Abstract—L-type calcium channels (Ca\textsubscript{1.2}) inactivate in response to elevation of intracellular Ca\textsuperscript{2+} (Ca\textsuperscript{2+}-dependent inactivation) and additionally by conformational changes induced by membrane depolarization (fast and slow voltage-dependent inactivation). Molecular determinants of inactivation play an essential role in channel inhibition by phenylalkylamines (PAAs). The relative impacts, however, of Ca\textsuperscript{2+}-dependent and voltage-dependent inactivation in Ca\textsubscript{1.2} sensitivity for PAAs remain unknown. In order to analyze the role of the different inactivation processes, we expressed Ca\textsubscript{1.2} constructs composed of different \(\beta\)-subunits (\(\beta_{1a}^\text{-}, \beta_{2a}^\text{-}, \) or \(\beta_{3}^\text{-}\)-subunit) in Xenopus oocytes and estimated their (-)gallopamil sensitivity by means of the two-microelectrode voltage clamp with either Ba\textsuperscript{2+} or Ca\textsuperscript{2+} as charge carrier. Ca\textsubscript{1.2} consisting of the \(\beta_{3}^\text{-}\)-subunit displayed the slowest inactivation and the lowest apparent sensitivity for the PAA (-)gallopamil. A significantly higher apparent (-)gallopamil-sensitivity with Ca\textsuperscript{2+} as charge carrier was observed for all 3 \(\beta\)-subunit compositions. The kinetics of Ca\textsuperscript{2+}-dependent inactivation and slow voltage-dependent inactivation were not affected by drug. The higher sensitivity of the Ca\textsubscript{1.2} channels for (-)gallopamil with Ca\textsuperscript{2+} as charge carrier results from slower recovery (\(t_{\text{rec,Ca}}=15\) seconds versus \(t_{\text{rec,Ba}}=3\) to 5 seconds) from a PAA-induced channel conformation. We propose a model where (-)gallopamil promotes a fast voltage-dependent component in Ca\textsubscript{1.2} inactivation. The model reproduces the higher drug sensitivity in Ca\textsuperscript{2+} as well as the lower sensitivity of slowly inactivating Ca\textsubscript{1.2} composed of the \(\beta_{3}^\text{-}\)-subunit. (Circ Res. 2001;89:700-708.)

Key Words: Ca\textsubscript{1.2} \(\beta\)-subunit \(\text{Ca}^2\text{+-dependent inactivation} \| \text{voltage-dependent inactivation} \| \text{PAA (-)gallopamil}

L-type calcium (Ca\textsuperscript{2+}) channels (Ca\textsubscript{1.2}, see Ertel et al\textsuperscript{1} for nomenclature) are the prime targets of the three clinically relevant classes of Ca\textsuperscript{2+} antagonists, ie, phenylalkylamines (PAAs), benzothiazepines (ie, diltiazem) and 1,4-dihydropyridines (DHPs).\textsuperscript{2-5} About 20 years ago, several groups demonstrated that Ca\textsubscript{1.2} inhibition by PAAs and diltiazem occurs predominately during membrane depolarizations (“use-dependent” action) when the channels are primarily in the open or inactivated conformation.\textsuperscript{2-4,6} Since then, numerous studies in myocardial and smooth muscle cells have shown that Ca\textsuperscript{2+} channel inhibition by these compounds, as well as by the new compound mibebradil, reflect a balance between channel shut-off during membrane depolarization and subsequent recovery at rest.\textsuperscript{7-11} Over the last three years there has been substantial progress toward understanding the role of inactivation in Ca\textsuperscript{2+} channel inhibition by Ca\textsuperscript{2+} antagonists. Site directed mutagenesis enabled a detailed analysis of the impact of this process in PAA sensitivity. A comparison of the PAA sensitivities of Ca\textsubscript{2.1} mutants and chimeric channel constructs inactivating at different rates revealed a strong correlation between the rates of fast voltage-dependent inactivation and apparent PAA sensitivities.\textsuperscript{11-13} Similar findings were reported for Ca\textsuperscript{2+} channel block by diltiazem.\textsuperscript{11,14} Subsequently, the studies concentrated on the localization of inactivation determinants that are relevant for channel inhibition. Thus, inactivation determinants in the loop between domains I and II and \(\beta\)-subunit interaction have been shown to control PAA sensitivity of a Ca\textsubscript{2.1} mutant\textsuperscript{13} as well as the mibebradil-sensitivity of wild type Ca\textsubscript{2.1}.\textsuperscript{15} The underlying mechanism in voltage-dependent inactivation modulation of Ca\textsubscript{1.2} inhibition by PAAs remains poorly understood. In the present study, we undertake a systematic analysis of the role of fast voltage-dependent (\(\beta\)-subunit–modulated), slow voltage-dependent, and additional Ca\textsuperscript{2+}-dependent inactivation in PAA sensitivity of Ca\textsubscript{1.2}. We show here that the \(\beta\)-subunit-dependent inactivation of Ca\textsubscript{1.2} affects the channel inhibition by (-)gallopamil. Furthermore, the apparent PAA sensitivity of Ca\textsubscript{1.2} channels is dependent on the charge carrying ion. A significantly stronger use-dependent inhibition with Ca\textsuperscript{2+} than with Ba\textsuperscript{2+} as charge carrier for various \(\beta\)-subunit combinations was observed. Slow voltage-dependent inactivation was not affected by drug. By means of a mathematical model, we have simulated (-)gallopamil action on Ca\textsubscript{1.2}. Our data are consistent with

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the hypothesis that the drug promotes fast voltage-dependent inactivation.

Materials and Methods

Expression of the Ca_{1.2} Construct

A Ca_{1.2}-deduced Ca^{2+} channel construct (named herein \( \alpha_{a,b} \)) used in the present study corresponds to \( \alpha_{c,d} \), cDNA with part of the amino terminus replaced by carp \( \alpha_{c,d} \) sequence. The substitution results in a considerable increase of the expression density (see Grabner et al for details). We observed no significant differences in PAA sensivity of Ca^{2+} channels consisting either of \( \alpha_{a,b} \) or \( \alpha_{c,d} \)-subunits (data not shown). The \( \alpha_{a,b} \)-subunit was coexpressed with either \( \beta_{1a} \), \( \beta_{1b} \), \( \beta_{2a} \), or \( \beta_{2b} \) subunits. The constructs were inserted into the polyadenylating transcription plasmid pSPCBI-2.

Electrophysiology

Two-microelectrode voltage clamp of Xenopus oocytes was performed 2 to 7 days after microinjection of approximately equimolar mixtures of cRNAs: \( \alpha_{a,b} \), \( \beta_{a,b}/\beta_{c,d} \), or \( \alpha_{a,b}/\beta_{c,d} \) subunits (0.2 ng/50 nL) and \( \alpha_{c,d} \)-subunits (0.3 ng/50 nL), as previously described. All experiments were performed at room temperature in either 10 mmol/L Ba(OH)\(_2\), 95 mmol/L NaOH, 2 mmol/L CsOH, 5 mmol/L HEPES (called herein 10 mmol/L Ba\(^{2+}\) solution), or 10 mmol/L Ca(OH)\(_2\), 95 mmol/L NaOH, 2 mmol/L CsOH, 5 mmol/L HEPES (10 mmol/L Ca\(^{2+}\) solution). pH was adjusted to 7.4 with methanesulfonic acid.

Activation of endogenous Ca\(^{2+}\)-activated Cl\(^-\) conductance was eliminated by injecting 25 to 50 nL of 0.1 mol/L BAPTA solution into the oocytes 30 to 60 minutes before the experiments. Leakage current correction was performed by using average values of scaled currents elicited by a 10 mV hyperpolarizing voltage step.

The potentials of maximal activation of \( \alpha_{a,b}/\beta_{a,b}/\beta_{c,d} \) channels ranged between 0 and 10 mV in 10 mmol/L Ba\(^{2+}\) solution and between 10 and 20 mV in 10 mmol/L Ca\(^{2+}\) solution.

Drug-sensitivity was estimated as use-dependent peak current inhibition during 10 test pulses (1 second) applied at 0.5 Hz from \(-80\) mV to the peak potential of the corresponding current-voltage relationship. Use-dependent inhibition was measured after a 3 minutes equilibration of the oocytes in the 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.

To accurately estimate Ca\(^{2+}\)-dependent and voltage-dependent components in \( \alpha_{a,b}/\beta_{a,b} \), current decay, a protocol with flexible sampling interval was used (see Figure 4A). During the first 400 ms of current decay, data points were sampled with 1 kHz, allowing a high resolution of the Ca\(^{2+}\)-dependent component. The subsequent part of the 40-second-long pulse was sampled at 20 Hz.

Recovery from fast voltage-dependent inactivation and inhibition by PAA was studied at a holding potential of \(-80\) mV after depolarizing Ca\(^{2+}\) channels during a 3-second prepulse to 10 mV (in 10 Ba\(^{2+}\) solution) or 20 mV (in 10 Ca\(^{2+}\) solution) by applying 30-ms test pulses at various time intervals after the conditioning prepulse. Peak current values were normalized to the peak current measured during the prepulse. The time course of \( I_{\text{Ba}} \) or \( I_{\text{Ca,1.2}} \) recovery was fitted to a mono- or double-exponential function (Figure 5).

Recovery from slow voltage-dependent inactivation was studied by a similar double-pulse protocol, with the 30-ms test pulses applied at 10, 20, 30, 60, 90, 120, and 180 seconds after the 30-second conditioning pulse to +10 mV (in Ba\(^{2+}\)). Peak \( I_{\text{Ba}} \) during recovery was normalized to peak of the last test pulse. The data were fitted with a single-exponential function. All data are given as mean±SEM, if not specified, n=4. Statistical significance was calculated according to Student’s unpaired t test (P<0.05).

Results

\( \beta \)-Subunit Composition of Ca_{1.2} Affects Sensitivity for (-)Gallopamil

Coexpressing the Ca_{1.2} construct (\( \alpha_{a,b} \)) with the \( \beta_{2a} \)-subunit (corresponding channels named herein \( \alpha_{a,b}/\beta_{2a} \)) induced a slow rate of voltage-dependent inactivation of \( I_{\text{Ba}} \), whereas coexpression of the \( \beta_{3a} \) and \( \beta_{3b} \)-isoforms (\( \alpha_{a,b}/\beta_{3a} \) and \( \alpha_{a,b}/\beta_{3b} \) channels) promoted a more rapid time course of inactivation (Figure 1A, see also Cens et al for details). In order to quantify the inactivation kinetics of the different constructs, we used the

**Figure 1.** Inactivation kinetics of Ca_{1.2} composed of the \( \alpha_{a,b} \), \( \alpha_{a,b} \)-, and different \( \beta \)-subunits. Normalized representative currents elicited by 3-second steps from \(-80\) mV to +10 mV in 10 mmol/L Ba\(^{2+}\) solution (A) and to +20 mV in 10 mmol/L Ca\(^{2+}\) solution (C). The different inactivation rates in Ba\(^{2+}\) (B) and Ca\(^{2+}\) (D) are expressed by the coefficient \( r_{9000} \) (fraction of noninactivated current during a 1-second step with respect to peak current).
coefficient $r_{1000}$ (ie, the current remaining after 1000 ms normalized to the peak current, Figure 1B).

$\text{Ca}^{2+}$-dependent inactivation caused an additional rapid component in the current decay in all constructs (Figure 1C). Irrespective of the charge carrier, $\alpha_{1L}/\beta_{2a}$-channels inactivated considerably slower than $\alpha_{1L}/\beta_{1a}$- and $\alpha_{1L}/\beta_{3}$-channels (Figures 1B and 1D).

PAA sensitivity was estimated as use-dependent peak current inhibition during 10 test pulses applied at 0.5 Hz (Figure 2, see Materials and Methods for details). (-)Gallopamil (10 $\mu$mol/L) inhibited $I_{\text{Ba}}$ through $\alpha_{1L}/\beta_{2a}$ less efficiently (34 $\pm$ 2%, n=10) than through $\alpha_{1L}/\beta_{1a}$ (54 $\pm$ 5%, n=6) and $\alpha_{1L}/\beta_{3}$ (62 $\pm$ 2%, n=5) channels (Figure 2B). This finding is in accordance with our previous observations on PAA-sensitive Ca$_{v}$1.2 mutants. 13

With 10 mmol/L Ca$^{2+}$ as charge carrier, a significantly stronger peak current inhibition for all Ca$_{v}$1.2 constructs ($\alpha_{1L}/\beta_{2a}$: 69 $\pm$ 4%; $\alpha_{1L}/\beta_{1a}$: 54 $\pm$ 3%; $\alpha_{1L}/\beta_{3}$: 71 $\pm$ 2%; n=6, Figure 2) was observed. The effect was most prominent for $\alpha_{1L}/\beta_{2a}$ channels exhibiting in 10 mmol/L Ca$^{2+}$ solution a remarkably higher PAA sensitivity than in 10 mmol/L Ba$^{2+}$.

The order of PAA sensitivity was found to be similar for both ionic conditions (ie, $\alpha_{1L}/\beta_{3}$ > $\alpha_{1L}/\beta_{1a}$). $\alpha_{1L}/\beta_{2a}$).

Role of Ca$^{2+}$- and Voltage-Dependent Inactivation in Ca$_{v}$1.2 Inhibition by (-)Gallopamil

It is well-established that Ca$_{v}$1.2 inactivates in a Ca$^{2+}$- and voltage-dependent manner. 19–21 Figure 3A illustrates representative $I_{\text{Ca}}$ through $\alpha_{1L}/\beta_{3}$ during 3-second depolarizing steps to different potentials. The time constants of the Ca$^{2+}$-dependent (B, squares, n=12) and voltage-dependent (C, triangles, n=12) components were estimated by fitting the current decay by a double-exponential function. The U-shaped dependence on voltage is observed only for the fast component ($\tau_{\text{Ca}}$).

The order of PAA sensitivity was found to be similar for both ionic conditions (ie, $\alpha_{1L}/\beta_{3}$ > $\alpha_{1L}/\beta_{1a}$). $\alpha_{1L}/\beta_{2a}$).
The different kinetics of Ca\(^{2+}\)- and voltage-dependent inactivation enabled us to study (-)gallopamil effects on both processes individually. Because of their slow rate of voltage-dependent inactivation, \(\alpha_{B}/\beta_{B}\) channels are particularly suitable for this analysis (see Figure 4A and inset for representative currents at 0 mV where Ca\(^{2+}\)-dependent inactivation occurs at its maximal rate). We observed no acceleration of the Ca\(^{2+}\)-dependent phase of current decay by 10 \(\mu\)mol/L or 50 \(\mu\)mol/L (-)gallopamil in \(\alpha_{B}/\beta_{B}\) (\(\tau_{Ca,0}\mu mol/L-10 mV=35\pm2 \, ms\) versus \(\tau_{Ca,control}-10 mV=32\pm3 \, ms\), \(\tau_{Ca,50\mu mol/L0 mV=45\pm6 \, ms\) versus \(\tau_{Ca,control0 mV=41\pm4 \, ms}, \, P>0.05\), Figure 4C, inset). Similar observations were made for \(\alpha_{B}/\beta_{B}\) and \(\alpha_{B}/\beta_{B}\) channels (data not shown).

(-)Gallopamil induced a concentration-dependent acceleration of the late phase of the \(I_{Ca}\) decay (\(\tau_{slow}\), Figures 4A and 4C) and accelerated the decay of \(I_{Ba}\) (Figures 4B and 4D). Interestingly, drug-induced and intrinsic voltage-dependent inactivation (Figures 4C, 4D, and 4D inset) displayed an almost identical voltage-dependence.

**Permeant Ion Determines Recovery From the Drug-Induced Channel Conformation**

To further elucidate the role of the charge carrying ion, we studied the recovery kinetics of \(I_{Ca}\) and \(I_{Ba}\) from inactivation in control and in the presence of (-)gallopamil (3 \(\mu\)mol/L to 50 \(\mu\)mol/L, Figure 5). In the absence of drug, \(I_{Ba}\) through \(\alpha_{B}B_{B}\) and \(\alpha_{B}/\beta_{B}\) inactivated during a 3-second depolarisation to +10 mV between 15% to 25% (Figures 5A and 5B). Recovery at -80 mV was well-described by a single-exponential function with \(\tau_{Ba,Ba}=1.7\pm0.2\) seconds (\(\alpha_{B}B_{B}\), \(n=5\)) and \(\tau_{Ba,Ba}=1.4\pm0.2\) seconds (\(\alpha_{B}B_{B}\), \(n=6\) Figures 5A and 5B, squares; Figure 5E and 5F, white bars). (-)Gallopamil increased the fraction of nonconducting channels at the end of the conditioning pulse in a concentration-dependent manner (Figure 5A through 5D). Recovery of drug-modified channels in 10 mmol/L Ba\(^{2+}\) was approximately monoeponential with corresponding time constants \(\tau_{Ba,Ba}=-2.8\pm0.3\) seconds, \(n=14\) and \(\tau_{Ba,\beta_{B}}=4.9\pm0.8\) seconds, \(n=10\) (Figures 5A, 5B, 5E, and 5F).

Because of Ca\(^{2+}\)-dependent inactivation, a substantially larger fraction of channels inactivated during a 3-second test pulse to +20 mV in 10 mmol/L Ca\(^{2+}\) solution. The predominant fraction of channels recovered rapidly from Ca\(^{2+}\)-dependent inactivation with respective time constants \(\tau_{Ca,Ba}=114\pm7\) ms and \(\tau_{Ca,Ba}=104\pm13\) ms (\(n=6\) (Figure 5C and 5D). The second component in recovery occurred at a comparable rate as in 10 mmol/L Ba\(^{2+}\) solution and is, therefore, likely to reflect recovery from the voltage-dependent inactivation.

Two findings distinguished the recovery from channel inhibition by (-)gallopamil with Ca\(^{2+}\) as charge carrier. First, (-)gallopamil diminished the fraction of channels recovering from Ca\(^{2+}\)-dependent inactivation in a concentration-dependent manner (Figures 5C and 5D, see insets). Second, recovery of drug-modified channels in 10 mmol/L Ca\(^{2+}\) solution occurred at a significantly slower rate (15\pm3 seconds versus 2.8\pm0.3 seconds [\(\alpha_{B}B_{B}\)], 16\pm3 seconds versus 4.9\pm0.8 seconds [\(\alpha_{B}B_{B}\)], Figures 5E and 5F) than in Ba\(^{2+}\).

These observations suggest the possibility that Ca\(^{2+}\)-dependent inactivation is modulated by (-)gallopamil. Indeed, one could hypothesize that the channels enter the Ca\(^{2+}\)-dependent inactivated state at a similar rate as in control but recover slowly from a drug-modulated Ca\(^{2+}\)-dependent inactivated state. To test this hypothesis, we analyzed the recovery from Ca\(^{2+}\)-dependent inactivation of \(\alpha_{B}B_{B}\) after a short (100-ms) depolarizing conditioning pulse (Figure 6). In the absence of drug, a 100-ms pulse induced almost exclusively Ca\(^{2+}\)-dependent inactivation (see rapid and complete recovery in Figure 6, squares). If (-)gallopamil were to change recovery from Ca\(^{2+}\)-dependent inactivation, it would occur at a comparably slow rate as observed in Figure 5D (illustrated by the dashed line in Figure 6).

This was, however, not the case (Figure 6, circles). Despite the high drug concentration the predominant fraction of the channels recovered at a fast rate that is typical for recovery...
from Ca$^{2+}$-dependent inactivation in the absence of drug ($\tau_{Ca,2}\beta_3 = 95\pm3$ ms, $\tau_{Ca,3,0 \mu M} = 90\pm4$ ms, $n=5$). A similar observation was made in the presence of 100 $\mu$mol/L (-)gallupamil (data not shown). We, therefore, conclude that (-)gallupamil does not affect the recovery kinetics from Ca$^{2+}$-dependent inactivation. The small fraction (4\%1\%) of slowly repriming channels apparently reflects recovery from PAA-induced inactivation accumulated during the 100 ms depolarizing pulse.

**Slow Voltage-Dependent Inactivation and Ca,1.2 Inhibition by (-)Gallupamil**

To analyze a possible role of slow inactivation in Ca,1.2 inhibition by (-)gallupamil, we extended the conditioning pulse to 30 seconds. The double-pulse protocol used for the estimation of recovery from slow inactivation (see Materials and Methods) incorporated a 10-second gap between the conditioning pulse and the first test pulse, enabling almost complete recovery of $\alpha_{1L}/\beta_2a$ and $\alpha_{1L}/\beta_3$ channels from the fast voltage-dependent inactivation and inhibition by (-)gallupamil ($\tau_{Ca,2,0 \mu M} = 2.8\pm0.3$ seconds, $\tau_{Ca,3,0 \mu M} = 4.9\pm0.8$ seconds, in Figures 5A and 5B). The remaining fraction of inactivated channels (between 20\% and 30\%) recovered from slow voltage-dependent inactivation. This recovery was monoexponential and occurred in control and in the presence of 10 $\mu$mol/L (-)gallupamil with identical kinetics ($\tau_{slow,2a,control} = 25\pm3$ seconds, $\tau_{slow,2a,10 \mu M} = 27\pm4$ seconds, $P>0.05$, Figure 7A; $\tau_{slow,3,control} = 17\pm2$ seconds, $\tau_{slow,3,10 \mu M} = 19\pm4$ seconds, $P>0.05$, Figure 7B).

We observed neither (-)gallupamil-induced changes in the kinetics of recovery from slow voltage-dependent inactivation, nor a drug-induced increase in the amount of slow-inactivated channels. Thus, PAA-induced inactivation and slow voltage-dependent inactivation are clearly distinguishable with respect to their recovery kinetics and, therefore, are likely to represent distinct processes.

**Discussion**

**$\beta$-Subunit Composition of Ca,1.2 Affects Voltage-Dependent Inactivation and Apparent PAA Sensitivity**

Our results demonstrate that the $\beta$-subunit composition of Ca,1.2 determines not only voltage-dependent inactivation kinetics but also their sensitivity for the PAA (-)gallupamil (Figures 1 and 2). First evidence for $\beta$-subunit–dependent
PAA sensitivity was reported by Lacinova et al., who observed a higher sensitivity of Ca,1.2 for (−)-gallopamil and verapamil when the α,1.2-subunit was coexpressed together with the β,1-subunit compared with cells transfected solely with α,1.2. We have later demonstrated that the PAA sensitivity of a mutant α,2.1 construct is determined by β-subunit–dependent inactivation properties (α,1L/β,b,2a). The results of the present study extend those previous observations to Ca,1.2 channels. Our data show that Ca,1.2 constructs formed by β,1L– and β,2a-subunits (promoting fast voltage-dependent inactivation) are more efficiently inhibited by (−)-gallopamil than α,1L/β,b,2a channels (Figure 2).

Selective Interaction of (−)-Gallopamil With Fast Voltage-Dependent Inactivation

Our data suggest a selective modulation of the fast voltage-dependent inactivation mechanism by (−)-gallopamil. (1) In line with earlier studies, we found no drug-induced acceleration of the Ca2+-dependent component of Ic decay (Figures 4A and 4C). (2) The kinetics of recovery from Ca2+-dependent inactivation were unaffected even by high drug concentrations (50 to 100 μmol/L Figure 6). (3) (−)-Gallopamil concentration dependently increased a slow component in channel recovery (likely to represent recovery from the fast voltage-dependent inactivation) with Ca2+ and Ba2+ as charge carriers (Figure 5). (4) In accordance with early work of Lee and Tsien and other groups, we observed a pronounced drug-induced acceleration of the voltage-dependent component of current decay with Ba2+ and Ca2+ as charge carriers (Figures 4A through 4D). (5) The impaired fast voltage-dependent inactivation produced by the αL-Lβb,2 interaction correlates with the decrease in the apparent (−)-gallopamil sensitivity (Figure 2). (6) PAA-induced acceleration of the current decay and intrinsic fast inactivation displayed a similar voltage-dependence (Figures 4C and 4D). (7) Finally, neither the amount of slow inactivated channels nor the kinetics of recovery from slow inactivation were affected by (−)-gallopamil (Figure 7).

Together, these data show that fast voltage-dependent inactivation is of key importance for the drug-channel interaction, whereas Ca2+-dependent and slow inactivation are not modified by (−)-gallopamil.

Role of the Permeant Ion in PAA Sensitivity

If Ca2+-dependent inactivation is virtually unaffected by (−)-gallopamil, how can the enhanced use-dependent inhibition of Ca,1.2 constructs by (−)-gallopamil with Ca2+ as charge carrier (Figure 2) be explained? Our data demonstrate that Ic recover from inhibition by (−)-gallopamil at a rate about 3 to 5 times slower than Ia (Figures 5E and 5F), suggesting that a decelerated recovery from PAA inhibition is the mechanism underlying the enhanced apparent PAA sensitivity in Ca2+ versus Ba2+ (Figure 2, see also Lee and Tsien and Nawrath and Wegener).

One possible interpretation of this finding is that an interaction of Ca2+ with a putative drug binding site increases the affinity of the receptor for the drug (as originally postulated by Glossman and Ferry resulting in a higher stability of the drug-channel complex. Alternatively, the
interaction of the channel with a divalent cation could affect the ability of the drug to induce inactivation (efficacy, see Colquhoun \textsuperscript{30} for review). Our experimental data do not allow these possibilities to be distinguished between. Moreover, it is currently not clear if the increase in apparent drug sensitivity with Ca\textsuperscript{2+} is accomplished by the ion interaction with pore determinants (eg, Glu\textsuperscript{118} and Glu\textsuperscript{1419}; see Hockerman et al\textsuperscript{31}) or, alternatively, with a site that is located in the region of the C-terminus.\textsuperscript{22,24,32,33}

**Simulation of PAA-Induced Effects on Channel Inactivation**

The molecular mechanism of Ca\textsubscript{1.2} inhibition by PAAs is conventionally explained by high-affinity drug binding to open or inactivated channels.\textsuperscript{2–4,7,8,26,27,34}

A high-affinity drug binding to the inactivated channel state would result in a substantial shift of the inactivation curve to more negative potentials. However, if the inactivation curve is measured under conditions close to steady-state the observed shift is hardly significant\textsuperscript{35} (see also Aczel et al\textsuperscript{10} and Degtiar et al\textsuperscript{36} for an overview of the steady-state problem).

The open-channel block hypothesis does not account for the recently observed correlation between the rate of intrinsic channel inactivation and the apparent drug sensitivity.\textsuperscript{11,12,28}

Most strikingly, the considerably larger fraction of open channels in Ca\textsubscript{1.2} composed of \(\beta_{2a}\) (compared with \(\beta_{1a}\) and \(\beta_{2c}\) subunits) did not result in a higher PAA sensitivity. Instead, a marked reduction of use-dependent channel inhibition was observed (Figure 2, see also Motoike et al\textsuperscript{11} Hering et al\textsuperscript{12} and Figure 1D in Sokolov et al\textsuperscript{13}). These data suggest an important role of the inactivation machinery in the drug-channel interaction.

An alternative concept to explain the action of \((\cdot)\)-gallolpamil is to assume that the drug promotes intrinsic fast voltage-dependent inactivation. In the model illustrated in Figure 8A, the drug binding/unbinding to the channel is assumed to occur in a state-independent manner. Equilibrating a cell membrane in drug produces a certain steady-state fraction of drug-modified channels. Therefore, at each particular drug concentration two populations of channels (modified and non-modified channels) coexist in a dynamic equilibrium.

The basic assumption of the model is that \((\cdot)\)-gallolpamil stabilizes the fast voltage-dependent inactivation. Consequently, the drug-induced acceleration of the current decay is simulated by increasing the onset of fast inactivation (rate \(\gamma\)) (\(\alpha_{\text{II}}/\beta_{3}\); \(\gamma_{\text{bas,control}}=0.13\) s\textsuperscript{-1} versus \(\gamma_{\text{bas,drug}}=0.85\) s\textsuperscript{-1}) and the slower recovery by a deceleration of the recovery (rate \(\delta\)) in the fraction of drug-modified channels (\(\alpha_{\text{II}}/\beta_{3}\); \(\delta_{\text{bas,control}}=1.25\) s\textsuperscript{-1} versus \(\delta_{\text{bas,drug}}=0.2\) s\textsuperscript{-1}, see online data supplement for full set of fit parameters, available at http://www.circresaha.org).

To account for Ca\textsuperscript{2+}-dependent inactivation, we have extended our previous inactivation model\textsuperscript{37} by introducing a Ca\textsuperscript{2+}-dependent inactivated state. Although \((\cdot)\)-gallolpamil does not affect the microscopic rates of Ca\textsuperscript{2+}-dependent inactivation (rate \(\alpha\)) and recovery (rate \(\beta\)) (see Figure 4A and 4C inset and Figure 6), the model nicely reproduces the higher use-dependent channel block in Ca\textsuperscript{2+} compared with Ba\textsuperscript{2+} (Figure 8B). The \(I_{\text{Ba}}\) recovery kinetics in the presence of 3, 10, and 50 \(\mu\text{mol/L}\) \((\cdot)\)-gallolpamil (corresponding to increasing fractions of drug-modified channels) are simulated in Figure 8C, left panel (data from Figure 5B). As shown in the right panel of Figure 8C, the slower recovery of drug-modified channels in Ca\textsuperscript{2+} (data from Figure 5D) can be reproduced by a further reduction of the microscopic rate \(\delta\) at hyperpolarized voltages (\(\alpha_{\text{II}}/\beta_{3}\); \(\delta_{\text{bas,drug}}=0.2\) s\textsuperscript{-1} versus \(\delta_{\text{Cas,drug}}=0.06\) s\textsuperscript{-1}).

One of the intriguing results of our simulation is that the difference in apparent PAA sensitivity of \(\alpha_{\text{II}}\beta_{3}\) and \(\alpha_{\text{II}}\beta_{3}\) channels does not require to assume that the \(\beta\)-subunit interaction affects the affinity of the channel for \((\cdot)\)-gallolpamil (ie, drug binding step). Instead, the lower apparent sensitivity of \(\alpha_{\text{II}}\beta_{3}\) channels may be equally well-explained by an interplay of the kinetic rates \(\gamma\) and \(\delta\). This finding is
illustrated in Figure 8B, where the β2-subunit effect on channel gating was simulated as a destabilization of the fast inactivated state (see also previous observations of Berjukow et al36 in Ca1,2). Decreasing γ and increasing δ reproduced not only the slower inactivation kinetics of α1L/β2 in control but simultaneously resulted in less use-dependent channel inhibition in the presence of drug (see simulated α1L/β2 traces in Figure 8B).

Taken together, we present experimental evidence that different β-subunit compositions of Ca1,2 affect not only the channel inactivation kinetics but also the PAA sensitivity. This finding may be of high pharmacological relevance in different tissues. 39 It will be interesting to analyze if differences in drug-sensitivity of Ca1,2 (eg, in heart and smooth muscle preparations) are related to a different β-subunit composition. The precise β-subunit composition of Ca1,2 under physiological conditions is, however, mostly unknown. Immunoprecipitation studies suggest that only about 50% of α1L in porcine heart but almost all in the uterus are associated with the β2-isofrom. 39 Recent functional studies of Wei et al40 demonstrated, however, that other isoforms than β2 are functionally dominant in native rat heart. In smooth muscle (trachea, aorta) immunoprecipitation studies suggest an interaction with unknown β-subunits. Thus, more detailed knowledge about the β-subunit composition of Ca1,2 is required before the data presented here can be correlated with a pharmacological data obtained from native tissues.

Irrespective of their β-subunit composition we observed a higher PAA sensitivity with Ca2+ than with Ba2+ as charge carrier reflecting a Ca2+-mediated stabilization of a PAA-induced conformation. We show here that only the voltage-dependent phase of ICa decay (as well as the voltage-dependent decay of Ba2+ currents) is accelerated by (-)gallopamil. This finding explains contradictions in a number of previous studies where some authors working in Ba2+ solution reported a pronounced PAA-induced acceleration of current kinetics,4.7,11,12,28 whereas the others working with short pulses in Ca2+ observed no acceleration. 26,27 Our simulation study demonstrated that the molecular mechanism of (-)gallopamil action is well explained by a drug-induced stabilization of the fast voltage-dependent inactivated state (Figure 8).

Acknowledgments
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References


On the Role of Ca$^{2+}$- and Voltage-Dependent Inactivation in Ca$_v$1.2 Sensitivity for the Phenylalkylamine (-)Gallopamil
Stanislav Sokolov, Eugen Timin and Steffen Hering

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Table 1. Simulation fit parameters for the kinetic model of PAA action.

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<th>Hyperpolarisation</th>
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<th>β</th>
<th>γ</th>
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<th>ε</th>
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<td></td>
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<td>-</td>
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<td>0.001</td>
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</table>

* Fraction of channels modified by designated drug concentration

# Recovery rates at hyperpolarisation