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Voltage-dependent acceleration of $Ca_v1.2$ channel current decay by (+)- and (−)-isradipine

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1 Inhibition of $Ca_v1.2$ by antagonist 1,4 dihydropyridines (DHPs) is associated with a drug-induced acceleration of the calcium (Ca^{2+}) channel current decay. This feature is contradictorily interpreted as open channel block or as drug-induced inactivation. To elucidate the underlying molecular mechanism we investigated the effects of (+)- and (−)-isradipine on $Ca_v1.2$ inactivation gating at different membrane potentials.

2 $\alpha_1$1.2 Constructs were expressed together with α_2-δ- and β_{1a}- subunits in *Xenopus* oocytes and drug-induced changes in barium current (I_{Ba}) kinetics analysed with the two microelectrode voltage clamp technique. To study isradipine effects on I_{Ba} decay without contamination by intrinsic inactivation we expressed a mutant (V1504A) lacking fast voltage-dependent inactivation.

3 At a subthreshold potential of −30 mV a 200-times higher concentration of (−)-isradipine was required to induce a comparable amount of inactivation as by (+)-isradipine. At +20 mV the two enantiomers were equally efficient in accelerating the I_{Ba} decay.

4 Faster recovery from (−)- than from (+)-isradipine-induced inactivation at −80 mV in a $Ca_v1.2$ construct ($\tau_{(-)\text{-isr.(Cav1.2)}} = 0.74$ s $< \tau_{(+)\text{-isr.(Cav1.2)}} = 2.85$ s) and even more rapid recovery of V1504A ($\tau_{(-)\text{-isr.(V1504A)}} = 0.39$ s $< \tau_{(+)\text{-isr.(V1504A)}} = 1.98$ s) indicated that drug-induced determinants and determinants of intrinsic inactivation (V1504) stabilize the DHP-induced channel conformation in an additive manner.

5 In the voltage range between −25 and 20 mV where the channels inactivate predominantly from the open state the (+)- and (−)-isradipine-induced acceleration of the I_{Ba} decay in V1504A displayed similar voltage-dependence as intrinsic fast inactivation of $Ca_v1.2$.

6 Our data suggest that the isradipine-induced acceleration of the $Ca_v1.2$ current decay reflects enhanced fast voltage-dependent inactivation and not open channel block.

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Abbreviations: DHP, 1,4-dihydropyridines; I_{Ba}, Ba^{2+} current

Introduction

The calcium (Ca^{2+}) channel blocker (or Ca^{2+} antagonist) isradipine is widely used for the treatment of hypertension and angina (Frishman & Michaelson, 1997). Isradipine belongs to the chemical class of the 1,4 dihydropyridines (DHPs) and exerts its therapeutic effect primarily by blocking $Ca_v1.2$ (L-type Ca^{2+} channels, see Ertel *et al*., 2000 for nomenclature) in the vascular system (Lund-Johansen, 1993).

$Ca_v1.2$ are hetero-oligomeric protein complexes consisting of a pore forming α_1-subunit, auxiliary β- (β_1-β_4) and α_2-δ-subunits modulating voltage-dependence, expression density and channel kinetics (Catterall, 1994; Walker & De Waard, 1998; Hering *et al*., 2000). The receptor determinants for DHPs are localized on the pore forming α_1-subunit ($\alpha_1$1.2) of $Ca_v1.2$ (Striessnig *et al*., 1998). Mutational analysis revealed nine amino acid residues in segments IIIS5, IIIS6 and IVS6 of $\alpha_1$2.1 that confer high affinity and stereoselective interaction with isradipine (Tang *et al*., 1993; Grabner *et al*., 1996; Peterson *et al*., 1996; 1997; Sinnegger *et al*., 1997; Ito *et al*., 1997).

Enantioselectivity is a hallmark of the interaction of isradipine with the $Ca_v1.m$ superfamily (Glossmann & Ferry, 1985). High concentrations of isradipine also inhibit low threshold calcium channels. This low affinity interaction of isradipine with non-$Ca_v1.m$ (non-L-type channels) occurs, however, in a non-stereoselective manner (Berjukow *et al*., 1996).

$Ca_v1.2$ inhibition by DHPs is commonly described in terms of a modulated receptor mechanism (Hille, 1978). A popular version of the modulated receptor hypothesis suggests high affinity DHP-binding to the inactivated Ca^{2+} channel conformation. This hypothesis is based on the well-documented DHP-induced leftward shift of the of the $Ca_v1.2$ availability curve (Bean, 1984; Sanguinetti & Kass, 1984).

However, Ca^{2+} channels inactivate by at least two ('fast' and 'slow') voltage-dependent mechanisms and in some channel types by an additional Ca^{2+}-dependent inactivation (Brehm & Eckert, 1978; Lee *et al*., 1999; Hering *et al*., 2000 for review). Most previous studies did not differentiate between DHP interactions with different inactivated channel conformations. We have recently shown that the high affinity (+)-enantiomer of isradipine promotes a channel

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state resembling intrinsic fast inactivation (Berjukow *et al.*, 2000).

The mechanism of the DHP-induced changes in Ca^{2+} channel gating remains, however, unclear. In particular, the DHP-induced acceleration of the I_{Ba} decay observed in many previous studies is controversially interpreted as either reflecting open channel block or, alternatively, drug-induced inactivation.

In the present study we have, therefore, analysed the effects of the two isradipine enantiomers on channel inactivation at different membrane potentials. We report here that (+)- and (−)-isradipine enhance channel transitions to a (drug-induced) inactivated channel conformation at subthreshold voltages where $Ca_v1.2$ inactivate predominantly from the resting state. At more depolarized voltages where $Ca_v1.2$ inactivate predominantly *via* the open channel conformation drug-effects on the current decay display a similar voltage-dependency as intrinsic fast inactivation. Our data suggest that the DHP-induced acceleration of the I_{Ba} decay at depolarized voltages reflects enhanced inactivation and not open channel block. An analysis of the recovery from drug-induced inactivation revealed that intrinsic determinants of fast inactivation (V1504) and drug-induced inactivation determinants contribute in an additive manner to the stability of the DHP-induced channel state.

Methods

α_1 cDNAs

The study was performed on the $Ca_v1.2$ channel construct Lh (named herein α_{1L}, Grabner *et al.*, 1996). α_{1L} is a construct corresponding to rabbit cardiac $\alpha_{1C\text{-}a}$ cDNA (Mikami *et al.*, 1989) with part of the amino terminus replaced by carp $\alpha_1 1.1$ sequence as described (Grabner *et al.*, 1996; Berjukow *et al.*, 1999). Mutation V1504A was previously described in Berjukow *et al.*, 1999. Amino acid numbering of V1504A is according to $\alpha_{1C\text{-}a}$ cDNA sequence.

Electrophysiology

Inward barium currents (I_{Ba}) were studied with two microelectrode voltage-clamp of *Xenopus* oocytes 2–7 days after microinjection of approximately equimolar cRNA mixtures of α_{1L} or V1504A (Grabner *et al.*, 1996) (0.3 ng–50 nl) together with α_2-δ(0.2 ng–50 nl) and β_{1a}(0.1 ng–50 nl) cRNA as described previously (Grabner *et al.*, 1996). The corresponding constructs were named herein α_{1L}/α_2-δ/β_{1a} or V1504A channels.

All experiments were carried out at room temperature in a bath solution with the following composition (mM): $Ba(OH)_2$ 40, NaOH 50, HEPES 5, CsOH 2 (pH adjusted to 7.4 with methanesulphonic acid). 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 0.4–2.2 MΩ. Tonic (resting-state-dependent) Ca^{2+} channel block was estimated as steady-state peak I_{Ba} inhibition during short (100 ms) test pulses from −80 to 20 mV at a frequency of 0.033 Hz. Drug effects on I_{Ba} inactivation were analysed as isradipine-induced current decay during 3 s test pulses from −80 to 20 mV. The dose-response curves of I_{Ba} inhibition were fitted using the

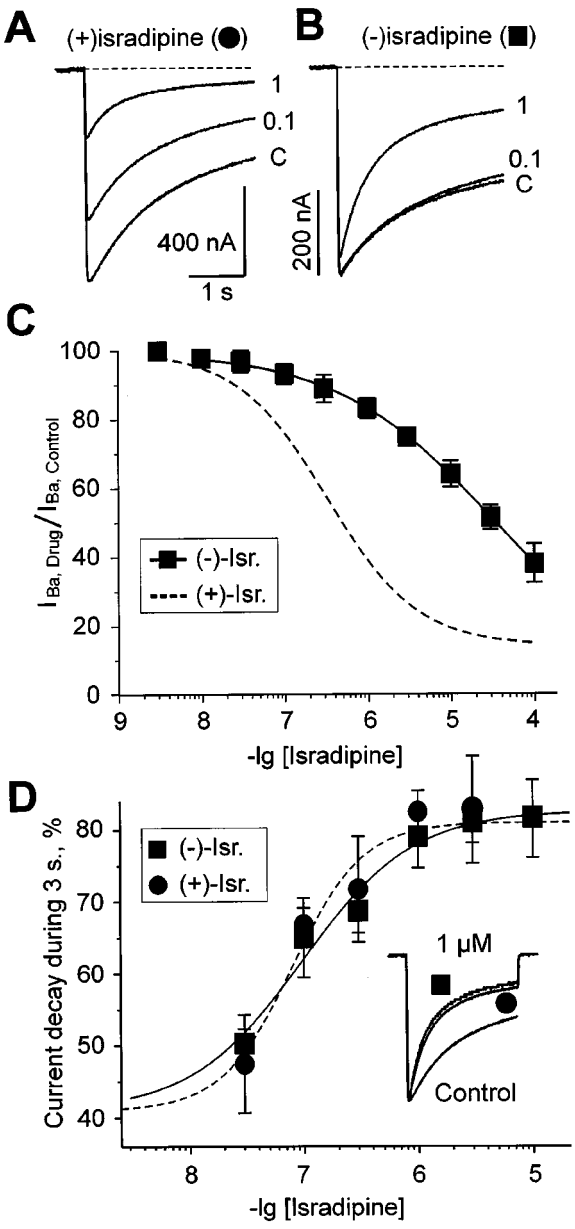

Figure 1 Peak current inhibition and modulation of inactivation kinetics in α_{1L}/α_2-δ/β_{1a} channels by (+)- and (−)-isradipine. (A,B) Barium currents through α_{1L}/α_2-δ/β_{1a} channels during membrane depolarizations from −80 mV to 20 mV in control and in the presence of different concentrations of (+)- and (−)-isradipine (in μM). (C) Concentration-response relationships of peak I_{Ba} inhibition of α_{1L}/α_2-δ/β_{1a} channels by (+)- (dashed line, from Berjukow *et al.*, 2000) and (−)-isradipine. Channel block was estimated as the ratio of peak current in the presence of the respective enantiomer compared to peak I_{Ba} in control. Data points represent the mean values from 4–11 experiments. The IC_{50} and the Hill coefficient (n_H) for peak current block by (−)-isradipine were obtained by best fit of the data points to the general dose-response equation (see Methods) yielding: $IC_{50} = 34 \pm 8$ μM, $n_H = 0.45 \pm 0.03$, $n = 4$. (D) Acceleration of I_{Ba} decay by (+)- and (−)-isradipine estimated as late current inhibition during a 3 s depolarizing test pulse from −80 to 20 mV. The inset shows three superimposed normalized I_{Ba} through α_{1L}/α_2-δ/β_{1a} in control and the presence of 1 μM (+)- and (−)-isradipine.

Hill equation: $I_{Ba,drug}/I_{Ba,control}$ (in %) = (100-A)/(1 + (C/IC_{50})^{nH}) + A, where IC_{50} is the concentration at which I_{Ba} inhibition is half maximal, C is the applied drug concentration, A is the fraction of I_{Ba} that is not blocked and n_H is the Hill coefficient.

Recovery from inactivation was studied with a conventional double-pulse protocol after depolarizing Ca^{2+} channels during a 3 s prepulse (to 20 mV) at a holding potential of −80 mV. 30 ms-test pulses (to 20 mV) were applied at various time intervals after the conditioning prepulse. Peak I_{Ba} values were normalized to the peak current measured during the prepulse and the time course of I_{Ba} recovery from inactivation was fitted to a mono- or biexponential function ($I_{Ba,recovery} = A_{fast} \times \exp(-t/\tau_{fast}) + A_{slow} \times \exp(-t/\tau_{slow}) + C$). The time constants of I_{Ba} inactivation were estimated by fitting the current decay to a mono or biexponential function. Data are given as mean ± s.e.mean statistical significance was calculated according to Student's unpaired *t*-test.

Results

(+)- and (−)-isradipine effects on peak and late current of $Ca_v1.2$

Isradipine binding to $Ca_v1.m$ is highly stereoselective with the (+)-enantiomer being more potent than (−)-isradipine (Glossmann & Ferry, 1985). To elucidate the functional consequences of this stereoselective drug-channel interaction we compared the inhibition of barium currents through $Ca_v1.2$ constructs by both enantiomers. First we expressed the α_{1L}-subunit (Berjukow *et al.*, 1999) together with the auxiliary α_2-δ- and β_{1a}-subunits in *Xenopus* oocytes and studied (+)- and (−)-isradipine effects on I_{Ba} under two microelectrode voltage clamp (see Methods). Peak I_{Ba} through α_{1L}/α_2-δ/β_{1a} channels were much less efficiently inhibited by (−)-isradipine (IC_{50} = 34 ± 8 μM, Figure 1A–C) than by the (+)-enantiomer (IC_{50} = 327 ± 41 nM, Berjukow *et al.*, 2000) which is in line with our previous observations

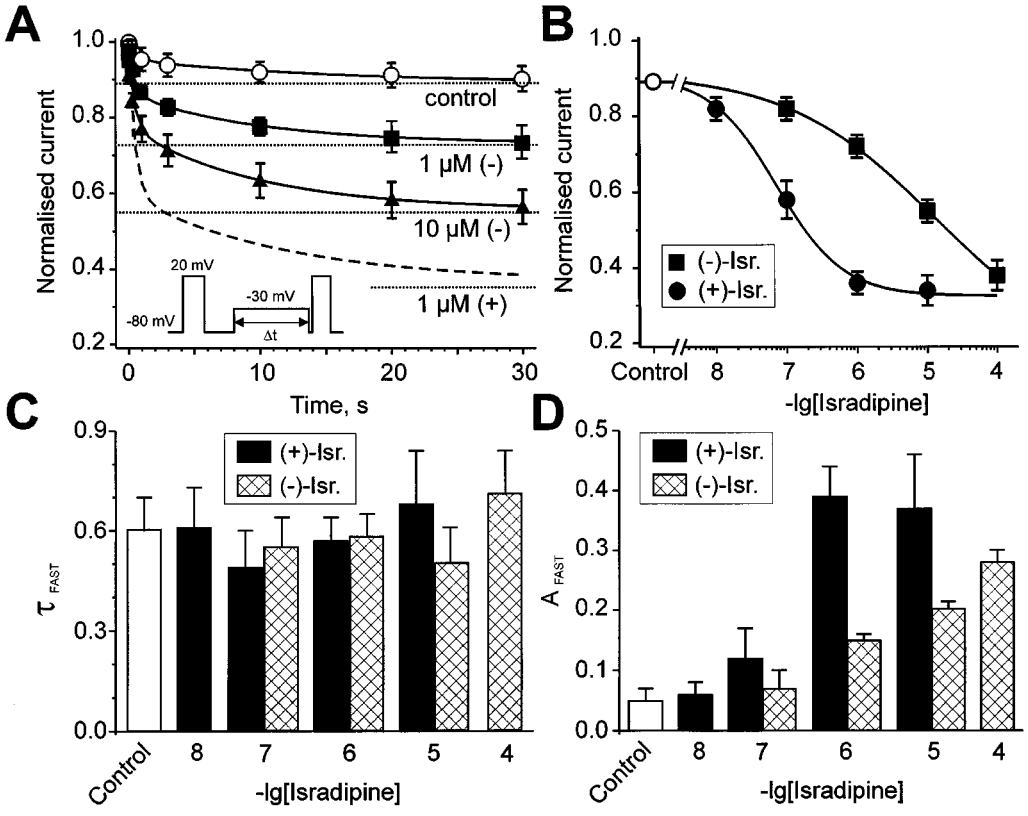

Figure 2 Drug-induced closed-state inactivation of α_{1L}/α_2-δ/β_{1a} channels at the subthreshold potential of −30 mV. (A) Effect of prepulses of variable duration from −80 mV to −30 mV on the peak current evoked by a subsequent test pulse to 20 mV (see inset). The smooth curves are biexponential functions fitted to the time course of mean I_{Ba} inactivation of α_{1L}/α_2-δ/β_{1a} channels ($I_{Ba/normalised} = A_{fast} \times \exp(-\tau/\tau_{fast}) + A_{slow} \times \exp(-\tau/\tau_{slow}) + C$). Asymptotic values are represented by the dotted lines. The parameters of the fit in control: A_{fast} = 0.05, τ_{fast} = 0.62 s, A_{slow} = 0.06, τ_{slow} = 13.0 s, C = 0.89; in 1 μM (−)-isradipine: A_{fast} = 0.15, τ_{fast} = 0.58 s, A_{slow} = 0.1, τ_{slow} = 14.0 s, C = 0.73; in 10 μM (−)-isradipine: A_{fast} = 0.21, τ_{fast} = 0.49 s, A_{slow} = 0.22, τ_{slow} = 11 s, C = 0.56. The time course of the biexponential fit to 1 μM (+)-isradipine-induced inactivation (dashed line from Berjukow *et al.*, 2000) is shown for comparison. (B) Drug-induced steady-state inactivation (dotted lines in A) induced by different concentrations of (+)- and (−)-isradipine is plotted as a function of the applied drug-concentration. Fitting of the data points to the dose-response equation yielded for (−)-isradipine: IC_{50} = 13 ± 5 μM, n_H = 0.41 ± 0.03 (n = 4) and for (+)-isradipine: IC_{50} = 72 ± 5 nM, n_H = 0.92 ± 14 (n = 3). (C) Kinetics of the fast component of drug-induced inactivation by (+)- and (−)-isradipine. The corresponding mean time constants (τ_{fast}) of the biexponential fits (shown in A) are indicated for different drug-concentrations. (D) The amplitude coefficient of the fast component (A_{fast}, see A) is illustrated for different drug concentration. (+)- (black bars) and (−)-isradipine- (hatched bars) induced fast inactivation at −30 mV are illustrated at different drug concentrations.

(Berjukow *et al*., 1996) and data of Handrock *et al*. (1999). Ca^{2+} channel inhibition by both enantiomers was accompanied by a drug-induced acceleration of the current decay (Figure 1A,B). In order to quantify this effect we plotted the I_{Ba} decay during a 3 s test pulse *versus* the applied drug concentrations. Surprisingly, as shown in Figure 1D, (+)- and (−)-isradipine displayed no enantioselectivity with respect to their effects on I_{Ba} decay ($IC_{50,(+)\text{-isr.}} = 78 \pm 18$ nM, $IC_{50,(-)\text{-isr.}} = 95 \pm 22$ nM, $P > 0.05$).

Next we investigate if the different potencies of (+)- and (−)-isradipine to inhibit peak I_{Ba} (Figure 1A–C) would correlate with their abilities to induce inactivation at subthreshold potentials. (+)- and (−)-isradipine induced an additional component in the onset of resting state inactivation of $\alpha_{1L}/\alpha_2\text{-}\delta/\beta_{1a}$ (Figure 2A, see also Berjukow *et al*., 2000). The mean time constant of the (−)-isradipine-induced inactivation at −30 mV ranged between 0.5 and 0.7 s (100 nM – 100 μM) which was quite similar to the rates observed for the (+)-enantiomer (between 0.5 and 0.73 s in 10 nM – 10 μM, see Figure 2C). The corresponding amplitude coefficients (A_{fast}) of this drug-induced component in channel inactivation increased with increasing drug concentrations (Figure 2D). Plotting A_{fast} *versus* the applied (+)- and (−)-isradipine concentrations revealed a significantly higher potency of the (+)-enantiomer. As shown in Figure 2B, an about 200-times higher concentration of (−)-isradipine was required to induce a comparable inactivation of resting channels as by (+)-enantiomer (IC_{50} 72 ± 5 nM for (+)-isradipine ($n = 3$) and 13 ± 4 μM for (−)-isradipine ($n = 4$)).

(+)- and (−)-isradipine-induced acceleration of I_{Ba} decay is voltage-dependent

To gain further insights into the molecular mechanism underlying the isradipine effects on I_{Ba} decay we made use of mutant V1504A lacking fast voltage-dependent inactivation (Berjukow *et al*., 1999; 2000). This enabled us to investigate drug effects on I_{Ba} decay without contamination by intrinsic voltage-dependent inactivation.

Peak current inhibition of V1504A by (−)-isradipine was more pronounced ($IC_{50} = 2.2 \pm 0.2$ μM, $n = 4$) than in $\alpha_{1L}/\alpha_2\text{-}\delta/\beta_{1a}$ channels ($IC_{50} = 34 \pm 8$ μM, $n = 4$, Figure 3A,B, see also Berjukow *et al*., 2000). Both enantiomers accelerated the I_{Ba} decay to comparable extents (Figure 3B, see also Figure 1D for similar result in the $Ca_v1.2$ construct).

Plotting the time constants of (+)- and (−)-isradipine (1 μM) induced I_{Ba} decay *versus* the applied test potentials revealed that both enantiomers accelerate the current decay in a voltage-dependent manner. Moreover, the drug-induced component of I_{Ba} decay in mutant V1504A and the time constants of intrinsic fast voltage-dependent inactivation of $\alpha_{1L}/\alpha_2\text{-}\delta/\beta_{1a}$ displayed almost identical voltage-dependence. These data suggest that both enantiomers restore fast voltage-dependent inactivation in mutant V1504A rather than blocking open channels (Figure 4).

Different stabilities of (+)- and (−)-isradipine-induced inactivated states

The stability of the DHP-induced channel conformation can be assessed by analysing the recovery from drug-induced

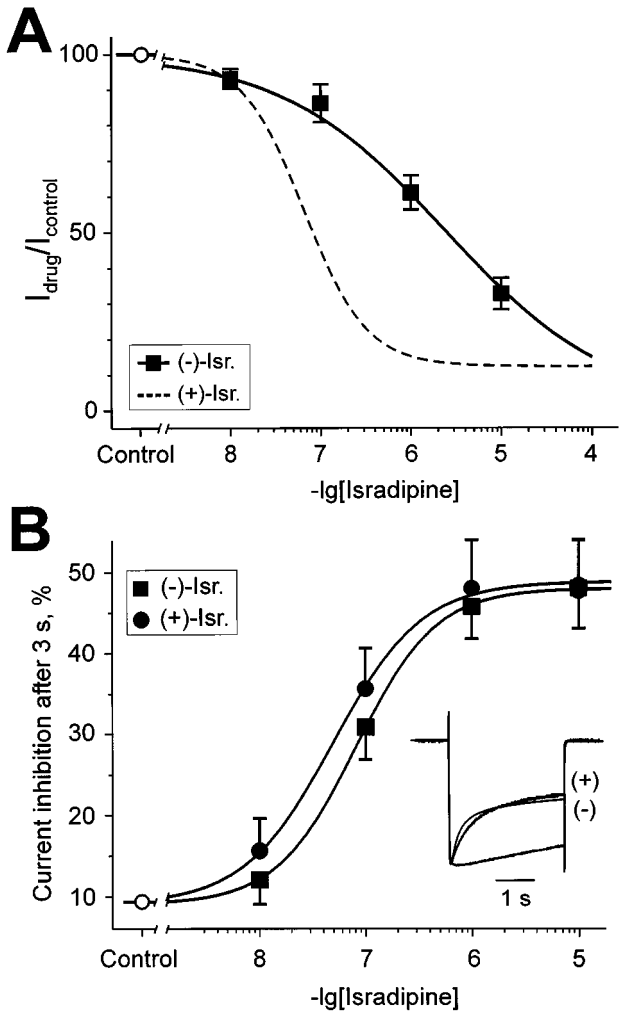

Figure 3 Inhibition of peak I_{Ba} and (+)- and (−)-isradipine-induced inactivation in mutant V1504A. (A) Concentration-response relationships of peak I_{Ba} inhibition of V1504A channels by (−)-isradipine. Data points represent the mean values from 3–5 experiments. The IC_{50} and the Hill coefficient (n_H) for peak current block by (−)-isradipine were obtained by best fit of the data points to the dose-response equation yielding: $IC_{50} = 2.2 \pm 0.2$ μM, $n_H = 0.53 \pm 0.03$; The dashed line represents the fitted concentration response curve for channel block by (+)-isradipine (from Berjukow *et al*., 2000). (B) (+)- and (−)-isradipine accelerate I_{Ba} inactivation of V1504A. (+)- and (−)-induced effects on I_{Ba} decay were estimated as current inhibition during a 3 s depolarizing test pulse from −80 to 20 mV in per cent. Inset: Superimposed normalized I_{Ba} illustrating (+)- and (−)-isradipine-induced acceleration of the current decay compared to control.

inactivation (Berjukow *et al*., 2000). In the present study we compared the stabilities of the (−)- and (+)-isradipine-induced channel states. As shown in Figure 5, I_{Ba} of $\alpha_{1L}/\alpha_2\text{-}\delta/\beta_{1a}$ recover at −80 mV in control with monoexponential kinetics ($\tau_{rec.control} \approx 0.65$ s). Recovery of $\alpha_{1L}/\alpha_2\text{-}\delta/\beta_{1a}$ channels from (+)-isradipine-induced inactivation at −80 mV (1 μM (+)-isradipine) was previously found to occur at an about four times slower rate (illustrated by the dashed line in Figure 5A, from Berjukow *et al*., 2000). The faster recovery of $\alpha_{1L}/\alpha_2\text{-}\delta/\beta_{1a}$ channels from (−)-isradipine-induced inactivation (Figure 5A,C, $\tau_{rec,(-)\text{-israd.}}$ (1 μM) = 0.74 ± 0.07, $n = 4$) suggested that the (−)-isradi-

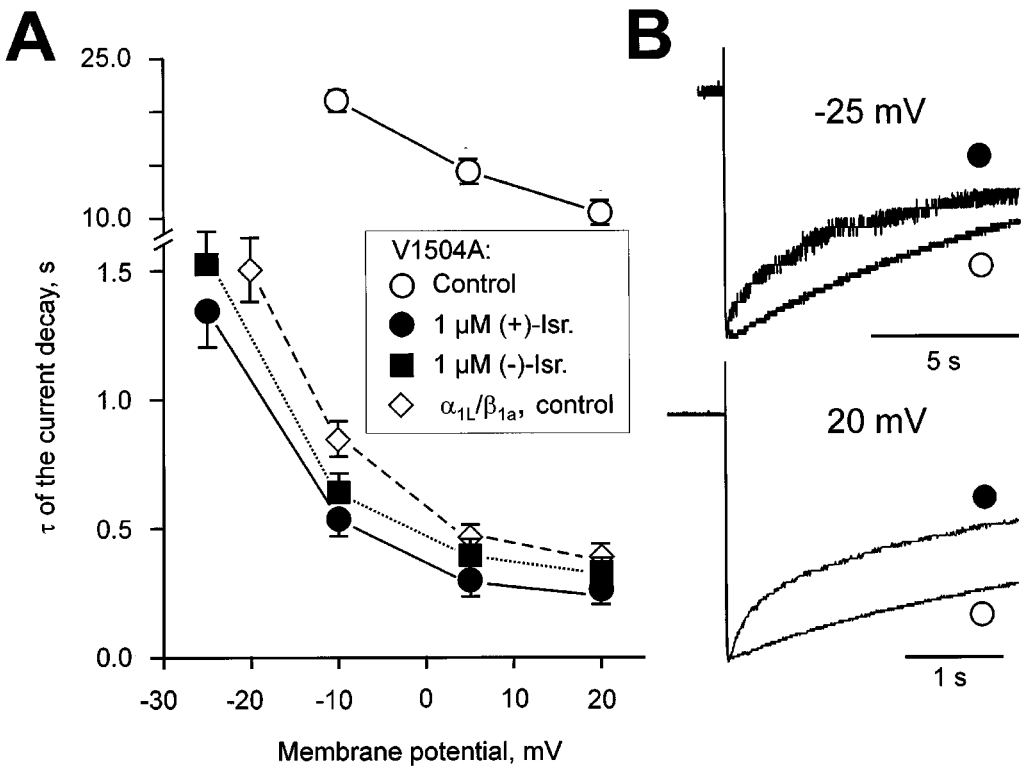

Figure 4 Voltage-dependent acceleration of the I_{Ba} decay by (+)- and (−)-isradipine. (A) The time constants of I_{Ba} decay of V1504A and α_{1L}/α_2-δ/β_{1a} were estimated by fitting single (V1504A, control) or bi-exponential functions to the current decay. Currents were elicited by 30 s steps from a holding potential −80 mV to the indicated voltages. Open circles represent the time constant of V1504A inactivation in control, filled circles and squares represent the (+)- and (−)-isradipine induced current decay time constants in V1504A. Open diamonds show the fast voltage-dependent inactivation time constant of α_{1L}/α_2-δ/β_{1a} in control. (B) Representative I_{Ba} of the mutant V1504A elicited by 10 s depolarizing steps to −25 mV and 3 s steps to 20 mV from −80 mV in control (open circles) or the presence of 1 μM (+)-isradipine (filled circles).

pine-induced channel conformation is less stable than the (+)-isradipine-induced state.

A similar trend was observed for construct V1504A displaying a significantly faster recovery from (−)-isradipine-induced inactivation ($\tau_{rec,(-)\text{-israd.}}$ (1 μM) = 0.39 ± 0.09 s, n = 3) compared to (+)-enantiomer ($\tau_{rec,(+)\text{-israd.}}$ (1 μM) = 1.98 ± 0.48 s, n = 5, Berjukow *et al*., 2000) (Figure 5B). Interestingly, recovery of V1504A from (+)- and (−)-isradipine-induced inactivation occurred at a significantly faster rate than corresponding recovery of α_{1L}/α_2-δ/β_{1a} (Figure 5C). These data suggest that (+)- and (−)-isradipine stabilize the drug-induced channel conformation to different extents. Furthermore, residue V1504 has obviously an additional stabilizing effect on the DHP-induced channel state.

Discussion

Mutational analysis of voltage-gated Ca^{2+} channels enabled first insight into the localization of putative DHP-binding determinants (Striessnig *et al*., 1998 for review). The molecular mechanism of Ca^{2+} channel block by DHPs is, however, still controversial (Hering *et al*., 1998). Recent studies on Ca^{2+} channel mutants with impaired inactivation enable for the first time a profound analysis of the role of the inactivated channel state in block by DHPs. In a preceding study we have demonstrated that the high affinity (+)-enantiomer of isradipine stabilizes an inactivated channel conformation resembling fast voltage-dependent inactivation (Berjukow *et al*., 2000).

However, the molecular mechanisms of antagonist DHP-induced changes in Ca^{2+} channel kinetics are still controversial. Drug-induced changes in Ca^{2+} channel gating are either interpreted as open channel block (e.g. Lacinova & Hofmann, 1998; Handrock *et al*., 1999) or, alternatively, as drug-induced channel inactivation (Berjukow *et al*., 2000).

We made, therefore, an attempt to characterize the role of intrinsic fast voltage-dependent channel inactivation in DHP action by analysing the I_{Ba} inhibition by the two isradipine enantiomers (Figure 1A–C, see also previous results of Berjukow *et al*., 1996; Handrock *et al*., 1999). Particular attention was paid to the molecular mechanism underlying the drug-induced changes in Ca^{2+} channel current decay.

Ca^{2+} channel current inhibition by isradipine correlates with resting-state-dependent inactivation

A key observation of our study was the close correlation between the different potencies of the two enantiomers to inhibit peak I_{Ba} and their different effectiveness to promote inactivation of resting channels (Figures 1 and 2). Hence, at −30 mV where $Ca_v1.2$ enter the inactivated state predominantly from the closed resting state an almost 200 times

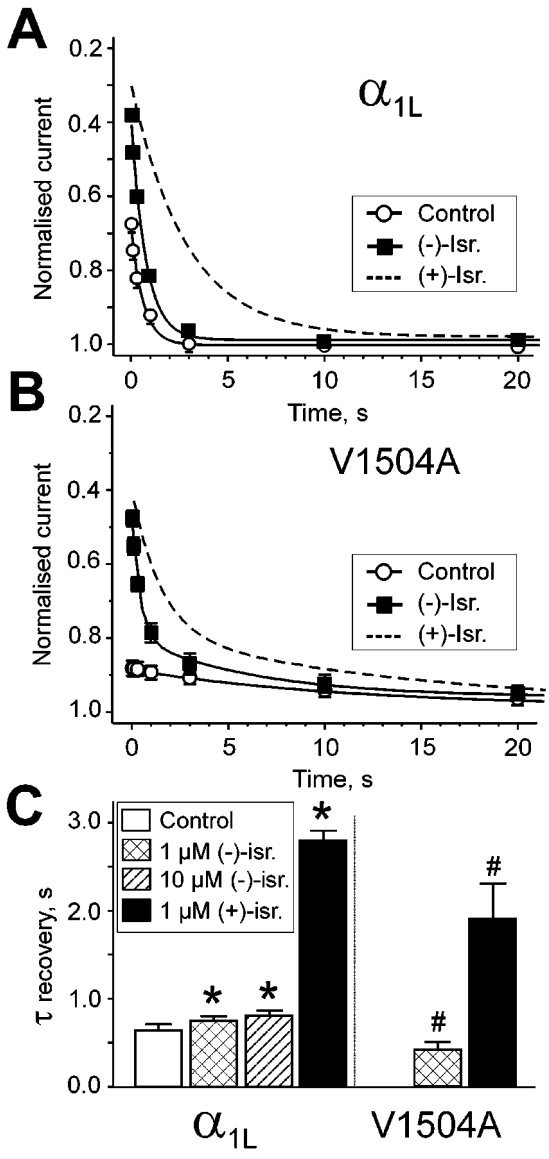

Figure 5 Recovery of $\alpha_{1L}/\alpha_2\text{-}\delta/\beta_{1a}$ and V1504A channels from (+)- and (−)-isradipine-induced inactivation. Time course of I_{Ba} recovery from (−)-isradipine-induced inactivation after a 3 s conditioning prepulses to 20 mV (holding potential −80 mV). Test pulses to 20 mV were applied at various time intervals after the conditioning pulses. Peak I_{Ba} during the test pulses were normalized to peak I_{Ba} measured during the conditioning prepulse. Smooth curves in panels represent mono- or biexponential functions fitted to I_{Ba} recovery of $\alpha_{1L}/\alpha_2\text{-}\delta/\beta_{1a}$ (A) and V1504A (B) channels in control and in 1 μM (−)-isradipine. The parameters of fit were in (A) for control recovery (○): A = 0.33, τ = 0.61 s, C = 1; recovery in 1 μM (−)-isradipine: A = 0.63, τ = 0.74 s, C = 0.97. Broken line illustrates the corresponding time course of I_{Ba} recovery in 1 μM (+)-isradipine (fitted curve form Berjukow *et al*., 2000). (B) Recovery of V1504A in control (○): A_{slow} = 0.11, τ_{slow} = 13.4 s, C = 0.98 and the presence of 1 μM (−)-isradipine: A_{fast} = 0.42, τ_{fast} = 0.39 s, A_{slow} = 0.21, τ_{slow} = 11.1 s, C = 0.98. Dashed line illustrates recovery of I_{Ba} in 1 μM (+)-isradipine (data from Berjukow *et al*., 2000). (C) Time constant of recovery from fast inactivation in control (white column), 1 μM and 10 μM (−)-isradipine (hatched columns). Black columns illustrate the corresponding recovery from (+)-isradipine-induced inactivation (data from Berjukow *et al*., 2000). $\alpha_{1L}/\alpha_2\text{-}\delta/\beta_{1a}$ and V1504A channels recover significantly faster from (−)-isradipine-induced inactivation compared to (+)-isradipine. Note that the inactivation deficient mutant V1504A recovered faster from (+)- and (−)-isradipine-induced inactivation than $\alpha_{1L}/\alpha_2\text{-}\delta/\beta_{1a}$ channels (*P < 0.01 compared to $\alpha_{1L}/\alpha_2\text{-}\delta/\beta_{1a}$ in control. #P < 0.01 compared to recovery of the respective enantiomers in $\alpha_{1L}/\alpha_2\text{-}\delta/\beta_{1a}$).

higher concentration of the (−)-enantiomer was required to inactivate the channels by a similar extent as by (+)-isradipine ($IC_{50,(+)\text{-isr.}}$ = 72 ± 5 nM *vs* $IC_{50,(-)\text{-isr.}}$ = 13 ± 5 μM) (Figure 2B). This corresponds to the stereoselective inhibition of peak current by the two enantiomers ($IC_{50,(+)\text{-isr.}}$ = 327 ± 41 nM *vs* $IC_{50,(-)\text{-isr.}}$ = 34 ± 8 μM). A correlation between resting channel block (at −80 mV, Figure 1A) and isradipine-induced inactivation at −30 mV (Figure 2B) supports the hypothesis that the two block components are closely interrelated (Figure 5). It is tempting to speculate that the two isradipine enantiomers bind to resting channels with different affinities and enhance voltage-dependent inactivation during subsequent membrane depolarizations. Such a scenario is in line with the results of our preceding study demonstrating that (+)-isradipine promotes inactivation in $Ca_v1.2$ without necessarily binding with high affinity to an inactivated channel conformation (Berjukow *et al*., 2000).

Open channel block or drug-induced inactivation?

At more positive voltages where $Ca_v1.2$ inactivate predominantly *via* the open state drug-induced changes in the current decay are more difficult to interpret. Some authors believe that DHP-effects on I_{Ba} decay reflect predominantly the rate of open channel block (Lacinova & Hofmann, 1998; Handrock *et al*., 1999) while others interpret the kinetics of drug-induced acceleration of the Ca^{2+} channel current decay as enhanced inactivation (Berjukow *et al*., 2000). We have, therefore, compared the concentration dependencies of (+)- and (−)-isradipine effects on the I_{Ba} decay and corresponding drug-induced inactivation at −30 mV where channel inactivation occurs predominantly from the resting state.

(+)-Isradipine-induced inactivation at −30 mV (IC_{50} = 72 ± 2 nM, n = 3) and the corresponding acceleration of the current decay at 20 mV (IC_{50} = 79 ± 18 nM, n = 3) displayed similar concentration dependencies (Figures 1D and 2B). At least for the high affinity (+)-enantiomer this finding supports the view that the drug effects at both potentials (−30 and 20 mV) reflect drug-induced transitions to an inactivated state (see Figures 1D, 2A and 3B) and not an open channel block.

This hypothesis is also indirectly supported by experiments of Lacinova & Hofmann (1998), indicating that open channel block by isradipine requires two orders of magnitude higher concentrations of isradipine than drug-induced inactivation.

However, a comparison of the effects of (+)- and (−)-isradipine on the I_{Ba} decay of $\alpha_{1L}/\alpha_2\text{-}\delta/\beta_{1a}$ and V1504A at 20 mV revealed a loss in enantioselectivity. At this potential both enantiomers were almost equally efficient in accelerating the calcium channel current decay (Figures 1D and 3B).

The most interesting finding of our study was, however, the prominent voltage-dependency of (+)- and (−)-isradipine effects on the current decay. This result provides for the first time convincing evidence that the isradipine effects on the I_{Ba} decay reflect voltage-dependent state

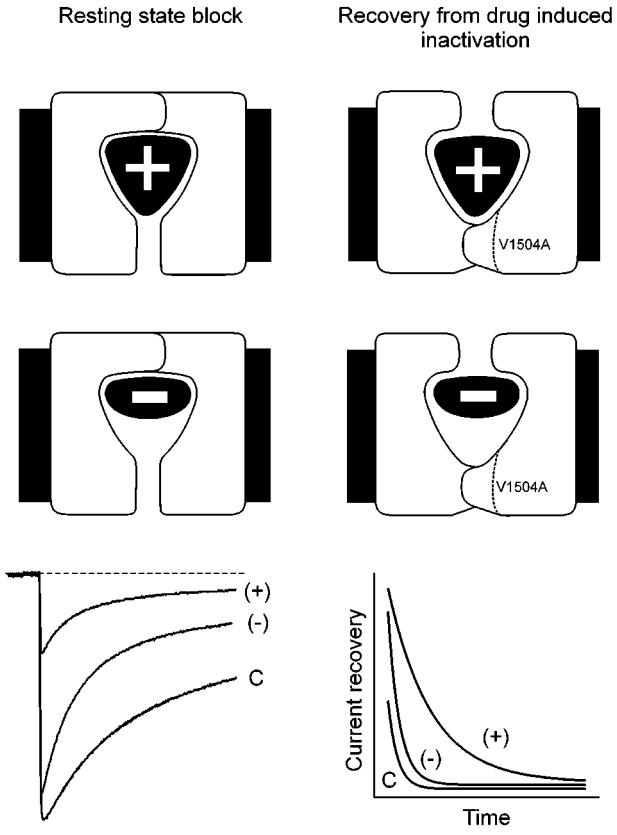

Figure 6 The two isradipine enantiomers differentially affect the stability of the drug-induced channel conformation. The scheme summarizes the principle findings of the present study. The left column illustrates the stronger tonic block of construct α_{1L}/α_2-δ/β_{1a} by (+)- compared to the (−)-enantiomer (illustrated by different fit of the two components in a schematic binding pocket). Superimposed currents in control (c), 1 μM (+)- and 1 μM (−)-isradipine (lower panel) are from Figure 1. The scheme on the right illustrates the lower stability of the (−)-isradipine-induced inactivated state in constructs α_{1L}/α_2-δ/β_{1a} and V1504A (Figure 5). The (−)-enantiomer is suggested to induce an inactivated state that is less stable than the channel conformation induced by (+)-isradipine. The experimental evidence for this hypothesis is illustrated in the lower panel summarizing the slower recovery of α_{1L}/α_2-δ/β_{1a} from (+)- than from (−)-isradipine-induced inactivation (data from Figure 5).

transitions to an inactivated channel conformation and not an open channel block (Figure 4). Making use of mutant V1504A we were able to analyse the kinetics of (+)- and (−)-isradipine-induced changes in I_{Ba} kinetics during 30 s depolarizing test pulses to different voltages. In the absence of drug I_{Ba} of V1504A decayed at a slow rate that could be approximated by fitting a single exponential function ($\tau_{inact.} = 22$ s at -10 mV and $\tau_{inact.} = 13$ s at 20 mV). (+)- and (−)-isradipine (1 μM) induced an additional voltage-dependent component in the I_{Ba} decay with a time constant $\tau_{inact.,(-)\text{-isr.}} = 1.5 \pm 0.2$ s and $\tau_{inact.,(+)\text{-isr.}} = 1.4 \pm 0.1$ s at -25 mV and $\tau_{inact.,(-)\text{-isr.}} = 0.33 \pm 0.03$ s and $\tau_{inact.,(+)\text{-isr.}} = 0.29 \pm 0.04$ s at 20 mV. A comparison with intrinsic fast inactivation of α_{1L}/α_2-δ/β_{1a} revealed an almost identical voltage-dependence (Figure 4).

The (+)-isradipine-induced acceleration of the I_{Ba} decay observed in this and our previous study (Berjukow *et al.*, 2000, Figures 1, 3 and 4B) is in contrast to results of Handrock *et al.* (1999) who observed similar effects only in the presence of high concentrations of the (−)-enantiomer. Handrock *et al.* (1999) considered the possibility that the lack of effect of (+)-isradipine in their study could be due to the strong block of I_{peak} by the (+)-enantiomer, which might have masked the effect on the current decay. The lower DHP-sensitivity of $Ca_v1.2$ expressed in *Xenopus* oocytes (i.e. less pronounced peak current inhibition compared to the more highly sensitive mammalian expression system used by Handrock *et al.*, 1999) apparently provided more optimal conditions to analyse drug effects on current kinetics.

Drug-induced and intrinsic determinants of $Ca_v1.2$ inactivation

Our finding that α_{1L}/α_2-δ/β_{1a} recover faster from (−)- than from (+)-isradipine-induced inactivation and the observation that impaired fast voltage-dependent inactivation of mutant V1504A is associated with an even faster recovery from (+)- and (−)-isradipine-induced inactivation provided important new insights into the molecular mechanism of DHP action. These data suggest that the stability of the inactivated states induced by the two enantiomers is crucially dependent on determinants of intrinsic fast voltage-dependent inactivation (i.e. V1504). The faster I_{Ba} recovery from (−)-isradipine-induced inactivation (compared to (+)-isradipine, Figures 2 and 5) illustrates the different efficiency of the two enantiomers to stabilize the drug-induced channel conformation (Figure 5).

Taken together, our data support the hypothesis that the drug-induced acceleration in the current decay at depolarized voltages (-25 – $+20$ mV) reflect the kinetics of (+)- and (−)-isradipine-induced inactivation rather than open channel block. The differences in repriming kinetics in the presence of the two enantiomers apparently characterize the different stabilities of the drug-induced inactivated channel conformations. Similarities between the (+)- and (−)-isradipine-induced conformational changes in $Ca_v1.2$ were highlighted by the almost identical onset kinetics (Figure 2A).

A distinction between DHP-effects on fast and slow inactivation of Ca^{2+} channels in further studies appears to be essential for a deeper understanding of their interaction with the $Ca_v1.2$ molecule. Our present results support a model where (+)- and (−)-isradipine promote fast voltage-dependent inactivation, although with different efficiency (Figure 6).

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