The Ca\(^{2+}\) Channel Inhibitor NNC 55-0396 Inhibits Voltage-Dependent K\(^{+}\) Channels in Rabbit Coronary Arterial Smooth Muscle Cells

Youn Kyoung Son\(^{1,\#}\), Da Hye Hong\(^{1,\#}\), Hongliang Li\(^{1}\), Dae-Joong Kim\(^{2}\), Sung Hun Na\(^{3}\), Hongzoo Park\(^{4}\), Won-Kyo Jung\(^{5}\), Il-Whan Choi\(^{6,\ast, a}\), and Won Sun Park\(^{1,\ast, b}\)

\(^{1}\)Institute of Medical Sciences, Department of Physiology, Kangwon National University School of Medicine, Chuncheon 200-701, Korea
\(^{2}\)Institute of Medical Sciences, Department of Anatomy and Cell Biology, Kangwon National University School of Medicine, Chuncheon 200-701, Korea
\(^{3}\)Institute of Medical Sciences, Department of Obstetrics and Gynecology, Kangwon National University Hospital, School of Medicine, Kangwon National University, Chuncheon 200-701, Korea
\(^{4}\)Department of Biomedical Engineering, and Center for Marine-Integrated Biomedical Technology (BK21 Plus) Pukyong National University, Busan 608-737, Korea
\(^{5}\)Department of Microbiology, Inje University College of Medicine, Busan 614-735, Korea

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Abstract. We demonstrated the inhibitory effect of NNC 55-0396, a T-type Ca\(^{2+}\) channel inhibitor, on voltage-dependent K\(^{+}\) (K\(_{V}\)) channels in freshly isolated rabbit coronary arterial smooth muscle cells. NNC 55-0396 decreased the amplitude of K\(_{V}\) currents in a concentration-dependent manner, with an IC\(_{50}\) of 0.080 \(\mu\)M and a Hill coefficient of 0.76. NNC 55-0396 did not affect steady-state activation and inactivation curves, indicating that the compound does not affect the voltage sensitivity of K\(_{V}\) channel gating. Both the K\(_{V}\) currents and the inhibitory effect of NNC 55-0396 on K\(_{V}\) channels were not altered by depletion of extracellular Ca\(^{2+}\) or intracellular ATP, suggesting that the inhibitory effect of NNC 55-0396 is independent of Ca\(^{2+}\)-channel activity and phosphorylation-dependent signaling cascades. From these results, we concluded that NNC 55-0396 dose-dependently inhibits K\(_{V}\) currents, independently of Ca\(^{2+}\)-channel activity and intracellular signaling cascades.

Keywords: NNC 55-0396, voltage-dependent K\(^{+}\) channel, coronary artery

Introduction

Calcium (Ca\(^{2+}\)) is a key factor in cellular signaling and function, and Ca\(^{2+}\) influx via Ca\(^{2+}\) channels increases the intracellular Ca\(^{2+}\) concentrations by promoting Ca\(^{2+}\) release from the intracellular Ca\(^{2+}\) stores. L-, T-, N-, P-, and Q-type Ca\(^{2+}\) channels have been identified based on their biophysical and electrophysiological characteristics, structure, localization, and pharmacological sensitivities (1). Among these, the T-type Ca\(^{2+}\) channel, which is found in neurons, endocrine cells, the kidney, cardiac myocytes, and vascular smooth muscle cells, is distinguished by its low conductance and its transient, low-voltage activation (2 – 6). Moreover, T-type Ca\(^{2+}\) channels contribute to numerous physiological functions, including neuronal firing, hormone secretion, myoblast fusion, fertilization, and smooth muscle contraction (6 – 12). In the cardiovascular system, modulation of the T-type Ca\(^{2+}\) channel has been identified as a key therapeutic target for cardiovascular diseases such as hypertension, hypertrophy, and chronic angina pectoris (13, 14).

In past decades, several T-type Ca\(^{2+}\)-channel inhibitors have been developed. Mibefradil, a tetralol derivative, was developed as a specific T-type Ca\(^{2+}\)-channel inhibitor in the 1990s (15). Although this drug effectively reduced hypertension and ameliorated ischemic heart disease (14, 16, 17), it also inhibited L-type Ca\(^{2+}\) channels via the production of an active metabolite through intracellular hydrolysis (18). Furthermore, it inhibited voltage-
dependent K⁺ (Kᵥ) channels, independently of Ca²⁺ channel inhibition (19). This issue led to the development of NNC 55-0396, a mibefradil derivative that does not simultaneously affect L-type Ca²⁺ channels (20). However, based on the similarity of chemical structure of mibefradil and NNC 55-0396, NNC 55-0396 could also have similar effects on the Kᵥ channel.

Four types of K⁺ channels are currently known to be expressed in vascular smooth muscle cells: 1) voltage-dependent K⁺ (Kᵥ) channels, 2) ATP-dependent K⁺ (KᵥATP) channels, 3) big-conductance Ca²⁺-activated K⁺ (BKCa) channels, and 4) inwardly rectifying K⁺ (Kᵢ) channels (21, 22). Among these, the Kᵥ channel plays a key role in regulating resting membrane potential and thereby maintaining basal vascular tone (21, 23, 24). In fact, the inhibition of Kᵥ channels produces membrane depolarization and vasoconstriction in some arteries. Moreover, impairment of Kᵥ channel is closely related to vascular dysfunction in diseases such as diabetes, hypertension, hypoxia, and cardiac disease (25 – 29).

In this study, we investigated the effect of NNC 55-0396 on the Kᵥ channel in freshly isolated rabbit coronary arterial smooth muscle cells. NNC 55-0396 inhibited Kᵥ current in a concentration-dependent manner independently of Ca²⁺ channel inhibition and phosphorylation-dependent signaling cascades.

Materials and Methods

Cell isolation

New Zealand White rabbits (2.0 – 2.5 kg) were anesthetized with simultaneous injection of zolletil (15 mg/kg), rompun (0.5 mg/kg), and heparin (100 U/kg). The procedure was conducted in accordance with guidelines established by the Committee for Animal Experiments at Kangwon National University. Under a stereomicroscope, the left descending coronary artery was dissected out of the heart and cleaned of the surrounding tissues in normal Tyrode’s solution. The isolated arteries were digested in Ca²⁺-free normal Tyrode’s solution containing papain (1.0 mg/mL), BSA (1.5 mg/mL), and DTT (1.0 mg/mL) for 20 min and then in Ca²⁺-free normal Tyrode’s solution containing collagenase (2.8 mg/mL), BSA (1.0 mg/mL), and DTT (1.5 mg/mL) for 20 min at 37°C.

Electrophysiology

Whole-cell currents were recorded from single arterial smooth muscle cells using the patch-clamp technique. Data were recorded using an EPC-8 amplifier (Medical System Corp., Darmstadt, Germany) controlled by an IBM-compatible computer via a digital interface (NI-DAQ 7; National Instruments, Union, CA, USA) using PatchPro software. The electrode tip had a resistance in the range of 2 – 4 MΩ when filled with pipette solution.

Solutions and drugs

Normal Tyrode’s solution contained 140 mM NaCl, 5.4 mM KCl, 0.33 mM NaH₂PO₄, 1.8 mM CaCl₂, 0.5 mM MgCl₂, 5 mM HEPES, 16.6 mM glucose, adjusted to pH 7.4 with NaOH. KB solution contained: 70 mM KOH, 50 mM L-glutamate, 20 mM KH₂PO₄, 55 mM KCl, 20 mM taurine, 3 mM MgCl₂, 20 mM glucose, 10 mM HEPES, 0.5 mM EGTA, adjusted to pH 7.3 with KOH. The pipette solution contained 115 mM K-aspartate, 25 mM KCl, 5 mM NaCl, 1 mM MgCl₂, 4 mM Mg-ATP, 10 mM EGTA, and 10 mM HEPES, adjusted to pH 7.2 with KOH.

Data analyses

We used the Origin 7.5 software package (Microcal Software, Inc., Northampton, MA, USA) for data analysis. To describe the kinetics of drug–ion channel interaction, a first-order blocking scheme was used (30, 31). The IC₅₀ and Hill coefficient were obtained using the least-squares method to fit the dose-dependence data to following Hill equation:

\[ f = 1 / \left(1 + \left(\frac{IC_{50}}{[D]}\right)^n\right) \]  (eq. 1)

where \( f \) is the fractional current block (\( f = I_{\text{drug}} / I_{\text{control}} \)) at the test potential, \( n \) is the Hill coefficient, and \([D]\) is the drug concentration.

Steady-state activation curve was obtained from tail currents. The tail currents were elicited by returning to a potential of −40 mV and were normalized by the maximal current. The activation curve was fitted with a Boltzmann equation, as follows:

\[ y = 1 / \left(1 + \exp \left(-\frac{(V - V_{1/2})}{k}\right)\right) \]  (eq. 2)

where \( k \) is the slope factor, \( V \) is the test potential, and \( V_{1/2} \) is the voltage of half-maximal activation.

The steady-state inactivation curve was obtained using a double-pulse protocol. After the application of preconditioning pulses at various potentials from −80 to +30 mV for 7 s, the steady-state current at the test potential (+40 mV for 600 ms) was obtained. The steady-state inactivation curve was fitted with another Boltzmann equation, as follows:

\[ y = 1 / \left(1 + \exp \left((V - V_{1/2}) / k\right)\right) \]  (eq. 3)

where \( V \) represents the preconditioning potential, \( V_{1/2} \) is the potential corresponding to the half-maximal of inactivation point, and \( k \) is the slope value.
Results

Inhibition of $K_v$ current by NNC 55-0396

We tested the effect of NNC 55-0396 on $K_v$ channels in freshly isolated coronary arterial smooth muscle cells from rabbit heart. BK$_{Ca}$ and K$_{ATP}$ currents were effectively prevented by inclusion of EGTA and ATP in the pipette solution. $K_v$ currents were elicited by 10-mV depolarizing step pulses from $-60$ mV to $+60$ mV for 600 ms from a holding potential of $-60$ mV. The $K_v$ current peaked rapidly and then showed a slow, partial intrinsic inactivation. $K_v$ current had decreased within 1 min after exposure to 0.1 $\mu$M NNC 55-0396, as shown in Fig. 1B. The inhibition by NNC 55-0396 was partially washed out (approximately 30%). The current–voltage ($I-V$) relationships at peak and steady-state $K_v$ currents are shown in Fig. 1, C and D, respectively. The application of NNC 55-0396 decreased the peak $K_v$ current by 39.92% ± 0.67% and steady-state current by 46.11% ± 0.87%.

NNC 55-0396 inhibits $K_v$ currents in a concentration-dependent manner

Various concentrations of NNC 55-0396 (0, 0.001, 0.01, 0.1, 1, or 10 $\mu$M) were applied to $K_v$ channels to test whether the observed inhibition was concentration-dependent. Currents were elicited by applying a +60-mV depolarizing pulse from a holding potential of $-60$ mV, and the representative traces were superimposed as shown in Fig. 2A. With increasing NNC 55-0396 con-
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centrations, the inhibition of \( K_V \) current also increased. The results of least-squares fitting using the Hill equation (eq. 1) yielded an IC\(_{50} \) of 0.08 ± 0.01 \( \mu \)M and a Hill coefficient of 0.76 ± 0.03 (Fig. 2B). These results demonstrate that NNC 55-0396 inhibits \( K_V \) channels in a concentration-dependent manner.

Effect of NNC 55-0396 on steady-state activation and inactivation of \( K_V \) currents

To determine whether NNC 55-0396 affected the gating properties of the \( K_V \) channel, we examined the effect of NNC 55-0396 on steady-state activation and inactivation curves. The steady-state activation curve was obtained from the tail current elicited by a two-pulse protocol, as described in Materials and Methods. The tail currents were normalized to the maximum current and plotted against the membrane potential, which were fitted with the Boltzmann function (eq. 2) as shown in Fig. 3A. Application of 0.1 \( \mu \)M NNC 55-0396 did not affect the activation curve of the \( K_V \) channel. The half-maximal potential (\( V_{1/2} \)) and slope value (\( k \)) were \(-11.30 \pm 0.43 \) mV and \( 7.54 \pm 0.20 \) under the control condition, and \(-10.09 \pm 0.76 \) mV and \( 7.65 \pm 0.35 \), respectively, in the presence of 0.1 \( \mu \)M NNC 55-0396.

The steady-state inactivation curve was calculated from the conventional double-pulse protocol. The steady-state currents elicited by the second pulse were obtained and normalized to the maximum current. The data were plotted against the membrane potential and fitted with a second Boltzmann equation (eq. 3) as described in the Materials and Methods. Similar to the activation curve, 0.1 \( \mu \)M NNC 55-0396 did not change the steady-state inactivation curve of \( K_V \) channels. The half-maximal potential (\( V_{1/2} \)) and slope value (\( k \)) were \(-32.32 \pm 1.53 \) mV and \( 7.81 \pm 0.69 \) under control conditions and \(-34.45 \pm 1.37 \) mV and \( 7.08 \pm 0.52 \), respectively, in the presence of 0.1 \( \mu \)M NNC 55-0396. These results suggest that NNC 55-0396 does not affect the voltage sensitivity of gating in \( K_V \) channels.

Effect of NNC 55-0396 on \( K_V \) channels in the absence of extracellular \( Ca^{2+} \) or intracellular ATP

To determine whether the inhibitory effect of NNC 55-0396 on \( K_V \) channel is related to \( T \)-type \( Ca^{2+} \) channel inhibition, we recorded the effect of NNC 55-0396 on \( K_V \) channels under \( Ca^{2+} \)-free conditions. As shown in Fig. 4, A and B, \( Ca^{2+} \)-free conditions did not affect \( K_V \) channels or the NNC 55-0396-induced inhibition of \( K_V \) channels. The current density of \( K_V \) channels was \( 43.35 \pm 0.97 \) pA/pF under the \( Ca^{2+} \)-free condition and \( 22.73 \pm 1.25 \) pA/pF after the application of 0.1 \( \mu \)M NNC 55-0396. The percentage inhibition of \( K_V \) channels due to NNC 55-0396 treatment under the \( Ca^{2+} \)-free condition did not differ compared to that observed under normal \( Ca^{2+} \) concentration.

We also assessed the effect of NNC 55-0396 on \( K_V \) channels in the absence of intracellular ATP to exclude the involvement of intracellular signaling cascades. As shown in Fig. 4, C and D, the inhibitory effect of NNC 55-0396 was not affected by the exclusion of intracellular ATP. The current density of \( K_V \) channels was \( 42.43 \pm 1.43 \) pA/pF under the ATP-free condition and \( 23.84 \pm 1.88 \) pA/pF after exposure to 0.1 \( \mu \)M NNC 55-0396. The percentage inhibition of \( K_V \) channels by NNC 55-0396 under ATP-free conditions showed no difference compared to that under normal condition. These results suggest that neither \( Ca^{2+} \)-channel activity nor the intracellular signaling cascade was involved in the inhibitory effect of NNC 55-0396 on \( K_V \) channels.
Discussion

This study demonstrated the inhibitory effect of NNC 55-0396 on K_V channels in freshly isolated rabbit coronary arterial smooth muscle cells. Based on several findings of this study, NNC 55-0396 appears to inhibit K_V channels directly regardless of its own target. First, inhibition of K_V channels in the presence of NNC 55-0396 occurred rapidly and reached steady-state within 1 min. This rapid response suggests that inhibition was not due to signaling cascades regulating K_V channels, but rather mediated by direct interaction. Second, the depletion of extracellular Ca^{2+} did not alter the inhibitory effect of NNC 55-0396 on K_V channels (Fig. 4: A and B). This suggests that the activity of the Ca^{2+} channel is not related to K_V-channel inhibition, given that the presence of Ca^{2+} ions is essential to activating any type of Ca^{2+} channel. Third, the inhibitory effect of NNC 55-0396 on K_V channel was sustained even under the ATP-free condition (Fig. 4: C and D), suggesting that intracellular signaling cascades are not involved in inhibition. Finally, the IC_{50} of NNC 55-0396 on the K_V channel is 0.080 μM, which is below the range necessary for inhibition of the T-type Ca^{2+} channel (IC_{50} = 6.8 μM for T-type Ca^{2+} channels examined in HEK/α1G cells) (20). From these results, we conclude that the inhibition of the K_V channel by NNC 55-0396 was independent of Ca^{2+}-channel activity and intracellular signaling cascades.

The regulation of Ca^{2+} channels, specifically L-type Ca^{2+} channels, has been intensively researched as a potential therapeutic target in cardiovascular diseases such as hypertension and hypertrophy. However, recent work has shown that several other types of Ca^{2+} channel are also promising therapeutic targets for cardiovascular disease (32), particularly N-type and T-type Ca^{2+} channels. N-type Ca^{2+} channels are involved in noradrenaline release in nerve cells (33), and T-type Ca^{2+} channels contribute to the regulation of heart rate in cardiac cells and vascular tone in arterial smooth muscle (34 – 37). Mibefradil was developed as a non-dihydropyridine T-type Ca^{2+}-channel inhibitor (14), but was subsequently withdrawn from the market in 1998 because it induced QT-interval prolongation and torsade de pointes (38). Mibefradil has also been shown to inhibit L-type Ca^{2+} channels, through a metabolite of intracellular hydrolysis (18). Currently, mibefradil is used only for laboratory purposes.

NNC 55-0396 is a specific T-type Ca^{2+}-channel inhibitor, developed through modification of mibefradil. In contrast to mibefradil, NNC 55-0396 does not produce the metabolite responsible for inhibiting the L-type Ca^{2+} channel (20) and was shown to improve tremor and myogenic tone (39, 40). However, previous work and the present study have revealed that both mibefradil and NNC 55-0396 inhibit K_V channels in arterial smooth muscle cells (19), suggesting that the structural similarities between mibefradil and NNC 55-0396 may contribute to K_V-channel inhibition.

Generally, K_V channel inhibitors contain a protonated ammonium atom as the key structure of the drug (30, 41 – 44). This tertiary amine structure is found in both mibefradil and NNC 55-0396 and is located near the
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center of the molecules. This amine group is critical for K\textsubscript{v}-channel inhibition. Although neither drug altered the voltage-dependent activation or inactivation curve for K\textsubscript{v} channels, their inhibitory mechanisms are likely distinct; mibefradil accelerated the intrinsic decay of the current, whereas NNC 55-0396 induced slightly more inhibition for steady-state current than for peak current (Fig. 1). This study was not designed to elucidate the detailed inhibitory mechanisms of these related drugs; however, distinct side chains are likely a key factor in the differing mechanisms of inhibition of K\textsubscript{v} channels.

K\textsubscript{v} channels expressed in vascular smooth muscle play a central role in the maintenance of resting membrane potential, and thereby resting tone (21, 22). Furthermore, K\textsubscript{v} channels are closely related to the agonist-induced and/or protein-kinase–dependent signaling cascade. For this reason, the effect of drugs on the K\textsubscript{v} channel must be clearly and thoroughly understood to correctly interpret vascular functional data. In fact, many studies, including our own, have reported that a number of compounds affect K\textsubscript{v} channels independently of their original targets. For example, mibefradil, LY294002, curcumin, verapamil, YC-1, and BIM (I) were shown to decrease K\textsuperscript{v} current directly while in the open state (i.e., by accelerating the intrinsic decay of the current), without affecting voltage-dependent gating properties (19, 45–49). Efodipidine, genistein, and H-89 also reduced K\textsubscript{v} current, but elicited this effect through sustained intrinsic decay of the current, suggesting that the inhibitory mechanism was not open-state block (50–52). Efodipidine shifted the steady-state inactivation curve to more negative potentials, but genistein and H-89 had no effect on either the steady-state activation or inactivation curves. Staurosporine was reported to inhibit K\textsubscript{v} currents through both open- and closed-state interactions (53). Indeed, staurosporine accelerated the intrinsic decay of current and shifted the voltage-dependent activation curve toward a positive potential. In the current study, we found that NNC 55-0396 reduced K\textsubscript{v} currents through a sustained intrinsic decay of the current. In addition, voltage-dependent activation and inactivation curves were not affected by NNC 55-0396, suggesting that the inhibitory action of NNC 55-0396 was not via an open-state block and that it had no effect on the voltage-dependent gating properties. This was similar to our observations for both genistein and H-89.

In this study, we demonstrated for the first time that NNC 55-0396 inhibits K\textsubscript{v} channels as well as T-type Ca\textsuperscript{2+} channels. Therefore, caution is required when using NNC 55-0396 to inhibit T-type Ca\textsuperscript{2+} channels for vascular functional studies.

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