, pH 7.4, 10 mM EDTA, 5 mM ATP, 2 mM DTT, 1 mM benzamidine, 2 μg/ml aprotinin, 0.3 mM PMSF, and 0.1% Triton X-100) and centrifuged for 40,000*g* at 4 °C for 20 min. The resulting supernatants were used for immunoprecipitation assays, slightly modified from Costa et al. [4]. One milliliter of each supernatant was first incubated with 25 μl of Pansorbin cells for 1 h at room temperature and then spun down for 5 min in a Nanofuge (Hoefer, San Francisco, CA). The supernatant was then incubated with 44 μg of anti-M-Va for 1 h, followed by the addition of 25 μl of Pansorbin, incubated for another 15 min, and then centrifuged in the Nanofuge. The resulting pellets were washed with TBS–T (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20). Initial supernatants and washed pellets were prepared for SDS–PAGE.

Other methods. Calpain cleavage of M-Va purified from chick brains was performed as described by Nascimento et al. [7]. SDS–PAGE was performed using 5–20% gradient minigels [16]. Immunoblotting was done as described by Towbin et al. [17], using HRP-conjugated secondary antibodies and developed by chemiluminescence, using the ECL kit from Amersham Biosciences. Blots were scanned and bands analyzed and quantified by the Image QuaNT software (Molecular Dynamics). All protein concentrations were determined by the method of Bradford [18] using bovine serum albumin, fraction V, as a standard.

Results

The morphological integrity of the synaptosomes was verified by electron microscopy of chemically fixed preparations. The ultrastructural images revealed membrane-enclosed synaptosomes with a large number of synaptic vesicles and one or more mitochondria (Fig. 1A). Since the antigenicity for our M-Va antibodies was not well preserved under these conditions, we applied the immunogold technique to lightly fixed and rapidly frozen synaptosomes. Structures clearly recognized as synaptosomes could be identified, most of which contained gold particles (Fig. 1B). From 36 electron micrographs taken from regions where the synaptosome morphology was best preserved, 796 out of 1206 gold particles (66%) were counted within clearly defined synaptosomes, thus giving structural evidence for the presence of M-Va in nerve endings.

Depolarization of isolated synaptosomes with 50 mM KCl in the presence of calcium resulted in the time-dependent appearance of an 80 k polypeptide, detected by the anti-M-Va antibody on Western blots (Fig. 2A), whereas little or no 80 k band was detected in experiments where calcium in the medium was depleted by EGTA (Fig. 2B). The relative intensities of the 80 k immunoreactive band in these experiments, determined by chemiluminescence labeling and densitometry in

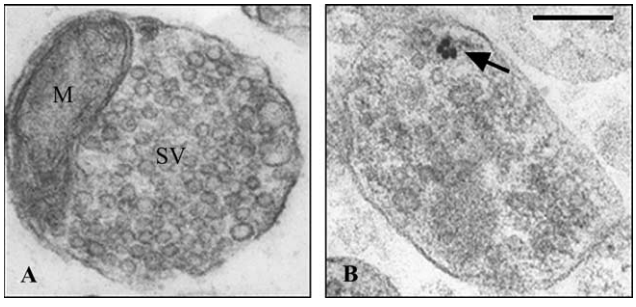

Fig. 1. Immunolocalization of M-Va within synaptosomes. Synaptosomes from rat brain were prepared for electron microscopy as described under Materials and methods for morphology (A) or for antigenicity with immunogold labeling of anti-M-Va (B) and visualized by electron microscopy. M, mitochondrion; SV, synaptic vesicles. The image in B illustrates M-Va immunoreactivity with 15-nm gold particles (arrow) within a synaptosome. Bar = 0.2 μm.

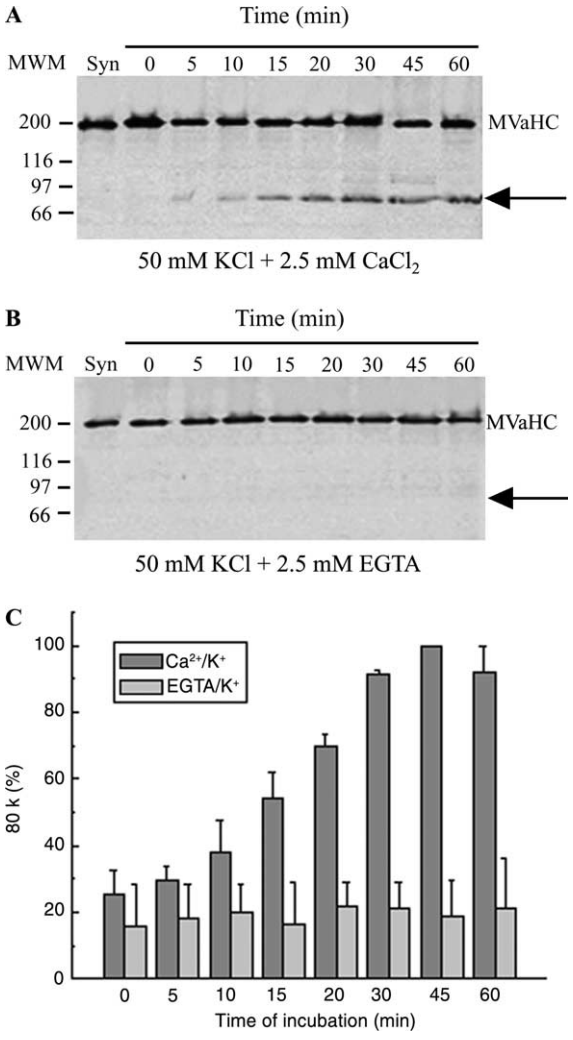

Fig. 2. Formation time course of an 80 k immunoreactive fragment from M-Va in depolarized synaptosomes. Protein fractions from synaptosomes that were incubated at 25 °C for the times indicated with 50 mM KCl in the presence of 2.5 mM $CaCl_2$ (A) or 2.5 mM EGTA (B) were analyzed on Western blots, probed with anti-M-Va antibody. The arrows to the right of the figures indicate the position of the 80 k immunoreactive band. (C) The 80 k bands from three independent experiments were scanned and the normalized density profile, expressed as percentage of the maximum density for each experiment in the presence of calcium, 80 k (%), plotted against the period of depolarization. Syn, non-depolarized synaptosomes; MVaHC, heavy chain of M-Va; and MWM, molecular weight markers.

three independent experiments, are shown in Fig. 2C, which illustrates the graded increase in 80 k formation with time of depolarization. Immunoprecipitation of M-Va from extracts of depolarized synaptosomes with anti-M-Va medial tail also brings down the 80 k polypeptide, but only when calcium was present during depolarization (Fig. 3A). The heavy chain of M-Va, but not the 80 k fragment, was immunoprecipitated by an antibody against the M-Va head domain (data not shown). Finally, the 80 k polypeptide from depolarized synaptosomes exactly comigrates on SDS–PAGE with

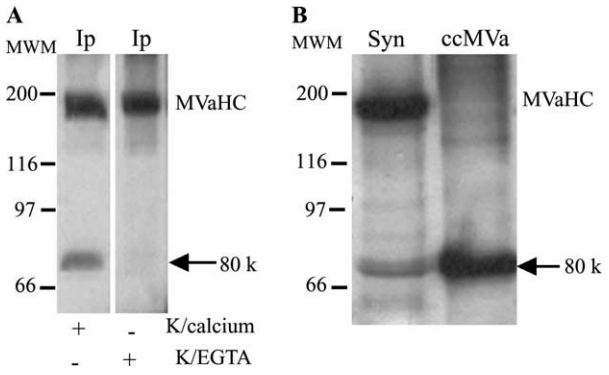

Fig. 3. Immunoprecipitation of the 80 k fragment with anti-M-Va. (A) Western blots are shown of the immunoprecipitates (Ip) by anti-M-Va on extracts from synaptosomes depolarized for 15 min in the presence and absence of calcium. (B) A direct comparison of mobility on SDS–PAGE is presented between the 80 k immunoreactive band from depolarized synaptosomes (Syn) and the 80 k fragment of chick M-Va generated by calpain cleavage in vitro (ccMVa). MVaHC, heavy chain of M-Va; MWM, molecular weight markers.

the well-characterized, 80 k proteolytic fragment [7] from the in vitro cleavage of M-Va by calpain (Fig. 3B). Together, these findings suggest that the 80 k polypeptide generated in depolarized synaptosomes is a product of endogenous calpain activity on the M-Va molecule, activated by calcium influx into the nerve endings.

To verify the degree to which depolarization vs calcium affects the formation of the 80 k fragment, synaptosomes were incubated in the presence of calcium, with or without KCl. As shown in Fig. 4, depolarization was necessary for the maximum production of the 80 k fragment, although calcium by itself promoted its formation. We speculate that this is due to membrane

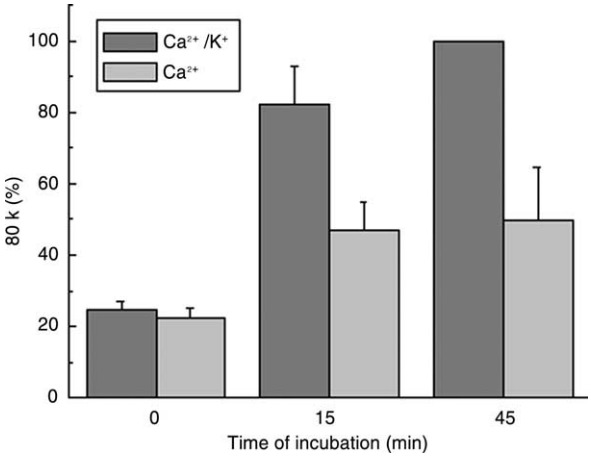

Fig. 4. Depolarization is necessary for maximum production of the 80 k fragment. Synaptosomes were suspended in media containing 2.5 mM $CaCl_2$ (Ca^{2+}) or 50 mM KCl plus 2.5 mM $CaCl_2$ (Ca^{2+}/K^+) and incubated for the periods indicated. The 80 k bands from Western blots of three independent experiments were scanned and the density profiles, expressed as percentage of the maximum density at 45 min for each experiment, 80 k (%), plotted against the period of depolarization.

leakage and/or spontaneous calcium uptake in isolated synaptosomes, as has been reported by others [19,20].

As a further test on the hypothesis that the appearance of the 80 k fragment corresponds to endogenous calpain activity, we pre-incubated synaptosomes with protease inhibitors: calpastatin, a potent and specific inhibitor of calpain [21], calpastatin peptide, the active portion of calpastatin [22], leupeptin, a tripeptide, and general inhibitor of thiol proteases, and aprotinin, a serine protease inhibitor. In in vitro assays, all of these

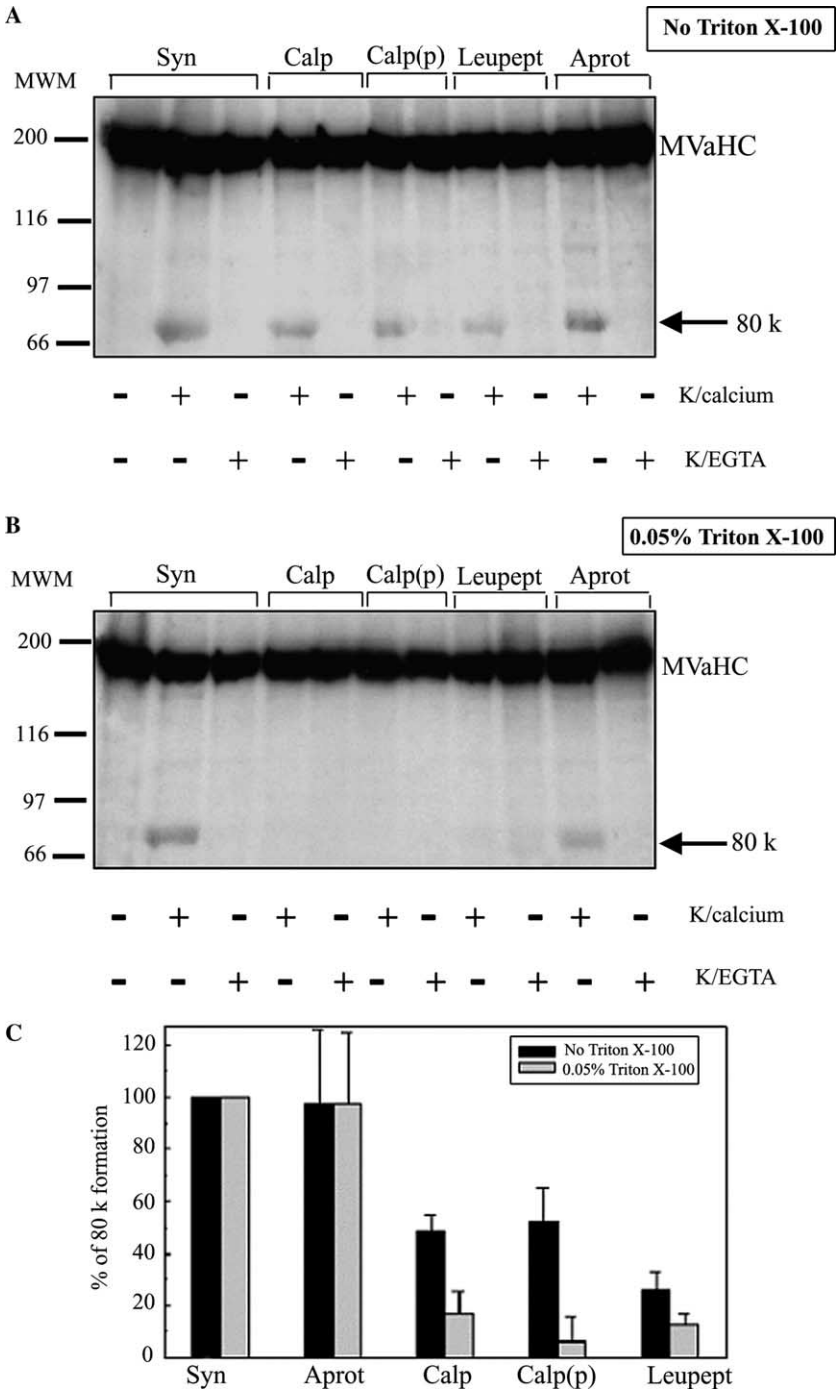

Fig. 5. Calpain inhibitors block the formation of the 80 k fragment. Western blots of synaptosome proteins were probed with anti-M-Va medial tail antibody. (A) Protein fractions from synaptosomes pre-incubated for 10 min without (Syn), or with 5 μM of the following protease inhibitors: calpastatin (Calp), calpastatin peptide (Calp(p)), leupeptin (Leupept) or aprotinin (Aprot) and then depolarized with 50 mM KCl for 15 min at 25 °C in the presence of 2.5 mM $CaCl_2$ (K/calcium) or 2.5 mM EGTA (K/EGTA). (B) The same protocol as in (A) except that the synaptosomes were pre-treated for 10 min with 0.05% Triton X-100 before treatment with protease inhibitors. MVaHC, heavy chain of M-Va; MWM, molecular weight markers. Arrows indicate the position of the 80 k fragment. (C) The 80 k bands from three independent experiments, similar to those in (A) and (B), were scanned and the density profile, 80 k (%), expressed as the % of 80 k fragment formed relative to that in the absence of inhibitors.

inhibitors, except aprotinin, blocked the cleavage of purified M-Va by calpain (data not shown). In intact synaptosomes, depolarized in the presence of calcium, all inhibitors, except aprotinin, partially inhibited the 80 k formation, leupeptin being the most potent (Fig. 5A). This correlates well with the membrane permeability of these inhibitors, leupeptin more easily passing through cell membranes. On the other hand, when the synaptosomes were pre-treated with 0.05% Triton X-100 to increase membrane permeability, all inhibitors, except aprotinin, essentially blocked the appearance of the 80 k fragment (Fig. 5B). Densitometric analysis from three independent experiments quantify and confirm these observations (Fig. 5C). Thus, these data suggest that endogenous calpain, activated by calcium influx, proteolyses M-Va localized to the synaptic region, generating the 80 k fragment, which corresponds to the C-terminal "tail" portion of the molecule [7].

Discussion

Calpain, a calcium-dependent cysteine protease, is considered to be a modulating intracellular protease that has been implicated in a large number of physiological processes including long-term potentiation, calcium-mediated signaling pathways, and pathological conditions, such as cataract formation, ischemia, and Alzheimer's disease [23,24]. Calpain has been associated with rapid degradation of specific targets including cytoskeletal proteins like fodrin, neurofilaments, talin, and MAP-2 [23,25]. This rapid and specific degradation is related to its recognition and binding to specific sequences known as PEST sites. M-Va possesses a PEST sequence in its tail domain and is cleaved by calpain in vitro at a site just downstream from the PEST region [7], which make it a likely candidate for intracellular proteolysis, mediated by calpain. In the present study, we demonstrate calcium-dependent, in situ proteolysis of M-Va in depolarized nerve endings that generates an 80 k tail-domain fragment of M-Va characteristic of its in vitro cleavage by calpain. Calpain specific inhibitors block the proteolysis, thus supporting the hypothesis that endogenous calpain, activated by calcium influx, targets M-Va for cleavage.

Recent evidence suggests that M-Va may have an important role in synaptic events. It is localized to nerve endings and binds to synaptic and/or endosomal vesicles and several vesicle-associated proteins, including synaptobrevin, synaptophysin, CaMKII, and syntaxin [2,4,9,26,27]. More recently, evidence has been presented for a role of M-Va in chromaffin vesicle secretion, demonstrating its association with chromaffin vesicles, and the inhibition of secretion by antibodies against M-Va [5].

These findings suggest that M-Va is the molecular motor responsible for the transport and/or anchoring of synaptic vesicles near the active zones in nerve endings. The demonstration here that M-Va is susceptible to calpain cleavage in depolarized synaptosomes suggests that synaptic activity includes proteolytic processing of M-Va during synaptic vesicle cycling. Similarly, fodrin, a major component of the subplasmic-membrane cytoskeleton, is a substrate for calpain and suffers proteolysis in synaptosomes upon calcium influx [28]. These latter authors suggested that calpain activation may modulate memory and neural transmission. Our studies add another potential target of this proteolytic system for synaptic modulation.

What could be the objective of proteolytic cleavage of M-Va at a site in its tail domain? A large amount of evidence suggests that class V myosins are involved in organelle transport over short distances and/or in tethering vesicles at the cell periphery. The regulation of binding and release of molecular motors to their specific organelles is an important feature of their cellular function [29], yet very little is actually known about it. Cell-cycle regulated binding of myosin V to pigment granules has been elegantly demonstrated in *Xenopus* melanophores [30] and is dependent on the phosphorylation of a specific site in the tail domain. The association of synaptic protein complexes with M-Va is often regulated by calcium ions [2,4,5,9], although the underlying mechanism of this regulation is not clear. Phosphorylation may be involved, since Costa et al. [4] have demonstrated a higher affinity of M-Va binding to CaMKII when this kinase is autophosphorylated. A second, albeit, non-reversible mechanism to release cargo from its motor may be calpain-directed proteolysis at the cargo-binding tail domain. In this case, the down regulation of the motor's role at the synapse will be sustained.

Finally, we would like to point out that biochemical studies have indicated that M-Va is regulated in vitro, directly and indirectly, by calcium ions in multiple ways—its ATPase activity [11], motility [31], binding to F-actin [8], phosphorylation by CaMKII [4], and site-specific proteolysis by calpain [7]. The present paper gives support to the notion that intracellular M-Va is quite well suited to respond to intracellular calcium signaling in specialized areas such as the synaptic region.

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