Biochemical and Biophysical Research Communications 388 (2009) 549–553

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Calneuron I inhibits Ca^{2+} channel activity in bovine chromaffin cells

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ARTICLE INFO

Article history:
Received 1 August 2009
Available online 12 August 2009

Keywords:
Calcium binding protein
Calcium current
EF-hand motif
Calcium signaling

ABSTRACT

Calneuron I (CalnI) is a calmodulin-like protein that contains two functional EF-hand motifs at the N-terminal and a hydrophobic segment at the C-terminal. CalnI was cloned from the adult rat cortex and fused with GFP at its N-terminal. When expressed in bovine chromaffin cells, wild-type CalnI was localized at the plasma membrane. However, a mutant that lacked the hydrophobic segment was localized in the cytosol and nucleus, while a Ca^{2+}-binding-deficient mutant was found in the cytosol and at the plasma membrane. Evaluation using the whole-cell patch-clamp technique revealed that Ca^{2+} currents were inhibited by both wild-type CalnI and the Ca^{2+}-binding-deficient mutant. When the bovine N-type Ca^{2+} channel was expressed in 293T cells, Ca^{2+} currents were mostly inhibited by co-expression of CalnI, but not by the mutant without the hydrophobic tail. These results suggest that CalnI attenuates Ca^{2+} channel activity and that its subcellular localization is important for this effect.

Introduction

Changes in the amounts of freely available Ca^{2+} are vital to the activity of many proteins with Ca^{2+} binding abilities [1,2]. Calmodulin (CaM) has four EF-hand motifs for Ca^{2+} binding and is transformed into an active form only when bound with Ca^{2+}, allowing it to modulate many physiological functions [3,4]. Recently, many proteins with structures similar to calmodulin have been identified and shown to modulate Ca^{2+} signaling pathways [5,6].

While CaM is ubiquitously expressed in almost every cell studied, most CaM-like Ca^{2+} binding proteins (CaBPs) are highly expressed in neurons, which hints at their roles in modulating neuron specific activities. These CaBPs have four putative EF-hand Ca^{2+} binding motifs; however, usually only two or three of them are functional in chelating Ca^{2+} [5,6]. Calneuron I (CalnI) was first identified in 2001, when Wu et al. [7] screened candidate genes potentially responsible for Williams syndrome, yet CalnI is localized outside of the region in the genome known to be responsible for this syndrome. CalnI has a sequence that is similar to CaM; however, only two EF-hand motifs, located in the N-terminal, are conserved for Ca^{2+} binding in CalnI. Additionally, CalnI is expressed in a brain-specific manner late in development, after P15 [7].

Unlike other CaBPs, CalnI displays a 38-a.a. hydrophobic extension located at the C-terminal, and has two EF-hand motifs with a Ca^{2+} binding affinity at 0.2–0.5 μM [8]. It is possible that CalnI is activated under physiological $[Ca^{2+}]_i$ to modulate Ca^{2+} signaling pathways. To verify how CalnI modulates cell activities, CalnI was cloned from the adult rat brain and expressed in bovine chromaffin cells. Our results demonstrate that CalnI localizes to the plasma membrane and inhibits Ca^{2+} currents. Therefore, CalnI may play an inhibitory role in neurotransmitter release and Ca^{2+} signaling.

Materials and methods

Chemicals. Hank's balanced salt solution (HBSS), Dulbecco's modified Eagle's medium (DMEM), and all other reagents for cell culture were purchased from Invitrogen Inc. (Carlsbad, CA, USA). All other chemicals were reagent grade and purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA), unless otherwise indicated.

Solutions. The normal HBSS bath buffer contained (in mM): 138 NaCl, 5.3 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 0.4 $MgSO_4$, 4 $NaHCO_3$, 0.34 Na_2HPO_4, 0.44 KH_2PO_4, 10 Hepes, and 5.6 glucose, pH 7.3. To measure Ca^{2+} currents (I_{Ca}), the patch pipette was filled with a Cs^+-containing solution composed of (in mM): 130 Cs-aspartate, 20 KCl, 1 $MgCl_2$, 0.1 EGTA, 3 Na_2ATP, 0.1 Na_2GTP, and 20 Hepes, pH 7.3. To evaluate the I_{Ca}, the cell was incubated in an *N*-methyl-D-glucamine (NMG) solution containing (in mM): 130 NMG, 2 KCl, 5 $CaCl_2$, 1 $MgCl_2$, 5.6 glucose, and 10 Hepes, pH 7.3.

Abbreviations: BES, *N*-bis[2-hydroxyethyl]2-aminoethanesulfonic acid; CaM, calmodulin; CalnI, calneuron I; CaBP, calcium binding protein; DAPI, 4′,6-diamidino-2-phenylindole; NMG, *N*-methyl-D-glucamine; HBSS, Hank's balanced salt solution.

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doi:10.1016/j.bbrc.2009.08.046

Cell preparation. Chromaffin cells were prepared by digesting bovine adrenal glands, which were obtained from local slaughterhouses, with collagenase (0.5 mg/mL). The cells were then purified by density gradient centrifugation, as described previously [9]. The cells were plated at a density of 2×10^5 on one 22 mm or three 10 mm poly-L-lysine-coated coverslip(s) in a 35-mm culture dish and in DMEM supplemented with 10% fetal bovine serum. The medium was replaced every two days. All experiments were carried out between days 3 and 10 after cell isolation.

Human embryonic kidney 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum in a humidified incubator with 5% CO_2.

Constructs and molecular biology. The plasmids for bovine N-type Ca^{2+} channels and their accessory subunits were generously provided by Dr. Aaron P. Fox (University of Chicago) [10]. The protocol used to prepare chromaffin cell cDNA was described previously [11]. Specific primer set with linkers (in parenthesis) containing restriction enzyme cutting sites (underlined) were designed according to cloned human and mouse CaInI [7] and the rat ETS data bank in order to amplify the coding region of CaInI from adult rat brain cDNA: (SalI) 5′-(GTCGACC)ATGCCGTTCCACCATGTAAC TGC-3′ and (Not I) 5′-(CGCGGCCGC)AACTCCATGCCGCTCCGCAGG-3′ for rat CaInI. The mutant without the C-terminal hydrophobic domain (CaInIΔHT) was constructed using the following reverse primer (Not I): (TGCGGCCGC)AACTTGCGGACGCAGGTCTGC. These constructs were first subcloned into a yT&A vector (Viogen Inc., Taipei, Taiwan), and then transferred into a pEGFP-C3 (Clontech, USA) vector by SalI and BamHI restriction enzymes. The mutants without functional EF-hand motifs [12] were constructed by PCR to replace the first two aspartate residues in both the N-terminal EF-hand motifs with alanine (D2A) with the following forward primers: 5′-CGTGTTCTGGCCAGAGCTGGG and 5′-GCCATGGCCGG AGCTGGGCA for 1st and 2nd EF-hand, respectively; to replace the last glutamate in both motifs with glutamine (E2Q) with the following forward primers: 5′-ATCTCTAAGCAGCAGCTGGGCAT and 5′-GTAGATTTTGATCAATTCATGACG for 1st and 2nd EF-hand, respectively; or to incorporate all of these changes (DE2AQ) (these residues are highlighted in Fig. 1A). All of these clones were verified by DNA sequencing.

Transfection. To transiently express those constructs in chromaffin or human embryonic kidney 293T cells, calcium phosphate transfection was used as described previously [13]. In brief, the plasmid (3 μg) was mixed for 30 min with 25 μL 1 M $CaCl_2$, 50 μL 2X BES-buffered saline, and water to make a final volume of 100 μL. To co-express the N-type Ca^{2+} channel with GFP or GFP-CaInI, GFP/GFP-CaInI, α1B, β2a, and α2δ were applied in a weight ratio of 1:2:2:3. This mixture was used to transfect cultured chromaffin cells as described previously [11]. Transfected cells with low GFP fluorescence signals, based on visual detection methods, were selected for recording.

Fluorescence imaging. To prepare the cells for fluorescent staining, chromaffin cells were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 30 min. The fixed cells were then permeabilized and stained with rhodamine-conjugated phalloidin (Invitrogen Inc., USA) and 4′,6-diamidino-2-phenylindole (DAPI) as described previously [11]. The stained cells were observed on a Leica TCS SP5 confocal microscope with a 100× objective.

Electrophysiological measurements. The recording setup was described previously [11]. In brief, the cell was incubated in NMG bath buffer containing 10 mM $CaCl_2$ and whole-cell voltage-clamped at −70 mV in order to measure the Ca^{2+} current. The cell was depolarized to various potentials for 100 ms once every 15 s. The maximal inward currents during depolarizations were normalized to the cell surface area represented by the capacitance compensation to represent the Ca^{2+} current for the current–voltage relationship.

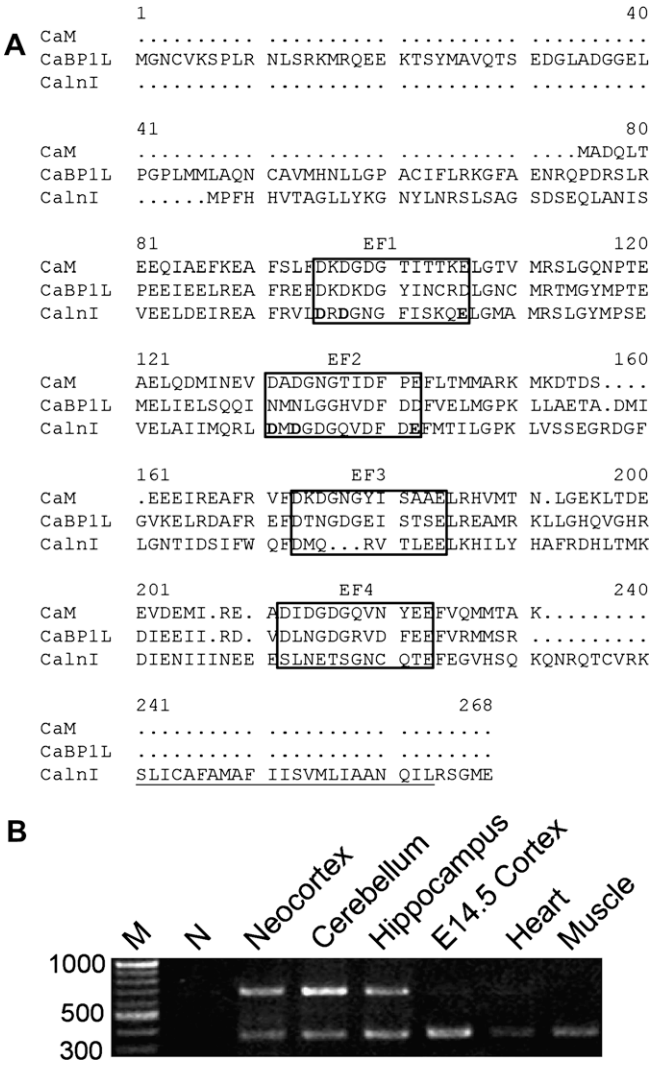

Fig. 1. CaInI expressed in the adult brain. (A) Amino acid sequence alignment of rat CaM, L-CaBP1, and CaInI. The putative EF-hand motifs are boxed and the conserved negatively charged a.a. in 1st and 2nd motifs for Ca^{2+} binding are in bold letters; the hydrophobic segment at the C-terminal of CaInI is underlined. (B) PCR amplification of CaInI from brain tissue. Specific primers, which covered the coding region (660 bp) of CaInI, were used to amplify the gene from cDNA synthesized from the adult rat neocortex, hippocampus, and cerebellum, as well as from the embryonic cortex (E14.5 cortex), adult heart and muscle. The band at approximately 350 bp, corresponding to actin, served as an internal control. M: 100 bp marker; N: negative control without cDNA.

Data analysis. Signals of current measurement were low-pass filtered at 3 kHz and stored in a Pentium III-based computer. The activation curve was plotted by normalizing the tail currents obtained at each depolarization potential to the current acquired at +60 mV. Normalized activation curves were fitted to a Boltzmann function, using the least-squares method according to: $A/\{1 + \exp[(V - V_{1/2})/\text{slope}] + b\}$, where V is the test voltage, $V_{1/2}$ is the midpoint of the activation curve, A is the amplitude and b is the baseline. All results are presented as means ± SEM and were analyzed using the Student's t test.

Results

CaInI expression in the adult brain

The predicted coding sequence of rat CaInI was deduced from the rat EST database deposited in the National Center for