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LONG SPLICE VARIANT N TYPE CALCIUM CHANNELS ARE CLUSTERED AT PRESYNAPTIC TRANSMITTER RELEASE SITES WITHOUT MODULAR ADAPTOR PROTEINS

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Abstract—The presynaptic N type Ca channel (CaV2.2) is associated with the transmitter release site apparatus and plays a critical role in the gating of transmitter release. It has been suggested that a distinct CaV2.2 long C terminal splice variant is targeted to the nerve terminal and is anchored at the release face by calcium/calmodulin-dependent serine protein kinase (CASK) and Munc-18-interacting protein (MINT), two modular adaptor proteins. We used the isolated chick ciliary ganglion calyx terminal together with two new antibodies (L4569, L4570) selective for CaV2.2 long C terminal splice variant to test these hypotheses. CaV2.2 long C terminal splice variant was present at the presynaptic transmitter release sites, as identified by Rab3a-interacting molecule (RIM) co-staining and quantitative immunocytochemistry. CASK was also present at the terminal both in conjunction with, and independent of its binding partner, MINT. Immunoprecipitation of CaV2.2 long C terminal splice variant from brain lysate coprecipitated CASK, confirming that these two proteins can form a complex. However, CASK was not colocalized either with CaV2.2 long C terminal splice variant or the transmitter release site marker RIM at the calyx terminal release face. Neither was MINT colocalized with CaV2.2 long C terminal splice variant. Our results show that native CaV2.2 long C terminal splice variant is targeted to the transmitter release sites at an intact presynaptic terminal. However, the lack of enrichment of CASK at the release site combined with the failure of this protein or its partner MINT to colocalize with CaV2.2 argues against the idea that these modular adaptor proteins anchor CaV2.2 at presynaptic nerve terminals.

Key words: CaV2.2, CASK, chick ciliary ganglion, transmitter release site, MINT, RIM.

The N type Ca channel (CaV2.2) is known to play a critical role in the gating of transmitter release at presynaptic nerve terminals. Physiological (Llinas et al., 1981; Stanley, 1993; Wachman et al., 2004), morphological (Robitaille et al., 1990) and ultrastructural (Heuser et al., 1974; Pumplin et al., 1981) findings suggest that these channels are clustered in close apposition to the transmitter release apparatus (Stanley, 1997).

CaV2.2 is known to exist in both short and long splice variant forms and the long variant, termed here CaV2.2-L, has an extended C terminal region with an additional 211 amino acids (Lu and Dunlap, 1999). This extended tail region harbors binding sites for two 'modular adaptor' proteins, calcium/calmodulin-dependent serine protein kinase (CASK) and Munc-18-interacting protein (MINT) (Maximov et al., 1999). It has been reported that this region is a key factor for the targeting of CaV2.2 to the presynaptic terminal (Maximov and Bezprozvanny, 2002). Thus, deletion of the modular adaptor binding sites reduced (but did not eliminate) CaV2.2 channels from axonal presynaptic-like varicosities in the hippocampal neuron cultures (Maximov and Bezprozvanny, 2002).

The above findings have also been used to support the idea that MINT and CASK not only assist in the trafficking CaV2.2-L to the nerve terminal but also serve as anchors at the transmitter release face (Davis and Bezprozvanny, 2001; Maximov et al., 1999; Maximov and Bezprozvanny, 2002; Zamponi, 2003). The evidence for channel attachment by these proteins is not, however, as compelling as that for targeting. CASK and MINT can bind the CaV2.2-L and are present at presynaptic nerve terminals (Hsueh et al., 1998; Maximov and Bezprozvanny, 2002). In addition, suppression of CASK and MINT by CASK RNAi or peptides corresponding to the MINT or CASK binding regions markedly interferes with transmitter release (Spafford et al., 2003). As assessed by recent reviews in the field, an anchoring role for the modular adaptor proteins has gained wide acceptance (Davis and Bezprozvanny, 2001; Zamponi, 2003; Zhai et al., 2001; Zhai and Bellen, 2004; Ziv and Garner, 2004). However, a direct association of these proteins at the release site has not been confirmed while interference in channel transport alone could account for the observed functional consequences on transmitter release.

We set out to test two main hypotheses: first, that CaV2.2-L is targeted to the transmitter release sites at a native and intact synapse and, second, to test if CASK and MINT are viable candidates as CaV2.2-L anchors at the transmitter release face. We developed two new high-affinity antibodies, L4569 and L4570, directed against a unique region within the long splice C terminal of CaV2.2-L. We used immunocytochemistry to localize

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Abbreviations: CASK, calcium/calmodulin-dependent serine protein kinase; CaV2.2, N type Ca channel alpha subunit; CaV2.2-L, long splice-variant of N type Ca channel alpha subunit; ICA, intensity correlation analysis; ICQ, intensity correlation quotient; MINT, Munc-18-interacting protein; RIM, Rab3a-interacting molecule.

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Table 1. Antibodies used in this study

Antibody	Source	IF[a]	Dilutions	
			IP	WB
CASK (m)	Chemicon Int. (Temecula, CA, USA)	1:20–100	1:1000	1:500
CASK (m)	BD Transduction (Mississauga, ON, Canada)	1:100	1:1000	1:1000
CaV2.2-L:				
L4569 (r)	This study[b]	1:100	1:500	1:500
L4570 (r)	This study[b]	1:100	1:500	1:500
MINT1 (p)	Stressgen Inc. (Victoria, BC, Canada)	1:50	1:1000	1:2000
pan CaV2.2 (r)	Li et al., 2004	1:100	1:200	1:750
RIM2[c] (r)	Synaptic Systems, Göttingen, FDR	1:100	–	–
Syntaxin 1 (m)	Sigma (St. Louis, MO, USA)	1:100	–	–

[a] Abbreviations used: IF, immunofluorescence; IP, immunoprecipitation; WB, Western blot; m, monoclonal; r, rabbit polyclonal; –, not tested here.
[b] Two rabbit polyclonal antibodies, L4569 and L4570, were generated using the same peptide: GTGRSYYHEADEDDWC (GenBank Accession #AAD51821) from a unique region of the long-splice variant CaV2.2 C-terminal (Invitrogen, Mississauga, ON, Canada) and purified by affinity chromatography.
[c] The RIM2 antibody detects multiple splice variants of the two main RIM genes—RIM1 and RIM2 (Wang et al., 2000, 2002; Wang and Sudhof, 2003).

the channels at the chick ciliary ganglion calyx-type presynaptic terminal. This preparation was used for several reasons: we have developed this isolated nerve terminal preparation for near light-limited resolution immunocytochemistry using high numerical aperture lenses. Further, the calyx has an extensive transmitter release face with multiple transmitter release sites at which the Ca channels are almost exclusively N type (Chan and Stanley, 2003; Stanley, 1991; Stanley and Goping, 1991; Yawo and Chuhma, 1994). We compared the location of immunostained proteins by a novel quantitative coimmunostaining analysis method, intensity correlation analysis (ICA), that can be used to test not only if the two target proteins are colocalized but if their staining intensities vary in synchrony, as expected for two proteins that are components of the same complex or sub-cellular structure (Li et al., 2004). We report that CaV2.2-L is, as predicted, localized to the transmitter release face and, based on co-staining with the marker Rab3a-interacting molecule (RIM) (Wang et al., 2002), is associated with the transmitter release sites.

A scaffold protein responsible for long term maintenance of a target protein must satisfy two minimal criteria: first, it must interact directly or indirectly with the target protein in a molecular complex and, second, the two proteins must be colocalized at the cellular region of interest. Thus, to test whether CASK and MINT are candidate CaV2.2-L release site anchors we first carried out binding studies to see if these proteins associate with CaV2.2-L. We then used quantitative immunocytochemistry to test if the two proteins colocalize with CaV2.2 and/or RIM at the nerve terminal transmitter release face. Our results support the first hypothesis since immunoprecipitation of CaV2.2-L coprecipitated both CASK and MINT. However, we failed to support the second hypothesis since neither protein colocalized with either the channel or RIM. The latter finding argues against the hypothesis that the modular adaptor proteins serve as CaV2.2 anchors at the transmitter release site.

EXPERIMENTAL PROCEDURES

Antibodies: see Table 1 for a description of antibodies used

Biochemical procedures. *Chick brain lysate* was prepared from 15 day chicken embryos (Frey's Hatchery, St. Jacobs, Ontario, Canada) homogenized on ice for 30–45 min in modified RIPA lysis buffer: 50 mM Tris–HCl, pH 8, 1% NP-40 (Igepal), 150 mM NaCl, 0.5% Na deoxycholate, and 1 mM EDTA, and

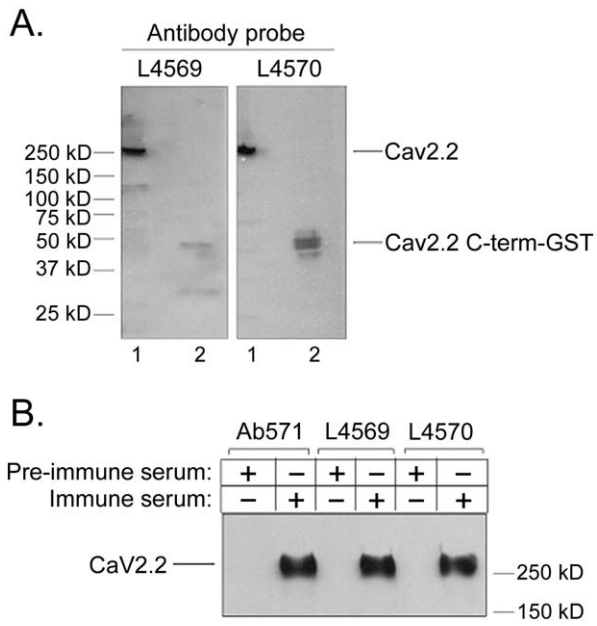

Fig. 1. Antibodies to the long splice region of CaV2.2 identify the full length protein. (A) Western blot of chick brain lysate (*lane 1*) probed with L4569 (*left panel*) or L4570 (*right panel*) reveal a high molecular weight band consistent with chick CaV2.2. Both antibodies also identified a CaV2.2-L, C-terminal fusion protein (*lane 2*). Molecular weight markers are as indicated (kD, kilodaltons). (B) Immunoprecipitations (IPs) from chick brain lysate with L4569, L4570 or Ab571 yielded an ~250 kD band specific for CaV2.2 as identified by the pan-CaV2.2 antibody, Ab571 (Li et al., 2004). Control IPs with the pre-immune serum from all three antibodies yielded no bands.