Selective Inhibitor of T-Type Calcium Channels

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ABSTRACT

Mibefradil is a Ca\(^{2+}\) channel antagonist that inhibits both T-type and high-voltage-activated Ca\(^{2+}\) channels. We previously showed that block of high-voltage-activated channels by mibefradil occurs through the production of an active metabolite by intracellular hydrolysis. In the present study, we modified the structure of mibefradil to develop a nonhydrolyzable analog, (1S,2S)-2-(2-(N-[(3-benzimidazol-2-yl)propyl]-N-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl cyclopropanecarboxylate dihydrochloride (NNC 55-0396), that exerts a selective inhibitory effect on T-type channels. The acute IC\(_{50}\) of NNC 55-0396 to block recombinant \(\alpha\)\(_{1G}\) T-type channels in human embryonic kidney 293 cells was \(\sim 7 \mu M\), whereas 100 \(\mu M\) NNC 55-0396 had no detectable effect on high-voltage-activated channels in INS-1 cells. NNC 55-0396 did not affect the voltage-dependent activation of T-type Ca\(^{2+}\) currents but changed the slope of the steady-state inactivation curve. Block of T-type Ca\(^{2+}\) current was partially relieved by membrane hyperpolarization and enhanced at a high-stimulus frequency. Washing NNC 55-0396 out of the recording chamber did not reverse the T-type Ca\(^{2+}\) current activity, suggesting that the compound dissolves in or passes through the plasma membrane to exert its effect; however, intracellular perfusion of the compound did not block T-type Ca\(^{2+}\) currents, arguing against a cytoplasmic route of action. After incubating cells from an insulin-secreting cell line (INS-1) with NNC 55-0396 for 20 min, mass spectrometry did not detect the mibefradil metabolite that causes L-type Ca\(^{2+}\) channel inhibition. We conclude that NNC 55-0396, by virtue of its modified structure, does not produce the metabolite that causes inhibition of L-type Ca\(^{2+}\) channels, thus rendering it more selective to T-type Ca\(^{2+}\) channels.

Voltage-gated Ca\(^{2+}\) channels are transmembrane proteins involved in the regulation of cellular excitability and intracellular Ca\(^{2+}\) signaling. Calcium channels have been divided into various categories based on functional and pharmacological criteria. High-voltage-activated (HVA) channels, which have been further subdivided into L-, N-, P/Q-, and R-types, require strong depolarizations for activation, whereas low-voltage-activated or T-type channels activate over a much more negative voltage range and exhibit unique inactivation and deactivation kinetics (Armstrong and Matteson, 1985; Catterall, 1998; Perez-Reyes, 1998). The main structural component of the voltage-gated calcium channel is the \(\alpha\) 1 subunit.
subunit, which forms the pore and the channel gates. Molecular cloning has identified 10 $\alpha_1$ subtypes. $\alpha_1$A–$\alpha_1$E and $\alpha_1$S encode HVA channels, whereas $\alpha_1$G–$\alpha_1$I encode T-type channels. Pharmacological agents that act selectively on $\alpha_1$ subtypes have been key in studying calcium channel function in many physiological systems; however, a selective antagonist of T-type Ca$^{2+}$ channels is not yet available.

Mibepradil, a tetralide derivative chemically distinct from other Ca$^{2+}$ channel antagonists, has been reported to block T-type Ca$^{2+}$ channel currents in many tissues, including heart (Madle et al., 2001), brain (McDonough and Bean, 1998), and vascular smooth muscle (Bian and Hermsmeyer, 1993; Mishra and Hermsmeyer, 1994; Schmitt et al., 1995). Mibepradil was also reported to block HVA Ca$^{2+}$1B subtypes. However, it is unclear whether the unique effects of mibepradil are caused by the blockade of T-type Ca$^{2+}$ channels since mibepradil also blocks the L-type Ca$^{2+}$ currents in the cardiomyocytes (Leuranguer et al., 2001). Therefore, identifying a more selective T-type Ca$^{2+}$ channel antagonist will be useful for the study of cardiac voltage-gated calcium channels and may promote the development of a new class of therapeutically beneficial compounds.

In a previous study, we demonstrated that hydrolysis of the ester side chain of mibepradil resulted in a compound (des-methoxyacetyl mibepradil or Ro 40-5966) that exhibited a channel-specific inhibitory effect (Wu et al., 2000).

The cardiovascular effects of mibepradil are interesting; for instance, it decreases heart rate without a negative inotropic effect (Osterrieder and Holck, 1989; Cremers et al., 1997), and its action is not associated with the reflex activation of neurohormonal and sympathetic systems (Ernst and Kelly, 1998). These properties differ from other clinically important Ca$^{2+}$ antagonists such as nifedipine, diltiazem, and verapamil, which in the heart selectively inhibit L-type ($\alpha_1$C) Ca$^{2+}$ channels. However, it is unclear whether the unique effects of mibepradil are caused by the blockade of T-type Ca$^{2+}$ channels since mibepradil also blocks the L-type Ca$^{2+}$ currents in the cardiomyocytes (Leuranguer et al., 2001).

To test this hypothesis, we examined the effects of several novel mibepradil derivatives on T-type and HVA Ca$^{2+}$ channel currents in whole-cell voltage-clamp recordings. We used HEK 293 cells stably transfected with the $\alpha_1$G cDNA originally cloned from rat pancreatic $\beta$-cells (Zhuang et al., 2000) in vector pcDNA3.1 (Invitrogen, Carlsbad, CA) to transfect into HEK cells using the FuGENE kit (Roche Diagnostics, Indianapolis, IN). Cell lines stably expressing Ca$_{\alpha,3.1}$ were obtained after transfection using standard cell cloning techniques (Freshney, 1983). HEK 293 cells stably transfected with $\alpha_1$G cDNA (HEK 293/$\alpha_1$G) were incubated in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, and 0.5 mg/ml hygromycin-B in an atmosphere of 5% CO$_2$ in air at 37°C for 2 to 5 days before recording.

**Electrophysiological Recording.** Whole-cell recordings were carried out by the standard “giga-seal” patch-clamp technique. The whole-cell recording pipettes were made of hemocapillary micropipettes with a resistance of 5-10 MOhm. The cell bodies were filled with an intracellular solution composed of 140 mM K$_2$SO$_4$, 1 mM MgCl$_2$, 10 mM HEPES, and 40 mM sucrose. The intracellular solution contained 10 mM CaCl$_2$, 110 mM tetraethylammonium-Cl, 10 mM CsCl, 10 mM HEPES, 10 mM MgCl$_2$, and 1 mM Ca(OH)$_2$, with pH adjusted to 7.4 with methanesulfonate. Mg-ATP (2 mM) was included in the pipette solution to minimize rundown of L-type Ca$^{2+}$ currents. For perforated-patch experiments, 200 $\mu$g/ml nystatin was used. The pipette was filled with nystatin-containing intracellular solution, and gentle suction was used to achieve gigaohm resistance. The access resistance gradually decreased within 5 min after the gigaohm-seal formation, and then currents were recorded after stabilization. The extracellular solution contained 26 mM sucrose, 130 mM tetraethylammonium-Cl, 10 mM CsCl, 10 mM HEPES, 10 mM MgCl$_2$, and 1 mM Ca(OH)$_2$, with pH 7.4.

**Mass Spectrometric Analysis.** Mass spectrometric analysis was performed on a PerSeptive Voyager-DE MALDI-TOF instrument (Applied Biosystems, Foster City, CA). Cultured INS-1 cells were treated with 20 $\mu$M NNC 55-0396 for various lengths of time under each experimental condition. After incubation, cells were washed with PBS three times, scraped into Eppendorf tubes with 1 ml of PBS, and centrifuged at 1000g for 5 min. The cell pellets were collected and redissolved by sonication (10 s) in 0.5 ml of PBS for mass spectrometric analysis. The sample was mixed with 150 $\mu$l of matrix. Ten microliters of each sample were mixed with 70 $\mu$l of the matrix. One microliter of the mixture was spotted on a plate for analysis on the MALDI-TOF instrument. Several positive ion spectra were recorded in the mass range m/z 820 to 99 at a mass resolution of 1000 and a scan speed of 2 s/decade. For NNC 55-0396, m/z 492 was the dominant ion (M + H$^+$. For calibration, a standard solution of 50 $\mu$M NNC 55-0396 was subjected to mass spectrometric analysis. The relative amount of NNC 55-0396 was determined by calibrating the intensities of NNC 55-0396 with the intensity of standard solution.

**Materials and Methods**

**Cell Culture.** INS-1 cells, an insulin-secreting cell line derived from rat pancreatic $\beta$-cells (Asfari et al., 1992) were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, and 50 $\mu$M mercaptoethanol in an atmosphere of 5% CO$_2$ in air at 37°C for 2 to 5 days before recording.
Statistics and Curve Fitting. Nonlinear regression analysis was used to fit concentration-response data to a sigmoid relationship, \( Y = 100/(1 + 10^{(log IC_{50} - X)}) \), where slope is the Hill slope parameter, \( IC_{50} \) is the concentration producing 50% blockage, and \( X \) is the drug concentration. Voltage-dependent activation and steady-state inactivation curves were generated by normalizing the currents with the maximal amplitude in each cell and fitting the data with the Boltzmann equation, \( 1/(1 + \exp((V - V_{1/2})/k)) \), where \( k \) represents the slope and \( V_{1/2} \) represents the voltage corresponding to half-activation of the channels. Student’s \( t \) test was used to compare \( V_{1/2} \) and \( k \) values determined from fits of the data with this equation.

The data fitting and statistical analysis were performed with Prism version 4 (GraphPad Software, San Diego, CA). In the figures where the data are presented as symbols and error bars, the values are mean ± S.E.M.

General Chemical Procedure. Reagents, starting materials, and solvents were purchased from common commercial suppliers and were used as received. All dry solvents were dried over molecular sieves (0.3 or 0.4 mm). Evaporation was carried out on a rotary evaporator at bath temperatures <40°C and under appropriate vacuum. Flash chromatography was carried out on a Bio-Rad FLASH 40 (Biotage, Uppsala, Sweden) using Bio-Rad FLASH columns (KPSIL 60 Å particle size, 32–63 μm). Melting points were determined with a Büchi B545 apparatus (Büchi, Flawil, Switzerland) and are uncorrected. Proton NMR spectra were recorded at ambient temperature using a Bruker AVANCE DPX 200 (Bruker, Newark, DE) (200 MHz) and DPX 300 (300 MHz), with tetramethylsilane as an internal standard in proton spectra. Chemical shifts are given in ppm (δ), and splitting patterns are designated as follows: s, singlet; d, doublet; dd, double doublet; t, triplet; dt, double triplet; q, quartet; quint, quintet; m, multiplet; and br, broad. The 70-electron-volt EI solid mass spectra were recorded on a Finnigan MAT TSQ 70 mass spectrometer (Thermo Finnigan, San Jose, CA). Reactions were followed by thin layer chromatography performed on silica gel 60F254 (Merck, Darmstadt, Germany) or ALUGRAM SIL G/UV254 (Macherey-Nagel, Düren, Germany) thin layer chromatography aluminum sheets.

Synthesis of NNC 55-0396. Methoxyacetic acid (2S)-[2-[N-(3-(2-benzimidazol-2-yl)propyl)-N-methylamino]ethyl]-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl ester dihydrochloride (mibefradil, 0.570 g) in ethanol (96%, 5 ml) and aqueous sodium hydroxide (1 N, 5 ml) was refluxed for 2 h. The cold reaction mixture was concentrated in vacuo. The residue was partitioned between water and dichloromethane. The aqueous layer was extracted with dichloromethane. The combined organic layers were dried with sodium sulfate and concentrated to give 2-(2-[[3-(1-benzimidazol-2-yl)propyl]methylamino]ethyl)-6-fluoro-1,2,3,4-tetrahydro-2-naphthalinol as a clear syrup (100%, 0.43 g). \(^1H\) NMR (CDCl_3): δ 7.57 (br, 2H); 7.23 (m, 2H); 6.97 (m, 1H); 6.58 (m, 2H); 3.07–2.83 (m, 3H); 2.75 (m, 1H); 2.6 (m, 4H); 2.5–2.22 (s + m, 3H + 3H); 2.06 (quint, 2H); 1.81 (br dd, 1H); 1.50 (m, 2H); 1.20 (d, 3H); 0.53 ppm (d, 3H).

Synthesis of NNC 55-0396 and Other Mibefradil Analogues. We synthesized three analogs of mibefradil by replacing the ester chain (methoxyacetyl) group NNC 55-0395, NNC 55-0396, and NNC 55-0397 (Fig. 1). All compounds were synthesized from mibefradil in two steps. Alkaline hydrolysis of mibefradil and subsequent treatment with valeryl chloride, cyclopropanecarboxyl chloride, and isobutyryl chloride gave the desired compounds NNC 55-0395, NNC 55-0396, and NNC 55-0397 upon workup, respectively.

Synthesis of (1S,2S)-2-(2-[[3-(1-benzimidazol-2-yl)propyl]-N-methylamino]ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl isobutyrate dihydrochloride (NNC 55-0397). NNC 55-0397 was prepared by an analogous procedure: Mp 114 to 117°C. EI SP/MS: 503 (M+). \(^1H\) NMR (DMSO-d_6): δ 7.77 (m, 2H); 7.49 (m, 2H); 7.08 (m, 1H); 6.96 (br d, 2H); 3.15 (4H, 3H); 2.7 (s, 3H); 2.48 (dt, 2H); 2.23 (m, 2H); 2.0 (m, 4H); 1.50 (p, 2H); 1.35 (quint, 2H); 1.02 (d, 3H); 0.90 (t, 3H); 0.35 ppm (d, 3H).

Synthesis of NNC 55-0396 and Other Analogues from Mibefradil. We synthesized three analogs of mibefradil by replacing the ester chain (methoxyacetyl) group NNC 55-0395, NNC 55-0396, and NNC 55-0397 (Fig. 1). All compounds were synthesized from mibefradil in two steps. Alkaline hydrolysis of mibefradil and subsequent treatment with valeroyl chloride, cyclopropanecarboxyl chloride, and isobutyryl chloride gave the desired compounds NNC 55-0395, NNC 55-0396, and NNC 55-0397 upon workup, respectively.

Results

Synthesis of NNC 55-0396 and Other Mibefradil Analogues. We synthesized three analogs of mibefradil by replacing the ester chain (methoxyacetyl) group NNC 55-0395, NNC 55-0396, and NNC 55-0397 (Fig. 1). All compounds were synthesized from mibefradil in two steps. Alkaline hydrolysis of mibefradil and subsequent treatment with valeryl chloride, cyclopropanecarboxyl chloride, and isobutyryl chloride gave the desired compounds NNC 55-0395, NNC 55-0396, and NNC 55-0397 upon workup, respectively.

Pharmacological-Screening Effect of NNC 55-0395, NNC 55-0396, and NNC 55-0397 on T-Type and HVA Ca\(^{2+}\) Currents. We used perforated whole-cell patch-clamp to examine the effects of NNC 55-0395, NNC 55-0396, and NNC 55-0397 on T-Type and HVA \( Ca^{2+} \) currents. In these experiments, the resting membrane potential was held at −40 mV to eliminate T-type \( Ca^{2+} \) current. The inhibitory potency of the mibefradil analogs on HVA \( Ca^{2+} \) current was examined at dosages of 0.1, 1, 10, and 100 μM approximately 10 min after the drug perfusion. The results showed that both NNC 55-0395 and NNC 55-0397 had an inhibitory effect on HVA \( Ca^{2+} \) currents at 100 μM (Fig. 2, A and C), whereas no inhibition on the HVA \( Ca^{2+} \) current was detected with NNC 55-0396 at the same concentration (Fig. 2B).

![Fig. 1.](image_url) Synthesis of NNC 55-0396 and other analogs from mibefradil and chemical structures of NNC 55-0395, NNC 55-0396, and NNC 55-0397. The side chain of mibefradil inside the dashed box is replaced by the new structures indicated by arrows.
Next, we determined the effects of NNC 55-0395, NNC 55-0396, and NNC 55-0397 on T-type Ca$^{2+}$ current. These experiments were conducted by measuring the effects of the compounds on whole-cell current in HEK 293/1G cells. The dose-dependent inhibition of compounds NNC 55-0395, NNC 55-0396 and NNC 55-0397 on T-type Ca$^{2+}$ current was examined at dosages ranging from 1 to 100 μM. NNC 55-0396 and NNC 55-0397 blocked more than 50% of the T-type Ca$^{2+}$ current at 8 μM (Fig. 2, E and F), whereas NNC 55-0395 inhibited less than 50% of the T-type Ca$^{2+}$ current at 64 μM (Fig. 2D).

Both NNC 55-0395 and NNC 55-0397 blocked HVA Ca$^{2+}$ channel currents in our screening experiments and were thus eliminated from further characterization. Compound NNC 55-0396, which blocked T-type Ca$^{2+}$ current but not HVA Ca$^{2+}$ currents, was selected for subsequent analysis.

Characterization of the Inhibitory Effects of NNC 55-0396 on T-Type Ca$^{2+}$ Current. To further characterize NNC 55-0396, we used whole-cell patch-clamp and a bath perfusion system to examine its dose-dependent effects on T-type and HVA Ca$^{2+}$ currents. At 8 μM, NNC 55-0396 reduced more than 50% of the peak of T-type Ca$^{2+}$ current compared with the control in HEK 293/αG cells (Fig. 3A); however, NNC 55-0396 at 100 μM did not block the HVA Ca$^{2+}$ current in INS-1 cells (Fig. 3B), whereas this HVA Ca$^{2+}$ current could be inhibited by 10 μM nifedipine, a selective L-type Ca$^{2+}$ channel blocker (Fig. 3C). Pooled data of the effects of NNC 55-0396 on T-type and HVA Ca$^{2+}$ currents are shown in Fig. 3D. After bathing HEK 293/αG cells with NNC 55-0396 at 8 μM or bathing INS-1 cells with NNC 55-0396 at 100 μM for over 8 min, T-type Ca$^{2+}$ currents were inhibited by 60%, whereas no decrease in the HVA Ca$^{2+}$ current was observed.

The inhibitory potency of NNC 55-0396 on T-type Ca$^{2+}$ currents in HEK 293/αG was also compared with that of mibefradil, as shown in Fig. 3E. By fitting the data with a sigmoid dose-response relationship equation, NNC 55-0396 and mibefradil blocked T-type Ca$^{2+}$ current with IC$_{50}$ values of 6.8 and 10.08, respectively. This result suggests that NNC 55-0396 retains potency similar to mibefradil as an inhibitor of T-type Ca$^{2+}$ current.

Long-term exposure of HEK/αG cells to NNC 55-0396 showed a decrease in current density of T-type Ca$^{2+}$ channels. Using whole-cell patch-clamp, we measured T-type Ca$^{2+}$ current density in the HEK/αG cells that had bathed in the extracellular solutions containing 0, 10, 100, and 1000 nM NNC 55-0396 for 30 to 60 min (Fig. 3F). All peak-current amplitudes and slow capacitances were obtained during the first minute after breaking in; thus, the effect of the frequency-dependent inhibitory effect was minimal. Our data showed a slow yet more potent inhibitory effect of NNC 55-0396 on the T-type Ca$^{2+}$ currents.

Figure 4A illustrates that there was no significant difference in the conductance-voltage relationship ($G/V$) curves of...
Fig. 3. The effects of NNC 55-0396 on T-type and HVA Ca^{2+} channels. A, current traces of T-type Ca^{2+} currents in an HEK 293/α1G cell before and after application of 8 μM NNC 55-0396. The holding potential was −70 mV, and the test potential was −20 mV. B, current traces of T-type and HVA Ca^{2+} currents in an INS-1 cell before and after 100 μM NNC 55-0396. The currents were recorded at 10 mV for 200 ms when held at −40 with perforated whole-cell patch-clamp. The traces were filtered at 1 kHz. C, Ca^{2+} current was inhibited by 10 μM nifedipine in an INS-1 cell. The currents were measured at 10 mV before and after nifedipine (Nif) administration with a holding potential of −70 mV. D, time-dependent effects of NNC 55-0396 on HVA and T-type Ca^{2+} currents. The open circles represent relative currents of HVA Ca^{2+} channels recorded in INS-1 cells before and after 100 μM NNC 55-0396 administration (n = 6), whereas the solid circles represent relative currents recorded in HEK 293/α1G cells before and after 8 μM NNC 55-0396 administration (n = 4). NNC 55-0396 was applied to the bath 3 min after the beginning of the recording. E, dose-dependent inhibitory effects of NNC 55-0396 (open circles, n = 6) and mibefradil (solid circles, n = 4) on T-type Ca^{2+} currents recorded in HEK/α1G cells. The smooth lines are consistent with the Hill equation, with IC_{50} values of 6.8 and 10.08 μM for NNC 55-0396 and mibefradil, respectively. The Hill slopes are −2.39 and −4.26 for NNC 55-0396 and mibefradil, respectively. F, comparison of current densities (pA/pF) among HEK/α1G cells incubated in various concentrations of NNC 55-0396 for 30 to 60 min. The peak-current amplitudes were measured at −20 mV when held at −70 mV (n = 7–11 for each set of data). The asterisk represents p < 0.01 of the data compared with the control (0 nM NNC 55-0396).

T-type Ca^{2+} currents between the control cells and cells incubated with 8 μM NNC 55-0396. The conductance (G) was calculated from the current (I) divided by the driving force (V_{drive} = V_{membrane} − V_{reversal}), where the V_{reversal} was hypothetically assigned as 70 mV. Curves were generated by fitting the data with the Boltzmann equation. The V_{1/2} and k values are −32.61 ± 1.0 and 4.16 ± 0.9 for the controls (n = 3) and −32.12 ± 0.8 and 4.33 ± 0.73 for cells incubated with NNC 55-0396 (n = 3), respectively. The steady-state inactivation curves of T-type Ca^{2+} current were also generated with the Boltzmann equation (Fig. 4B). The V_{1/2} values are −59.21 ± 0.38 and −62.84 ± 0.94 for the controls (n = 4) and cells incubated with NNC 55-0396 (n = 6), respectively. The slope k values are 4.92 ± 0.33 with a 95% confidence interval between 4.16 to 5.69 for the controls and 7.95 ± 0.72 with a 95% confidence interval between 6.31 to 9.58 for cells incubated with NNC 55-0396, respectively. Therefore, the steady-state inactivation curve of T-type Ca^{2+} current is flattened when NNC 55-0396 is present.

State-Dependent Block of T-Type Ca^{2+} Current by NNC 55-0396. The inhibitory mechanism of NNC 55-0396 on T-type Ca^{2+} current was further investigated by testing the effect of changing the holding potential and stimulation frequency. As shown in Fig. 5A, in the absence of NNC 55-0396, changing the holding potential from −120 to −80 mV did not alter the peak amplitude of T-type Ca^{2+} current in an HEK 293/α1G cell, elicited by depolarizations to −10 mV every 10 s. In contrast, after the addition of 8 μM NNC 55-0396, switching the membrane potential from −80 to −120 mV caused an increase in the current amplitude, suggesting a partial relief of block at the more hyperpolarized holding potential. Similarly, increasing the pulse rate from 0.05 to 0.5 Hz had little effect on current amplitude without NNC 55-0396.

Fig. 4. Effects of NNC 55-0396 on voltage-dependent activation (G/V) and steady-state inactivation of T-type Ca^{2+} channel. A, voltage-dependent activation (G/V) curves of T-type Ca^{2+} channel with (solid circles) and without (open circles) the presence of 8 μM NNC 55-0396 were fit by the Boltzmann equation (n = 3). B, steady-state inactivation curves of T-type Ca^{2+} channel with (solid circles) and without (open circles) the presence of 8 μM NNC 55-0396 were fit by the Boltzmann equation (n = 6). Steady-state inactivation was determined by applying a prestimulating pulse of 1.5 s at various voltages immediately before the test pulse at −20 mV. For both A and B, currents were recorded in HEK 293/α1G cells, and the cell membrane was held at −70 mV.
55-0396, whereas the rate of inhibition of T-type Ca\(^{2+}\) current was accelerated by increasing the frequency of stimulation in the presence of the drug (Fig. 5B). Similar results of both voltage-dependent and frequency-dependent blockade were observed in four experiments.

**Mass Spectrometric Analysis of NNC 55-0396-Treated INS-1 Cells.** Previously, we found that mibefradil blocked both T-type and HVA Ca\(^{2+}\) current in INS-1 cells (Wu et al., 2000). After entering the cells, mibefradil is broken down into metabolites. One of them is des-methoxyacetyl mibefradil, which exerts an inhibitory effect on HVA Ca\(^{2+}\) channels by acting on the channels from inside the cell. To investigate why NNC 55-0396 and mibefradil have different effects on HVA Ca\(^{2+}\) channels, we used mass spectrometric analysis to examine whether des-methoxyacetyl mibefradil was produced intracellularly when the cells were treated with NNC 55-0396. Figure 6A shows a section of mass spectrum of molecules in INS-1 cells that had been incubated with NNC 55-0396 for 20 min. Notably, no des-methoxyacetyl mibefradil (which peaks at 424 m/z) was detected in this preparation. This finding suggests that, unlike mibefradil, the compound NNC 55-0396 is not hydrolyzed into des-methoxyacetyl mibefradil. Thus, NNC 55-0396 does not inhibit HVA Ca\(^{2+}\) channels as mibefradil does. The amount of NNC 55-0396 (which peaks at 492 m/z), however, increased in the cells for longer periods than 10 min as shown in Fig. 6B.

**Accessibility of NNC 55-0396 on the T-Type Ca\(^{2+}\) Channel.** The slow onset of NNC 55-0396 inhibition on T-type Ca\(^{2+}\) current and the increasing NNC 55-0396 accumulation in the cells with time suggest that this compound dissolves in or passes through the plasma membrane to exert its effect. To test this hypothesis, we examined the reversibility of the NNC 55-0396 blockade of T-type Ca\(^{2+}\) current. Whole-cell currents were evoked in HEK 293/\(\alpha_{2}\)G cells by test pulses to \(-10\) mV from a holding potential of \(-70\) mV. After establishing a steady current, small volumes of 8 \(\mu M\) NNC 55-0396 were delivered in close proximity to the recording cell with a quartz capillary positioned by a micromanipulator. The drug was washed out after more than a 50\% inhibition of T-type Ca\(^{2+}\) current was achieved. As shown in Fig. 7A, the blockade of the T-type Ca\(^{2+}\) current by NNC 55-0396 was poorly reversible during a 10-min washout period in these experiments. Since the NNC 55-0396 blocks T-type Ca\(^{2+}\) current at a relatively slow rate and is poorly reversible by washing out, the drug binding site of the channel may be within transmembrane or intracellular domains of the channel.

If the site of action of NNC 55-0396 is on the intracellular side of the membrane, we would expect that intracellular perfusion of the drug would effectively block T-type Ca\(^{2+}\) current. To test this possibility, whole-cell currents were recorded in HEK 293/\(\alpha_{2}\)G cells with 8 \(\mu M\) NNC 55-0396 added to the pipette solution. As shown in Fig. 7B, there was...
Development of a specific T-type Ca$^{2+}$ inhibitor and thus have potentially important scientific and therapeutic implications.

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References


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