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The action of calcium channel blockers on recombinant L-type calcium channel α_1-subunits

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1 CHO cells expressing the $\alpha_{1C\text{-}a}$ subunit (cardiac isoform) and the $\alpha_{1C\text{-}b}$ subunit (vascular isoform) of the voltage-dependent L-type Ca^{2+} channel were used to investigate whether tissue selectivity of Ca^{2+} channel blockers could be related to different affinities for α_{1C} isoforms.

2 Inward current evoked by the transfected α_1 subunit was recorded by the patch-clamp technique in the whole-cell configuration.

3 Neutral dihydropyridines (nifedipine, nisoldipine, (+)-PN200-110) were more potent inhibitors of $\alpha_{1C\text{-}b}$-subunit than of $\alpha_{1C\text{-}a}$-subunit. This difference was more marked at a holding potential of −100 mV than at −50 mV. SDZ 207-180 (an ionized dihydropyridine) exhibited the same potency on the two isoforms.

4 Pinaverium (ionized non-dihydropyridine derivative) was 2 and 4 fold more potent on $\alpha_{1C\text{-}a}$ than on $\alpha_{1C\text{-}b}$ subunit at Vh of −100 mV and −50 mV, respectively. Effects of verapamil were identical on the two isoforms at both voltages.

5 [^{3}H]-(+)-PN 200-110 binding experiments showed that neutral dihydropyridines had a higher affinity for the $\alpha_{1C\text{-}b}$ than for the $\alpha_{1C\text{-}a}$ subunit. SDZ 207-180 had the same affinity for the two isoforms and pinaverium had a higher affinity for the $\alpha_{1C\text{-}a}$ subunit than for the $\alpha_{1C\text{-}b}$ subunit.

6 These results indicate marked differences among Ca^{2+} channel blockers in their selectivity for the $\alpha_{1C\text{-}a}$ and $\alpha_{1C\text{-}b}$ subunits of the Ca^{2+} channel.

Keywords: L-type Ca^{2+} channels; α_1-subunit; Ca^{2+} channel blocker; tissue selectivity; dihydropyridine; verapamil; patch-clamp

Introduction

Tissue selectivity constitutes the main guideline for the development of new generations of Ca^{2+} channel blockers. It determines the therapeutic indications of the molecule and its counter-indications. The more studied is the vascular versus cardiac selectivity, for which great differences between drugs are reported (Spedding *et al*., 1990; Godfraind *et al*., 1992; Sun & Triggle, 1995). Extensive *in vivo* and *in vitro* studies have shown that tissue selectivity may be related to several factors, including physicochemical properties of the drug and electrophysiological characteristics of the tissue (Wibo, 1989). Recently, molecular biology studies have identified several isoforms of the α_1 subunit of the L-type Ca^{2+} channel, issued from different genes and produced by various splicing processes (Perez-Reyes & Schneider, 1995). Their relative expression is tissue-dependent and is regulated with development (Feron *et al*., 1994).

Two transcripts coding for the IS6 segment of the $\alpha_{1C\text{-}a}$ subunit and for the IS6 segment of the $\alpha_{1C\text{-}b}$ subunit, have been shown to be selectively expressed in cardiac and vascular smooth muscle, respectively (Welling *et al*., 1997). Interestingly, nisoldipine, a dihydropyridine (DHP) Ca^{2+} channel blocker with a high vascular selectivity (Godfraind *et al*., 1992), is a more potent blocker of inward current in cells transfected with $\alpha_{1C\text{-}b}$ isoform cDNA than in those transfected with $\alpha_{1C\text{-}a}$ isoform cDNA (Welling *et al*., 1993). These results suggest that vascular versus cardiac selectivity of nisoldipine could be related to the tissue-specific expression of isoforms of the α_{1C} gene. However, there is as yet no evidence that this property is shared by other DHP and non-DHP Ca^{2+} channel blockers in a way that could account for their particular tissue selectivity.

The aim of the present study was to investigate whether differences in the affinity of Ca^{2+} channel blockers for isoforms of the α_1 subunit could be involved in their tissue selectivity. We used Chinese hamster ovary cells (CHO) transfected either with cDNA encoding for the $\alpha_{1C\text{-}a}$ or with cDNA encoding for the $\alpha_{1C\text{-}b}$ subunit of the L-type Ca^{2+} channel, issuing respectively from rabbit heart and lung smooth muscle (Welling *et al*., 1993). The Ca^{2+} channel blocking activity of three neutral DHP derivatives, (+)-PN 200-110, nifedipine and nisoldipine, which show different degrees of vascular selectivity (Godfraind *et al*., 1992), and one positively charged derivative SDZ 207-180 (Kass *et al*., 1991) was compared to that of the phenylalkylamine verapamil, which is equipotent in cardiac and vascular tissue (Sun & Triggle, 1995), and to that of pinaverium bromide, a non-DHP compound with a quaternary ammonium, reported to show intestinal selectivity (Christen, 1990). The voltage-dependent current mediated by the α_1 subunit of the L-type Ca^{2+} channel ($I_{\alpha 1}$) was recorded with the whole-cell configuration of the patch-clamp technique using barium ions as charge carrier. Binding affinity of Ca^{2+} channel blockers was also assessed in displacement studies using the Ca^{2+} channel ligand [^{3}H]-(+)-PN 200-110.

Results indicate that different affinity for isoforms of the $\alpha_{1C\text{-}a}$ subunit may only account for a part of the selectivity of the Ca^{2+} channel blockers identified in whole tissues. Some of the results have been presented to the Belgian Society for

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Fundamental and Clinical Pharmacology and Physiology (Feron *et al*., 1996; Morel *et al*., 1996).

Methods

Cells

Chinese hamster ovary (CHO) cells transfected with the $\alpha_{1C\text{-}a}$ (CHO-A) or with the $\alpha_{1C\text{-}b}$ (CHO-B) subunit of the L-type Ca^{2+} channel (Welling *et al*., 1993) were used. CHO-A cells were sub cultured using nutrient mixture Ham's F-12 (Gibco, Life Technologies, Ghent, Belgium) supplemented with streptomycin, penicillin, geneticin and foetal calf serum. CHO-B cells were sub cultured using Dulbecco's modified Eagle medium (Gibco, Life Technologies, Ghent, Belgium) supplemented with penicillin, streptomycin and foetal calf serum. Cells were used 2 days after plating.

Electrophysiology

Transmembrane currents were recorded at room temperature with the whole-cell configuration of the patch-clamp method (Hamill *et al*., 1981) using the List EPC-7 patch-clamp amplifier and pClamp software (Axon Instrument) as previously described (Buryi *et al*., 1995). The bath was continuously superfused with solution consisting of (mM): NaCl, 120; $BaCl_2$, 10; $MgCl_2$, 1.2; glucose, 10, HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]), 10; pH adjusted to 7.4 with NaOH. Patch pipettes had resistances of 3–5 MΩ. The internal pipette solution contained (mM): NaCl, 120; $MgCl_2$, 4; Na_2ATP, 5; glucose, 10; HEPES, 10; EGTA (ethylene glycol-bis (β-aminoethyl ether), N, N, N′, N′-tetra-acetic acid), 3; pH adjusted to 7.2 with NaOH. Substitution of K^+ by Na^+ in both the pipette and external solutions set the reversal potential of Na^+ and Cl^- close to 0 mV. For this reason the test voltage of current measurement was set at 0 mV. Control experiments with 1 μM tetrodotoxin added to the bath solution showed no contribution of Na^+ channel current in these cells.

[^{3}H]-(+)-PN 200-110 binding experiments

[^{3}H]-(+)-PN 200-110 binding was measured in CHO cells plated in 35 mm dishes and incubated in DME medium (Dulbecco's modified Eagle's, Life Technology) containing 5 mM or 50 mM KCl. Cells were incubated for 90 min at 37°C without serum in the presence of [^{3}H]-(+)-PN 200-110 (75 Ci mmol^{-1}) and various concentrations of unlabelled competitor. Non-specific binding was determined in the presence of nifedipine 1 μM, and subtracted from the total binding to obtain the specific binding. Reaction was stopped by washing the cells with a solution containing NaCl, 0.9%; DMSO, 5%; and bovine serum albumin, 1%. Cells were solubilized in 1 ml sodium dodecylsulphate 0.2% and the radioactivity was counted by liquid scintillation. Protein was determined according to Lowry *et al*. (1951).

Drugs

Nifedipine (Sigma), nisoldipine (Bayer AG, Leverkussen, Germany), (+)-PN 200-110 (Sandoz, Basel, Switzerland) and SDZ 207-180 (Sandoz, Basel, Switzerland) were dissolved in ethanol as 1 mM stock solution and diluted in bath solution immediately before use. [^{3}H]-(+)PN 200-110 (75 Ci mmol^{-1}) was from DuPont NEN Research Products Brussels, Belgium. Experiments with DHPs were always carried out under yellow light to prevent photo-inactivation. Verapamil hydrochloride (Sigma) and pinaverium bromide (4-(2-bromo-4,5-dimethoxybenzyl)-4-[2-{2(6,6 - dimethylnorpinan - 2 - yl) ethoxy} ethyl] morpholinium bromide, Solvay Pharma France, Paris, France) were dissolved in water as 10 mM stock solution.

Analysis

Dissociation constants of the Ca^{2+} channel blockers (K) are the concentrations inhibiting $I_{\alpha 1}$ by 50%. They were obtained for each cell according to the equation

$$I_{\alpha 1\ (\%\ \text{of control})} = 100^* \frac{K}{K + [A]} \qquad [1]$$

with [A] the concentration of the blocker.

The value of the dissociation constant of the blockers to the inactivated state of the Ca^{2+} channel (K_I) was determined according to the equation

$$\frac{1}{K_{-50\ mV}} = \frac{h}{K_R} + \frac{(1-h)}{K_I} \qquad \text{(Bean, 1984)} \qquad [2]$$

with $K_{-50\ mV}$ the dissociation constant measured at holding potential of −50 mV; h, the proportion of non-inactivated channels at holding potential of −50 mV; and K_R, the dissociation constant of the blocker binding to the resting channels measured at holding potential of −100 mV.

Inactivation curves were fitted to experimental data using the Boltzmann equation

$$\frac{1}{1 + \exp\frac{(V - V_{0.5})}{k}} \qquad [3]$$

with V, the potential, $V_{0.5}$, the midpoint of the curve and k the slope factor. K_I values were also calculated from the shift of the inactivation curves according to the equation

$$-\Delta V_{0.5} = k \ln\frac{1 + A/K_I}{1 + A/K_R} \qquad \text{(Bean, 1983)} \qquad [4]$$

where $\Delta V_{0.5}$ is the shift in the midpoint of the steady state availability curve, k is the slope factor determined from the control steady state availability curve, A the concentration of the blocker, K_I and K_R the dissociation constants for the binding to the inactivated and resting channels.

Data analysis was performed using Excel (Microsoft), Kaleidagraph (Synergy software, Reading, U.K.) and Multifit (Day Computing, Cambridge, U.K.) softwares on Macintosh.

In binding experiments, saturation isotherms were analysed by Scatchard and Hill plots. The dissociation constants and the maximum binding capacity were calculated by linear regression. Displacement curves were analysed by a sigmoid curve fitting programme (Munson & Rodbard, 1980). An interactive technique gave estimate of the concentration of competitor inhibiting 50% of the specific binding. The inhibitory constant (K_{inh}) values were calculated according to Cheng & Prusoff (1973).

Data are expressed as mean ± s.e.mean. Tests of significance were performed using Student's *t*-test.

Results

Effect of Ca^{2+} channel blockers on α_1 subunit current at a holding potential of −100 mV

In K^+-free solution using Ba^{2+} as charge carrier the current measured in CHO cells transfected with the $\alpha_{1C\text{-}a}$ (CHO-A) or

$\alpha_{1C\text{-}b}$-subunit (CHO-B) of the L-type Ca^{2+} channel was of maximal amplitude between 0 and 10 mV and inactivated when holding potential was set positive to −100 mV. The standardized protocol that was used to assess the inhibition of $I_{\alpha 1}$ by the different antagonists consisted of recording the current elicited by 25 ms depolarizing pulses to 0 mV applied every 10 s from a holding potential of −100 mV (control condition) (Figure 1). The holding potential was thereafter set to −50 mV leading to a slowly developing partial inactivation of the current. At steady state, non-inactivated current was 63.6±1.6% ($n=54$) and 63.5±1.3% ($n=54$) of control current in CHO-A and CHO-B cells, respectively. Holding potential was then reset to −100 mV and the Ca^{2+} channel blocker was introduced in the perfusion solution when the current had completely recovered from inactivation. The same protocol was then applied in the continuous presence of the Ca^{2+} channel blocker.

All the compounds tested blocked $I_{\alpha 1}$ elicited from a holding potential of −100 mV in a dose-dependent manner (Figure 2). Dissociation constants ($K_{-100\ mV}$) are reported in Table 1. The neutral DHP derivatives nisoldipine, nifedipine and (+)-PN 200-110 were respectively 4, 5 and 7 fold more potent inhibitors of $I_{\alpha 1}$ in CHO-B compared to CHO-A cells, while no difference was noted in the potency of SDZ 207-180 (a/b ratio of $K_{-100\ mV}$ was 0.9). Pinaverium showed a higher potency in CHO-A compared to CHO-B cells (a/b ratio of $K_{-100\ mV}$ was 0.5, $P<0.01$). Verapamil was equipotent on the two isoforms.

Inhibition of α_1 subunit current by Ca^{2+} channel blockers at a holding potential of −50 mV

Since the action of most Ca^{2+} channel blockers is voltage-dependent (Méry *et al*., 1996), the inhibition of $I_{\alpha 1}$ was also tested at a Vh of −50 mV. The DHPs concentration-effect curves determined at −50 mV were shifted to lower concentrations compared to the curves obtained at −100 mV. Values of the dissociation constants ($K_{-50\ mV}$) are reported in Table 1. Among neutral DHPs, the voltage-dependent increase in potency was the largest in CHO-A cells with (+)-PN 200-110, with a ratio of $K_{-100\ mV}$ on $K_{-50\ mV}$ equal to 18. The $K_{-100\ mV}/K_{-50\ mV}$ ratio was 7 and 6 for nifedipine and nisoldipine, respectively. For these molecules the voltage-dependence was less pronounced in CHO-B cells than in CHO-A cells, with $K_{-100\ mV}/K_{-50\ mV}$ ratios in CHO-B cells between 5 ((+)-PN 200-110) and 2.5 (nifedipine). Voltage-dependence was similar with SDZ 207-180 ($K_{-100\ mV}/K_{-50\ mV}$ ratio was 4.5 in CHO-A cells and 3 in CHO-B cells). The inhibition of $I_{\alpha 1}$ by pinaverium was also voltage-dependent; its inhibitory potency was increased by a factor of 5 and 2, in CHO-A and CHO-B cells, respectively. Verapamil was markedly more potent at a holding potential of −50 mV compared to −100 mV, with a 30 fold and a 24 fold decrease of the K value when holding potential was depolarized from −100 mV to −50 mV, in CHO-A and in CHO-B cells, respectively.

As was observed at a Vh of −100 mV, there was no significant difference between CHO-A cells and CHO-B cells at

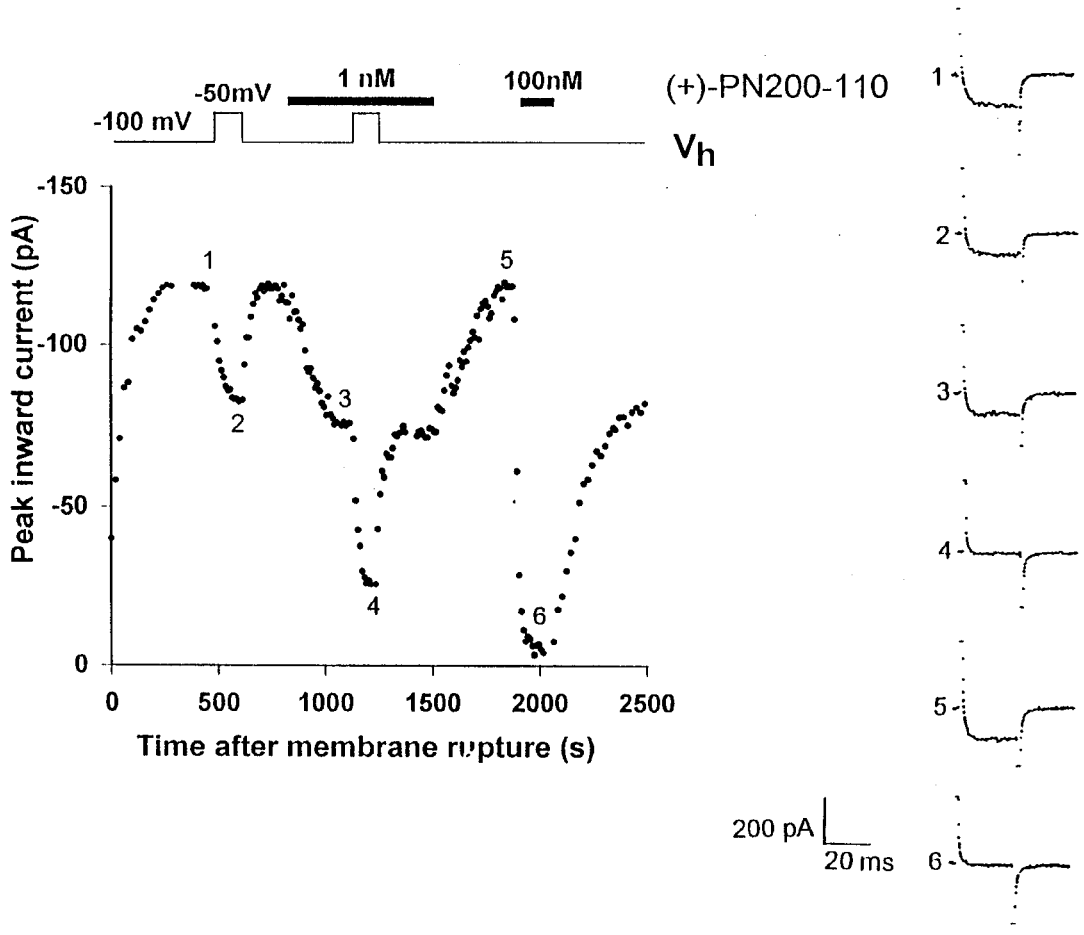

Figure 1 Standardized protocol for measuring the effect of Ca^{2+} channel blocker on Ca^{2+} channel current. Each point represents the current mediated by the $\alpha_{1C\text{-}b}$ subunit of the Ca^{2+} channel elicited from a holding potential of −100 mV or −50 mV to test the potential of 0 mV without or with 1 or 100 nM (+)-PN 200-110 in the perfusion solution, as indicated on the top of the figure. The pulse duration was 25 ms. Numbers in the plot refer to the typical current traces shown in inset.

−50 mV in the inhibitory potency of the charged DHP SDZ 207-180 while the neutral DHPs were significantly more potent in CHO-B cells compared to CHO-A cells. However, the difference in the potency of DHPs between CHO-A and CHO-B cells was less pronounced at −50 mV than at −100 mV: a/b ratios of $K_{-50\ mV}$ were about 2. Conversely, pinaverium was more potent in CHO-A than in CHO-B cells: the a/b ratio of $K_{-50\ mV}$ was equal to 0.25 suggesting that the difference in affinity was larger at −50 mV than at −100 mV. For verapamil, no difference was noted between CHO-A and CHO-B cells.

The values of the dissociation constant for the inactivated state of the channel (K_I), calculated from equation [2], are reported in Table 1, taking the $K_{-100\ mV}$ value as the

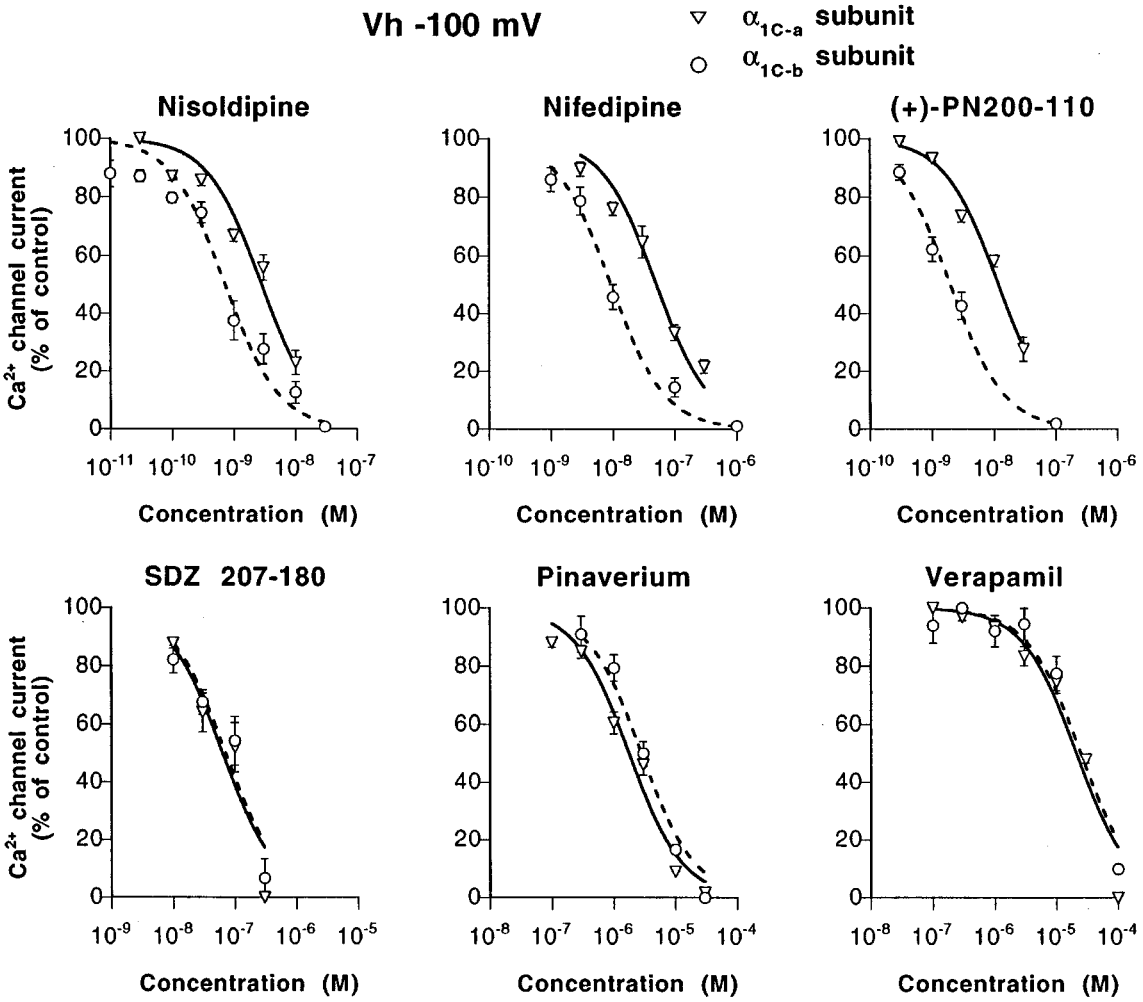

Figure 2 Concentration-effect curves of the inhibition of Ca^{2+} channel current by Ca^{2+} channel blockers in CHO cells transfected with α_{1C-a} and α_{1C-b} subunit of the L-type Ca^{2+} channel at holding potential of −100 mV. Each point is the mean±s.e.mean of four to ten determinations. Curves were drawn according to equation [1].

Table 1 Dissociation constants of the Ca^{2+} channel blockers on recombinant α_{1C-a} and α_{1C-b} subunit of the L-type Ca^{2+} channel

	$K_{-100\ mV}$		$K_{-50\ mV}$		K_I	
	α_{1C-a}	α_{1C-b}	α_{1C-a}	α_{1C-b}	α_{1C-a}	α_{1C-b}
Nisoldipine	2.1±0.5 nM (18)	0.56±0.10 nM (17)[a]	0.33±0.07 nM (14)	0.15±0.03 nM (14)[b]	0.16±0.03 nM (14)	0.08±0.02 nM (14)[a]
Nifedipine	47±5 nM (27)	10±2 nM (25)[a]	6.9±0.8 nM (12)	3.7±0.6 nM (11)[a]	2.9±0.4 nM (12)	1.9±0.4 nM (11)
(+)-PN200-110	15±3 nM (23)	2.2±0.2 nM (11)[a]	0.82±0.07 nM (12)	0.46±0.05 nM (7)[a]	0.41±0.05 nM (12)	0.19±0.02 nM (7)[a]
SDZ 207-180	91±15 nM (12)	100±20 nM (21)	20±4 nM (13)	32±8 nM (18)	6.2±1.1 nM (13)	11±3 nM (18)
Pinaverium	1.8±0.2 μM (26)	3.3±0.4 μM (19)[a]	0.34±0.07 μM (13)	1.3±0.2 μM (10)[a]	0.11±0.04 μM (13)	0.62±0.10 μM (10)[a]
Verapamil	29±6 μM (31)	23±6 μM (14)	0.95±0.21 μM (20)	0.96±0.16 μM (14)	0.46±0.10 μM (20)	0.35±0.05 μM (13)

$K_{-100\ mV}$ and $K_{-50\ mV}$ are the dissociation constants obtained from the inhibition of the Ca^{2+} channel current at holding potential of –100 mV and –50 mV. K_I are the dissociation constants for the inactivated state of the channel, calculated according to the equation [2]. Values are means±s.e.mean from (*n*) cells. [a]$P<0.01$; [b]$P<0.05$, compared to α_{1C-a} subunit.

dissociation constant for the resting state of the channel (K_R). K_I a/b ratios of about 2 were obtained for DHPs. K_I values of SDZ 207-180 and of verapamil were similar in CHO-A and in CHO-B cells. On the other hand, the K_I value of pinaverium was 5.5 times higher in CHO-B than in CHO-A cells ($P<0.01$).

Effect of Ca^{2+} channel blockers on the steady state voltage-dependent inactivation of α_1 subunit current

Steady state inactivation of $I_{\alpha 1}$ was measured after applying conditioning potential varying from -100 mV to -10 mV for 90 s. Half maximal inactivation was observed at -45.5 ± 0.3 mV ($n=5$ cells) and -44.5 ± 1.2 mV ($n=5$ cells), in CHO-A and CHO-B cells, respectively. The effect of the Ca^{2+} channel blockers on the availability curve was determined after equilibration of the cell in the presence of the drug at a holding potential of -100 mV. In the presence of a Ca^{2+} channel blocker, the steady state availability curve of $I_{\alpha 1}$ was shifted to more negative potentials as indicated by the change in the voltage of half-inactivation, $V_{0.5}$ (Table 2). Slope factor was slightly higher but this difference did not reach statistical significance. The shift in the availability curve allowed to estimate the value of K_R and of K_I of the blocker (Bean, 1983). Those values are reported in Table 2. They were similar to the values calculated from the concentration-dependent inhibition of the current reported in Table 1.

Effect of Ca^{2+} channel blockers on the current-voltage relationship of α_1 subunit current in CHO-A and CHO-B cells

I-V curves were established by applying pulses to voltages varying from -80 to $+50$ mV from a holding potential of -100 mV. In both CHO-A and CHO-B cells, none of the blockers tested changed the position of the maximum of the I-V relation and the threshold potential of the activation of the current. Figure 3 shows a representative experiment where the effect of nisoldipine on the I-V curve was recorded. Similar results were obtained with the other Ca^{2+} channel blockers.

[^{3}H]-(+)-PN 200-110 binding in CHO cells transfected with the $\alpha_{1C\text{-}a}$ and the $\alpha_{1C\text{-}b}$ subunit of the L-type Ca^{2+} channel

[^{3}H]-(+)-PN 200-110 specific binding was measured in intact CHO cells. The experiments were performed in medium containing either 5 mM KCl (physiological medium) or 50 mM KCl (depolarizing medium). No detectable specific binding of [^{3}H]-(+)-PN 200-110 was identified in non-transfected cells. In transfected cells the non-specific binding of [^{3}H]-(+)-PN 200-110 amounted to 10–20% of the total binding. Binding parameters were calculated from displacement curves using increasing concentration of the unlabelled ligand. Incubation of the cells in depolarizing medium containing 50 mM KCl decreased the dissociation constant of [^{3}H]-(+)-PN 200-100 (Table 3) without affecting the maximum binding capacity (CHO-A: 84.7 ± 3.5 fmol mg^{-1} protein in 50 mM KCl compared to 106.0 ± 16.7 fmol mg^{-1} protein in 5 mM KCl; CHO-B: 127.0 ± 4.6 fmol mg^{-1} protein in 50 mM KCl compared to 157.0 ± 24.6 fmol mg^{-1} protein in 5 mM KCl; mean values from three independent determinations). Higher binding capacity of [^{3}H]-(+)-PN 200-110 in CHO-B than in CHO-A cells probably reflects variation in the transfection efficiency.

Table 3 reports the values of the inhibition constants (K_{inh}) of different Ca^{2+} channel blockers derived from the concentration for half maximal inhibition of the specific binding of [^{3}H]-(+)-PN 200-110 measured in CHO-A and CHO-B cells incubated in physiological medium (KCl 5 mM) (Figure 4). Nisoldipine and nifedipine exhibited a higher affinity (lower K_{inh}) for the recombinant $\alpha_{1C\text{-}b}$ than for the $\alpha_{1C\text{-}a}$ subunit. Inhibition of [^{3}H]-(+)-PN 200-110 binding by SDZ 207-180 was not significantly different between CHO-A and CHO-B cells. The non-DHP compound pinaverium also displaced [^{3}H]-(+)-PN 200-110 specific binding. Contrary to DHPs, pinaverium was more potent in CHO-A than in CHO-B cells (Table 3).

Discussion

Results showed marked differences between Ca^{2+} channel blockers in their respective affinity for the $\alpha_{1C\text{-}a}$ and $\alpha_{1C\text{-}b}$ subunit of the L-type Ca^{2+} channel. The α_1 subunits expressed in CHO cells were isolated from rabbit heart and lung smooth muscle (Welling *et al*., 1993; Bosse *et al*., 1992) and are splicing products from the same gene (CaCh 2, α_{1C}) (Biel *et al*., 1991). In agreement with the previous report (Welling *et al*., 1992), we did not note significant difference between the two isoforms in terms of functional characteristics: such as kinetics and voltage-dependence of activation and inactivation. Both α_1 subunits were sensitive to the Ca^{2+} channel blockers. The potency of the drugs tested was in the order nisoldipine > (+)-

Table 2 Dissociation constants of Ca^{2+} channel blockers estimated from the steady state inactivation parameters of Ca^{2+} channel current

$\alpha_{1C\text{-}a}$ subunit	n	*$V_{0.5}$ (mV)*	*k (mV)*	*K_R*	*K_I*
Control	5	-45.5 ± 0.3	8.9 ± 0.9		
Nisoldipine (3 nM)	3	-63.4 ± 0.9[a]	10.6 ± 0.8	4.9 ± 1.8 nM	0.25 ± 0.01 nM
Nifedipine (10 nM)	3	-60.2 ± 2.3[a]	10.1 ± 1.6	35 ± 3 nM	1.9 ± 0.6 nM
Pinaverium (1 μM)	4	-61.8 ± 2.8[a]	9.9 ± 1.9	1.6 ± 0.2 μM	0.15 ± 0.07 μM
$\alpha_{1C\text{-}b}$ subunit	n	*$V_{0.5}$ (mV)*	*k (mV)*	*K_R*	*K_I*
Control	5	-44.5 ± 1.2	10.0 ± 0.7		
Nisoldipine (3 nM)	3	-69.6 ± 1.4[a]	13.2 ± 2.0	1.2 ± 0.3 nM	0.07 ± 0.02 nM[b]
Nifedipine (10 nM)	3	-62.1 ± 4.5[a]	12.0 ± 0.7	17 ± 5 nM[b]	1.2 ± 0.4 nM
Pinaverium (3 μM)	4	-61.6 ± 2.3[a]	13.3 ± 1.8	3.9 ± 0.8 μM[b]	0.33 ± 0.06 μM[b]
Verapamil (3 μM)	3	-66.9 ± 0.5[a]	11.7 ± 1.3	26 ± 1 μM	0.31 ± 0.02 μM

$V_{0.5}$ (voltage of half-inactivation) and k (slope factor) were calculated from steady state inactivation curves. K_R (dissociation constant for the resting state of the channel) and K_I (dissociation constant for the inactivated state of the channel) were calculated from the difference in $V_{0.5}$ of the inactivation curves (equation [4]). Values are means from *n* cells. [a]$P<0.01$ versus control; [b]$P<0.05$ versus dissociation constant on $\alpha_{1C\text{-}a}$ subunit.

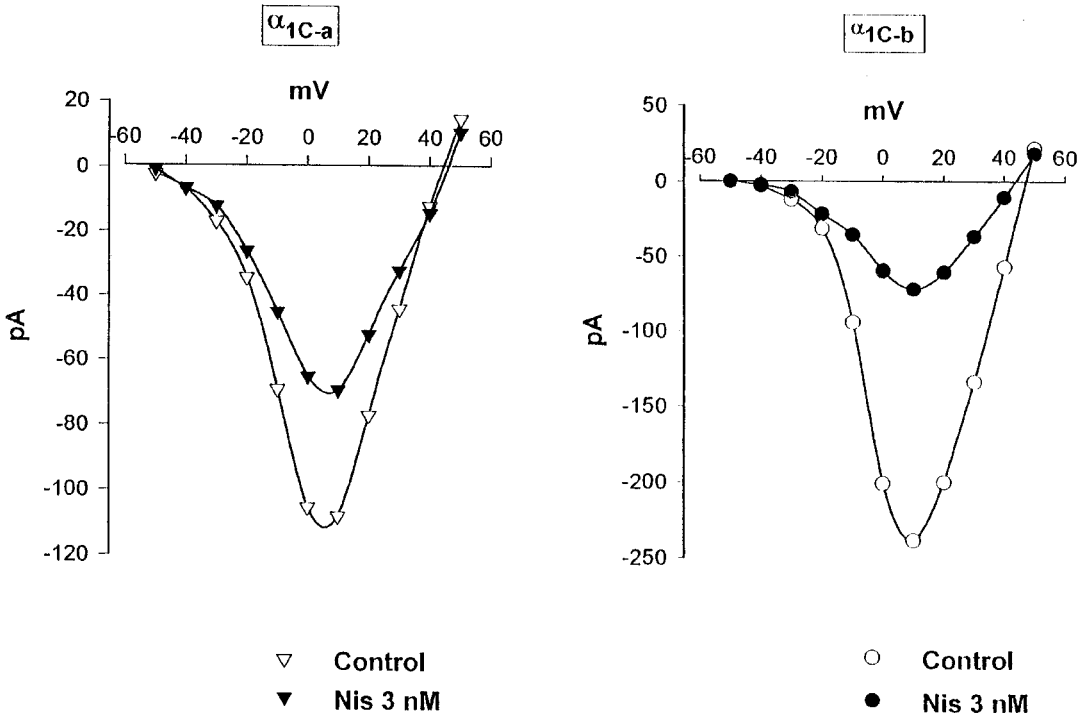

Figure 3 Effect of nisoldipine on the current-voltage relationship for Ca^{2+} channel current measured in CHO cells expressing the $\alpha_{1C\text{-}a}$ or the $\alpha_{1C\text{-}b}$ subunit of the L-type Ca^{2+} channel. Ca^{2+} channel current was activated from holding potential of -100 mV.

Table 3 Dissociation constants (K_D) or inhibition constants (K_{inh}) of different Ca^{2+} channel blockers on recombinant $\alpha_{1C\text{-}a}$ subunit and $\alpha_{1C\text{-}b}$ subunit of the Ca^{2+} channel

	Incubation condition KCl (mM)	*K_D or K_{inh}* (nM) $\alpha_{1C\text{-}a}$ *subunit*	$\alpha_{1C\text{-}b}$ *subunit*	
(+)PN 200-110	5	1.17±0.11	0.40±0.09	$P<0.01$
	50	0.42±0.06	0.15±0.01	$P<0.01$
Nifedipine	5	15.6±2.3	6.51±0.21	$P<0.05$
Nisoldipine	5	2.7±0.5	0.62±0.11	$P<0.01$
SDZ 207-180	5	30.2±8.1	18.0±3.8	n.s.
Pinaverium	5	1500±140	2920±220	$P<0.01$

K_D value of (+)-PN 200-110 was calculated from Scatchard plot of the binding data. K_{inh} of nifedipine, nisoldipine, SDZ 207-180 and pinaverium were calculated from the concentrations inhibiting 50% of the specific binding of [^{3}H]-(+)-PN 200-110. Each value is the mean±s.e.mean of three to six determinations. n.s., not significantly different.

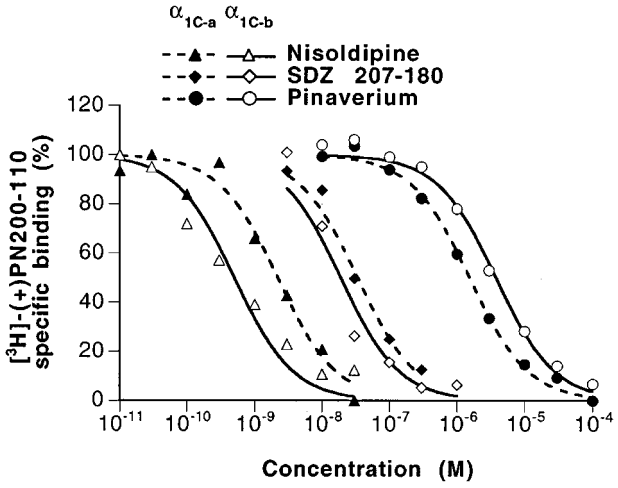

Figure 4 Displacement of the specific binding of [^{3}H]-(+)PN 200-110 in CHO cells expressing the $\alpha_{1C\text{-}a}$ (CHO-A) or the $\alpha_{1C\text{-}b}$ subunit (CHO-B) of the L-type Ca^{2+} channel. Cells were incubated in KCl 5 mM medium in the presence of [^{3}H]-(+)-PN 200-110 (100 pM) and different concentrations of competitor. Points are mean from three to five independent experiments (s.e.mean smaller than the symbols).

PN 200-110 > nifedipine > SDZ 207-180 > pinaverium > verapamil, in agreement with what has been observed in native channels either in electrophysiological studies, in functional studies or in radioligand binding studies (Godfraind *et al.*, 1986, 1992; Kass *et al.*, 1991; Méry *et al.*, 1996).

Dihydropyridines

Effect of binding of DHPs in CHO cells transfected with $\alpha_{1C\text{-}a}$ and with $\alpha_{1C\text{-}b}$ subunit were voltage-dependent, in agreement with functional and binding studies in isolated tissues or in isolated cells (Morel & Godfraind, 1987, 1991; Kokubun *et al.*, 1986; Wei *et al.*, 1989). For a number of DHPs, a 10–300 fold reduction in IC_{50} or in dissociation constant is reported when the membrane potential is changed from -80 mV to -40/-20 mV (Méry *et al.*, 1996). The $K_{-100\text{ mV}}/K_{-50\text{ mV}}$ ratios that were observed in the present study were in that range, although voltage-dependence of nisoldipine ($K_{-100\text{ mV}}/K_{-50\text{ mV}}$ ratio of 7, or K_R/K_I ratio of about 10) appeared lower than that reported in cardiac cells where a 1000 fold increase in affinity with channel inactivation has been observed (Sanguinetti & Kass, 1984). With all DHP derivatives used the increase in affinity with channel inactivation was larger with the $\alpha_{1C\text{-}a}$ subunit (13–37 fold increase) than with the $\alpha_{1C\text{-}b}$ subunit (6–12 fold increase). The most voltage-dependent compound was (+)-PN 200-100. Voltage-dependence was confirmed by the shift in the availability curves.

Nisoldipine has been shown to be a more potent blocker of the Ca^{2+} channel activity expressed by transfection of the $\alpha_{1C\text{-}b}$ cDNA than of channel activity expressed by transfection of the $\alpha_{1C\text{-}a}$ cDNA (Welling *et al*., 1993). The present results showed that the higher affinity of nisoldipine for the $\alpha_{1C\text{-}b}$ subunit was shared by the neutral DHP derivatives nifedipine and (+)-PN 200-110, but not by the ionized derivative SDZ 207-180. The isoform selectivity of neutral DHPs was voltage-dependent: the 4–7 fold difference in dissociation constant at hyperpolarized potentials, which reflects the binding to the resting state of the channel, was reduced to a 2 fold difference at inactivated state of the channel. A similar observation was reported with (+)-PN 200-110 in natural splice variants of the human fibroblast α_{1C} subunit of the L-type Ca^{2+} channel (Soldatov *et al*., 1995). It is also in agreement with the comparison of DHP action on calcium currents in rat mesenteric artery and cardiac cells indicating a 2–3 fold higher binding of nitrendipine to the resting state of the smooth muscle channel, although its binding to the inactivated state of the channel is very similar in cardiac and mesenteric artery cells (Bean *et al*., 1986). In contrast to neutral DHP derivatives, the permanently charged DHP SDZ 207-180 did not display significant isoform selectivity. The important role played by the positive charge present in ionized DHP molecules has been underlined by Bangalore *et al*. (1994). It may be hypothesized that the positive charge anchors the molecule in the membrane in such a position that it is less affected by the conformational difference existing between the $\alpha_{1C\text{-}a}$ and the $\alpha_{1C\text{-}b}$ subunits.

Pinaverium

The non-DHP positively charged derivative pinaverium interacts competitively with [^{3}H]-(+)-PN 200-110 binding (Feron *et al*., 1992). Blockade by pinaverium of the Ca^{2+} channel current mediated by the $\alpha_{1C\text{-}a}$ as well as the $\alpha_{1C\text{-}b}$ subunit was voltage-dependent. Very little shift in the availability curve has been reported with pinaverium in smooth muscle cells from the longitudinal muscle of the rabbit jejunum when 10 s conditioning pulses were used (Beech *et al*., 1990). However we noted that, using 90 s conditioning pulses, the availability curve was significantly shifted towards more negative values of potential. Since pinaverium blockade is not use-dependent (Beech *et al*., 1990), this shift reflects the voltage-dependence of the action of pinaverium. An important difference between pinaverium and DHP was noted in the present study in their opposite affinity ratio for the $\alpha_{1C\text{-}a}$ and $\alpha_{1C\text{-}b}$ subunits of the Ca^{2+} channel. Moreover, while the affinity ratio of DHPs was higher at hyperpolarized potentials, pinaverium more strongly discriminated between the two isoforms of the α_1 subunit at depolarized potentials.

Verapamil

I_{z1} block by verapamil was markedly voltage-dependent, in agreement with the voltage-dependent block of the native Ca^{2+} channel (Sanguinetti & Kass, 1984). In contrast to neutral DHPs and pinaverium, verapamil potency was not affected by the changes in the molecular structure of the α_1 subunit evoked by the transfection of the cells with $\alpha_{1C\text{-}a}$ and $\alpha_{1C\text{-}b}$ cDNA. Since verapamil is positively charged at physiological pH, further experiments with more compounds will be necessary to determine whether the absence of isoform selectivity of verapamil is related to its positive charge or to a different susceptibility of the phenylalkylamine binding site to the amino acid variations existing between the $\alpha_{1C\text{-}a}$ and the $\alpha_{1C\text{-}b}$ subunits.

Relation to tissue selectivity of Ca^{2+} channel blockers

Could the different affinities of Ca^{2+} channel blockers for the isoforms of the α_1-protein be involved in the tissue selectivity of these drugs? On the basis of extensive *in vitro* studies, nisoldipine appears to be the most vascular selective DHP with a ratio IC_{50} heart/IC_{50} vessels of about 1000, followed by (+)-PN 200-110 (ratio of about 100) and nifedipine (ratio of about 10) while verapamil and diltiazem are equipotent in vascular and cardiac tissues (Godfraind *et al*., 1992; Zheng *et al*., 1992; Sun & Triggle, 1995). The pharmacological approach that was followed in the present study confirmed the higher affinity of nisoldipine for the $\alpha_{1C\text{-}b}$ isoform compared to the $\alpha_{1C\text{-}a}$ isoform (Welling *et al*., 1993) and extended this property to other neutral DHPs whereas verapamil was equiactive on both $\alpha_{1C\text{-}a}$ and $\alpha_{1C\text{-}b}$ isoforms. The last observation indicates that absence of selectivity could be related to absence of difference in the affinity for the $\alpha_{1C\text{-}a}$ and $\alpha_{1C\text{-}b}$ isoforms. On the other hand, the largest ratio of DHP potency between $\alpha_{1C\text{-}b}$ and $\alpha_{1C\text{-}a}$ subunits was about 7. This ratio is increased when the difference in resting potentials of cardiac and vascular cells is taken into account: the affinity of DHP in the heart would indeed be well reflected by the affinity constant at -100 mV, while the affinity in smooth muscle would be best reflected by the affinity constant at -50 mV. The comparison of the concentration-effect curves obtained at -100 mV with cells expressing the $\alpha_{1C\text{-}a}$ and at -50 mV with cells expressing the $\alpha_{1C\text{-}b}$ proteins showed that the ratio of DHP potency was of 26, 13 and 15 for (+)-PN 200-110, nifedipine and nisoldipine, respectively. This value is close to the vascular selectivity of nifedipine, but it is far from the heart/vessel IC_{50} ratio observed with nisoldipine or (+)-PN 200-110 in functional studies. Moreover, no marked difference was noted among the DHP derivatives while significant differences in the tissue selectivity, covering a 30–100 fold range, are apparent in the DHP series and have been reported by several authors (Sun & Triggle, 1995). Thus, the high vascular selectivity of nisoldipine compared to nifedipine can be better predicted considering both the kinetic parameters of the binding of DHPs (Wibo *et al*., 1988), the voltage-dependence of their binding and effect (Morel & Godfraind, 1987, 1991; Sun & Triggle, 1995) and the electrophysiological characteristics of the tissue, in particular the pattern of the stimulation (Godfraind *et al*., 1992; Wibo, 1989). Additionally, the role of the auxiliary subunits of the Ca^{2+} channel in the sensitivity to the blockers may not be neglected (Wei *et al*., 1995). Nevertheless, the particular behaviour of pinaverium, with a higher potency against the $\alpha_{1C\text{-}a}$ splice variant, could account for its typical pharmacological profile and could explain the selective inhibition of calcium-induced contraction of the intestine by this compound (Szekeres & Papp, 1989) and its interesting effect in the treatment of the hypermotility of intestinal smooth muscle (Christen, 1990) since a large proportion of the $\alpha_{1C\text{-}a}$ isoform has been found in this tissue (Feron *et al*., 1994).

In conclusion, the present results show important differences between Ca^{2+} channel blockers in affinity ratio for the $\alpha_{1C\text{-}b}$ and $\alpha_{1C\text{-}a}$ subunits of the L-type voltage-dependent Ca^{2+} channel. Affinity ratio was not only function of the chemical family of the blocker, but was also influenced by the ionization of the molecule. The absence of isoform selectivity of the phenylalkylamine verapamil and the higher affinity of pinaverium for the $\alpha_{1C\text{-}a}$ isoform were in agreement with organ bath and *in vivo* data. On the other hand, the $\alpha_{1C\text{-}b}/\alpha_{1C\text{-}a}$ affinity ratio of neutral DHP was found to poorly correlate with their degree of vascular versus cardiac selectivity.

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