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The C-terminus of human $Ca_v2.3$ voltage-gated calcium channel interacts with alternatively spliced calmodulin-2 expressed in two human cell lines

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ABSTRACT

$Ca_v2.3$ containing voltage-activated Ca^{2+} channels are expressed in excitable cells and trigger neurotransmitter and peptide-hormone release. Their expression remote from the fast release sites leads to the accumulation of presynaptic Ca^{2+} which can both, facilitate and inhibit the influx of Ca^{2+} ions through $Ca_v2.3$. The facilitated Ca^{2+} influx was recently related to hippocampal postsynaptic facilitation and long term potentiation. To analyze Ca^{2+} mediated modulation of cellular processes more in detail, protein partners of the carboxy terminal tail of $Ca_v2.3$ were identified by yeast-2-hybrid screening, leading in two human cell lines to the detection of a novel, extended and rarely occurring splice variant of calmodulin-2 (CaM-2), called CaM-2-extended (CaM-2-ext). CaM-2-ext interacts biochemically with the C-terminus of $Ca_v2.3$ similar to the classical CaM-2 as shown by co-immunoprecipitation. Functionally, only CaM-2-ext reduces whole cell inward currents significantly. The insertion of the novel 46 nts long exon and the consecutive expression of CaM-2-ext must be dependent on a new upstream translation initiation site which is only rarely used in the tested human cell lines. The structure of the N-terminal extension is predicted to be more hydrophobic than the remaining CaM-2-ext protein, suggesting that it may help to dock it to the lipophilic membrane surrounding.

1. Introduction

Cytosolic Ca^{2+} ions modulate important cellular processes either by direct binding to an effector protein or more often by forming complexes with Ca^{2+}-dependent regulators which subsequently support muscle contraction, vesicle fusion for secretion and neurotransmitter release or gene transcription [1]. As the regulated increase of cytosolic Ca^{2+} concentration is very crucial for the living cell, the influx through plasmalemma Ca^{2+} channels is tightly controlled, by the incoming Ca^{2+} itself, which is known as Ca^{2+}-dependent inactivation (CDI) [2,3]. On the other hand, Ca^{2+} dependent facilitation (CDF) is a common feature of neuronal voltage gated Ca^{2+} channels (VGCC) which supports the increase in synaptic strength also by E-/R-type Ca^{2+} channels [4,5]. Based on structural data, calmodulin (CaM) in complex with Ca^{2+} channels may constitute a prototype for Ca^{2+} sensors that are intimately colocalized with Ca^{2+} sources [6–8].

For high-voltage gated (HVA) Ca^{2+} channels, at least two major interaction sites at the channel protein are known for Ca^{2+} activated processes. The interaction site with synaptic vesicle proteins was mapped to the II–III loop, which is related to excitation–contraction coupling in skeletal muscle and heart, and couples excitation through VGCC to secretion and neurotransmitter release [9–11]. Excitation transcription coupling however was found to be linked to a C-terminal peptide derived from HVA Ca^{2+} channels, which binds to a nuclear protein, associates with a promoter and regulates gene expression [12].

For $Ca_v2.3$ E-/R-type Ca^{2+} channels, splice variants differ by an arginine-rich segment within the II–III loop. This confers a unique

Abbreviations: $Ca_v2.3$, ion conducting α1 subunit of E-type voltage-gated Ca^{2+} channel; CDF, Ca^{2+} dependent facilitation; CDI, Ca^{2+} dependent inactivation; FLAG, a fusion tag consisting of eight amino acids (DYKDDDDK) including an enterokinase-cleavage site; HVA, high-voltage activated; myc, a fusion tag consisting of 11 amino acids (EQKLISEEDL); LTD, long term depression; LTP, long term potentiation; PKC, protein kinase C; RT, reverse transcription; VGCC, voltage-gated Ca^{2+} channel

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Ca^{2+}-sensitivity unrelated to Ca^{2+}/CaM and the C-terminal IQ-site to the major neuronal $Ca_v2.3$ splice variants [13–15].

The isoleucine–glutamine (IQ) domain on the channel C-terminus and adjacent regions interact with Ca^{2+}/CaM and have a preeminent role during Ca^{2+}regulated processes for Ca_v1 and Ca_v2 VGCCs [16,17]. X-ray crystallographic structures revealed opposite orientations of the bilobed Ca^{2+}/CaM protein complex for both subfamilies [6].

In vivo, in CA1 pyramidal neurons, Ca^{2+}/CaM regulates two opposing Ca^{2+}-dependent processes that underlie memory formation. The induction of long term potentiation (LTP) and long term depression (LTD) require activation of Ca^{2+}/CaM-dependent enzymes, such as the Ca^{2+}/CaM-dependent kinase II and calcineurin, respectively. Obviously, both lobes of CaM function as distinct Ca^{2+} sensors that differentially transduce Ca^{2+} signals to the downstream targets [18,19].

In the present study we have screened for interaction partners of the $Ca_v2.3$ C-terminus. By yeast two-hybrid (Y2H) screening, we have identified a novel calmodulin-2 (CaM-2) isoform that differs from known CaMs by an extended N-terminus. Transcripts containing an additional exon of 46 nts were identified in two different human cell lines. Further, by RACE-PCR, the upstream sequence was determined and found to be identical with the classical 5′-end of CaM-2 [20]. Both variants of CaM-2 interact biochemically with the C-terminus of $Ca_v2.3$, but only CaM-2-ext interacts functionally by reducing whole cell inward current in HEK-293T cells.

2. Material and methods

2.1. Yeast two-hybrid screen

Vectors, yeast strains, reagents, and methods were derived from the MATCHMAKER two-hybrid system (Clontech, Palo Alto, CA). The yeast strains *Saccharomyces cerevisiae* AH109 and Y187 (Clontech) were used as hosts (AH109 contains 2 nutritional reporter genes for adenine and histidine; both yeast strains contain the *LacZ* reporter gene). The DNA encoding the cytosolic linker between domains II and III and the C-terminal tail were amplified from vector pHB239 encoding the human E-type Ca^{2+} channel [21]. AH109 cells were transformed with vectors containing the bait. After transformation of bait and mating with library-strain, cells were plated on synthetic dropout (SD) to test for leucine, tryptophane, histidine and adenine prototrophy, and transactivation of α-galactosidase activity.

2.2. RNA isolation and reverse transcription-polymerase chain reaction

Total RNA was isolated from cell cultures and brain using standard protocols. Small tissue samples from cell lines were processed using Tri-reagent (Sigma, Munich/Germany). Fragments of cDNA were amplified by polymerase chain reaction (PCR, Biometra, Heidelberg/Germany) after reverse transcription (RT) of total RNA [21,22].

For the detection of both, the canonical and the extended calmodulin, oligonucleotide CaMextfwd was used as the forward primer, and oligonucleotide CaMextrev as reverse primer (Table 1). In controls, the amplified DNA-fragment was digested with HindIII and EcoRI. For the detection of the extended CaM alone, the former reverse primer CaMextrev and the specific primer CaMinsertfwd located in the novel exon 1a were used (Table 1). Oligonucleotide primers were purchased from Eurogentec (Belgium). The identity of amplified extended calmodulin cDNA was confirmed by digestion with indicative restriction enzymes yielding the predicted sizes of cDNA-fragments.

2.3. Sequencing and digestion with restriction enzymes

Amplified cDNA fragments were processed using a PCR Purification Kit (Qiagen, Hilden/Germany) and sequenced on an ABI Prism™ 377 DNA Sequencer (Perkin Elmer/Applied Biosystems) using the Taq FS dyeDeoxy-terminator cycle sequencing chemistry.

2.4. Rapid amplification of cDNA ends (RACE) PCR

The oligonucleotide primers, which were designed for the detection of the novel and the canonical 5′ end of CaM-2, are listed in Table 1. One microgram of HEK-293 total RNA, 1 μl of 12 μM 5′-RACE CDS Primer A, 1 μl of 12 μM linker oligonucleotide (SMART IIA; Clontech), and nuclease-free water to 5 μl were incubated for

Table 1
Oligonucleotide primer sequences.

Step / #	Primer	Sequence	Miscelaneous
RACE-PCR			
	SMART IIA	5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′	for RT
	5′-PCR Primer	5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′	RACE, fwd
	RACECaM2EX1a/1rv	5′-GTACAAAGCTAACCATGCTGCAAGC-3′	RACE, rev
	RACECaM2EX1arv	5′-CTTGCAGGGTTGTTGTGCAAAGTG-3′	RACE, rev
	CaMkl5racerva	5′-CTCTTCAGTCAGTTGGTCAGCCAT GCTGCAAGC-3′	RACE, rev
	CaMkl5racervb	5′-CTGCTCTTCAGTCAGTTGGTCAGCCATGCTGCAAGCG-3′	RACE, rev
RT-PCR			
	CaMextfwd	5′-TGG TTG TGT GGT CGC GTC TCG G –3′	Fig. 3B
	CaMextrev	5′-CCA TCA TTG TCA GAA ATT CAG GG-3′	Fig. 3B, 3D
	CaMinsertfwd	5′-GTTAGCTTTGTACATAGCATCTCAC-3′	Fig. 3D (in exon1a)
			U94725:
# 1	CaM2UTRfw1	5′-ATCTGTGGAGTTAGAAATCC-3′	6 – 25
# 2	CaM2UTRfw2	5′-GCAAAGAACGCGAAGAGG-3′	384 – 401
# 3	CaM2specfw1b	5′-GCAGGTACAAACCAGCCAAT-3′	804 – 823
# 4	CaM2specfw1	5′-CCACCGGCCTTTTAACGG-3′	910 – 927
# 5	CaM2specrv1b	5′-CGGATGACGTAAGTGGGTTT-3′	960 – 941
# 6	CaM2specfw2	5′-GGCGGAGGGATCTGGCGG-3′	1085 – 1102
# 7a	CaMext5lnfwd	5′-GAATTAGTCCGAGTGGA-3′	1140 – 1156
# 7b	CaMext5shfwd	5′-AGTCCGAGTGGAGAGAG-3′	1145 – 1161
# 8	CaMext5comrv	5′-CATGCTGCAAGCGCTAC-3′	1215 – 1199
# 9	CaM2specfw2b	5′-TTGCAGCATGGTTAGCTTTG-3′	1206 – exon1a
# 10	CaM2specrev2	5′-CAAAGCTAACCATGCTGCAA-3′	exon1a − 1206
# 11	CaM2specrev	5′-GCAGGGTTGTTGTGCAAAGTG-3′	in exon1a