Mibefradil Inhibition of T-Type Calcium Channels in Cerebellar Purkinje Neurons

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ABSTRACT

The antihypertensive agent mibefradil completely and reversibly inhibited T-type calcium channels in freshly isolated rat cerebellar Purkinje neurons. The potency of mibefradil was increased at less hyperpolarized holding potentials, and the apparent affinity was correlated with the degree of channel inactivation. At 35°C, the apparent dissociation constant $K_{a}$ was 1 μM at a holding voltage of −110 mV (corresponding to noninactivated channels) and 83 nm at a holding voltage of −70 mV (corresponding to 65% inactivation). The increased affinity was attributable mainly to a decreased off-rate. Mibefradil also inhibited P-type calcium channels in Purkinje neurons, but inhibition was much less potent. At a holding potential of −70 mV, the $K_{a}$ for mibefradil inhibition of P-type channels was ~200-fold higher than that for inhibition of T-type channels. Mibefradil should be a useful compound for distinguishing T-type channels from high voltage-activated calcium channels in neurons studied in vitro.

Voltage-dependent calcium channels in neurons have diverse functions, including crucial roles in excitability (Huguenard, 1996), neurotransmission (Dunlap et al., 1995), activation of intracellular signaling pathways, and regulation of gene expression (Ghosh and Greenberg, 1995). Multiple types of voltage-gated calcium channels in neurons have been distinguished by kinetics, voltage dependence, single-channel properties, and pharmacological characteristics (Tsien et al., 1995). Some types of calcium channels distinguished in native cells have been convincingly identified with particular gene products, namely N-type channels with α1B subunits, L-type channels with α1C and α1D subunits, and P-type and Q-type channels (which have many similarities) with α1A gene products (reviewed by Hofmann et al., 1994; Mori, 1994; Dunlap et al., 1995; Randall, 1998). The α1E gene encodes the “R-type” current in central neurons (Zhang et al., 1993; Wakamori et al., 1994; Randall and Tsien, 1997), but the correspondence is less certain because of a lack of distinguishing pharmacological agents.

Of native calcium channels, T-type (low-voltage-activated) channels are the most distinctive in terms of voltage dependence, kinetics, and single-channel properties (Tsien et al., 1995; Huguenard, 1996; Erkel and Erkel, 1997). Such channels, which are characterized by rapid inactivation kinetics, inactivation at moderately depolarized holding potentials, slow deactivation kinetics, and small single-channel conductances, are present in a wide range of excitable cells, including neurons, neuroendocrine cells, cardiac muscle, and smooth muscle, as well as some nonexcitable cells (reviewed by Erkel et al., 1997). Recently, Perez-Reyes and colleagues (1998) discovered a new calcium channel α1 subunit, termed α1G, that seems to correspond to T-type channels. Although no pharmacological correspondence has been established, currents from the cloned channels have the distinctive fast inactivation, slow deactivation, and small single-channel conductance characteristic of native T-type channels.

The identification of the new clone will bring renewed attention to T-type channels, including investigations of their pharmacological characteristics. There is currently no peptide toxin for T-type channels comparable to those targeting N-type or P-type channels, and there are no organic molecules with potency and selectivity comparable to those of dihydropyridines for L-type channels. Amiloride, which is mainly known as a blocker of epithelial sodium channels, is somewhat selective for T-type channels over other types of calcium channels but requires concentrations of ~1 mM for complete inhibition (Tang et al., 1988; McCobb et al., 1989; Takahashi et al., 1989). Nickel is somewhat selective for T-type current in some cell types (Fox et al., 1987) but has little selectivity in others (Regan, 1991; Huguenard, 1996).

Mibefradil (Ro 40–5967; Posicor) is an antihypertensive drug that is structurally different from other classes of calcium channel blockers (Clozel et al., 1991, 1997; Triggle, 1996). The antihypertensive action of mibefradil probably

ABBREVIATIONS: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid.

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reflects vasodilating effects (Osterrieder and Holck, 1989), which are hypothesized to result from block of calcium channels in vascular smooth muscle cells (Bian and Hersmeyer, 1993). In both vascular smooth muscle cells and cardiac muscle cells, mibebradil is more potent in blocking T-type calcium channels than L-type calcium channels (Mishra and Hersmeyer, 1994a; Bénardeau and Ertel, 1998). The half-maximal blocking concentration (EC50) for T-type channels in vascular muscle is ~100 nM (Mishra and Hersmeyer, 1994a). In contrast, mibebradil inhibition of cloned a1A, a1B, a1C, and a1E calcium channels and native L-type channels is much weaker (EC50 = 3–21 µM) (Mishra and Hersmeyer, 1994b; Bezprozvanny and Tsien, 1995; Bénardeau and Ertel, 1998). Mibebradil has not yet been tested with a1G channels.

Mibebradil has been found to inhibit T-type calcium channels in several neuronal preparations, including neuroblasto
toma cells (Randall and Tsien, 1997), sensory neurons (Todorovic and Lingle, 1998), and spinal motoneurons (Viana et al., 1997). In neurons, unlike vascular or cardiac muscle, block of T-type channels seems to be no more potent than that of various high-threshold calcium channels (Randall and Tsien, 1997; Viana et al., 1997). However, comparisons were made either across cell types or on overall calcium currents, where distinction among components may be difficult.

We investigated the action of mibebradil on cerebellar Purkinje neurons under conditions in which homogeneous T-type calcium currents could be obtained. We found that mibebradil inhibited T-type calcium channels in Purkinje neurons with greater potency, compared with all other calcium currents examined, including T-type currents in vascular muscle. The potency of mibebradil depended strongly on the holding potential; at physiological resting potentials, mibebradil eliminated T-type current with minimal effects on P-type current. Thus, mibebradil should be a useful tool for analyzing the cellular function of neuronal T-type calcium currents and for correlating expressed cDNA with native T-type currents.

Materials and Methods
Preparation of Purkinje neurons. Cerebellar Purkinje neurons were isolated from the brains of 9–16-day-old Long-Evans rats with a slight modification of previously described procedures (Mintz et al., 1992; McDonough et al., 1996). Rats were anesthetized with methoxyflurane, and the hearts were perfused with ice-cold Ca2+-free Tyrode’s solution (150 mM NaCl, 4 mM KCl, 2 mM MgCl2, 10 mM HEPES, 10 mM glucose, pH adjusted to 7.4 with NaOH) to rapidly cool the brain tissue. Cerebellar chunks were removed with fine scissors and minced with a razor blade in cold oxygenated dissociation solution (82 mM Na2SO4,30 mM K2SO4,5 mM MgCl2,10 mM HEPES, 10 mM glucose, pH adjusted to 7.4 with NaOH). Tissue was then transferred into dissociation solution with 3 mg/ml protease XXIII (Sigma) (pH readjusted to 7.4 with NaOH) and incubated at 35° for 7–8 min, under a continual stream of pure oxygen. Tissue was then transferred to dissociation solution with 1 mg/ml trypsin inhibitor (Sigma) and 1 mg/ml bovine serum albumin (Sigma) (pH adjusted to 7.4 with NaOH) and was allowed to cool to room temperature. Tissue was maintained in this solution or in Tyrode’s solution (composition as described above but with 2 mM CaCl2) for up to 8 hr, with light oxygenation. Tissue chunks were triturated in Tyrode’s solution as needed, and Purkinje neurons were identified morphologically (Regan, 1991).

Recording of calcium channel currents. Calcium channel currents were recorded using the whole-cell configuration of the patch-clamp technique, using patch pipettes made from borosilicate glass tubing (100-µl Boralex capillaries; Dynalab, Rochester, NY) and coated with Sylgard (Dow Corning Corp., Midland, MI). Pipettes had resistances of 1–3 MΩ when filled with internal solution. After a stable whole-cell recording in Tyrode’s solution was obtained, the cell was lifted off the bottom of the dish and positioned directly in front of a gravity-fed array of 12 perfusion tubes made of 250-µm (i.d.) quartz tubing (with Teflon tubing) to glass reservoirs.

Currents were recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA), filtered with a corner frequency of 5 kHz (four-pole Bessel filter), digitized at 10 kHz using a Digidata 1200 interface and pClamp6 software (Axon Instruments), and stored on a computer. Compensation (typically ~80%) for series resistance (typically ~2.5 times higher than the pipette resistance) was used. Only data from cells with current small enough to yield a voltage error of <5 mV were analyzed. Calcium channel currents were corrected for leak and capacitative currents by subtracting a scaled current elicited by a 10-mV step from the holding potential. The 10-mV offset was typically a hyperpolarizing step, except at a holding potential of ~110 mV, where it was a depolarizing step.

The intracellular (pipette) solution contained 56 mM CsCl, 68 mM CsF, 2.2 mM MgCl2, 4.5 mM EGTA, 9 mM HEPES, 4 mM MgATP, 14 mM creatine phosphate (Tris salt), and 0.3 mM GTP (Tris salt) (pH adjusted to 7.4 with CsOH). The external solution contained 160 mM tetraethylammonium chloride, 10 mM HEPES, 5 mM BaCl2, 600 nM tetrodotoxin, and 1 mg/ml cytochrome c (from horse heart; Sigma). Standard external solution also contained 1 µM nimodipine (3–5 µM nimodipine in a few experiments) to block L-type currents and, for isolation of T-type currents, 10 µM ω-conotoxin-MVIIC (Bachem California, Torrance, CA) to block P-type currents (McDonough et al., 1996). External solutions were exchanged in <1 sec by moving the cell between continuously flowing solutions from the perfusion tubes. Reported potentials were not corrected for a junction potential of ~2 mV between the pipette solution and the Tyrode’s solution in which the offset potential was zeroed before seal formation.

Most experiments were performed at 35°, at which T-type currents were consistently larger than at room temperature. Some of the increased current amplitude at holding potentials of ~80 to ~90 mV was the result of a temperature dependence of steady state inactivation (Boltzmann fit parameters for inactivation curves: 23°, V1/2 = –81 ± 0.3 mV, k = 9.5 ± 1 mV, n = 8; 35°, V1/2 = –74 ± 0.6 mV, k = 7.4 ± 0.4 mV, n = 6) (see Fig. 5). In addition, maximal currents from strongly hyperpolarized holding potentials were also consistently larger at 35° than at room temperature.

Mibebradil was the kind gift of Dr. Jean-Paul Clozel and Dr. Eric Ertel (F. Hoffmann-La Roche, Basel, Switzerland). Mibebradil was prepared as a 10 mM stock solution in water. The potency of mibebradil (tested on T-type channels in Purkinje neurons) in this stock solution changed little in approximately 1 month with storage at 4°. However, after 4–12 months of storage the potency of the drug was greatly diminished (by at least a factor of 5 after 12 months). Loss of potency occurred for powder stored at room temperature for 4 months, for powder stored at ~20° for 12 months (in a sealed vial inside a desiccator), and for a 10 mM stock solution in distilled water stored at 4°. All of the experiments reported here were performed with drug used within 5 weeks of receipt.

Determination of the dose-response relationship for mibebradil at 35° was complicated by run-down of calcium currents. As evident from Fig. 2, there was often a decline of ~10% in the magnitude of T-type current over several minutes. We estimate the resulting errors in fitted half-blocking concentrations as <10%. Values are reported as mean ± standard error.

Results
Inhibition of T-type channels by mibebradil. Cerebellar Purkinje neurons were previously found to exhibit rapidly inactivating, T-type calcium currents (Kaneda et al., 1990;
Regan, 1991; Mouginit et al., 1997), as well as more slowly inactivating, higher-threshold calcium currents that are mainly (85–100%) attributable to P-type channels, with small contributions from L-type and N-type channels (Mintz et al., 1992). To record currents from T-type channels in isolation, we used a combination of the L-type channel blocker nimodipine and the peptide toxin ω-conotoxin-MVIIC, which blocks both P-type and N-type currents (Hillyard et al., 1992; McDonough et al., 1996). As shown in Fig. 1, 10 μM ω-conotoxin-MVIIC completely blocked the slowly inactivating component of current (in the presence of 5 μM nimodipine), leaving in isolation a rapidly inactivating component of current. Such currents were present in 70 of 70 Purkinje neurons tested under these conditions (5 mM Ba$^{2+}$ as charge carrier, 35°). These currents exhibited all of the characteristics expected of T-type currents, including rapid inactivation, slow deactivation, and high sensitivity to inactivation by moderately depolarized holding potentials (complete inactivation at a holding potential of −50 mV).

The T-type current in Purkinje neurons isolated in this way was inhibited rapidly, completely, and reversibly by micromolar concentrations of mibefradil. Fig. 2 shows the nearly complete block by 2 μM mibefradil of current elicited by a step from −80 mV to −30 mV (in the continuous presence of nimodipine and ω-conotoxin-MVIIC). Inhibition developed within seconds and reversed within a few minutes. T-type current was inhibited equally well at test potentials from −60 mV to 0 mV (Fig. 3).

**Inhibition of T-type channels at different holding potentials.** The potency of mibefradil depended strongly on the holding potential (Fang and Osterrieder, 1991; Bezprozvanny and Tsien, 1995). Fig. 4 shows the dose dependence of mibefradil block of T-type current at holding potentials of −110 mV and −70 mV. The concentration required for half-maximal block was −2 μM when current was elicited from −110 mV but only −0.1 μM when current was elicited from −70 mV.

The enhanced potency of mibefradil at more depolarized holding potentials could reflect a direct effect of voltage on drug binding. Alternatively, it could reflect more potent binding of drug to inactivated states of the channel (which predominate at −70 mV, where current is approximately 70% inactivated) than to closed resting states. To distinguish between these possibilities, we quantitatively compared the potency of inhibition and the voltage dependence of inactivation. The potency of inhibition was determined for a range of potentials from −110 mV to −70 mV, using concentrations of mibefradil of 20 nM to 10 μM. The dose-response relationship for inhibition could be fit reasonably well by the equation $1/(1 + [\text{mibefradil}]/K_{\text{app}})$, the relationship expected for 1:1 binding of drug to receptor (Fig. 5). Values for $K_{\text{app}}$ increased with more hyperpolarized holding voltages and approached saturation at holding voltages of −100 mV (849 nM) and −110 mV (1000 nM).

If the voltage dependence of mibefradil potency arises from different affinities of drug binding to inactivated versus resting states of the channel, it should be possible to predict how the potency would vary with voltage. Considering only a single resting state and a single inactivated state, the apparent affinity of drug binding (i.e., the inverse of the apparent dissociation constant or $1/K_{\text{app}}$) would be a weighted average of the affinities for binding to the resting state ($1/K_R$) and to the inactivated state ($1/K_I$), weighted by the fraction of channels in each of those states in the absence of drug, i.e., $1/K_{\text{app}}$.
was not obviously different at different voltages (data not shown). However, the rate of inhibition may not reflect only the interaction between mibefradil and the channel, because as the mibefradil concentration was increased 100-fold, from 500 nM to 5 μM, the time constant of inhibition decreased only modestly, from 8.2 to 2.7 sec (n = 3; tested at a holding potential of −100 mV), and the relationship between the rate of inhibition and the drug concentration was highly nonlinear. One possibility is that the onset of mibefradil inhibition is limited by a step such as mibefradil partitioning into the membrane, rather than interaction with the channel. Thus, we cannot rule out the possibility that the on-rate for drug binding to the channel is voltage-dependent.

The rate of recovery from mibefradil inhibition clearly did vary with membrane potential. In the experiment shown in Fig. 7, A and B, after a brief application of mibefradil (5 μM for −4 sec) recovery was much faster at −100 mV (τ = 19 sec) than at −80 mV (τ = 42 sec). In combined results from multiple cells measured with the same protocol (Fig. 7C), the effective rate constant for recovery increased approximately 8-fold from −70 mV (0.009 ± 0.003 sec⁻¹) to −110 mV (0.07 ± 0.005 sec⁻¹). This suggests faster unbinding of drug from resting channels than from inactivated channels. The 8-fold change in the off-rate can account for most of the change in the apparent dissociation constant between −70 and −110 mV (−12-fold) (Fig. 5). The effective rate constant for recovery from block probably does not accurately measure the actual unbinding of drug molecules, because the rate of recovery depended somewhat on the mibefradil concentration and the length of exposure. For example, in one cell the rate of recovery exhibited predominant time constants of 31, 46, and 53 sec after application of 5 μM mibefradil for 4, 14,
or 34 sec (measured at −90 mV). Also, for the longest application, washout was clearly nonexponential. Reversal after prolonged mibebradil application may be slowed because of accumulation of mibebradil within the membrane or the cell. For this reason, the results in Fig. 7 were determined with short (~4-sec) exposures.

**Selectivity for T-type over P-type channels in Purkinje neurons.** We directly compared the potency of mibebradil at T-type channels and P-type calcium channels. Fig. 8 shows combined T-type and P-type currents recorded from a Purkinje neuron (with no ω-conotoxin-MVIIC in the external solution) at 35°C, with a holding voltage of −70 mV. Physiologically, the resting potential of Purkinje neurons is typically positive to −70 mV (Raman and Bean, 1997). The transient current, corresponding to T-type current, was abolished by 500 nM mibebradil, but the steady state current, corresponding to P-type current, was inhibited only ∼7% at a test voltage of −20 mV. The effects of mibebradil were fully reversible (data not shown).

Inhibition of P-type currents was characterized further at room temperature, where currents through P-type channels were more stable than at 35°C. P-type currents were recorded at a holding voltage of −70 mV to inactivate most T-type currents and were measured near the end of test pulses to avoid the small remaining T-type currents. Sequential applications of increasing concentrations of mibebradil to P-type channels at a holding potential of −70 mV showed a half-blocking concentration of ∼3 μM (Fig. 9A). For comparison, the dose-response relationship for inhibition of T-type currents was determined under identical conditions (except with 10 μM ω-conotoxin-MVIIC included in the external solution). Mibebradil potency for T-type currents was somewhat higher at room temperature, with a half-blocking concentration of ∼20 nM, than at 35°C (half-block by ∼85 nM). Fig. 10 shows combined dose-response results for block of P-type current and T-type current at −70 mV at room temperature. Curves were fitted to the equation $1/(1 + [mibebradil]/K_{app})$; best fits gave $K_{app}$ values of 3 μM for mibebradil at P-type channels and 14 nM for mibebradil at T-type channels. Thus, the selectivity of mibebradil for T-type over P-type channels was ∼200-fold, when currents were measured under the same conditions.

![Fig. 6. Comparison of the voltage dependence of $K_{app}$ with the voltage dependence of channel inactivation. A, Inactivation of T-type channels as a function of holding potential. Peak currents evoked by a 30-msec test pulse to −30 mV were measured after 4 sec at the indicated holding potential and were normalized to the current elicited at −110 mV ($n$ = 6 cells). The fraction of channels not inactivated ($h$) is plotted versus holding voltage. The fitted curve is described by the equation $h = 1/[1 + \exp(V_{hold} - V_{1/2})/k]$, with $V_{1/2} = −75$ mV and $k = 7.3$ mV. B, $K_{app}$ as a function of holding potential. $K_{app}$ values were taken from Fig. 5. The fitted line is the best fit to the equation $K_{app} = 1/[h/K_{p} + (1 - h)/K_{i}]$, with $h$ as in A, $K_{p} = 1.2$ μM, and $K_{i} = 77$ nM. R, resting state; I, inactive state; M, mibebradil. Measurements were made at 35°C.](image)

![Fig. 7. Rates of recovery from mibebradil inhibition at two different holding potentials. A and B, Recovery from inhibition by 5 μM mibebradil applied for −4 sec, measured at holding voltages of −100 mV (A) and −80 mV (B), in the same cell. Current values are peak currents from a 30-msec pulse to −30 mV. Traces at right, currents before mibebradil, 4 sec after mibebradil application, and after recovery. Curves, single-exponential function fits to the recovery phase of the time course, with time constants of 19 sec (−100 mV) and 42 sec (−80 mV). C, Rate of recovery ($1/\tau_{off}$) plotted versus holding voltage. $\tau_{off}$ was measured after application of 5 μM mibebradil for −4 sec. The number of measurements for each voltage was as follows: −70 mV, $n$ = 3; −80 mV, $n$ = 5; −90 mV, $n$ = 3; −100 mV, $n$ = 5; −110 mV, $n$ = 4. Data are from a total of eight cells. Measurements were made at 35°C.](image)
Discussion

Voltage dependence and kinetics. Our results show that mibefradil produces potent and selective block of T-type calcium channels in cerebellar Purkinje neurons. Any quantitative consideration of the potency or selectivity of mibefradil must take into account the pronounced voltage dependence of its action, whereby inhibition is more powerful at less hyperpolarized holding voltages. This voltage dependence seems to reflect voltage-dependent gating of the channel and preferential binding of drug molecules to particular gating states. In particular, the voltage dependence of drug potency closely mirrors the voltage dependence of inactivation, suggesting high affinity binding to inactivated states of the channel.

The strong voltage dependence of mibefradil potency in Purkinje neurons contrasts with the mild voltage dependence (and weaker block) of T-type channels in neuroblastoma cells (Randall and Tsien, 1997) and other cell lines (Mehrke et al., 1994). Interestingly, little or no voltage dependence for block of calcium channels was also reported for vascular muscle (Mishra and Hermsmeyer, 1994b). In contrast, there was strong voltage dependence for inhibition of cardiac T-type channels (Benardeau and Ertel, 1998), similar to our results. Further characterization of the molecular biological characteristics of T-type channels should reveal whether the differences in the voltage dependence of block among various cell types are correlated with expression of different gene products or splice variants. The 15-fold difference in affinity for inactivated versus resting channels suggested by our data (Fig. 7C) can be compared with the 30–70-fold differences seen for mibefradil block of cloned α1A, α1B, α1C, and α1E channels (Bezprozvanny and Tsien, 1995), although the absolute potency of the drug at those channels was 1 order of magnitude lower than that at T-type channels in Purkinje neurons.

The tighter binding of drug to inactivated channels is at least partly attributable to a slower rate of dissociation from inactivated channels, because the apparent off-rate was 8-fold faster with a holding voltage of −110 mV (resting channels) than with a holding voltage of −70 mV (mostly inactivated channels). A similar difference in the apparent off-rate was found for cloned α1A, α1B, α1C, and α1E channels (Bezprozvanny and Tsien, 1995), consistent with fundamental similarities in the basis of the voltage dependence of mibefradil. In our experiments, the weak dependence of the apparent on-rate on the mibefradil concentration and the concentration dependence of the apparent off-rate suggest that the situation is more complicated than direct bimolecular interaction of mibefradil molecules in the extracellular space.

Fig. 8. Effect of mibefradil on a mixed population of T-type and high voltage-activated (mainly P-type) calcium currents in the same cell. Currents were evoked by a 30-msec step to the indicated test voltage from a holding potential of −70 mV, under control conditions and after steady state inhibition by 500 nM mibefradil (*). The external solution contained 1 μM nimodipine but no ω-conotoxin-MVIIC. Measurements were made at 35°C.

Fig. 9. Potency of mibefradil for T-type and P-type calcium channels under identical conditions. A, P-type currents in response to sequential application of 1, 3, 10, 30, and 100 μM mibefradil. The external solution contained 1 μM nimodipine but no ω-conotoxin-MVIIC. Currents were measured every 2 sec from the last 5 msec of a 30-msec step to 0 mV, delivered from a holding voltage of −70 mV, at 22°C. B, Peak T-type currents in response to sequential application of 20, 50, and 100 nM mibefradil. The external solution included 10 μM ω-conotoxin-MVIIC and 1 μM nimodipine to remove high voltage-activated currents. Currents were measured every 2 sec with a 30-msec step to −20 mV from a holding voltage of −70 mV. For the cell shown, inactivation of T-type channels was fit well by a Boltzmann function with $V_{1/2} = −81$ mV and slope $k = 10$ mV. Measurements were made at 22°C.
solution with binding sites. Mibefradil might gain access to its binding site by partitioning into the cell membrane. This could explain the complexities of the on and off kinetics. Given these complexities, the results are probably consistent with the voltage dependence of drug binding arising entirely from changes in the rate of unbinding.

**Potency.** The potency of mibefradil in inhibiting T-type channels in Purkinje neurons is greater than previously reported for other types of calcium channels, including T-type channels in other cell types. In previous studies of T-type currents in various neuronal cells, the concentration of mibefradil producing 40–60% block was 1 µM in neuroblastoma cells (Randall and Tsien, 1997), 3 µM in rat sensory neurons (Todorovic and Lingle, 1998), and 1 µM in spinal motoneurons (Viana et al., 1997); only mild voltage dependence was reported in these cases. We found half-block with concentrations ranging from 14 nM to 1 µM, depending on the holding potential and the temperature. Interestingly, this high potency is closer to that observed in vascular smooth muscle (half-block by ~100 nM at a holding potential of ~80 mV) (Mishra and Hermensmeyer, 1994a) than to potencies observed in other neuronal preparations. These results seem to indicate that the T-type channels in Purkinje neurons may be different from those in neuroblastoma cells, sensory neurons, or spinal motoneurons. However, in making these comparisons, the loss of potency that we noted when the drug had been stored for many months at either room temperature or 20°C must be taken into consideration. It was important that we made our measurements within approximately 1 month of receiving fresh drug; the condition of the drug may be an important variable in different studies.

**Selectivity.** Our results strongly support the idea that mibefradil is a selective blocker of T-type calcium channels, compared with other calcium channels (Clozel et al., 1997; Ertel et al., 1997; Hermensmeyer et al., 1997), and they indicate that this selectivity is also found in neurons. The direct comparison of block of T-type channels and P-type channels at the same physiological holding potential showed that concentrations of 200–500 nM could produce essentially complete inhibition of T-type channels, with <5% inhibition of P-type channels. We did not attempt to quantify the sensitivity of L-type or N-type currents, which each contribute <5–10% of the overall high-threshold current in Purkinje neurons. However, because Bezprozvanny and Tsien (1995) found that cloned α1A and α1B channels showed similar sensitivities (both similar to our measurements of native P-type channels) and that α1C channels were considerably less sensitive, there is no reason to expect that native N-type or L-type channels would be any more sensitive than P-type channels. Consistent with this, Viana et al. (1997) found relatively low sensitivity (EC50 ~ 3 µM) of L-type currents in spinal motoneurons.

**Utility.** Mibefradil is the first pharmacological agent with enough selectivity to inhibit neuronal T-type currents without significantly affecting other types of calcium channels. It should be useful for studies of the functional roles of neuronal T-type channels, which are still incompletely understood. The lack of a high affinity ligand for T-type channels is one reason why no biochemical studies of these channel proteins have been possible. Because channels are presumably maximally inactivated in membrane preparations, they would be in the high affinity state in such preparations. Our results suggest a dissociation constant at least as low as ~15–50 nM, possibly near the range allowing radioligand studies.

Mibefradil does not cross-the blood-brain barrier, so no effects on central neurons should occur with oral administration. If mibefradil or a membrane-permeant analog did gain access to the central nervous system, it is conceivable that potent block of T-type channels might produce anticonvulsant effects similar to those of ethosuximide (Coulter et al., 1990; Huguenard, 1996). In fact, mibefradil block of neuronal T-type channels is much more potent than that by ethosuximide (or any other known blocker), and the weak activity against other calcium channel types should leave their functions (including normal neurotransmission) unperturbed.

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