Journal of Physiology (1993), **471**, *pp.* 749–765
With 8 figures
Printed in Great Britain

STABLE CO-EXPRESSION OF CALCIUM CHANNEL α_1, β AND α_2/δ SUBUNITS IN A SOMATIC CELL LINE

BY ANDREA WELLING, EVA BOSSE, ADOLFO CAVALIÉ*, RONALD BOTTLENDER, ANDREAS LUDWIG, WOLFGANG NASTAINCZYK†, VEIT FLOCKERZI AND FRANZ HOFMANN

*From the Institut für Pharmakologie und Toxikologie, Technische Universität München, Biedersteiner Straße 29, 80802 München, Germany, *Max-Planck-Institut für Psychiatrie, Am Klopferspitz 18a, 82152 Martinsried, Germany and †Medizinische Biochemie, Universität des Saarlandes, 66424 Homburg, Germany*

(*Received 11 February 1993*)

SUMMARY

1. The high-voltage-activated L-type calcium channel is a multi-protein complex of α_1, α_2/δ, β and γ subunits. The α_1 subunit contains the voltage-dependent calcium-conducting pore. Chinese hamster ovary (CHO) cells were stably transfected with the complementary DNA of the α_1, β and α_2/δ subunit. These subunits were not detected in wild-type CHO cells.

2. The α_1 (CaCh2b) subunit itself directed the expression of functional calcium channels which bound calcium channel blockers and showed voltage-dependent activation and inactivation.

3. The co-expression of the α_1 subunit with the β subunit (CaB1 gene) enhanced the density of the dihydropyridine binding sites 2- to 3-fold and increased dihydropyridine-sensitive barium inward currents (I_{Ba}) up to 3·5-fold from $-13{\cdot}3$ μA/cm^2 (α_1 subunit) to $-46{\cdot}7$ μA/cm^2 (α_1 and β subunits).

4. Co-expression of the β subunit did not change the sensitivity of I_{Ba} towards dihydropyridines, but accelerated current activation and inactivation and shifted the half-maximal steady-state activation and inactivation to slightly more hyperpolarizing potentials.

5. The co-expression of the α_2/δ subunit together with α_1 and β subunits accelerated the inactivation kinetics of the channel without a major effect on the other parameters.

6. These results indicate that the β and α_2/δ subunit interact with the α_1 subunit and modulate thereby the properties of the α_1 subunit-dependent inward current.

INTRODUCTION

Voltage-dependent calcium channels play a crucial role in excitation–contraction coupling and other cellular processes like motility, secretion and excitability. The L-type calcium channel from skeletal muscle is a complex of four proteins: the α_1

subunit (212018 Da), which contains the binding sites for all known calcium channel blockers and the calcium-conducting pore; the intracellularly located β subunit (57868 Da); the transmembrane γ subunit (25058 Da) and the α_2/δ subunit, a disulphide-linked dimer of 125018 Da (see Hofmann, Biel & Flockerzi, 1994, and references cited therein). Complementary DNAs for the skeletal muscle calcium channel were isolated on the basis of peptide sequences derived from the purified proteins (Tanabe *et al.* 1987; Ellis *et al.* 1988; Ruth *et al.* 1989; Bosse *et al.* 1990; Jay *et al.* 1990). Using these cDNAs as probes distinct gene products encoding α_1 and β subunits have been cloned from heart, smooth muscle, kidney, fibroblasts, endocrine and neuronal cells (see Hofmann *et al.* 1994 and references cited therein). By contrast, the γ subunit appears to be expressed exclusively in skeletal muscle. The α_2/δ subunit is highly conserved in most tissues, including brain, cardiac and smooth muscle, indicating that calcium channels in these tissues are hetero-oligomers formed from a common α_2/δ and different α_1 and β subunits.

The L-type calcium channels from cardiac muscle (Mikami *et al.* 1989) and smooth muscle (Biel *et al.* 1990) are splice variants of one gene, CaCh2 (Perez-Reyes, Wei, Castellano & Birnbaumer, 1990; Biel *et al.* 1991; Hofmann *et al.* 1994), which differs from the genes of the α_1 subunits from skeletal muscle (CaCh1) and brain (CaCh3, CaCh4, CaCh5 and CaCh6). Expression of smooth or cardiac muscle α_1 subunits in *Xenopus* oocytes (Mikami *et al.* 1989; Biel *et al.* 1990, 1991; Singer, Biel, Lotan, Flockerzi, Hofmann & Dascal, 1991; Wei, Perez-Reyes, Lacerda, Schuster, Brown & Birnbaumer, 1991) or in chinese hamster ovary (CHO) cells (Bosse *et al.* 1992) led to the formation of dihydropyridine-sensitive inward currents (I_{Ba}) with properties similar to those recorded in native cells. The expression of the skeletal muscle α_1 subunit in mouse fibroblasts (L-cells) generated cell lines expressing dihydropyridine-sensitive calcium channels with activation kinetics that are about 100 times slower than expected for skeletal muscle calcium currents (Perez-Reyes *et al.* 1989). Co-expression of the calcium channel β subunit from the same tissue with skeletal muscle α_1 accelerated channel activation and led to calcium currents with physiological activation kinetics (Lacerda *et al.* 1991; Varadi, Lory, Schultz, Varadi & Schwartz, 1991). In addition, the expression of the β subunit was coincident with an increase in the number of dihydropyridine binding sites whereas I_{Ba} remained unchanged (Lacerda *et al.* 1991) or decreased (Varadi *et al.* 1991). Furthermore it was reported that in L-cells the co-expression of all four skeletal muscle calcium channel subunits (Varadi *et al.* 1991) or an excess of the β subunit over the α_1 subunit (Lory, Varadi & Schwartz, 1992) resulted in a decreased amplitude of the barium current and in a diminished response towards the calcium channel agonist BayK8644. As in fibroblasts, the co-expression of the α_2/δ or β subunit alone or in combination accelerated the speed of activation of I_{Ba} directed by the cardiac α_1 subunit in *Xenopus* oocytes (Singer *et al.* 1991; Wei *et al.* 1991). However, and in contrast to the results with mouse fibroblasts, the expression of β subunit in oocytes coincided with an increase in α_1-directed I_{Ba} which remained sensitive towards dihydropyridines.

In an attempt to resolve the inconsistencies in the results obtained with L-cells and *Xenopus* oocytes the calcium channel β and α_2/δ subunits were introduced stably into CHO cells already expressing the smooth muscle α_1 subunit (Bosse *et al.* 1992). In these cells the β subunit protein increased the maximal number of binding sites for

dihydropyridines and, as in *Xenopus* oocytes, enhanced the density of I_{Ba} without changing significantly its sensitivity towards dihydropyridines. Co-expression of the α_2/δ protein together with the smooth muscle α_1 and skeletal muscle β subunit accelerated channel inactivation kinetics.

METHODS

Construction and transfection of calcium channel α_1, β and α_2/δ subunit expression plasmids

The construction of the expression plasmid p91$\alpha_1$2b carrying the complete protein-coding region of the smooth muscle α_1 subunit has been described previously (Bosse *et al.* 1992). To obtain the expression plasmid p91B1 carrying the entire protein-coding region of the calcium channel CaB1 β subunit (Ruth *et al.* 1989) the 1·63 kb EcoRI fragment of pCaB1 (Singer *et al.* 1991) was inserted into the EcoRI site of p91023(B) in the same orientation with respect to the adenovirus late promotor to yield p91B1. To obtain a second-expression plasmid carrying the β subunit cDNA, the expression plasmid pKNH (Nukuda, Mishina & Numa, 1987) containing the neomycin-resistance gene (the expression of which makes mammalian cells resistant to the antibiotic G418) was cleaved with HindIII and blunted with the large fragment of DNA polymerase I in the presence of the four dNTPs. The 1·63 kb EcoRI fragment from pCaB1 was blunted with the large fragment of DNA polymerase I in the presence of desoxyadenosine-5′-triphosphate (dATP) and thymidine-5′-triphosphate (TTP) and inserted into the blunt-end site of pKNH in the same orientation with respect to SV40 early gene transcription to generate pKNHB1. To obtain the recombinant plasmid pKNHA1 the 3·5 kb cDNA fragment from pCaCA1 (Mikami *et al.* 1989) containing the protein-coding region of the skeletal muscle α_2/δ subunit was ligated into the blunt-end site of pKNH. All cells (10^7 cells for each experiment) were transfected with 10 μg cDNA by electroporation using gene pulser transfection apparatus (Bio-Rad Laboratories). The recombinant expression plasmids used to obtain the different cell lines are summarized in Table 1.

Cell culture

CHO cells were grown in Ham's F12 medium in the presence of 10% fetal bovine serum, streptomycin (30 μg/ml) and penicillin (30 units/ml). CHOβ10 cells were grown in the same medium except that for selection and maintenance G418 was added at 400 and 200 μg/ml, respectively. CHOCa9 cells were grown in Dulbecco's modified Eagle's medium, in the presence of 10% dialysed fetal bovine serum, streptomycin (30 μg/ml), penicillin (30 units/ml) and non-essential amino acids. In the cases of co-transfections with pKNHB1 and pKNHA1, G418 was included at the concentrations indicated above. CHOCa9β3 cells were co-transfected with pKNHA1 and the histidinol dehydrogenase gene (Hariman & Mulligan, 1988) and transfectants were selected in the presence of 2·5 mM L-histidinol. After selection of CHOCa9β3α2/δ4 cells histidinol could be omitted from the medium without a change in α_2/δ protein expression. Before collecting and testing, cells were plated at the equivalent density of $\approx 10^6$ per 175 cm^2 flask and grown to confluence (5 days) to densities of $\approx 10^7$ cells per 175 cm^2 flask. After washing with phosphate-buffered saline, cells were collected for immunoblots or binding of calcium channel blockers to membranes, or used for electrophysiological analysis of calcium channel activities.

SDS–PAGE and immunoblot analysis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using the system of Laemmli (1970) on 0·75 mm thick 7·5% polyacrylamide gels. Gels were either stained with Coommassie or transferred to an Immobilon-P-membrane using the semi-dry method (1 h at 0·8 mA/cm^2 gel). Blots were blocked with 2% non-fat dry milk (Glücksklee®, Nestlé) in buffer A (10 mM Tris–HCl, pH 8·0, 150 mM NaCl, 0·05% (w/v) Tween 20) for 2 h at 20 °C. The monoclonal antibodies 7C3 and 5H9, which are directed against the skeletal muscle β and α_2/δ subunit respectively (Nastainczyk, Ludwig & Hofmann, 1990), were diluted 1:10000 in buffer A containing 1% non-fat dry milk and were added to the blot. After incubation for 2 h at 20 °C the membrane was washed 3 times with buffer A containing 1% non-fat dry milk and incubated with alkaline phosphatase-linked anti-mouse secondary antibody for 1 h at room temperature (diluted 1:7500 in

buffer A containing 1% non-fat dry milk). Blots were washed 3 times again and then developed using 0·01% 4-nitro-blue-tetrazolium and 0·005% 5-bromo-4-chloro-3-indolylphosphate as substrates in buffer B (100 mM Tris–HCl, pH 9·5, 100 mM NaCl, 5 mM $MgCl_2$).

Current recordings

The CHO cells were grown on coverslips for 2 days as described above. Before recording Ba^{2+} currents the coverslips were rinsed three times with phosphate-buffered saline solution and placed into an experimental chamber containing (mM): NaCl, 82; TEA, 20; $BaCl_2$, 30; CsCl, 5·4; $MgCl_2$, 1; glucose, 10; Hepes, 5; adjusted to pH 7·4 with NaOH. In some experiments $BaCl_2$ was replaced by $CaCl_2$. The pipette solution contained (mM): CsCl, 112; $MgCl_2$, 1; Na_2ATP, 3; Hepes, 5; EGTA, 10; adjusted to pH 7·4 with CsOH. Ba^{2+} and Ca^{2+} currents were recorded in the whole-cell mode of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) at room temperature using an EPC-9 patch-clamp amplifier (Heka Electronics, Lamprecht, Germany). Patch pipettes (borosicilate glass) had resistances of 1·5–3·0 MΩ when filled with the pipette solution. Data were sampled once every 0·1 ms and filtered at 1–3 kHz. The whole-cell capacitance (C_m), determined by the compensation needed to cancel the capacitive artifact, ranged from 20 to 35 pF. The inward currents were recorded by applying a test pulse of 140 ms duration every 5 s from a holding potential (V_H) of −80 to +10 mV. The current density was calculated from the individual cell capacitance, assuming a specific membrane capacitance of 1 $\mu F/cm^2$. The activation curve and the current–voltage relations were determined by stepping the membrane potential to the indicated potentials from a V_H of −80 mV every 5 s. The steady-state inactivation was determined using a 1 s conditioning pulse to indicated membrane potentials and a test pulse to +20 mV. Activation and inactivation curves were fitted according to the Boltzmann equation $I/I_{max} = 1/[1+\exp\{(V-V_{0.5})/k\}]$, where I is the current at each potential (V), I_{max} is the maximal current, $V_{0.5}$ is the half-maximal activation or inactivation voltage, respectively, and k is the slope factor. The leak current component was obtained by hyperpolarizing voltage steps or using the P/n procedure with n between 6 and 10 and was subtracted from all current traces.

Miscellaneous

The cDNAs of the calcium channel subunits were originally cloned from rabbit lung (the α_1 subunit of the calcium channel gene CaCh2b) and rabbit skeletal muscle (the β subunit of gene CaB1 and the α_2/δ subunit of gene CaA_21). Isradipine binding and protein concentrations were determined according to Bosse *et al.* (1992) and Smith *et al.* (1985), respectively.

RESULTS

Stable co-transfection of CHO⁻ cells with calcium channel α_1 and β subunits

We previously described the construction of the CHOCa9 or α_1 cell line that resulted from transfecting the cDNA of the type CaCh2b calcium channel α_1 subunit into CHO^- cells (Bosse *et al.* 1992), which are deficient in dihydrofolate reductase. This calcium channel subunit is a splice variant of the calcium channel α_1 gene 2 (CaCh2) and has been isolated from rabbit lung, a tissue rich in smooth muscle fibres (Biel *et al.* 1990). In CHOCa9 cells this α_1 subunit directs the formation of functional calcium channels which bind calcium channel blockers and show the voltage-dependent activation and slow inactivation and unitary current conductance characteristic of calcium channels in smooth muscle (Bosse *et al.* 1992).

These α_1 cells were stably co-transfected with the cDNA of the calcium channel β subunit and individual cell clones were tested by immunoblot analysis for the expression of the β protein (Fig. 1). No endogenous proteins reactive to a specific monoclonal antibody directed against the skeletal muscle β subunit were detected in CHO^- and CHOCa9 cells (Fig. 1, lanes 1 and 2) demonstrating that these cells are devoid of a CaB1-type β subunit. The β subunit protein is expressed to a different

extent in CHOCa9β15 and CHOCa9βK4 cells (Fig. 1, lanes 3 and 4). The β protein is expressed about 5–10 times higher in CHOCa9β15 cells than in CHOCa9βK4 cells, if one takes into account the difference in colour development (see Fig. 1, lanes 3 and 4) and the fact that 4 times more protein was applied to lane 4 than to lane 3.

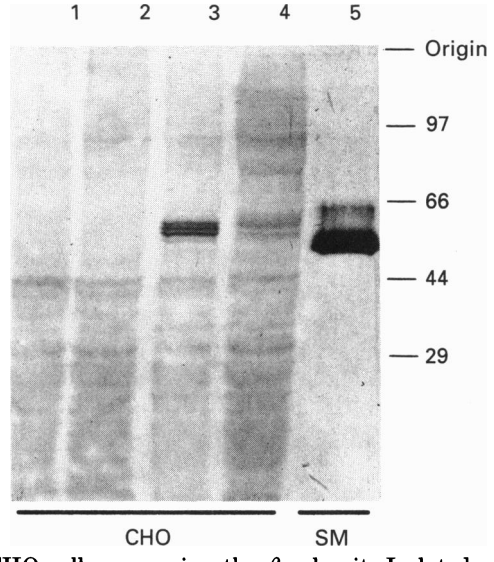

Fig. 1. Immunoblot of CHO cells expressing the β subunit. Isolated microsomes from CHO$^-$ (75 μg, lane 1), CHOCa9 (20 μg, lane 2), CHOCa9β15 (20 μg, lane 3), CHOCa9βK4 (75 μg, lane 4) and 0·19 μg purified rabbit skeletal muscle (SM) dihydropyridine receptor (lane 5) were subjected to SDS–PAGE under reducing conditions and transferred to Immobilon-P-membranes. The blot was stained with the monoclonal anti-β subunit antibody. The molecular mass is indicated to the right in kDa.

The β subunit increases the number of functional calcium channels

The possible effects of the β subunit protein on the density of the dihydropyridine binding sites and on calcium channel current were assayed by ligand binding studies using the tritiated dihydropyridine-type calcium channel blocker isradipine and by the patch-clamp technique in the whole-cell configuration, respectively. For the measurements four cell lines were selected which contained either the β subunit but no α_1 subunit (CHOβ10) or the α_1 subunit and different amounts of the β protein (CHOCa9βK4, CHOCa9β15 and CHOCa9β3). Table 1 summarizes the equilibrium binding constants and mean Ba^{2+} current densities in non-transfected CHO$^-$ cells (wild type) and CHO cells stably transfected with the skeletal muscle β (CHOβ10), smooth muscle α_1 (CHOCa9 cells) and co-transfected with α_1 and skeletal muscle β subunit (CHOCa9βK4, CHOCa9β15 and CHOCa9β3). As described non-transfected (CHO$^-$) cells and cells transfected with the β subunit (CHOβ10) did not contain saturable isradipine binding sites and did not produce a voltage-activated I_{Ba} (Table 1). CHOCa9 cells expressing the smooth muscle α_1 subunit alone revealed specific reversible high-affinity binding sites with K_D and B_{max} values of 0·13 nM and 141 fmol/mg protein. Coincident with the expression of the β subunit protein the

TABLE 1. Equilibrium binding constants and Ba^{2+} inward current (I_{Ba}) in non-transfected Chinese hamster ovary cells (CHO^-) and CHO cells stably transfected with the smooth muscle α_1 (CaCh2b) and the skeletal muscle β (CaB1) calcium channel subunits

Cell line	Subunits expressed	Expression Vector	Isradipine binding K_D (nM)	Isradipine binding B_{max} (fmol/mg)	Current density ($\mu A/cm^2$) −BayK8644	Current density ($\mu A/cm^2$) +BayK8644
CHO^-	—		n.m.	n.m.	n.d.	$-1{\cdot}4 \pm 0{\cdot}8$ (6)
CHOβ10	β	pKNHB1	n.m.	n.m.	n.d.	$-3{\cdot}1 \pm 1{\cdot}9$ (5)*
CHOCa9	α_1	p91$\alpha_1$2b	$0{\cdot}13 \pm 0{\cdot}04$	141 ± 3 (3)	$-13{\cdot}3 \pm 1{\cdot}9$ (11)	$-46{\cdot}7 \pm 5{\cdot}3$ (14)
CHOCa9βK4	$\alpha_1\beta$	p91$\alpha_1$2b + p91B1	0·17	155	$-20{\cdot}2 \pm 2{\cdot}3$ (4)	$-55{\cdot}2 \pm 5{\cdot}9$ (4)
CHOCa9β15	$\alpha_1\beta$	p91$\alpha_1$2b + pKNHB1	$0{\cdot}12 \pm 0{\cdot}03$	240 ± 6 (3)	$-30{\cdot}1 \pm 4{\cdot}5$ (9)	$-68{\cdot}3 \pm 6{\cdot}2$ (9)
CHOCa9β3	$\alpha_1\beta$	p91$\alpha_1$2b + pKNHB1	0·13	364	$-46{\cdot}6 \pm 8{\cdot}30$ (13)	$-101{\cdot}3 \pm 13{\cdot}2$ (8)

The current densities were determined from the maximum of the *I–V* curves with 30 mM Ba^{2+} as divalent charge carrier in the absence and presence of 2 μM BayK8644. The entries are means ± S.E.M. with numbers of binding experiments and cells used in parentheses. Binding experiments were conducted in triplicate. *, In six other cells no inward current larger than $-0{\cdot}5$ $\mu A/cm^2$ was detected. n.m., not measureable; n.d., not detected.

TABLE 2. The β subunit affects I_{Ba} directed by the expression of the CaCh2b α_1 subunit

Cell line	CHOCa9		CHOCa9β15		CHOCa9β3	
Subunit expressed	α_1		α_1/β		α_1/β	
BayK8644 (2 μM)	—	+	—	+	—	+
I_{Ba} ($\mu A/cm^2$)	$-13{\cdot}3 \pm 1{\cdot}9$ (11)	$-46{\cdot}7 \pm 5{\cdot}3$ (14)	$-30{\cdot}1 \pm 4{\cdot}5$ (9)	$-68{\cdot}3 \pm 6{\cdot}2$ (9)	$-46{\cdot}6 \pm 8{\cdot}3$ (13)	$-101{\cdot}3 \pm 13{\cdot}2$ (8)
90% ttp (ms)	$12{\cdot}5 \pm 1{\cdot}5$ (12)	$18{\cdot}3 \pm 0{\cdot}8$ (14)	$8{\cdot}6 \pm 0{\cdot}7$ (12)	$12{\cdot}5 \pm 0{\cdot}7$ (12)	$7{\cdot}2 \pm 0{\cdot}6$ (12)	$8{\cdot}8 \pm 0{\cdot}8$ (7)
dec_{100} (%)	$84{\cdot}7 \pm 1{\cdot}8$ (11)	$86{\cdot}4 \pm 1{\cdot}3$ (14)	$71{\cdot}3 \pm 2{\cdot}9$ (12)	$69{\cdot}1 \pm 3{\cdot}4$ (12)	$46{\cdot}3 \pm 4{\cdot}4$ (13)	$44{\cdot}1 \pm 4{\cdot}5$ (8)
dec_{770} (%)	$37{\cdot}6 \pm 7{\cdot}1$ (6)	$16{\cdot}2 \pm 3{\cdot}6$ (10)	$19{\cdot}6 \pm 3{\cdot}5$ (9)	$17{\cdot}6 \pm 3{\cdot}3$ (9)*	$4{\cdot}1 \pm 1{\cdot}4$ (9)	$5{\cdot}6 \pm 1{\cdot}5$ (6)
$V_{0{\cdot}5a}$ (mV)	$9{\cdot}5 \pm 1{\cdot}7$ (5)	$-7{\cdot}4 \pm 2{\cdot}1$ (5)	$4{\cdot}5 \pm 0{\cdot}6$ (5)	$-5{\cdot}4 \pm 0{\cdot}5$ (5)*	$0{\cdot}8 \pm 1{\cdot}6$ (6)	$-12{\cdot}6 \pm 1{\cdot}0$ (5)*
$V_{0{\cdot}5i}$ (mV)	$5{\cdot}1 \pm 4{\cdot}0$ (5)	$-7{\cdot}6 \pm 2{\cdot}1$ (5)	$-7{\cdot}3 \pm 1{\cdot}7$ (5)	$-11{\cdot}5 \pm 2{\cdot}3$ (5)	$-13{\cdot}2 \pm 1{\cdot}2$ (6)	$-22{\cdot}3 \pm 1{\cdot}5$ (5)

I_{Ba} was determined from the maximum of the *I–V* curves. All other values were obtained at +10 mV with 30 mM Ba^{2+} as divalent charge carrier. The entries are means ± S.E.M. with the numbers of cells in parentheses. 90% ttp, the time by which I_{Ba} reaches 90% of its peak amplitude; dec_{100} and dec_{770}, I_{Ba} 100 and 770 ms after the beginning of depolarization, expressed as percentage of peak amplitude; $V_{0{\cdot}5a}$ and $V_{0{\cdot}5i}$ are the voltages of half-maximal activation and inactivation, respectively. *, These values are not significantly different from the corresponding values of the CHOCa9 cell line. All other values are significantly different from those of the CHOCa9 cell line at $P < 0{\cdot}05$ or $P < 0{\cdot}001$.

binding site densities increased by factors of 1·1, 1·6 and 2·6 to 155, 238 and 364 fmol/mg protein in CHOCa9βK4, CHOCa9β15 and CHOCa9β3 cells, respectively (Table 1). Scatchard analysis of dihydropyridine binding indicated that the β subunit increased the maximal number of detectable binding sites without altering their affinity (Table 1).

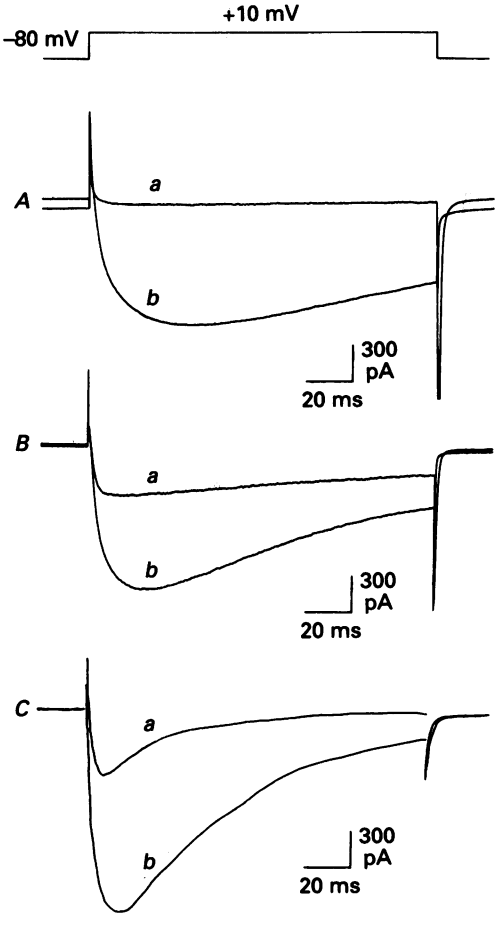

Fig. 2. Co-expression of β subunit enhances α_1 subunit-directed I_{Ba}. Representative current traces were recorded from a CHOCa9 cell (α_1 subunit) (*A*), CHOCa9β15 cell ($\alpha_1\beta$ subunits) (*B*) and CHOCa9β3 cell ($\alpha_1\beta$ subunits) (*C*) in the absence (trace *a*) and presence (trace *b*) of 2 μM BayK8644.

Co-expression of α_1 and β subunit influences I_{Ba}

Inward currents through voltage-dependent calcium channels were measured in the presence of 30 mM $BaCl_2$. CHOCa9 cells and cells co-expressing the α_1 and the β subunit had inward currents (I_{Ba}) that activated and then slowly inactivated in response to a depolarizing voltage step. Figure 2 shows representative current traces of α_1-(CHOCa9, *A*) and $\alpha_1\beta$-(CHOCa9β15, *B*, and CHOCa9β3, *C*) expressing cells in

the absence (Fig. 2, traces *a*) and presence (Fig. 2, traces *b*) of BayK8644. Voltage-activated I_{Ba} was observed in all cells tested of the three cell lines and remained stable through more than forty-one (CHOCa9) and twenty-three (CHO9βK4, CHOCa9β15 and CHOCa9β3) passages. The mean maximal current amplitude in

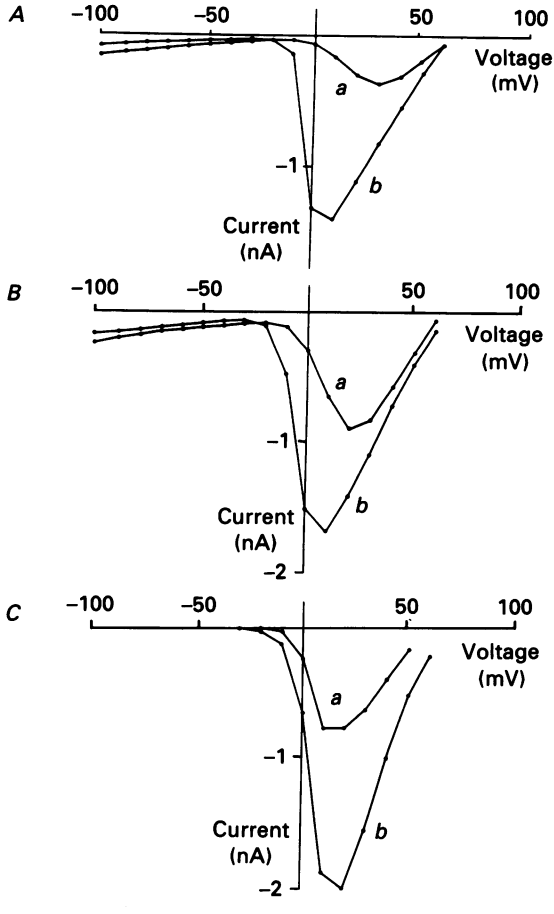

Fig. 3. Whole-cell current–voltage relations. The *I–V* curves of the CHOCa9 (α_1 subunit) (*A*), CHOCa9β15 ($\alpha_1\beta$ subunits) (*B*) and CHOCa9β3 ($\alpha_1\beta$ subunits) (*C*) cells were obtained in the absence (*a*) and presence (*b*) of 2 μM BayK8644. The divalent charge carrier was 30 mM Ba^{2+}. In *A* and *B* leak current was not subtracted.

eleven different cells of the CHOCa9 clone was -310 pA, resulting in a current density of $-13{\cdot}3$ $\mu A/cm^2$ (Table 1). I_{Ba} was reversibly blocked and increased 3·5-fold in the presence of 1 μM isradipine (Bosse *et al.* 1992) and 2 μM BayK8644 (Fig. 2 and Table 2), respectively. In general I_{Ba} was evident at membrane potentials between -20 and -10 mV and peaked between $+10$ and $+25$ mV (Fig. 3). Similar to smooth muscle cells in primary culture, the voltage curve was shifted to more hyperpolarizing potentials in the presence of BayK8644. This effect was most prominent in CHOCa9 cells (Fig. 3*A*) and disappeared almost completely in CHOCa9β3 cells (Fig. 3*C*). In

parallel to the changes in binding site density, stable co-expression of the β subunit resulted in an 1·5- to 3·5-fold increase in current densities (Table 1 and Fig. 4). Co-expression with skeletal muscle β subunit did not significantly alter the α_1 response to Bay K 8644 (Fig. 4).

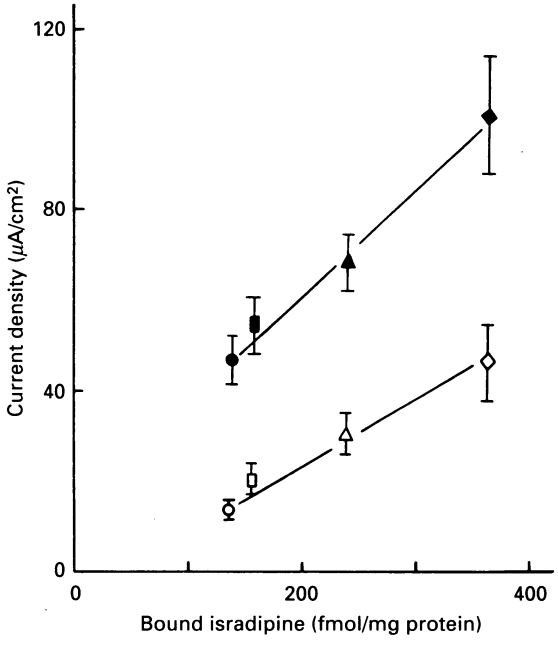

Fig. 4. Correlation between the density of dihydropyridine binding sites and I_{Ba}. The peak I_{Ba} densities were recorded in the absence (open symbols) and presence of 2 μM Bay K 8644 (filled symbols) using CHOCa9 (α_1 cells ○, ●) and $\alpha_1\beta$ cells (CHOCa9βK4, □, ■; CHOCa9β3, △, ▲; CHOCa9β15, ◇, ◆). The values are taken from Tables 1 and 2.

The co-expression of the β subunit accelerated the activation and inactivation kinetics of I_{Ba} (Fig. 2). The activation time was measured as the time needed to reach 90% of the peak amplitude (90% ttp). At +10 mV, the 90% ttp value was slowest in α_1 subunit-expressing cells (12·5 ms) and decreased with increasing amount of expressed β subunit protein to 7 ms in the CHOCa9β3 cell line (Table 2). The decay rate of I_{Ba} was quantified at 100 and 770 ms after the beginning of depolarization (dec_{100} and dec_{770}). I_{Ba} was slowest in CHOCa9 cells and became faster in cells co-expressing the β subunit protein (Table 2). The decrease in 90% ttp and increase in current decay were significantly different between the three cell lines. These differences could be due to the fact that maximal I_{Ba} occurred at different membrane potentials in the three cell lines. However, similar values were obtained in the presence of Bay K 8644 (Table 2), which shifted the maximal I_{Ba} to +10 mV in all cell lines (see Fig. 3), and presumably allowed activation of all available channels at +10 mV.

CHO^- cells and cells expressing the β subunit alone (CHOβ10) did not produce a similar voltage-activated I_{Ba}. In the presence of 2 μM Bay K 8644 five out of eleven

CHOβ10 cells produced I_{Ba} which was 6·6% of the basal current measured in the CHOCa9 cells expressing the α_1 subunit alone. An apparently identical I_{Ba} was recorded in some non-transfected cells (Table 1).

Steady-state activation and inactivation of I_{Ba}

Co-expression of the α_1 subunit together with the β subunit also modulated the steady-state activation and inactivation measured in the presence of 30 mM $BaCl_2$

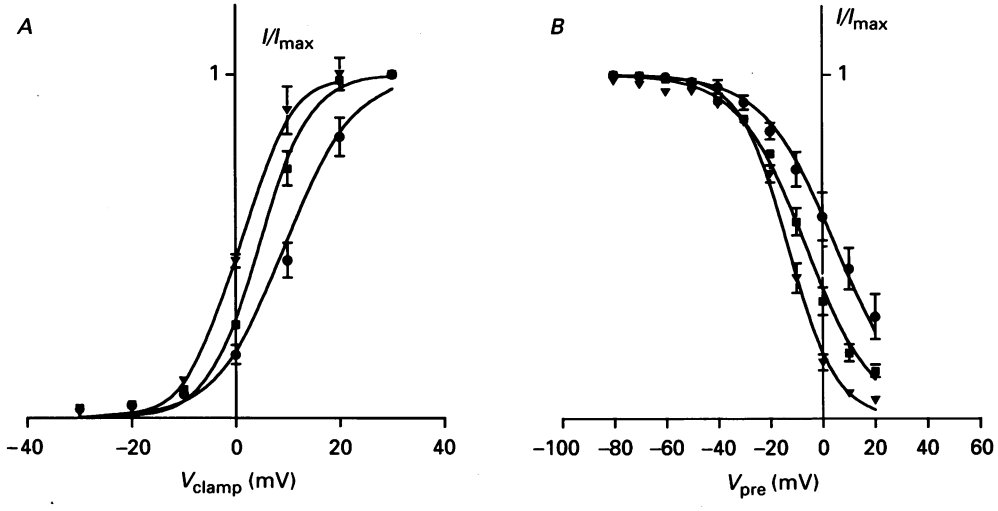

Fig. 5. Steady-state activation (*A*) and inactivation (*B*) is affected by the β subunit. The entries are means ± S.E.M. using data from five (CHOCa9, ●; CHOCa9β15, ■) and six (CHOCa9β3, ▲) independent experiments. The divalent charge carrier was 30 mM Ba^{2+}.

(Fig. 5). The half-maximal activation ($V_{0.5a}$) of the α_1 subunit-directed current occurred at 9·5 ± 1·7 mV (Fig. 5*A* and Table 2) with a slope factor of −6·6 mV. Co-expression of the β subunit shifted $V_{0.5a}$ to 4·5 ± 0·6 and 0·8 ± 1·6 mV in CHOCa9β15 and CHOCa9β3 cells, respectively (Table 2). Co-expression of the β subunit slightly reduced the apparent slope factor k_a to −5 mV (Fig. 5*A*). In accordance with the current–voltage curves BayK 8644 shifted the $V_{0.5a}$ values to negative potentials in all cell lines and obliterated the differences between α_1 and $\alpha_1\beta$ subunit-containing cells (Table 2). The steady-state half-maximal inactivation ($V_{0.5i}$) of the α_1 subunit-directed current occurred at 5·1 ± 4 mV with a slope factor of 13·7 mV. Co-expression of the β subunit shifted the $V_{0.5i}$ to −13·2 ± 1·2 mV in CHOCa9β3 cells (Table 2 and Fig. 4). These differences in $V_{0.5i}$ remained significant in the presence of BayK 8644 (Table 2).

Effects of α_2/δ on calcium channel activity in α_1/β cells

Northern blot analysis (Ellis *et al.* 1988; Biel *et al.* 1991) showed that heart, as well as lung, trachea and aorta, which are rich in smooth muscle cells, express three calcium channel subunits, namely the α_1 subunit, a β subunit and the α_2/δ subunit.

An expression of the γ subunit has not been detected in these tissues so far. Therefore, it was of interest to study in detail the potential contribution of the α_2/δ subunit to I_{Ba} in cells stably expressing the $\alpha_1\beta$ subunits. To mimic the physiological conditions and to compare the data with those previously obtained in cardiac or smooth muscle myocytes these experiments were carried out in the presence of Ca^{2+} or Ba^{2+}.

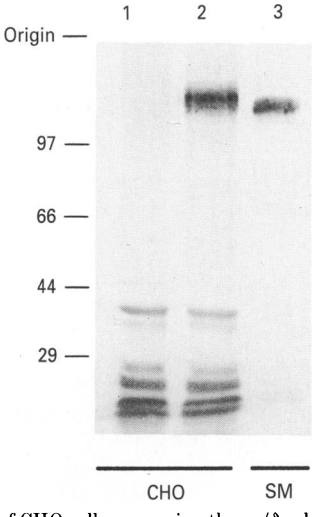

Fig. 6. Immunoblot of CHO cells expressing the α_2/δ subunit. Isolated microsomes from CHOCa9β3 ($\alpha_1\beta$, 50 μg, lane 1). Ca9β3α2/δ ($\alpha_1\beta\alpha_2/\delta$, 50 μg, lane 2) and rabbit skeletal muscle (SM) microsomes (25 μg, lane 3) were subjected to SDS–PAGE under non-reducing conditions and transferred to Immobilon-P-membranes. The blot was stained with the monoclonal anti-α2/δ subunit antibody. The molecular mass is indicated to the left in kDa.

The CHOCa9β3 cell line was stably transfected with the α_2/δ cDNA. The α_2 and δ proteins are linked by a disulphide bridge and immunoblot analysis under non-reducing conditions using a specific monoclonal antibody for the α_2 protein revealed the expression of the α_2/δ dimer in Ca9β3α2/δ4 ($\alpha_1\beta\alpha_2/\delta$) cells (Fig. 6, lane 2). After treatment with sulphydryl reducing agents the mobility of the α_2 protein on SDS–PAGE increased due to the dissociation of the δ protein (data not shown). No endogenous proteins of the size of α_2/δ were labelled in CHOCa9 cells (Fig. 6, lane 1) or CHO^- cells, demonstrating that these cells are devoid of an α_2/δ protein. The antibody cross-reacts with unknown low molecular weight proteins in all CHO cells tested but not in skeletal muscle microsomes (Fig. 6, lane 3).

Expression of the α_2/δ protein in addition to the α_1 and β subunits increased the number of binding sites for the dihydropyridine isradipine about twofold to 810 fmol/mg protein without altering the affinity (K_D = 0·15 nM). In contrast, inward current densities were identical in the CHOCa9β3α2δ4 and CHOCa9β3 cell clones (Table 3). Reducing the Ba^{2+} concentration in the bathing solution from 30 to

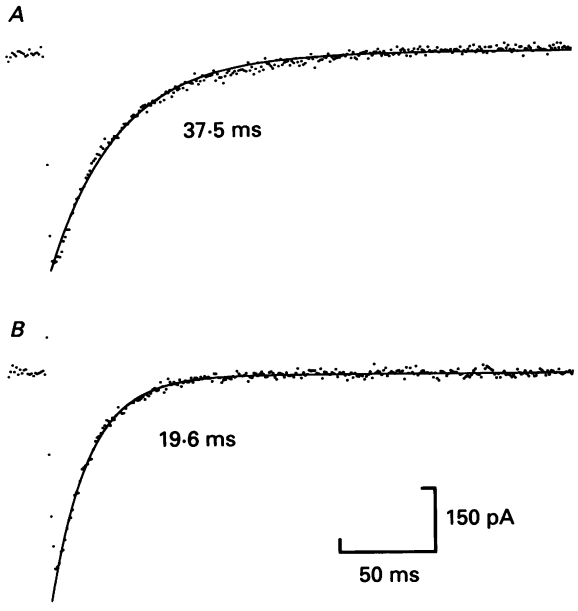

Fig. 7. Time course of Ca^{2+} current decay in CHOCa9β3 (*A*) and CHOCA9β3α2/δ (*B*) cells. Whole-cell currents were elicited in solutions containing 10 mM Ca^{2+} by pulses from −75 to +20 mV. The smooth curves represent monoexponential functions fitted to the data with the time constants indicated close to each trace.

TABLE 3. Inactivation parameters of calcium channels in $\alpha_1\beta$ and $\alpha_1\beta\alpha_2/\delta$ cells in the presence of Ba^{2+} and Ca^{2+}.

Cell line	CHOCa9β3			CHOCa9β3α2/δ4
Subunit expressed	$\alpha_1\beta$			$\alpha_1\beta\alpha_2/\delta$
	30 mM Ba^{2+}	10 mM Ba^{2+}	10 mM Ca^{2+}	10 mM Ca^{2+}
Current density (μA/cm^2)	−46·6±8·3 (13)	−16·0±4·3 (9)	−13·1±5·6 (12)	−14·2±8·5 (23)
$V_{0.5i}$ (mV)	−13·2±1·2 (6)	−15·6±0·8 (7)	−12·8±3·8 (8)*	−18·9±4·5 (15)*
k_i (mV)	9·1±1·7 (6)	6·3±1·6 (7)	7·1±2·1 (8)	8·8±2·1 (15)
τ (ms)	> 250	> 250	35·9±7·1 (9)†	18·9±2·8 (19)†

The current densities were determined as in Table 2. $V_{0.5i}$ and k_i are the potential of half-maximal steady-state inactivation and its slope factor, respectively. τ, the time constant of current decay at +20 mV. I_{Ba} inactivated slowly and no more than 15% during 250 ms steps to +20 mV, indicating that τ > 250 ms. *n*, number of cells. Statistically significant differences ($P < 0.05$) are indicated (* and †) for $V_{0.5i}$ and τ values in CHOCa9β3 and CHOCa9β3α2/δ4 cells.

10 mM decreased I_{Ba} in CHOCa9β3 cells about threefold from −46·6 to −16 μA/cm^2 (Table 3). Replacement of Ba^{2+} by Ca^{2+} as charge carrier yielded densities of I_{Ca} in CHOCa9β3 and CHOCA9β3α2/δ cells similar to those of I_{Ba} (Table 3). Devapamil, a phenylalkylamine-type calcium channel blocker, reversibly blocked I_{Ca} by 100% at a concentration of 1 μM (data not shown). BayK8644 at a concentration of 2 μM increased I_{Ca} 2·9- (CHOCa9β3) and 2·5-fold (CHOCa9β3α2/δ4).

In both cell lines, the CHOCa9β3 and CHOCa9β3α2/δ4 cells, the activation threshold of I_{Ca} was between -10 and 0 mV and calcium currents were maximal between $+10$ and $+25$ mV. Co-expression of the α_2/δ subunit did not influence steady-state activation of I_{Ca} when compared with cells expressing the α_1 and β

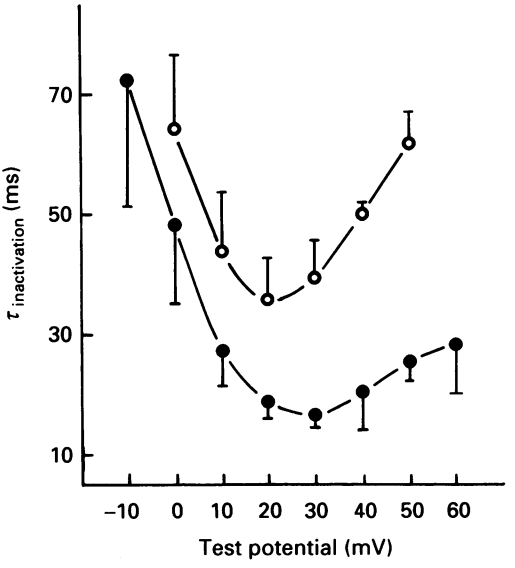

Fig. 8. Voltage dependence of I_{Ca} decay in CHOCa9β3 (○) and CHOCa9β3α2/δ4 cells (●). The time constant of current decay (τ) was determined at the indicated potentials in the presence of 10 mM Ca^{2+}. The values are means ± S.D. for five independent experiments.

subunits. However, co-expression of the α_2/δ protein accelerated the inactivation of I_{Ca} (Fig. 7). The inactivation curves of both cell lines could be fitted by a monoexponential function. At a membrane potential of $+20$ mV, the time constant of current decay (τ) was 37·5 ms in CHOCa9β3 cells and 19·5 ms in CHOCa9β3α2/δ4 cells (Table 3). Current decay depended on voltage (Fig. 8). Accelerated inactivation was more evident at potentials positive to $+10$ mV than at the other potentials. In addition, co-expression of the α_2/δ subunit protein shifted the $V_{0.5i}$ value to slightly more negative potentials without changing the slope factor (Table 3).

DISCUSSION

The present work shows that stable co-expression of a calcium channel β subunit in CHO cells modified barium currents through calcium channels generated by the expression of the CaCh2b-type α_1 subunit. The modulatory effects of the β subunit included (i) an increase in the density of dihydropyridine binding sites, (ii) an increase in peak barium inward current (I_{Ba}), (iii) an apparent acceleration of activation and inactivation kinetics and (iv) a shift in the steady-state activation and inactivation curves of I_{Ba} to slightly more hyperpolarizing membrane potentials. The additional expression of the third calcium channel subunit α_2/δ in cells containing the α_1 and β subunits (i) increased further the density of dihydropyridine binding

sites, (ii) accelerated the inactivation kinetics of the channel and (iii) shifted the steady-state inactivation curve further to more hyperpolarizing potentials. The inward currents generated in CHO cells by the expression of the α_1 subunit alone or in combination with the β subunit or the β and α_2/δ subunits were sensitive to dihydropyridines and devapamil.

Expression of the β subunit alone in CHO cells did not induce significant calcium currents, indicating that these cells contain endogenous calcium channels only at very low levels, if at all. In agreement with these results, Northern blots did not reveal α_1 subunit-specific mRNA species in CHO^- cells (Bosse *et al.* 1992). This is in contrast to the findings obtained in *Xenopus* oocytes in which the skeletal muscle β subunit associated with an endogenous α_1 subunit and increased barium currents through the endogenous calcium channel (Singer *et al.* 1991; Klöckner, Itagaki, Bodi & Schwartz, 1992; Dascal, Lotan, Karni & Gigi, 1992; Williams *et al.* 1992).

The β and α_2/δ subunits used in these experiments were originally isolated from skeletal muscle. The β subunit cDNA was derived from the CaB1-type β subunit gene and transcripts of this gene are present in skeletal muscle, heart and brain (Powers, Liu, Hogan & Gregg, 1992). The α_2/δ mRNA is expressed in brain, heart and smooth muscle. The same α_2/δ and β proteins are able to interact functionally with the α_1 subunits encoded by the genes CaCh1 to CaCh5 (see Hofmann *et al.* 1994 for references). It is therefore likely that in native tissues the same proteins form functional calcium channels with similar properties.

An increase in the number of binding sites for dihydropyridines has been observed previously when the β subunit was co-expressed with the skeletal muscle α_1 (CaCh1 gene) in mouse L-cells (Lacerda *et al.* 1991; Varadi *et al.* 1991). Like in CHO cells, the β subunit increased the maximal number of detectable binding sites in L-cells without altering their affinity towards calcium channel blockers. In the $\alpha_1\beta$ CHO cells the increased dihydropyridine binding capacity is directly correlated with an enhancement of I_{Ba} in the absence and presence of Bay K 8644 (Fig. 4) whereas in L-cells α_1 subunit-directed current densities remained unchanged (Lacerda *et al.* 1991) or even decreased after co-expression of the β subunit (Varadi *et al.* 1991; Lory *et al.* 1992). Co-expression of the skeletal muscle β subunit protein and the α_1 subunits from heart (Singer *et al.* 1991; Wei *et al.* 1991) or brain (Mori *et al.* 1991; Williams *et al.* 1992) increased severalfold the calcium currents directed by the α_1 subunit in *Xenopus* oocytes.

The mechanisms by which the β subunit modulates calcium channel density remain to be determined. From immunoblot analysis it appears that the amount of β protein expressed is directly related to the number of dihydropyridine binding sites and I_{Ba}. In cells where expression of the β protein was low, such as CHOCa9βK4 cells, the density of the binding sites and I_{Ba} was only slightly increased compared to α_1 cells. A higher expression rate of the β protein as in CHOCa9β15 cells correlated significantly with an enhanced number of binding sites and current density. The changes in current density could be caused by an increase in the proportion of 'available' channels without changing the total number of channel molecules. However, the increase in the total number of dihydropyridine binding sites induced by the co-expression of the β subunit strongly favours the interpretation that the β subunit increases the number of channel molecules present in the plasma membrane.

All β subunits identified so far contain α helical domains with hydrophobic segments arranged as heptad repeats (Ruth *et al.* 1989; Hullin *et al.* 1992). Such heptad repeats of hydrophobic residues are thought to participate in the interaction between cytoskeletal proteins (Fuchs & Hanukoglu, 1983) implying that the β subunit binds to the cytoskeleton. By simultaneous interaction with the α_1 subunit the β subunit protein might increase the number of α_1 subunit molecules at the cell surface by anchoring it to the cytoskeleton. Co-expression of calcium channel α_1 and β subunits also led to an acceleration of channel activation kinetics. The time by which I_{Ba} reached 90% of its peak amplitude decreased slightly from 12·5 to 7·2 ms in the presence of the β subunit. This acceleration of channel opening is smaller than the 100-fold increase in channel opening observed when the β subunit was co-expressed with the skeletal muscle α_1 subunit (Perez-Reyes *et al.* 1989). As observed with the peak current–voltage relationships, the β subunit also shifted the voltage dependence of steady-state activation and inactivation to negative membrane potentials. Apparently, the magnitude of the shift depended on the concentration of the expressed β subunit protein. This dependence is not readily explained by a 1:1 stoichiometry of the α_1 and β proteins. It is possible that more than one β protein interacts with one α_1 subunit but additional experiments are necessary to confirm this possibility.

The expression of α_2/δ protein increased the dihydropyridine binding capacity in these cells without significantly changing current densities. A similar discrepancy has been noted before for the dihydropyridine receptor in skeletal muscle (Schwartz, McCleskey & Almers, 1985) and L-cells expressing the skeletal muscle α_1 and β subunits (Lacerda *et al.* 1991; Varadi *et al.* 1991). It is possible that in the $\alpha_1\beta\alpha_2/\delta$ cells the number of dihydropyridine binding sites increased but was not accompanied by the functional maturation of the binding sites as an ion channel because of impaired post-translational modifications. This possibility is in accordance with the observations (i) that the truncated skeletal muscle α_1 subunit (DeJongh, Warner, Colvin & Catterall, 1991) reconstitutes normal calcium channel functions in dysgenic myocytes (Beam, Adams, Niidome, Numa & Tanabe, 1992) and (ii) that proteolysis increases barium current in L-cells expressing skeletal muscle α_1 and β subunits (Lory *et al.* 1992).

The α_2/δ protein affected the inactivation but not the activation kinetics of the channel. The α_2/δ protein speeded up the inactivation and shifted the potential at which 50% of the channels were inactivated under steady-state conditions. Since the slope factor k_i remained unchanged, the latter observation strengthens the idea that the α_2/δ subunit increases the sensitivity of calcium channels to voltage-dependent inactivation (Singer *et al.* 1991).

For some high-voltage-activated calcium channels it has been shown that channel inactivation results from a local rise of intracellular free calcium as calcium ions flow into the cell during a depolarizing pulse (Chad & Eckert, 1984). One line of evidence in favour of a calcium-dependent inactivation of the expressed calcium channel is the very slow inactivation in the presence of Ba^{2+} and the very fast inactivation in the presence of Ca^{2+}. However, the expected recovery from inactivation at positive potentials, usually taken as an indication for calcium-dependent inactivation (Lee, Marban & Tsien, 1985), was not readily observed in most cells. In summary, these

findings demonstrate that the β and α_2/δ subunits interact in specific ways with the α_1 subunit and directly participate in calcium channel function.

We thank T. Tanabe and R. J. Kaufman for generously providing pKNH and p91023(B), respectively, and Mrs Roller and Mrs Jackiw for graphical work. This work was supported in part by grants from Deutsche Forschungsgemeinschaft, Thyssen and Fond der Chemischen Industrie to F. H. and V. F.

REFERENCES

BEAM, K. G., ADAMS, B. A., NIIDOME, T., NUMA, S. & TANABE, T. (1992). Function of a truncated dihydropyridine receptor as both voltage sensor and calcium channel. *Nature* **360**, 169–171.

BIEL, M., HULLIN, R., FREUNDNER, ST, SINGER, D., DASCAL, N., FLOCKERZI, V. & HOFMANN, F. (1991). Tissue-specific expression of high-voltage-activated dihydropyridine-sensitive L-type calcium channels. *European Journal of Biochemistry* **200**, 81–88.

BIEL, M., RUTH, P., BOSSE, E., HULLIN, R., STÜHMER, W., FLOCKERZI, V. & HOFMANN, F. (1990). Primary structure and functional expression of a high voltage activated calcium channel from rabbit lung. *FEBS Letters* **269**, 409–412.

BOSSE, E., BOTTLENDER, R., KLEPPISCH, T., HESCHELER, J., WELLING, A., HOFMANN, F. & FLOCKERZI, V. (1992). Stable and functional expression of the calcium channel α_1 subunit from smooth muscle in somatic cell lines. *EMBO Journal* **11**, 2033–2038.

BOSSE, E., REGULLA, S., BIEL, M., RUTH, P., MEYER, H. E., FLOCKERZI, V. & HOFMANN, F. (1990). The cDNA and deduced amino acid sequence of the γ subunit of the L-type calcium channel from rabbit skeletal muscle. *FEBS Letters* **267**, 153–156.

CHAD, J. E. & ECKERT, R. (1984). Calcium domains associated with individual channels can account for anomalous voltage relations of Ca-dependent responses. *Biophysical Journal* **45**, 993–999.

DASCAL, N., LOTAN, I., KARNI, E. & GIGI, A. (1992). Calcium channel currents in *Xenopus* oocytes injected with rat skeletal muscle RNA. *Journal of Physiology* **450**, 469–490.

DEJONGH, K. S., WARNER, C., COLVIN, A. A. & CATTERALL, W. A. (1991). Characterization of the two size forms of the α_1 subunit of skeletal muscle L-type calcium channels. *Proceedings of the National Academy of Sciences of the USA* **88**, 10778–10782.

ELLIS, S. B., WILLIAMS, M. E., WAYS, N. R., BRENNER, R., SHARP, A. H., LEUNG, A. T., CAMPBELL, K. P., MCKENNA, E., KOCH, W. J., HUI, A., SCHWARTZ, A. & HARPOLD, M. M. (1988). Sequence and expression of mRNAs encoding the α_1 and α_2 subunits of a DHP-sensitive calcium channel. *Science* **241**, 1661–1664.

FUCHS, E. & HANUKOGLU, I. (1983). Unravelling the structure of the intermediate filaments. *Cell* **34**, 332–334.

HAMILL, O. P., MARTY, E., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

HARIMAN, S. C. & MULLIGAN, R. C. (1988). Two dominant-acting selectable markers for gene transfer studies in mammalian cells. *Proceedings of the National Academy of Sciences of the USA* **85**, 8047–8051.

HOFMANN, F., BIEL, M. & FLOCKERZI, V. (1994). Molecular basis of Ca^{2+} channel diversity. *Annual Review of Neurosciences* **17** (in the Press).

HULLIN, R., SINGER-LAHAT, D., FREICHEL, M., BIEL, M., DASCAL, N., HOFMANN, F. & FLOCKERZI, V. (1992). Calcium channel β subunit heterogeneity: functional expression of cloned cDNA from heart, aorta and brain. *EMBO Journal* **11**, 885–890.

JAY, S. D., ELLIS, S. B., MCCUE, A. F., WILLIAMS, M. E., VEDVICK, T. S., HARPOLD, M. M. & CAMPBELL, K. (1990). Primary structure of the γ subunit of the DHP-sensitive calcium channel from skeletal muscle. *Science* **248**, 490–492.

KLÖCKNER, U., ITAGAKI, K., BODI, I. & SCHWARTZ, A. (1992). β-Subunit expression is required for cAMP-dependent increase of cloned cardiac and vascular calcium channel currents. *Pflügers Archiv* **420**, 413–415.

LACERDA, A. E., KIM, H. S., RUTH, P., PEREZ-REYES, E., FLOCKERZI, V., HOFMANN, F., BIRNBAUER, L. & BROWN, A. M. (1991). Normalization of current kinetics by interaction between the α_1 and β subunits of the skeletal muscle dihydropyridine-sensitive Ca^{2+} channel. *Nature* **352**, 527–530.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

LEE, K. S., MARBAN, E. & TSIEN, R. W. (1985). Inactivation of calcium channels in mammalian heart cells: joint dependence on membrane potential and intracellular calcium. *Journal of Physiology* **364**, 395–411.

LORY, P., VARADI, G. & SCHWARTZ, A. (1992). The β subunit controls the gating and dihydropyridine sensitivity of the skeletal muscle Ca^{2+} channel. *Biophysical Journal* **63**, 1421–1424.

MIKAMI, A., IMOTO, K., TANABE, T., NIIDOME, T., MORI, Y., TAKESHIMA, H., NARUMIYA, S. & NUMA, S. (1989). Primary structure and functional expression of the cardiac dihydropyridine-sensitive calcium channel. *Nature* **340**, 230–233.

MORI, Y., FRIEDRICH, T., KIM, M.-S., MIKAMI, A., NAKAI, J., RUTH, P., BOSSE, E., HOFMANN, F., FLOCKERZI, V., FURUICHI, T., MIKOSHIBA, K., IMOTO, K., TANABE, T. & NUMA, S. (1991). Primary structure and functional expression from complementary DNA of a brain calcium channel. *Nature* **350**, 398–402.

NASTAINCZYK, W., LUDWIG, A. & HOFMANN, F. (1990). The dihydropyridine-sensitive calcium channel of the skeletal muscle: biochemistry and structure. *General Physiology and Biophysics* **9**, 321–329.

NUKADA, T., MISHINA, M. & NUMA, S. (1987). Functional expression of cloned cDNA encoding the α subunit of adenylate cyclase-stimulating G-protein. *FEBS Letters* **211**, 5–9.

PEREZ-REYES, E., KIM, H. S., LACERDA, A. E., HORNE, W., WEI, X., RAMPE, D., CAMPBELL, K. P., BROWN, A. M. & BIRNBAUMER, L. (1989). Induction of calcium currents by the expression of the α_1 subunit of the dihydropyridine receptor from skeletal muscle. *Nature* **340**, 233–236.

PEREZ-REYES, E., WEI, X., CASTELLANO, A. & BIRNBAUMER, L. (1990). Molecular diversity of L-type calcium channel. Evidence for alternative splicing of the transcripts of three non-allelic genes. *Journal of Biological Chemistry* **265**, 20430–20436.

POWERS, P. A., LIU, S., HOGAN, K. & GREGG, R. G. (1992). Skeletal muscle and brain isoforms of a β-subunit of human voltage-dependent calcium channels are encoded by a single gene. *Journal of Biological Chemistry* **267**, 22967–22972.

RUTH, P., RÖHRKASTEN, A., BIEL, M., BOSSE, E., REGULLA, S., MEYER, H. E., FLOCKERZI, V. & HOFMANN, F. (1989). Primary structure of the β subunit of the DHP-sensitive calcium channel from skeletal muscle. *Science* **245**, 1115–1118.

SCHWARTZ, L. M., MCCLESKEY, E. W. & ALMERS, W. (1985). Dihydropyridine receptors in muscle are voltage-dependent but most are not functional calcium channels. *Nature* **314**, 747–751.

SINGER, D., BIEL, M., LOTAN, I., FLOCKERZI, V., HOFMANN, F. & DASCAL, N. (1991). The roles of the subunits in the function of the calcium channel. *Science* **253**, 1553–1557.

SMITH, P. K., KROHN, R. I., HERMANSON, G. T., MALLIA, H. K., GARTNER, F. H., PROVENZANO, M. D., FUJIMOTO, E. K., GOEKE, N. H., OLSON, B. J. & KLENK, D. C. (1985). Measurement of protein using bicinichoninic acid. *Analytical Biochemistry* **150**, 76–85.

TANABE, T., TAKESHIMA, H., MIKAMI, A., FLOCKERZI, V., TAKAHASHI, H., KANGAWA, K., KOJIMA, M., MATSUO, H., HIROSE, T. & NUMA, S. (1987). Primary structure of the receptor for calcium channel blockers from skeletal muscle. *Nature* **328**, 313–318.

VARADI, G., LORY, P., SCHULTZ, D., VARADI, M. & SCHWARTZ, A. (1991). Acceleration of activation and inactivation by the β subunit of the skeletal muscle calcium channel. *Nature* **352**, 159–162.

WEI, X., PEREZ-RYES, E., LACERDA, A. E., SCHUSTER, G., BROWN, A. M. & BIRNBAUMER, L. (1991). Heterologous regulation of the cardiac Ca^{2+} channel α_1 subunit by skeletal muscle β and γ subunits. *Journal of Biological Chemistry* **266**, 21943–21947.

WILLIAMS, M. E., FELDMAN, D. H., MCCUE, A. F., BRENNER, R., VELICELEBI, G., ELLIS, S. B. & HARPOLD, M. M. (1992). Structure and functional expression of α_1, α_2, and β subunits of a novel human neuronal calcium channel subtype. *Neuron* **8**, 71–84.