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Mechanism of voltage- and use-dependent block of class A Ca^{2+} channels by mibefradil

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1 The action of mibefradil was studied on wild type class A calcium (Ca^{2+}) channels and various class A/L-type channel chimaeras expressed in *Xenopus* oocytes. The mechanism of Ca^{2+} channel block by mibefradil was evaluated with two microelectrode voltage clamp.

2 Resting-state dependent block (or initial block) of barium currents (I_{Ba}) through class A Ca^{2+} channels was concentration dependent with an IC_{50} value of 208 ± 23 μM.

3 Mibefradil (50 μM) did not significantly affect the midpoint voltage of the steady-state inactivation curve suggesting that inactivation does not promote Ca^{2+} channel block. Chimaeric class A/L-type Ca^{2+} channels inactivating with faster or slower kinetics than wild type class A channels were equally well inhibited by mibefradil as wild type class A channels.

4 Frequent Ca^{2+} channel activation facilitated I_{Ba} inhibition by mibefradil (use-dependent block). Recovery from use-dependent block was voltage-dependent, being slower at depolarized membrane potentials ($\tau = 75 \pm 15$ s at -70 mV, ($n = 6$) *vs* $\tau = 20 \pm 2$ s at -100 mV, ($n = 6$), $P < 0.05$).

5 We suggest that use-dependent block of class A Ca^{2+} channels by mibefradil occurs because of slow recovery from open channel block (SROB) and not because of drug binding to inactivated channels.

6 Voltage-dependent slow recovery from open state-dependent block provides a molecular basis for understanding the cardiovascular profile of mibefradil such as selectivity for vasculature and relative lack of negative inotropic effects.

Keywords: Ca^{2+} channels; mibefradil; use-dependent block; open state block; calcium channel inactivation

Introduction

The pore forming α_1 subunit of L-type calcium (Ca^{2+}) channels (classes C(α_{1C}), D(α_{1D}) and S(α_{1S})) is the molecular target of Ca^{2+} channel blockers such as phenylalkylamines (PAAs)[1], 1,4 dihydropyridines (DHPs) and benzothiazepines (BTZ) (for review see Striessnig *et al*., 1998). Mibefradil represents a novel class of Ca^{2+} antagonists with antihypertensive and antianginal properties (Triggle, 1996). Radioligand binding studies suggest the existence of a distinct receptor site for mibefradil on L-type Ca^{2+} channels (Osterrieder & Holck, 1989; Rutledge & Triggle, 1995).

The drug relaxes coronary arteries and has only weak negative inotropic effects (Clozel *et al*., 1989; Osterrieder & Holck, 1989). The first clinical trials suggested that mibefradil might be effective in the treatment of hypertension and angina (Braun *et al*., 1996, Bernik *et al*., 1996).

Functional studies have revealed that T-type Ca^{2+} channels in vascular smooth muscle cells (Mishra & Hermsmeyer, 1994) and human medullary thyroid carcinoma cells (Mehrke *et al*., 1994) are more effectively blocked by mibefradil than L-type currents. It was therefore proposed that the selective action of mibefradil on vascular tissue is related to inhibition of T-type Ca^{2+} channels (Mishra & Hermsmeyer, 1994). According to Mehrke *et al*. (1994) T-type Ca^{2+} channels are predominantly blocked in the resting (closed) state, whereas Randall & Tsien, (1997) observed a higher degree of block at more depolarized voltages.

Mibefradil has characteristics of a non-selective Ca^{2+} channel blocker, inhibiting α_{1A} (P/Q-type), α_{1C} (L-type), α_{1B} (N-type) and α_{1E} (R-type) channels in micromolar concentrations (Bezprozvanny & Tsien, 1995). T-type channels in a neuronally derived cell line (NG 108-15 cells) are somewhat less effectively blocked by mibefradil than α_{1E} channels in cerebellar granule cells (Randall & Tsien, 1997).

The molecular mechanism of mibefradil interaction with closed, open and inactivated channels is still controversial. A voltage dependent block of L-type channels by mibefradil was first observed by Fang & Osterrieder (1991) and later confirmed for the cardiac $\alpha_{1c\text{-}a}$ (Lacinova *et al*., 1995) and smooth muscle $\alpha_{1C\text{-}b}$ subunits expressed in CHO cells (Mehrke *et al*., 1994; Welling *et al*., 1995).

In terms of the modulated receptor hypothesis (Hille, 1997) the higher efficiency of mibefradil to block α_{1C}, α_{1A}, α_{1B} and α_{1E} Ca^{2+} channels at depolarized voltages was interpreted as predominant drug binding to inactivated Ca^{2+} channels (Mehrke *et al*., 1994; Lacinova *et al*., 1995; Welling *et al*., 1995; Bezprozvanny & Tsien, 1995) providing a further explanation for mibefradil's selectivity for tissues with less negative resting potentials (vascular smooth muscle cells cf. ventricular myocytes).

Surprisingly, in vascular smooth muscle cells the drug does not affect the steady-state inactivation curve of L-type Ca^{2+} channels. The channels are predominantly blocked in the resting closed conformation (Mishra & Hermsmeyer, 1994). The latter finding does not agree with a high affinity block of inactivated channels. Furthermore, open channel block by mibefradil was indicated by drug induced acceleration of the current decay (Bezprozvanny & Tsien, 1995).

To clarify the role of different channel conformations for use-dependent block by mibefradil we systematically investigated the inhibition of class A Ca^{2+} channels under conditions favouring selective drug interaction with either open, resting or inactivated channels. The role of channel inactivation for channel block was analysed in class A/L-type Ca^{2+} channel chimaeras inactivating with faster or slower kinetics than wild

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type class A channels. Here we report that enhanced class A Ca^{2+} channel inhibition by mibefradil upon membrane depolarization is due to slow recovery from open state-dependent block and not to enhanced drug binding to channels in the inactivated state.

Methods

Electrophysiology

Inward barium currents were studied with two microelectrode voltage-clamp of *Xenopus* oocytes after microinjection of cRNAs (2–7 days) in approximately equimolar mixtures of α_1 (0.3 ng/50 nl)/β_{1a} (Ruth *et al*., 1989) and α_2-δ (Ellis *et al*., 1988) (0.2 ng/50 nl) as previously described (Grabner *et al*., 1996; Hering *et al*., 1996). All experiments were carried out at room temperature in a bath solution with the following composition: 40 mM $Ba(OH)_2$, 40 mM N-methyl-D-glucamine, 10 mM HEPES, 10 mM glucose (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 0.3–2 MΩ. Activation of endogenous Ca^{2+}-activated Cl^--conductance by barium influx through Ca^{2-} channels was eliminated by injecting the oocytes 20–40 min before the voltage-clamp experiments with 50–100 nl of a 0.1 M BAPTA solution. Oocytes with current amplitudes larger than 2.5 μA were excluded from the analysis.

The recording chamber (150 μl total volume) was continuously perfused at a flow rate of 1 ml/min with control or drug-containing solutions. Data were digitized at 2 kHz, filtered at 0.5 kHz and stored on a computer hard disk. Leakage current correction was performed by using average values of scaled leakage currents elicited by a 10 mV hyperpolarizing voltage step. The pClamp software package (version 6.0 Axon Instruments, Inc.) was used for data acquisition and analysis.

Initial current inhibition ('resting-state dependent block') was measured as peak I_{Ba} inhibition during the first pulse after a 3 min equilibration in drug containing solution at a given holding potential. Initial current inhibition was maximal after 3 min (data not shown). Use-dependent current inhibition was subsequently estimated during trains of 30 or 100 ms test pulses applied at frequencies from 0.1–1 Hz.

Recovery of I_{Ba} from inactivation was studied by depolarizing Ca^{2+} channels during a 120 s prepulse to 20 mV and subsequent application of a second test pulse from a given holding potential to a test potential of 20 mV at various time intervals after the conditioning prepulse. Peak I_{Ba} values were normalized to the peak current measured during the prepulse. I_{Ba} recovered between 90 and 100% during a subsequent 3 min rest at −120 mV. The time course of I_{Ba} recovery from inactivation was fitted to a biexponential function $I_{Ba,\ recovery} = A*\exp(-t/\tau_{rec,fast}) + B*\exp(-t/\tau_{rec,slow}) + C$.

The inactivation curves were estimated by measuring peak I_{Ba} during a 200 ms test pulse to 20 mV following a 30 s conditioning prepulse after an interpulse interval of 3 ms. Unless otherwise stated the membrane potential was held at −120 mV and conditioning and test pulses were applied only once every 3 min to enable maximal unblock of channels. The voltage of half-maximal inactivation ($V_{0.5}$) and the slope factor (k) describing the steepness of the Ca^{2+} channel inactivation curve were obtained by fitting the data to the Boltzmann function: $I/I_{max} = 1 + \exp(V - V_{0.5}/k)$, where V is the membrane potential and I/I_{max} the fraction of available I_{Ba} for a given prepulse potential.

The dose-response data of initial I_{Ba} inhibition were fitted using the Hill equation: $I_{Ba,drug}/I_{Ba,control}$ (in %) = [1/(1 + (C/IC_{50})^{n_H})]*100, where IC_{50} is the concentration at which I_{Ba} inhibition is half maximal, C the applied drug concentration and n_H the Hill coefficient. All pooled data are reported as mean ± standard error of the mean. Statistical significance was assessed by two-sided, paired *t*-test, with $P < 0.05$ taken as the minimal level of significance.

Molecular Biology

The construction of the L-type chimaera Lh (repeats I-IV from $\alpha_{1C\text{-}a}$, Mikami *et al*., 1989), N-terminus replaced by the corresponding sequences from carp skeletal muscle α_{1S} (Grabner *et al*., 1991) was described by Grabner *et al*. (1996). The rapidly inactivating class A/L-type chimaera AL23 (IVS6 in α_{1A} replaced by L-type sequence from carp skeletal muscle α_{1S}, Döring *et al*., 1996) and AL25 (three residues in IVS6 of α_{1A} replaced by corresponding L-type amino acids, Hering *et al*., 1996) and the slowly inactivating chimaeras AL20 (segments IIIS5, IIIS6 and connecting IIIS4-IIIS5 and IIIS5-IIIS6 linkers in α_{1A} replaced by α_{1S}, Hering *et al*., 1996) and the mutant chimaera IF19,20AA (Hering *et al*., 1997) were also previously described.

Results

Resting state- and use-dependent block of class A Ca^{2+} channels by mibefradil

To evaluate the state-dependent interaction of mibefradil with class A Ca^{2+} channels we expressed the α_{1A} subunit (Mori *et al*., 1991) together with the β_{1a} (Ruth *et al*., 1989) and α_2-δ (Ellis *et al*., 1988) subunits in *Xenopus* oocytes. Class A Ca^{2+} channels are particular appropriate for long lasting experiments as this channel type displays no or only minimal run-down. Figure 1A displays I_{Ba} through wild type class A channels evoked by pulses from a holding potential of −80 mV to a test potential of 20 mV in control and in the presence of 100 μM mibefradil respectively. We observed an initial block (defined as peak I_{Ba} inhibition during the first pulse after a 3 min equilibration in drug containing solution, also called resting-state-dependent block) and an additional use-dependent block (measured during 0.1 Hz trains of 100 ms test pulses). Both, initial and use-dependent block increased with increasing drug concentration (Figure 1B). The concentration-response curve of the initial block of class A Ca^{2+} channels yielded an IC_{50} value of 208 ± 23 μM ($n \geqslant 3$, Figure 2).

Use-dependent block of open Ca^{2+} channels by mibefradil

Upon membrane depolarization, Ca^{2+} channels pass through the open and subsequently the inactivated channel conformation. Use-dependent I_{Ba} inhibition during a train of depolarizing test pulses (see Figure 1A) could, therefore, reflect drug interaction with either, or both, channel conformations.

To investigate a possible drug interaction with inactivated class A Ca^{2+} channels we analysed the steady-state inactivation curve of wild type class A Ca^{2+} channels in controls and the presence of mibefradil (50 μM), respectively. This drug concentration caused significant initial block and more than 60% use-dependent I_{Ba} inhibition (see Figures 2 and 4A,B).

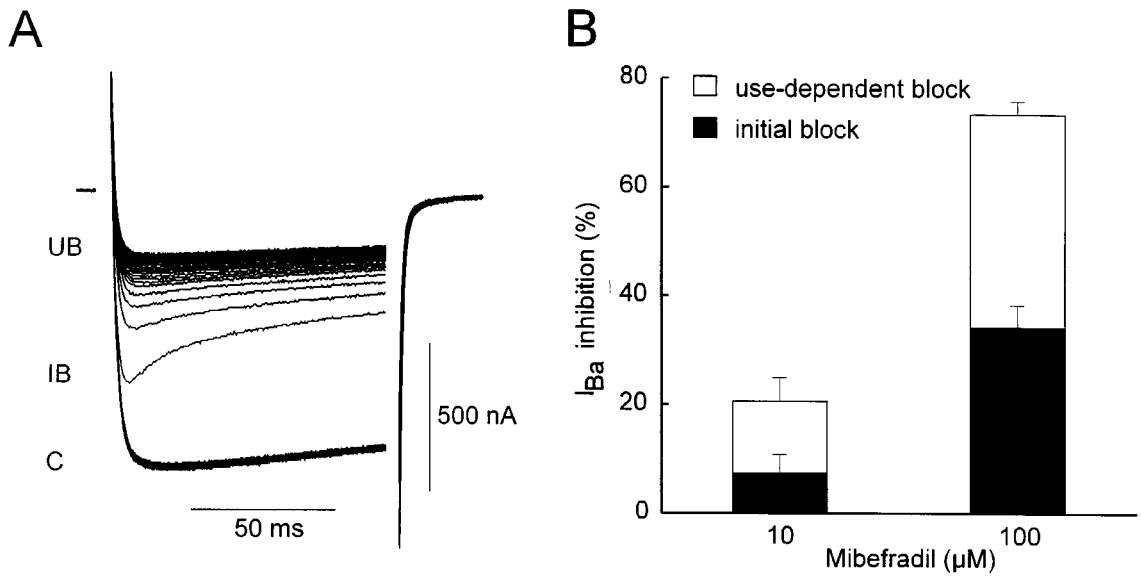

Figure 1 Resting state- and use-dependent block of class A Ca^{2+} channels by mibefradil in *Xenopus* oocytes. (A) Peak I_{Ba} inhibition during the first test pulse after a 3 min incubation in drug (100 μM mibefradil) compared to control (C) was defined as initial block (IB). Use-dependent block (UB) was subsequently measured as cumulative current inhibition (in %) during 15 depolarizing pulses (100 ms, 0.1 Hz) from -80 mV to 20 mV (see Methods). No I_{Ba} inhibition during a similar train was observed in the absence of drug (C). (B) Bar graphs show mean values of the initial block (black columns) and use-dependent I_{Ba} inhibition of wild type class A channels (white columns) by 10 and 100 μM mibefradil ($n \geqslant 4$).

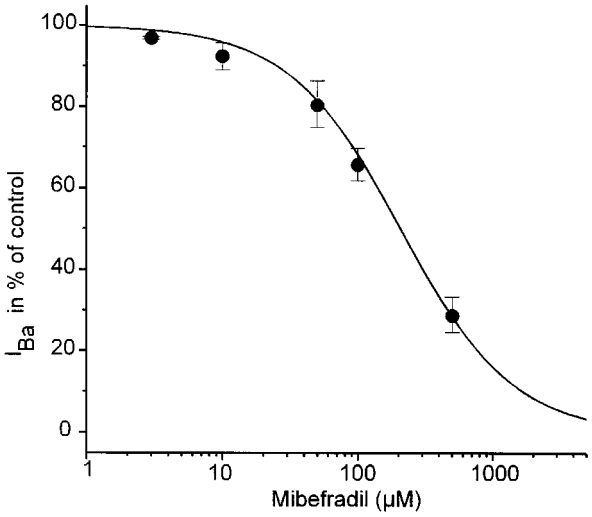

Figure 2 Concentration-response curve for initial I_{Ba} block through class A Ca^{2+} channels by mibefradil. Mean values for initial I_{Ba} inhibition by mibefradil (similar conditions as described in Figure 1) were obtained from three to six experiments on different cells. The solid line represents the best fit to a dose-response equation (see Methods). The fit yielded an $IC_{50} = 208 \pm 23$ μM and a Hill coefficient of 1.04 ± 0.1.

Maintained Ca^{2+} channel inactivation did not enhance channel block. This is clearly demonstrated in Figure 3A where long-lasting (30 second) depolarizations of the membrane did not induce a significant shift of the midpoint voltage of the availability curve of class A Ca^{2+} channels ($V_{0.5,control} = -20.1 \pm 2.5$ mV versus $V_{0.5,Mibefradil} = -24.5 \pm 2.9$ mV, $n = 6$ with $P > 0.05$). However, under non-steady-state conditions (double pulses applied at 0.05 Hz from a holding potential of -60 mV), mibefradil causes not only apparent shifts of the midpoint voltage but also drastic deformations in the shape of the 'non steady-state inactivation curve' to more negative potentials (Figure 3B).

Use-dependent block of open Ca^{2+} channels is enhanced at depolarized membrane voltages

Previous studies have shown that Ca^{2+} channel block by mibefradil is attenuated if the membrane potential is shifted during a train of test pulses to more depolarizing voltages (Lacinova *et al*., 1995; Welling *et al*., 1995; Bezprozvanny & Tsien, 1995). Here we systematically analysed the initial and use-dependent block during short (30 ms) pulse trains applied at 1 Hz from various holding potentials (-80 mV, -100 mV, -120 mV). As shown in Figure 4A, little macroscopic current inactivation occurred during a 30 ms test pulse and no significant I_{Ba} inactivation developed during the 1 Hz pulse train in the absence of drug. In line with the results shown in Figure 1, mibefradil (50 μM) induced significant resting state-dependent I_{Ba} inhibition. Use-dependent block was much more dependent on the holding potential ($10.4 \pm 1.2\%$ at -120 mV *vs* $42.1 \pm 3.7\%$ at -60 mV, $n = 3$) than the initial block ($12.8 \pm 1.9\%$ at -120 mV *vs* $26.6 \pm 8.4\%$ at -60 mV, $n = 3$).

Voltage-dependent recovery from block at rest

The data shown in Figure 3A clearly demonstrate that channel inactivation does not enhance Ca^{2+} channel block by mibefradil. This result does, however, not exclude a role of inactivation in use-dependent Ca^{2+} channel block. We have recently shown that PAA access their receptor site in the pore of L-type and mutant class A Ca^{2+} channels *via* the open state without significantly shifting the inactivation curve of mutant class A and L-type Ca^{2+} channels (Degtiar *et al*., 1997). However, PAA-dissociation is modulated by structural determinants of an inactivation mechanism at the inner mouth of the pore (Hering *et al*., 1997). If such a mechanism would be valid for mibefradil, recovery from inactivation is expected to be rate limiting for recovery from use-dependent block.

We have, therefore, compared the kinetics of I_{Ba} recovery from open-state-dependent block and from inactivation. Use-dependent block was induced by a series of 100 ms test pulses applied at 0.2 Hz from −80 mV to 20 mV. In the absence of drug the pulse train did not cause accumulation of channels in inactivation which enabled a clear separation of both recovery processes (Figure 5). Recovery from use-dependent block was monoexponential ($\tau_{rec\text{-}use}$) and voltage dependent (Figure 5 and 6B). I_{Ba} recovery from inactivation was biexponential with a fast recovery time constant τ_{fast} (ranging between 2 and 5 s) and a second slow recovery process (τ_{slow}) that was significantly accelerated at more negative membrane holding potentials (Figure 6B). In the range of the membrane resting potential (−70 mV) Ca^{2+} channels recovered about 2 fold faster from slow inactivation than from use-dependent block by mibefradil (50 μM) (Figure 6A,B). The fast time constant was not significantly affected by mibefradil ($\tau_{fast,control} = 4.4 \pm 0.4$ *vs* $\tau_{fast,drug}$ 4.9 ± 0.4, $P > 0.05$).

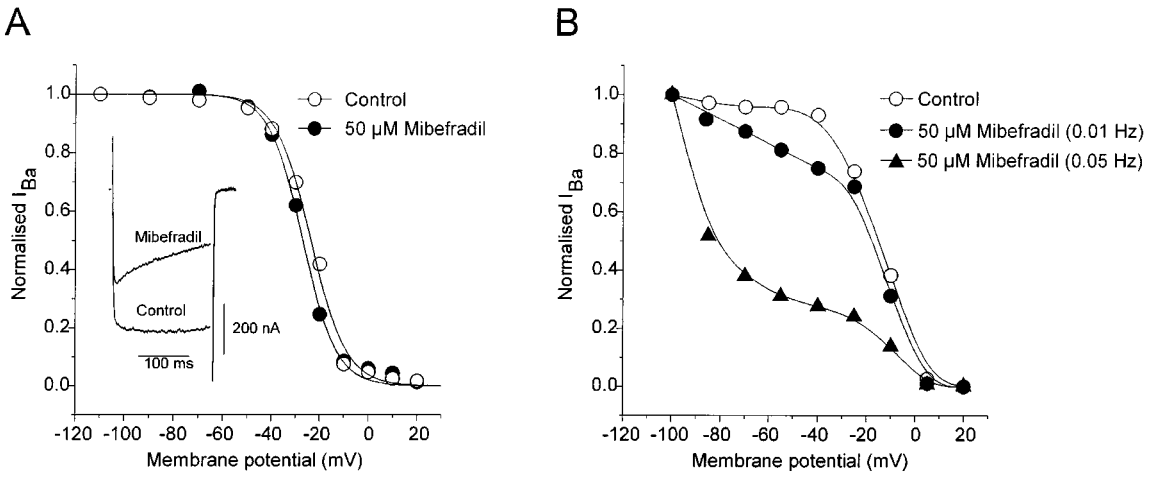

Figure 3 Mibefradil action on voltage dependence of class A Ca^{2+} channel steady-state inactivation. (A) Inactivation curves of class A Ca^{2+} channels were estimated from normalised peak I_{Ba} in control and the presence of 50 μM mibefradil. Conditioning prepulses (30 s) to the indicated membrane potentials and subsequent test pulses to 20 mV were applied every 120 s from a holding potential of −120 mV (see Methods). Lines represent best fit to a Boltzmann function (see Methods) yielding in control $V_{0.5} = -23.6$ mV and $k = 7.4$ mV and in drug $V_{0.5} = -26.9$ mV and $k = 7.0$ mV. I_{Ba} in control and in the presence of 50 μM mibefradil after conditioning pulses to −30 mV are illustrated in the inset. (B) Inactivation curve of class A Ca^{2+} channels measured under non-steady-state conditions. The membrane holding potential was adjusted to −60 mV. A 5 s conditioning prepulse and a 200 ms-test pulse were applied every 90 s (0.01 Hz) or every 20 s (0.05 Hz). The solid lines are drawn by a spline function.

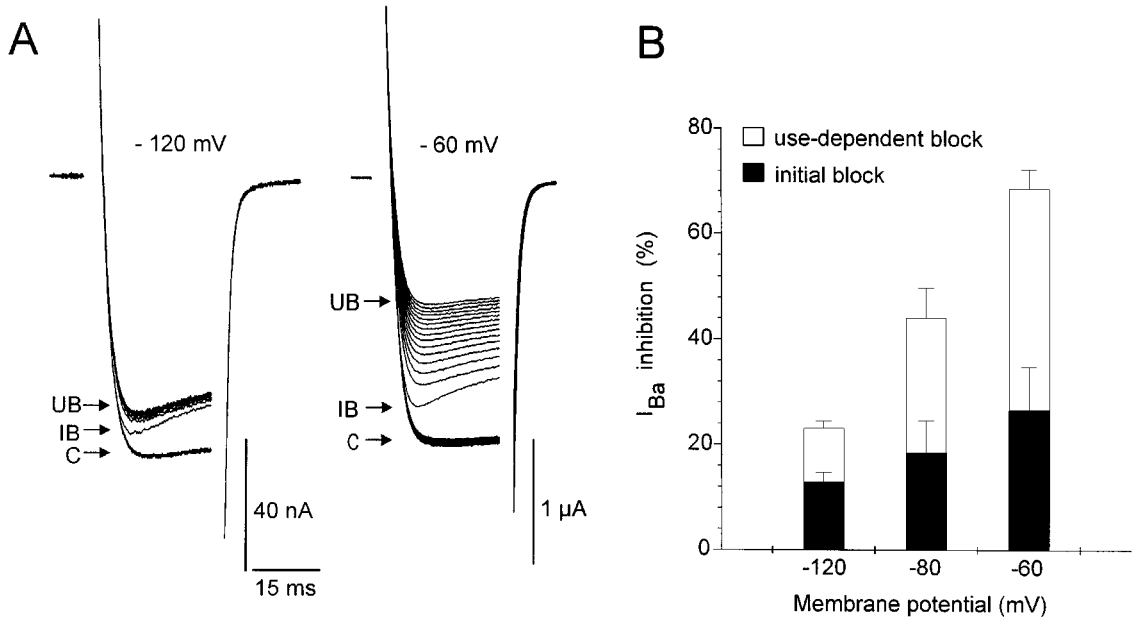

Figure 4 Voltage dependence of initial- and use-dependent inhibition of class A Ca^{2+} channels by mibefradil (50 μM). (A) Estimation of initial (IB) and use-dependent I_{Ba} block (UB) at −120 and −60 mV during trains of 15 pulses (30 ms) applied to 20 mV. (B) Mean values of initial and use-dependent block at −120, −80 and −60 mV ($n = 3$).

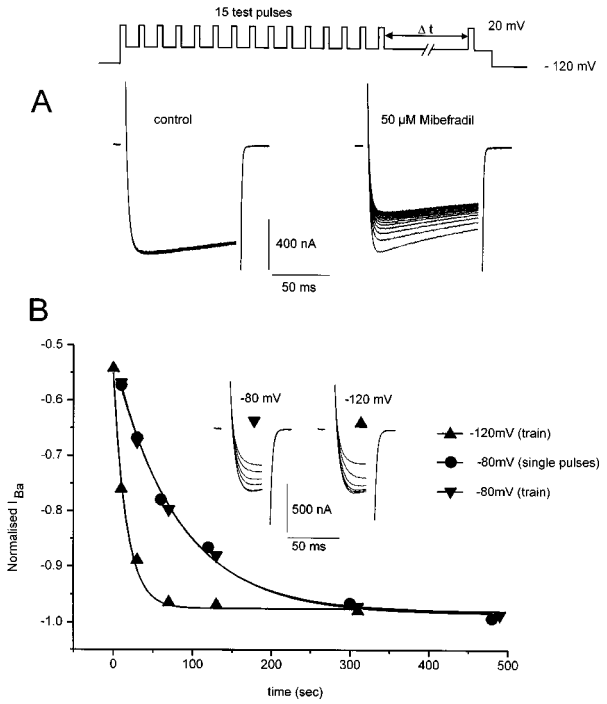

Figure 5 Voltage dependent I_{Ba} recovery from use-dependent block by miberadil. (A) I_{Ba} were elicited by 100 ms test pulse trains (0.1 Hz) from −80 mV to 20 mV in the absence (control) and presence of mibefradil (50 μM). The recovery protocol is schematically illustrated in the upper panel. (B) Time course of peak I_{Ba} recovery from use-dependent block by mibefradil at −120 mV and −80 mV. I_{Ba} were normalized to peak I_{Ba} of the first pulse in the train. The time constant of channel unblock was estimated from peak current values of a series of short (50 ms) test pulses applied at the indicated time intervals (train recovery protocol). Alternatively, single pulses were applied at a given interval (Δt) after six individual conditioning pulse trains (single pulse recovery protocol, see illustration in A). The inset illustrates I_{Ba} recovery from block that was monitored during 50 ms pulses at holding potentials of −80 and −120 mV, respectively. To induce maximal unblock the membrane voltage was held for 3 min at −120 mV between individual trains. Curves represent monoexponential fits for the experimental points by the function: $I_{Ba,recovery} = 1\text{-}exp(-t/\tau_{recovery})$ yielding recovery time constants of 16.5 (train recovery protocol at −120 mV), 79.2 (single pulse recovery protocol at −80 mV) and 81.4 s (train recovery protocol at −80 mV). Both methods yielded similar recovery time constants. Mean time constants for recovery from use-dependent block at different voltages are shown in Figure 6B.

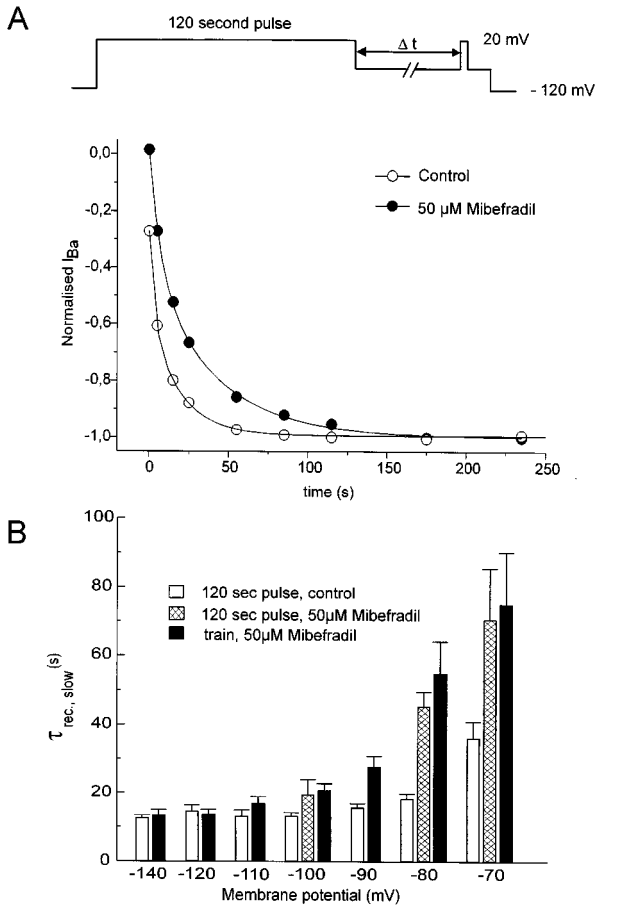

Figure 6 I_{Ba} recovery from slow inactivation and from use-dependent block by mibefradil. (A) Time course of peak I_{Ba} recovery from slow inactivation at −80 mV after a 120 s depolarizing test pulse to 20 mV in control and the presence of 50 μM mibefradil in oocytes expressing class A Ca^{2+} channels. Peak current values were normalized to peak I_{Ba} of the prepulse. Time courses of I_{Ba} recovery were fitted to a biexponential function yielding in control $\tau_{fast} = 3$ s and $\tau_{slow} = 18.5$ s and in mibefradil (50 μM) $\tau_{fast} = 4.2$ s and $\tau_{slow} = 40$ s. (B) Mean time constants of voltage dependence of I_{Ba} recovery from slow inactivation ($n > 5$) after a 120 s conditioning pulse to 20 mV in control and in mibefradil (50 μM) compared to the time constant of recovery from use-dependent inhibition during a train of 15 test pulses (from −80 mV to 20 mV at 0.1 Hz, experiments were performed as described in Figure 5).

Fast inactivation and Ca^{2+} channel block by mibefradil

Bezprozvanny & Tsien (1995) demonstrated that both, class A and L-type Ca^{2+} channels, carry the receptor site for mibefradil. Consequently, chimeric constructs between class A and L-type channels inactivating with different kinetics are interesting models to study the role of inactivation in channel block. We have analysed the initial and use-dependent block in chimaeras AL23 and AL25 (inactivating with faster kinetics than wild type class A channels, Döring *et al*., 1996; Hering *et al*., 1996) and chimaeras AL20 and IF19,20AA (inactivating slower than wild type class A channels, Hering *et al*., 1996; Hering *et al*., 1997). Despite of their different inactivation kinetics all channel constructs were similarly blocked by mibefradil (100 μM) (Figure 7). The rapidly inactivating PAA and BTZ sensitive chimaera AL23 was equally well inhibited as the slowly inactivating constructs IF 19, 20AA (Figure 7A) and AL20. The initial block was stronger in AL23 than in wild type class A and other chimeric channel constructs ($P < 0.05$, Figure 7B).

Discussion

Slow recovery from open channel block (SROB)

We have systematically studied the role of the resting, open and inactivated states for use-dependent inhibition of class A Ca^{2+} channels by mibefradil. Enhanced Ca^{2+} channel block by mibefradil at depolarised voltages was previously interpreted in terms of the modulated receptor hypothesis as high affinity block of inactivated channels (Fang & Osterrieder, 1991; Lacinova *et al*., 1995; Mehrke *et al*., 1994; Welling *et al*., 1995; Bezprozvanny & Tsien, 1995). Our data indicate a different mechanism for mibefradil interaction with class A Ca^{2+} channels. As demonstrated in Figure 3, channel inactivation does not induce additional block. Use-dependent block of class A Ca^{2+} channels by mibefradil is, therefore, caused by slow recovery from open channel block and not by enhanced drug binding to inactivated channels. This result is in line with previous results of Mishra & Hermsmeyer (1994) who demonstrated that mibefradil does

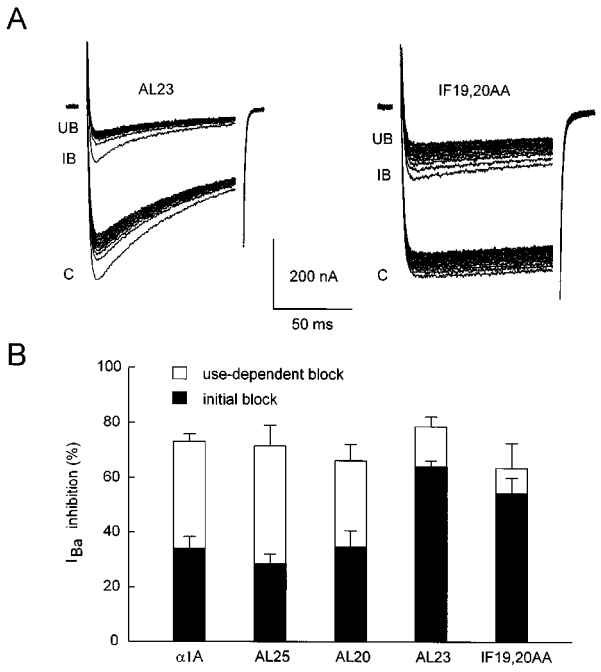

Figure 7 Block of chimeric class A/L-type Ca^{2+} channels by mibefradil. Initial block and use-dependent I_{Ba} inhibition of wild type class A channels and class A/L-type chimaeras AL25, AL20, AL23 and IF19, 20AA (see Methods) during 100 ms pulse trains (0.1 Hz, from −80 to 20 mV) by 100 μM mibefradil. Despite the marked differences in I_{Ba} inactivation of chimaeras AL23 (Döring *et al*., 1996) and IF19, 20AA (Hering *et al*., 1997) the total I_{Ba} inhibition (initial- and use-dependent) was not significantly different ($P>0.05$). Initial block of AL23 was significantly larger than in wild type class A channels ($P<0.05$).

not shift the midpoint voltage of the L-type Ca^{2+} channel inactivation curve.

Evidence for a single drug blocked state comes also from a comparison of the recovery time courses after a 100 ms pulse train and a single 2 min conditioning prepulse (Figure 6B). Despite the fact that both protocols produce a dramatically different amount of depolarization favouring inactivation (1.5 s during the train in Figure 5 *vs* 120 s conditioning during the prepulse in Figure 6A), recovery from block occurred at the same rate after both protocols (Figure 6B).

Our data clearly demonstrate that open state-dependent block does not exclude enhanced channel block at depolarized voltages (Figure 4B). Once mibefradil gained access to its receptor site, recovery from block is voltage-dependent and significantly slower at depolarized voltages (i.e. $\tau_{recovery}=75\pm15$ at −70 mV *vs* $\tau_{recovery}=20\pm2$ at −100 mV, $P<0.05$, Figure 6). Shifting the membrane potential to more depolarized voltages will, therefore, inevitably induce additional block if the time for unblock exceeds the interpulse interval of the train (Figure 4).

It is interesting that SROB at depolarised voltages may cause dramatic changes in the 'inactivation curve' if the channels are not allowed to recover from block after a double pulse protocol. Thus, the results shown in Figure 3B add further evidence that enhanced drug interaction with inactivated channels (resulting in scaling and shift of the midpoint voltage of the availability curve) can only be concluded from measurements that were performed under steady state conditions (Degtiar *et al*., 1997, see also Hering & Timin, 1993 for theoretical discussion of open channel block induced changes in the inactivation curve).

Mishra & Hermsmeyer, (1994) reported pure resting state-dependent block of L-type channels in vascular smooth muscle cells. In their experiments pulse trains were applied from a negative holding potential (−80 mV). Other authors report a pronounced use-dependent block of Ca^{2+} channels by mibefradil if the membrane potential is shifted during a train to more depolarized potentials (−40 mV) (Lacinova *et al*., 1995, Welling *et al*., 1995). This apparent discrepancy can be explained by slow recovery from open state-dependent block at more depolarized voltages (Figure 4 and 5). Hence, holding the membrane potential negative enough will favour rapid unblock between the individual pulses of the train and therefore prevent use-dependent accumulation of channels in a non conducting state.

Chimaera IF19,20AA with a double alanine substitution in segment IIIS6 at the inner mouth of the channel pore was equally well inhibited by mibefradil than the wild type class A channel (Figure 7). Compared to Ca^{2+} channel block by PAA, where residues in segment IIIS6 at the inner mouth of the channel pore affect PAA action (Hering *et al*., 1997) those determinants appeared to be less important for block by mibefradil.

Lacinova *et al*. (1995) have demonstrated that Ca^{2+} channel block by verapamil but not by mibefradil is facilitated by coexpression of the 'fast inactivating' β_3 subunit. This result is in line with our present findings suggesting that chimeric class A/L-type channels are blocked by mibefradil irrespectively of their inactivation kinetics (Figure 7). Thus, unlike PAA action, inactivation determinants appear to be less significant in guarding mibefradil's access to the receptor site.

Resting state-dependent block by mibefradil

Resting state-dependent block is usually defined as peak current inhibition during the first test pulse in drug. However, there is clear evidence that part of the peak I_{Ba} inhibition during the first pulse in mibefradil is caused by rapid open channel block (see acceleration in I_{Ba} kinetics in Figures 1A and 4A) and does not exclusively reflect drug interaction with the resting closed channel state. The initial I_{Ba} block reflects, therefore, partially open state block. It is, nevertheless, likely that mibefradil is able to reach its receptor site in the pore by penetrating the activation gate barrier at rest (see concentration-dependent I_{Ba} inhibition in Figure 2). A similar kind of resting state block was previously described for high concentrations of permanently charged PAA and BTZ applied to the extracellular side of the membrane (Hering *et al*., 1993; Berjukov *et al*., 1996). A low affinity interaction of mibefradil with a distinct binding site at the closed resting channel conformation can, however, not be excluded.

Comparison of mibefradil- and PAA-action

Taken together, our data reveal a high degree of similarity between the action of mibefradil and PAAs (Degtiar *et al*., 1997; Hering *et al*., 1997). Both drugs access their receptor determinants *via* the open channel conformation and the subsequent unblock at rest is voltage-dependent and slower at more depolarized membrane potentials. At rest, recovery from block by PAA is rate limited by recovery of the channels from slow inactivation (Hering *et al*., 1997). Our data suggest that unblock of mibefradil occurs at an even slower rate (Figure 6B). The latter finding provides an explanation for the about 10 fold higher voltage dependency of Ca^{2+} channel block by mibefradil compared to verapamil (Fang & Osterrieder, 1991).

Clinical relevance

It remains to be determined if the cardiovascular profile of mibefradil – selectivity for coronary vasculature and relative lack of negative inotropic effects – is caused by a selective T-type channel block in smooth muscle cells or by voltage dependent recovery of L-type channels. A rapid unblock of L-type channels at the more negative resting potentials in the working myocardium compared to slow unblock of L-type Ca^{2+} channels in the depolarized smooth muscle cells provides an alternative explanation.

The available functional data suggest a high degree of similarity between L-type and class A Ca^{2+} channel block by mibefradil (Bezprozvanny & Tsien, 1995). Significant use-dependent block of class A Ca^{2+} channels by mibefradil occures when the drug is applied in therapeutically-relevant concentrations (i.e. 3 μM mibefradil induce $7.3 \pm 1.9\%$ use-dependent I_{Ba} inhibition during a train of 15 100 ms-pulses from -80 to 20 mV at 0.2 Hz). It is, therefore, tempting to suggest that the proposed mechanism of mibefradil-action (SROB) provides a molecular basis for an understanding of unwanted side effects such as sinus bradycardia and first degree-atrioventricular block (Bernik *et al*., 1996; Viscoper *et al*., 1997; Braun *et al*., 1996; Rosenquist *et al*., 1997). Hence, a slower channel unblock in the more depolarized sinus node and atrial tissue than in the polarised working myocardium might be expected.

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