Inactivation determinant in the I–II loop of the Ca^{2+} channel α_1-subunit and β-subunit interaction affect sensitivity for the phenylalkylamine (−)gallopamil

Stanislav Sokolov, Regina G. Weiß, Brigitte Kurka, Franz Gapp and Steffen Hering

Institut für Biochemische Pharmakologie, Peter-Mayr-Straße 1, A-6020 Innsbruck, Austria

(Received 8 April 1999; accepted after revision 2 June 1999)

1. The role of calcium (Ca^{2+}) channel inactivation in the molecular mechanism of channel block by phenylalkylamines (PAAs) was analysed in a PAA-sensitive rabbit brain class A Ca^{2+} channel mutant ($\alpha_{1A\text{-}PAA}$). Use-dependent barium current (I_{Ba}) inhibition of $\alpha_{1A\text{-}PAA}$ by (−)gallopamil and Ca^{2+} channel recovery from inactivation and block were studied with two-microelectrode voltage clamp after expression of $\alpha_{1A\text{-}PAA}$ and auxiliary α_2-δ- and β_{1a}- or β_{2a}-subunits in *Xenopus* oocytes.

2. Mutation Arg387Glu (α_{1A} numbering) in the intracellular loop connecting domains I and II of $\alpha_{1A\text{-}PAA}$ slowed the inactivation kinetics and reduced use-dependent inhibition (100ms test pulses at 0·2 Hz from −80 to 20 mV) of the resulting mutant $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$ channels by 100 μM (−)gallopamil (53 ± 2%, $\alpha_{1A\text{-}PAA}/\beta_{1a}$ *vs.* 31 ± 2%, $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$, $n \geqslant 4$). This amino acid substitution simultaneously accelerated the recovery of channels from inactivation and from block by (−)gallopamil.

3. Coexpression of $\alpha_{1A\text{-}PAA}$ with the β_{2a}-subunit reduced fast I_{Ba} inactivation and induced a substantial reduction in use-dependent I_{Ba} inhibition by (−)gallopamil (25 ± 4%, $\alpha_{1A\text{-}PAA}/\beta_{2a}$; 13 ± 1%, $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{2a}$). The time constant of recovery from block at rest was not significantly affected.

4. These results demonstrate that changes in channel inactivation induced by Arg387Glu or β_{2a}-α_1-subunit interaction affect the drug–channel interaction.

Calcium (Ca^{2+}) channel inhibition by drugs such as phenylalkylamines (PAAs), benzothiazepines (BTZs) and mibefradil increases during repetitive depolarisation of the membrane (Lee & Tsien 1983; McDonald *et al.* 1984; Bezprozvanny & Tsien 1995; Aczél *et al.* 1998). Such a 'use-dependent' channel inhibition reflects distinct drug interactions with the resting, open and inactivated channel states. It is believed that state-dependent Ca^{2+} channel block plays an important role in the therapeutic action of PAAs and BTZs as antiarrhythmics (Hondeghem & Katzung, 1984).

Functional studies on mutant Ca^{2+} channels enabled the first insights into the molecular architecture of the Ca^{2+} channel drug-binding domains (see Hockerman *et al.* 1997*b* and Striessnig *et al.* 1998 for review). The available data suggest that three different classes of Ca^{2+} channel antagonists (PAAs, BTZs and 1,4-dihydropyridines) bind in close proximity within the pore region of L-type Ca^{2+} channel α_1-subunits (Striessnig *et al.* 1998). Three amino acids in segment IVS6 (Tyr1463, Ala1467, Ile1470) and four residues in transmembrane segment IIIS6 (Tyr1152, Ile1153, Phe1164 and Val1165) have been identified as crucial L-type determinants of the PAA sensitivity (Hockerman *et al.* 1995, 1997*a*). Insertion of three 'L-type-specific' residues (Tyr1463, Ala1467 and Ile1470) into segment IVS6 of the only weakly PAA sensitive class A (α_{1A}) Ca^{2+} channel transferred PAA sensitivity to the corresponding α_{1A} mutant (here called $\alpha_{1A\text{-}PAA}$; Hering *et al.* 1996).

An unequivocal identification of the PAA binding determinants by mutational analysis of α_1 Ca^{2+} channel subunits is, however, complicated by an apparent interdependence between Ca^{2+} channel block and inactivation gating (see Hering *et al.* 1998 for review). In particular, transfer of the IVS6 L-type determinants of PAA sensitivity (Tyr1463, Ala1467 and Ile1470) to class A Ca^{2+} channels accelerated inactivation (Hering *et al.* 1996; Degtiar *et al.* 1997). Moreover, introduction of an additional L-type amino acid (Met1464 into IVS6 of $\alpha_{1A\text{-}PAA}$) facilitated channel inactivation and enhanced use-dependent channel block by (−)gallopamil (Hering *et al.* 1996). Accordingly, substitution of a PAA determinant in $\alpha_{1A\text{-}PAA}$ (Ile1470 by the corresponding class A channel Met, $\alpha_{1C\text{-}a}$ numbering) which substantially reduced channel

inactivation induced an about 30-fold decrease of the apparent association rate for (−)devapamil (Degtiar *et al.* 1997) and alanine substitutions of three L-type amino acids localised close to the inner channel mouth on segment IIIS6 and IVS6 reduced Ca^{2+} channel inactivation and simultaneously BTZ and PAA sensitivity (Hering *et al.* 1997; Berjukow *et al.* 1999).

In our previous studies on the role of Ca^{2+} channel inactivation in channel block by PAAs and BTZs we have focused on residues that are located on segments IIIS6 and IVS6 (Degtiar *et al.* 1997; Hering *et al.* 1997; Berjukow *et al.* 1999). Here we analyse in a PAA-sensitive class A Ca^{2+} channel mutant ($\alpha_{1A\text{-}PAA}$) expressed in *Xenopus* oocytes if inactivation determinants localised outside the channel pore of an α_1-subunit influence Ca^{2+} channel block by (−)gallopamil.

We demonstrate that a single amino acid substitution (Arg387Glu, α_{1A} numbering) in the intracellular loop between domains I and II slows channel inactivation and reduces sensitivity for (−)gallopamil. Furthermore, a reduced inactivation caused by coexpression of $\alpha_{1A\text{-}PAA}$ with β_{2a}- instead of the β_{1a}-subunit reduced Ca^{2+} channel block by (−)gallopamil even more dramatically. Our study clearly demonstrates that inactivation determinants that are localised outside the putative drug binding regions in the channel pore affect the molecular mechanism of use-dependent Ca^{2+} channel block by (−)gallopamil.

METHODS

Generation of α_{1A}-constructs

The construction of the PAA-sensitive triple rabbit brain class A Ca^{2+} channel mutant AL25 (named herein $\alpha_{1A\text{-}PAA}$) was previously described (Hering *et al.* 1996). The derived mutant $\alpha_{1A\text{-}PAA/R\text{-}E}$ was constructed by introducing a single point mutation (R387E, α_{1A} numbering) into $\alpha_{1A\text{-}PAA}$ cDNA by the 'gene SOEing' technique (Horton *et al.* 1989). The point mutation was verified by sequence analysis. All constructs were inserted into the polyadenylating transcription plasmids pSPCBI 2 (a kind gift of Dr O. Pongs, University of Hamburg).

Electrophysiology

Female *Xenopus laevis* (NASCO, Fort Atkinson, WI, USA) were anaesthetised by exposing them for 15 min to a 0·2% MS-222 (methane sulfonate salt of 3-aminobenzoic acid ethyl ester; Sandoz) solution before surgically removing parts of the ovaries.The frogs were then allowed to recover and returned to their tank. Each frog was reused up to two times and subsequently killed by decapitation under anaesthesia. The interval between the operations was longer than 4 months. Follicle membranes from isolated oocytes were enzymatically digested with 2 mg ml^{-1} collagenase (Type 1A, Sigma). Calcium channel currents (I_{Ba}) were studied 2 to 7 days after microinjection of approximately equimolar cRNA mixtures of α_1 (0·3 ng per 50 nl)–β_{1a}(β_{2a}) (0·1 ng per 50 nl)–$\alpha_2\delta$ (0·2 ng per 50 nl) with two-microelectrode voltage clamp of *Xenopus* oocytes with 40 mM Ba^{2+} as charge carrier in a bath solution containing (mM): 40 $Ba(OH)_2$, 40 *N*-methyl-D-glucamine, 10 Hepes, 10 glucose, adjusted to pH 7·4 with methanesulfonic acid as previously described (Grabner *et al.* 1996). Endogenous chloride currents of the oocytes were suppressed by injecting 20–40 nl of a 0·1 M BAPTA solution 30–240 min before the voltage clamp measurements. Voltage-recording and current-injecting microelectrodes were filled with 2·8 M CsCl, 0·2 M CsOH, 10 mM EGTA, 10 mM Hepes (pH 7·4) and had resistances of 0·3–2 MΩ.

Drug sensitivity was estimated as use-dependent Ca^{2+} channel block during 20 test pulses (100 ms) applied at 0·2 Hz from −80 mV to 20 mV corresponding to the peak current voltage of the current–voltage relationships of all studied Ca^{2+} channel mutants. Use-dependent block was measured after a 3 min equilibration of the oocytes in drug-containing solution. To estimate the accumulation of Ca^{2+} channels in inactivation under control conditions similar pulse trains were applied in the absence of drug. Resting channel block was measured in an individual set of experiments as peak I_{Ba} inhibition during 100 ms test pulses from −80 to 20 mV after a 5 min equilibration in drug-containing solution.

Recovery from inactivation was studied at a holding potential of −80 mV after depolarising Ca^{2+}channels during a 3 s prepulse to 20 mV by applying 30 ms test pulses to 20 mV at various time intervals after the conditioning prepulse. Peak I_{Ba} values were normalised to the peak current measured during the prepulse. The time course of I_{Ba} recovery from inactivation was fitted to a biexponential function:

$$I_{Ba}\ \text{recovery} = A\exp(-t/\tau_{fast}) + B\exp(-t/\tau_{slow}) + C.$$

Initial rates of I_{Ba} decay (see Fig. 1*B*) were estimated by calculating the maximum derivative of mono- ($\alpha_{1A\text{-}PAA}/\beta_{2a}$, $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{2a}$) or biexponential ($\alpha_{1A\text{-}PAA}/\beta_{1a}$, $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$) fits to current inactivation during a 3 s depolarisation from −80 to 20 mV.

Voltage dependence of I_{Ba} inactivation ('inactivation curve') was measured as normalised peak current during a 30 ms test pulse that was applied 3 ms after a 3 s conditioning prepulse to a given voltage. Conditioning and test pulses were applied every 60 s from a holding potential of −100 mV. Inactivation curves were fitted to the equation:

$$I/I_{max} = I_{ss} + (1 - I_{ss})/(1 + \exp[(V - V_{0.5})/k]).$$

Data are given as means ± S.E.M. Statistical significance was calculated according to Student's unpaired *t* test ($P < 0.05$).

RESULTS

Mutation Arg387Glu in the domain I–II loop of the PAA-sensitive $\alpha_{1A\text{-}PAA}$-subunit and β_{2a}-subunit interaction affect channel inactivation and sensitivity for (−)gallopamil

We have previously reported that amino acid substitutions in transmembrane segments IIIS6 and IVS6 of class A and class C α_1-subunits affect Ca^{2+} channel inactivation kinetics and simultaneously sensitivity for PAA and BTZ (Hering *et al.* 1998). In order to further characterise the role of channel inactivation in Ca^{2+} channel block by PAAs we substituted arginine by glutamine in position 387 of the intracellular loop between domains I and II of the PAA-sensitive class A channel mutant $\alpha_{1A\text{-}PAA}$ (see Herlitze *et al.* 1997).

As expected from previous studies on wild-type class A channels (Herlitze *et al.* 1997), the I–II loop mutation Arg387Glu reduced the rate of current decay of the resulting quadruple mutant $\alpha_{1A\text{-}PAA/R\text{-}E}$ and shifted the mid-point of the inactivation curve to more positive

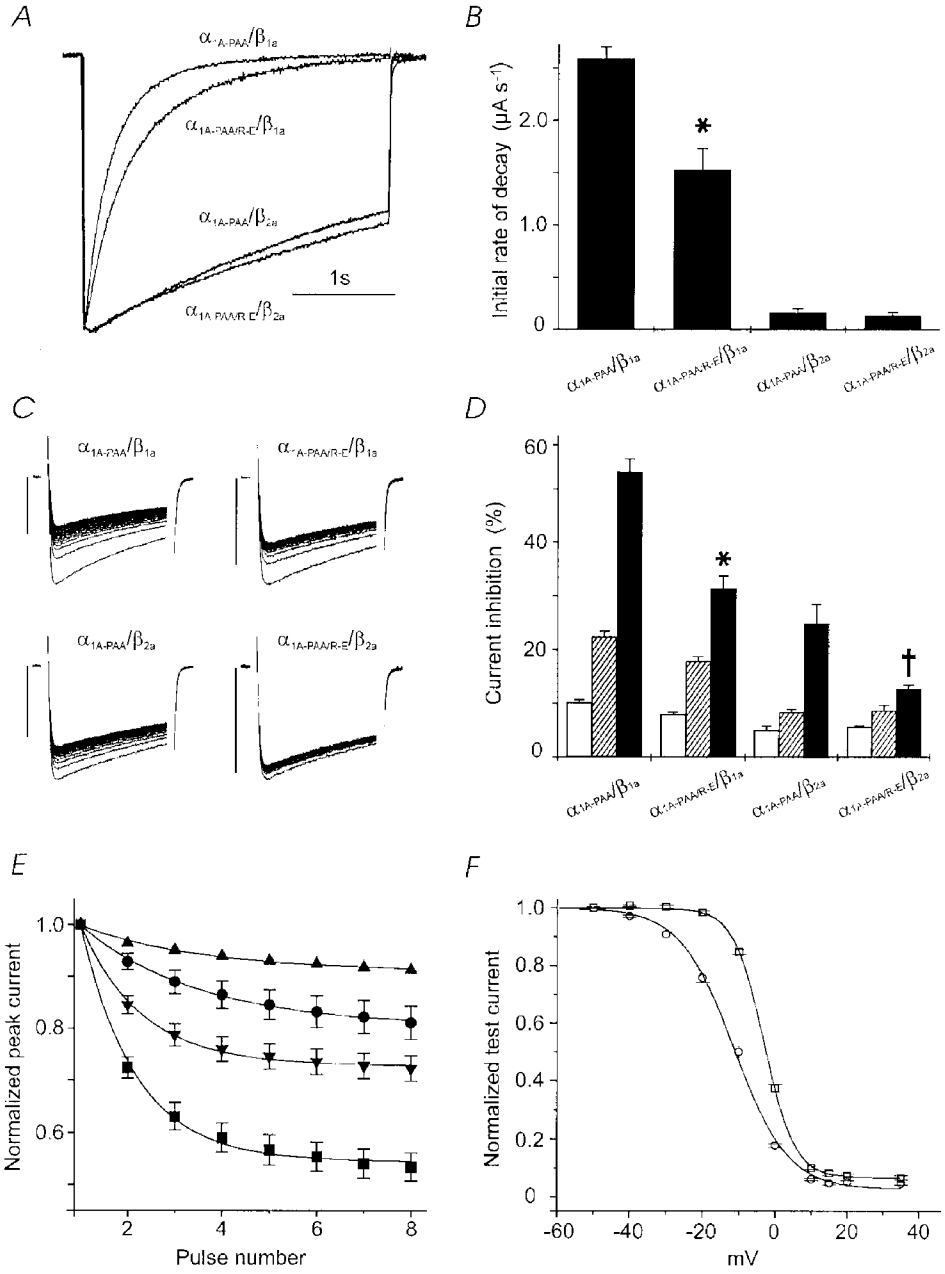

Figure 1. Mutation Arg387Glu and α_1-β_{2a} -subunit interaction reduce channel block by (−)gallopamil

A, normalised I_{Ba} of mutants $\alpha_{1A\text{-}PAA}/\beta_{1a}$, $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$, $\alpha_{1A\text{-}PAA}/\beta_{2a}$ and $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{2a}$ during 3 s depolarising test pulses applied from a holding potential of −80 mV to 20 mV. *B*, comparison of the time course of I_{Ba} inactivation of mutants $\alpha_{1A\text{-}PAA}/\beta_{1a}$, $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$, $\alpha_{1A\text{-}PAA}/\beta_{2a}$ and $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{2a}$ (in control) measured as initial rate for I_{Ba} decay during a 3 s pulse from −80 mV to 20 mV ($n \geqslant 7$, see Methods). Statistically significant reduction in the rate of current inactivation compared with $\alpha_{1A\text{-}PAA}/\beta_{1a}$ is indicated by the asterisk (*$P < 0{\cdot}05$). *C*, use-dependent I_{Ba} inhibition of $\alpha_{1A\text{-}PAA}/\beta_{1a}$, $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$, $\alpha_{1A\text{-}PAA}/\beta_{2a}$ and $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{2a}$ by 100 μM (−)gallopamil during trains of 20 pulses (100 ms) applied at 0·2 Hz from a holding potential of −80 mV to 20 mV. Vertical bars, 0·5 μA. *D*, comparison of the use-dependent I_{Ba} block of $\alpha_{1A\text{-}PAA}$ and $\alpha_{1A\text{-}PAA/R\text{-}E}$ expressed with either the β_{1a}- or β_{2a}-subunit by 10 and 100 μM (−)gallopamil. The block of I_{Ba} was measured as cumulative peak current inhibition (as a percentage) during 20 depolarising pulses (100 ms, 0·2 Hz) in control (□) or in the presence of 10 μM (▨) and 100 μM (−)gallopamil (■). Bars represent the means ± S.E.M. ($n = 5$–8). * Significantly different from I_{Ba} block of $\alpha_{1A\text{-}PAA}/\beta_{1a}$; † Significantly different from $\alpha_{1A\text{-}PAA}/\beta_{2a}$. *E*, initial rate of peak I_{Ba} inhibition by 100 μM (−)gallopamil (see *C* for details of the pulse protocol). Smooth lines are single-exponential fits to averaged peak current decays ($n \geqslant 5$) with time constants of 13·0 ± 2 s (▲, $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{2a}$), 12·5 ± 1 s (●, $\alpha_{1A\text{-}PAA}/\beta_{2a}$), 6·5 ± 0·5 s (▼, $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$) and 6·0 ± 0·5 s (■, $\alpha_{1A\text{-}PAA}/\beta_{1a}$). *F*, inactivation curves of $\alpha_{1A\text{-}PAA}/\beta_{1a}$ (○) and $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$ channels (□) in control. Curves are drawn according to the equation $I/I_{max} = I_{ss} + (1 - I_{ss})/(1 + \exp[(V - V_{0\cdot5})/k])$ (see Methods) with $V_{0\cdot5} = -11{\cdot}3 \pm 0{\cdot}5$ mV, $k = 7{\cdot}4 \pm 0{\cdot}4$ mV, $I_{ss} = 0{\cdot}03 \pm 0{\cdot}01$, $n = 5$ for $\alpha_{1A\text{-}PAA}/\beta_{1a}$ and $V_{0\cdot5} = -3{\cdot}1 \pm 0{\cdot}1$ mV, $k = 4{\cdot}20 \pm 0{\cdot}08$ mV, $I_{ss} = 0{\cdot}07 \pm 0{\cdot}01$, $n = 4$ for $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$.

potentials (Fig. 1*A*, *B* and *F*). I_{Ba} of $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$ displayed less use-dependent I_{Ba} inhibition by (−)gallopamil (10 and 100 μM) compared with $\alpha_{1A\text{-}PAA}/\beta_{1a}$ (Fig. 1*C* and *D*).

Next we determined if a modulation of the current decay by different β-subunits would affect Ca^{2+} channel block by (−)gallopamil. It is well established that coexpression of the β_{2a}-isoform induces a slow rate of voltage-dependent inactivation of Ca^{2+} channels (Stea *et al.* 1994). Accordingly, coexpression of the mutants $\alpha_{1A\text{-}PAA}$ and $\alpha_{1A\text{-}PAA/R\text{-}E}$ with the β_{2a}-subunit dramatically decreased I_{Ba} inactivation (Fig. 1*A* and *B*). Again, slower inactivation kinetics induced significantly less use-dependent block of $\alpha_{1A\text{-}PAA}/\beta_{2a}$ and $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{2a}$ compared with $\alpha_{1A\text{-}PAA}/\beta_{1a}$ and $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$ channels (Fig. 1*C* and *D*). Interestingly, peak I_{Ba} inhibition by (−)gallopamil occurred at a faster rate if Ca^{2+} channels were coexpressed with the β_{1a}-subunit (Fig. 1*E*). There was little additional effect of the point mutation on inactivation rate once the β_{2a}-subunit was coexpressed (Fig. 1*A*); however, there was still a substantial effect on drug sensitivity (Fig. 1*D*).

We did not observe significant resting channel block (<5%) by 10 μM (−)gallopamil. Resting channel inhibition induced by 100 μM (−)gallopamil was 15·0 ± 1·7% ($\alpha_{1A\text{-}PAA}/\beta_{1a}$), 6·7 ± 1·1% ($\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$, $P < 0{\cdot}01$ compared with $\alpha_{1A\text{-}PAA}/\beta_{1a}$), 20·1 ± 1% ($\alpha_{1A\text{-}PAA}/\beta_{2a}$) and 18·2 ± 1% ($\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{2a}$) ($n \geqslant 4$).

Mutation Arg387Glu and α_1-β_{2a}-subunit interaction differently affect recovery of Ca^{2+} channels from block

To elucidate the molecular basis of the different (−)gallopamil sensitivities of $\alpha_{1A\text{-}PAA}$ and $\alpha_{1A\text{-}PAA/R\text{-}E}$ coexpressed either with the β_{1a}- or β_{2a}-subunit, we analysed the drug-induced changes in I_{Ba} recovery from inactivation and block. The time courses of I_{Ba} recovery were fitted to a double-exponential function (see Methods). Under control conditions the slow component in Ca^{2+} channel repriming reflects recovery of Ca^{2+} channels from a slow or ultra-slow inactivated state (Boyett *et al.* 1994). (−)Gallopamil dose-dependently slowed recovery in all mutants (Fig. 2) whereas the time constant of recovery from fast inactivation was not significantly affected by the drug. The latter finding suggests that slow recovery reflects the fraction of drug-modified Ca^{2+} channels.

Mutation Arg387Glu did more than just slow the rate of current inactivation (see Fig. 1*A*). As shown in Fig. 2*A* and *B*, $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$ channels recovered significantly faster from inactivation ($\tau_{fast} = 0{\cdot}20 \pm 0{\cdot}04$ s, $\tau_{slow} = 1{\cdot}9 \pm 0{\cdot}3$ s, $n = 7$) compared with $\alpha_{1A\text{-}PAA}/\beta_{1a}$ ($\tau_{fast} = 0{\cdot}34 \pm 0{\cdot}04$ s,

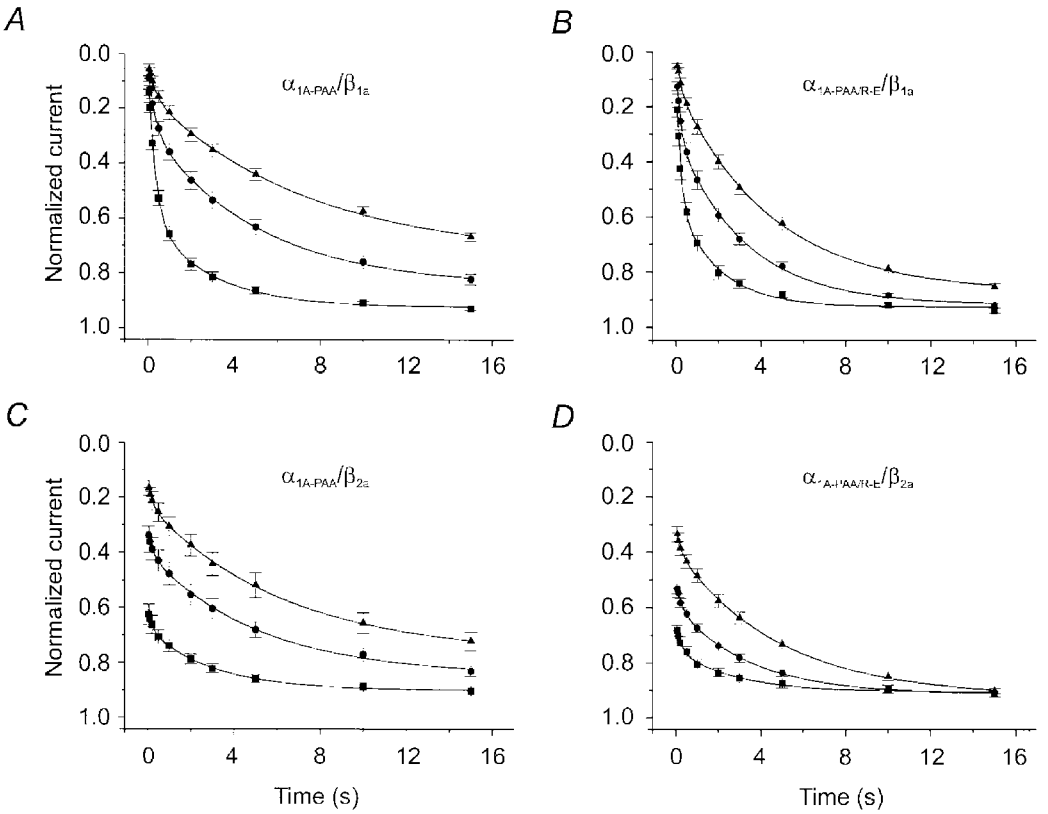

Figure 2. Recovery of Ca^{2+} channels from inactivation and block by (−)gallopamil

I_{Ba} recovery of $\alpha_{1A\text{-}PAA}/\beta_{1a}$ (*A*), $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$ (*B*), $\alpha_{1A\text{-}PAA}/\beta_{2a}$ (*C*) and $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{2a}$ (*D*) was measured by a two-pulse protocol in the absence (■) and presence of 10 (●) and 100 μM (−)gallopamil (▲). Test pulses were applied at various times (between 50 ms and 15 s) after a conditioning prepulse (see Methods). I_{Ba} values were normalised to peak I_{Ba} of the conditioning prepulse and plotted as a function of time. Data points are fitted by a double-exponential function (see Table 1 for mean values).

Table 1. Time constants (τ, s) and corresponding amplitude coefficients (A) of double-exponential I_{Ba} recovery from inactivation (see Fig. 2)

Construct	Conditions	τ_{fast}	A_{fast}	τ_{slow}	A_{slow}	n
$\alpha_{1A\text{-}PAA}/\beta_{1A}$	Control	0·34 ± 0·04	0·54 ± 0·03	2·8 ± 0·4	0·33 ± 0·03	7
	10 μM	0·34 ± 0·06	0·21 ± 0·02	5·0 ± 0·3	0·57 ± 0·01	5
	100 μM	0·34*	0·11 ± 0·02	7·3 ± 0·6	0·59 ± 0·01	6
$\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1A}$	Control	0·20 ± 0·04	0·43 ± 0·04	1·9 ± 0·3	0·39 ± 0·04	7
	10 μM	0·23 ± 0·04	0·24 ± 0·02	3·3 ± 0·2	0·61 ± 0·02	4
	100 μM	0·2*	0·10 ± 0·01	4·4 ± 0·2	0·75 ± 0·01	6
$\alpha_{1A\text{-}PAA}/\beta_{2A}$	Control	0·23 ± 0·05	0·073 ± 0·007	3·0 ± 0·2	0·221 ± 0·007	4
	10 μM	0·4 ± 0·2	0·09 ± 0·02	5·1 ± 0·4	0·43 ± 0·02	4
	100 μM	0·23*	0·08 ± 0·01	6·3 ± 0·4	0·54 ± 0·01	4
$\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{2A}$	Control	0·24 ± 0·07	0·09 ± 0·01	2·8 ± 0·4	0·15 ± 0·01	5
	10 μM	0·28 ± 0·09	0·08 ± 0·01	3·5 ± 0·2	0·32 ± 0·01	5
	100 μM	0·24*	0·071 ± 0·008	4·9 ± 0·2	0·53 ± 0·01	5

Data were obtained by fitting the kinetics of I_{Ba} recovery to a biexponential function (Fig. 2, see Methods).
* In the presence of 100 μM (−)gallopamil τ_{fast} was fixed to $\tau_{fast,control}$.

$\tau_{slow} = 2{\cdot}8 \pm 0{\cdot}4$ s, $n = 7$, $P < 0{\cdot}05$, Table 1). Faster I_{Ba} recovery from inactivation was accompanied by significantly faster recovery of $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$ channels from block by (−)gallopamil ($\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$: τ_{slow}, 100 μM = 4·4 ± 0·2 s, $n = 6$; $\alpha_{1A\text{-}PAA}/\beta_{1a}$: τ_{slow}, 100 μM = 7·3 ± 0·6 s, $n = 6$, $P < 0{\cdot}05$, Table 1).

As shown in Fig. 1*A*, coexpressing $\alpha_{1A\text{-}PAA}$ and $\alpha_{1A\text{-}PAA/R\text{-}E}$ with the β_{2a}-subunit almost completely diminished fast I_{Ba} inactivation. This finding was confirmed by the corresponding recovery experiments. During the 3 s conditioning pulse less than 10% of $\alpha_{1A\text{-}PAA}/\beta_{2a}$ or $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{2a}$ channels accumulated in fast inactivation

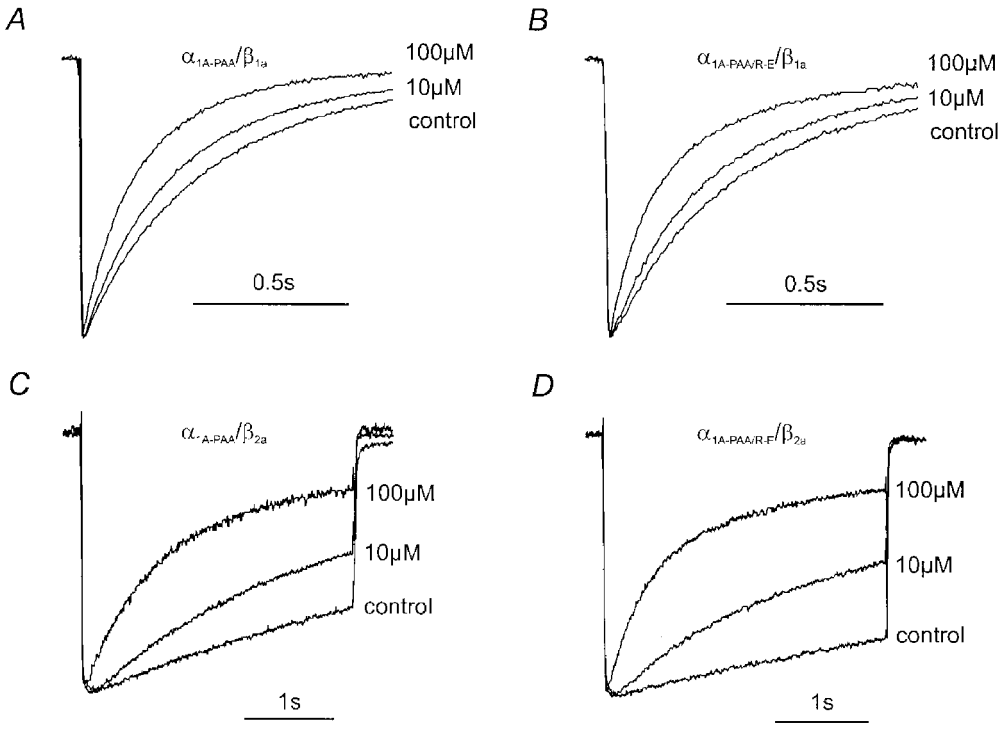

Figure 3. (−)Gallopamil-induced acceleration of I_{Ba} decay

Normalised I_{Ba} of $\alpha_{1A\text{-}PAA}/\beta_{1a}$, $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$, $\alpha_{1A\text{-}PAA}/\beta_{2a}$ and $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{2a}$ in control and in the presence of 10 and 100 μM (−)gallopamil are superimposed to illustrate channel block during 1 s ($\alpha_{1A\text{-}PAA}/\beta_{1a}$, $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$, *A* and *B*) or 3 s ($\alpha_{1A\text{-}PAA}/\beta_{2a}$, $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{2a}$, *C* and *D*) test pulses from −80 mV to 20 mV.

compared with more than 50% of $\alpha_{1A\text{-}PAA}/\beta_{1a}$ and $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$ (Fig. 2). Subsequent application of 10 or 100 μM (−)gallopamil substantially accelerated the I_{Ba} decay of all channel constructs (Fig. 3) and attenuated the slow component in I_{Ba} recovery (Fig. 2). Furthermore, drug-induced acceleration of the current decay was more prominent in $\alpha_{1A\text{-}PAA}/\beta_{2a}$ and $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{2a}$ channels. As previously observed for $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$, recovery of $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{2a}$ channels from block was more rapid than recovery of $\alpha_{1A\text{-}PAA}/\beta_{2a}$ (Fig. 2 and Table 1).

DISCUSSION

Photoaffinity labelling experiments, radioligand binding studies and alanine scanning mutagenesis of L-type transmembrane segments IIIS6 and IVS6 suggest that the drug binding pockets for PAA, BTZ and 1,4-dihydropyridines are located near the Ca^{2+} channel pore (see Striessnig *et al.* 1998 for review). However, substitutions of inactivation determinants that have been identified in close proximity to the putative drug binding determinants on pore forming S6 segments significantly modulate sensitivity for PAA (see Hering *et al.* 1997) and BTZ (Berjukow *et al.* 1999). The latter findings suggest that those amino acids form either part of the drug-binding site or, alternatively, affect PAA and BTZ sensitivity in an indirect manner (i.e. via conformational changes modulating drug access, drug trapping or the steric orientation of the receptor determinants in the pore region, see Hering *et al.* 1998 for review). We have, therefore, investigated if inactivation determinants that are localised outside the putative drug-binding region have similar modulatory effects on sensitivity for the phenylalkylamine (−)gallopamil.

Here we demonstrate that an amino acid substitution (Arg387Glu) in the intracellular loop between domains I and II of the PAA-sensitive class A Ca^{2+} channel mutant $\alpha_{1A\text{-}PAA}$ and coexpression of $\alpha_{1A\text{-}PAA}$ with the β_{2a}-subunit slow the rate of I_{Ba} inactivation and, simultaneously, reduce use-dependent Ca^{2+} channel block by (−)gallopamil (Fig. 1).

It is highly unlikely that Arg387 forms part of the PAA-binding site. Hence, arginine in position 387 of the $\alpha_{1A\text{-}PAA}$-subunit was mutated to the corresponding glutamate of the L-type $\alpha_{1C\text{-}a}$-subunit. Since L-type channels carry the high-affinity PAA-binding site, transfer of a L-type amino acid to $\alpha_{1A\text{-}PAA}$ would be expected to increase and not, as shown here, to decrease PAA sensitivity (Fig. 1*C*, *D* and *E*).

It appears more likely that mutation Arg387Glu and the association of the β_{2a}-subunit with a motif on the I–II loop known as the 'alpha interaction domain' (AID; Pragnell *et al.* 1994; see also Walker *et al.* 1998 for a second β-interaction motif located on the carboxyl terminus of α_{1A}) affect drug sensitivity indirectly by modulating channel inactivation. In other words, our data are consistent with the hypothesis that the observed effects on gallopamil sensitivity are secondary to the change in inactivation and recovery rates. It appears, therefore, likely that the different inactivation rates of naturally occurring Ca^{2+} channel splice variants (Zuhlke *et al.* 1998; Bourinet *et al.* 1999) affect their pharmacological properties.

Such a mechanism clearly differs from observations of Zamponi *et al.* (1996) indicating that the I–II loop of class A channels forms part of the piperidine receptor.

As shown in Fig. 2*B*, $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$ channels display a faster recovery from inactivation than $\alpha_{1A\text{-}PAA}/\beta_{1a}$ (see also Table 1). $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$ also recovered more rapidly from block by (−)gallopamil suggesting that mutation Arg387Glu affects sensitivity for (−)gallopamil by accelerating channel unblock at rest (Table 1).

The β_{2a}-subunit-induced changes in $\alpha_{1A\text{-}PAA}$ inactivation (Fig. 1) and the consequences for use-dependent channel block by (−)gallopamil were even more dramatic (Fig. 2). Coexpression of the β_{2a}-subunit almost completely diminished fast inactivation during a depolarising test pulse (Fig. 1*A*). However, the strong time-dependent block of $\alpha_{1A\text{-}PAA}/\beta_{2a}$ channels indicates that nearly complete lack of fast inactivation does not prevent Ca^{2+} channel block (Fig. 3*C* and *D*). This finding is in line with the comparable resting channel inhibition by 100 μM (−)gallopamil observed for $\alpha_{1A\text{-}PAA}/\beta_{2a}$, $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{2a}$ and $\alpha_{1A\text{-}PAA}/\beta_{1a}$.

The time constants of $\alpha_{1A\text{-}PAA}/\beta_{2a}$ recovery from fast and slow inactivation did not significantly differ from $\alpha_{1A\text{-}PAA}/\beta_{1a}$. Accordingly, drug bound $\alpha_{1A\text{-}PAA}/\beta_{2a}$ channels recovered at nearly the same rate as the 'higher sensitive' $\alpha_{1A\text{-}PAA}/\beta_{1a}$ channels (Fig. 2 and Table 1), and the reduced use-dependent inhibition of $\alpha_{1A\text{-}PAA}/\beta_{2a}$ channels is, in line with the slower rate of I_{Ba} inhibition (Fig. 1*E*), caused by a reduced block development during membrane depolarisation.

Arg387Glu also diminished use-dependent channel block by (−)gallopamil (Fig. 1*D*). However, contrary to the effect of the β_{2a}-subunit, this amino acid substitution affected not only the rate of block development but also the rate of recovery from block at rest. 'Lower PAA sensitivity' of $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$ (see Fig. 1) is, therefore, mainly caused by a faster channel unblock between individual pulses (Fig. 2*A* and *B*). This hypothesis fits nicely with Arg387Glu-induced changes in channel inactivation (Fig. 1*B* and *F*) that would reduce drug trapping in inactivation and facilitate recovery from block at rest (Fig. 2*A* and *B*).

Under control conditions, the effect of the Arg387Glu substitution on I_{Ba} recovery of $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{2a}$ was less evident than in $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$ channels. However, a crucial role of Arg387Glu for channel unblock was confirmed by significantly faster recovery of $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{2a}$ (compared with $\alpha_{1A\text{-}PAA}/\beta_{2a}$) in the presence of 10 and 100 μM (−)gallopamil (Fig. 2 and Table 1). In other words, reduced use-dependent block of $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{2a}$ (compared with $\alpha_{1A\text{-}PAA}/\beta_{2a}$, Fig. 1*D*) is also due to facilitated channel unblock between individual test pulses of a train (Table 1).

Taken together, use-dependent block was reduced either by slowing channel inactivation (caused by β_{2a}-interaction) or

by speeding recovery (mutation Arg387Glu). The additive and kinetically different effects of the β_{2a}-subunit interaction and mutation Arg387Glu on channel block (Fig. 1*D*) and recovery (Fig. 2) indicate that the corresponding conformational changes in the α_1-subunit are distinct and independent. Interestingly, only $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{1a}$, but not $\alpha_{1A\text{-}PAA}/\beta_{2a}$ or $\alpha_{1A\text{-}PAA/R\text{-}E}/\beta_{2a}$, displayed significantly different resting channel block compared with $\alpha_{1A\text{-}PAA}/\beta_{1a}$ channels.

It is tempting to speculate that reduced use-dependent block of $\alpha_{1A\text{-}PAA}/\beta_{2a}$ channels during a train (Fig. 1*D*) reflects β_{2a}-induced changes in the steric orientation of the putative PAA binding determinants on pore forming S6-segments during a membrane depolarisation whereas Arg387Glu-induced changes in inactivation promote accelerated channel unblock. A detailed characterisation of the functional consequences of an amino acid substitution (i.e. possible modulation of fast and/or slow inactivation gating) is, therefore, a key requirement for future mutational studies directed towards the identification of drug-binding domains.

Aczél, S., Kurka, B. & Hering, S. (1998). Mechanism of voltage- and use-dependent block of class A Ca^{2+} channels by mibefradil. *British Journal of Pharmacology* **125**, 447–454.

Berjukow, S., Gapp, F., Aczél, S., Sinnegger, M. J., Mitterdorfer, J., Glossmann, H. & Hering, S. (1999). Sequence differences between α_{1C} and α_{1S} Ca^{2+} channel subunits reveal structural determinants of a guarded and modulated benzothiazepine receptor. *Journal of Biological Chemistry* **274**, 6154–6160.

Bezprozvanny, I. & Tsien, R. W. (1995). Voltage-dependent blockade of diverse types of voltage-gated Ca^{2+} channels expressed in *Xenopus* oocytes by the Ca^{2+} channel antagonist mibefradil (Ro 40-5967). *Molecular Pharmacology* **48**, 540–549.

Bourinet, E., Soong, T. W., Sutton, K., Slaymaker, S., Mathews, E., Monteil, A., Zamponi, G. W., Nargeot, J. & Snutch, T. P. (1999). Splicing of α_{1A} subunit gene generates phenotypic variants of P- and Q-type calcium channels. *Nature Neuroscience* 2/5, 407–415.

Boyett, M. R., Honjo, H., Harrison, S. M., Zang, W. J. & Kirby, M. S. (1994). Ultra-slow voltage-dependent inactivation of the calcium current in guinea-pig and ferret ventricular myocytes. *Pflügers Archiv* **428**, 39–50.

Degtiar, V. E., Aczél, S., Doring, F., Timin, E. N., Berjukow, S., Kimball, D., Mitterdorfer, J. & Hering, S. (1997). Calcium channel block by (−)devapamil is affected by the sequence environment and composition of the phenylalkylamine receptor site. *Biophysical Journal* **73**, 157–167.

Grabner, M., Wang, Z., Hering, S., Striessnig, J. & Glossmann, H. (1996). Transfer of 1,4-dihydropyridine sensitivity from L-type to class A (BI) calcium channels. *Neuron* **16**, 207–218.

Hering, S., Aczél, S., Grabner, M., Doring, F., Berjukow, S., Mitterdorfer, J., Sinnegger, M. J., Striessnig, J., Degtiar, V. E., Wang, Z. & Glossmann, H. (1996). Transfer of high sensitivity for benzothiazepines from L-type to class A (BI) calcium channels. *Journal of Biological Chemistry* **271**, 24471–24475.

Hering, S., Aczél, S., Kraus, R. L., Berjukow, S., Striessnig, J. & Timin, E. N. (1997). Molecular mechanism of use-dependent calcium channel block by phenylalkylamines: role of inactivation. *Proceedings of the National Academy of Sciences of the USA* **94**, 13323–13328.

Hering, S., Berjukow, S., Aczél, S. & Timin, E. N. (1998). Calcium channel block and inactivation: common molecular determinants. *Trends in Pharmacological Sciences* **19**, 439–443.

Herlitze, S., Hockerman, H. G., Scheuer, T. & Catterall, W. A. (1997). Molecular determinants of inactivation and G protein modulation in the intracellular loop connecting domains I and II of the calcium channel α_{1A} subunit. *Proceedings of the National Academy of Sciences of the USA* **94**, 1512–1516.

Hockerman, G. H., Johnson, B. D., Abbott, M. R., Scheuer, T. & Catterall, W. A. (1997*a*). Molecular determinants of high affinity phenylalkylamine block of L-type calcium channels in transmembrane segment IIIS6 and the pore region of the alpha1 subunit. *Journal of Biological Chemistry* **272**, 18759–18765.

Hockerman, G. H., Johnson, B. D., Scheuer, T. & Catterall, W. A. (1995). Molecular determinants of high affinity phenylalkylamine block of L-type calcium channels. *Journal of Biological Chemistry* **270**, 22119–22122.

Hockerman, G. H., Peterson, B. Z., Johnson, B. D. & Catteral, W. A. (1997*b*). Molecular determinants of drug binding and action on L-type calcium channels. *Annual Review of Pharmacology and Toxicology* **37**, 361–396.

Hondeghem, L. M. & Katzung, B. G. (1984). Antiarrhythmic agents: the modulated receptor mechanism of action of sodium and calcium channel-blocking drugs. *Annual Review in Pharmacology and Toxicology* **24**, 387–423.

Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K. & Pease, L. R. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**, 61–68.

Lee, K. S. & Tsien, R. W. (1983). Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nitrendipine in single dialysed heart cells. *Nature* **302**, 790–794.

McDonald, T. F., Pelzer, D. & Trautwein, W. (1984). Cat ventricular muscle treated with D600: characteristics of calcium channel block and unblock. *Journal of Physiology* **352**, 217–241.

Pragnell, M., De Waard, M., Mori, Y., Tanabe, T., Snutch, T. P. & Campbell, K. P. (1994). Calcium channel β-subunit binds to a conserved motif in the I-II cytoplasmic linker of the α_1-subunit. *Nature* **368**, 67–70.

Stea, A., Tomlinson, W. J., Soong, T. W., Bourinet, E., Dubel, S. J., Vincent, S. R. & Snutch, T. P. (1994). Localisation and functional properties of a rat brain α_{1A} calcium channel reflect similarities to neuronal Q- and P-type channels. *Proceedings of the National Academy of Sciences of the USA* **91**, 10576–10580.

Striessnig, J., Grabner, M., Mitterdorfer, J., Hering, S., Sinnegger, M. J. & Glossmann, H. (1998). Structural basis of drug binding to L Ca^{2+} channels. *Trends in Pharmacological Sciences* **19**, 108–115.

Walker, D., Bichet, D., Campbell, K. P. & De Waard, M. (1998). A β4 isoform-specific interaction site in the carboxyl-terminal region of the voltage-dependent Ca^{2+} channel α1A subunit. *Journal of Biological Chemistry* **273**, 2361–2367.

Zamponi, G. W., Soong, T. W., Bourinet, E. & Snutch, T. P. (1996). β subunit coexpression and the α_1 subunit domain I-II linker affect piperidine block of neuronal calcium channels. *Journal of Neuroscience* **16**, 2430–2443.

Zuhlke, R. D., Bouron, A., Soldatov, N. M. & Reuter, H. (1998). Ca^{2+} channel sensitivity towards the blocker isradipine is affected by alternative splicing of the human α_{1C} subunit gene. *FEBS Letters* **427**, 220–224.

Acknowledgements

We thank Professor H. Glossmann for continuous support of our work and Dr Stanislav Berjukow for comments on the manuscript. We also thank Drs Y. Mori and K. Imoto for the gift of the α_{1A} cDNA, Dr A. Schwartz for providing the α_2/δ cDNA, Dr L. Birnbaumer for providing the β_{2a} cDNA and Dr Traut (Knoll AG, Ludwigshafen, Germany) for providing (−)gallopamil. This work was supported by grants from the Fonds zur Förderung der Wissenschaftlichen Forschung P12649 (S.H.), a grant from the Else Kröner Fresenius Stiftung (S.H.), and a grant from the Österreichische Nationalbank (S.H.), and is part of the thesis of S.S.

Corresponding author

S. Hering: Institut für Biochemische Pharmakologie, Peter-Mayr-Straße 1, A-6020 Innsbruck, Austria.

Email: steffen.hering@uibk.ac.at