

# Characterization of $\beta$ subunit modulation of a rabbit cardiac L-type $\text{Ca}^{2+}$ channel $\alpha_1$ subunit as expressed in mouse L cells

Philippe Lory<sup>a</sup>, Gyula Varadi<sup>a</sup>, Donald F. Slish<sup>b</sup>, Marie Varadi<sup>a</sup> and Arnold Schwartz<sup>a</sup>

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

Received 16 October 1992; revised version received 16 November 1992

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

Mouse L cell;  $\text{Ca}^{2+}$  channel expression; Cardiac  $\alpha_1$  subunit;  $\beta$  Subunit

## 1. INTRODUCTION

The high-voltage activated (L-type)  $\text{Ca}^{2+}$  channel is the primary pathway for  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  channel subunits ( $\alpha_1$ ,  $\alpha_2/\delta$ ,  $\beta$  and  $\gamma$ ) have been cloned [2–5]. Several  $\alpha_1$ -isoforms have also been cloned from cardiac ( $\text{CARD}\alpha_1$ ) [6–8], smooth muscle [9,10] and brain [11–14], as well as various  $\beta$  isoforms, from rat brain [15,16], human brain [13], rabbit heart [17] and human heart [18].

Expression of  $\text{SKM}\alpha_1$  and  $\text{CARD}\alpha_1$  has been assayed in several systems. It appears that the functional properties of tissue-specific  $\alpha_1$  isoforms may depend on the recipient cell [19–21]. For example, transfection of the  $\text{SKM}\alpha_1$  subunit in the mouse L cell, results in the expression of DHP-sensitive  $\text{Ca}^{2+}$  channels and DHP binding [22] but such expression is absent in *Xenopus* oocytes. In contrast, the  $\text{CARD}\alpha_1$  subunit expresses functional  $\text{Ca}^{2+}$  channels in *Xenopus* oocytes [6,8,23,24].

Several recent studies provide evidence that SKM  $\beta$   $\alpha_2/\delta$  and  $\gamma$  subunits have a regulatory role on  $\text{SKM}\alpha_1$  subunit activity [25–27]. It has been shown that the  $\beta$  subunit enhances  $\text{CARD}\alpha_1$  activity when expressed in

*Xenopus* oocytes [8,16,17,23,24].  $\beta$  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  $\text{Ca}^{2+}$  current which is enhanced by  $\text{SKM}\beta$  and/or  $\text{SKM}\alpha_2/\beta$  subunits [13,23]. Thus, it is still unclear whether these studies demonstrate a specific  $\text{CARD}\alpha_1$ - $\beta$  interaction or an influence of the recipient cell on exogenous  $\text{Ca}^{2+}$  channel activity.

In the present study we investigate the properties of the  $\text{CARD}\alpha_1$  expression in mouse L cell. This cell line, in contrast to other expression systems, does not exhibit any endogenous  $\text{Ca}^{2+}$  channel activity and lacks expression of  $\text{Ca}^{2+}$  channel subunit transcripts [25]. We compare here the functional properties of  $\text{CARD}\alpha_1$  and  $\text{SKM}\alpha_1$  L-type  $\text{Ca}^{2+}$  channels expressed in L cells, and present the effects of a  $\beta$  subunit on  $\text{CARD}\alpha_1$  activity.

## 2. MATERIALS AND METHODS

### 2.1. Isolation and construction of a full-length rabbit cardiac $\alpha_1$ cDNA

A mixed primed (random primer-oligodT) rabbit cardiac library, constructed in  $\lambda$ ZAPII 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'-HindIII site and the 3'-NotI site, ligated into the corresponding cloning sites of the pAGS-3 mammalian expression vector [29] and designated pAGS-3HT $\alpha_1$ .

### 2.2. Transfection of Ltk-cells

Mouse L cells were cotransfected with pSV2neo and pAGS-3HT $\alpha_1$

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.

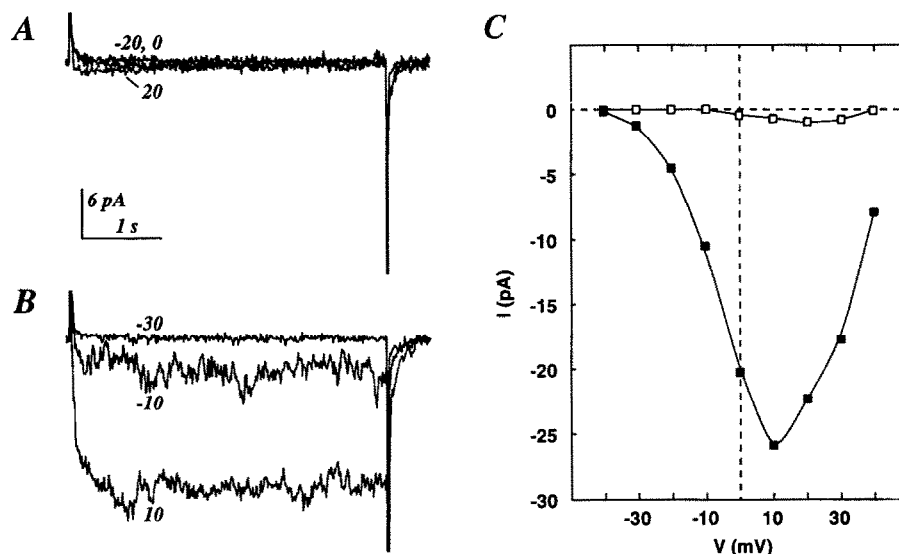


Fig. 1.  $\text{Ba}^{2+}$  current activity in a cell expressing the cardiac  $\alpha_1$  subunit (LH18 cell). (A) The family of current traces illustrates the basal current activity ( $V_c$ 's are indicated on the traces). (B)  $\text{Ba}^{2+}$  currents recorded on the same cell after application of Bay K 8644 (1  $\mu\text{M}$ ) in the bath. (C) Current-voltage relationships for control currents ( $\square$ ) and in the presence of Bay K 8644 ( $\blacksquare$ ), respectively.

at a molar ratio of 1:10 [30]. After 2 to 3 weeks of G418 selection (400  $\mu\text{g}/\text{ml}$ ), 20 individual clones were isolated and screened by PCR methodology for the insertion of the  $\text{CARD}\alpha_1$  cDNA into the genome. Northern blot analysis was used to test the level of expression of the  $\text{CARD}\alpha_1$  message. A cell line, designated LH18, showed the highest expression for  $\text{CARD}\alpha_1$ , and was further cotransfected with pSVTK [31] and pSG-5 $\beta$ , an expression plasmid for the SKM $\beta$  subunit [25], using the conditions described above. Selection was done in a HAT-G418 medium (Hypoxanthine, 0.1 mM; Aminopterin, 0.4  $\mu\text{M}$ ; thymidine, 16  $\mu\text{M}$ ; G418, 200  $\mu\text{g}/\text{ml}$ ). 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  $\text{Ba}^{2+}$  as charge carrier, in the whole-cell configuration [32] at room temperature (20–22°C). The bathing solution was in (mM):  $\text{Ba}(\text{OH})_2$ , 40; glutamate, 40; *N*-methyl *D*-glucamine, 80; HEPES, 10;  $\text{MgCl}_2$ , 2; pH adjusted to 7.4 with  $\text{CH}_3\text{SO}_3\text{H}$ . The pipette solution contained the following (in mM): *N*-methyl *D*-glucamine, 110;  $\text{MgCl}_2$ , 2; EGTA, 15; HEPES, 15; pH adjusted to 7.3 with  $\text{CH}_3\text{SO}_3\text{H}$ . 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).  $\text{Ba}^{2+}$  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*<sup>-</sup> cells were characterized for their expression of  $\text{CARD}\alpha_1$  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  $\text{Ca}^{2+}$  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  $\text{Ba}^{2+}$  currents were very low or not detectable under control conditions in cells expressing  $\text{CARD}\alpha_1$  (Fig. 1A). In fact,  $\text{Ba}^{2+}$  currents were detecta-

Table I

Comparison of  $\text{CARD}\alpha_1$ -,  $\text{CARD}\alpha_1\beta$ - and SKM $\alpha_1$ - $\text{Ba}^{2+}$  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  $\text{CARD}\alpha_1$ - and  $\text{CARD}\alpha_1\beta$ - $\text{Ba}^{2+}$  currents, at +20 mV for SKM $\alpha_1$ - $\text{Ba}^{2+}$  currents) correspond to currents obtained in the presence of 1  $\mu\text{M}$  Bay K 8644.

	% of Cells with Ctrl I <sub>Ba</sub>	I <sub>Ba</sub> density (pA/pF)		Act. threshold* (mV)	Time to peak* (ms)
		Ctrl	1 $\mu\text{M}$ Bay K		
SKM $\alpha_1$	75% (8 <sup>#</sup> )	0.57 ± 0.2 (8)	1.45 ± 0.15 (8)	-20	4.1 ± 0.2 s (6)
CARD $\alpha_1$	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  $\mu\text{M}$  Bay K 8644 in the bath.

<sup>#</sup>In agreement with our previous reports [25,27].

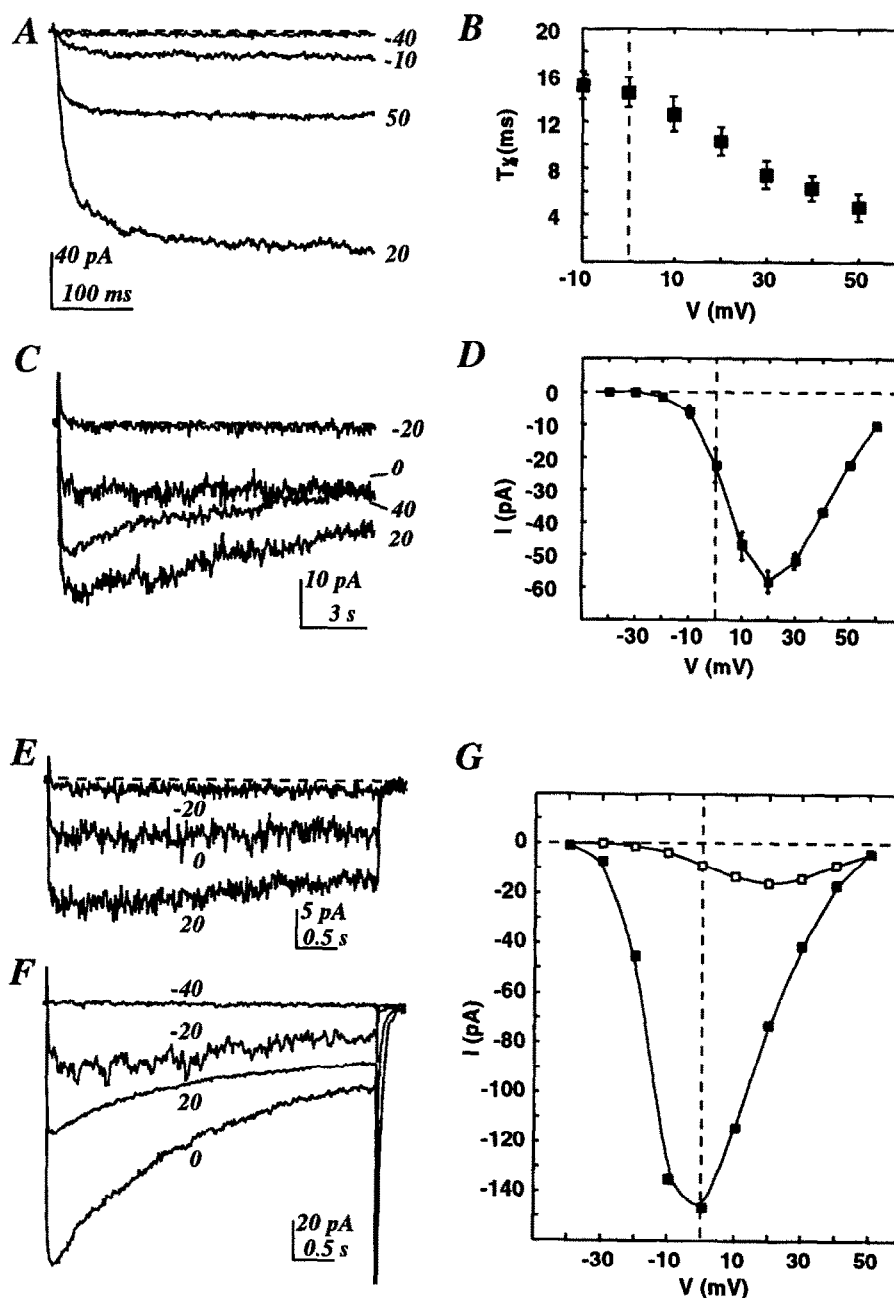


Fig. 2. Modulation of the basal  $\text{CARD}\alpha_1\text{-Ba}^{2+}$  current activity by the  $\text{SKM}\beta$  subunit. (A) Family of  $\text{Ba}^{2+}$  currents recorded in a LHB3 cell using a linear current subtraction procedure with 4 depolarizing subpulses enable to elicit active currents (P/-4 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  $\text{CARD}\alpha_1\text{-Ba}^{2+}$  currents. The values are from 7 cells (peak currents up to  $-50$  pA) using a protocol described in A for the  $\text{CARD}\alpha_1\text{-Ba}^{2+}$  current acquisition. The determination of the  $t_{1/2}$ -activation value was done using the CLAMPAN module of the pCLAMP software (presented as mean  $\pm$  S.E.M.). (C) Inactivation of  $\text{CARD}\alpha_1\text{-Ba}^{2+}$  current during 15-s  $V_c$  duration. At  $20$  mV, the percentage of current decay was  $26 \pm 7\%$  after 15 s (mean  $\pm$  S.E.M.,  $n = 12$ ). (D) Current-voltage relationship of  $\text{CARD}\alpha_1\text{-Ba}^{2+}$  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  $\text{CARD}\alpha_1\text{-Ba}^{2+}$  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  $1 \mu\text{M}$  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  $\text{Ba}^{2+}$  current in all of the cells. In the presence of Bay K 8644 ( $1 \mu\text{M}$ ),  $\text{CARD}\alpha_1\text{-Ba}^{2+}$  current activated at  $-30$  mV and peaked at  $+10$

mV (Fig. 1B,C). In contrast to  $\text{CARD}\alpha_1$ ,  $\text{SKM}\alpha_1 \text{Ba}^{2+}$  currents were routinely recorded in the absence of Bay K 8644 (Table I). In some experiments,  $\text{SKM}\alpha_1$  currents up to  $-200$  pA were observed (data not shown). Application of Bay K 8644 ( $1 \mu\text{M}$ ) in these experiments pro-

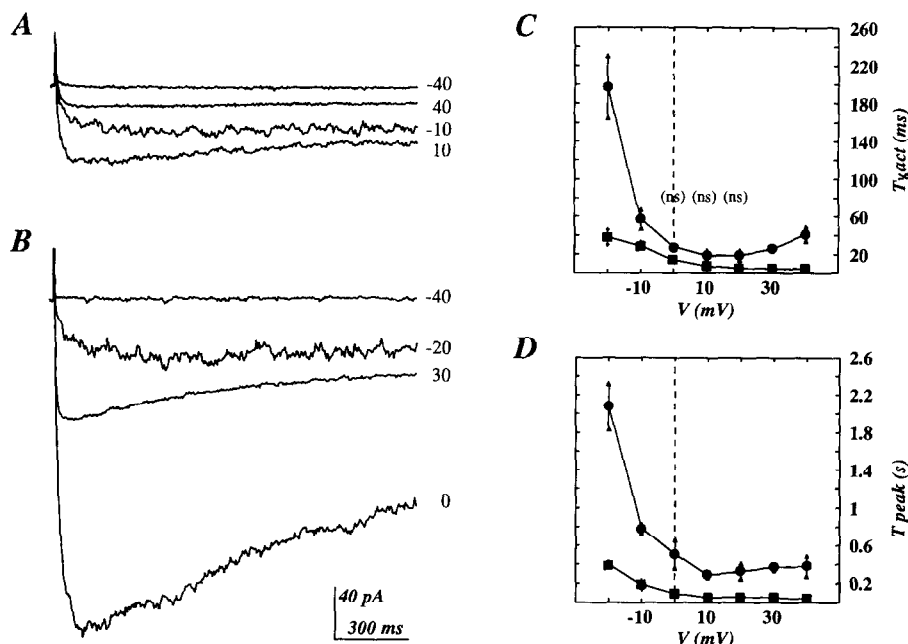


Fig. 3. Comparison of activation properties of CARD $\alpha_1$ - and CARD $\alpha_1\beta$ -Ba $^{2+}$  currents. (A) and (B) Representative CARD $\alpha_1$ -Ba $^{2+}$  currents and CARD $\alpha_1\beta$ -Ba $^{2+}$  currents, respectively, using a 1.5-s  $V_c$  duration. The bath solution contained 1  $\mu$ M Bay K 8644. The  $V_c$ 's are indicated on the traces. (C) Representation of the half-time activation-voltage relationships for CARD $\alpha_1$  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 CARD $\alpha_1$  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 SKM $\alpha_1$  currents (Table I).

Bay K 8644-stimulated CARD $\alpha_1$ -Ba $^{2+}$  currents displayed faster activation kinetics than SKM $\alpha_1$ -Ba $^{2+}$  currents (Table I). The time to peak for CARD $\alpha_1$  ( $350 \pm 12$  ms for a depolarizing pulse ( $V_c$ ) at 10 mV) was tenfold faster than for SKM $\alpha_1$  ( $4.1 \pm 2$  s for a  $V_c$  at 20 mV). In addition, CARD $\alpha_1$  current activated a more negative potentials than SKM $\alpha_1$  current using identical recording conditions [25,27].

Coexpression of SKM $\beta$  with CARD $\alpha_1$  in the LH18 cell line resulted in a large increase in current density (Fig. 2). For the cell line CARD $\alpha_1$ SKM $\beta$ /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  $3.1 \pm 1.8$  pA/pF ( $n = 32$ ). A similar level of current density was also observed for several other CARD $\alpha_1$ SKM $\beta$  cell lines. CARD $\alpha_1\beta$  currents 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  $\mu$ M 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 CARD $\alpha_1$  and CARD $\alpha_1\beta$  currents were also compared in the presence of 1  $\mu$ M 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 CARD $\alpha_1$  and CARD $\alpha_1\beta$  currents were not significantly different at  $+10$  mV ( $23.5 \pm 8$  ms ( $n = 4$ ) and  $11.7 \pm 4.2$  ms ( $n = 3$ ), respectively). However, time-to-peak values were significantly faster (3- to 4-fold) when the  $\beta$  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 CARD $\alpha_1$  (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 ( $\sim 25\%$  of the total current) remained after 15 s (Fig. 4C). A comparison of CARD $\alpha_1\beta$ - and CARD $\alpha_1$  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  $\beta$  subunit may participate in the induction of a fast component of the cardiac L-type  $\text{Ca}^{2+}$  channel inactivation.

#### 4. DISCUSSION

We have analysed how  $\text{Ca}^{2+}$  channels resulting from the expression of a cardiac  $\alpha_1$  subunit are affected by a  $\beta$  subunit using permanent expression strategies in the mouse L cell. Constitutively, L cells do not exhibit any  $\text{Ca}^{2+}$  channel activity or expression of any of the L-type  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  channel expression [14,33] as well as for *Xenopus* oocytes [23,24]. In the case of *Xenopus* oocytes, the 'auxiliary' subunits ( $\alpha_2/\delta$  and  $\beta$ ) influence endogenous  $\text{Ca}^{2+}$  channel activity [14,23]. Whether or not,  $\text{CARD}\alpha_1$  subunit introduced in expression systems having  $\text{Ca}^{2+}$  channels, e.g. *Xenopus* oocytes, are under the control of endogenous  $\alpha_2/\delta$  and  $\beta$  subunits is unknown. We observed that the transfection of the  $\text{CARD}\alpha_1$  subunit in L cells results in an extremely low level of  $\text{Ca}^{2+}$  channel activity. The low channel activity seen with  $\text{CARD}\alpha_1$  in L cells might be related to the lack of auxiliary  $\text{Ca}^{2+}$  channel subunit expression in this particular expression system. However, this further provides an opportunity for subunit interaction studies.

We demonstrated that the  $\beta$  subunit is essential in determining the basal level of  $\text{CARD}\alpha_1$  activity (up to 20-fold increase). This is contrary to our previous observations in L cells transfected with combinations of  $\text{SKM}\alpha_1$  and  $\beta$  subunits, in which  $\text{Ba}^{2+}$  current amplitude was reduced upon coexpression of  $\text{SKM}\beta$  subunit [25,27]. However, the  $\beta$  subunit was very effective in increasing the apparent level of expression of the  $\text{SKM}\alpha_1$  protein as judged by the increase in DHP binding activity ( $B_{\text{max}}$ ) [25,26].

In the present study, the  $\beta$  subunit appears to have only a minor effect on  $\text{CARD}\alpha_1$ - $\text{Ba}^{2+}$  current kinetics, inducing a fast component of the current inactivation. This is in contrast with the significant acceleration of  $\text{SKM}\alpha_1$  current activation and inactivation kinetics produced by the  $\beta$  subunit [25]. Taken together, the data show that in L cells, the  $\beta$  subunit has a dramatically different effect on  $\text{CARD}\alpha_1$  compared to  $\text{SKM}\alpha_1$ .

The use of a  $\text{SKM}\beta$  isoform for coexpression studies with a  $\text{CARD}\alpha_1$  subunit [8,23,24] does not represent a limitation for the study of  $\beta$  subunit modulation of  $\text{CARD}\alpha_1$ , since the  $\text{SKM}\beta$  isoform and a cardiac isoform ( $\beta_2$ ) induce similar changes in  $\text{CARD}\alpha_1$ - $\text{Ba}^{2+}$  current properties [16]. Similarly, the use of another isoform of the  $\beta$  subunit present in cardiac tissue (CAB3) did not result in any change other than a larger increase in  $\text{CARD}\alpha_1$ - $\text{Ba}^{2+}$  current density [17]. Moreover, there is now evidence for the expression of the

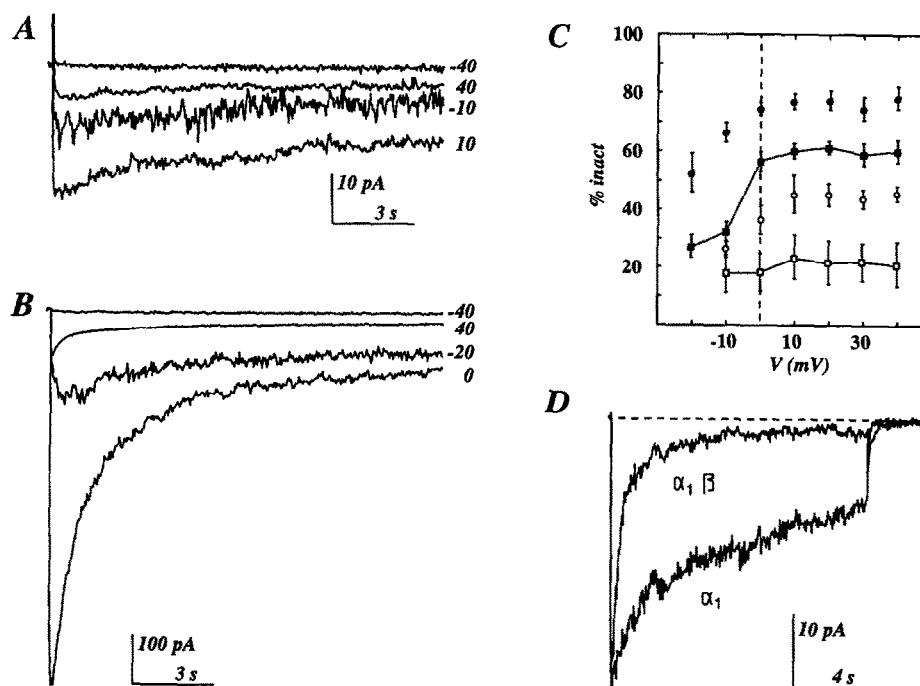


Fig. 4. Comparison of inactivation properties of  $\text{CARD}\alpha_1$ - and  $\text{CARD}\alpha_1\beta$ - $\text{Ba}^{2+}$  currents. (A) and (B) Representative  $\text{CARD}\alpha_1\beta$ - $\text{Ba}^{2+}$  currents and  $\text{CARD}\alpha_1\beta$ - $\text{Ba}^{2+}$  currents, respectively, using a 15-s  $V_c$  duration. Similar to the experimental conditions described in Fig. 3 legend, the bath solution contained 1  $\mu\text{M}$  Bay K 8644 and the  $V_c$ 's are indicated on the traces. (C) Representation of the half-time inactivation-voltage relationships for  $\text{CARD}\alpha_1$  current (open symbols,  $n = 6$ ) and  $\text{CARD}\alpha_1\beta$  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  $\text{CARD}\alpha_1$ - $\text{Ba}^{2+}$  current and  $\text{CARD}\alpha_1\beta$ - $\text{Ba}^{2+}$  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  $\beta$  subunit in human heart [18].

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

**Acknowledgements:** We thank Dr. S.H. Bryant for fruitful discussions and review of the manuscript. We thank Dr. L. Birnbaumer and Dr. K.P. Campbell for LCa.11 cells and skeletal muscle  $\beta$  cDNA, respectively, and Dr. J.I. Miyazaki for providing the pAGS-3 vector. This research was supported by NIH Grants HL-43231, HL-22619 and T32 HL07382, and Tanabe Seiyaku Fund (to A.S.) and a postdoctoral fellowship from Institut de la Santé et de la Recherche Médicale, France (to P.L.).

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