α_1-β interaction in voltage-gated cardiac L-type calcium channels

Andrea Marquart, Veit Flockerzi*

Institut für Pharmakologie, Universität Heidelberg, Im Neuenheimer Feld 366, D-69120 Heidelberg, Germany

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Abstract The β subunits of voltage-gated calcium channels normalize current amplitude, kinetics and voltage dependence of these channels by interacting with the channel's pore forming subunit α_1. By screening an epitope expression library of α_{1Ca} fusion proteins, a β2a binding site of 22 amino acids was identified within the I–II cytoplasmic linker but not on other cytoplasmic sequences of α_{1Ca}. This binding site overlaps by 14 amino acids with the conserved 18 amino acid peptide assumed to be essential for α_1-β interaction. The common 14 amino acid motif of α_{1Ca} is sufficient to bind β2a, and in addition β1a, β3 and β4.

Key words: High-voltage-gated calcium channel; Subunit composition; Protein interaction; Epitope library; Protein probing

1. Introduction

High-voltage-gated calcium channels are expressed in cells as multimeric protein complexes consisting of a principal subunit, α_1, which is responsible for the voltage-gated ion conductance and pharmacological sensitivity of the channel, and three or four auxiliary subunits, β, α_2, δ and γ [1]. So far, four β subunits have been characterized (β1, β2, β3 and β4). Coexpression of the β1 cDNA with α_{1S}, α_{1C} or α_{1A} increased current amplitude, shifted the activation threshold in the hyperpolarizing direction and affected activation and inactivation kinetics of the channel [2–6]. Similar modulating properties especially on activation of α_1-induced currents could be assigned to the β2, β3 and β4 subunits suggesting that the mechanisms whereby β subunits regulate the Ca^{2+} entry is largely conserved among all voltage-gated calcium channels. A first step towards an understanding of the structural basis of the interactions between α_1 and β subunits was made by Pragnell et al. [7], who identified a sequence motif of the intracellular loop of α_1 that connects the first and second of the four repeat domains as a critical point of contact with β. This motif is highly conserved in all α_1 subunits known so far. However, although it is known from expression studies that α_1 subunits reconstitute a functional channel with every β subunit, it seems unlikely that channel complexes are formed by random association via conserved binding sites. Rather it appears that in addition to distinct expression patterns of α_1 and β subunits the binding sites are determinant in α_1-β interaction. Additional amino acids yet to be identified as well as non-conserved residues within the conserved motif [7] may contribute to the preferential interaction of a given α_1 and β subunit and thereby influence the composition of native calcium channels and their functional properties. In addition, results obtained by expressing the channel in *Xenopus* oocytes [8,9] showed that activation and inactivation of α_1-induced currents by β are separable events, indicating that β subunits interact with at least one more site of α_1 in addition to the one found thus far.

We therefore started to define the structural requirements for α_1-β interaction using the cardiac L-type calcium channel as a model. The cardiac channel has been studied in detail and comprises the a-type splice variants of the β2 and α_{1C} gene products [1]. By constructing an α_{1Ca} epitope library which was probed with an in vitro synthesized ^{35}S-labelled β2a subunit we found that β binds to a cytoplasmic 22 amino acid motif in α_{1Ca} which contains 14 amino acids of the conserved α_1-β interaction site [7]. No other β binding site in α_{1Ca} was detected. Fusion proteins containing this common 14 amino acid motif bind β2a, and in addition β1a, β3 and β4, indicating that this motif fulfills the structural requirements for α_1-β interaction in general.

2. Materials and methods

2.1. *In vitro synthesis of the ^{35}S-labelled β subunit probes*

The [^{35}S]methionine-labelled β1a [10], β2a [11], β3 [11] and β4 [12] subunits were synthesized by coupled in vitro transcription and translation in the TNT system (Promega). Fig. 1A shows fluorograms of the in vitro translated ^{35}S-β probes. A protease inhibitor mix (antipain, aprotinin, chymostatin, leupeptin and pepstatin A, 0.1 ng/μl each), yeast t-RNA (40 ng/μl) and RNasin (0.8 U/μl, Promega) were added to the reaction to minimize proteolysis and reduce background translation.

2.2. *Construction and screening of an α_{1Ca} subunit epitope library*

To obtain randomly cleaved α_{1Ca} cDNA fragments (GenBank accession number X15539), 10 μg of cDNA subcloned in pcDNAI was incubated in the presence of 50 mM Tris-HCl, pH 7.5, 50 μg/ml BSA, 10 mM $MnCl_2$ and deoxyribonuclease I (DNase I) at concentrations of 1, 0.67 and 0.4 U/ml for 10 min at room temperature. Under these conditions cleavage is random [13] and fragment sizes can be controlled by varying the enzyme concentration. The reactions were stopped by addition of EDTA, pH 8.0 and glycerol at final concentrations of 16.7 mM and 5% (v/v), respectively. Aliquots of the reaction mixtures were analyzed by agarose gel electrophoresis (Fig. 1B). Reaction mixtures containing fragments of ~50–220 bp length were pooled, loaded onto a 2% agarose gel and electrophoresed. The DNA fragments were electroeluted. After extraction with phenol/chloroform the DNA was precipitated in the presence of sodium acetate, pH 7.0 (0.3 M) and ethanol, dried and resuspended. Assuming an average size of 135 bp 22.5 pmol of DNA ends were blunted by T4 DNA polymerase (1 U) in the presence of dNTPs (0.1 mM each dCTP, dGTP, dTTP, 1 mM dATP) for 20 min at 11°C. After inactivation of the enzyme (10 min, 75°C) the DNA was incubated in the presence of

*Corresponding author. Fax: (49) (6221) 546623.

Abbreviations: aa, amino acids; DNase I, deoxyribonuclease I; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecylsulfate

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0.6 U *Tth* DNA polymerase for 15 min at 70°C, which preferentially adds single dA residues to the 3′ ends of DNA. 1.2 pmol of dA-tailed DNA fragments were subcloned into 57 fmol of the pTOPE-1b vector (Novagen), which had been cleaved with *Eco*RV followed by the addition of single dT residues at its 3′ ends. The pTOPE-1b vector is designed for expression of inserts as stable fusion proteins driven by T7 RNA polymerase. This system is based on the pET (plasmid for expression by T7 RNA polymerase) system developed by Studier and coworkers [14]. In the pTOPE-1b vector cDNA clones are fused to a gene fragment of the T7 capsid protein T10 under control of the T7 promoter. The recombinant plasmids were transferred to *E. coli* NovaBlue(DE3) which contains a chromosomal copy of the T7 RNA polymerase gene under the control of the inducible *lacUV5* promotor. Colonies were plated on nylon filters (laid on LB plates containing 50 μg/ml ampicillin) at a density of 1×10^4 colonies per filter and incubated for 24 h at 37°C. Replicate filters were prepared and placed into a chloroform vapor chamber for 15 min. After lysis of the bacteria the filters were put on Whatman 3MM paper saturated with 20 mM Tris-HCl, pH 7.9, 6 M urea, 0.5 M NaCl. The filters were then blocked in 50 mM sodium phosphate, pH 7.4, 150 mM NaCl (PBS) containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat dry milk and washed twice with the same solution. For screening with the ^{35}S-labelled β2a probe the filters were incubated overnight at room temperature with 1 μl transcription/translation mix per ml PBS containing 5% (w/v) BSA, 0.5% (w/v) non-fat dry milk and the protease inhibitor mix (0.1 μg/ml each inhibitor). The filters were washed three times with PBS, 0.1% (v/v) Tween 20 and 5% (w/v) BSA, dried and exposed to an X-ray film (Hyperfilm MP, Amersham). For isolation of single positive colonies two rescreens were performed according to the protocol above. The recombinant plasmids of positive clones were isolated by standard methods and cDNA inserts sequenced in both directions by the dideoxy chain termination method using [α-^{35}S]dATP. By screening $\sim1.2\times10^5$ clones five independent positive clones were isolated. According to the size of the DNA fragments used in comparison with the full length α_{1Ca} cDNA a large number of independent clones is to be expected. However, different growth rates of individual transfected *E. coli* clones were observed apparently resulting in a reduced amount of fusion protein produced by a considerable number of bacterial clones, which escaped detection by the β probe.

2.3. Construction and expression of a glutathione S-transferase fusion protein

Fusion protein epitopes of the α_{1Ca} subunit were constructed by amplifying base pairs 1348–1413 ($\alpha_{1Ca/22aa}$) and 1372–1413 ($\alpha_{1Ca/14aa}$) with the following primers: 5′-AAGGATCCGATTTCCAGAAGTTG-3′and 5′-AGGGAATTCGATCCAGTCCAGGTA-3′, ($\alpha_{1Ca/22aa}$); 5′-AAGGATCCCAGCAGCTGGAAGAG-3′and 5′-AGGGAATTCGATCCAGTCCAGGTA-3′ ($\alpha_{1Ca/14aa}$). These PCR products were digested with *Bam*HI and *Eco*RI, subcloned in pGEX-2T vector (Pharmacia) and sequenced on both strands by the dideoxy chain termination method. Recombinant pGEX vectors were introduced into *E. coli* BL21 cells. Overnight cultures of the pGEX epitope constructs were diluted 1:10 and incubated at 37°C until OD_{578} reached 0.8–1. Expression of fusion proteins was induced by adding isopropyl β-D-thiogalactoside at a final concentration of 0.1 mM. Cells were incubated for an additional 4 h at 37°C before they were sedimented by centrifugation, resuspended in 0.05 volumes of ice-cold PBS containing 1 μg/ml antipain, 1 mM benzamidine, 1 mM iodoacetamide, 1 μg/ml leupeptin, 1 mM *ortho*-phenanthroline, 1 mM pepstatin A, 0.1 mM PMSF and 50 mM EDTA, pH 8.0. The extracts were sonicated for 3 min and thereafter incubated with gentle shaking for 15 min at room temperature in the presence of Triton X-100 at a final concentration of 1% (v/v). After centrifugation aliquots of the supernatants were mixed with sodium dodecylsulfate (SDS) sample buffer and proteins separated electrophoretically on 12% SDS-polyacrylamide gels.

2.4. Overlay experiments

After separation by SDS-polyacrylamide gel electrophoresis proteins were transferred to nitrocellulose filters (Schleicher & Schüll). The blots were blocked in PBS, 0.1% (v/v) Tween 20 and 5% (w/v) non-fat dry milk followed by incubation (16 h at 4°C) in the presence of 1 μl ([^{35}S]β2a or [^{35}S]β1a), 6 μl ([^{35}S]β3) or 4 μl ([^{35}S]β4) transcription/translation mix per ml of the blocking solution containing the protease inhibitor mix (0.1 μg/ml each inhibitor). The filters were washed three times with the blocking solution, dried and exposed to a film.

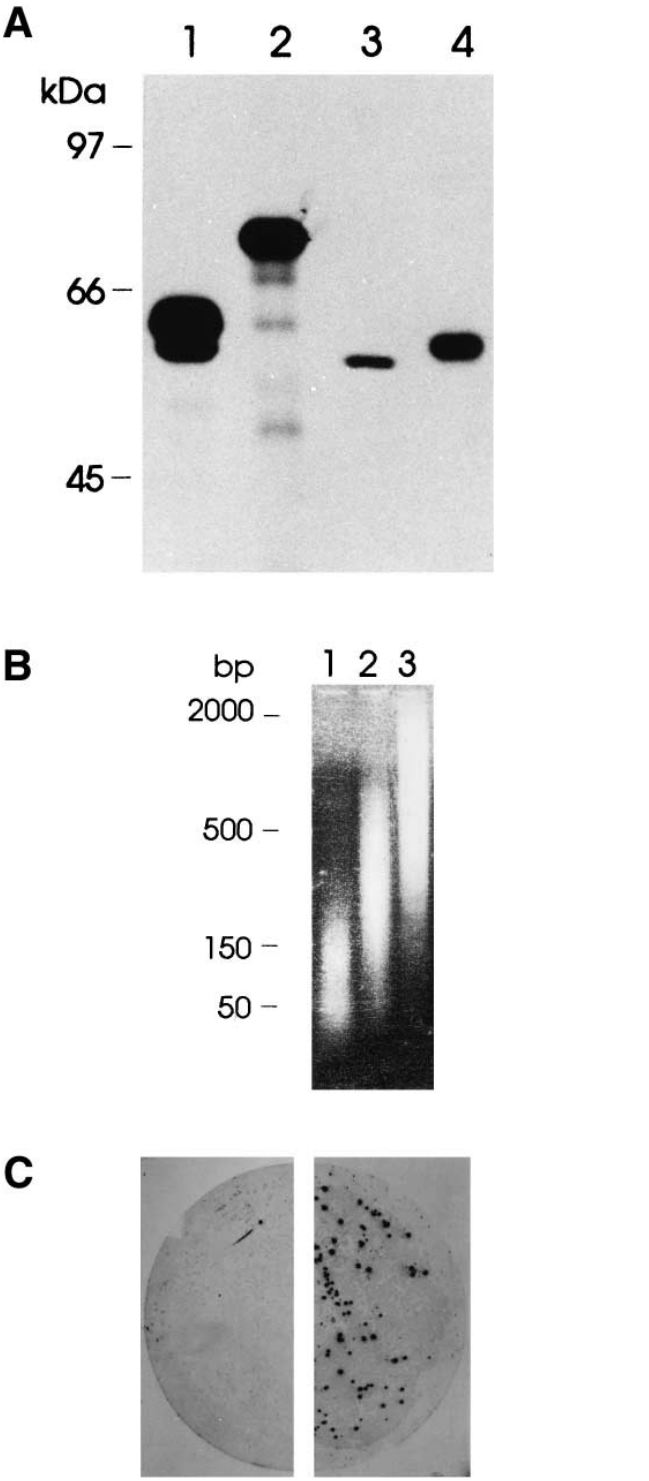

Fig. 1. Epitope expression library of α_{1Ca} fusion proteins. A: Fluorogram of the in vitro translated [^{35}S]β1a (lane 1), β2a (lane 2), β3 (lane 3) and β4 (lane 4) probes. 1 μl of each translation reaction was electrophoresed on a 7% SDS-polyacrylamide gel, dried in the presence of sodium salicylate and methanol and exposed to a film. B: α_{1Ca} cDNA fragments electrophoresed on a 2% agarose gel after treatment with 1, 0.67 and 0.4 U DNase I per ml assay volume (lanes 1–3, respectively). An increase of the enzyme concentration leads to a decrease of cDNA fragment size. C: Autoradiogram of a nylon filter containing a single positive colony isolated by screening a cardiac muscle α_{1Ca} subunit epitope library with the in vitro translated [^{35}S]β2a subunit probe; left, first screen; right, second screen.

$\alpha 1_{Ca}$ $_{424}$FFVLNLVLGVLSGEFSKEREKAKARG**DFQKLREKQQLEEDLKGYLDWI**TQAEDIDPENEDEGMD$_{487}$
rbr1 FFVLNLVLGVLSGEFSKEREKAKARG**DFQKLREKQQLEEDLKGYLDWI**TQAEDIDPENEDEG
rar1 EFSKEREKAKARG**DFQKLREKQQLEEDLKGYLDWI**TQAEDIDPENEDEG
rb17 SKEREKAKARG**DFQKLREKQQLEEDLKGYLDWI**TQAE
rbr6 G**DFQKLREKQQLEEDLKGYLDWI**
rar3 **DFQKLREKQQLEEDLKGYLDWI**TQAEDIDPENEDEGMD

common sequence $_{450}$**DFQKLREKQQLEEDLKGYLDWI**$_{471}$

β binding site $_{458}$**QQLEEDLKGYLDWI**$_{471}$

Fig. 2. Identification of the β subunit binding site of the α_{1Ca} protein. Alignment of amino acid sequences of fusion α_{1Ca} epitopes as deduced from the five independent clones obtained. Bold letters represent the common 22 amino acid β binding site encoded by all clones. Underlined is the conserved 18 amino acid sequence motif of the β binding site in α_1 delineated by Pragnell and coworkers ([7], amino acids 458–475 in α_{1Ca}). A minimal β binding site of 14 residues can be deduced from the overlap of this conserved motif and the 22 amino acid sequence of this study. The first and last amino acids are numbered according to their location in the primary structure of α_{1Ca}.

3. Results and discussion

By screening a T7 polymerase-based epitope library of the rabbit α_{1Ca} subunit with the ^{35}S-labelled β2a protein five independent positive clones were obtained (Fig. 1C). These clones were selected, reprobed and isolated. Sequence analysis revealed that all clones contained cDNAs in the appropriate reading frame and encoded peptide sequences derived from the α_{1Ca} subunit. The sizes of the peptide epitopes ranged from 23 to 62 amino acids (Fig. 2). The smallest epitope sufficient to bind the β subunit is encoded by clone rbr6 (Fig. 2), comprises 23 amino acids and extends from residue 449 to 471 of α_{1Ca}. Interestingly, it contains 22 residues (amino acids 450–471) which are also included in the derived sequences of the four other clones which were isolated, indicating that this 22 amino acid motif represents the β2a binding site within α_{1Ca}. No clones were obtained encoding peptide sequences derived from other regions of the α_{1Ca} protein. Analysis of the transmembrane topology of the α_{1Ca} subunit maps this β2a subunit binding site to the putative cytoplasmic

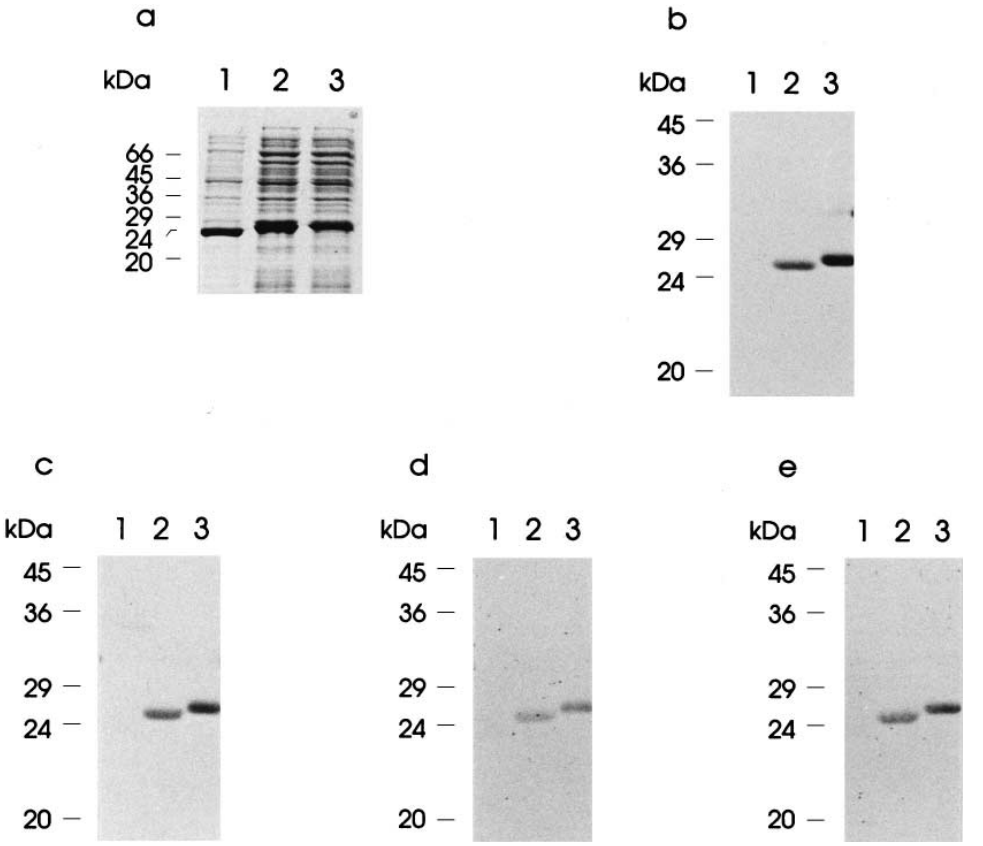

Fig. 3. Expression of glutathione *S*-transferase (GST) fusion proteins in total *E. coli* lysate: Coomassie stained 12% SDS-polyacrylamide gel (a) and the autoradiograms of the corresponding overlay of the nitrocellulose immobilized proteins with the ^{35}S-labelled β2a (b), β1a (c), β3 (d) or the β4 (e) subunit. Lanes 1, GST control; lanes 2, α_{1Ca} 14 amino acid epitope expressed as GST fusion protein; lanes 3, α_{1Ca} 22 amino acid epitope expressed as GST fusion protein.

linker between repeats I and II, positioned 15 amino acids from the C-terminal end of IS6 transmembrane domain of the α_{1Ca} subunit.

Independent verification of this interaction was obtained by constructing a glutathione *S*-transferase (GST) fusion protein expressing amino acids 450–471 of the α_{1Ca} protein (Fig. 3a). Among the bacterial cell proteins only the fusion protein was recognized by ^{35}S-labelled β2a but not other bacterial proteins or GST itself (Fig. 3b, lanes 3 and 1). In addition to β2a ^{35}S-labelled β1a, β3 and β4 (Fig. 3c–e, lanes 3) bind to the 22 amino acid motif demonstrating specific interaction of β2a, β1a, β3 and β4 with α_{1Ca}.

Using an α_{1Ca} λgt11 expression library Pragnell and coworkers [7] mapped the b-type splice variant of the β1 subunit gene to the same location on the I–II cytoplasmic linker of the α_{1Ca} subunit. The smallest of two overlapping clones from the α_{1Ca} λgt11 expression library [7] encoded an 84 amino acid epitope which extends from amino acids 434 to 517 of this subunit and contains the 22 amino acid β binding site defined in this study. Further studies by Pragnell and coworkers [7] showed that the β1b binding site maps to the same location on the I–II cytoplasmic linker of even distantly related α_1 proteins. They identified a 45 amino acid sequence of α_{1S}, a 56 amino acid sequence of α_{1B}, and a 51 amino acid sequence of α_{1A} to be responsible for β1b binding. Sequence comparison of these β1b binding sites [7] identified a conserved motif QQ-E--L-GY--WI---E (amino acids 458–475 in α_{1Ca}, Fig. 2) and evidence was given that this motif represents the binding site for β subunits within even distantly related α_1 proteins [15–17]. The β binding site described in this study overlaps by 14 amino acids with this conserved motif, apparently reducing the α_1-β interaction site to 14 residues (Q_{458}–I_{471}). Accordingly the 14 amino acid peptide was expressed as part of a GST fusion protein. As shown in Fig. 3 (lane 2) this fusion protein binds β2a, β1a, β3 as well as β4 demonstrating that it is sufficient for β binding.

Eight of the 14 amino acid residues present in α_{1Ca} (QQ-E--L-GY--WI, Fig. 2) are conserved in all mammalian α_1 subunits identified so far (α_{1S}, α_{1A}, α_{1B}, α_{1C}, α_{1D}, and α_{1E}) as well as in the α_1 subunits isolated from *Drosophila melanogaster* and *Musca domestica*. These conserved residues may play a more general role in binding of β to α_1. The six non-conserved amino acids interspersed among the conserved then may contribute to some of the differences in affinity between α1 and β subunits as has already been observed between α_{1A} and several β subunits [15].

In summary, the results of this study demonstrate that a short sequence of only 14 amino acids in α_{1Ca} is sufficient to bind β1a, β2, β3 or β4 proteins. No additional β binding motif was detected although there is evidence for a second α_1-β interaction site [8,9]. One possibility is that the interaction of the apparent second site with β is to weak to be detected by the experimental approach used in this study. Alternatively α_1-β interactions might depend on posttranslational modifications and this interaction might not be readily identified either because bacteria do not perform the modifications of α_{1Ca} in an equivalent manner to mammalian cells or because modifications of the in vitro synthesized β2a protein are not properly accomplished by the reticulocyte extract.

Finally, the cDNA library based method to detect proteins by protein probing used in this study offers several advantages over traditional protein purification or immunochemical methods. It allows direct identification of the cDNAs encoding target proteins of interest and does not require purified target protein or antibodies. Additionally and in contrast to the two hybrid assay, it allows identification of peptide epitopes as small as 17 (Trost and Flockerzi, to be published) to 23 (this study) amino acids.

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