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The IVS6 segment of the L-type calcium channel is critical for the action of dihydropyridines and phenylalkylamines

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The current through the L-type calcium channel is inhibited and stimulated by distinct dihydropyridines at very low concentrations. The molecular determinants for the high affinity block and stimulation were investigated using chimeras between the class C and E calcium channels. Mutation of three amino acids in the last putative transmembrane segment (IVS6) of the α_{1C} subunit decreased the affinity for (+)isradipine 100-fold without significantly affecting the basic properties of the expressed channel. Mutation of two of these three amino acids completely abolished the stimulatory effect of the calcium channel agonist Bay K 8644. These mutations only slightly affected the blocking efficacy of mibefradil and the phenylalkylamine devapamil. Three distinct but adjacently located amino acids mediated the high affinity block by devapamil. These results suggest that the IVS6 segment of the α_{1C} subunit is critical for the high affinity interaction between the L-type calcium channel and the calcium channel agonist Bay K 8644 and the two antagonists isradipine and devapamil.

Keywords: channel agonist/dihydropyridines/L-type calcium channel/phenylalkylamines

Introduction

The contraction of the cardiac, vascular and non-vascular muscles depends largely on the influx of calcium through L-type calcium channels (Reuter, 1983). These channels are the therapeutic targets of a large class of drugs, the calcium channel blockers and agonists. It was shown that calcium channels are multimeric protein complexes composed of up to four different proteins, the α_{1C}, α_2/δ and β subunit (for details see Hofmann *et al.*, 1994). The α_1 subunit is the principal subunit, which contains the channel pore and the voltage sensor, whereas the other proteins are auxiliary subunits with modulatory functions. The cDNA of the α_{1C} gene, which has been expressed singly or in combination with other subunits in *Xenopus laevis* oocytes, CHO and HEK cells (Singer *et al.*, 1991; Wei *et al.*, 1991; Welling *et al.*, 1993; Zong *et al.*, 1995), induces a regular voltage-dependent L-type barium current. The current induced by the α_{1C} subunit alone is blocked by all calcium channel blockers and is increased by calcium channel agonists, suggesting that the α_{1C} subunit is the major target for the known calcium channel ligands such as dihydropyridines (DHPs), phenylalkylamines (PAAs) and benzothiazepines (for details, see Tsien *et al.*, 1991; Catterall and Striessnig, 1992). Photoaffinity labeling studies of the skeletal muscle calcium channel still embedded in the native membrane indicated that the DHPs bind to the extracellular site of the pore region in repeats III and IV (Nakajama *et al.*, 1991; Striessnig *et al.*, 1991; Kalasz *et al.*, 1993). Photoaffinity labeling of the purified skeletal muscle L-type calcium channel by two different DHPs resulted in incorporation of the side chain of the compounds in a peptide located directly after the last transmembrane segment IVS6 (Regulla *et al.*, 1991). The importance of repeat IV was supported further by work with chimeric channels in which parts of repeats III and IV were exchanged with the corresponding sequences of the class A channel cDNA (Tang *et al.*, 1993). These chimeras suggested that inhibition of the induced current by low concentrations of the DHP antagonist isradipine required an intact repeat IV of the α_{1C} subunit. Here we investigated the role of repeat IV of the L-type calcium channel α_{1C} subunit in the high affinity interaction with its agonists and antagonists. Mutation of three amino acids in the last putative transmembrane segment (IVS6) of the α_{1C} subunit drastically reduced the high affinity interaction with isradipine. Mutation of two of these three amino acids completely abolished the stimulatory effect of the agonist Bay K 8644. Three amino acids adjacent to that identified for the DHP block were required for the high affinity block by PAA. The observed decrease in sensitivity was specific for the investigated drugs, since none of these mutations affected the affinity of the channel for the calcium channel blocker mibefradil.

Results and discussion

Identification of sites important for the DHP block

Initial studies were done with chimera 4 (Ch 4), in which the α_{1C} sequence (Biel *et al.*, 1990) was replaced in repeat IV between S4 and the cytosolic end of S6 by the homologous sequence of the DHP-insensitive class α_{1E} subunit (Schneider *et al.*, 1994). The expressed chimera had a greatly reduced sensitivity to isradipine and was not stimulated by Bay K 8644 (Figure 1). The exchange of various parts of this relatively large sequence showed that the exchange of the sequence between amino acids 1485 and 1493 (Ch 20), which is located approximately in the middle of IVS6 of the α_{1C} subunit, was sufficient to prevent high affinity block or stimulation of the expressed current (Figure 1A). The IC_{50} values for (+)isradipine

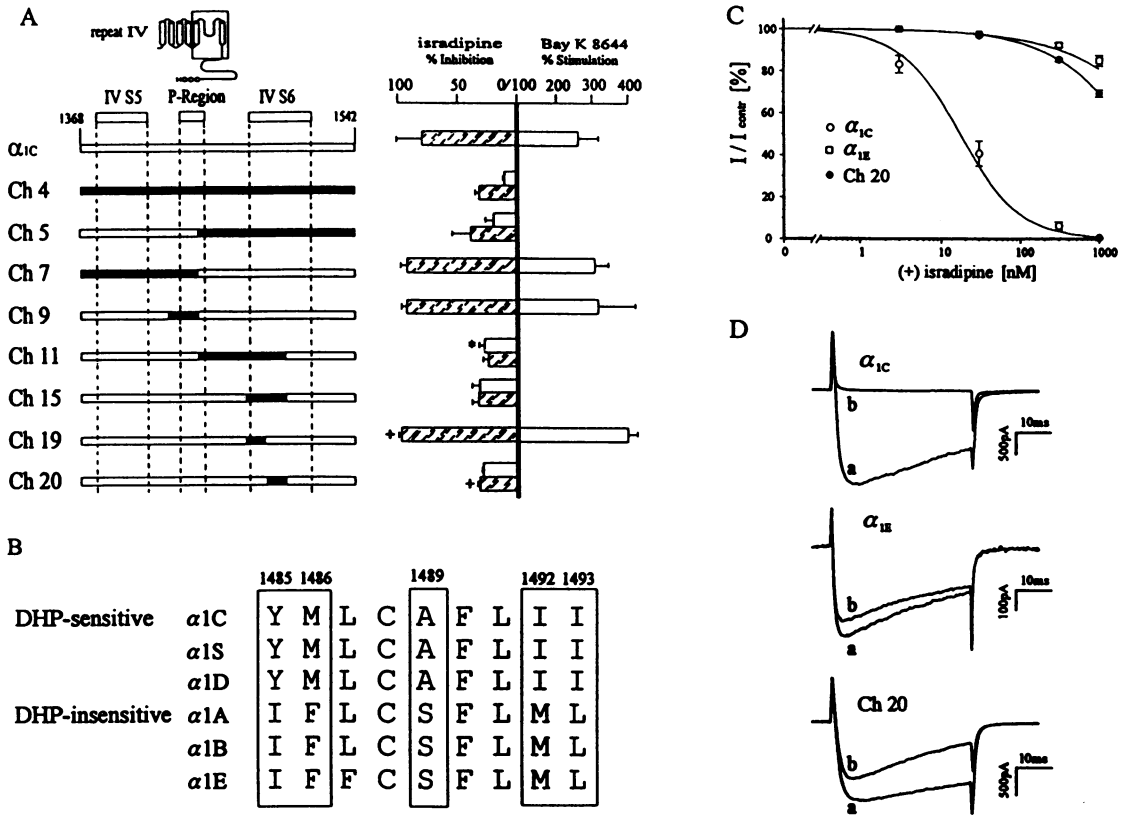


Fig. 1. (**A**) Left side: scheme of repeat IV of the calcium channel α_1 subunit. The area marked by a rectangle in repeat IV and the bars indicate the regions replaced in the α_{1C} subunit (open bar) by the corresponding sequence of the α_{1E} subunit (filled bar). Right side: inhibition and stimulation of the expressed channels by 1 μM (±)isradipine (shadowed bars) and 1 μM (±)Bay K 8644 (open bars). All values are expressed as percent of the control I_{Ba} ± SEM for 5–10 individual cells. +, the block of I_{Ba} through Ch 19 and 20 was tested in the presence of 1 μM (+)isradipine. *, Bay K 8644 stimulated I_{Ba} 1.1-fold in two out of eight cells. (**B**) Alignment of the sequence of Ch 20 for the α_1 subunit of genes C, S and D (DHP-sensitive) and of genes A, B and E (DHP-insensitive). Boxed residues indicate the differences between the groups. The numbers refer to the α_{1C} sequence (Biel *et al.*, 1990). (**C**) Dose–response curves of the inhibition of the α_{1C}, α_{1E} and Ch 20 channel by (+)isradipine. The smooth lines are either fits of experimental data to the Hill equation (α_{1C} and Ch 20) or were drawn by eye (α_{1E}). The points are means ± SEM. (**D**) Individual I_{Ba} traces for the α_{1C}, α_{1E} and Ch 20 channel in the absence (a) and presence (b) of 1 μM (+)isradipine.

were 16 ± 2 nM (n = 10) and 2.7 ± 0.5 μM (n = 10) for the wild-type channel and Ch 20, respectively, showing a >100-fold decrease in the affinity for isradipine (Figure 1C).

The alignment of the corresponding sequences of the six identified calcium channel genes (Figure 1B) indicated that this sequence is identical in the three DHP-sensitive calcium channels (class C, S and D), but differs at five or six positions in the DHP-insensitive channels (class A, B and E). To identify the amino acids involved in the high affinity isradipine block, individual amino acids were exchanged singly or in combination (Figure 2). The single mutation of Tyr1485 (Ch 17) and Ala1489 (Ch 21) did not affect significantly the block of I_{Ba} by 1 μM (+)isradipine. In contrast, the single mutation of Met1486 (Ch 22), the double mutation of Ile1492/3 (Ch 18) and Tyr/Met1485/6 (Ch 23) and the triple mutations of Ch 24 and Ch 25 resulted in a 5- to 10-fold decrease in affinity for isradipine. A combination of Ch 23 and Ch 18 yielding Ch 28 shifted the IC_{50} value 100-fold, indicating that Tyr1485, Met1486, Ile1492 and Ile1493 were required for the high affinity block. A further refinement of this chimera showed that Tyr1485 contributed less to the high affinity block than Met1486 (compare Ch 26 with Ch 27). Ile1492 is apparently not involved in the high affinity block since its mutation did not change significantly the IC_{50} value

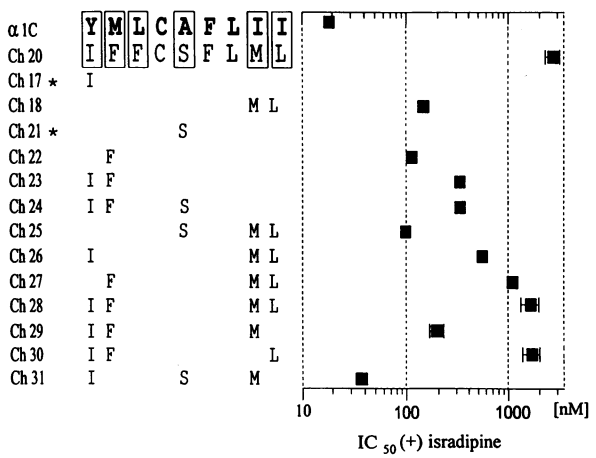


Fig. 2. Localization of the amino acids responsible for the high affinity block by (+)isradipine (left part). The IC_{50} values (right part) were calculated from complete dose–response curves as shown in Figure 1C. The error bars (SEM) are only indicated if they were larger than the symbols. *, the block of Ch 17 and Ch 21 was tested only at 1 μM (+)isradipine and was indistinguishable from that of the wild-type α_{1C} channel.

obtained with the mutation of Tyr/Met1485/6 alone (compare Ch 29 with Ch 23). In contrast, Ile1493 is important for the high affinity block since its mutation to Leu

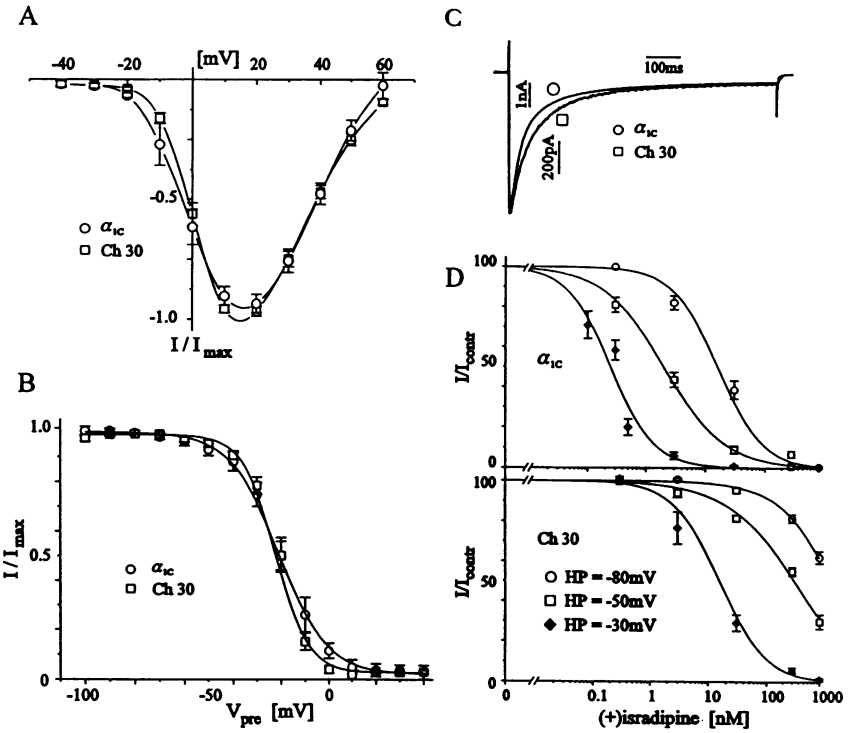


Fig. 3. (**A**) The current–voltage relationships were measured with 40 ms voltage clamp steps from the HP of –80 mV to the indicated potentials. The I_{Ba} amplitudes of individual cells (α_{1C}, n = 8; Ch 30, n = 13) were first normalized to the maximal amplitude and then averaged. Smooth lines were drawn by eye. (**B**) Steady-state inactivation of I_{Ba} was measured from a holding potential (HP) of –80 mV using a 5 s long pre-pulse to the indicated potentials (V_{pre}) followed by a 10 ms return to –80 mV followed by a 100 ms long test pulse to +10 or +20 mV (peak IV for individual cells) at 0.04 Hz. The amplitudes of I_{Ba} of individual cells were first normalized to the maximal amplitude and then averaged. The smooth lines represent the fits of experimental points to the Boltzmann equation with a half-maximal inactivation potential of –21.0 ± 1.5 mV (n = 8) and –22.3 ± 0.8 (n = 13) (not significantly different) and slope factors of 10.0 ± 0.9 and 6.8 ± 0.5 (P<0.01) for the α_{1C} and Ch 30 channel. (**C**) The time course of I_{Ba} inactivation during an 800 ms pulse from a HP of –80 mV to +20 mV was fitted optimally by the sum of two exponentials with time constants of 76 ± 10 ms and 0.5 ± 0.1 s (n = 8) for the α_{1C} channel and 73 ± 6 ms and 0.70 ± 0.05 s (n = 13) for the Ch 30 channel. These time constants are not significantly different between channels. The relative amplitudes (in %) for the fast inactivating component were 58 ± 5 and 72 ± 3 for the α_{1C} and Ch 30 channel, respectively. These values are different at P<0.05. Current traces are scaled to the same amplitude to facilitate comparison. (**D**) Dose–response curves for α_{1C} (upper part) and Ch 30 (lower part) channel measured at HPs of –80, –50 and –30 mV. The smooth lines are fits of experimental data to the Hill equation. All points are mean ± SEM for 6–11 different cells.

resulting in Ch 30 increased the IC_{50} value to 1.7 ± 0.3 μM. These results suggested that the three amino acids Tyr1485, Met1486 and Ile1493 are important for a high affinity block of the L-type calcium channel by isradipine.

The extent of the DHP block of L-type calcium channels depends on the holding potential and increases with more depolarized membrane potentials at which the channels start to inactivate (Bean, 1984; Sanguinetti and Kass, 1984). It was possible that the decreased DHP sensitivity of Ch 30 was caused by a shift of the voltage-dependent activation and inactivation to more positive membrane potentials. The analysis showed no shift in the current–voltage relationship when compared with the wild-type channel (Figure 3A). In addition, the mutations of Ch 30 did not affect significantly the steady-state availability of the channel (Figure 3B) or its inactivation during a long depolarizing pulse (Figure 3C). The ~100-fold shift of the IC_{50} value was not modified drastically when the holding potential was shifted from –80 mV to –50 mV and –30 mV (Figure 3D). As expected, the IC_{50} values depended on the membrane potential and decreased from 16 ± 2 to 2.0 ± 0.3 and 0.25 ± 0.03 nM (n = 6–11 experiments) for the wild-type channel and from 1731 ± 327 to 338 ± 35 and 16 ± 2 nM (n = 6–11 experiments) for Ch 30. From these results, we concluded that the 100-fold decrease in the sensitivity of the Ch 30 to isradipine was not caused by a modification of the basic electrophysiological properties of the α_{1C} channel nor by a grossly altered voltage dependence of the channel–drug interaction (but see also next paragraph). The location of the DHP interaction site approximately half way between the extracellular and intracellular membrane side is in excellent agreement with the observation that DHP analogs, in which a side chain of variable length contained a permanent positive charge, are optimal channel blockers when the active moiety of the DHP can interact with a site which is ~11–14 Å away from the extracellular surface (Bangalore *et al.*, 1994).

Closer inspection of the traces obtained in the absence and presence of isradipine (e.g. see Figure 1D) indicated that the DHP increased the current decay of the Ch 20 channel. A detailed comparison of the current decay was done with the wild-type α_{1C} and the Ch 30 channels. For this analysis, experiments were used in which the concentration of isradipine was close to the IC_{50} value for each channel, being 30 nM and 1 μM for the α_{1C} and Ch 30 channel, respectively. Isradipine increased the current decay in Ch 30 but not in the wild-type channel (Table I). The increased current decay was observed only at isradipine concentrations >0.3 μM. A cautious interpreta-

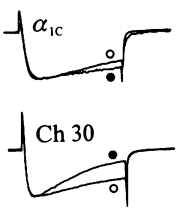
tion of this finding raises the possibility that isradipine interacted with a second, low affinity site in the Ch 30 channel and thereby facilitated an open channel block. This type of block was not observed with the wild-type channel since the current was already blocked by the occupancy of the high affinity DHP site. This possibility is strengthened by the finding (see Table II) that the mutations of Ch 30 had no effect on the low affinity site used by the novel calcium channel blocker mibefradil. It is possible, therefore, that the mutations of Ch 30 allow the detection of a second, low affinity site for isradipine which cannot be identified easily in the wild-type channel, since occupancy of the high affinity site occurs already at concentrations which do not affect this putative low affinity site.

Requirements for Bay K 8644 stimulation

The initial experiments of Figure 1 suggested that stimulation and inhibition of I_{Ba} was abolished in the same chimeras. Similarly to Ch 20 (Figure 4B), the Ch 30 channel was not stimulated but slightly inhibited by Bay K 8644 (Figure 4D). In contrast to the high affinity isradipine block, the stimulation of the L-type current by Bay K 8644 was already abolished by the mutation of the two amino acids Tyr1485 and Met1486 yielding Ch 23 (Figure 4C). The single mutation of Tyr1485 or Met1486 was not sufficient to abolish I_{Ba} stimulation (not shown). The known properties of Bay K 8644 (Hess *et al.*, 1984; Nowycky *et al.*, 1985; Kass, 1987; Bechem and Hoffmann, 1993; Hering *et al.*, 1993), i.e. the shift of the current–voltage relationship to more negative potentials and the prolongation of the tail current (see Figure 4A), were not observed with the chimeric channels (Figure 4B–D). To rule out effects of the (+)enantiomer, a channel antagonist, the experiments of Figure 4 were repeated with (–)Bay K 8644, a channel agonist. As observed already with (±)Bay K 8644 (Figure 4C), the stimulatory effect of (–)Bay K 8644 was converted to an inhibitory effect in Ch 23. This result is in agreement with previous reports (Kass, 1987; Bechem and Hoffmann, 1993) that the (–)Bay K 8644 enantiomer also has antagonistic properties. The same results were obtained with Ch 30, indicating that the agonistic interaction of (–)Bay K 8644 was abolished in both chimeras. However, 1 μM (–)Bay K 8644 blocked the I_{Ba} of Ch 23 and Ch 30 by 63 ± 4% (n = 4) and 17 ± 3% (n = 5), respectively, suggesting that the inhibitory effect of (–)Bay K 8644 required the interaction with Ile1493.

Table I. Isradipine increases current decay in Ch 30

	Current decay (%)		*P*
	(+)Isradipine		
	–	+	
α_{1C}	22 ± 4	19 ± 3	n.s.
Ch 30	21 ± 4	68 ± 3	<0.001



Current decay was calculated by dividing the current remaining at the end of a 40 ms long pulse from a HP of –80 mV to +20 mV by the peak current. (+)Isradipine was present at 30 nM (α_{1C}) or 1 μM (Ch 30). These concentrations are close to the corresponding IC_{50} values for (+)isradipine. The values are the means ± SEM of nine experiments for each channel. The right side shows representative current traces at control conditions (○) or in the presence of (+)isradipine (●). To facilitate comparison, the traces were scaled to the maximal amplitude of the Ch 30 control trace.

Differentiation of the amino acids necessary for high affinity isradipine and devapamil block

The specificity of these amino acids for the DHP agonist and antagonist was ascertained further by studying the PAA (–)devapamil and the newly introduced calcium channel blocker mibefradil (Mehrke *et al.*, 1994; Bezprozvanny and Tsien, 1995; Lacinová *et al.*, 1995). The half-maximal inhibition of Ch 20 and Ch 30 required 32- and 6-fold higher concentrations of (–)devapamil, respectively, than did that of the wild-type channel (Table II). A potential separation of the two interaction sites was achieved by Ch 31 in which Tyr1485 was changed to an Ile, Ala1489 to a Ser and Ile1492 to a Met (see Figure 2). The affinity for (–)devapamil and (+)isradipine was decreased 31- and only 2.4-fold with the Ch 31 channel, respectively. The reversed sensitivity of Ch 30 and Ch 31 for devapamil and isradipine supports the notion that the sites for isradipine and devapamil are located adjacently on the IVS6 segment. These sites are specific for the PAA and DHPs used, since mibefradil blocked the wild-type, the Ch 20 and the Ch 30 channel half-maximally at 2.5, 3.3 and 4.2 μM (Table II). The results clearly indicate that the three amino acids Tyr1485, Met1486 and Ile1493 are required for high affinity block by isradipine, the first two amino acids Tyr1485 and Met1486 for stimulation of the L-type channel by Bay K 8644, and Tyr1485, Ala1489 and Ile1492 for the high affinity block by the PAA devapamil. The experiments with mibefradil show that other inhibitory site(s) exist on the L-type calcium channel which are not affected by these mutations. It is possible that isradipine at micromolar concentrations blocked the

Table II. Isradipine and devapamil block Ch 20, Ch 30 and Ch 31 with different affinities

Drug	HP (mV)	IC_{50} (fold decrease)				*n*
		WT	Ch 20	Ch 30	Ch 31	
(+)Isradipine	–80	0.016 ± 0.002 (1)	2.7 ± 0.5 (168)	1.7 ± 0.3 (106)	0.038 ± 0.004 (2.4)	6–11
(–)Devapamil	–60	0.5 ± 0.2 (1)	16 ± 2.0 (32)	3.2 ± 0.5 (6)	15.5 ± 1.7 (31)	5–12
Mibefradil	–80	2.5 ± 0.3 (1)	3.3 ± 0.4 (1.3)	4.2 ± 0.4 (1.7)	n.t.	5–9

For identity of the chimeras see Figure 2. The IC_{50} values were obtained as described in Figure 1 and are given in μM ± SEM. (fold decrease), the value was obtained by dividing the IC_{50} of the chimera by that of the wild-type (WT) channel. *n*, range of the number of independent concentration–response curves per value; n.t., not tested.

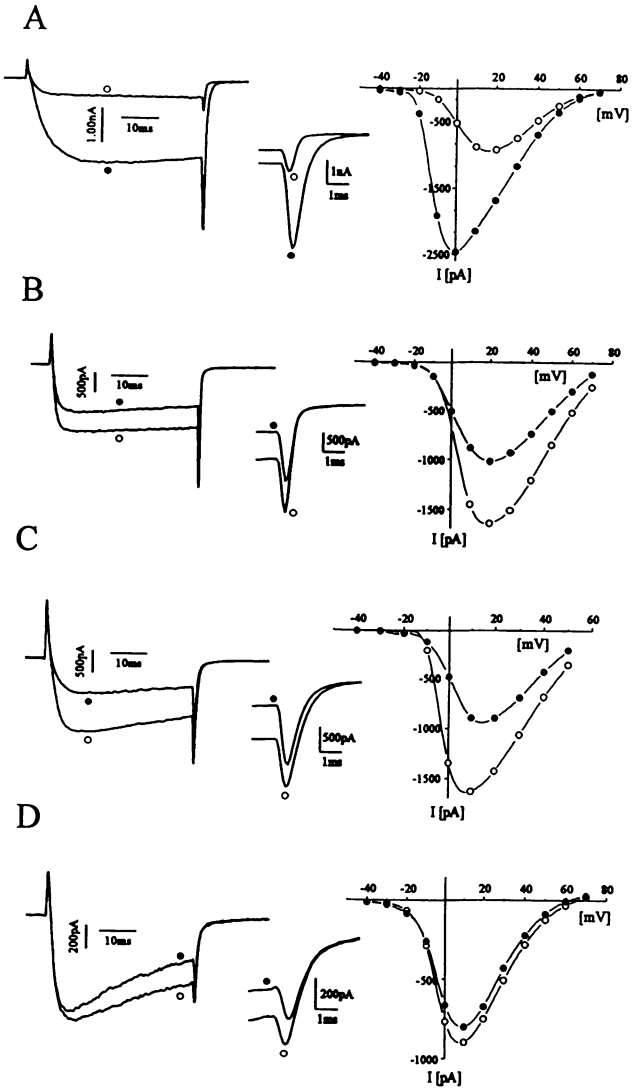


Fig. 4. The I_{Ba} of cells expressing the α_{1C} (**A**), the Ch 20 (**B**), the Ch 23 (**C**) and the Ch 30 (**D**) channel was measured in the absence (○) or presence (●) of 1 μM (±)Bay K 8644. From left to right are shown: an example of I_{Ba} measured during a depolarizing step from –80 mV to 0 mV (A), +20 mV (B) and +10 mV (C and D); an enlargment of the corresponding tail current; the complete current–voltage curves. For the amino acids exchanged in Ch 20, Ch 23 and Ch 30 see Figure 2. (±)Bay K 8644 stimulated I_{Ba} by 260 ± 60% (n = 6) in A (α_{1C}), but inhibited I_{Ba} by 28 ± 1% (n = 2) in (B) (Ch 20), by 46 ± 4% (n = 5) in (C) (Ch 23) and by 21 ± 9% (n = 6) in (D) (Ch 30). The current–voltage relationship was shifted by –20 mV, 0 mV, +5 mV and 0 mV in the wild-type (A), Ch 20 (B), Ch 23 (C) and Ch 30 (D) channel. The tail current was prolonged only in the cells expressing the wild-type channel (A) but not in those expressing the chimeric channels (B–D).

Ch 30 channel by binding to this or a similar site (see above and Table I).

Our experiments identified the basic requirement for stimulation and high affinity block of the L-type calcium channel by DHPs and PAAs. Recently, Tyr1485, Ala1489 and Ile1492 have also been identified as being crucial for the high affinity devapamil block (Hockerman *et al.*, 1995). This location and the findings that (i) both DHPs (Bangalore *et al.*, 1994) and PAAs (Wegener and Nawrath, 1995) block the channel from the extracellular side, (ii) compounds from both classes photoaffinity labeled peptides close to (Regulla *et al.*, 1991) or containing the IVS6 segment (Catterall and Striessnig, 1992) and (iii) early results suggested that compounds from both classes compete for the same or very similar sites (Galizzi *et al.*, 1984, 1986), are consistent with the concept that the IVS6 segment is in close contact with the pore of the L-type calcium channel. The availability of the chimeric constructs separating these sites will greatly facilitate our understanding of the molecular basis of channel block by these clinically important compounds.

Materials and methods

Construction of calcium channel chimeras

For construction of the chimeras, the sequence between *Sac*II (nt 4101) and *Bst*EII (nt 4626) sites of the rabbit α_{1Cb} cDNA (Biel *et al.*, 1990) was subcloned into the pT7-Blue vector (Novagen). Two silent mutations were introduced to create an *Sph*I (nt 4212) and a *Hin*dIII (nt 4322) restriction site. The complete insert or part of it was replaced by the corresponding sequence of the α_{1E} cDNA (from nt 4765 to 5289) (Schneider *et al.*, 1994). Replacement sequences were obtained by standard PCR overlap extension techniques. When appropriate, the α_{1E} sequences were amplified with primers containing the *Sac*II, *Sph*I, *Hin*dIII and *Bst*EII restriction sites. The sequence of each chimera was verified by sequence analysis. The following amino acid sequences of the α_{1C} subunit were replaced with the corresponding amino acid sequence of the α_{1E} subunit (numbers according to the α_{1Cb} sequence): Ch 4 1369–1542, Ch 5 1445–1542, Ch 7 1369–1437, Ch 9 1421–1437, Ch 11 1445–1493, Ch 15 1474–1493, Ch 19 1474–1482, Ch 20 1485–1493. The point mutations of chimeras 17, 18 and 21–31 are indicated in Figure 2.

Transfection of HEK 293 cells and electrophysiological recordings

For transient expression in HEK 293 cells, the full-length cDNAs of the chimeric constructs were cloned into the pcDNA 3 vector (Invitrogen). HEK 293 cells were transfected with either wild-type or chimeric plasmids together with the cDNA plasmids encoding the β2a and α2/δ subunits and the green fluorescence protein (GFP) by lipofection with LipofectAMINE™ (Gibco BRL, Life Technologies) at a DNA mass ratio of 1:1:1:0.1.

The ionic currents were recorded under whole-cell patch clamp conditions with Ba^{2+} as a charge carrier as described in Lacinová *et al.* (1995). The extracellular bath solution contained (in mM): NaCl, 82; TEACl, 20; $BaCl_2$, 30; CsCl, 5.4; $MgCl_2$, 1; EGTA, 0.1; HEPES, 5; glucose, 10; pH 7.4 (NaOH). Current through the α_{1E} channel was measured in the presence of 110 mM NaCl and 2 mM $BaCl_2$. The intracellular solution contained (in mM): CsCl, 102; TEACl, 10; EGTA, 10; $MgCl_2$, 1; Na_2ATP, 3; HEPES, 5; pH 7.4 (CsOH). Drugs were applied by a rapid solution changer and reached the cell membrane within 1 s (Lacinová *et al.*, 1995). The current density in all investigated cells ranged between –10 and –100 pA/pF with a mean value for both wild-type class C and Ch 30 channels of –50 pA/ pF. The effects of the DHPs were tested with 40 ms long voltage clamp steps from a holding potential (HP) of –80 mV to +10 or +20 mV (peak of current–voltage relationship for each individual cell) at 0.2 Hz. The new steady-state condition was reached 1 and 4 min after application of Bay K 8644 and (+)isradipine, respectively.

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