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Requirement of the calcium channel β subunit for functional conformation

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The cardiac dihydropyridine-sensitive L-type calcium channel was stably expressed in Chinese hamster ovary cells by transfecting the rabbit cardiac calcium channel α_1 subunit cDNA with or without coexpression of the β subunit of skeletal muscle calcium channel. Whereas coexpression of the β subunit significantly increased DHP binding activity and calcium channel activity, it did not affect the amount of the α_1 subunit expressed, as judged by RNA blot hybridization analysis and immunoblotting analysis The results suggest that association with the β subunit is necessary for the α_1 subunit protein to take a proper conformation suitable for a functional calcium channel.

Calcium channel; CHO cell, cDNA expression; Immunoblotting; β Subunit

1. INTRODUCTION

Voltage-dependent calcium channels are essential for many cellular functions, including muscle contraction and secretory processes. In skeletal muscle, the dihydropyridine (DHP)-sensitive L-type calcium channel is a complex of five subunits, α_1, α_2, β, γ and δ [1]. The cDNAs of these subunits have been cloned [2–6] and the δ subunit has been shown to derive from a precursor common to the α_2 subunit [7,8]. Other types of calcium channel, whether L-type or non-L-type, are considered to be composed of a similar complex of polypeptides [1]. Although the α_1 subunit embodies the essential properties as a calcium channel, coexpression of small subunits results in significant increase in calcium channel activity [9,10]. Particularly, effects of coexpression of the β subunit have been well studied, which results in significant increases in DHP binding and current density and also accelerates activation and inactivation kinetics of the calcium channel current [11–18]. The mechanisms underlying the increased channel activity caused by coexpression of small subunits have been postulated to be facilitation of anchoring of the α_1 subunit, protein stabilization and direct subunit modulation [9–18]. However, there is no experimental evidence to prove any of these mechanisms. We report here characterization of rabbit cardiac calcium channel protein expressed in CHO cells with and without coexpression of the β subunit, and discuss the functional role of the β subunit.

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Abbreviations: CHO cell, Chinese hamster ovary cell; DHP, dihydropyridine.

2. MATERIALS AND METHODS

2.1 *Construction of expression plasmids and isolation of transformants*

The expression plasmid pCCAR carrying the cDNA for the rabbit cardiac DHP-sensitive calcium channel α_1 subunit was described previously [19]. The expression plasmids pCAS7, pCAS14 and pCAS15, which carry the cDNAs encoding the rabbit skeletal muscle calcium channel α_2 [9], β [4] and γ subunits [6], respectively, were constructed as follows. The plasmid $pKCRH_2$ [20] was cleaved by *Hin*dIII and blunted by T_4 DNA polymerase. With the resulting DNA fragment, the 3.5-kilobase-pair (kb) *Eco*RI–*Eco*RI fragment from pSPCA1 [9], the 1.6-kb *Eco*RI–*Eco*RI fragment from pCaB1 [10] and the 0.75-kb *Xba*I–*Hin*dIII fragment from pCaG1 [10] were ligated after being blunted by T_4 DNA polymerase, in the same orientation with respect to SV40 early gene transcription, to yield pCAS7, pCAS14 and pCAS15, respectively. Clones of Chinese hamster ovary (CHO) cells, CCAR2823 and CCAR3217, which were transformed with the plasmid pCCAR [19], were described previously [19]. Clones CCARβ3 and CCARβ609 were isolated by cotransfecting CHO cells with the expression plasmids pCCAR and pCAS14. Clones CCAR$\alpha_2\beta\gamma$8918 and CCAR$\alpha_2\beta\gamma$9024 were isolated by cotransfecting CHO cells with the expression plasmids pCCAR, pCAS7, pCAS14 and pCAS15. All transformants were isolated by screening G418-resistant clones using RNA blot hybridization analysis. All the CHO clones were established after cloning twice by limiting dilution.

2.2. *RNA blot hybridization analysis*

Total RNA was extracted from transformants by the LiCl–urea method [21]. RNA blot hybridization analysis was carried out essentially as described previously [22]. The hybridization probes used were the 7.0-kb *Hin*dIII–*Hin*dIII fragment from pCCAR [19], the 3.5-kb *Eco*RI–*Eco*RI fragment from pSPCA1 [9], the 1.6-kb *Eco*RI–*Eco*RI fragment from pCaB1 [10] and the 0.75-kb *Xba*I–*Hin*dIII fragment

from pCaG1 [10]. Autoradiography was performed at −70°C for 24 h with an intensifying screen An RNA ladder (Bethesda Research Laboratories) was used as size marker (in kb).

2.3. *Immunoblotting analysis*

Membrane preparations from all clones were electrophoresed on SDS-6% polyacrylamide gel and transferred [23] to GVHP filter (Millipore). The filter was incubated with polyclonal antibody (CR2) as described previously [19] and then with 0.5 μCi/ml of [^{125}I]anti-rabbit IgG (Amersham). After washing, the filter was subjected to autoradiography at −70°C for 30 h with an intensifying screen. Size markers were from Sigma and Bromophenol blue served as tracking dye.

2.4. *DHP binding assay*

[^{3}H](+)PN200–110 (74.1 Ci/mmol; New England Nuclear) binding was assayed with membrane preparations (100 μg protein) in a solution (0.2 ml) containing 50 mM Tris-HCl (pH 7.4), 3 mM $CaCl_2$ and various concentrations (0.25–4 nM) of [^{3}H](+)PN200–110. After incubation for 40 min at room temperature, the samples were collected on GF/C filters (Whatman) and washed with a solution containing 0.2 M choline chloride and 20 mM Tris-HCl (pH 7.4). The filters were then counted for radioactivity. Nonspecific binding was determined in the presence of a large amount of unlabelled nifedipine.

2.5. *Electrophysiological measurements*

Ba^{2+} currents through the calcium channel were measured using the patch-clamp technique in the whole-cell configuration at room temperature, in the absence of calcium channel agonists. The pipette solution contained (in mM) 120 CsCl, 3 $MgCl_2$, 2 Na-ATP, 10 EGTA and 5 HEPES (pH adjusted to 7.4 with CsOH). Cells were bathed in a solution containing (in mM) 120 NaCl, 10.8 $BaCl_2$, 1 $MgCl_2$, 5.4 CsCl, 10 glucose and 10 HEPES (pH adjusted to 7.4 with NaOH). Tetrodotoxin was included in the bath solution when an endogenous Na^+ current was present. Current records were sampled at 5 or 20 kHz after low-pass filtering at 2 kHz (−3 dB). Leakage and capacitive transients were eliminated by a P/4 method.

3. RESULTS AND DISCUSSION

CHO cells were transfected with the cardiac calcium channel α_1 subunit cDNA alone or in combination with an expression plasmid for the skeletal muscle β subunit cDNA or with expression plasmids for the skeletal muscle α_2, β and γ subunit cDNAs. RNA blot hybridization analyses of total RNA preparations from various cell lines confirmed that the transformants successfully express mRNA species of a reasonable size corresponding to the introduced cDNAs and that the parental CHO cells expressed no detectable transcripts for the cardiac α_1 subunit or the skeletal muscle α_2, β or γ subunits (Fig. 1A–D).

The calcium channel α_1 subunit protein expressed in

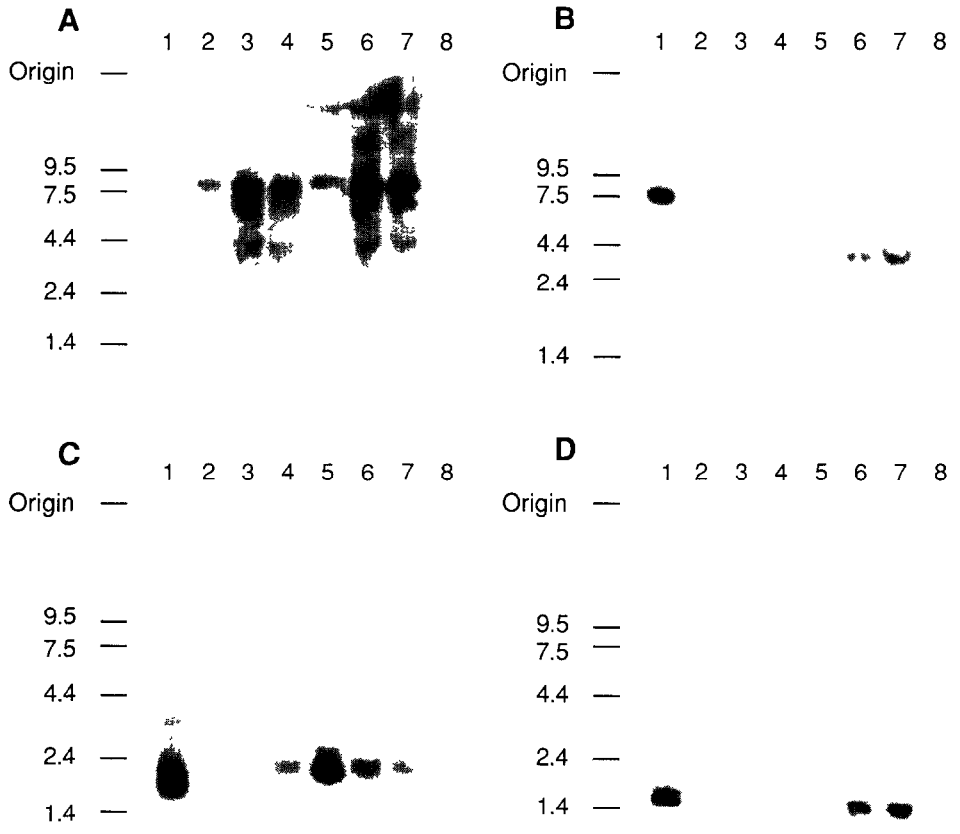

Fig. 1. Autoradiograms of blot hybridization analysis of RNA from transformants with cDNA probes specific for cardiac calcium channel α_1- (A), skeletal muscle calcium channel α_2- (B), β- (C) and γ- (D) subunit mRNAs. Poly(A)$^+$ RNA (2 μg each) prepared from cardiac muscle (A, lane 1) and skeletal muscle (B–D, lane 1) from rabbit, total RNA (20 μg each, A–D) from CCAR2823 cells (lane 2), CCAR3217 (lane 3), CCARβ3 (lane 4), CCARβ609 (lane 5), CCAR$\alpha_2\beta\gamma$8918 (lane 6), CCAR$\alpha_2\beta\gamma$9024 (lane 7) and non-transfected CHO cells (lane 8) were analysed. The hybridization probes used were described in section 2.

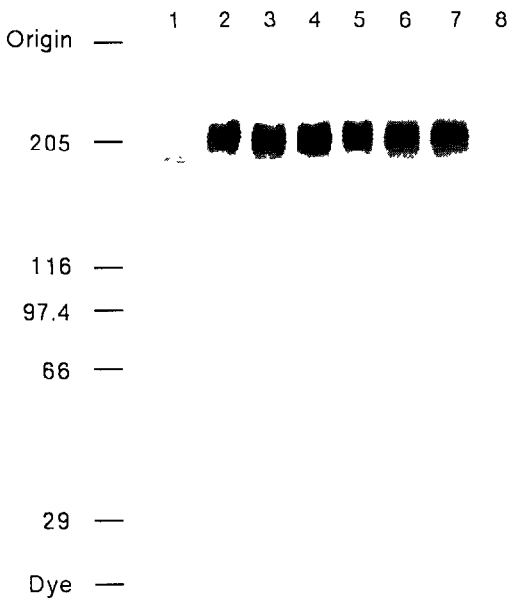

Fig. 2. Expression of the rabbit cardiac calcium channel α_1 subunit protein in CHO cells. Immunoblotting analysis of the membrane preparations (100 μg protein each) from rabbit heart (lane 1), CCAR2823 (lane 2), CCAR3217 (lane 3), CCARβ3 (lane 4), CCARβ609 (lane 5), CCAR$\alpha_2\beta\gamma$8918 (lane 6), CCAR$\alpha_2\beta\gamma$9024 cells (lane 7) and non-transfected CHO cells (lane 8) using polyclonal antibody CR2 against the rabbit cardiac calcium channel α_1 subunit.

transformed cells was characterized by immunoblotting analysis of membrane preparations using polyclonal antibody (CR2), which was raised against the C-terminal peptide of the rabbit cardiac calcium channel α_1 subunit [19]. The immunoblotting revealed that the transformed clones produced a protein similar in Mr to the larger form of the 250- and 200-kDa α_1 subunit polypeptides from rabbit heart [19] (Fig. 2). Furthermore, there was no appreciable difference in amount of the α_1 subunit protein produced among the transfected cell lines.

Membranes from transformed CHO cells were assayed for binding of [^{3}H](+)PN200-110, a DHP antagonist (Table I). Membrane preparations of the transformants showed saturation binding with similar apparent dissociation constants (K_d), ranging from 1.08 nM to 1.33 nM. These values were in the same range as the K_d measured with microsomes prepared from rabbit heart (0.9 nM; mean of three measurements). Although membrane preparations of the transformed cells without the β subunit (CCAR2823 and CCAR3217) showed only small DHP binding, coexpression of the β subunit resulted in a marked increase in the total number of binding sites (B_{max}).

All the transformants displayed Ba^{2+} currents evoked by depolarization (Table I). While CCAR2823 and CCAR3217 showed only small currents, the presence of the β subunit enhanced the current amplitude by up to 100-fold.

It is now generally accepted that the voltage-dependent calcium channel complex, whether L type or non-L type, is composed of the α_1 subunit and small subunits. There has been accumulating evidence that functional properties of the calcium channel activity alter when small subunits are coexpressed [9–18]. However, the molecular mechanisms underlying the effects of the small subunits have remained to be elucidated. In our present study, coexpression of the calcium channel α_1 subunit with the β subunit results in significant increases in DHP binding capacity. Scatchard analysis of DHP binding has revealed that the β subunit increases the total number of binding sites B_{max} without altering the apparent dissociation constant K_d. Furthermore, coexpression of the β subunit causes a marked increase in voltage-dependent calcium channel activity. However, the amount of the α_1 subunit protein is not affected by the β subunit, as judged by immunoblotting analysis. Thus our results clearly demonstrate that the increase in functional calcium channel activity caused by coexpression of the β subunit does not result from an increase in amount of the expressed α_1 subunit protein, which rules out the possibility that the β subunit stabilizes the a1 subunit and protects it from degradation.

Patch-clamp recording provides direct measurements of the amount of functional channel on the cell surface,

Table I

Effects of coexpression of small subunits on DHP-binding and channel activity

Subunit combinations	Clone	DHP binding B_{max} (fmol per mg protein)	DHP binding K_d (nM)	Number of responsive cells/ Number of cells tested	Mean amplitude (pA)
α_1	CCAR2823	42	1.08	7/8	34 ± 10
	CCAR3217	68	1.23	17/19	29 ± 11
α_1, β	CCARβ3	2005	1.15	11/11	3827 ± 2545
	CCARβ609	391	1.33	10/10	221 ± 187
$\alpha_1, \alpha_2, \beta, \gamma$	CCAR$\alpha_2\beta\gamma$8918	2147	1 16	11/11	1972 ± 1262
	CCAR$\alpha_2\beta\gamma$9024	2838	1.19	11/11	2501 ± 2296

Values of B_{max} and K_d are means of at least two measurements. Currents were evoked by depolarization to + 20 mV from a holding potential of −80 mV. Data for peak current amplitudes are given as means ± S.D.; these values have been calculated only for the responsive cells.

whereas DHP binding measures the amount of functional protein in total membrane fraction, probably including intracellular components. Comparison of the estimates of number of the channels from the current amplitude and the DHP binding suggests that only a small portion of the DHP binding protein is electrophysiologically active. This observation is at least partly accounted for by the fact that only part of the mature protein has been transported onto the cell surface. Because the ratio of the current amplitude to the DHP binding seems independent of the presence of the β subunit, it is unlikely that the major role of the β subunit is to help the calcium channel protein move onto the surface membrane.

The most likely explanation of our results regarding the functional role of the β subunit is that it binds to and helps the α_1 subunit take a proper conformation suitable for a functional calcium channel. Therefore, the β subunit may play a critical role in the regulation of functional activity of the calcium channel. Since there are multiple types of calcium channel α_1 subunit, existence of at least four β-subunit genes [18] may add more complexity to the regulation of calcium channel activity, particularly in brain.

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