0026-895X/04/6606-1652–1661$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 66:1652–1661, 2004

Vol. 66, No. 6
3219/1184979
Printed in U.S.A.

State-Dependent Mibefradil Block of Na^+ Channels

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Received January 15, 2004; accepted September 10, 2004

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Mibefradil is a T-type Ca^{2+} channel antagonist with reported cross-reactivity with other classes of ion channels, including K^+, Cl^-, and Na^+ channels. Using whole-cell voltage clamp, we examined mibefradil block of four Na^+ channel isoforms expressed in human embryonic kidney cells: $Na_v1.5$ (cardiac), $Na_v1.4$ (skeletal muscle), $Na_v1.2$ (brain), and $Na_v1.7$ (peripheral nerve). Mibefradil blocked $Na_v1.5$ in a use/frequency-dependent manner, indicating preferential binding to states visited during depolarization. Mibefradil blocked currents of all Na^+ channel isoforms with similar affinity and a dependence on holding potential, and drug off-rate was slowed at depolarized potentials (k_{off} was 0.024/s at −130 mV and 0.007/s at −100 mV for $Na_v1.5$). We further probed the interaction of mibefradil with inactivated $Na_v1.5$ channels. Neither the degree nor the time course of block was dependent on the stimulus duration, which dramatically changed the residency time of channels in the fast-inactivated state. In addition, inhibiting the binding of the fast inactivation lid ($Na_v1.5$ ICM + MTSET) did not alter mibefradil block, confirming that the drug does not preferentially interact with the fast-inactivated state. We also tested whether mibefradil interacted with slow-inactivated state(s). When selectively applied to channels after inducing slow inactivation with a 60-s pulse to −10 mV, mibefradil (1 μM) produced 45% fractional block in $Na_v1.5$ and greater block (88%) in an isoform ($Na_v1.4$) that slow-inactivates more completely. Our results suggest that mibefradil blocks Na^+ channels in a state-dependent manner that does not depend on fast inactivation but probably involves interaction with one or more slow-inactivated state(s).

Mibefradil (Ro 40-5967) is a novel Ca^{2+} channel antagonist that was marketed briefly as Posicor (Roche Pharmaceuticals, Nutley, NJ) to treat hypertension. Its effective hemodynamic profile, which included reductions in vascular resistance, heart rate, and neointima formation after vascular injury, prompted its consideration in treating progression to heart failure. Mibefradil blocks both classes of Ca^{2+} channels in the cardiovascular system (i.e., L- and T-type), exhibiting a 5- to 15-fold higher affinity for T-type channels (Mehrke et al., 1994; Mishra and Hermsmeyer, 1994b; Clozel et al., 1997; Martin et al., 2000). Despite its therapeutic benefits, mibefradil was withdrawn from the market because of a fatal drug interaction involving inhibition of a cytochrome P450 enzyme (Welker et al., 1998). Mibefradil, however, remains an intriguing compound and an important prototype drug for structural studies of its channel targets.

Although it is best known for its Ca^{2+} channel-blocking properties, mibefradil also has micromolar affinity for other channels, including native and recombinant K^+ channels (Chouabe et al., 1998; Liu et al., 1999; Perchenet and Clement-Chomienne, 2000) and Ca^{2+}-activated and volume-activated Cl^- channels in macrovascular endothelial cells (Nilius et al., 1998). Mibefradil has also been reported to block Na^+ channels found in GH3 cells (Eller et al., 2000) and cardiac Na^+ channels expressed in HEK 293 cells (Guatimosim et al., 2001). Such cross-reactivity suggests that the cardiovascular effects of mibefradil might involve interaction with multiple ion channels rather than selective inhibition of T-type Ca^{2+} currents and also points to a potentially common mibefradil-binding motif across targets.

The isoform specificity of the action of mibefradil in Ca^{2+} channels, often exploited to elucidate isoform-specific physiological roles for different classes of Ca^{2+} channels, prompted us to ask whether mibefradil interacts with Na^+ channels in an isoform-dependent manner. So far, 10 isoforms of the Na^+ channel α1 subunit have been cloned and characterized ($Na_v1.1$–1.9, Na_x) (for review, see Goldin, 2001). Despite high amino acid sequence similarity within the Na^+ channel subfamily, isoforms vary in their pharmacological profiles. For example, six isoforms ($Na_v1.1$, 1.2, 1.3, 1.4, 1.6, and 1.7) are highly tetrodotoxin-sensitive, whereas the others ($Na_v1.5$, 1.8, 1.9, and Na_x) exhibit varying degrees of tetrodotoxin resistance (Goldin, 2001). In addition, the cardiac isoform,

This work was supported by an American Heart Association PreDoctoral Fellowship (to M.M.M.) and by National Institutes of Health grant R01-HL65680 (to D.A.H.).

This work has been presented in part in abstract form: McNulty MM and Hanck DA. State dependent-isoform independent block of Na channels by mibefradil. *Biophys J* **82:**85a (2002).

ABBREVIATIONS: HEK, human embryonic kidney; MTSET, methanethiosulfonate ethyltrimethylammonium; MTS, methanethiosulfonate.

$Na_v1.5$, is more sensitive to lidocaine block than the skeletal muscle channel, $Na_v1.4$ (Nuss et al., 1995) and the CNS isoforms (Bean et al., 1983). In this study, we examined mibefradil block of Na^+ channel isoforms cloned from four different tissues: $Na_v1.5$ (heart), $Na_v1.4$ (skeletal muscle), $Na_v1.2$ (brain), and $Na_v1.7$ (peripheral nerve). Mibefradil had a similar affinity across all four isoforms, suggesting that a common binding site is formed.

Several of the studies examining mibefradil block across multiple targets provide evidence for state dependence (i.e., preferential binding to certain channel states over others). State-dependent drug binding is a common feature of several cardiovascular drugs and often accounts for their therapeutic effectiveness. Studies of T-type Ca^{2+} current in human medullary carcinoma hMTC cells (Mehrke et al., 1994; Klugbauer et al., 1998) and rat vascular muscle (Mishra and Hermsmeyer, 1994a) found no use dependence of mibefradil block, suggesting that mibefradil blocked the rested state, whereas studies of T current in cardiac myocytes (Benardeau and Ertel, 1998; Leuranguer et al., 2001) and dorsal root ganglion cells (Todorovic and Lingle, 1998) reported use-dependent block pointing to preferential interaction with activated and/or inactivated conformations of the channel. Use-dependent block has also been noted for other channel targets such as K^+ channels (Perchenet and Clement-Chomienne, 2000) and Na^+ channels (Eller et al., 2000). Despite the evidence demonstrating state-dependent drug interactions, the identity of the highest affinity binding state is still under question. Na^+ channels have long served as a useful model for studying channel-drug interactions, because many of the structural features responsible for Na^+ channel gating, especially those involved in channel inactivation, have been extensively characterized, whereas the structural details underlying Ca^{2+} channel gating are less well understood. Therefore, we chose to further investigate the state-dependence of mibefradil in Na^+ channels. We found that block of Na^+ channels was dependent on holding potential but was not dependent on the residency time in the fast-inactivated state nor on the binding of the fast inactivation gate ($Na_v1.5$ ICM + MTSET), suggesting that mibefradil does not preferentially target the fast-inactivated state. However, selective exposure of mibefradil to slow-inactivated channels inhibited current with an affinity similar similar to that of modestly or frequently depolarized channels. As a whole, our data suggest that mibefradil blocks Na^+ channels in a novel state-dependent manner that does not depend on fast inactivation; rather, it probably involves interaction with one or more slow-inactivated conformations. Some of these data have been reported in abstract form (McNulty and Hanck, 2002).

Materials and Methods

Heterologous Expression and Site-Directed Mutagenesis. The cDNA for the human heart voltage-gated sodium channel, $Na_v1.5$ (hH1a), was kindly provided by H. Hartmann and A. Brown (Hartmann et al., 1994). The mutation of the phenylalanine in the IFM motif within the III-IV linker to a cysteine has been described previously (Sheets et al., 2000). $Na_v1.4$ (skeletal muscle, $\mu1$) cDNA was originally from Gail Mandel (Stony Brook University, Stony Brook, NY) and $Na_v1.2$ (BrIIA) cDNA was kindly provided by A. Goldin (Auld et al., 1988). For creation of stable cell lines, cDNA for $Na_v1.5$ was subcloned into the mammalian expression vectors pRcCMV or pcDNA5/FRT and $Na_v1.4$, $Na_v1.2$ and $Na_v1.5$ ICM were subcloned into pRcCMV (Invitrogen, Carlsbad, CA). Stable lines were created using G418 selection (Invitrogen) (600 μg/ml for selection and 200 μg/ml for continued maintenance) in HEK293 cells (American Type Culture Collection, Manassas, VA) or Hygromycin B (Sigma, St. Louis, MO) selection in HEK293/FLP cells (Invitrogen) (100 μg/ml for selection, 50 μg/ml for maintenance). HEK293 cells stably expressing a human homolog of $Na_v1.7$ (hNE/PN1) were kindly provided by T. Cummins (Cummins et al., 1998). Cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and selection antibiotic in 100-mm Corning (Acton, MA) culture dishes. Media were changed every 48 h, and cells were passaged weekly. Whole-cell voltage clamp was performed on trypsinized cells (0.25% Trypsin-EDTA; Invitrogen) 3 to 6 days after plating.

Solutions and Chemicals. Pipette solution contained 100 mM CsF, 40 mM CsCl, 10 mM NaCl, and 10 mM HEPES titrated to pH 7.4 with CsOH. For most experiments, 10 mM EGTA was added to the pipette solution to improve the tightness of seals. The addition of EGTA into the pipette solution affected neither the magnitude nor the kinetics of the Na^+ currents; as a result, data recorded using both sets of pipette solutions were pooled. The bath solution for recording $Na_v1.7$ and $Na_v1.2$ currents contained 140 mM NaCl, 2 mM $CaCl_2$, and 10 mM HEPES titrated to pH 7.4 with NaOH. In general, $Na_v1.5$ and $Na_v1.4$ currents were too large to control in 140 mM NaCl, so a lower $[NaCl]_o$ was used (70 mM for $Na_v1.4$; 35 or 10 mM for $Na_v1.5$) substituting NaCl with CsCl. When a lower [NaCl] was used in the bath solution, pipette NaCl was lowered to 5 or 1 mM (replaced with CsCl). Neither the degree nor the kinetics of drug block was affected by the concentration of extracellular Na^+ (data not shown). Mibefradil, obtained as a kind gift from Roche Pharmaceuticals, was used for most of the experiments until it became available commercially (Sigma, St. Louis, MO). Mibefradil was diluted in bath solution to the desired concentration (100 nM-10 μM) from a stock solution (1 mM in distilled water stored in the dark). The methanethiosulfonate compound, MTSET, was obtained from Toronto Research Chemicals (North York, ON, Canada) and was freshly added to the pipette solution just before the experiment.

Electrophysiological Recordings and Analysis. All recordings were made using the whole-cell voltage clamp technique. Patch pipettes were pulled from microhematocrit glass capillary tubes (Fisherbrand) using the Flaming/Brown micropipette puller P97 (Sutter Instruments, Novato, CA) and had resistances of 0.5 to 1.2 MΩ when filled with pipette solution. All recordings were made using pClamp software 8.1 and an Axopatch 200B amplifier with a Digidata 1322A Interface (Axon Instruments Inc., Union City, CA). Data were filtered at 5 or 10 kHz with an eight-pole, low-pass Bessel filter and sampled at two times the filter frequency. Recordings were made at room temperature (22–25°C). Because time-dependent shifts in Na^+ channel gating were most rapid within the first several minutes after patch rupture, recordings were generally initiated approximately 3 to 5 min after whole-cell access, and care was taken to compare data at similar time points after patch rupture.

Drug was applied using either a two-chamber bath in which cells sealed to the patch pipette were lifted and moved from one chamber (without drug) to a second (with drug) and vice versa or a single chamber bath in which solutions were exchanged using the cFLOW perfusion system (Cell Microcontrols, Virginia Beach, VA) and/or the SF77 Perfusion Fast-Step system (Warner Instruments, Hamden, CT). Both methods of drug application yielded similar results. For most experiments, the effect of mibefradil was assessed by depolarizing cells for 20 to 30 ms once every 5 s (0.2 Hz) from a holding potential of −130 mV. Details of other protocols are given under *Results*.

All currents were capacity-corrected using 8 to 16 subthreshold responses (voltage steps of 10 or 20 mV) and leak-subtracted, based on linear leak resistance calculated at hyperpolarized voltages (−80 mV and below) or linear interpolation between the current at the holding potential and 0 mV. Only cells with a leak resistance of 500

MΩ or greater were included in pooled data. Curve fitting and statistical analyses (Student's t test) were performed using MatLab (The Mathworks, Inc., Natick, MA) and Origin software (OriginLab Corp, Northampton, MA). A p value of <0.05 was considered significant. Data are depicted as means ± S.E.M.

Results

Tonic and Use-Dependent Mibefradil Block of $Na_v1.5$ Channels. A previous study that reported mibefradil block of Na^+ channels (Eller et al., 2000) raised the possibility that mibefradil block is state-dependent; i.e., it preferentially targets certain channel states over others. To further investigate the state origin of mibefradil block, we tested for rested-state and use-dependent mibefradil block of $Na_v1.5$ channels. Rested-state block was evaluated by holding cells at a negative potential that assured channels resided in the resting state (−130 mV) and exposing them for 1 min to 10 μM mibefradil using a rapid perfusion system (see *Materials and Methods*), which changes the solution flowing across the cell in a 20-ms time frame. After 1 min, during which time cells were not depolarized, a train of pulses was initiated. Forty-one percent of the current was blocked in the first pulse (Fig. 1A). Lengthening exposure time to 5 min did not increase fractional block; therefore, the amount of block after a 1-min exposure was taken to represent steady state rested-state block. Assuming a single binding site and using the Langmuir isotherm this degree of block predicted a K_D of 14 μM for the rested, or closed, state. With further infrequent depolarizations to −30 mV once every 5 s (0.2 Hz), an additional block of 40% developed leading to a total reduction in peak current of 84% (Fig. 1A, right). Even though cells were depolarized for 20 ms only once every 5 s, additional block accumulated, indicating that depolarization revealed one or more higher affinity, periodically accessible, binding conformations with slow drug on- and off-rates.

The accumulation of block at a low frequency of stimulation (1 pulse every 5 s) predicted greater fractional block with shorter recovery intervals between pulses (i.e., higher stimulation frequencies). This behavior could be better appreciated using a lower concentration (1 μM) of mibefradil and Fig. 1B shows that the fraction of current that could be blocked was sensitive to stimulation frequency. Representative traces in the absence of drug and after block with 1 μM mibefradil illustrate steady-state block at two stimulation frequencies, 0.2 and 10 Hz (Fig. 1B, left). At 1 μM, mibefradil produced greater block across a 10-fold change in stimulation frequency (0.2 to 20 Hz) (Fig. 1B, right). The effect was maximal at 10 Hz as stimulating at 20 Hz did not further increase fractional block. At a 20-Hz stimulation frequency, which corresponds to a 100-fold increase in the amount of time spent depolarized every second and an approximately 17-fold decrease in the recovery time per second (compared with 0.2 Hz), mibefradil produced 3-fold greater block (20% block at 0.2 Hz to 60% block at 20 Hz).

Even though it is generally believed that mibefradil blocks its channel targets by binding in the inner pore, the binding site for mibefradil is molecularly unknown. With a central tertiary amine and a pK_a of approximately 8.8, mibefradil is partially charged at physiological pH. A charged drug will not easily cross the lipid membrane; therefore, if it is responsible for blocking the channel, it must do so at an extracellular site. However, it would be expected that the uncharged fraction of drug crosses the membrane, and drug could then gain access to its binding site from the intracellular side of the channel. To address this question, mibefradil was applied under acidic (bath solution, pH 6.6) conditions, and block induced by high frequency stimulation (10 Hz) was evaluated. A greater fraction of mibefradil will remain charged in an acidic environment and would be less likely to cross the membrane. Block was significantly disfavored at low pH (Fig. 1B, right), suggesting that uncharged mibefradil must cross the membrane and block the channel from the intracellular side.

Mibefradil Blocked Four Na^+ Channel Isoforms in a Holding Potential-Dependent Manner. The use-dependence of mibefradil action on Na^+ channels (Fig. 1) (Eller et al., 2000) was indicative of state-dependent binding interactions, which could arise from voltage-dependent changes in the conformation of the drug binding site (Hille, 1977). We, therefore, evaluated mibefradil block at different membrane

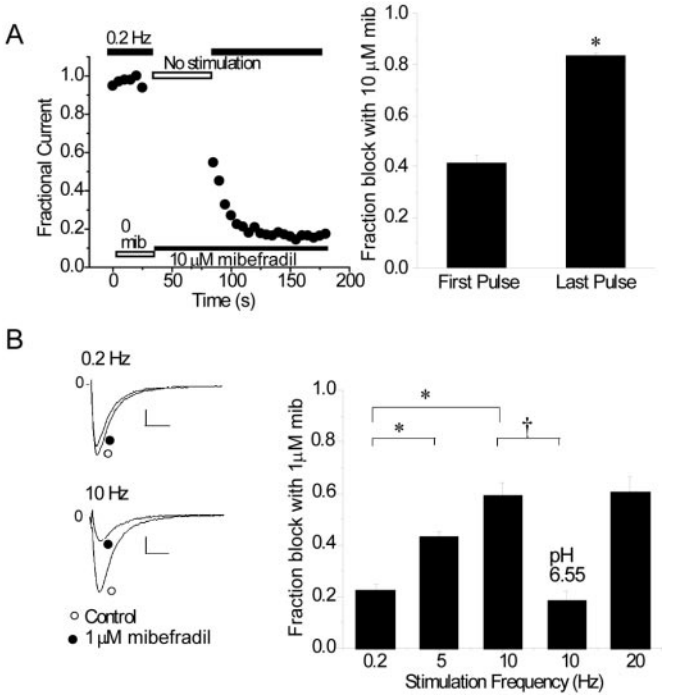

Fig. 1. Tonic and use-dependent mibefradil block of $Na_v1.5$. A, a representative cell and pooled data showing both tonic and use-dependent block of $Na_v1.5$. After measuring peak current at −30 mV from a holding potential of −130 mV in the absence of drug, the cell was exposed, via a rapid perfusion system (see *Materials and Methods*) to 10 μM mibefradil for 1 min while holding the cell at −130 mV. After 1 min, trains of 20-ms depolarizations to −30 mV were applied at 0.2 Hz in the presence of mibefradil. The degree of block with the first pulse after drug application indicates the degree of rested-state block. Additional block (i.e., use-dependent block) was observed upon repeated depolarizations to −30 mV before eventually reaching a plateau. Pooled data (right) compare the average block during the first pulse after equilibration with drug and after the drug effect reaches steady state during a 0.2 Hz depolarization (indicated by last pulse) (first pulse, 41.4 ± 3.3%; last pulse, 83.5 ± 1.1%; $n = 5$). *, $p < 0.05$. B, representative current traces depict peak current in the absence of mibefradil (○) and after the effect of 1 μM mibefradil has reached steady state (●) at two stimulation frequencies: 0.2 and 10 Hz. *XY* scale bars, 1 ms (x) and 500 pA (y). Pooled data at four frequencies demonstrate more block during higher frequencies: 0.2 Hz, 22.3 ± 2.6% block ($n = 6$); 5 Hz, 40.0 ± 3.6% block ($n = 4$); 10 Hz, 56.7 ± 3.3% block ($n = 4$); 20 Hz, 60.5 ± 5.9% block ($n = 3$); *, $p < 0.05$ compared with 0.2 Hz. Fractional block at 10 Hz was also tested in low pH (pH 6.55 compared with pH 7.4): 18.3 ± 3.7 ($n = 3$). †, $p < 0.05$.

holding potentials. Representative traces of $Na_v1.5$ (Fig. 2A) illustrate that greater block was observed when cells were held at a −100 mV than at −130 mV, and Fig. 2B shows the concentration-response data. Depolarizing the cells increased the affinity of mibefradil approximately 6-fold.

Drugs often target channels in an isoform-dependent manner and Na^+ channel isoforms exhibit different pharmacological profiles. To test for isoform specificity, we assayed mibefradil block of four Na^+ channel isoforms cloned from different tissues: $Na_v1.5$, $Na_v1.4$, $Na_v1.2$, and $Na_v1.7$. $Na_v1.5$ was cloned from human heart (Hartmann et al., 1994), and the $Na_v1.4$ we examined was cloned from rat skeletal muscle (Trimmer et al., 1989). $Na_v1.2$, a major brain isoform cloned from rat (Auld et al., 1988), and a human homolog of $Na_v1.7$, a channel highly expressed in dorsal root ganglia neurons (Cummins et al., 1998), were also examined. These channels gate over different voltage ranges (Fig. 3A). $Na_v1.5$ activated and inactivated over the most negative potential range and $Na_v1.2$ over the most positive. Therefore, although block at −130 mV, a potential that assured full availability, could be evaluated in all the isoforms, the holding potentials used to evaluate the effect of potential were selected for $Na_v1.4$, $Na_v1.2$, and $Na_v1.7$ to reflect their more positive gating range, i.e., −90 mV, −70 mV, and −90 mV, respectively.

Mibefradil (1 μM), assayed from a holding potential of −130 mV, blocked $Na_v1.5$, $Na_v1.4$, and $Na_v1.2$, similarly

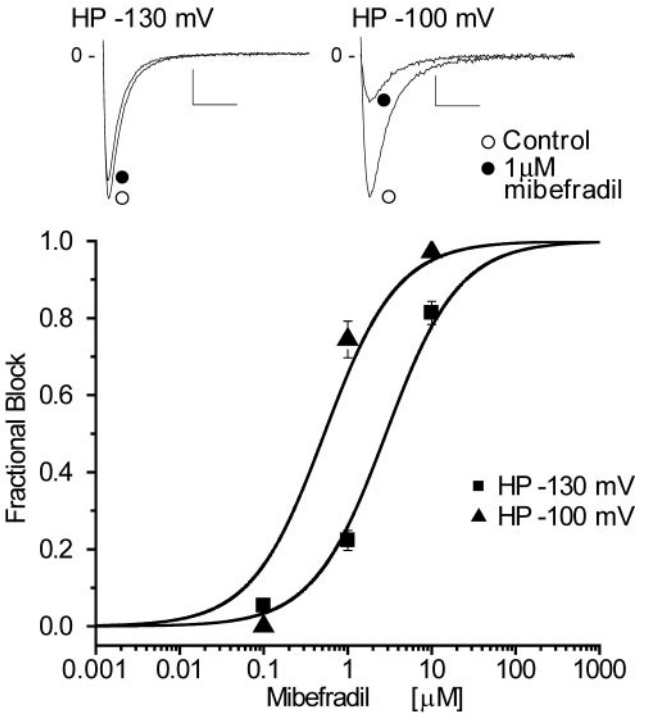

Fig. 2. Mibefradil block of $Na_v1.5$ is dependent on holding potential. A, traces from representative cells in response to −30 mV in absence of mibefradil (○) and after the effect of 1 μM mibefradil has reached steady state (●) with 0.2 Hz stimulation from a holding potential of −130 or −100 mV. *XY* scale bars, 2 ms (*x*) and 500 pA (*y*)(for 130 mV) and 100 pA (for −100 mV). B, concentration-response data for mibefradil block of $Na_v1.5$ at −130 mV (■)and −100 mV (●) holding potentials. Fractional block was determined by normalizing the peak current (average of at least 10 sweeps) after the drug effect has reached steady state to the peak current (average of at least four sweeps) in the absence of drug. Curves were fit with a Langmuir isotherm assuming a single binding site to obtain IC_{50}s at each holding potential: −130 mV, 2.9 ± 0.4 μM (n = 5–6); −100 mV, 0.5 ± 0.3 μM (n = 1–5).

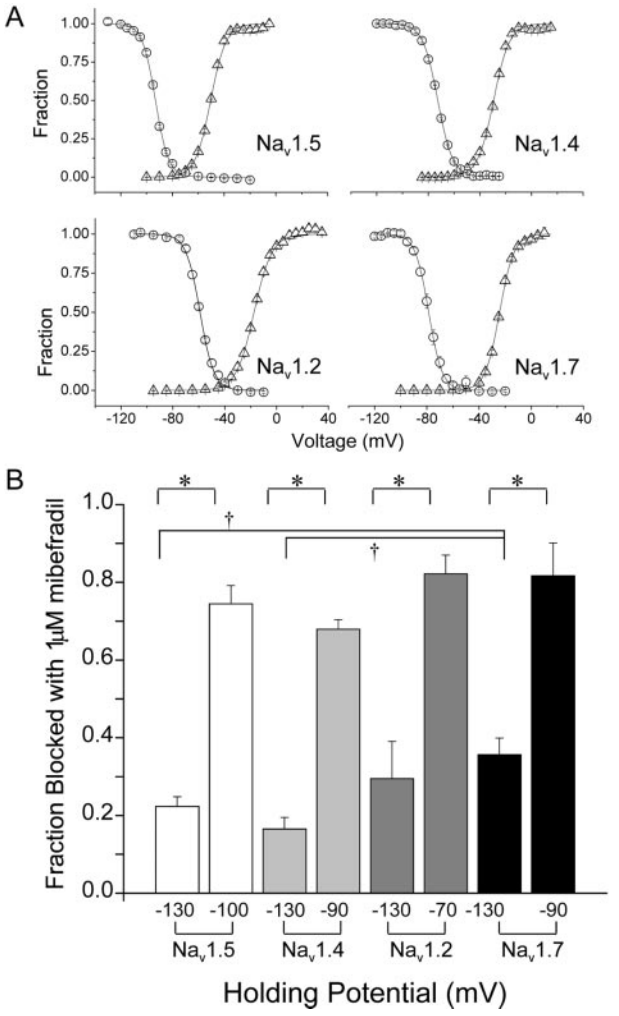

Fig. 3. Mibefradil block of three Na^+ channel isoforms. A, overlay of activation (△) and availability (○) curves for $Na_v1.5$, $Na_v1.4$, $Na_v1.2$, and $Na_v1.7$. Activation parameters were estimated by fitting current-voltage relationships with a transform of a Boltzmann distribution $I = (V - V_{rev}) \times G_{max}/1 + \exp^{(V_{½} - V)/k}$, where the peak Na^+ current (I) at each test voltage (V) was a function of the voltage for half-activation ($V_{1/2}$), k is the slope factor, V_{rev} is the reversal potential, and G_{max} is maximum peak conductance: $Na_v1.5$, $V_{1/2}$ activation, −50.6 ± 1.3 mV; k, 5.6 ± 0.3 mV (n = 8); $Na_v1.4$, $V_{1/2}$ activation, −29.6 ± 1.6 mV; k, 6.1 ± 0.35 (n = 9); $Na_v1.2$, $V_{1/2}$ activation, −17.6 ± 2.9 mV; k, 6.9 ± 0.7 mV (n = 8); $Na_v1.7$, $V_{1/2}$ activation, −24.3 ± 3.0 mV; k, 5.2 ± 0.2 mV (n = 6). Voltage-dependent channel availability was measured with a two-pulse protocol in which cells were first cells were depolarized for 1 s at potentials ranging from −110 to −30 mV from a holding potential of −130 mV or −150 mV before measuring Na^+ currents every 5 s at −30 mV for $Na_v1.5$, −10 mV for $Na_v1.2$ and $Na_v1.7$, and 0 mV for $Na_v1.4$. Availability was fit with the following Boltzmann equation $I/I_{max} = 1/1 + \exp^{(V - V_{½})/k}$, where I is the peak current, I_{max} is the maximum peak current, V is the prepulse voltage, $V_{1/2}$ is the voltage for half-inactivation, and k is the slope factor: $Na_v1.5$: $V_{1/2}$ inactivation, −93.1 ± 1.7 mV; k, 5.0 ± 0.2 mV (n = 8); $Na_v1.4$: $V_{1/2}$ inactivation, −72.8 ± 2.1 mV; k, 5.9 ± 0.3 mV (n = 9); $Na_v1.2$: $V_{1/2}$ inactivation, −58.9 ± 1.7 mV; k, 5.5 ± 0.5 mV (n = 8); $Na_v1.7$: $V_{1/2}$ inactivation, −78.4 mV ± 1.0 mV; k, 5.4 ± 0.3 mV (n = 6). B, fractional mibefradil block of $Na_v1.5$, $Na_v1.4$, $Na_v1.2$, and $Na_v1.7$ at different holding potentials. Fractional block at −130 mV: $Na_v1.5$: 22.3 ± 2.6% block (n = 6); $Na_v1.4$: 16.5 ± 3.1% block (n = 5); $Na_v1.2$: 29.5 ± 9.5% block (n = 3); $Na_v1.7$: 35.7 ± 4.3%, (n = 4). Mibefradil blocked significantly more current at modestly depolarized potentials (*, $p < 0.05$ compared with the fractional block of each isoform at −130 mV): $Na_v1.5$, 61.0 ± 4.1% block at −100 mV (n = 4); $Na_v1.4$, 67.9 ± 5.0% block at −90 mV (n = 4); $Na_v1.2$, 82.1 ± 4.9% block at −70 mV (n = 4); $Na_v1.7$, 81.7 ± 8.5% block at −90 mV (n = 4). Between isoforms, fractional block of $Na_v1.5$ and $Na_v1.4$ at −130 mV was significantly (†, $p < 0.05$) smaller than block of $Na_v1.7$ at the same potential. At the depolarized potentials, block of $Na_v1.2$ differed significantly ($p < 0.05$) from block of $Na_v1.5$ and $Na_v1.4$.

(Fig. 3B). From this holding potential, significantly more $Na_v1.7$ current was blocked than $Na_v1.5$ (36% versus 22%) and $Na_v1.4$ (36% versus 16%), suggesting that mibefradil had a roughly 2-fold higher rested-state affinity for the peripheral neuronal isoform. For all four Na^+ channel isoforms, mibefradil was significantly more effective at the more depolarized potentials. Block of $Na_v1.2$ at −70 mV and $Na_v1.7$ at −90 mV was modestly greater than $Na_v1.5$ at −100 mV and $Na_v1.4$ at −90 mV; block predicted approximately 2.5-fold greater affinity for the neuronal isoforms. These differences were small, however. All four channels were blocked by mibefradil in the same concentration range suggesting a similar binding motif.

The increased affinity at the more depolarized holding potential could be the result of a slower drug off-rate and/or a faster on-rate. Off-rates can be directly calculated from the washout of drug. Peak currents during the washout plotted as a function of time were fit with a single exponential to determine time constants for drug washout. Washout was well described by single exponentials and was usually complete at a holding potential of −130 mV. Washouts at −100 mV were also well described by single exponentials; it is interesting, however, that they were only rarely complete, suggesting the presence of a state or states with an especially high affinity. The reciprocals of the time constants for drug washout predicted mibefradil off-rates of 0.024 s^{-1} and 0.007 s^{-1} at −130 mV and −100 mV, respectively, consistent with a higher affinity binding site at positive holding potentials.

Mibefradil Block of $Na_v1.5$ Was Not Dependent on Fast Inactivation. Enhanced block at more positive holding potentials is usually interpreted to mean that the drug has a higher affinity for inactivated channels than for closed channels. With depolarization, Na^+ channels exhibit multiple components of inactivation that fall into two types differentiated by their time courses, fast and slow (for review, see Goldin, 2003). Fast inactivation occurs within milliseconds after brief depolarizations and recovers with similar kinetics. Slow inactivation develops and recovers over a much longer time (i.e., on the order of seconds to minutes). To determine whether the dependence of mibefradil on holding potential and stimulation frequency reflected preferential interaction with the fast-inactivated state, we examined block of $Na_v1.5$ under conditions that changed the residency time of channels in the fast-inactivated conformation. Trains (0.2 Hz) of different durations (5, 20, and 100 ms) from a holding potential of −130 mV were applied during the wash-on of 10 μM mibefradil. At this low frequency of stimulation, the recovery interval varied by less than 2% across stimulus durations. In addition, because channels inactivated within 5 ms, the proportion of time channels spent in the open state was the same under all conditions. Therefore, it was principally the residency time of the fast-inactivated state that varied. There was a 20-fold difference in the percentage of time spent depolarized between the shortest and longest durations (0.1% with 5-ms duration, 0.4% with 20-ms duration, and 2% with 100-ms duration). The slow time course of accumulation of block in the trains suggested that this would be adequate to reveal a role for the fast-inactivated state. However, there was no difference in the degree or time course of the development of block across all durations (Fig. 5, B and C). Allowing channels to spend more time in the fast-inactivated state did not enhance mibefradil block.

Although this strongly suggested that fast inactivation did not affect mibefradil block, we designed a second test to probe for mibefradil interaction with fast-inactivated channels. Block was assessed in $Na_v1.5$ channels in which the putative inactivation gate was modified. Drugs such as lidocaine exhibit drastically reduced affinity under this condition (Bennett et al., 1995). Fast inactivation of voltage-gated sodium channels depends on the binding of a triplet of residues in the Domain III-IV linker (the IFM motif) with sites on or near the inner vestibule of the channel pore (West et al., 1992). We took advantage of a technique previously developed in which

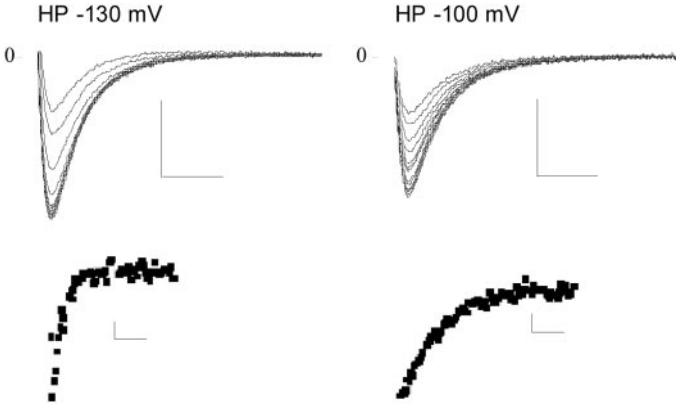

Fig. 4. Mibefradil off-rate is slowed at depolarized holding potentials. Representative wash-out traces depicting the removal of mibefradil from $Na_v1.5$ with 20-ms depolarizations (0.2 Hz) to −30 mV from a holding potential of −130 mV and −100 mV. *XY* scale bars, 2 ms (*x*) and 500 pA (*y*). Peak currents during washout were plotted as a function of pulse number and are shown beneath the traces. *XY* scale bars, 100 s (*x*) and 100 pA (*y*). Data (peak currents versus time) were fit with single exponential functions to derive time constants: −130 mV, τ 42.4 ± 2.3 s (n = 17); −100 mV, τ 144 ± 9.1 s (n = 8).

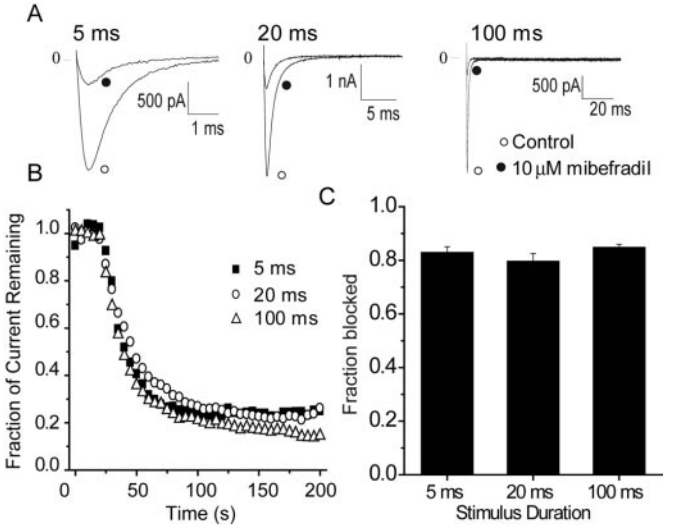

Fig. 5. Mibefradil block of $Na_v1.5$ does not depend on stimulus duration. Mibefradil block of channels at three different depolarization durations that changed the residency time of fast-inactivated channels: 5, 20, and 100 ms. Cells were depolarized at 0.2 Hz from a holding potential of −130 mV. A, representative traces at each stimulus duration in absence (○) of drug and after the effect of 10 μM mibefradil has reached steady state (●) B, representative cells depicting the time course for the development of block with 10 μM mibefradil and 0.2 Hz depolarization frequency each stimulation duration (5, 20, and 100 ms). C, pooled data depicting fractional block with 10 μM mibefradil as a function of stimulus duration: 5 ms, 82.9 ± 2.2% block (n = 5); 20 ms, 79.6 ± 2.9% (n = 5); 100 ms, 84.8 ± 1.2% (n = 5).

the phenylalanine of the IFM motif is mutated to a cysteine and the intracellular face of the channel is exposed to cysteine-modifying methanethiosulfonate (MTS) derivatives. MTS reagents covalently modify accessible cysteinyl sulfhydryls by the transfer of chemical groups to the cysteine. Depending on the MTS reagent used, this transfer may alter size, charge, and/or the chemical nature of the cysteine residue. Modification of ICM has been successfully used to modify fast inactivation (Kellenberger et al., 1996; Chahine et al., 1997). For these experiments, inactivation was removed by the intracellular addition of approximately 1 mM MTSET, which transfers a positively charged group to the cysteine. After acquiring whole cell access, approximately 3 to 5 min were required for MTSET to covalently link to the cysteine in the ICM motif and abolish inactivation. Representative traces of $Na_v1.5$ ICM currents are shown in Fig. 6 and compared with $Na_v1.5$ ICM currents in the presence of MTSET. $Na_v1.5$ ICM currents decayed only modestly more slowly than wild-type $Na_v1.5$. The decay phase was almost entirely eliminated upon exposure to MTSET. At −130 mV and a stimulation frequency of 0.2 Hz, mibefradil (10 μM) was as effective at blocking MTS-modified $Na_v1.5$ ICM currents as wild-type control and $Na_v1.5$ ICM (without MTSET) currents. The time course of the development of block was also the same (data not shown). The effect of mibefradil did not depend on the binding of the inactivation "hinged lid", and we must conclude that mibefradil does not preferentially target the fast-inactivated state.

Mibefradil Interacted with Slow-Inactivated Channels. With prolonged depolarization, Na^+ channels can also occupy one or more slow-inactivated states from which recovery requires several seconds (Goldin, 2003). Although the molecular basis of slow inactivation is unknown, it seems clear that transitions to slow-inactivated states do not rely on the fast inactivation gate in the III-IV linker; rather, they involve rearrangements in other regions of the channel, including various transmembrane segments and portions of the outer pore (Balser, 2001). $Na_v1.5$ channels expressed in HEK cells exhibit multiple components of inactivation after prolonged depolarizations and develop slow inactivation as a function of potential with slow inactivation at potentials negative to channel opening and maximal near −10 mV (Townsend and Horn, 1997; O'Reilly et al., 1999). We therefore chose −10 mV to evaluate whether mibefradil interacts with slow-inactivated channels. To provide a context for the pharmacological experiment, we first determined the magnitude and time course of the development of slow inactivation under our experimental conditions (Fig. 7A). Cells were held for increasing durations at −130, −100, and −10 mV, and the extent of slow-inactivated channels was assayed in a step to −30 mV after a brief (40 ms) hyperpolarization to −140 mV to ensure full recovery of fast inactivated channels. At −130 mV, no slow inactivation was evident (data not shown). At −100 mV, availability decreased with a single exponential time constant of greater than 20 s, predicting at steady state a decrease in channel availability of approximately 20%. At −10 mV, 62% of channels became unavailable, with a single exponential time constant of 8.0 s. Recovery at −140 mV from slow inactivation at −10 mV was evaluated in $Na_v1.5$ after a prolonged depolarization of 100 s, which was chosen to encompass the durations of drug exposure tested. $Na_v1.5$ channels recovered with a bi-exponential time course with a faster time constant of 140 ms and a slower τ of 1.7 s, representing 56% and 44% of the total, respectively (Fig. 7B).

To evaluate whether mibefradil exhibited a preference for slow-inactivated channels, we again used a rapid drug application perfusion system (see *Materials and Methods*) and a voltage protocol that allowed us to selectively apply mibefradil only when channels were maximally slow-inactivated;

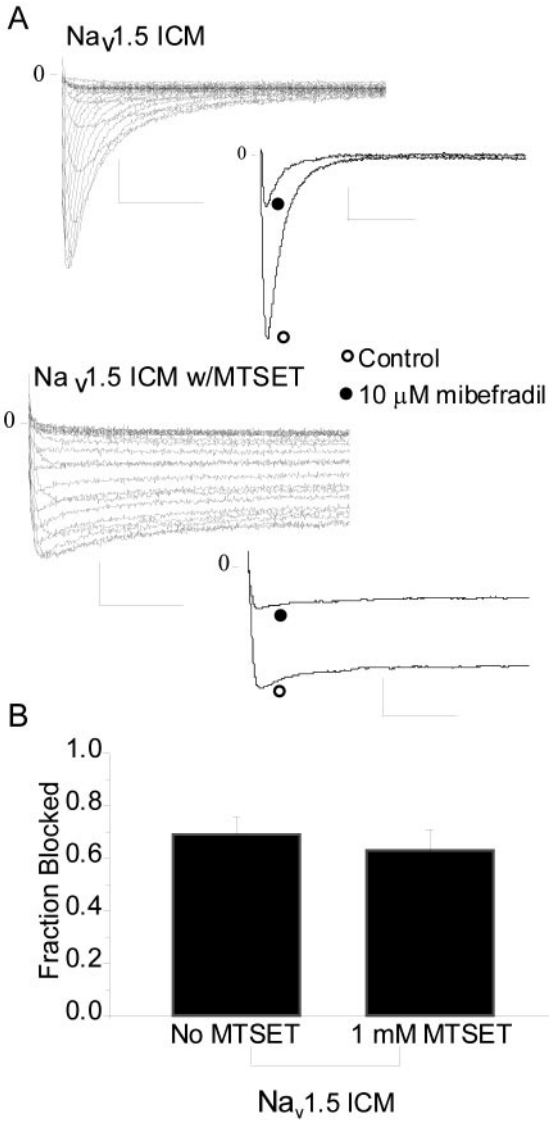

Fig. 6. Mibefradil block of $Na_v1.5$ does not depend on binding of the IFM inactivation particle. A, the phenylalanine of the IFM motif found in the III-IV linker of $Na_v1.5$ was mutated to cysteine to generate $Na_v1.5$ ICM channels. Top, representative current traces of $Na_v1.5$ ICM in the absence of MTSET in response to a set of depolarizations (at 5-mV increments) from a holding potential of −130 mV. Peak currents in the absence of drug and after the effect of 10 μM mibefradil has reached steady state. *XY* scale bars, 5 ms (x) and 1 nA (y). Bottom, traces of currents through $Na_v1.5$ ICM + MTSET channels. Application of 1 mM MTSET intracellularly covalently modified the cysteine within the ICM motif and prevented binding of the inactivation lid. Modified channels exhibited a loss of current decay. Representative traces show currents in response to −30 mV test pulses from a holding potential of −130 mV in absence (○) of drug and after the effect of 10 μM mibefradil has reached steady state during a 0.2-Hz stimulation (●). *XY* scale bars, 5 ms (x) and 1 nA (y). C, pooled data of fractional block with 10 μM mibefradil of $Na_v1.5$ ICM in the absence and presence of 1 mM intracellular MTSET: $Na_v1.5$ ICM, no MTSET, 69.2 ± 6.7% block (n = 3); $Na_v1.5$ ICM + MTSET, 63.3 ± 7.7% (n = 6).

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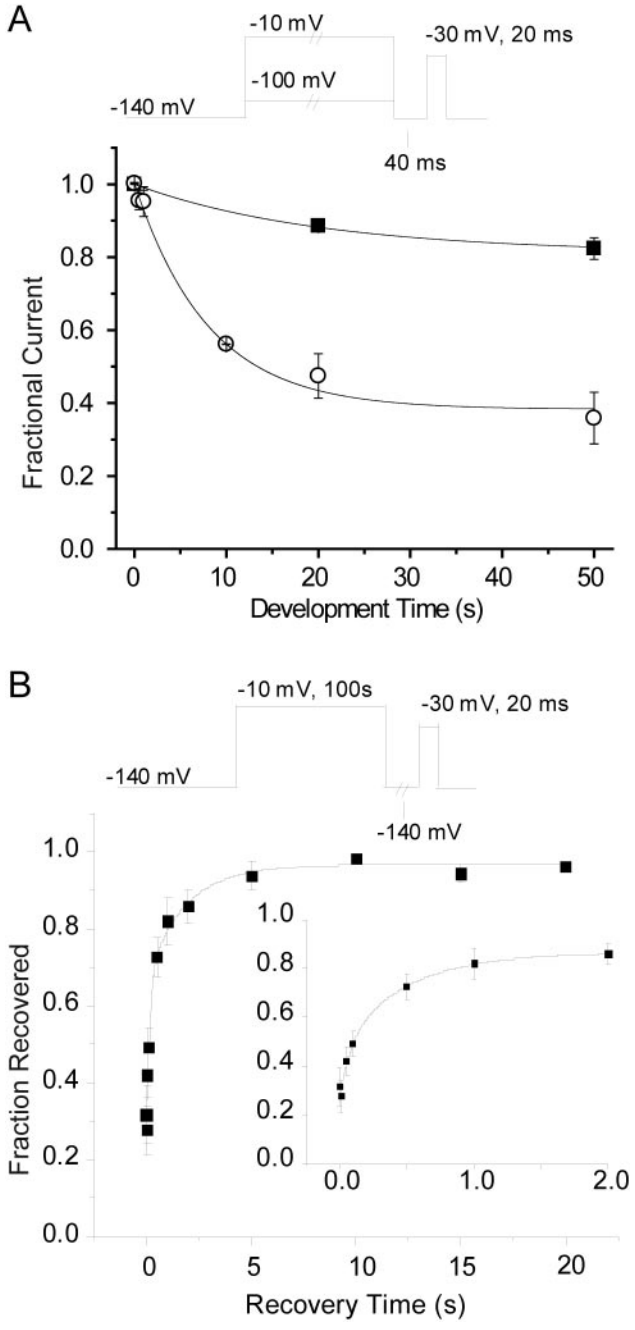

Fig. 7. Development of slow inactivation and Recovery from inactivation of $Na_v1.5$ channels. A, development of slow inactivation was examined using a two-pulse protocol in which channels were depolarized to either −100 (■) or −10 mV (○) for varying durations, briefly hyperpolarized (40 ms) to −140 mV to recover fast inactivated channels, and tested for current at −30 mV. Currents for both protocols were normalized to peak test currents elicited in the absence of inactivating prepulses. These test currents and capacitance measurements were performed before and after slow inactivation protocols to ensure changes in peak current were not caused by time-dependent changes in gating and/or cell size. A linear regression describing time-dependent decreases in cell capacitance, and hence peak current, was used to normalize slow inactivation data in cases in which peak currents decreased in the absence of inactivating prepulses. Averaged data were fit with a single exponential function (−10 mV, $n = 3$; −100 mV, $n = 4$). B, recovery from inactivation: After a prolonged (100-s) depolarization to −10 mV, channels were allowed to recover at −140 mV for varying intervals before testing for recovered current at −30 mV. Inset, expanded version of recovery graph to depict the early (0–2 s) time points. Averaged data were fit with a double exponential function ($n = 5$–7 for each time point).

i.e., mibefradil was applied for increasing durations between 8 and 40 s, after a 60-s depolarization to −10 mV (Fig. 8A). This duration was 6 × τ for the development of slow inactivation we measured at −10 mV (Fig. 7A) and therefore sufficiently long enough to achieve steady-state slow inactivation. Drug was removed just before a hyperpolarization to −140 mV, where the cell was allowed to recover from inactivation for 1 s. Based on our recovery from inactivation data after a 100-s depolarization to −10 mV (Fig. 7B), this duration represented ~5 of the faster time constant of recovery and only 0.25 of the slower time constant. Overall, we would predict that approximately 82% of channels would have recovered during the 1-s hyperpolarization, and 18% would remain inactivated. To take into account the fact that a proportion of the channels would remain inactivated after a 1-s hyperpolarization, for each cell tested, this two pulse protocol was repeated both in the absence and presence of mibefradil, and data collected in the presence of drug were normalized to currents recorded in the absence of drug. This

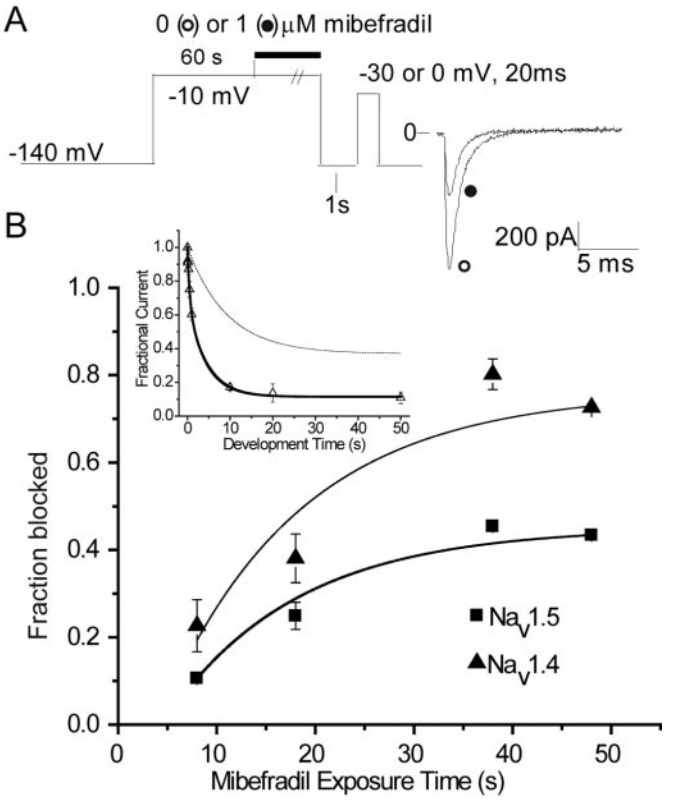

Fig. 8. Mibefradil interacts with slow-inactivated $Na_v1.5$ and $Na_v1.4$ channels. A, to evaluate mibefradil's interaction with slow-inactivated state(s), 1 μM mibefradil was selectively applied to cells after a 50- to 60-s depolarization to −10 mV using rapid perfusion system (see *Materials and Methods*). Mibefradil was removed 2 s before a brief (1 s) hyperpolarization to −140 mV and subsequent test pulse to −30 mV. Representative current traces are shown before (○) and after (●) application of 1 μM mibefradil. B, inset, development of slow inactivation at −10 mV for $Na_v1.4$ (△) using the protocol described in Fig. 7A. The dashed line represents the best fit single exponential line for $Na_v1.5$ development data at −10 mV (shown in Fig. 7A) except a test pulse to 0 mV (instead of −30 mV) was used. Using the same protocol diagrammed in A, the mibefradil exposure time was varied to determine the time course for the development of mibefradil block for $Na_v1.5$ (■) and $Na_v1.4$ (△) slow-inactivated channels, which could be described by a single exponential for both isoforms: $Na_v1.5$, $\tau = 13.2 \pm 3.1$ s ($n = 1$–3); $Na_v1.4$, $\tau = 14.0 \pm 5.1$ s ($n = 1$–6). The degree of block at the two longest exposure durations were statistically different between $Na_v1.5$ and $Na_v1.4$, $\tau\ p < 0.05$.

method allowed a direct calculation of the preference of mibefradil for the slow-inactivated state(s) present after a 60-s depolarization. Summary data are shown in Fig. 8B. As the duration of exposure to 1 μM mibefradil was increased, fractional block increased with a single exponential time course with a time constant of 13 s. Maximal block was evident after 35 s, reaching 45%. These data suggest that mibefradil has affinity for one or more slow-inactivated states in which channels reside during prolonged (60–100 s) depolarization.

To more definitively correlate mibefradil block with the presence of slow-inactivated channel states, we examined the interaction of mibefradil with slow-inactivated $Na_v1.4$ channels, an isoform shown to slow-inactivate to a greater extent than $Na_v1.5$ (Richmond et al., 1998; O'Reilly et al., 1999). Supporting the previously published data, under our experimental conditions, $Na_v1.4$ channels expressed in HEK cells developed slow inactivation more quickly and completely than $Na_v1.5$ at −10 mV. Approximately 88% of channels developed slow inactivation with a time course of development that was best described by a double exponential (Fig. 8B, inset): a faster time constant of 536 ms and a longer time constant of 4.4 s, representing 37% and 63% of the total, respectively. The greater proportion of channels residing in one or more slow-inactivated states during a prolonged depolarization in $Na_v1.4$ compared with $Na_v1.5$ predicts that mibefradil should block $Na_v1.4$ currents to a greater extent when drug is selectively applied to slow-inactivated channels. After this prediction, block of slow-inactivated $Na_v1.4$ channels with 1 μM mibefradil was approximately 2-fold greater than $Na_v1.5$, developing with a single time constant of 14 s. These data provide further evidence that mibefradil has affinity for slow-inactivated states present during prolonged depolarizations.

Discussion

Mibefradil has long been considered an intriguing compound based on its therapeutic effectiveness and its high affinity for T-type Ca^{2+} channels. We report here that mibefradil blocks multiple isoforms of Na^+ channels with micromolar affinity that is similar to that reported for other non–T-type channels (Nilius et al., 1997; Chouabe et al., 1998; Liu et al., 1999; Perchenet and Clement-Chomienne, 2000). There were small differences in fractional block across isoforms, which could reflect actual small differences in affinities; on the other hand, they could reflect differences in gating between isoforms that were incompletely accounted for with our protocols. Data for $Na_v1.5$, $Na_v1.4$, $Na_v1.2$, and $Na_v1.7$ were also qualitatively similar to the previous report of mibefradil block of I_{Na}, in which 1 μM mibefradil was sufficient to block greater than 50% of Na^+ current in GH3 cells from a holding potential of −80 mV (Eller et al., 2000), a cell type for which the isoform responsible for the Na^+ current is not known. Our results are also consistent with the submicromolar affinity reported for $Na_v1.5$ expressed in HEK cells (Guatimosim et al., 2001) at −100 mV. The similarity in the action of mibefradil across isoforms suggests a common binding motif is formed. It is interesting that this IC_{50} in the micromolar range is actually lower than the IC_{50} for the classic Na channel blocker, lidocaine, which blocks current in the 20 to 200 μM range (Makielski and Falleroni, 1991; Hanck et al., 2000), and the kinetics of the development of and recovery from mibefradil block is markedly slower compared with lidocaine.

Many ion channel drugs exhibit preferential block of one or more channel state(s) over others. For the Na^+ channel, rested state, or closed channel, block by mibefradil was apparent, although even low frequency stimulation increased block, suggesting that channel states visited during depolarizations reveal higher affinity conformations compared with the closed state in which channels reside at negative potentials. Furthermore, modest depolarization increased the affinity of mibefradil by approximately 6-fold. Mibefradil inhibition of the T-type Ca^{2+} channel, $Ca_v3.2$, (Martin et al., 2000) and of native T channels in rat cerebellar Purkinje neurons (McDonough and Bean, 1998) and guinea pig atrial myocytes (Benardeau and Ertel, 1998) was also dependent on holding potential. Such dependence on holding potential has been traditionally interpreted to indicate that the drug preferentially targets inactivated channels.

Fast inactivation of Na^+ channels occurs rapidly (within milliseconds) after depolarization and involves binding of a motif (IFM) in the domain III-IV linker (West et al., 1992). Given, however, that a 20-fold increase in the time channels spent fast-inactivated in pulse trains did not affect mibefradil block, it seems unlikely that mibefradil targeted the fast-inactivated conformation. Similar insensitivity to pulse duration was also reported for I_{Na} in GH3 cells (Eller et al., 2000). A more stringent test of whether mibefradil block was affected by fast inactivation was devised in which fast inactivation was inhibited in lid-deficient Na^+ channels. For these channels mibefradil block, both in duration and time course, were like WT, dramatically demonstrating that mibefradil did not preferentially target the fast-inactivated state. Moreover, these surprising data also indicate that mibefradil must not preferentially target a closed state or states close to the open state or the open state itself, because after removal of the lid these states are more preferentially visited during depolarizations. This result was particularly surprising given that removal of the fast-inactivation gate profoundly alters the affinity of other Na^+ channel blockers. For example, removal of fast-inactivation in cardiac Na^+ channels by either limited proteolysis or site-directed mutagenesis of the IFM motif resulted in a greater than 10-fold reduction in lidocaine affinity (Bennett et al., 1995). Likewise, mutations of the interdomain III-IV linker also abolished the high affinity component of cocaine inhibition of cardiac Na^+ channels (O'Leary and Chahine, 2002). Therefore, mibefradil not only interacts with Na^+ channels with a higher apparent affinity than local anesthetics, but it does so with a distinct mechanism and, perhaps, at a different site.

In addition to fast inactivation, Na^+ channels also slow inactivate (i.e., with prolonged depolarization, Na^+ channels progressively occupy one or more relatively stable conformations from which development and recovery kinetics are on the order of several seconds) (for review, see Balser, 2001; Goldin, 2003). Although the molecular details of slow inactivation are unclear, available data suggest that slow inactivation does not depend on the same structural components (i.e., DIII-IV IFM motif) as fast inactivation. Under our experimental conditions, $Na_v1.5$ channels expressed in HEK cells developed and recovered from slow-inactivation with similar kinetics, as has been previously reported (Townsend and Horn, 1997; O'Reilly et al., 1999; Vilin et al., 1999). Our

data also are consistent with published evidence demonstrating development of slow inactivation at potentials negative to channel opening. Small quantitative differences between our data and previously published data can probably be accounted for by differences in experimental conditions, such as choice of expression system and/or the concentration of external Na^+ ions, both of which have been shown to influence the kinetics and degree of entry into slow-inactivated states.

Recent studies have also suggested slow-inactivated channel conformations may serve as important substrates for drug binding. A link between components of Na^+ channel slow inactivation and use-dependent lidocaine action, for example, has been proposed (Kambouris et al., 1998; Chen et al., 2000). Therefore, we chose to determine whether mibefradil interacts with slow-inactivated Na^+ channels using a rapid drug perfusion exchange system and selectively exposing channels slow-inactivated by a 60-s depolarization to mibefradil. Consistent with this possibility, mibefradil blocked maximally slow-inactivated $Na_v1.5$ channels in the low micromolar range. Skeletal muscle ($Na_v1.4$) channels slow-inactivate more completely than the heart isoform (Richmond et al., 1998; O'Reilly et al., 1999; current study), and mibefradil blocked maximally slow-inactivated $Na_v1.4$ channels to a greater extent than $Na_v1.5$. This enhanced block of Nav1.4 is not likely to be a result of isoform-specific differences in the mibefradil binding site, given that a similar affinity was observed when block was measured from more negative holding potentials (Fig. 3), potentials at which the extent of slow inactivation has been shown to be similar between the two isoforms (Richmond et al., 1998; O'Reilly et al., 1999). Rather, the degree of block correlated with the extent of slow inactivation, and this raises the possibility that the slow rate of development of block in pulse trains results from interaction with slow-inactivated channels. Because Na^+ channels do not fully slow-inactivate, it is possible that development of block results from slow binding to one or more activated and/or inactivated conformations. Further experiments are necessary to definitively identify the highest affinity conformation.

Because slow inactivation can develop at potentials negative to channel opening, and mibefradil blocks slow-inactivated channels in the low to submicromolar range, it is tempting to speculate that the higher apparent affinity of mibefradil for channels modestly (−100 or −90 mV) depolarized is caused by preferential binding of mibefradil to slow-inactivated states occupied at those potentials. It is not possible, however, to calculate the affinity for slow-inactivated states from these data because neither $Na_v1.4$ nor $Na_v1.5$ channels completely slow inactivate. In addition, even with a 100-s depolarization, channels continue to occupy multiple nonconducting channel conformations, and mibefradil could potentially interact with some of these or affect occupancy indirectly by mass action. Multiple kinetically distinct, but overlapping, types of slow inactivation have been identified and characterized [intermediate, slow, and ultraslow (for review, see Balser, 2001)], and there is evidence that drugs can exhibit varying affinity for the different components of slow inactivation. Lidocaine, for example, has a seemingly higher affinity for the inactivated state with "intermediate kinetics" (I_M) than for the classic "slow" inactivated state (Kambouris et al., 1998). With these caveats in mind, however, and if we make the assumption that mibefradil is only interacting with the channels that have developed into some form of a slow-inactivated conformation during a 100-s pulse to −10 mV (62% for $Na_v1.5$; 88% for $Na_v1.4$), we can make a prediction regarding the affinity of mibefradil for slow-inactivated channels by dividing the fractional block measured at the end of a prolonged depolarization (0.45 for $Na_v1.5$; 0.76 for $Na_v1.4$, from Fig. 8) by the fraction of channels slow-inactivated during that depolarization in absence of drug (0.62 for $Na_v1.5$; 0.88 for $Na_v1.4$, from Fig. 7). Using this method, the affinity of mibefradil for slow-inactivated channels is in the range of 0.15 μM (for $Na_v1.4$) to 0.38 μM (for $Na_v1.5$), which is near the apparent affinities estimated from block of modestly depolarized and frequently depolarized channels.

Although none of our data supports preferential binding of mibefradil to the open state, it should not be concluded that mibefradil does not bind to open channels. Single channel analysis of recombinant T-type Ca^{2+} channels and native hMTC T-type channels have demonstrated a slight shortening of open time upon exposure to mibefradil, implicating open channel block (Michels et al., 2002). The same studies, however, also revealed a reduced fraction of active sweeps suggesting the existence of a kinetically different and long-lived (>150 ms) channel-drug interaction. Therefore, it is likely that at least two mechanisms of block are involved, although preferential interaction with the open state cannot be responsible for mibefradil's higher affinity at depolarized potentials because the proportion of channels and their residency time in the open state is the same during both protocols testing the effect of holding potential. In addition, a drug selectively targeting the open state would have only a brief opportunity to access its receptor during depolarization before the "hinged lid" inactivated the channel. If this were the case for mibefradil, disabling inactivation via covalent modification of the inactivation gate motif (Nav1.5 ICM + MTSET) should have increased the degree of block because mibefradil would have a greater and longer-lived access to its open channel binding site. We did not observe a difference in block; therefore, the open state is probably not mibefradil's highest affinity target.

All together, our data support a model whereby state-dependent mibefradil block of Na^+ channels is not caused by binding to fast-inactivated or open channels but rather to one or more slow-inactivated states more prevalent at more positive voltages. The dose range and novel state-dependent profile of mibefradil block makes it well suited for structure/function studies of Na^+ channels and potentially for identification of common binding motifs between ion channels and in determination of how drug binding is affected by conformational changes during channel gating.

Acknowledgments

We thank Constance Mlecko, Gayle Tonkovich, and J. R. Liu for technical assistance and John Kyle and Harry Fozzard for helpful discussion.

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