Effect of mibefradil on sodium and calcium currents

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Interstitial cells of Cajal (ICC) generate the electrical slow wave. The ionic conductances that contribute to the slow wave appear to vary among species. In humans, a tetrodotoxin-resistant Na$^+$ current (Nav1.5) encoded by SCN5A contributes to the rising phase of the slow wave, whereas T-type Ca$^{2+}$ currents have been reported from cultured mouse intestine ICC and also from canine colonic ICC. Mibefradil has a higher affinity for T-type over L-type Ca$^{2+}$ channels, and the drug has been used in the gastrointestinal tract to identify T-type currents. However, the selectivity of mibefradil for T-type Ca$^{2+}$ currents over ICC and smooth muscle Na$^+$ channels has not been clearly demonstrated. The aim of this study was to determine the effect of mibefradil on T-type and L-type Ca$^{2+}$ and Na$^+$ currents expressed in HEK-293 cells. Whole cell currents were recorded from HEK-293 cells coexpressing green fluorescent protein with either the rat brain T-type Ca$^{2+}$ channel $\alpha_{1C}3.3b + \beta_2$, the human intestinal L-type Ca$^{2+}$ channel subunits $\alpha_1$ and $\beta_2$, or Nav1.5. Mibefradil significantly reduced expressed T-type Ca$^{2+}$ current at concentrations $\geq 0.1$ μM (IC$_{50}=0.29$ μM), L-type Ca$^{2+}$ current at $> 1$ μM (IC$_{50}=2.7$ μM), and Na$^+$ current at $\geq 0.3$ μM (IC$_{50}=0.98$ μM). In conclusion, mibefradil inhibits the human intestinal tetrodotoxin-resistant Na$^+$ channel at submicromolar concentrations. Ca$^{2+}$ channels are expressed in several ion channel classes are coexpressed.

MATERIALS AND METHODS

Expression vector construction and HEK-293 transfection. The expression vectors containing the human jejunal L-type Ca$^{2+}$ channel $\alpha_{1C}$ (α1,2)- and β-subunits, the rat brain T-type Ca$^{2+}$ channel $\alpha_3$,β3b-subunit, or the human jejunal Na$^+$ channel SCN5A α-subunit were described previously (14, 17, 18). The $\alpha_3$,β3b-subunit clone was a gift from Dr E. Perez-Reyes. Using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA), the green fluorescent protein (GFP) pEGFP-C1 (Clontech, Palo Alto, CA), and either the sodium or L- or T-type calcium channel, expression vectors were transiently cotransfected into HEK-293 cells (American Type Culture Collection, Manassas, VA). HEK cells expressing L- or T-type Ca$^{2+}$ channel subunits were maintained for 2 days, whereas cells expressing SCN5A were cultured for 1 day posttransfection. Transfected cells were identified by fluorescent microscopy and patch clamped to record whole cell currents.

Patch clamp recordings. Patch clamp recordings were obtained at 22°C using standard whole cell techniques (5, 6). Microelectrodes were pulled from Kimble KG-12 glass on a P-97 puller (Sutter Instruments, Novato, CA). Electrodes were coated with R6101 (Dow Corning, Midland, MI) and fire polished to a final resistance of 3 to 5 MΩ. Currents were amplified, digitized, and processed using an Axopatch 200B amplifier, Digidata 1322A, and pCLAMP 8 software (Axon Instruments, Foster City, CA). Whole cell records were sampled at 10 kHz and filtered at 5 kHz with an eight-pole Bessel filter. In T-type Ca$^{2+}$ current experiments, cells were held at $-100$ mV and pulsed from $-90$ to $+35$ mV in 5-mV intervals for 150 ms each. To record L-type Ca$^{2+}$ current, cells were held at $-100$ mV and pulsed from $-60$ to $+40$ mV in 5-mV intervals for 50 ms each. For Na$^+$ current experiments, we used a holding potential of between $-100$ to $-75$ mV and stepped from $-80$ to $+35$ mV in 5-mV intervals for 50 ms each. Most cells were held at $-100$ or $-90$ mV, but when the Na$^+$ currents were too large to be recorded accurately, the holding voltage was changed to a more depolarized voltage. Three cells were held at $-80$ mV and two cells at $-75$ mV. A 70–80% series resistance compensation (lag of 60 μs) was applied during each recording. Cell capacitance of HEK-293 cells transfected with $\alpha_3$,β3b-β was $23 \pm 4$ pF (n = 12), α$\alpha_{1C}$ + β2-β was $19 \pm 3$ pF (n = 10), and SCN5A was $20 \pm 2$ pF (n = 18). The differences in cell capacitance between these groups were not significant ($P > 0.05$). Resistance after access was established was 5–10 MΩ.

Drug and solutions. Intracellular solution contained in (mM) 130 Cs$^+$, 125 methanesulfonate, 20 Cl$^-$, 5 Na$^+$, 5 Mg$^2+$, 5 HEPES, 2 EGTA, 2.5 ATP, and 0.1 GTP. Extracellular solution was normal Ringer solution (in mM): 149.2 Na$^+$, 159 Cl$^-$, 4.74 K$^+$, 2.54 Ca$^{2+}$, 5 HEPES, and 5 glucose, and replaced with increasing concentrations.

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of mibefradil 0, 0.03, 0.1, 0.3, 1, 3, and 10, and back to the control solution without the drug (Sigma-Aldrich, St. Louis, MO). Up to three concentrations were applied to any one cell. The bath chamber was rinsed with 1.5 ml NaCl Ringer + mibefradil at 10 ml/min for ~9 s. Two recordings were taken at each concentration immediately after rinse and 2 min later. Intra- and extracellular solutions were equilibrated to pH 7.0, 300 mmol/kg and pH 7.35, 300 mmol/kg, respectively. We did not normalize for rundown in our experiments. We did not apply more than three concentrations to a particular cell to avoid long experiments. We have not observed rundown in cells transfected with SCN5A. Rundown for the L-type Ca2+ current was about 15% over 10 min after peak inward current.

Data analysis. Data were analyzed using Clampfit (Axon Instruments, Union City, CA), Excel (Microsoft, Redmond, WA), and SigmaPlot (SPSS, Chicago, IL). For current-voltage graphs, the peak inward current of a single control trace was normalized to one using the equation $I_{\text{norm}} = -1 \times (I/I_{\text{max}})$, where $I_{\text{norm}}$ is normalized current, $I$ is measured current at a given voltage, and $I_{\text{max}}$ is maximum peak inward current of the control trace. Thus peaks of all other traces per cell were expressed as a fraction of 1. Statistical comparisons were made using a paired, two-tailed Student’s t-test. Statistical significance was accepted when $P$ values were $< 0.05$. Data are expressed as means ± SE. Sigmoidal dose-response curves were built in SigmaPlot by inserting normalized peak current data into the equation:

$$y = \min + (\max - \min)/[1 + 10^{\log EC_{50} - x/\text{Hillslope}}].$$

RESULTS

Effect of mibefradil on heterologously expressed T-type Ca2+ channel subunit $\alpha_{3.3b}$. To compare the selectivity of mibefradil against L-type Ca2+ or Na+ currents, we first determined its effects on the T-type current under the same recording conditions. After coexpressing GFP with rat brain T-type Ca2+ channel $\alpha$-subunit $\alpha_{3.3b}$ and a Ca2+ channel $\beta_2$-subunit in HEK-293 cells, we recorded standard whole cell currents (Fig. 1). Adding extracellular solution containing 0.03 M mibefradil did not change expressed T-type Ca2+ current ($-804 ± 180$ pA 0 μM control, to $-752 ± 192$ pA 0.03 μM, 8 ± 7% decrease, $n = 6$, $P > 0.05$). However, 0.1 μM mibefradil significantly reduced T-type Ca2+ current by 24 ± 6% compared with controls ($-485 ± 143$ pA 0 μM control, to $-398.5 ± 126$ pA 0.1 μM, $n = 6$, $P < 0.05$). Subsequent additions of 0.3, 1, 3, and then 10 μM mibefradil further reduced the current by 52 ± 5, 75 ± 7, 94 ± 1, and 98 ± 4%, respectively, compared with predrug ($-644 ± 119%$ pA control, to $-395 ± 111$ pA 0.3 μM, to $-131.5 ± 52$ pA 1 μM, to $-48 ± 9$ pA 3 μM, and $-8 ± 2$ pA 10 μM, $n = 6$ for each concentration, $P < 0.05$ at 0.1–10 μM). Only 10 ± 3% of the control current returned 2 min after washing out mibefradil with multiple exchanges of fresh Ringer solution ($-78 ± 25$ pA, $n = 6$, $P < 0.05$ compared with predrug).

Effect of mibefradil on heterologously expressed human jejunum L-type Ca2+ channel subunit. Previous studies testing the effects of mibefradil on L-type Ca2+ channels used Ba2+ instead of Ca2+ as the charge carrier (summarized in Ref. 15). To make a direct comparison between the doses necessary to block Ca2+ current of T- and L-type channels, we used Ca2+ in the extracellular solution. HEK-293 cells were transfected with the human jejunum L-type subunits $\alpha_{1C}$ and $\beta_2$ (Fig. 2), and whole cell currents were measured. Concentrations of 0.1 or 0.3 μM mibefradil did not significantly change L-type Ca2+ current [-157 ± 39 pA control; to $-151 ± 36$ pA at 0.1 μM (1 ± 6% decrease), to $-147 ± 40$ pA at 0.3 μM (9 ± 6% decrease), $n = 5$; $P > 0.05$]. In separate experiments adding 1 μM mibefradil to the extracellular solution resulted in a non-

![Fig. 1](image-url)
A 10% decrease in L-type Ca$^{2+}$ current by the Na$^+$ channel SCN5A (IC$_{50} = 0.29 \mu$M), followed by the Na$^+$ channel SCN5A (IC$_{50} = 0.98 \mu$M), and the L-type Ca$^{2+}$ channel $\alpha_{1C} + \beta_2$ (IC$_{50} = 2.7 \mu$M).

**DISCUSSION**

Coordinated gastrointestinal motility requires a complex interaction between several cell types, including nerves, smooth muscle cells, and ICC. Different classes of ICC are present in the gastrointestinal tract and appear to subserve different functions. At least three different functions have been ascribed to ICC (1, 9, 20, 23–25). ICC act to modulate neuronal signals to smooth muscle cells (1, 23, 25), may serve as mechanotransducers (20), and generate and amplify the pacemaker signal and the slow wave (9, 24). The slow wave recorded from smooth muscle cells originates from ICC and is
an important determinant of smooth muscle function, with the frequency of the slow wave setting the frequency of smooth muscle contraction, and the amplitude of the slow wave contributing to the control of the strength of smooth muscle contractions. The rhythmic depolarization of smooth muscle membrane potential opens L-type Ca\textsuperscript{2+} channels expressed in smooth muscle allowing Ca\textsuperscript{2+} entry and initiation of contractions (4).

Ionic conductances that underlie the pacemaker signal and upstroke of the slow wave are not well understood and appear to vary between species. In cultured mouse small intestinal ICC, both chloride channels (10, 26) and Ca\textsuperscript{2+}-permeable nonselective cation channels of the TRP family have been proposed as the pacemaker channel (22). In dogs (13) and mice (11), a T-type channel has been reported to be expressed in ICC and in humans, a Na\textsuperscript{+} channel (20). The Na\textsuperscript{+} channel is not the pacemaker channel but contributes to the upstroke of the slow wave and its regulation by stretch (19, 20). With several different candidates with overlapping electrophysiological properties, it is difficult to interpret intact muscle strip experiments, yet these experiments are necessary to integrate information obtained at a channel level often under culture conditions that may alter the expression of ion channels. Pharmacological and electrophysiological experiments at the muscle strip level are therefore often needed, and drugs that discriminate between the candidate ion channels are required. QX314 is a quaternary membrane-impermeant derivative of lidocaine that is used as a Na\textsuperscript{+} channel blocker (3, 7, 21) and when applied externally has different effects on neuronal and cardiac Na\textsuperscript{+} channels, including Nav1.5 (2, 8, 20). QX314 is therefore useful to discriminate between nonselective cation channels, Ca\textsuperscript{2+}-selective channels, and Na\textsuperscript{+} channels; however, QX314 at 500 nM also inhibits about 30% of T-type Ca\textsuperscript{2+} current (unpublished data) and its mechanism of action...
requires a prolonged application time (8, 20). Mibebradil has been used as a selective T-type Ca\(^{2+}\) channel blocker (11, 12), and the data provided in this report and the literature (summarized in Ref. 15) suggests that at concentrations below 1 \(\mu\)M it indeed can discriminate between \(\alpha_{1C}\)-L-type and the \(\alpha_{1,3,3b}\)-T-type Ca\(^{2+}\) channels.

Published data for the IC\(_{50}\) (in 2 mM Ca\(^{2+}\)) for \(\alpha_{1,3.1}\) (\(\alpha_{1G}\), 0.27 \(\mu\)M) and \(\alpha_{1,3.2}\) (\(\alpha_{1H}\), 0.14 \(\mu\)M) also suggest that mibebradil can discriminate between T-type Ca\(^{2+}\) channels containing other \(\alpha\)-subunits and \(\alpha_{1C}\)-L-type Ca\(^{2+}\) channels (15). However, the data from this report and a recently published report (16) also suggest that, with an IC\(_{50}\) of 0.29 \(\mu\)M for the \(\alpha_{1,3.3b}\)-T-type Ca\(^{2+}\) channel and an IC\(_{50}\) of 0.98 \(\mu\)M for Na\(_{1.5}\), mibebradil cannot be used to discriminate between the two channel types, and caution must be used in the interpretation of the effects of mibebradil as a blocker of T-type Ca\(^{2+}\) channels when, as is nearly always the case, more than one channel type is expressed in a given cell type.

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