Long C terminal splice variant Ca\textsubscript{v}2.2 identified in presynaptic membrane by mass spectrometric analysis

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\textbf{Ca}_{2.2} voltage-gated calcium channels play a key role in the gating of transmitter release at presynaptic terminals. Recently we used mass spectrometry (MS) to analyze the protein complex associated with Ca\textsubscript{v}2.2 in purified presynaptic terminal membranes. A number of known and new Ca\textsubscript{v}2.2-associated proteins were identified, but not the channel itself. Here we set out to explore this anomaly. As previously, we used antibody \textit{Ab571} to capture the channel from purified synaptosome membrane lysate. We prepared a brain membrane lysate enriched for presynaptic active zones using standard methods to fractionate purified synaptosomes. These were osmotically lysed to generate a fraction enriched in presynaptic surface membranes. The lysate was solubilized in modified RIPA buffer and was passed over anti-Ca\textsubscript{v}2.2 antibody covalently bonded to immunoprecipitation beads. Captured complexes on the beads were then stripped of weakly-bound proteins by exposure to high salt to enrich the channel fraction. Proteins remaining bound to the sample were recovered in high concentration urea and the sample was subjected to standard enzyme digestion and MS analysis. We identified 12 distinct Ca\textsubscript{v}2.2 peptides, but no other ion channel peptides, in the lysate-exposed bead sample but no other ion channel peptides were recovered. Interestingly one of the channel peptides was derived from the alternatively spliced, long-C terminal region. Hence, confidence in identification of Ca\textsubscript{v}2.2 was beyond reasonable doubt. The identification of the long-splice Ca\textsubscript{v}2.2 provides compelling evidence that this variant is targeted to the presynaptic terminal, as we and others have suggested.

\textbf{Introduction}

Voltage-gated calcium channels (Ca\textsubscript{v}2.2) play a key role in the gating of transmitter release at presynaptic terminals and are known to be associated with the secretory vesicle fusion machinery.\textsuperscript{1,4} Recently, as part of the goal of characterizing the presynaptic Ca\textsubscript{v}2.2 scaffold, we carried out a mass spectrometric (MS) analysis of the proteins captured with this channel from purified synaptosome membrane lysates. The channel was captured with a well-characterized, high-affinity anti-Ca\textsubscript{v}2.2 antibody \textit{Ab571} immobilized on a gel matrix. We employed low-stringency solubilization and wash conditions in order to capture a large number of associated proteins. The experiment successfully identified many known and also new Ca\textsubscript{v}2.2-associated proteins but failed to detect the channel itself. Although unexpected, it was not inconsistent with previous work as no previous MS-based analyses of neuronal cell membranes or presynaptic terminals that we are aware of has successfully detected Ca\textsubscript{v}2-family (Ca\textsubscript{v}2.1, Ca\textsubscript{v}2.2, Ca\textsubscript{v}2.3; herein Ca\textsubscript{v}2.x) calcium channels. Indeed, only Ca\textsubscript{v}2.3 has been detected in any cellular proteomic analysis.\textsuperscript{3}

In this study we set out to test the hypothesis that the reason the Ca\textsubscript{v}2.2 channel was missed in our previous study was that, despite being the target of our
antibody, it was still at a low concentration compared to the other proteins in the complex. At first sight, this idea may sound strange considering that it is the primary protein identified by the antibody. However, we have shown previously that immunoprecipitation (IP) of CaV2.2 from presynaptic terminals actually recovers a large fraction of the transmitter release site complex (Fig. 1) and that this complex can be imaged by ultra-high-resolution (4pi) light microscopy with a diameter of ~200 nm. Such a large structure, which we hypothesize reflects a ‘release site organelle’ could contain far more copies of the co-IP proteins than the channels themselves; proteins that we suppose dominate the mass spectrometric analysis. We have previously analyzed relative binding strength, with respect to electrostatic interactions of proteins in a complex by a method we termed ‘Fractional Recovery’ (FR)6. In this method, we expose the immobilized complex to a high-salt challenge and quantify the relative recovery of associated proteins and we have shown that this method dissociates a large fraction of CaV2.2-associated proteins.7 We reasoned that with FR treatment we would increase the relative concentration of the channel itself, perhaps to a point where we could detect it by MS/MS analysis.

Results

Immobilized antibody capture of CaV2.2. Ab571 was immobilized by cross-linking to protein-A plus gel using a standard protocol (see Methods). As in our previous study our objective was to examine CaV2.2 channels associated with transmitter release sites on nerve terminal surface membranes. For this we fractionated brain lysates and isolated synaptosome membranes. For this we fractionated brain lysates and isolated synaptosome membranes. As described previously. This also served to concentrate CaV2.2 in our sample compared to whole brain. ~40 chick brains were dissected for each experiment and the purified synaptosome membrane fraction was solubilized in RIPA buffer and incubated with the immobilized antibody. As shown previously, a western blot of this lysate probed with Ab571 (or an unrelated anti-CaV2.2 antibody) exhibited prominent protein bands corresponding to the molecular weight of CaV2.2 (Fig. 1, lane 1).

High salt displacement of weakly bound proteins. After treatment with lysate the immobilized antibody and its bound protein complexes were exposed to wash buffer with elevated NaCl (1,150 mM).6 We have previously demonstrated that this treatment dissociates proteins that are associated with the channel via relatively weak electrostatic bonds.4,7 As shown previously, the high salt treatment does not displace the channel itself from the antibody (Fig. 1, lane 2).5,7

Protein recovery from the gel. Proteins were eluted from the gel by incubation in high concentration urea (8 M). This method was preferred to the previously employed denaturing of all the gel-associated proteins by boiling in SDS since we found that it permits reconstitution and reuse of the immobilized antibody, a significant technical improvement considering our dwindling antibody stocks. We confirmed that the channel was eluted from the Ab571, but not control IgG, gel by western blot (Fig. 1, lanes 4 and 5).

Mass spectrometric analysis. The urea-eluted proteins were subjected to trypsin digestion and were analyzed by standard LC MS/MS mass spectrometry (Fig. 2). In two separate experiments we identified 12 peptide fragments attributed to CaV2.2 (Table 1). In experiment 1 we identified 11 CaV2.2 peptides. Four peptides were detected in experiment 2. Three reproduced the same as in experiment 1 while one was a novel sequence. These peptides were all from intracellular domains of the protein but otherwise their origins span almost the full-length of the protein, including two in the N terminus (#1–2), four in the II–III loop (#3–6), and seven in the C terminus. One CaV2.2 peptide (#12) heralded from the alternatively spliced, long-C terminal variant provides additional evidence that this variant is targeted to the presynaptic terminal, an idea that was originally proposed on the basis of expressed CaV2.2 channel C terminal fragments11 and demonstrated for native channels using an anti-long-splice-specific antibody.8 This finding is consistent with the idea that the targeting motif for presynaptic terminals is present in the long splice region, as suggested previously.11 While we cannot rule out the possibility that the short-C terminal splice channel is also targeted to the release face, we can hypothesize that the targeting motif is associated with this region, consistent with the idea that the presynaptic CaV2.2 channel is a specific variant, uniquely adapted for this role.

This study was prompted by our earlier report in which we listed CaV2.2-binding proteins and yet were unable to detect the channel itself. We hypothesized that the channel was missed due to high concentrations of co-precipitated proteins.12 Here we set out to reduce these associated proteins from the CaV2.2 complex by a high-salt wash prior to eluting the proteins captured by the gel.4,6 The demonstration that CaV2.2 captured by Ab571 can be detected by mass spectrometric analysis provides additional confidence in the list.

Discussion

In this study we used a high-affinity anti-CaV2.2 antibody as a tool to capture the channel from purified chick synaptosome membrane lysate. The captured proteins were subjected to high salt to dissociate loosely bound complex partners and were then solubilized, digested and subjected to mass spectrometric analysis. CaV2.2 was identified with very high confidence by the characterization of 12 peptides that originated from virtually the full length of the channel. This is the first report of mass spectrometric identification of the CaV2.2 channel. Its detection was made possible by three main factors: first the purification of synaptosome membrane enriched for the protein; second, the availability of a specific and high-affinity anti-CaV2.2 antibody, Ab571, and third, the shedding of channel-associated proteins by means of a high-salt wash.

The detection of a peptide from the alternatively spliced, long-C terminal variant provides additional evidence that this variant is targeted to the presynaptic terminal, an idea that was originally proposed on the basis of expressed CaV2.2 channel C terminal fragments11 and demonstrated for native channels using an anti-long-splice-specific antibody.8 This finding is consistent with the idea that the targeting motif for presynaptic terminals is present in the long splice region, as suggested previously.11 While we cannot rule out the possibility that the short-C terminal splice channel is also targeted to the release face, we can hypothesize that the targeting motif is associated with this region, consistent with the idea that the presynaptic CaV2.2 channel is a specific variant, uniquely adapted for this role.
Figure 1. Immobilized Ab571 captures Ca_{2.2} from synaptosome membrane lysates. Antibody Ab571 identifies a protein band corresponding to the molecular weight of Ca_{2.2} channels in a western blot of synaptosome membrane (SM) lysate (−4% IP sample; left lane). Ab571 or control rabbit IgG (RlgG) antibody immobilized on gels were incubated with SM lysate to capture the channel. Treatment of the gels with high salt (1,150 mM) did not elute Ca_{2.2} (lane 2). However, the channel was eluted from the Ab571 (lane 4) but not control RlgG (lane 5) by subsequent treatment with high urea (8 M). Molecular weight markers are shown on the left in kDa (kilodaltons).

Table 1. Identified Ca_{2.2} peptides.

| Peptide sequence       | Ca_{2.2} AA* | Ca_{2.2} domain | Sequest cross correlation score | X!Tandem score |
|-----------------------|-------------|-----------------|---------------------------------|--------------|
| (1) FGDDLPLTR         | 4           | N-terminal      | 2.57                            |              |
| (2) QNCFTVNR          | 58          | N-terminal      | 2.20                            |              |
| (3) ASCALYNELDPEER    | 798         | II-III loop     | 3.61                            | 3.41         |
| (4) THLDRPLVEPR       | 827         | II-III loop     | 2.23                            | 2.41         |
| (5) LSPVDQEVEQTK      | 848         | II-III loop     | 2.91                            | 4.55         |
| (6) VPEQPEDADNQK      | 1073        | C-terminal      | 2.98                            |              |
| (7) ATLEQTQPSAFSSAK   | 1917        | C-terminal      | 3.87                            | 8.92         |
| (8) SSSALNNGTLPAPEGGIK| 1938        | C-terminal      | 5.54                            |              |
| (9) TQDVYETR          | 1966        | C-terminal      | 1.77                            | 1.68         |
| (10) GHSIEPIER        | 1981        | C-terminal      | 2.65                            | 5.29         |
| (11) TTHQGLADGEAVAQSGESSLK | 2099 | C-terminal | 5.66                            | 11.51        |
| (12) GLSEHINALLR      | 2246        | C-terminal      | 1.72                            | 1.96         |

* Amino acid numbers refer to chick Ca_{2.2} splice variant cdB1 (Accession# AAD51815); cy, cytoplasmic; “Balgh et al. 2008.

Figure 2. MS/MS spectrum example for a Ca_{2.2} peptide. Proteins eluted as in Figure 1 were subjected to reduction/alkylation followed by overnight trypsin digestion. The digests were analyzed by LC MS/MS. The peptide sequence corresponds to ATLEQTQPSAFSSAK (see Table 1).
of channel-associated proteins itemized in the earlier report. Further, the high salt-exposure method may serve as a general approach to characterize the primary, antibody-directed, protein by MS/MS.

Methods

Animals. All research was carried out on chick embryo neural tissue from pre-terminal (15 day) eggs and do not require animal protocol approval according to Canadian and International guidelines.

Synaptosome preparation. Whole E15-17 chick brains were dissected and homogenized in homogenization buffer (0.32 M sucrose, 10 mM HEPES pH 7.4, 2 mM EDTA, supplemented with a protease inhibitor cocktail) using a glass Teflon handheld homogenizer. The homogenate was centrifuged at 1,000 x g for 15 minutes to remove cell debris, followed by a 30 minute high-speed spin at 200,000 x g. The pellet was washed by resuspension in homogenization buffer and recovered by centrifugation, which was repeated to yield a crude membrane pellet used for further fractionation. Resuspended crude membrane fraction was gently layered onto a discontinuous sucrose gradient (0.8 M/1.2 M sucrose) and centrifuged for 2 hours at 100,000 x g in a swinging bucket rotor (SW41Ti). The gradient interphase (0.8 M/1.2 M) containing purified synaptosomes was collected. The purified synaptosome fraction was lysed by osmotic shock (10 mM HEPES, 2 mM EDTA, pH 7.4), and was mixed with wash buffer (150 mM NaCl, 2 mM EDTA, pH 7.4) and was incubated with disuccinimidyl suberate (DSS; Pierce). 15 µl of DSS was added to the antibody/gel mixture and incubated at room temperature for 45 minutes with gentle shaking. All the wash steps were carried out as described in the manufacturer’s protocol. The immobilized antibody was stored in binding buffer supplemented with 0.02% sodium azide.

Immunoprecipitation. Chick SM lysates were centrifuged at 10,000 x g for 10 minutes to pellet the insoluble fraction and were then pre-cleared with protein A plus gel (Pierce; 25 µl of 50% slurry) for 1 hour at 4°C. The cleared lysates were incubated with immobilized Ab571 or RIgG overnight at 4°C with gentle rotation. The gel was then washed 3X with wash buffer (Pierce; 0.14 M NaCl; 0.008 M sodium phosphate, 0.002 M potassium phosphate, and 0.01 M KCl, pH 7.4) and was mixed with ~100 µl packed volume of Protein A plus gel (Pierce). The antibody/Protein A gel mixture was incubated overnight at 4°C with gentle rotation allowing antibody binding to Protein A plus gel. After the binding reaction, both Ab571 and RIgG were cross-linked using disuccinimidyl suberate (DSS; Pierce). 15 µl of DSS was added to the antibody/gel mixture and incubated at room temperature for 45 minutes with gentle shaking. All the wash steps were carried out as described in the manufacturer’s protocol. The immobilized antibody was stored in binding buffer supplemented with 0.02% sodium azide.

High salt treatment and protein recovery. The antibody/protein complexes on the gel were incubated with a 100 µl of high salt wash buffer (NaCl concentration in the Pierce wash buffer was increased to 1,150 mM) for 15 minutes and the supernatant was reserved for western blot analysis. Protein complexes bound to the immobilized antibodies after the high salt treatment were recovered by a 30 minute incubation with 8 M urea at room temperature. The supernatant containing the eluted proteins was reserved for mass spectrometric and western blot analysis. The protein samples were subjected to in-solution reduction, alkylation and trypsin digestion prior to mass spectrometric analysis as described below.

Western blotting. Protein samples were diluted with Laemmli sample buffer (Biorad) supplemented with 5% β-mercaptoethanol (Sigma-Aldrich) using a 1:1 ratio and boiled at 95°C for 5 minutes followed by SDS-PAGE separation and western blotting using standard procedures.

Reduction, alkylation and proteolytic digestion. The urea eluted protein samples were reduced with 2.5 mM Dithiothreitol (DTT; BioShop) for 30 minutes at 50°C, which was followed by alkylation at 25°C for 30 minutes in the dark using 50 mM iodoacetamide (Sigma-Aldrich). Both DTT and iodoacetamide were dissolved in 50 mM ammonium bicarbonate (pH 8.0) immediately before use. The reduced/alkylated protein samples were diluted in 50 mM ammonium bicarbonate (pH 8.0) to lower the final urea concentration to 4 M and were then incubated with 0.3 µg of sequencing grade modified trypsin (Promega) overnight at 37°C. Tryptsin solution was prepared according to the manufacturer’s protocol (Promega).

Mass spectrometric analysis. The peptides were suspended in 0.1% formic acid prior to mass spectrometry analysis. Mass spectrometry was performed on a linear ion-trap/orbitrap hybrid instrument (Thermo-Fisher Scientific, San Jose CA) using a split-free nano-LC system (EASY nLC, Proxeon Biosciences, Odense Denmark). The peptides were concentrated using an on-line C18 trapping column and separated using a 75 µm ID column packed with Magic C-18 resin (Michrom Biosciences) with a gradient of 0 to 40% acetonitrile over 100 minutes. MS data was acquired at 60,000 fwhm resolution in the Orbitrap and MS/MS data was acquired in the linear ion-trap in a data-dependent fashion. Carbamidomethyl-cysteine was considered as a static modification, the oxidation of methionine and the deamidation of asparagine and glutamine were considered as partial modifications. Parent ion accuracy was 5 ppm and fragment ion accuracy was set at 0.5 Da. The search engine results were analyzed using the Protein Prophet and Peptide Prophet algorithms contained within the Scaffold platform (Proteome Software, Portland, OR). In addition, all spectra representing Ca,2.2 were examined manually.
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