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N TYPE Ca^{2+} CHANNELS AND RIM SCAFFOLD PROTEIN COVARY AT THE PRESYNAPTIC TRANSMITTER RELEASE FACE BUT ARE COMPONENTS OF INDEPENDENT PROTEIN COMPLEXES

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Abstract—Fast neurotransmitter release at presynaptic terminals occurs at specialized transmitter release sites where docked secretory vesicles are triggered to fuse with the membrane by the influx of Ca^{2+} ions that enter through local N type (CaV2.2) calcium channels. Thus, neurosecretion involves two key processes: the docking of vesicles at the transmitter release site, a process that involves the scaffold protein RIM (Rab3A interacting molecule) and its binding partner Munc-13, and the subsequent gating of vesicle fusion by activation of the Ca^{2+} channels. It is not known, however, whether the vesicle fusion complex with its attached Ca^{2+} channels and the vesicle docking complex are parts of a single multifunctional entity. The Ca^{2+} channel itself and RIM were used as markers for these two elements to address this question. We carried out immunostaining at the giant calyx-type synapse of the chick ciliary ganglion to localize the proteins at a native, undisturbed presynaptic nerve terminal. Quantitative immunostaining (intensity correlation analysis/intensity correlation quotient method) was used to test the relationship between these two proteins at the nerve terminal transmitter release face. The staining intensities for CaV2.2 and RIM covary strongly, consistent with the expectation that they are both components of the transmitter release sites. We then used immunoprecipitation to test if these proteins are also parts of a common molecular complex. However, precipitation of CaV2.2 failed to capture either RIM or Munc-13, a RIM binding partner. These findings indicate that although the vesicle fusion and the vesicle docking mechanisms coexist at the transmitter release face they are not parts of a common stable complex.

Key words: calcium channel, active zone, RIM, synaptic vesicle, cytomatrix, scaffold.

The rapid release of neurotransmitters from nerve terminals at synapses occurs at specialized areas on the presynaptic transmitter release face opposing postsynaptic receptor regions that are described morphologically as *active zones* or functionally as *transmitter release sites* (TRSs). Synaptic vesicles are transported to these sites and are triggered to fuse with the membrane and discharge their contents by the influx of Ca^{2+} ions through surface membrane Ca^{2+} channels. A number of previous structural and functional studies suggest that a cluster of Ca^{2+} channels is sequestered within the TRS close to the synaptic vesicle fusion sites consistent with the hypothesis that transmitter release is gated by this local source of Ca^{2+} entry (Heuser et al., 1974; Llinas et al., 1981; Stanley, 1997; Gentile and Stanley, 2005).

The TRS is a multi-molecular complex at which many protein types contribute to its various functions (Sudhof, 2004). However, of these Rab3A interacting molecule (RIM) 1α or RIM2α (herein RIM) is one of the very few specifically located at the TRS (Wang et al., 2000). RIM is a large scaffolding protein with numerous protein interaction sites and a number of known binding partners that are mostly associated with synaptic vesicle docking into the release site or their priming for fusion (Calakos et al., 2004; Kaeser and Sudhof, 2005). These binding partners include Munc-13 (Betz et al., 2001; Dulubova et al., 2005), a cytoplasmic protein associated with synaptic vesicle priming.

The objective of this study was to test whether the presynaptic Ca^{2+} channel cluster, specifically the N type calcium channel (CaV2.2), which serves as a marker of the membrane-associated synaptic vesicle fusion mechanism, is part of a common multimolecular complex with RIM, which serves as a marker of the vesicle docking/priming mechanisms. Such an association is suggested by evidence for direct binding of RIM to an intracellular region of the channel (Coppola et al., 2001) or as a part of a larger complex (Hibino et al., 2002). We first tested whether CaV2.2 and RIM are co-localized at the transmitter release face of a presynaptic terminal. We used the isolated chick ciliary ganglion calyx synapse (Stanley, 1989; Stanley and Goping, 1991) to localize these proteins at an undisturbed, native presynaptic transmitter release face. This nerve terminal is ideal for such an analysis since virtually all the Ca^{2+} channels are N type (Stanley and Atrakchi, 1990; Stanley, 1991; Yawo and Chuhma, 1994), the transmitter release face can be imaged at near light-limited resolution (Stanley and Mirotznik, 1997; Li et al., 2004) and the Ca^{2+} channel clusters are sufficiently widely spaced to distinguish release site-associated proteins from those in other areas of the nerve terminal. The channel was identified using a well-characterized, high-affinity antibody Ab571

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Abbreviations: ICA, intensity correlation analysis; ICQ, intensity correlation quotient; IgG, immunoglobulin; RIM, Rab3A interacting molecule; ROI, region of interest; TRS, transmitter release site.

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reported previously (Li et al., 2004) and RIM was localized with a commercial antibody. Protein pair co-localization was analyzed by means of a novel quantitation method, intensity correlation analysis (ICA; Li et al., 2004). This method tests whether the intensity of two stains vary in synchrony which is evidence that the two proteins are parts of a common complex or sub-cellular entity. We used standard co-immunoprecipitation to test if the Ca^{2+} channels are also physically linked to the RIM complex by standard co-immunoprecipitation. Our studies suggest that the Ca^{2+} channel/synaptic vesicle fusion complex and the vesicle docking/priming complex are components of functionally interacting, yet physically separate molecular complexes.

EXPERIMENTAL PROCEDURES

Antibodies

Dilutions for antibodies used in this study are shown in Table 1.

Chick brain lysate and synaptosome preparation

Chick brain lysates were prepared as described previously (Li et al., 2004; Khanna et al., 2006). The only modification was the filtering of the lysate through a 0.22 μm syringe filter (Millipore, Cambridge, ON, Canada) prior to use. Protein concentrations were determined with BioRad protein assay reagent (Hercules, CA, USA).

Synaptosome preparation

A chicken brain synaptosome fraction was prepared as described previously for rat brain (Huttner et al., 1983) with minor modifications. Twenty 15 day old chicken embryo brains were dissected into 10 volumes ice-cold homogenization buffer (0.32 M sucrose, 10 mM HEPES pH 7.4, 2 mM EDTA, supplemented with protease inhibitors) and homogenized using 10–15 strokes of a glass Teflon handheld homogenizer. The homogenate was then spun at 1000×*g* 4 °C for 15 min to remove a nuclear fraction and cellular debris pellet. The supernatant from the low-speed spin was spun at 200,000×*g* 4 °C for 45 min. The pellet from this spin was re-suspended in homogenization buffer and spun again at 200,000×*g* for an additional 45 min. This second pellet (P2) was re-suspended in HEPES lysis buffer (50 mM HEPES pH 7.4, 2 mM EDTA plus protease inhibitors) and layered onto 4 ml of 1.2 M sucrose and centrifuged at 230,000×*g* (4 °C) for 30 min in a swinging bucket rotor. The gradient interphase was collected, diluted in 7–8 ml of ice-cold HEPES-buffered sucrose (0.32 M sucrose, 4 mM HEPES, pH 7.4) and layered onto 4 ml of 0.8 M sucrose and re-centrifuged at 230,000×*g* for 15 min (4 °C). The pellet from this spin, which contains synaptosomal proteins, was re-suspended in modified RIPA buffer; filtered through a 0.22 μm syringe filter before its protein concentration determined was determined and fractions were stored at −80 °C until use.

Immunoprecipitation and Western blotting

These procedures were performed exactly as described earlier (Li et al., 2004; Khanna et al., 2006).

Chick calyx synapse preparation

This has been described in detail (Stanley and Goping, 1991; Stanley, 1991; Sun and Stanley, 1996; Mirotznik et al., 2000; Li et al., 2004). After trituration of the ganglia the cells/terminal preparation was plated at 37 °C in a standard cell incubator for 45 min.

Immunostaining

This has been described in detail (Mirotznik et al., 2000; Li et al., 2004). Staining with two rabbit polyclonal antibodies was as described using the *pretty-poly* method (Morris and Stanley, 2003).

Microscopy

Microscopy techniques were as described (Li et al., 2004). Slides were imaged at 60× magnification with a 1.4 numerical aperture lens.

Iterative deconvolution deblurring

The Z Axiovision turn-key iterative deconvolution program was used off-line at its highest stringency using a theoretical point-spread function, as described (Li et al., 2004). Regions of interest (ROIs) were identified by eye from the sampled optical sections and, if needed, from neighboring sections.

ICA/ICQ

This analysis has been described in detail (Li et al., 2004). Basically, for the ICA we calculated the function $(A_i-a)(B_i-b)$, where a and b are the means of each pixel staining pair intensity values A_i and B_i. A_i or B_i was graphed in separate scatter plots against their respective $(A_i-a)(B_i-b)$ value. Distributions that skew to the right reflect dependent staining patterns (where the two pixel staining intensity values vary in synchrony), ones that are symmetrical about the 0 axis indicate random staining, while those that skew to the left reflect independent staining patterns, where the pixel staining intensity values vary inversely. Note that the analysis can be carried out for each stain separately so that a dependence of stain *A* on *B* but a lack of dependence of *B* on *A* can be identified and, further, that the plots permit detection of complex or mixed staining relations. The intensity correlation quotient (ICQ) reflects the ratio of the number of positive $(A_i-a)(B_i-b)$ values to the total number of pixels in the ROI, corrected to a −0.5 (independent staining) to +0.5 (dependent staining) range by subtracting 0.5. The ICQ

Table 1. Antibodies used in this study

Antibody	Source	Dilutions		
		IF	IP	WB
CaV2.2, Ab571[a]	Stanley	1:100	1:200	1:500
Munc-13-1 (m)	Synaptic Systems Labs (Göttingen, FDR)	1:50	1:300	1:300
pan-Munc-13 (p)	BD Transduction Labs (Mississauga, ON)	—	1:500	1:500
RIM2 (p)	Synaptic Systems Labs	1:100	1:500	1:200

Abbreviations used: IF, immunofluorescence; IP, immunoprecipitation; (m), monoclonal; (p), polyclonal; WB, Western blot; —, not used. See text for other abbreviations.

[a] (Li et. al, 2004).

provides a single value indication that can be used for statistical comparison. Typically, with $N>5$ ROIs, a mean ICQ value of $-.05$ to $+.05$ indicates random staining, $+.05$ to $+.10$, indicates a moderate covariance and $>.1$ a strong covariance. ICA/ICQ analysis was carried out by means of an automated graphic plugin (Image Correlation Analysis; for detailed protocol and source see Supplemental Material) for the public domain image analysis software. ImageJ (Wayne Rasband; Research Services Branch, National Institutes of Mental Health, National Institutes of Health, Bethesda MD).

Image presentation

Images were cut, background signals subtracted and brightness/contrast adjusted using ImageJ and PowerPoint software, applying each adjustment to the entire image. Dye overlay images and ROI lines were created and superimposed using ImageJ. No other image manipulations were made.

Statistical analysis

Student's t-test was used, except where stated. Values are either tested for two means between conditions (P) or for one mean for a difference from 0 ($P_{=0}$). Each calyx ICQ value reflects the mean of two determinations from image planes at least 400 nm apart (two z axis increments). In a few cases a single image plane was used where this was not possible. N values refer to calyx terminals examined. The sign test was used for statistical tests of ICQ values for single experiment examples, as in Fig. 1 (see: Li et al., 2004).

RESULTS

CaV2.2 and RIM co-localize at a presynaptic transmitter release face

We tested whether the transmitter release face CaV2.2 clusters are located at the TRSs by staining the terminals for the active zone-marker RIM using 'RIM2' antibody. This antibody detects RIM1, RIM2, RIMβ and a number of splice variants (Fig. 2A), as reported previously (Wang et al., 2000; Wang et al., 2002; Wang and Sudhof, 2003). An overlay of CaV2.2 staining, in green, with that for RIM, in red, generated yellow regions consistent with co-localization (Fig. 1A, top panel). However, green and red regions were also apparent, raising the possibility that the observed yellow regions result from random overlays. We therefore carried out a quantitative analysis of the staining patterns using the ICA/ICQ method reported previously (Li et al., 2004). This method tests whether the staining intensities of the two proteins vary in synchrony, as predicted for two proteins that are parts of the same molecular complex or subcellular organelle. To facilitate this analysis we have developed a plugin for the open-source image analysis program ImageJ (for download and instructions see: Supplemental Material). The ICA plots exhibited a prominent right skew (Fig. 1B, C), indicating that the two stains vary strongly in synchrony. We also calculated the ICQ, a value that can vary from -0.5 to $+0.5$. The mean ICQ value for seven calyces was positive, and highly significant (0.15 ± 0.02, $N=7$ calyces; $P_{=0}<0.001$) confirming that the staining for the two proteins covaries strongly. These results indicate that CaV2.2 and RIM are both co-localized at the transmitter release face and that in this region their concentrations covary.

We carried out a similar co-localization analysis for the RIM binding partner, Munc-13. The calyx terminals stained brightly for Munc-13. However, this staining did not co-localize with that for CaV2.2 (Fig. 1D). ICA plots were random or skewed to the left (Fig. 1E, F) with a mean ICQ value that was not significant (-0.018 ± 0.019, $N=5$; $P_{=0}>0.3$). Thus, our results suggest that Munc-13 is not preferentially co-localized with CaV2.2 at the transmitter release face. This does *not*, however, mean that Munc-13 is absent from the release site; in that case the ICQ value would be strongly negative. The strict interpretation is that it is randomly distributed relative to CaV2.2; it remains possible that this is a broadly expressed protein that is present in both release site and non-release site areas.

The synaptosomes Ca^{2+} channel does not co-precipitate with RIM or MUNC-13

The finding that CaV2.2 staining co-varies with RIM is evidence for a common molecular complex. In order to test for this directly we carried out a co-immunoprecipitation analysis using chick brain lysate. This analysis was made possible by the availability of Ab571, an antibody directed against the channel II–III loop with an affinity that is sufficiently high to permit capture of the channel (Li et al., 2004). We also tested for RIM co-precipitation using two new anti-CaV2.2 antibodies directed against the channel C terminal long-splice region (Khanna et al., 2006). The CaV2.2 long-splice variant has been reported to be preferentially targeted to the TRS (Maximov and Bezprozvanny, 2002). Immunoprecipitation of CaV2.2 failed to co-precipitate RIM (Fig. 2A) or Munc-13 (Fig. 2B) while the reverse, precipitation of RIM or Munc-13, also failed to co-precipitate CaV2.2 in five trials for RIM and 10 trials for Munc-13 (Figs. 2C, 3A). Similar results were obtained with rat brain synaptosomes (data not shown).

Co-localization of RIM with Munc-13. Since RIM is known to bind to Munc-13 we also tested whether these two proteins are co-localized at the presynaptic transmitter release face. Dye overlay resulted in yellow puncta (data not shown) while the ICA scatter plots were positively skewed with a positive mean ICQ (0.130 ± 0.042, $N=4$; $P_{=0}=0.05$). We also used co-immunoprecipitation to confirm that RIM and Munc-13 are present in a common complex (Fig. 2D).

The finding that the protein pairs CaV2.2/RIM, and RIM/Munc-13 are co-localized and yet CaV2.2/Munc-13 are not implies that the RIM/Munc-13 complex is not necessarily associated with the CaV2.2 channel clusters. The relatively broad distribution of Munc-13 within the terminal may ensure that it is always available for transient roles in the transmitter release process.

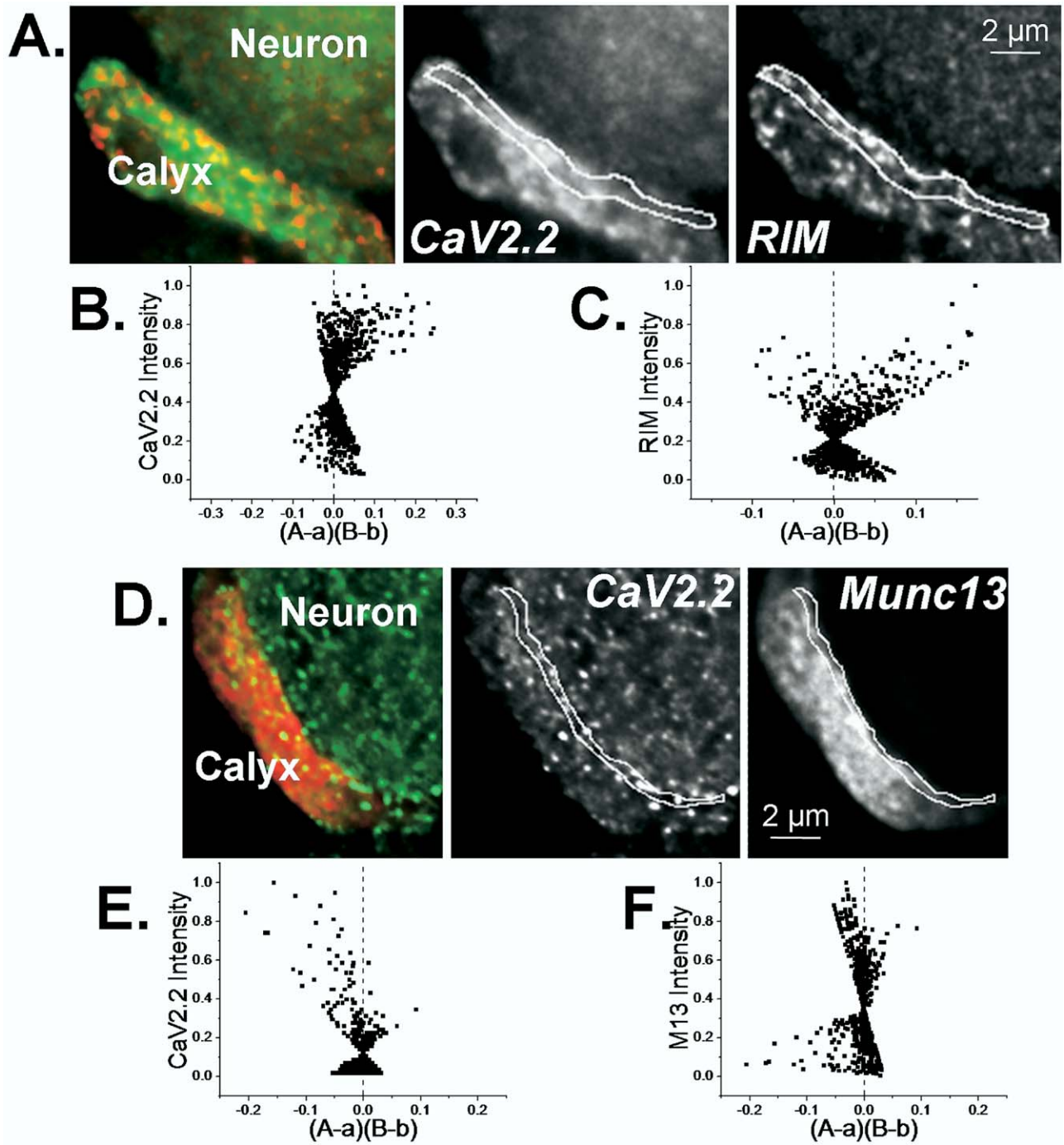

Fig. 1. CaV2.2 co-localizes with RIM but not Munc-13 at the calyx terminal transmitter release face. (A) Optical section through a calyx presynaptic terminal stained in the left panel for CaV2.2 (green) and RIM (red) giving yellow in regions stained by both dyes at high intensity. Intensity-proportional gray scale images are also shown in the right two panels with the release face ROI demarcated by continuous line. ICA analysis of these two staining pairs generated scatter plots with strong right skews for both CaV2.2 (B) and RIM (C). ICQ=0.214 ($P_{=0}<0.01$). (D–F) ICA analysis for CaV2.2 and Munc-13. Note the poor co-localization of the stains at the transmitter release face and the corresponding ICA plots that are skewed to negative values at high staining intensities. ICQ=−0.02 ($P_{=0}>0.1$).

Extrasynaptic linkages are not responsible for CaV2.2 and RIM co-localization

It has been suggested that presynaptic organization may be influenced or maintained by linkages from the postsynaptic cell or to the intervening extracellular matrix (Maximov and Bezprozvanny, 2002; Nishimune et al., 2004). In order to test the possibility that CaV2.2 and RIM are linked via an extracellular, trans-synaptic link we repeated the ICA analysis on calyces that were fully isolated from the postsynaptic neuron (Fig. 3). We have found that this isola-

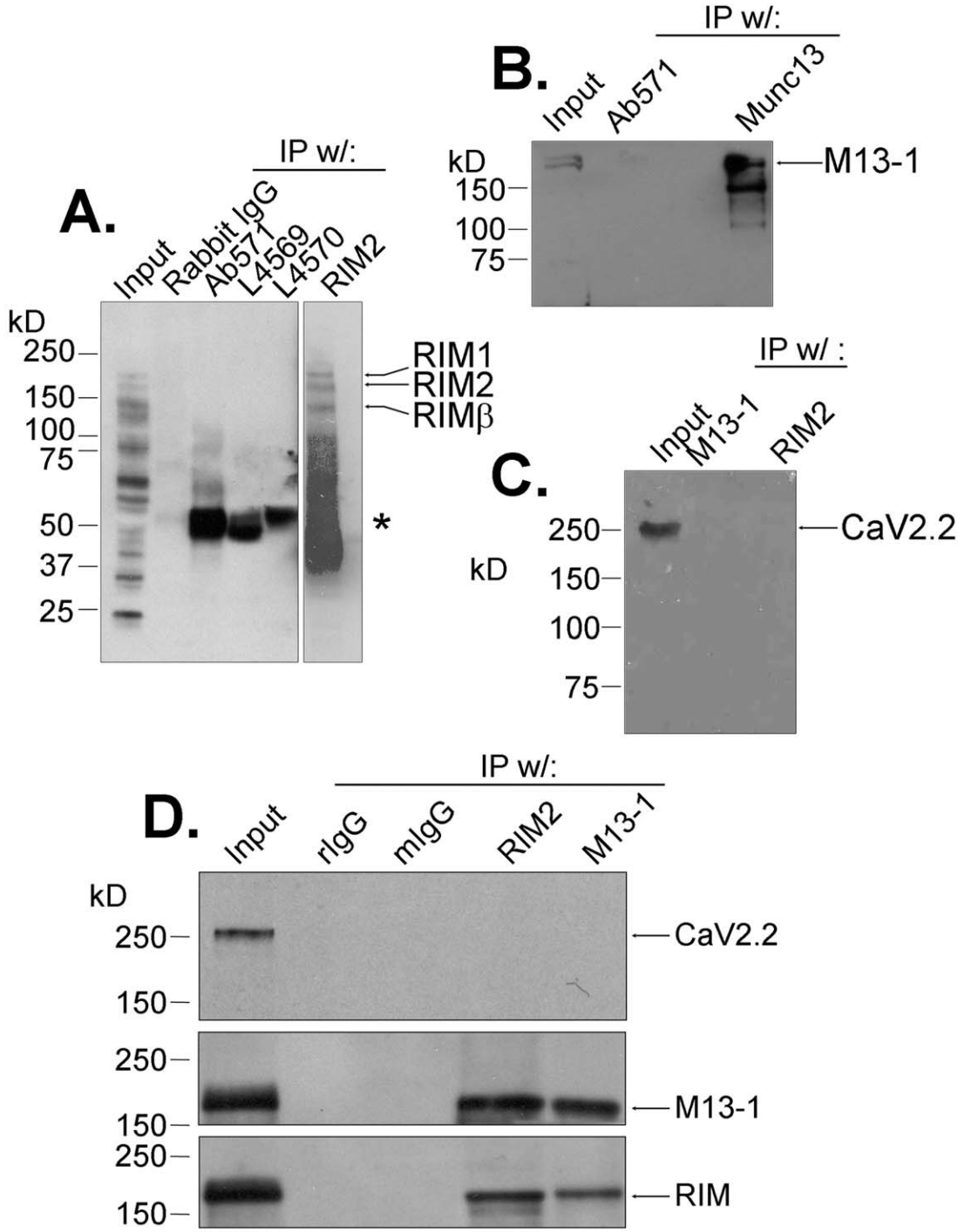

Fig. 2. RIM and Munc-13 co-precipitate with each other but not with CaV2.2. (A) Western blot analysis of chick brain lysates immunoprecipitated (i.p. w/:) with anti-CaV2.2 antibodies. The left lane (input) was loaded with 5% of the brain extract used for each of the immunoprecipitations. The antibody detects multiple RIM splice variants, three of which are labeled. Immunoprecipitation of CaV2.2 (Ab571) or the long C terminal splice variant CaV2.2 (L4569 and L4570 antibodies) failed to coprecipitate RIM (probed with 'RIM2' antibody). Note that the RIM2 antibody detects many members of the RIM protein family, as previously reported. The asterisk indicates the non-specific immunoglobulin (IgG) band. (B) Munc-13-1 (herein M13-1) is detected after precipitation with the polyclonal antibody against itself (Munc-13; far right lane) but is not coprecipitated with CaV2.2 (Ab571). Legend as in A. (C) Immunoprecipitation with two different anti-RIM antibodies (RIM1 and RIM2) or anti-Munc-13-1 failed to co-precipitate CaV2.2. (D) Western blot analyses of chick brain synaptosomes immunoprecipitated with antibodies against rabbit IgG control, mouse IgG control, polyclonal RIM2 and monoclonal Munc-13-1. The input lane represents 5% of the synaptosome extract used for the i.p. I.p. with RIM2 or Munc-13-1 failed to detect CaV2.2 while RIM2 and Munc-13-1 were detected reciprocally.

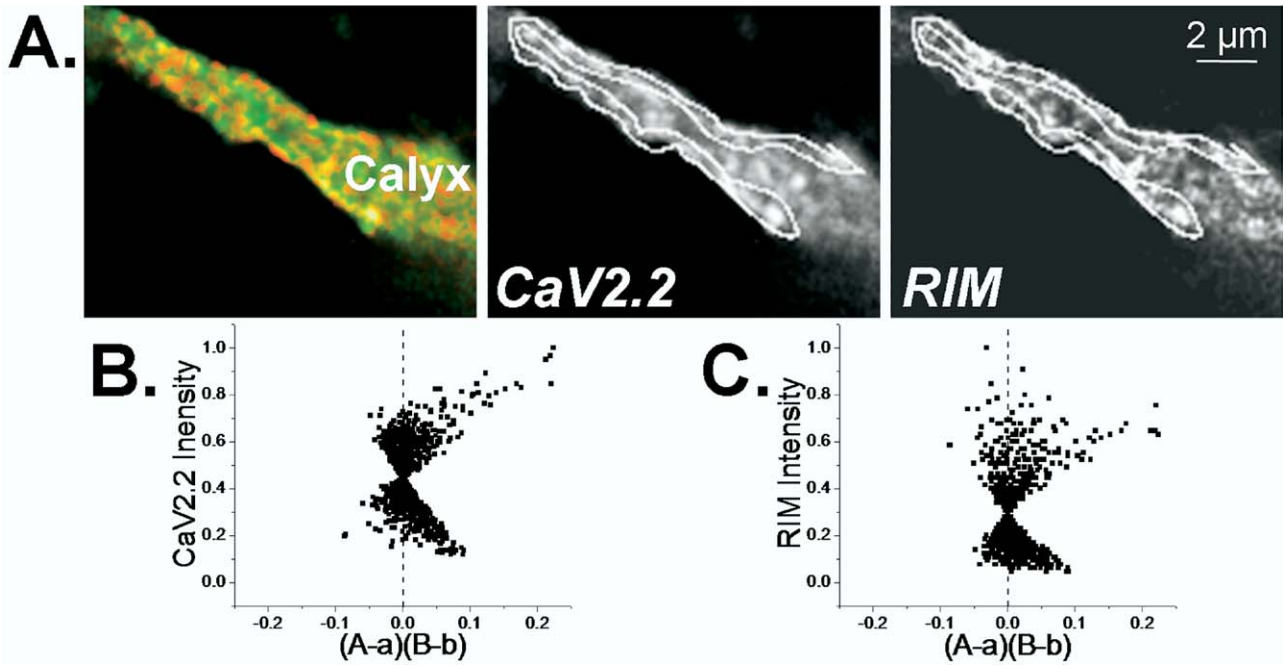

Fig. 3. (A–C) CaV2.2 remains co-localized with RIM in the fully isolated presynaptic terminal. CaV2.2 and RIM co-localization analysis was repeated as in Fig. 1A–C in calyx terminals that were fully dissociated from the postsynaptic terminal. Since the transmitter release face cannot be identified with confidence in fully isolated terminals (Stanley, 1991) we analyzed a contiguous area of the surface membrane that exhibited prominent CaV2.2 clusters. ICQ=0.12 ($P_{=0}<0.01$). Antibodies: CaV2.2, Ab571; RIM, RIM2.

tion method removes at least a significant part of the extracellular matrix (Sun et al., 2006). The staining intensity analysis resulted in strongly right-skewed ICA plots (Fig. 3B, C) and a positive mean ICQ (+0.19±0.02, $N=5$; $P_{=0}<0.001$) which was not significantly different from that for the attached calyces ($P>0.1$). Thus, the Ca^{2+} channels remain associated with RIM at the transmitter release face, even in the absence of the postsynaptic cell.

DISCUSSION

In this study we show that transmitter release face Ca^{2+} channel clusters are both co-localized and co-vary with RIM at the transmitter release face of intact presynaptic terminals. However, the finding that the two proteins do not co-precipitate suggests that they are not parts of a common molecular scaffold complex.

Ca^{2+} channels have been shown to be clustered at the transmitter release face by a number of experimental approaches including: freeze fracture (Heuser et al., 1974; Pumplin et al., 1981); imaging of Ca^{2+} influx (Llinas et al., 1992; Smith et al., 1993; Wachman et al., 2004; Photowala et al., 2005); light microscopy (Robitaille et al., 1990; Li et al., 2004); cell-attached patch clamp recording (Stanley, 1991), and surface membrane scanning at nanometer resolution (Haydon et al., 1994).

It is somewhat surprising that despite the highly critical, specific and evolutionarily conserved role of synaptic transmission, few proteins have been demonstrated to be exclusively associated with the TRS itself. Indeed, RIM (Wang et al., 1997) and the release site Ca^{2+} channel itself (Stanley, 1997) stand out as two of the few proteins that are sufficiently sequestered to the TRS to serve as markers. The channel is essential for the gated and local influx of Ca^{2+} ions to trigger the release mechanism and it is generally accepted that it is an integral element of the membrane-associated synaptic vesicle fusion apparatus (Stanley, 1997; Zhai and Bellen, 2004). In contrast, RIM was originally identified by its binding to Rab3, a small monomeric GTPase protein that is known to regulate synaptic vesicle dynamics. A number of studies have implicated RIM as a scaffolding protein involved with regulation of synaptic vesicle availability at the release site (Schoch et al., 2002). Recent studies including a RIM1 knockout suggest the main role of the protein is in the modulation of both short and long term potentiation via synaptic vesicle release site interactions or priming (Koushika et al., 2001; Calakos et al., 2004).

The association of RIM with the final steps in synaptic vesicle discharge should place it close to the Ca^{2+} channel cluster and its associated vesicle fusion sites. This physical co-localization combined with the functional association of both proteins with synaptic vesicle exocytosis is consistent with the hypothesis that they are parts of a common scaffold complex. Indeed, two studies have suggested that RIM is linked directly (Coppola et al., 2001) or indirectly (Hibino et al., 2002) to the Ca^{2+} channel. However, the first of these is based entirely on pull-down studies of the cytoplasmic C_2A and C_2B domains of RIM with a region from the II–III loop of the channel, a valid but yet relatively low stringency test for protein interactions, while the latter, based on a link via 'RIM binding protein' to a distal segment of the CaV2.2 C terminal, remains to be

confirmed. Our finding that RIM and calcium channel staining intensities co-vary is consistent with a common complex. However, the fact that they do not co-precipitate suggests that if such a link exists it is either of low affinity or it cannot survive the biochemical isolation procedure. The latter is unlikely since we have successfully co-precipitated a number of release site-associated proteins using this method (Li et al., 2004; Khanna et al., 2006) including the RIM/Munc-13 complex (Fig. 2D). Note that a failure to co-precipitate RIM cannot be attributed to occlusion of the RIM binding site by the antibody since anti-CaV2.2 antibodies with two totally distinct antigenic sites, the II–III linker and the extended C terminal, gave the same result (Fig. 2A). A second marker for synaptic vesicle interactions with the release site, Munc-13, also did not co-precipitate with the channel.

It is particularly interesting that the staining intensities for CaV2.2 and RIM exhibit a relatively high covariance and yet there is little evidence for a stable biochemical interaction. These findings suggest two independent protein complexes that interact with each other with a fixed stoichiometric ratio. Thus, the interaction between the synaptic vesicle fusion and synaptic vesicle docking mechanisms may be both restricted—such as in a one-to-one relationship—but reflect a dynamic interaction that allows the rapid coupling and uncoupling of the link. One possible advantage for a stoichiometric but loose linkage may be to provide a number of competing synaptic vesicle sources for each specific docking site, ensuring a supply that is not source limited.

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APPENDIX

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuroscience.2006.04.053.

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