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Original article

Identification of a cardiac isoform of the murine calcium channel α1C ($Ca_v1.2$-a) subunit and its preferential binding with the β2 subunit

Manabu Murakami [a,*], Takayoshi Ohba [b], Yoichiro Takahashi [b], Hiroyuki Watanabe [b], Ichiro Miyoshi [c], Shinsuke Nakayama [d], Kyoichi Ono [a], Hiroshi Ito [b], Toshihiko Iijima [a]

[a] *Department of Pharmacology, Akita University School of Medicine, Akita 010-8543, Japan*
[b] *Second Department of Internal Medicine, Akita University School of Medicine, Akita, Japan*
[c] *Center for Experimental Animal Science, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan*
[d] *Department of Cell Physiology, Nagoya University Graduate School of Medicine, Tsuruma-cho, Shouwa, Nagoya, Japan*

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Abstract

We describe a cardiac muscle isoform of the voltage-dependent calcium channel α1 subunit, which corresponds to the rabbit ortholog of α1C-a ($Ca_v1.2a$). We also cloned smooth muscle isoforms α1C-b ($Ca_v1.2b$) and α1C-d ($Ca_v1.2d$). Differences among these three isoforms lie within the N-terminal region (exon 1A or 1B), the sixth transmembrane segment of domain I (exon 8A or 8B), and the use of exon 10, which forms the intracellular loop between transmembrane domains I and II. Two-hybrid analysis revealed interactions among the three α1 isoforms and β subunits. In vitro overlay and immunoprecipitation analyses revealed preferential binding between α1C-a and β2, which is also expressed at a high level in the heart.

Keywords: Voltage-dependent calcium channel; α1 subunit; Cardiac muscle; cDNA; Overlay; β subunit

1. Introduction

Voltage-dependent calcium channels (VDCCs) are the main pathways by which calcium enters cells. VDCCs are multimeric complexes of a main pore-forming α1 subunit and other auxiliary subunits such as β, α2/δ, and γ. VDCCs play a crucial role in the control of calcium-linked cellular functions and determine the electrophysiological properties of cells. VDCCs are classified pharmacologically into five groups (T, L, N, R, and P/Q), and 10 genes have been described for the pore-forming α_1 subunits [1].

L-type calcium channels are crucial for excitation–contraction coupling, which is the main pathway for calcium entry into heart and smooth muscle tissue. L-type calcium channels are the targets of calcium channel blockers such as dihydropyridine, which are used widely to treat hypertension and angina pectoris. Among the 10 α1 subunits that have been identified, four genes encode L-type calcium channels ($Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$, and $Ca_v1.4$). Much attention has been paid to $Ca_v1.2$, which forms L-type calcium channels in cardiac and smooth muscle [1,2]. Although a single gene encodes pore-forming α1 subunits in these two clinically important tissues, the physiological properties of L-type channels in cardiac tissue differ from those in smooth muscle [3]. For example, smooth muscle L-type channels are much more sensitive to dihydropyridines, and this sensitivity depends on the membrane potential and other factors such as the channel assembly [4]. In addition, the $Ca_v1.2$ gene is responsible for Timothy syndrome, which is a multiorgan disorder of autosomal-dominant heredity with a long QT syndrome [5]. Although one cDNA isoform of $Ca_v1.2$ has been reported to exist in neurons [6], whether other isoforms of the $Ca_v1.2$ gene exist within the cardiovascular system has not been determined.

The channel pore-forming α1 subunit has four transmembrane domains (I–IV). It is widely accepted that a high-affinity interaction between α1 and β subunits occurs between two highly conserved domains in each molecule. Specifically, these

* Corresponding author. Tel./fax: +81 18 834 8930.
E-mail address: manabumurakami@excite.co.jp (M. Murakami).

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subunits interact via the alpha-interaction domain (AID), which is located between the first and second domains of the α1 subunit, and the beta-interaction domain (BID), which is located in the secondary conserved domain of β subunit [7]. Additional interaction sites may also exist [8].

The purpose of this study was to identify novel isoforms of $Ca_v1.2$ in murine cardiac tissue. We identified three novel splice variants of the $Ca_v1.2$ gene, which we designated $Ca_v1.2$-a, $Ca_v1.2$-b, and $Ca_v1.2$-d. Furthermore, we investigated the binding affinities of these novel isoforms of $Ca_v1.2$ for β subunits.

2. Materials and methods

2.1. RNA isolation and reverse transcriptase–polymerase chain reaction analysis

Total RNA was isolated from the heart, brain, and aorta of a C57/BL6 mouse using the RNeasy Kit (Qiagen, Valencia, CA, USA). Reverse transcription was carried out in a solution of 10 pmol oligo-dT primer, 1 μg RNA, 1× first strand cDNA buffer (Life Technologies, Rockville, MD, USA), 10 mM dithiothreitol, 0.4 mM dNTPs, 40 U RNasin, and 200 U Superscript II in a volume of 25 μl at 42 °C for 45 min. For the reverse transcriptase–polymerase chain reaction (RT-PCR), 2.0 μl of template was used. The full-length α1C-c specific sequence was amplified using primers H1s and (5′-ATGATTCGGGCCTTTGTTCAGCC-3′) and H2as (5′-CTACAGGTTGCTGACGTAGGACCT-3′), which correspond to the murine $Ca_v1.2$ subunit sequences M^1IRAFVQP^8 and R^{2163}SYVSNL^{2169} (including the stop codon), respectively. To amplify the full-length cDNA of α1C-c, we used primers H2as and C1s (5′-ATGGTCAATGAAAACACGAGGATG-3′), which corresponds to M^1VNENTRM^8. Splice-variant sequences (with/without exon 10) were amplified using primers 5s and (5′-ACCATGGAGGGCTGGACAGA-3′) and 4as (5′-AGACTCAGTCTCACTTGTGGG-3′), which correspond to the murine $Ca_v1.2$ subunit sequences T^{393}MEGWTD^{399} and G^{496}APAGLHD^{503}, respectively. The amplified sequences were subcloned into the pZero-2 plasmid (Invitrogen, Carlsbad, CA, USA) and were sequenced. The sequenced cDNA fragments were then inserted into DsRed-C1 (Clontech, Palo Alto, CA, USA) or pTRG (Stratagene, La Jolla, CA, USA) plasmids for expression analysis.

β2 and β3 subunit-specific sequences were amplified by PCR (35 cycles) with the following primers: B2S (5′-CTAGAGAACATGAGGCTACAG-3′) and B2A (5′-ACTGTTTGCACTGGGCTTAGG-3′), corresponding to the sequences of the murine β2 subunit L_{131}ENMRLQ$_{137}$ and P_{198}KPSANS$_{204}$, and B3S (5′-CTCAAACAGGAACAGAAGGCC-3′) and B3A (5′-CATAGCCTTTCAGAGAGGGTC-3′), corresponding to the sequences of the murine β3 subunit L_{129}KQEQKAR$_{136}$ and P_{185}SLKGYE$_{191}$.

To obtain constructs for the overlay analysis, we used primer 4as as well as the following primers:

7s (5′-AAAGAGAGGGAGAAAGCCAAA-3′; K^{440}ERE KAK^{446});
14s (5′-GCCCGAGGAGATTTCCAGAA-3′; A^{446}RGD FQK^{452});
13as (5′-TAGTTGCTGCTTCTCTCGAAG-3′; L^{453}REKQ QL^{459});
12as (5′-GTAGCCTTTGAGATCTTCTTCTAG-3′; L^{459}EED LKGY^{466});
11as (5′-TTCTGCCTGGGTGATCCAGTCC-3′; L^{467}DWIT GAE^{474});
8as (5′-ATCATGCAAGCCCGCTGGAGC-3′; RGAPAG LH);
9as (5′-CCAAGCAAACTTCCCTTTC-3′; DGKKGKFAW); and
10as (5′-CATGCTCACATGGGTTTCTGTA-3′; STETH VSM).

2.2. BacterioMatch two-hybrid assay

Full-length sequences of murine β2 and β3 were cloned into the pBT-bait plasmid (Stratagene), which contained λcI protein to activate the λ operator. PCR-amplified fragments of α1C-a, α1C-b, and α1C-d (amplified using primers 5s and 4as) were subcloned into the pTRG-target plasmid (Stratagene), which contained RNAP-α. These plasmids were used to transform the reporter strain (Stratagene), which was grown initially on agar plates that contained tetracycline, chloramphenicol, kanamycin, and carbenicillin (12.5, 34.0, 50.0, and 250.0 μg/ml, respectively; TCKC-agar plates). After this initial TCKC-agar plate-based screening, individual clones were further verified using a secondary marker, namely the β-galactosidase gene, on X-gal-containing (80 μg/ml) TCKC-agar plates [9].

2.3. Cell culture

Human embryonic kidney 293 (HEK-293) cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% dialyzed fetal bovine serum. HEK-293 cells were transfected with expression vectors that carried a portion of the cloned murine α1C sequence into the C-terminal region of DsRed in the pDsRed-C1 vector (Clontech). This permitted immunodetection and immunoprecipitation with anti-DsRed antibody (Clontech) after transient expression using the Lipofectamine Plus reagent (Invitrogen) [10].

2.4. Immunoprecipitation

Immunoprecipitation was performed using a protein G immunoprecipitation kit (Sigma, St. Louis, MO, USA). The cell pellet was re-suspended in 1.0 ml of lysis buffer (20 mM sodium phosphate, 150 mM sodium chloride, 10% glycerol, 1 mM ethylenediaminetetraacetic acid, 0.5% Triton-X 100, pH 7.2) and complete TM protease inhibitor cocktail (Roche, Basel, Switzerland) at a concentration of ~1 mg/ml. This suspension was set on ice for 1 h before being centrifuged at 10,000 × *g* at 4 °C for 15 min. The cleared lysate was incubated at 4 °C for 1 h with 2 μg of a polyclonal antibody directed against the DsRed-tagged protein, which was inserted at the N-terminus of each expression construct. Protein G sepharose (50 μl) was added to the samples, which were incubated for 16 h at 4 °C. The immunoprecipitate was washed five times