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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).