The block of the expressed L-type calcium channel is modulated by the β_3 subunit

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Abstract **The α_{1C} subunit of the L-type calcium channel was stable, expressed alone or in combination with the β_3 subunit in Chinese hamster ovary cells. The β_3 subunit enhanced significantly the inactivation of barium currents indicating that both subunits interacted with each other. The β_3 subunit decreased significantly the half-maximal inhibitory concentration of the calcium channel blockers (−)-gallopamil and verapamil, but did not affect significantly the block caused by isradipine and mibefradil at the holding potentials of −80 mV and −40 mV. These results suggest that the β_3 subunit affects distinctly the interaction of the expressed α_{1C} subunit with different classes of organic calcium channel blockers.**

Key words: Calcium channel; α_{1C} Subunit; β_3 Subunit; Calcium channel blockers; Patch clamp; CHO cell

1. Introduction

High voltage-activated dihydropyridine sensitive calcium channels are complexes of up to three proteins, the α_1, α_2/δ and β subunit [1]. The contribution of the α_2/δ and the different β subunits to the properties of the native channels is not clear at present. The expression of the α_{1C} subunit induces L-type calcium and barium currents (I_{Ba}) in CHO and HEK cells. These currents have many properties of the native current including the sensitivity against organic calcium channel blockers and agonists [2–7]. The binding site for dihydropyridines (DHP) and phenylalkylamines has been localized exclusively at the α_1 subunit [8,9]. In agreement with these biochemical and electrophysiological data, expression of the α_1 subunit alone was sufficient to restore allosterically regulated high affinity binding of isradipine [2,10]. Coexpression of the β_1 subunit increased in parallel the number of binding sites and the density of I_{Ba} without affecting significantly other DHP binding parameters [3]. Recently, it was reported that transient coexpression of the β_1 subunit together with a chimeric α_1 subunit containing the amino terminal part of the carp skeletal muscle α_{1S} clone and repeat I-IV and the carboxy-terminus of the rabbit cardiac $\alpha_{1C\text{-}a}$ clone in COS cells increased the affinity of the α_1 subunit for isradipine over 20-fold [11] suggesting that under some conditions the β subunit might affect the affinity of the channel for the organic calcium blockers. A drawback of these experiments was that no data were provided showing that the expressed α_1 subunit protein induced L-type current in the COS cells. Therefore, we addressed the question whether or not the β_3 subunit affects significantly the half-maximal concentration of several compounds needed to block the current of the expressed α_{1C} subunit. The β_3 subunit was choosen since this subunit is expressed with the α_{1C} subunit in vascular smooth muscle, which tissue is a therapeutical important target for the calcium channel blockers.

2. Materials and methods

2.1. Drugs and reagents

All chemicals were of the highest purity available. (±)-Verapamil and (−)-gallopamil were kindly provided by Knoll AG, Ludwigshafen. (±)-Isradipine and mibefradil (Ro 40-5967) were from Sandoz and Hoffmann-La Roche, respectively, Basel, Switzerland. The stock solution (10 mM) of mibefradil was prepared in bi-distilled water. The stock solutions of verapamil, gallopamil and isradipine were prepared in ethanol. They were stored at −20°C and diluted to the required concentrations in the extracellular solution. They were always used within a day.

2.2. Cell transfection and culture

The construction and selection of the CHOCa1 cell line, which contains the entire protein coding region of the rabbit cardiac $\alpha_{1C\text{-}a}$ subunit [12], and the CHOCα1β3 cell line, which expresses the coding region of the β_3 subunit [13] in addition to the α_1 subunit, were described in [4,5]. The electrophysiological characteristics of the CHOCα1 cell line has been described extensively in [4,5]. An β_3 subunit specific peptide antibody was raised as described in Ludwig et al. [14].

2.3. Electrophysiological recording and data analysis

The ion currents were recorded under whole-cell patch clamp conditions [15] using an EPC-9 amplifier (HEKA Elektronik GmbH). Ba^{2+} was used as a charge carrier and the holding potential was either −80 or −40 mV. For further details of recording see [5]. The extracellular bath solution contained (in mM): NaCl 82, TEA-Cl 20, $BaCl_2$ 30, CsCl 5.4, $MgCl_2$ 1, EGTA 0.1, HEPES 5, glucose 10, pH 7.4 (NaOH). The intracellular solution contained (in mM): CsCl 102, TEA-Cl 10, EGTA 10, $MgCl_2$ 1, Na_2-ATP 3, HEPES 5, pH 7.4 (CsOH). Drugs were applied by a rapid solution changer and reached the cell membrane within 1 s [5].

The data were analysed by the program package provided by HEKA Elektronik. The current–voltage relations were fitted to the Boltzmann function:

$$I/I_{max} = [g(TP - E)]/\{1 + \exp[-(TP - V_{0.5})/k]\} \quad (1)$$

where g is the normalized conductance, TP is the amplitude of a test pulse, E is the reversal potential, $V_{0.5}$ is the half-maximal activation voltage and k equals the activation slope. The steady-state inactivation curves were measured at 0.04 Hz using 5 s conditioning pulses followed by a 10 ms return to the holding potential of −80 mV followed by a 150 ms test pulse to +30 mV. These curves were fitted to the Boltzmann equation 2:

$$I/I_{max} = 1/\{1 + \exp[-(CP - V_{0.5})/k]\} \quad (2)$$

where CP is the amplitude of a conditioning prepulse, $V_{0.5}$ equals the

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Fig. 2. Basal biophysical characteristics of the CHOCα1 (α1) and the CHOCα1β3 (α1β3) cell line. (A) Family of I_{Ba} measured in the two (α1, upper part; α1β3, lower part) cell lines. Currents were activated by 100 ms long pulses from the holding potential of −80 mV to the voltages from −20 to +30 mV, step 10 mV (left part) and from +40 to +70 mV, step 10 mV (right part). Scale bars represent 100 pA and 20 ms. Cell capacity was 17 and 16 pF. (B) The current–voltage relations for both cell lines. Each individual *I/V* curve was normalized to the maximal inward current (peak *I/V*) and all normalized *I/V* curves were then averaged. The lines were obtained by fitting the points to the Eqn. 1 with following values: α1 cell line: $g = 0.026 \pm 0.001$, $V_{0.5} = 14.6 \pm 0.3$, $E = 70.2 \pm 0.4$, $k = 7.4 \pm 0.2$, $\chi^2 = 0.00013$, $n = 11$. α1β3 cell line: $g = 0.027 \pm 0.001$, $V_{0.5} = 11.5 \pm 0.6$, $E = 66.0 \pm 0.5$, $k = 7.2 \pm 0.3$, $\chi^2 = 0.00045$, $n = 16$. The inward currents activated by the depolarizing pulses to 0, +10 and +50 mV were significantly different in both cell lines. (C) The time courses of the I_{Ba} inactivation measured during a 800 ms long depolarizing pulse from the holding potential −80 mV to +30 mV. Traces were superimposed to facilitate a comparison. The transposed lines represent fits by the sum of two exponentials. Corresponding time constants are: α1 cell, $\tau 1 = 260$ ms, $\tau 2 = 2.70$ s, cell capacity 14 pF; α1β3 cell, $\tau 1 = 140$ ms, $\tau 2 = 0.92$ s, cell capacity 16 pF. Calibration bars represent (horizontal) 100 ms and (vertical) 50 pA for both α1 and α1β3. (D) The steady-state inactivation (SSI) curves for individual cells were measured as described in section 2. Each individual SSI curve was normalized to the maximal inward current measured during the test pulse. All normalized curves were then averaged. The lines were obtained by fitting the points to the Eqn. 2 with following values: α1 cell line, $V_{0.5} = -3.2 \pm 1.3$, $k = 11.2 \pm 0.9$, $n = 9$; α1β3 cell line, $V_{0.5} = -8.4 \pm 0.8$, $k = 9.4 \pm 0.5$, $n = 13$.

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half-maximal inactivation voltage and k is the inactivation slope. The dose-dependence curves were fitted to the Hill equation (3):

$$I/I_{max} = 100/[1 + (conc/IC_{50})^n] \quad (3)$$

where conc is the concentration of an investigated drug, IC_{50} is the drug concentration that suppresses the current amplitude by 50% and n is the Hill coefficient. If not mentioned otherwise, all values are means ± S.E.M. with the number of cells in brackets. The significance of difference between two sets of observations was evaluated by unpaired Student's t-test.

3. Results and discussion

3.1. Coexpression of the β_3 subunit affects I_{Ba}

The CHOCα1 and the CHOCα1β3 cells had the characteristic current of an L-type calcium channel (Fig. 2A) (see also [4,5]). The expression of the β_3 subunit protein in the CHOCα1β3 cells was confirmed by an immunoblot using an β_3 specific antibody (Fig. 1). Both cell lines bound similar amounts of isradipine (between 73. 5 and 99 fmol/mg in four different membrane preparations) with the same affinity (K_d between 0.15 and 0.2 nM). The β subunit was functional coupled to the α_1 subunit as indicated by several changes of the I_{Ba} kinetics (Fig. 2), although the density of I_{Ba} was not significantly altered in the presence of the β_3 subunit (Table 1). To compare voltage-dependent activation of I_{Ba} in both cell lines, the current–voltage relation of individual cells were normalized to the maximal inward current, averaged and fitted to the Eqn. 1 (see section 2). The average half-maximal activation voltage was shifted by 3.2 mV in the hyperpolarizing direction upon β_3 coexpression (Fig. 2B). The speed of the current inactivation during an 800 ms long depolarizing pulse to +30 mV was significantly enhanced in the presence of the β_3 subunit (Fig. 2C). The time course of the I_{Ba} inactivation could be best fitted by the sum of two exponentials. The two time constants were significantly different between the two cell lines (Table 1). Furthermore, the β_3 subunit shifted the half-maximal inactivation voltage by 5.2 mV to hyperpolarizing potentials (Fig. 2D and Table 1). Both steady-state inactivation curves were significantly different at the conditioning potentials positive to −20 mV at $P<0.05$ or better (Fig. 2D). These results strengthened the notion that the β_3 subunit affected inactivation and activation of the channel but had no effect on the density of I_{Ba} or DHP binding sites. These findings are in line with previous studies which had shown a wide variability of the regulatory effects of the coexpression of a β subunit on the kinetics of the expressed α_1 subunit [6,16–18].

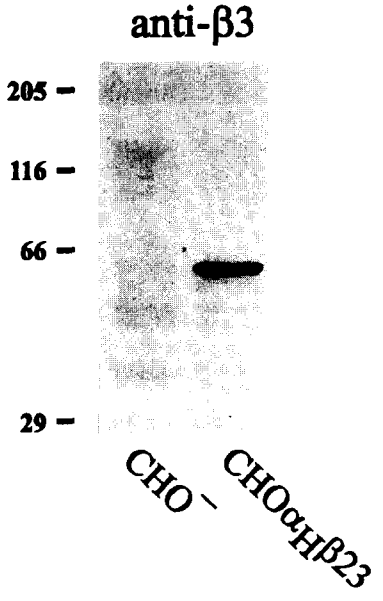
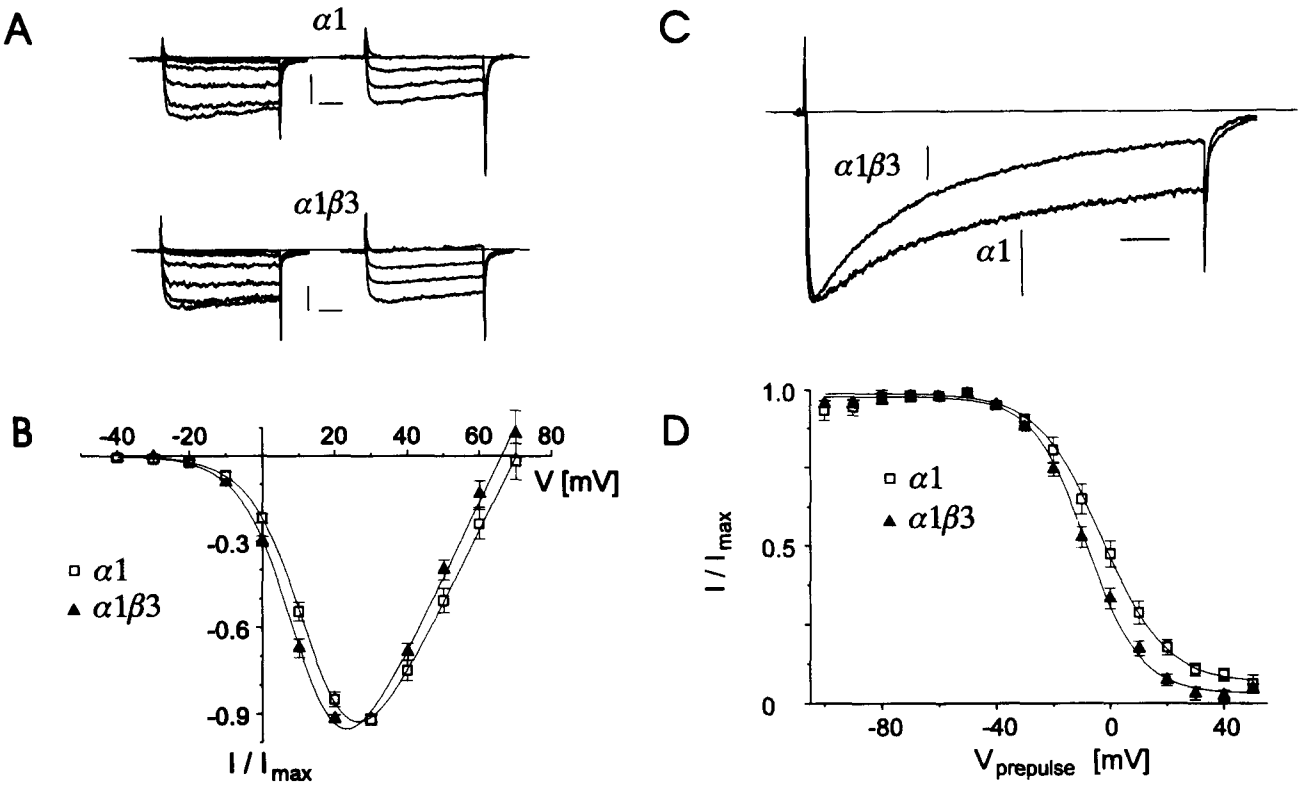
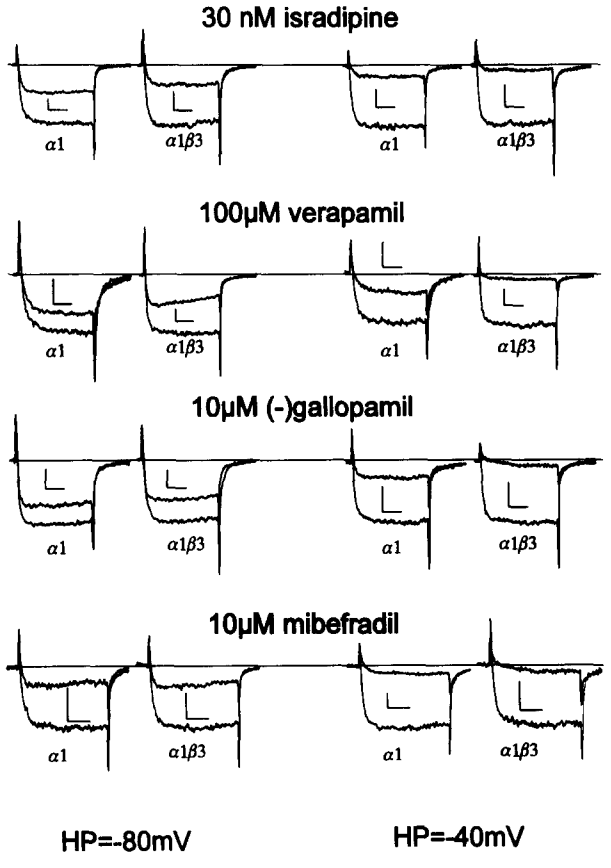


Fig. 1. Expression of the β_3 protein. Microsomal membranes (50 μg per lane) of a non-transfected CHO cell (CHO^-) and CHOCα1β3 cell ($CHO\alpha_{1H}\beta 23$) were separated on a 7.5% SDS gel and transferred to an immobilon membrane. The blot was probed with an β_3 subunit specific antibody and stained by a second alkaline phosphatase coupled anti-rabbit IgG antibody.

3.2. The β_3 subunit modifies the interaction of calcium channel blockers with the $\alpha_{1C\text{-}a}$ subunit

The inhibitory potency of the different calcium channel blockers was tested at holding potentials of −80 mV and −40 mV in both cell lines – examples of individual traces are given in Fig. 3 – because these drugs block the L-type calcium channel voltage-dependent [4–6,19,20]. At a holding potential of −80 mV, the β_3 subunit did not affect significantly the IC_{50} values for isradipine, a DHP, which were 25 nM and 20 nM in the absence and presence of the β subunit, respectively (Fig. 4A and Table 2). A shift in the holding potential from −80 mV to −40 mV increased the affinity for isradipine 9- and 20-fold in the CHOCα1 and CHOCα1β3 cells, respectively. The difference between the IC_{50} values at a holding potential of −40 mV was not significant at a $P<0.05$ level indicating that the β_3 subunit did not affect significantly the affinity for isradipine. These results are in agreement with earlier findings and suggestions that the affinity of the channel for this organic blocker depends





only on the α_1 subunit [4–6,10] and is not increased by the coexpression of a β subunit.

The above experiments were repeated with the phenylalkylamine verapamil. Surprisingly, the β subunit increased the affinity for verapamil 2.6- and 13.6-fold at holding potential −80 mV and −40 mV, respectively (Fig. 4C and Table 2). Verapamil is known to interact with several proteins in a semi-specific manner. To rule out 'non-specific' interactions of verapamil, the (−)-isomer of gallopamil was included in this study. At both holding potentials, the block by (−)-gallopamil was significantly enhanced in the presence of the β_3 subunit, although the effect of the β subunit was less pronounced than with verapamil (Fig. 4B). The β subunit shifted the IC_{50} values for (−)-gallopamil 2.7-fold at both holding potentials (Table 2).

These results suggested that the β_3 subunit affected the affinity for phenylalkylamines but not that for the dihydropyridine. To test the potential generality of this observation, the experiments were extended to include mibefradil, an organic calcium channel blocker which does not belong to the used two classes of calcium channel blockers. Mibefradil blocks high and low

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Fig. 3. Example of currents recorded in the absence and presence of the indicated drug concentrations in both cell lines at both holding potentials. In each pair of traces, the control trace was recorded just before the superfusion with the indicated drug started. The second trace shows I_{Ba} 3 min after drug application, when a new stable current amplitude was reached. The I_{Ba} was activated by 40 ms long pulse from the holding potential −80 mV to +30 mV, or from the holding potential −40 mV to +30 mV with the frequency of 0.2 Hz. Calibration bars represent 10 ms (horizontal) and 100 pA (vertical).

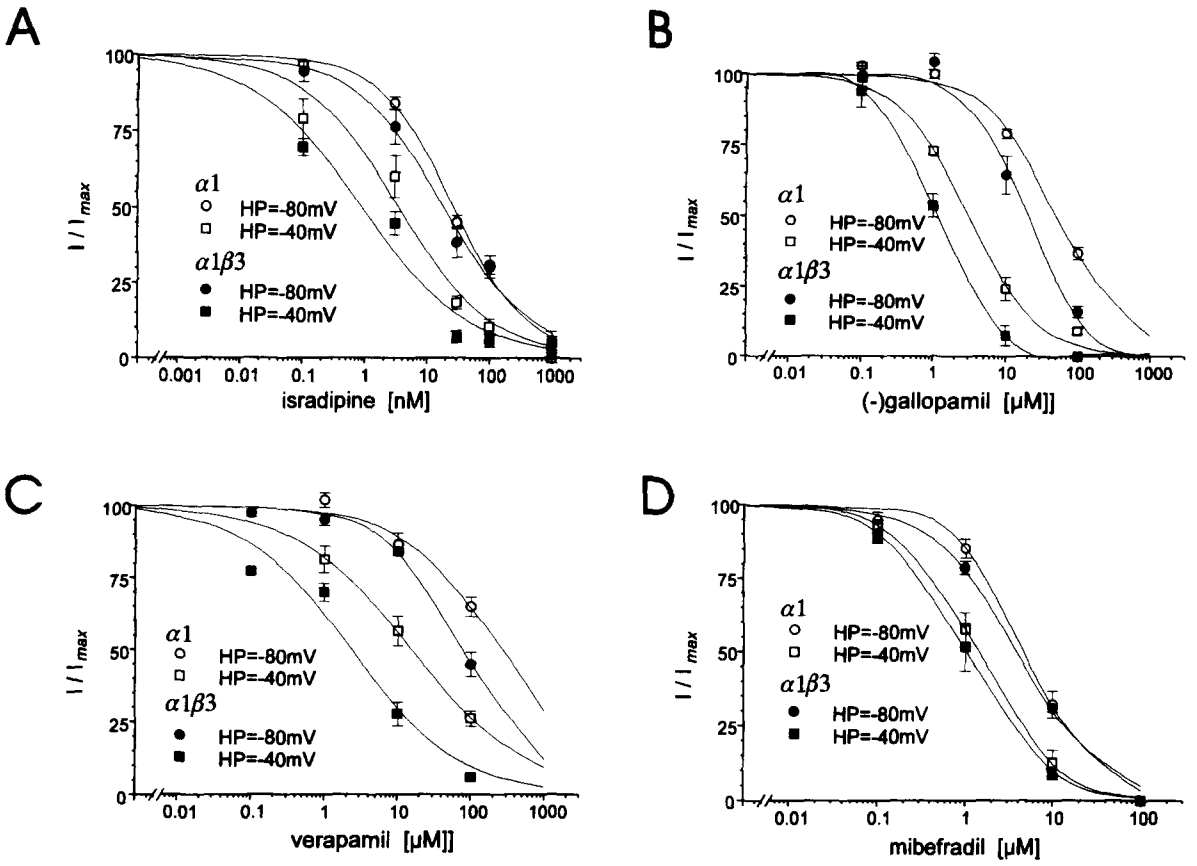

Fig. 4. Dose–response curves for isradipine (A), (−)-gallopamil (B), verapamil (C) and mibefradil (D). The I_{Ba} was activated by 40 ms long pulse from the holding potential −80 mV to +30 mV, or from the holding potential −40 mV to +30 mV with the frequency of 0.2 Hz. After the steady-state currents were reached in the presence of drug, the 'equilibrated' amplitude of the inward current in the presence of the drug was normalized to the amplitude in the absence of the drug. Between 5 and 10 measurements in different cells were averaged at each drug concentration and holding potential. The points were fitted to the equation 3. The Hill coefficients were not fixed in equation 3 and varied from 0.6 to 1.2 in individual curves.

voltage-activated calcium channels [21] and inhibits the current through all cloned and expressed high voltage-activated calcium channels with similar affinity [22] suggesting a common blocking mechanism. In both cell lines mibefradil blocked I_{Ba} at each holding potential (Fig. 4D). However, the coexpression of the β_3 subunit had no significant effect on the extent of block at all investigated concentrations (Table 2) suggesting that the β_3 subunit affected rather drug specific the block of the α_1 subunit. In this context it is of interest that the β subunit interacts with the α_1 subunit at the intracellular loop connecting repeat I and II [23] and that phenylalkylamines block the channel from the cytosol [24] and bind potentially to the intracellular side of the α_1 subunit at the IVS6 segment [9], whereas the DHPs approach the channel from the extracellular space [25].

Table 1
Basic electrophysiological characteristics of the two cell lines

Cell	Inactivation time constants			
	I_{max} (pA/pF)	τ_1 (ms)	τ_2 (s)	$V_{0.5}$ (mV)
CHOCα1	−11.3 ± 1.3 (11)	254 ± 20* (11)	2.8 ± 0.4*** (11)	−3.2 ± 1.3 (9)
CHOCα1β3	−13.8 ± 1.7 (16)	166 ± 14* (16)	0.90 ± 0.07*** (16)	−8.4 ± 0.8 (13)

I_{max} for each cell was calculated from the peak current of individual current–voltage relations and was normalized to the cell capacity. The inactivation time constants $\tau 1$ and $\tau 2$ were calculated from the double-exponential fits to the current traces measured during 800 ms depolarizing pulse from holding potential −80 mV to +30 mV. $V_{0.5}$ is the half-maximal inactivation voltage calculated according to the Eqn. 2. * and ***Indicate a significant difference between the two cell lines at $P < 0.05$ and $P < 0.001$, respectively.

Table 2
Inhibitory constants for various calcium channel blockers

Compound	Holding potential (mV)	IC_{50} (μM)		$P \leq$
		CHOCα1	CHOCα1β3	
Isradipine	−80	0.025 ± 0.002	0.020 ± 0.004	n.s.
	−40	0.0027 ± 0.0010	0.001 ± 0.001	n.s.
Gallopamil	−80	51 ± 4	23 ± 4	0.01
	−40	3.7 ± 0.3	1.1 ± 0.2	0.001
Verapamil	−80	225 ± 60	84 ± 15	0.05
	−40	15 ± 3	1.1 ± 0.2	0.01
Mibefradil	−80	4.9 ± 0.7	4.3 ± 0.3	n.s.
	−40	1.4 ± 0.3	0.9 ± 0.1	n.s.

For experimental details see legends to Figs. 3 and 4. The dose–response curves were fitted to Eqn. 3. The P values show non-significant (n.s.) and significant differences between the IC_{50} values of both cell lines.

The mechanism by which the β subunit affected the efficacy of the phenylalkylamines block is unknown. It is tempting to attribute the enhanced sensitivity to phenylalkylamines to the increased availability of the channel in the inactivated state, which should facilitate their interaction with the blockers. However, in this case one would expect an increased sensitivity of the channels towards the block by isradipine and mibefradil, too. On the other hand, a major conformational change of the phenylalkylamine binding site caused by the interaction of the β subunit with the α_1 subunit is unlikely, since the β subunit had no effect on the efficacy of the two other calcium channel blockers. A direct verification of this hypothesis was not possible, since non-transfected CHO cells have already a high affinity binding site for phenylalkylamines [6]. Therefore even if the above mechanism was not excluded, it can not be the only one responsible for the altered sensitivity of channel to the phenylalkylamine blockers either. It is more likely that the increase in blocking efficiency was caused by subtle changes in the basic biophysical properties of the channel. The binding of the β subunit to the α_1 subunit shifts the voltage for channel opening to negative membrane potentials [26]. One may expect therefore, regulatory effects of the binding of the β subunit on other regions of the α_1 subunit including the binding sites for organic calcium channel blockers. This interpretation is supported by the recent finding that the use of alternative exons of the α_{1C} gene, which are not involved in the areas identified as direct binding sites [9], has a marked effect on the efficacy of nisoldipine [4] and isradipine [27] as channel blockers. The results of this report together with these previous observations indicates, therefore, that the half-maximal inhibitory concentration of a specific compound is not only determined by the residues located at the binding site, but also by residues far apart from the binding site which affect the biophysical properties of the channel.

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