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Characterization of β subunit modulation of a rabbit cardiac L-type Ca^{2+} channel α_1 subunit as expressed in mouse L cells

Philippe Lory[a], Gyula Varadi[a], Donald F. Slish[b], Marie Varadi[a] and Arnold Schwartz[a]

[a]*Department of Pharmacology and Cell Biophysics, University of Cincinnati, 231 Bethesda Avenue, Cincinnati, OH 45267-0575, USA* and [b]*Harvard Medical School, Department of Cellular and Molecular Physiology, 25 Shattuck Street, Boston, MA 02115, USA*

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Functional properties of a rabbit cardiac α_1 Ca^{2+} channel subunit (CARDα_1) were investigated using the patch-clamp technique in mouse L cells, a recipient cell line which is devoid of any Ca^{2+} channel subunits. Cell lines resulting from stable transfection of the CARDα_1 subunit as well as in coexpression with a β subunit (CARD$\alpha_1\beta$) derived from skeletal muscle (SKMβ) were characterized. The results show that while the CARDα_1–Ca^{2+} channel activity is negligible, the Ba^{2+} current density is dramatically increased in the presence of β subunit (~20-fold). CARDα_1– and CARD$\alpha_1\beta$–Ba^{2+} currents were both sensitive to the 1,4-dihydropyridine (DHP) agonist, Bay K 8644 (5- to 8-fold increase). Activation kinetics of CARDα_1– and CARD$\alpha_1\beta$–Ba^{2+} currents were comparable. The inactivation time-course was faster (3- to 4-fold) for CARD$\alpha_1\beta$–Ba^{2+} currents. We conclude that the main role of the β subunit in heart is to modulate the L-type current density and present several lines of evidence that SKMα_1 and CARDα_1 are differentially regulated by the β subunit.

Mouse L cell; Ca^{2+} channel expression; Cardiac α_1 subunit; β Subunit

1. INTRODUCTION

The high-voltage activated (L-type) Ca^{2+} channel is the primary pathway for Ca^{2+} entry in cardiac tissue. Structurally, the cardiac L-type channel is similar to its skeletal muscle (SKM) counterpart which has been biochemically characterized [1]. The cDNAs for SKM Ca^{2+} channel subunits (α_1, α_2/δ, β and γ) have been cloned [2–5]. Several α_1-isoforms have also been cloned from cardiac (CARDα_1) [6–8], smooth muscle [9,10] and brain [11–14], as well as various β isoforms, from rat brain [15,16], human brain [13], rabbit heart [17] and human heart [18].

Expression of SKMα_1 and CARDα_1 has been assayed in several systems. It appears that the functional properties of tissue-specific α_1 isoforms may depend on the recipient cell [19–21]. For example, transfection of the SKMα_1 subunit in the mouse L cell, results in the expression of DHP-sensitive Ca^{2+} channels and DHP binding [22] but such expression is absent in *Xenopus* oocytes. In contrast, the CARDα_1 subunit expresses functional Ca^{2+} channels in *Xenopus* oocytes [6,8,23,24].

Several recent studies provide evidence that SKM β α_2/δ and γ subunits have a regulatory role on SKMα_1 subunit activity [25–27]. It has been shown that the β subunit enhances CARDα_1 activity when expressed in *Xenopus* oocytes [8,16,17,23,24]. β Subunit effect on cardiac current kinetics was found in some studies [8,16] but not in others [17,23,24]. As a complicating factor, *Xenopus* oocytes possess an endogenous Ca^{2+} current which is enhanced by SKMβ and/or SKMα_2/β subunits [13,23]. Thus, it is still unclear whether these studies demonstrate a specific CARDα_1–β interaction or an influence of the recipient cell on exogenous Ca^{2+} channel activity.

In the present study we investigate the properties of the CARDα_1 expression in mouse L cell. This cell line, in contrast to other expression systems, does not exhibit any endogenous Ca^{2+} channel activity and lacks expression of Ca^{2+} channel subunit transcripts [25]. We compare here the functional properties of CARDα_1 and SKMα_1 L-type Ca^{2+} channels expressed in L cells, and present the effects of a β subunit on CARDα_1 activity.

2. MATERIALS AND METHODS

2.1. *Isolation and construction of a full-length rabbit cardiac α_1 cDNA*

A mixed primed (random primer-oligodT) rabbit cardiac library, constructed in λZAPII vector, was screened using standard techniques [28]. Three overlapping cDNA clones (HTDHP4.15, HTDHP3.2 and HTDHP2.0) were assembled in pBluescript SK(+) vector (Stratagene, Inc., CA). The full-length cDNA, with the exception of 16 conservative amino-acid changes, is essentially identical to that published by Mikami et al. [6]. The cDNA was cleaved from pBluescript using the 5′-*Hin*dIII site and the 3′-*Not*I site, ligated into the corresponding cloning sites of the pAGS-3 mammalian expression vector [29] and designated pAGS-3HTα_1.

2.2. *Transfection of Ltk-cells*

Mouse L cells were cotransfected with pSV2neo and pAGS-3HTα_1

Correspondence address: Arnold Schwartz, Department of Pharmacology and Cell Biophysics, University of Cincinnati, 231 Bethesda Avenue, Cincinnati, OH 45267-0575, USA. Fax: (1) (513) 558 1778.

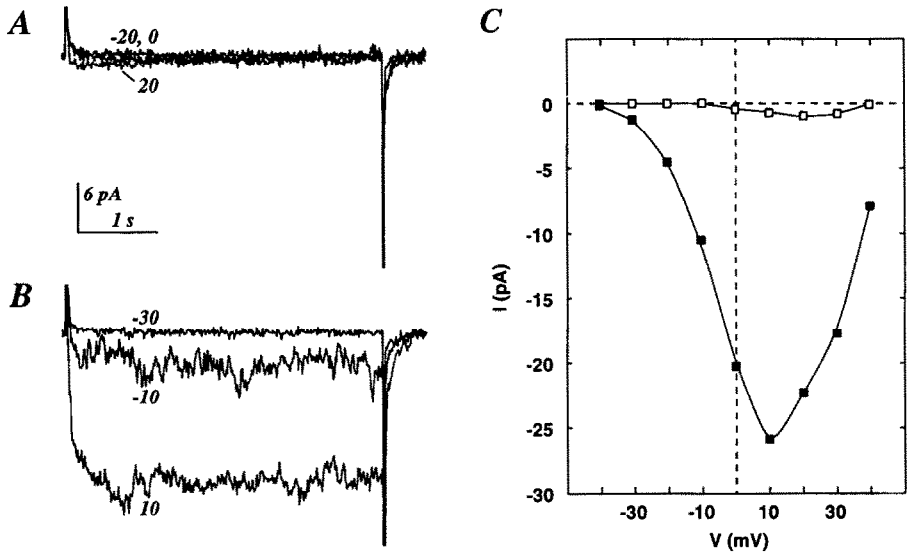

Fig. 1. Ba^{2+} current activity in a cell expressing the cardiac α_1 subunit (LH18 cell). (A) The family of current traces illustrates the basal current activity (V_c's are indicated on the traces). (B) Ba^{2+} currents recorded on the same cell after application of Bay K 8644 (1 μM) in the bath. (C) Current–voltage relationships for control currents (□) and in the presence of Bay K 8644 (■), respectively.

at a molar ratio of 1:10 [30]. After 2 to 3 weeks of G418 selection (400 μg/ml), 20 individual clones were isolated and screened by PCR methodology for the insertion of the CARDα_1 cDNA into the genome. Northern blot analysis was used to test the level of expression of the CARDα_1 message. A cell line, designated LH18, showed the highest expression for CARDα_1 and was further cotransfected with pSVTK [31] and pSG-5β, an expression plasmid for the SKMβ subunit [25], using the conditions described above. Selection was done in a HAT-G418 medium (Hypoxanthine, 0.1 mM; Aminopterine, 0.4 μM; thymidine, 16 μM; G418, 200 μg/ml). Isolation and characterization of these cell lines was performed as described above.

2.3. *Cell culture and electrophysiology*

Cells were grown on glass coverslips for 2 to 3 days [25,27]. Calcium channel currents were recorded, using Ba^{2+} as charge carrier, in the whole-cell configuration [32] at room temperature (20–22°C). The bathing solution was in (mM): $Ba(OH)_2$, 40; glutamate, 40; *N*-methyl D-glucamine, 80; HEPES, 10; $MgCl_2$, 2; pH adjusted to 7.4 with CH_3SO_3H. The pipette solution contained the following (in mM): *N*-methyl D-glucamine, 110; $MgCl_2$, 2; EGTA, 15; HEPES, 15; pH adjusted to 7.3 with CH_3SO_3H. Pipettes (ref. 7052; Garner, USA) had resistances between 3 to 5 MOhm. Capacitive transients were minimized using the analog circuitry of the amplifier (Axopatch 1B; Axon Instruments, CA). Ba^{2+} currents were recorded at various digitizing rates and filtered at 0.5 or 1 kHz using a four-pole Bessel filter. Stimulation of the cell, acquisition and analysis of the data were performed using the pCLAMP package (ver. 5.5; Axon Instruments, CA). Statistical comparisons between experimental groups of values (time for half-activation, time to peak, percentage of inactivation) were made using Student's unpaired *t*-test, where $P < 0.05$ was considered significant.

3. RESULTS

Twenty independent clonal cell lines derived from transfected mouse *Ltk*$^-$ cells were characterized for their expression of CARDα_1 mRNA by Northern blot analysis. More than 50% of these cell lines were positive for the expression of a 7.1 kb message as expected from the cDNA construct. Functional expression of DHP-sensitive L-type Ca^{2+} channels was detected by electrophysiology in four cell lines (LH4, LH18, LH32 and LH35). The cell line LH18 was used for further electrophysiological studies. The Ba^{2+} currents were very low or not detectable under control conditions in cells expressing CARDα_1 (Fig. 1A). In fact, Ba^{2+} currents were detecta-

Table I

Comparison of CARDα_1-, CARD$\alpha_1\beta$- and SKMα_1-Ba^{2+} currents in mouse L cells. The current density has been normalized with respect to the membrane capacitance. The number of cells is indicated in parenthesis ('nd' for not determined). Values for activation threshold and time to peak (at +10 mV for CARDα_1- and CARD$\alpha_1\beta$-Ba^{2+} currents, at +20 mV for SKMα_1-Ba^{2+} currents) correspond to currents obtained in the presence of 1 μM Bay K 8644.

	% of Cells with Ctrl IBa	IBa density (pA/pF)		Act. threshold* (mV)	Time to peak*
		Ctrl	1 μM Bay K		
SKMα_1	75% (8#)	0.57 ± 0.2 (8)	1.45 ± 0.15 (8)	−20	4.1 ± 0.2 s (6)
CARDα_1	8% (124)	< 0.02 (32)	0.4 ± 0.1 (32)	−30	350 ± 12 ms (13)
CARD$\alpha_1\beta$	100% (32)	3.1 ± 1.8 (32)	nd	−35	67 ± 12 ms (8)

*In the presence of 1 μM Bay K 8644 in the bath.
#In agreement with our previous reports [25,27].

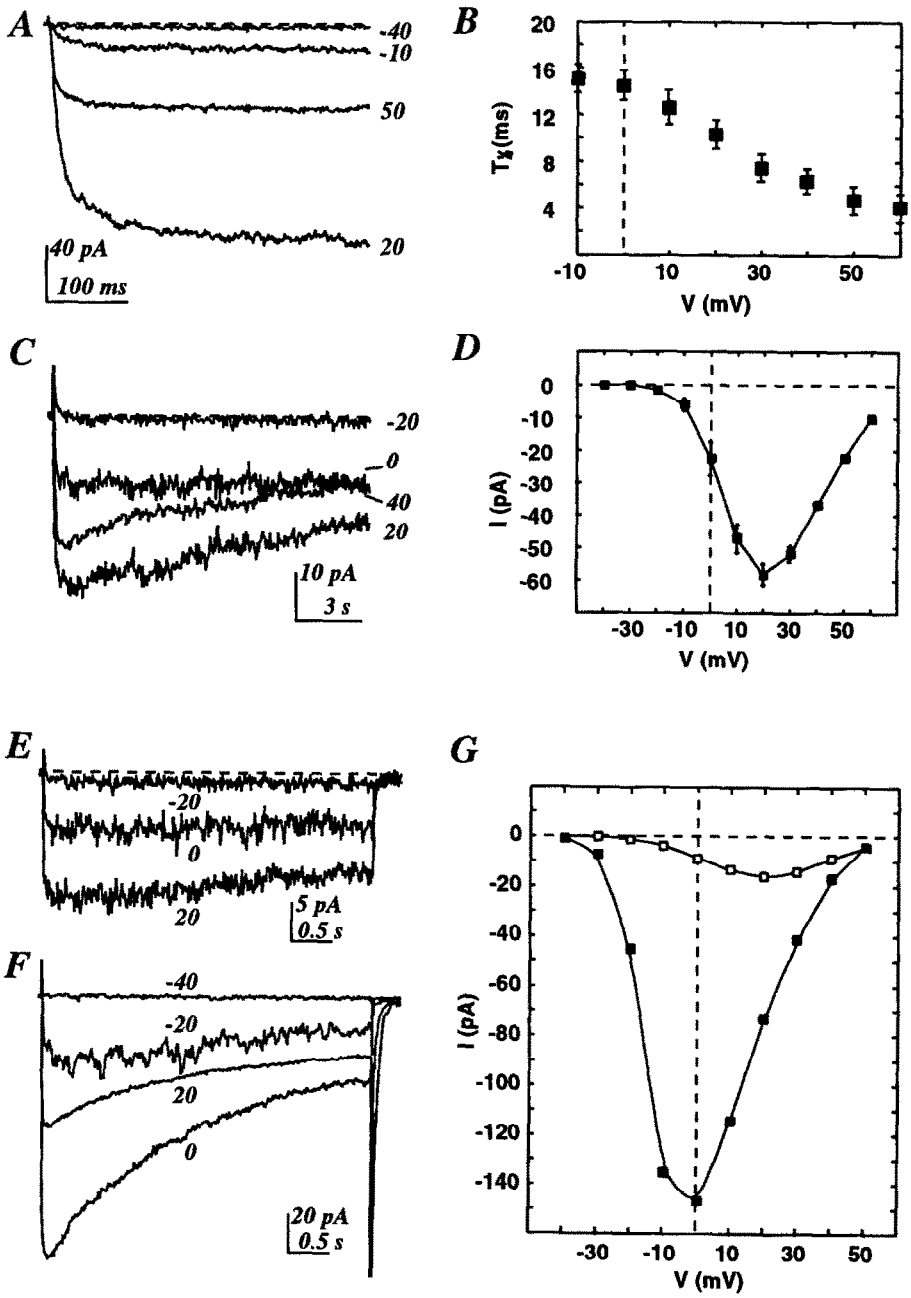

Fig. 2. Modulation of the basal CARDα_1-Ba^{2+} current activity by the SKMβ subunit. (A) Family of Ba^{2+} currents recorded in a LHB3 cell using a linear current subtraction procedure with 4 depolarizing subpulses enable to elicit active currents (P/-4 protocol; pCLAMP ver 5.5). The V_c's are indicated on the traces. The HP was −60 mV. (B) Half-time ($t_{1/2}$) for activation for CARD$\alpha_1\beta$-Ba^{2+} currents. The values are from 7 cells (peak currents up to −50 pA) using a protocol described in A for the CARD$\alpha_1\beta$-Ba^{2+} current acquisition. The determination of the $t_{1/2}$-activation value was done using the CLAMPAN module of the pCLAMP software (presented as mean ± S.E.M.). (C) Inactivation of CARD$\alpha_1\beta$-Ba^{2+} current during 15-s V_c duration. At 20 mV, the percentage of current decay was 26 ± 7% after 15 s (mean ± S.E.M., n = 12). (D) Current–voltage relationship of CARD$\alpha_1\beta$-Ba^{2+} current. The graph represents an average of 3 individual I–V curves obtained on 3 cells (13.5 pF, 17 pF and 21 pF). (E) Modulation of CARD$\alpha_1\beta$-Ba^{2+} currents by the agonist Bay K 8644 obtained on an LHB3 cell (C_m = 22.4 pF): control current traces and (F) in the presence of 1 μM Bay K 8644 (G) Current–voltage relationship of the traces presented in E and F.

ble in only 8% (n = 124) of the LH18 cells tested (Table I). However, the addition of the DHP agonist, Bay K 8644, revealed a measurable Ba^{2+} current in all of the cells. In the presence of Bay K 8644 (1 μM), CARDα_1-Ba^{2+} current activated at −30 mV and peaked at +10 mV (Fig. 1B,C). In contrast to CARDα_1, SKMα_1 Ba^{2+} currents were routinely recorded in the absence of Bay K 8644 (Table I). In some experiments, SKMα_1 currents up to −200 pA were observed (data not shown). Application of Bay K 8644 (1 μM) in these experiments pro-

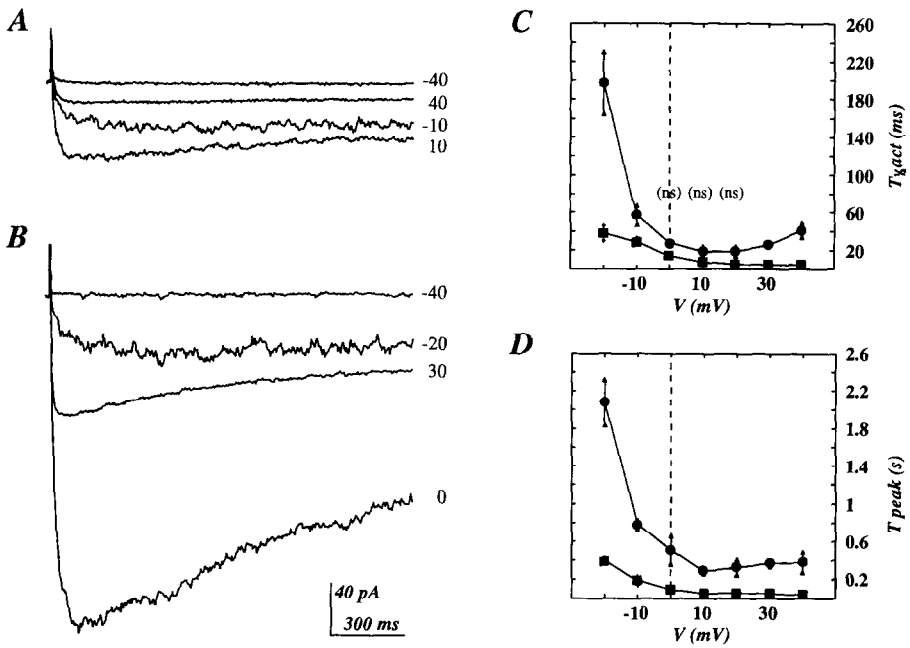

Fig. 3. Comparison of activation properties of CARDα_1- and CARD$\alpha_1\beta$-Ba^{2+} currents. (A) and (B) Representative CARD$\alpha_1\beta$-Ba^{2+} currents and CARD$\alpha_1\beta$-Ba^{2+} currents, respectively, using a 1.5-s V_c duration. The bath solution contained 1 μM Bay K 8644. The V_c's are indicated on the traces. (C) Representation of the half-time activation–voltage relationships for CARDα_1 current (filled circles, n = 4) and CARD$\alpha_1\beta$ current (filled squares, n = 7). (D) Representation of the time-to-peak–voltage relationships for CARDα_1 current (filled circles, n = 7) and CARD$\alpha_1\beta$ current (filled squares, n = 8). It is indicated on the graphs (ns) when the difference was not found statistically significant.

duced a 2.3-fold increase (n = 8) of SKMα_1 currents (Table I).

Bay K 8644-stimulated CARDα_1-Ba^{2+} currents displayed faster activation kinetics than SKMα_1-Ba^{2+} currents (Table I). The time to peak for CARDα_1 (350 ± 12 ms for a depolarizing pulse (V_c) at 10 mV) was tenfold faster than for SKMα_1 (4.1 ± 2 s for a V_c at 20 mV). In addition, CARDα_1 current activated a more negative potentials than SKMα_1 current using identical recording conditions [25,27].

Coexpression of SKMβ with CARDα_1 in the LH18 cell line resulted in a large increase in current density (Fig. 2). For the cell line *CARDα_1SKMβ/B3* (called LHB3), control currents (CARD$\alpha_1\beta$) up to −50 pA were routinely observed using a V_c at +20 mV (n = 26). In 5 cells, we measured Ba^{2+} currents up to −150 pA (Fig. 2A). The current density was 3.1 ± 1.8 pA/pF (n = 32). A similar level of current density was also observed for several other CARDα_1SKMβ cell lines. CARD$\alpha_1\beta$ currents obtained obtained for V_c's from −10 mV to +50 mV displayed time for half-activation ($t_{1/2}$) ranging from 15 ms to 4.1 ms (Fig. 2B). The time to peak was about 850 ms at +20 mV (ranging from 400 ms to 1.5 s, n = 18). Inactivation of CARD$\alpha_1\beta$ current was slow at all the potentials examined (Fig. 2C). The current–voltage relationship (Fig. 2D) shows that the Ba^{2+} current in LHB3 cells activated at −20 mV and peaked at +20 mV. CARD$\alpha_1\beta$ currents were enhanced 5- to 8-fold and the kinetics were faster in the presence of 1 μM Bay K 8644 (Fig. 2E,F). In addition, Bay K 8644 produced a marked leftward shift (up to 15 mV) of the current–voltage relationship (Fig. 2G).

Activation kinetics for CARDα_1 and CARD$\alpha_1\beta$ currents were also compared in the presence of 1 μM Bay K 8644 (Fig. 3A,B). The time for half activation ($t_{1/2}$, Fig. 3C) was not significantly different for currents obtained in the range of maximal peak current activation (0, +10, +20 mV). Similarly, activation time-constant values obtained after monoexponential fitting (CLAMPFIT ver. 5.51) of CARDα_1 and CARD$\alpha_1\beta$ currents were not significantly different at +10 mV (23.5 ± 8 ms (n = 4) and 11.7 ± 4.2 ms (n = 3), respectively. However, time-to-peak values were significantly faster (3- to 4-fold) when the β subunit was present (Fig. 3D).

Inactivation of CARD$\alpha_1\beta$ currents, measured after 3 seconds of V_c, was significantly faster at all potentials (3-fold) than for CARDα_1 (Fig. 4A,B). The faster inactivation time course may account for the change in time-to-peak values. However, CARD$\alpha_1\beta$ currents did not inactivate completely and a slow inward current (~ 25% of the total current) remained after 15 s (Fig. 4C). A comparison of CARD$\alpha_1\beta$- and CARDα_1 currents of similar amplitude revealed that a current-dependent phenomenon could not account for the acceleration in inactivation kinetics (Fig. 4D). The data indicate that

the β subunit may participate in the induction of a fast component of the cardiac L-type Ca^{2+} channel inactivation.

4. DISCUSSION

We have analysed how Ca^{2+} channels resulting from the expression of a cardiac α_1 subunit are affected by a β subunit using permanent expression strategies in the mouse L cell. Constitutively, L cells do not exhibit any Ca^{2+} channel activity or expression of any of the L-type Ca^{2+} channel subunit transcripts [22,25]. This is contrary to other mammalian cell lines such as CHO cells or HEK293 cells (data not shown) used for Ca^{2+} channel expression [14,33] as well as for *Xenopus* oocytes [23,24]. In the case of *Xenopus* oocytes, the 'auxilliary' subunits (α_2/δ and β) influence endogenous Ca^{2+} channel activity [14,23]. Whether or not, CARDα_1 subunit introduced in expression systems having Ca^{2+} channels, e.g. *Xenopus* oocytes, are under the control of endogenous α_2/δ and β subunits is unknown. We observed that the transfection of the CARDα_1 subunit in L cells results in an extremely low level of Ca^{2+} channel activity. The low channel activity seen with CARDα_1 in L cells might be related to the lack of auxilliary Ca^{2+} channel subunit expression in this particular expression system. However, this further provides an opportunity for subunit interaction studies.

We demonstrated that the β subunit is essential in determining the basal level of CARDα_1 activity (up to 20-fold increase). This is contrary to our previous observations in L cells transfected with combinations of SKMα_1 and β subunits, in which Ba^{2+} current amplitude was reduced upon coexpression of SKMβ subunit [25,27]. However, the β subunit was very effective in increasing the apparent level of expression of the SKMα_1 protein as judged by the increase in DHP binding activity (B_{max}) [25,26].

In the present study, the β subunit appears to have only a minor effect on CARDα_1-Ba^{2+} current kinetics, inducing a fast component of the current inactivation. This is in contrast with the significant acceleration of SKMα_1 current activation and inactivation kinetics produced by the β subunit [25]. Taken together, the data show that in L cells, the β subunit has a dramatically different effect on CARDα_1 compared to SKMα_1.

The use of a SKMβ isoform for coexpression studies with a CARDα_1 subunit [8,23,24] does not represent a limitation for the study of β subunit modulation of CARDα_1, since the SKMβ isoform and a cardiac isoform (β_2) induce similar changes in CARDα_1-Ba^{2+} current properties [16]. Similarly, the use of another isoform of the β subunit present in cardiac tissue (CAB3) did not result in any change other than a larger increase in CARDα_1-Ba^{2+} current density [17]. Moreover, there is now evidence for the expression of the

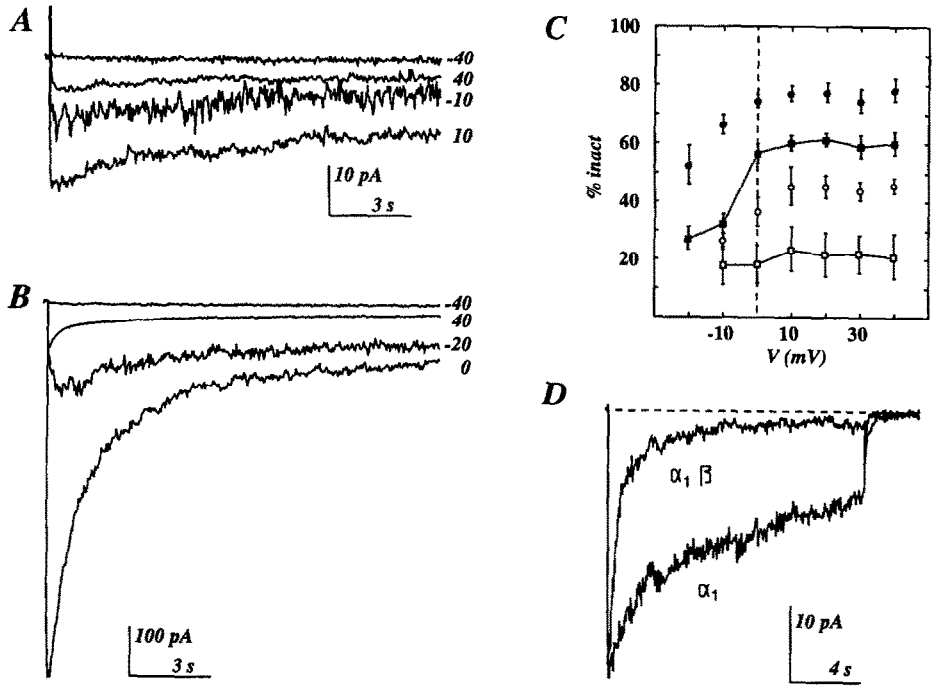

Fig. 4. Comparison of inactivation properties of CARDα_1- and CARD$\alpha_1\beta$-Ba^{2+} currents. (A) and (B) Representative CARD$\alpha_1\beta$-Ba^{2+} currents and CARD$\alpha_1\beta$-Ba^{2+} currents, respectively, using a 15-s V_c duration. Similar to the experimental conditions described in Fig. 3 legend, the bath solution contained 1 μM Bay K 8644 and the V_c's are indicated on the traces. (C) Representation of the half-time inactivation–voltage relationships for CARDα_1 current (open symbols, $n = 6$) and CARD$\alpha_1\beta$ current (filled symbols, $n = 5$). The solid lines represent the percentage of decrease in current amplitude after 3 s. The symbols without lines represent the percentage of decrease in current amplitude after 15 s. (D) Comparison of time-dependent decay of CARDα_1-Ba^{2+} current and CARD$\alpha_1\beta$-Ba^{2+} current recorded from two cells (LH18 and LHB3, respectively) having similar current density (15.2 pF and 16 pF). The V_c was 10 mV and the HP was −60 mV.

skeletal muscle isoform of the β subunit in human heart [18].

In summary, we present here evidence that in heart the β subunit has a crucial role in determining the basal activity of the L-type Ca^{2+} channel. It is now of interest to probe the physiological relevance of this regulation of the cardiac L-type activity.

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