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Bipartite syntaxin 1A interactions mediate $Ca_V2.2$ calcium channel regulation

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ABSTRACT

Functional interactions between syntaxin 1A and Ca_V2 calcium channels are critical for fast neurotransmitter release in the mammalian brain, and coexpression of syntaxin 1A with these channels not only regulates channel availability, but also promotes G-protein inhibition. Both the syntaxin 1A C-terminal H3 domain, and N-terminal Ha domain have been shown to interact with the $Ca_V2.2$ channel synprint region, suggesting a bipartite model of functional interaction, however the molecular determinants of this interaction have not been closely investigated. We used in vitro binding assays to assess interactions of syntaxin 1A truncation mutants with $Ca_V2.2$ synprint and $Ca_V2.3$ II–III linker regions. We identified two distinct interactions between the $Ca_V2.2$ synprint region and syntaxin 1A: the first between C-terminal H3c domain of syntaxin 1A and residues 822–872 of $Ca_V2.2$; and the second between the N-terminal 10 residues of the syntaxin 1A Ha region and residues 718–771 of $Ca_V2.2$. The N-terminal syntaxin 1A fragment also interacted with the $Ca_V2.3$ II–III linker. We then performed whole cell patch clamp recordings to test the effects of a putative interacting syntaxin 1A N-terminus peptide with $Ca_V2.2$ and $Ca_V2.3$ channels in a recombinant expression system. A YFP-tagged peptide corresponding to the N-terminal 10 residues of the syntaxin 1A Ha domain was sufficient to allosterically inhibit both $Ca_V2.2$ and $Ca_V2.3$ channel function but had no effect on G-protein mediated inhibition. Our results support a model of bipartite functional interactions between syntaxin 1A and $Ca_V2.2$ channels and add accuracy to the two putative interacting domains, consistent with previous studies. Furthermore, we highlight the syntaxin 1A N-terminus as the minimal determinant for functional regulation of $Ca_V2.2$ and $Ca_V2.3$ channels.

1. Introduction

The Ca_V2 family of voltage-dependent calcium channels plays a critical role in calcium-dependent, fast neurotransmitter release in the mammalian presynapse [1–4]. This process is tightly regulated, with the synaptic protein syntaxin 1A playing a central role in regulating Ca_V2 channel function bidirectionally, first by inhibiting the channel in the absence of a docked vesicle, and second, by permitting calcium entry only when a mature SNARE complex, primed for neurotransmitter release, is available [5] (for review see [6]). When Ca_V2 channel interactions with syntaxin 1A are perturbed [7] or abolished [8], neurotransmission is compromised.

Syntaxin 1A was first found to associate with the $Ca_V2.2$ channel at a motif on the channel II–III linker, coined the *syn*aptic *pro*tein *int*eraction (synprint) site [9,10]. These, and later studies, implicated the syntaxin 1A C-terminal H3 domain in this interaction [7,11,12]. The $Ca_V2.3$ channel interaction with syntaxin 1A was also thought to take place within the H3 domain [13], because binding and inhibition is lost following cleavage of syntaxin 1A by BoNTC1 [14]. It was argued that the H3 domain interaction was partially disrupted when syntaxin 1A switched from its 'closed' to 'open' configuration, becoming available for four-helical SNARE assembly, but remaining anchored to the channel. However, syntaxin 1A was later found able to bind multiple adjacent synprint motifs [15,16], with the N-terminal Ha region also interacting with synprint and causing functional inhibition of $Ca_V2.2$ [16–18]. This suggested that syntaxin 1A might mediate bipartite interactions with Ca_V2 channels [11,16].

Although several studies have examined the molecular determinants of interaction of either syntaxin 1A or the $Ca_V2.2$ synprint region in isolation, the putative sites of interaction and functional implications of a bipartite model have not been robustly investigated.

Here, we explore the molecular determinants of both syntaxin 1A and the $Ca_V2.2$ synprint region. Our results refine the putative location of two distinct sites of interaction between syntaxin 1A and the $Ca_V2.2$ synprint region. We also show that the N-terminal 10 residues of syntaxin 1A are sufficient to allosterically inhibit both $Ca_V2.2$ and $Ca_V2.3$, suggesting that channel inhibition and anchoring occur at two distinct sites.

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2. Materials and methods

2.1. Molecular biology

The $Ca_V2.2$ synprint region was subcloned into pTrcHisC to add an N-terminal Xpress epitope as previously described [17] then truncated from the C-terminus in approximately 50 residue intervals to produce coding sequences corresponding to (full length channel) residues 718–919 (ST4), 718–869 (ST3), 718–820 (ST2) and 718–767 (ST1) using site-directed mutagenesis [17]. The $Ca_V2.3$ II–III linker was subcloned into pTrcHisA at restriction sites 5′XhoI, 3′KpnI to add an N-terminal Xpress epitope. Cytoplasmic syntaxin 1A, cloned into pGex-4T-3 to add a GST-tag, and truncation mutants producing coding sequences 1–228, 1–183, 1–158, 1–109, and 1–69, are previously described [17,19]. GST-syntaxin 1A truncations within the Ha domain producing coding sequences for amino acids 1–59, 1–50, 1–40, 1–30, 1–20 and 1–10 were created by site-directed mutagenesis. The N-terminal 10 residues of syntaxin 1A M-KDRTQELRTA-K were scrambled to M-TLKATRRQDE-K as a negative control for $Ca_V2.2$ and Cav2.3 II–III linker binding specificity, created by annealing sense (5′-AAT TCC ATG ACC CTC AAG GCC ACG CGC CGA CAG GAC GAG AAG C-3′) and antisense (5′-GTA CTG GGA GTT CCG GTG CGC GGC TGT CCT GCT CTT CGA GCT-3′) oligonucleotides of the cut insert, at 50 °C for 30 min, then ligated directly into pGEX-5X-3 at restriction sites 5′EcoRI, 3′XhoI.

For confirmation of recombinant colocalization and electrophysiological analysis, the N-terminal 10 residues of syntaxin 1A were cloned into pEYFP-N at restriction sites 5′EcoRI, 3′XhoI to produce a peptide with a C-terminal YFP-tag (YFP-1A10), using Taq DNA polymerase (Invitrogen, Burlington, ON) and PCR steps as per manufacturer's recommendations.

2.2. In vitro binding assays

Protein lysate preparation and GST in vitro binding assays, PAGE and Western blotting were performed as previously described [17]. Protein interaction was confirmed by probing for Xpress-Ca_V2 using an anti-Xpress 1° antibody, then a HRP-conjugated anti-mouse 2° antibody (both Invitrogen, Burlington, ON). Xpress-tagged Ca_V2 proteins were visualized using standard ECL detection methods, developed and fixed. Blots were repeated a minimum of three times.

2.3. Electrophysiology

Characterization of $Ca_V2.2$ and $Ca_V2.3$ channels was conducted by cotransfecting rat cDNAs encoding their α_1, α_2-δ and β_{1b} subunits (3 μg each) into tsA-201 cells. Wildtype syntaxin 1A effects were investigated by cotransfection with rat full length syntaxin 1A cDNA (3 μg). The functional consequences syntaxin 1A N-terminal interaction was explored by coexpressing YFP-1A10 (5 μg). Cell culture and transfection techniques for all conditions are previously described [20]. Whole cell patch clamp recordings were conducted using external solution containing 20 mM $BaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 40 mM tetraethylammonium-chloride, 87.5 mM CsCl, 10 mM glucose (pH 7.7 with tetraethylammonium-OH) and internal solution containing 108 mM cesium-methanesulfonate, 4 mM $MgCl_2$, 9 mM EGTA, and 9 mM HEPES (pH 7.2 with CsOH). Cells expressing a GFP or YFP marker were selected for patching. Data was acquired using an Axopatch 200B amplifier running pClamp 9.0 software (Axon Instruments, Sunnyville, CA). Currents were low pass-filtered at 1 kHz and digitized at 10 kHz. Series resistance was compensated to 80%. Currents smaller than 80 ρA and larger than 2 nA were excluded from the data set.

Current–voltage relations for $Ca_V2.2$ and $Ca_V2.3$ were recorded by stepping to a test pulse of −60 mV to +60 mV from a holding potential of −100 mV. $Ca_V2.2$ steady-state inactivation data were recorded using a slow inactivation protocol as described previously by Degtiar et al. [21]. Currents were evoked at +10 mV for 20 ms before and immediately after a 30 s conditioning prepulse, applied at 10 mV increments from −100 mV to +10 mV. Channel recovery was promoted by holding the membrane at −100 mV for 60 s between sweeps. $Ca_V2.3$ steady-state inactivation data were recorded using a fast inactivation protocol (a 1500 ms conditioning prepulse applied at 10 mV increments from −100 mV to +10 mV). The steady-state inactivation data were fitted in Prism 5 (Graphpad, La Jolla, CA) using a modified Boltzmann equation. Voltage-dependence of inactivation (Vh) data were extracted from individual cell curve fit values calculated in SigmaPlot (Systat, San Jose, CA).

The ability of the N-terminal 10 residues of syntaxin 1A (YFP-1A10) or wildtype syntaxin 1A to elicit tonic G-protein inhibition of $Ca_V2.2$ and $Ca_V2.3$ channels was assessed as a ratio of peak current amplitude after (+PP) and 200 ms before (−PP) a strong depolarizing prepulse of +150 mV for 50 ms. Peak current amplitude was obtained from a test pulse of +10 mV for 15 ms.

Data analysis and offline leak subtraction was completed with Clampfit 9.0 (Axon Instruments, Sunnyville, CA). All statistical analysis was performed in Prism 5 (Graphpad, La Jolla, CA), using two-tailed unpaired *t*-test. Significance was taken as $p < 0.05$. All values are reported as mean ± standard error.

3. Results

3.1. Syntaxin 1A N- and C-terminal domains interact with distinct $Ca_V2.2$ synprint motifs

We previously demonstrated that full length syntaxin 1A and truncations removing domains H3c (228–268), H3b (183–268), H3a (158–268), Hc (109–268), Hb (69–268) interact with the $Ca_V2.2$ synprint region [17]. Here, we extended our work by assessing interactions between these and a further round of syntaxin 1A Ha domain truncation mutants (59, 50, 40, 30, 20 and 10) and C-terminal $Ca_V2.2$ synprint truncations removing residues 922–965 (ST4), 872–965 (ST3), 822–965 (ST2) and 771–965 (ST1; Fig. 1A). Interactions were determined by probing for Xpress-$Ca_V2.2$ synprint fragments that bound to GST-immobilized syntaxin 1A fragments in vitro. All syntaxin 1A truncations strongly interacted with ST4 (Fig. 1A, top panel) and ST3 (Fig. 1A, second top panel). The strong full length syntaxin 1A interactions with ST2 (Fig. 1A, second bottom panel) and ST1 (Fig. 1A, bottom panel) were weakened (1A228), and then lost (1A183, 1A158, 1A109) as successive syntaxin 1A domains were removed. This loss of binding suggests a first interaction site between the C-terminal H3c domain of syntaxin 1A and $Ca_V2.2$[822–872].

ST2 regained weak interactions within the Ha domain (1A69, 1A59, 1A50, 1A40, 1A30, 1A20) that recovered strength at the N-terminus (1A10), whereas ST1 regained strong interactions further C-terminal in the Ha domain (1A59, 1A50, 1A40, 1A30). This gain of binding suggests a second site of interaction between as little as the N-terminal 10 residues of the syntaxin 1A Ha region and $Ca_V2.2$[718–771].

To rule out the possibility of non-specific antibody interactions, pure Xpress-synprint protein lysate was run alongside the same protein bound to GST-syntaxin 1A (Fig. 1B). Reactivity in both lanes suggested a positive match for Xpress-synprint. Neither pure GST-syntaxin 1A nor GST protein lysate were detected by the anti-Xpress antibody.