Selective Inhibition of T-type Ca\(^{2+}\) Channels by Ro 40-5967

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Abstract The present study shows that the chemically novel nondihydropyridine Ca\(^{2+}\) antagonist, Ro 40-5967, blocks T-type divalent ion currents in vascular muscle cells. T-type Ca\(^{2+}\) channels were blocked selectively and completely by therapeutic concentrations of 1 to 10 \(\mu\)mol/L Ro 40-5967, at which there was only 25% to 70% block of L-type Ca\(^{2+}\) currents. Using the combination of Ro 40-5967 and nisoldipine, a dihydropyridine selective for L-type Ca\(^{2+}\) channels, we found that all Ca\(^{2+}\) current could be completely blocked; thus, Ro 40-5967 is the first Ca\(^{2+}\) channel blocker to eliminate dihydropyridine-insensitive voltage-dependent Ca\(^{2+}\) current at therapeutically useful concentrations. The stepwise sequential block of T- and L-type Ca\(^{2+}\) currents demonstrated in the present study fulfills the functional criterion for the separate identity of the two Ca\(^{2+}\) channel types, and introduces a pharmacological tool that promises to be important in the exploration of T-type Ca\(^{2+}\) channel function. (Circ Res. 1994;75:144-148.)

Key Words • Ca\(^{2+}\) antagonist • vascular muscle • vasodilators • dihydropyridine • nisoldipine

One surprising observation about Ca\(^{2+}\) channel antagonists that are presently used in cardiovascular medicine is that all of these drugs appear to act primarily on L-type (long-lasting, high-voltage-activated) Ca\(^{2+}\) channels, only one of multiple recognized classes found in mammalian cells.\(^{1,2}\) In contrast, the T-type (transient, low-voltage-activated) Ca\(^{2+}\) channel, which is less prevalent and only superficially understood, is found in relatively high density in spontaneously active vascular muscle and is always correlated with pacemaker (spontaneous contractile) function.\(^{3,4}\) Comparisons of T-type Ca\(^{2+}\) channels with L and N types show that although single-channel conductance, selectivity, and kinetics, along with the voltage domains and inactivation rates, provide multiparameter identification,\(^{5,6}\) sensitivity to a specific blocker is missing, and only inorganic ions (\(\text{Ni}^{2+}\)) or drugs with other primary mechanisms of action (eg, amiloride or alcohols) can even partially block T-type Ca\(^{2+}\) channels.\(^{5,6}\) Thus, T-type Ca\(^{2+}\) channels have not been identified with a characteristic blocker, as compared with L-type Ca\(^{2+}\) channels which are now referred to as dihydropyridine receptors.\(^{5,7}\) Selective block of T-type Ca\(^{2+}\) channels would appear to be a major goal.

Recent reports that T-type Ca\(^{2+}\) channels are associated with hypertrophy of ventricular myocardium\(^{8}\) may suggest that vascular muscle T-type Ca\(^{2+}\) channels could also have a role in the regulation of vascular growth, eg, carotid hypertrophy. Unfortunately, the function of T-type Ca\(^{2+}\) channels can only be inferred by correlations, because a selective drug to bind T-type Ca\(^{2+}\) channels is lacking. The present study shows that a chemically novel Ca\(^{2+}\) antagonist, Ro 40-5967, at concentrations that are effective antihypertensive therapy in humans,\(^{9}\) shows selectivity for T-type Ca\(^{2+}\) channels. Plasma concentrations of \(\approx 1 \mu\)mol/L are found 1 to 2 hours after an oral dose of 120 mg in healthy human volunteers, and the half-life was over 12 hours. At this dose, Ro 40-5967 reduced diastolic blood pressure by up to 20 mm Hg, was well-tolerated, did not decrease cardiac output or cause atrioventricular conduction block, and thus has a favorable profile. Ro 40-5967 appears to be the only compound for which there is selective block of T-type Ca\(^{2+}\) channels (essentially complete block of T-type currents at a concentration that only partially blocks L-type currents). The favorable pharmacological profile of Ro 40-5967\(^{10}\) may be a consequence of this T-type Ca\(^{2+}\) channel selectivity.

Materials and Methods

Solutions and Drugs

CV3M solution contained 4.0 mmol/L l-glutamine, 20 \(\mu\)g/mL gentamicin, 20 mmol/L HEPES (pH 7.3), and 16 mmol/L NaHCO\(_3\) dissolved in a mixture of 85% MEM/Earle’s salts and 15% horse serum. KG solution consisted of (mmol/L) potassium glutamate 140, HEPES (final pH 7.3) 25, NaHPO\(_4\) 0.5, glucose 5.5, NaHCO\(_3\) 16, and phenol red 0.014. For recording inward Ba\(^{2+}\) current (\(I_{\text{Ba}}\)), vascular muscle cells (VMCs) were suffused with Ba20TEA containing (mmol/L) BaCl\(_2\) 20, tetraethylammonium (TEA) chloride 125, HEPES acid 15, MgCl\(_2\) 1, and dextrose 5.5, with final pH adjusted to 7.4 with TEA OH. The pipette solution consisted of (mmol/L) cesium glutamate 140, MgCl\(_2\) 1, HEPES acid 10, EGTA 3.4, ATP dipotassium salt 0.5, and GTP Tris salt 0.5, pH adjusted to 7.2 with CsOH. All solutions were filtered (0.2 \(\mu\)m) immediately before use. Ro 40-5967 \(1(TS2S)-2-[2(3-2-benzimidazo[1,2-b]pyrrolyl)methylaminomethyl]-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl methoxycetate dihydrochloride] was a gift from Hoffmann-LaRoche Ltd. A stock solution of Ro 40-5967 (1 mmol/L) was prepared in ultrapure, filtered Milli-Q water and diluted with Ba20TEA solution to concentrations of 1 or 10 \(\mu\)mol/L. Cells were suffused with the drug for a

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period of 5 minutes before the effects on ion currents were evaluated.

**Cell Culture**

Primary cultures of VMCs were prepared on 14 separate dates from azygous veins of 1- to 3-day-old NIH/nihe neonatal rats, as described previously. In brief, azygous veins were surgically isolated and placed in 25°C CV3M solution. After a rinse in CV5M, the tissue was soaked for 10 minutes in KG solution, minced into ~1-mm pieces using fine dissecting scissors, and incubated at 37°C for 30 minutes in collagenase (30 mg in 10 mL KG solution containing 0.1 μmol/L Ca2+). Collagenase supernatant was discarded, and the sediment was sequentially exposed to three or four incubations of 15 minutes in 15 mL of 1 mg/mL trypsin in KG solution. After each incubation, the supernatant was removed and placed in 25 mL of CV3M on ice. Two combined supernatant fractions were centrifuged into pellets (200g for 15 minutes), and VMCs were resuspended in 10 mL ice-cold CV3M and centrifuged for 10 minutes at 200g. Supernatant was removed, and VMCs were resuspended in 10 mL 37°C CV3M and transferred to a T25 tissue-culture flask for 1 hour of sedimentation at 37°C. The nonattached VMCs were transferred to centrifuge tubes, spun for 5 minutes at 200g, resuspended in CV3M+250 μmol/L bromodeoxyuridine (BrdU), diluted to a density of 70,000 cells per milliliter, and plated on clean 9×22 mm glass coverslips (in plastic tissue-culture dishes). VMCs were kept in a 5% CO2/95% air incubator at 95% humidity and 37°C. BrdU was always removed at least 24 hours before contracting VMCs were used for electrophysiological experiments (after 3 to 14 days).

**Measurement of Membrane Currents**

Glass coverslips with attached VMCs were placed in a 0.3 mL laminar flow chamber on the stage of a Zeiss Axiovert microscope. VMCs were suffused at room temperature (23°C) with anionic solution at 0.6 mL/min. Patch pipettes were prepared from thin-wall 7052 glass tubing using a Brown-Flaming micropipette puller (model P87, Sutter Instruments Co.). Heat-polished pipettes with tip resistances of 2.5 to 5 MΩ were used in the whole-cell voltage-clamp configuration. The pipette was connected to the input stage of a high-speed, low-noise, current-to-voltage amplifier and was mounted on a three-dimensional hydraulic manipulator. Reference potential of the pipette was set to 0, a gigascale (4 to 10 GΩ) was established, holding potential Vh was set at −60 mV, and breakthrough to whole-cell recording was achieved by gentle suction.

Membrane currents were amplified by the Axopatch amplifier (Axon Instruments), low-pass Bessel-filtered at 1 kHz, monitored on a two-channel storage oscilloscope (Tektronik 5031), and digitized using a TL-1 analog-to-digital interface at a sampling frequency of 10 kHz with pCLAMP software (Axon Instruments) on a Hewlett-Packard Vectra computer. The protocol for isolation of T- and L-type Ca2+ channel currents was based on different holding (Vh) and test potentials (VT) that define the characteristic voltage domains of the two types, as described previously. Three VMC populations were possible during whole-cell recording: (1) cells with predominantly L-type currents, (2) cells with both T- and L-type currents, and (3) cells with mainly T-type currents. We separated T- and L-type Ca2+ channel currents in population 2 by use of −80 and −30 mV Vm as follows. Ih in Ba2+ as the charge carrier, were elicited with step depolarizations of 300-millisecond duration applied every 5 seconds from the two Vh. From Vm=−80 mV, Vm to −20 mV elicited transient Ih which decayed to 0 in <100 milliseconds. L-type currents are only slightly (or not) activated by Vh=−20 mV; however, from Vm=−30 mV, the step to Vm=−30 mV gave only L-type currents because T-type channels are inactivated completely by Vm=−30 mV. In essence, peak T-type current was determined by the −80 to −20 mV transition, and peak L-type current was determined by the depolarizing step from −30 to +30 mV.

**Data Analysis**

Data are given as mean±SEM (n=number of experiments). Statistical significance was determined by Student’s unpaired t test. Values of P<0.05 were considered statistically significant.

**Results**

Fig 1 shows the whole-cell patch-clamp recordings from a rat VMC, which separate transient (T-type) and longer-lasting (L-type) Ca2+ channel currents by use of Vm at −80 and −30 mV. Divalent ion channel inward currents were excited in single VMC, with Ba2+ as the charge carrier, by 300-millisecond step depolarizations applied every 5 seconds from two different Vh. From Vm=−80 mV, a Vm to −20 mV elicited T-type Ih, which decayed within 60 to 100 milliseconds (Fig 1A). The peak amplitude of T-type Ih (Vm=−80 mV, Vm=−20 mV) varied between 20 and 150 pA in seven VMC. Stepping from Vh=−80 mV to Vm=−20 mV elicted an L-type Ih which decayed slowly (sustained at >15% of peak for >300 milliseconds) and contained both T- and L-type currents. In contrast, when Vh was changed to −30 mV, there was no transient current at Vm=−20 mV, because of the voltage inactivation of the T-type channels (Fig 1B). Rather, pulses from Vh=−30 mV to Vm=−20 mV elicited an L-type Ih which reached maximum amplitudes of 100 to 400 pA at the Vm=+20 mV step.

In addition to identification on the basis of voltage domains and kinetics, we used pharmacological sensitivity to 30 mmol/L nisoldipine to distinguish L- from T-type Ca2+ channels. Fig 2 (upper tracings) shows that exposure to 30 mmol/L nisoldipine for ≥3 minutes had no effect on Ih elicited at Vm=−20 mV from Vh=−80 mV (the T-type current). However, Fig 2 (lower tracings) shows that 30 mmol/L nisoldipine completely blocked the Ih that would have been excited by depolarization to Vm=+20 mV from Vh=−30 mV (the L-type current). Most significantly, T-type current (upper tracings) was nearly completely inhibited by 1 mmol/L Ro 40-5967 but was not affected by nisoldipine. As shown in another cell, Ro 40-5967 (1 μmol/L) alone could reduce most of the T-type Ih while reducing L-type current much less (Fig 3). In Fig 3A, 5 minute suffusion of the VMCs with 1 μmol/L Ro 40-5967 inhibited Ih 80% during the step to Vm=−20 mV from Vh=−80 mV. However, at Vh=−30 mV which inactivated T-type channels, 1 μmol/L Ro 40-5967 caused only fractional inhibition (28%) of peak L-type channels on step depolarization to Vm=+20 mV (Fig 3B). Consistently, Ro 40-5967 blocked almost all T-type and one-fourth to one-third of the L-type currents during the step to Vm=+20 mV from Vh=−80 mV (Fig 3C).

Fig 4 shows the activation current-voltage relation and average peak Ih for combined T- and L-type Ca2+ channels in the presence and absence of 10 μmol/L Ro 40-5967. The preponderant inhibition of the T-type Ih resulted in suppression of the left part of the curve (Fig 4A). Reduction of the negative voltage domain current results from inhibition of T-type channels, as shown by no recorded Ih at Vm=−30 mV in 10 μmol/L Ro 40-5967, leaving the current-voltage curve as virtually pure (nisoldipine inhabitable) L-type current. Fig 4B
Fig 1. Appearance of vascular muscle T- and L-type Ca²⁺ channel currents from holding potentials (V₀) of −80 mV (A) and −30 mV (B). Inward currents were recorded in an isolated vascular muscle cell (VMC) in a solution with 20 mmol/L Ba²⁺ as the extracellular divalent ion. A (V₀ = −80 mV) shows a series of Ba²⁺ currents (Iₒ) with prominent transient (T-type) and longer-lasting (L-type) currents at test potentials (Vₜ) from −20 to +20 mV. B (V₀ = −30 mV) shows Iₒ of the L-type recorded exactly 2 minutes later at the same Vₜ. A step to Vₜ = −20 mV from a V₀ = −80 mV elicited only T-type Iₒ (completely inactivating within 100 milliseconds), whereas a step to Vₜ = −20 mV from V₀ = −30 mV triggered practically no Iₒ (because T-type currents were voltage-inactivated and Vₜ = −20 mV is just threshold for stimulating L-type Iₒ). However, a step to Vₜ = +20 mV from a V₀ = −30 mV activated L-type channel currents (which could be sustained for >3 seconds). The dotted lines represent 0 current, and the voltages near the end of each tracing show the Vₜ. All tracings are from the same VMC.

shows that average control T-type current amplitude, measured during depolarizations to Vₜ = −20 mV from V₀ = −80 mV, was 134 ± 19 pA (n = 7). A 5 minute treatment with 1 μmol/L Ro 40-5967 reduced peak amplitude by 85% to 11 ± 6 pA (n = 5). Complete block of the current during depolarizations to Vₜ = −20 mV from V₀ = −80 mV was found after 5 minute exposure to 10 μmol/L Ro 40-5967 (n = 4). In comparison, peak L-type currents obtained by step depolarizations to Vₜ = +20 mV from V₀ = −30 mV were 218 ± 20 (n = 7) for the control condition, 163 ± 11 pA (n = 5) (25% reduced) in 1 μmol/L Ro 40-5967, and 74 ± 6 (n = 4) (66% reduced) in 10 μmol/L Ro 40-5967.

Fig 5 shows the separate Ro 40-5967 concentration response curves for T-type, L-type, and the L- and T-type combination of currents using the voltage protocols of Fig 3. The higher sensitivity and greater maximum inhibition of T-type than L-type Iₒ to Ro 40-5967 is apparent. The concentration of Ro 40-5967 inhibiting T-type current by 50% appears to be ~100 nmol/L.

Discussion

The experiments presented here, using VMC that we earlier characterized as containing both T- and L-type Ca²⁺ channels, directly demonstrate the selective

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**Fig 2.** Sequential block of L- and T-type Ca²⁺ currents by 30 nmol/L nisoldipine, alone and in combination with 1 μmol/L Ro 40-5967. V₀ indicates holding potential; Vₜ, test potential. The upper tracings show that pure T-type Ba²⁺ current (Iₒ) (Vₜ = −20 mV from V₀ = −80 mV as shown in Fig 1) was not blocked by nisoldipine (30 nmol/L), whereas 1 μmol/L Ro 40-5967 nearly (>90%) abolished the nisoldipine-insensitive T-type Iₒ. On the other hand, the L-type Iₒ (Vₜ = +20 mV from V₀ = −30 mV) was completely abolished by 30 nmol/L nisoldipine (lower tracings). Iₒ remained completely blocked at all Vₜ from either V₀ = −80 or −30 mV by the combination of 30 nmol/L nisoldipine and 1 μmol/L Ro 40-5967, without any indication of a latent Ca²⁺ agonist action of Ro 40-5967. All tracings are from the same vascular muscle cell, with labels as in Fig 1.
inhibition of divalent ion current through the T-type Ca\(^{2+}\) channels. The protocol was chosen to rigorously test whether Ro 40-5967 would selectively block T-type Ca\(^{2+}\) channels, after the L-type Ca\(^{2+}\) channels were blocked by nisoldipine. Ro 40-5967 is the first compound to succeed in this protocol, which requires the ability to block T-type Ca\(^{2+}\) channel currents (remaining after block of L-type channels) at pharmacologically useful concentrations. This definitive demonstration of selective T-type Ca\(^{2+}\) channel block has not been found for any other drug. Selective block of T-type channels fulfills the necessary prediction of the hypothesis for the existence of independent Ca\(^{2+}\) channels.\(^{1-3,12,13,16}\)

VMC of the azygos vein of newborn rats were chosen for these experiments because of the previous extensive characterization we have made of these spontaneously active VMC, including T- and L-type channel identification by voltage and kinetics, pharmacology, permeability, and inactivation characteristics.\(^{4,12-15,17}\) Similar conclusions of separate T- and L-type channels have been reached for cardiac muscle, neuronal cells, and a variety of secretory cells in which Ca\(^{2+}\) channels can be found.\(^{3,16}\) T-type Ca\(^{2+}\) channels have an unknown role, but have recently been shown to predominate in young and hypertrophied adult myocardium.\(^{8}\) Separation of L- and T-type Ca\(^{2+}\) channels is based on rigorous biophysical data, which leave little doubt that two individual channel types exist. However, the identification of ion channels has been importantly assisted by the development of drugs with high affinity for a given ion channel type,\(^{7,18-20}\) and thus, the binding criterion is important to satisfy. With this selective T-type channel blocking tool, the key question of the role of T-type Ca\(^{2+}\) channels in cellular function may be more directly addressed. Surely more studies of different Ca\(^{2+}\) channel types are warranted in vascular muscle, as in the many types of cells showing this dynamic membrane signal mechanism.\(^{21}\)

Although Kuga et al.\(^{22}\) earlier claimed block of T-type channels (although at high concentrations) by the Ca\(^{2+}\)
antagonists, diltiazem, verapamil, nicardipine, and flunarizine, there has never been selectivity shown for T-over L-type channels until now. The interpretation of their data is controversial, because they used \( V_{H} = 60 \) mV and high (for VMC) frequencies of 0.5 Hz, which we believe results in a mixture of T- and L-type currents, in their attempt to isolate L-type from T-type currents. Kuga et al.\(^2\) relied on 5 mmol/L hydrofluoric acid (HF) as an absolute selection step for presumptive elimination of L-type currents (and chose \( V_{H} = 60 \) mV instead of \( V_{H} = -30 \) mV) but eschewed the channel kinetic, pharmacological, and permeability criteria for identification that are widely accepted,\(^1,1,3,12,15,16\) making their interpretations enigmatic. Their data would as readily be accounted for by a left shift of the current-voltage curve for L-type Ca\(^{2+}\) channels by HF and a mixture of L- and T-type currents in all of the data they showed. The expected action of HF (or AIF, which would be formed in cell cytoplasm) is adenylyl cyclase activation\(^23\) and cGMP activation through nonspecific activation of G proteins,\(^24\) probably leading to phosphorylation changes that might shift (either way depending on G protein ensemble and state) voltage dependence of the channel.\(^20\) The complete T-type channel block shown here is clearly different from the partial reductions in divalent ion currents of Kuga et al.\(^23\) Based on the voltage domains of VMC Ca\(^{2+}\) channels, the \( I_{Na} \) that they blocked, from \( V_{H} = 60 \) mV to \( V_{l} \) steps positive to \(-10 \) mV, can be accounted for within our definition of L-type Ca\(^{2+}\) channel block.\(^1,3,12,15,16\)

The search to find a T-type Ca\(^{2+}\) channel blocker is clearly important. Finding complete block of T-type currents at a therapeutically useful 1 \( \mu \)mol/L concentration, which is exactly equivalent to that normalizing blood pressure in hypertensive humans,\(^9\) makes the finding especially significant. Important actions of Ro 40-5967, such as reduced thickening of the subendothelium in aortae of spontaneously hypertensive rats,\(^26\) could possibly be explained by inhibiting T-type Ca\(^{2+}\) channels. Furthermore, developments to further characterize biochemical and functional parameters of T-type Ca\(^{2+}\) channels may be facilitated by this discovery. As with isolation of L-type Ca\(^{2+}\) channels by affinity chromatography with nitrendipine, the availability of high affinity binding of T-type channels may allow the isolation and cloning of proteins that compose the channel. If this were true, Ro 40-5967, or a derivative with higher affinity, may establish a new class of Ca\(^{2+}\) antagonist and be a valuable tool for molecular exploration of Ca\(^{2+}\) channels, in addition to being a valuable drug that may further extend the already remarkable usefulness of Ca\(^{2+}\) antagonists.

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