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The mitochondrial Ca^{2+}-activated K^+ channel activator, NS 1619 inhibits L-type Ca^{2+} channels in rat ventricular myocytes

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Abstract

We examined the effects of the mitochondrial Ca^{2+}-activated K^+ (mitoBK$_{Ca}$) channel activator NS 1619 on L-type Ca^{2+} channels in rat ventricular myocytes. NS 1619 inhibited the Ca^{2+} current in a dose-dependent manner. NS 1619 shifted the activation curve to more positive potentials, but did not have a significant effect on the inactivation curve. Pretreatment with inhibitors of membrane BK$_{Ca}$ channel, mitoBK$_{Ca}$ channel, protein kinase C, protein kinase A, and protein kinase G had little effect on the Ca^{2+} current and did not alter the inhibitory effect of NS 1619 significantly. The application of additional NS 1619 in the presence of isoproterenol, a selective β-adrenoreceptor agonist, reduced the Ca^{2+} current to approximately the same level as a single application of NS 1619. In conclusion, our results suggest that NS 1619 inhibits the Ca^{2+} current independent of the mitoBK$_{Ca}$ channel and protein kinases. Since NS 1619 is widely used to study mitoBK$_{Ca}$ channel function, it is essential to verify these unexpected effects of NS 1619 before experimental data can be interpreted accurately.

Keywords: Mitochondrial Ca^{2+}-activated K^+ channel; NS 1619; L-type Ca^{2+} channel; Ventricular myocytes

The mitochondrial large-conductance Ca^{2+}-activated K^+ (mitoBK$_{Ca}$) channel in cardiac myocytes is considered a key element in cardioprotection [1–3]. NS 1619, a specific BK$_{Ca}$ channel activator, is widely used to activate the mitoBK$_{Ca}$ channel in cardiac myocytes. Since cardiac myocytes do not express the Ca^{2+}-activated K^+ channel on the cell membrane [4], NS 1619 is very effective for studying mitoBK$_{Ca}$ channels. A previous study suggested that the effect of NS 1619 was very specific to activation of the mitoBK$_{Ca}$ channel, since the activation of the mitoBK$_{Ca}$ channel by NS 1619 was inhibited completely by a mitoBK$_{Ca}$ channel inhibitor (paxilline), but not by other mitochondrial K^+ channel inhibitors [5]. However, the usefulness of NS 1619 in intact cells is limited by its nonspecific actions on channels other than mitoBK$_{Ca}$ channels.

L-type Ca^{2+} channels play a central role in regulating the excitation–contraction (EC) coupling, action potential duration, and modulation of pacemaker activity [6–9]. Clinically, drugs that inhibit the Ca^{2+} influx through L-type Ca^{2+} channels are used to treat hypertension, angina pectoris, and certain cardiac arrhythmias [10,11]. Cardiac Ca^{2+} channels are strongly regulated by the intracellular second messenger system (e.g., protein kinases). For example, in cardiac muscle, protein kinase C (PKC) generally activates Ca^{2+} currents [12]. In addition, the activation of protein kinase A (PKA) induces an increase in Ca^{2+} channel activity [13]. Although several studies have reported protein kinase G (PKG)-mediated inhibition of Ca^{2+} channels

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[14], others have reported the opposite effect, particularly when cAMP levels are elevated [15]. Considering the importance of Ca^{2+} channels in modulating cardiac function and disease, it is essential to verify the nonspecific actions of the mitoBK$_{Ca}$ channels opener NS 1619 on other channels before experimental data can be interpreted accurately.

Therefore, we investigated the effects of NS 1619 on the Ca^{2+} current in freshly isolated from rat ventricular myocytes. We found that NS 1619 inhibited the Ca^{2+} current, and that this inhibitory effect was independent of the intracellular signal transduction mechanism and mitochondria activity.

Materials and methods

Cell isolation. Male Sprague–Dawley rats (250–280 g) were anesthetized by intraperitoneal injection of a mixture of pentobarbital sodium (50 mg/kg body weight) and heparin (300 U/ml). The heart was cannulated and then retrogradely perfused via the aorta on a Langendorff apparatus, and was perfused with enzyme solution containing 0.01% collagenase (1 mg/10 ml, Yakult) for 25–30 min. Following the isolation procedure, the left ventricle was dissected out and agitated mechanically in high-K^+, and low-Cl^- storage medium to obtain single myocytes.

Solutions and drugs. The composition of normal Tyrode and high-K^+, and low-Cl^- storage medium were described previously [3]. The recording solution contained (mM): 140, NaCl; 10, CsCl; 5, Hepes; 0.33, NaH_2PO_4; 0.5, $MgCl_2$; 16.6, glucose; 1.8, $CaCl_2$; titrated to pH 7.4 with NaOH. The pipette filling solution contained (mM): 106, CsCl; 20, TEA–Cl; 5, Mg–ATP; 5, NaCl; 10, Hepes; 10, EGTA; with the pH adjusted to 7.25 using CsOH. NS 1619, iberiotoxin, paxilline, isoproterenol, tetrodotoxin, GF 109203X, and isoproterenol were purchased from Sigma Chemical Co. (St Louis, MO). Rp-8-CPT-cAMPs and RP-8-BR-PET-cGMPs were purchased from Biologic Life Science Institute (Bremen, Germany).

Electrophysiological recordings. The membrane currents were recorded in the whole-cell configuration, using Axopatch-1C amplifier (Axon instruments Inc., Union, CA). The voltage signals were filtered at 1–2 kHz and were sampled at a rate of 2–4 kHz. All experiment parameters, such as pulse generation and data acquisition, were controlled using the PatchPro software, developed by our group. Recording electrodes were pulled from thin-walled borosilicate capillaries (Clark Electromedical Instruments, Pangbourne, UK) using Narishige PP-83 puller (Narishige Scientific Instrument Lab., Tokyo, Japan) and had a resistance of 3 MΩ when filled with pipette solution. Membrane capacitance was measured by integrating the area under the capacitance was measured series resistance compensation. To normalize for differences in total membrane area, current densities were calculated by dividing the total current by the membrane capacitance of the cell.

Data analysis. Origin 6.0 software (Microcal Software, Inc., Northampton, MA) was used for data analysis. Interaction kinetics between drugs and channels was described on the basis of a first-order blocking scheme, as described previously [16]. The apparent affinity constant (K_d) and Hill coefficient (n) were obtained by fitting concentration-dependence data to the following Hill equation:

$$f = 1/\{1 + (K_d/[D])n\}$$

in which f is the fractional inhibition ($f = 1 - I_{drug}/I_{control}$) at the test potential, and $[D]$ represents different drug concentrations.

Voltage-dependent activation was estimated from peak conductance.

$$G_{Ca} = I_{Ca}/(V_m - V_{rev})$$
$$d_{inf}(V) = G_{Ca}/G_{Camax}$$

where G_{Ca} is the peak conductance, I_{Ca} is the peak Ca^{2+} current, $d_{inf}(V)$ is the steady-state activation parameter, and $G_{Ca\ max}$ is the maximum value if G_{Ca}. V_{rev} is measured as the zero-current potential in the I–V relation.

Activation curves were fitted with the following Boltzmann equation:

$$y = 1/\{1 + \exp(-(V - V_{1/2})/k)\}$$

where k represents the slope factor, V represents the test potential, and $V_{1/2}$ is the voltage at which the conductance was half-maximal.

The steady-state voltage dependence of inactivation was investigated using a two-pulse voltage protocol; currents were measured with a 200-ms test potential to 0 mV, and 2-s preconditioning pulses were varied from −60 to −10 mV (in 10-mV steps) in the absence and presence of drugs. The resulting steady-state inactivation data were fitted with Boltzmann equation:

$$y = 1/\{1 + \exp((V - V_{1/2})/k)\}$$

where V is the preconditioning potential, $V_{1/2}$ represents the potential corresponding to the half-inactivation point, and k represents the slope value.

Statistics. Data are presented as means ± SEM. Statistical analyses were performed by student's t-test. A value of $P < 0.05$ was defined as statistically significant.

Results

Inhibition of the L-type Ca^{2+} current by NS 1619 in rat ventricular myocytes

We investigated the effect of NS 1619 on the L-type Ca^{2+} channels in rat ventricular myocytes. The Ca^{2+} current was isolated by blocking the K^+ current with internal and external Cs^+, and blocking the Na^+ and T-type Ca^{2+} currents with tetrodotoxin (20 μM) and a holding potential of −50 mV. Fig. 1A and B shows that the application of 10 μM NS 1619 inhibited the Ca^{2+} current in ventricular myocytes. Within 2 min of exposing the cells to NS 1619, the amplitude of the inward currents was greatly decreased throughout the entire range of the voltage. For example, at 0 mV, 10 μM NS 1619 significantly reduced the currents from -31.57 ± 2.65 pA/pF to -11.41 ± 1.99 pA/pF (Fig. 1C). This inhibitory effect was partially reversed (<65%, data not shown) when the NS 1619 was washed out.

Fig. 1D and E illustrates the concentration dependence of the effects of NS 1619 on the Ca^{2+} current. Examples showing inhibition of the Ca^{2+} current by 3, 10, and 30 μM are given in Fig. 1D. The concentration-dependence of the Ca^{2+} current inhibition is summarized in Fig. 1E. A nonlinear least-squares fit of the Hill equation to the concentration-dependence data yielded an apparent K_d value of 6.52 ± 0.27 μM and a Hill coefficient of 1.75 ± 0.07.

Effect of NS 1619 on the activation and steady-state inactivation of the Ca^{2+} current

The voltage-dependence of activation and steady-state inactivation was evaluated to determine whether the inhibition of Ca^{2+} current by NS 1619 was caused by a shift in the activation and/or inactivation curves. The activation curves were obtained by dividing the current amplitude by the electromotive force, and the data were fitted with

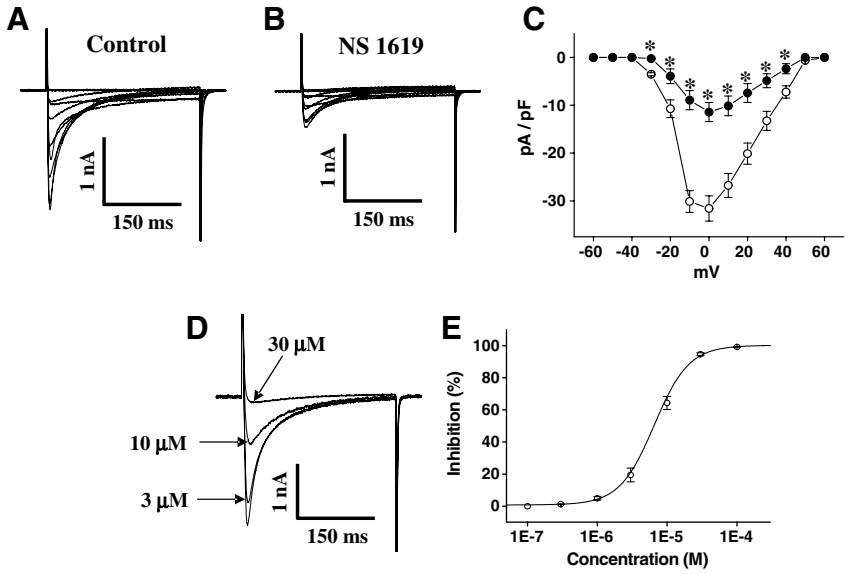

Fig. 1. Effect of NS 1619 on the whole-cell Ca^{2+} current. Superimposed current traces were elicited by 300-ms depolarizing pulses between −50 and +50 mV from a holding potential of −50 mV in steps of 10 mV under control conditions (A) and in the presence of 10 μM NS 1619 (B). (C) The current-voltage (*I–V*) relationships of the peak Ca^{2+} current in the absence (○) and presence (●) of 10 μM NS 1619 ($n = 4$). (D) Superimposed currents were elicited by applying 300-ms depolarizing pulses from a holding potential of −50 mV to 0 mV. The traces of the currents obtained in the presence of 3, 10, and 30 μM NS 1619. (E) The average percent inhibition of the Ca^{2+} current at 0 mV induced by different concentrations of NS 1619 in ventricular myocytes. All $n = 5$. $^{*}P < 0.05$.

a Boltzmann function. Fig. 2A shows the voltage-dependence of activation under control conditions and in the presence of 10 μM NS 1619. The application of 10 μM NS 1619 caused a significant positive shift in the activation curve. The half-maximal activation was shifted by +5 mV in the presence of NS 1619, but the slope of the activation curve was not altered. A potential of the half-maximal activation ($V_{1/2}$) and the slope value (k) were; -15.13 ± 0.26 mV and 4.76 ± 0.29, respectively, under control condition; -10.60 ± 0.30 mV and 5.12 ± 0.33, respectively, in the presence of 10 μM NS 1619.

The steady-state inactivation kinetics of the Ca^{2+} current in the absence and presence of 10 μM NS 1619 were investigated. The steady-state inactivation of the Ca^{2+} current was examined using a conventional double-pulse protocol. The values were plotted against the pre-pulse potentials, and fitted to the Boltzmann equation. As shown in Fig. 2B, NS 1619 did not shift the voltage-dependence of inactivation. The potential of the half inactivation ($V_{1/2}$) and the slope value (k) were; -31.30 ± 0.39 mV and 5.10 ± 0.36, respectively, under control condition; -31.54 ± 0.25 mV and 4.85 ± 0.23, respectively, in the presence of 10 μM NS 1619.

Effect of membBK$_{Ca}$ or mitoBK$_{Ca}$ channel inhibitors on the inhibition of Ca^{2+} channels by NS 1619

Although previous reports have suggested that cardiac myocytes do not express the BK_{Ca} channel in the cell membrane [4], we tested whether inhibition of the Ca^{2+} current

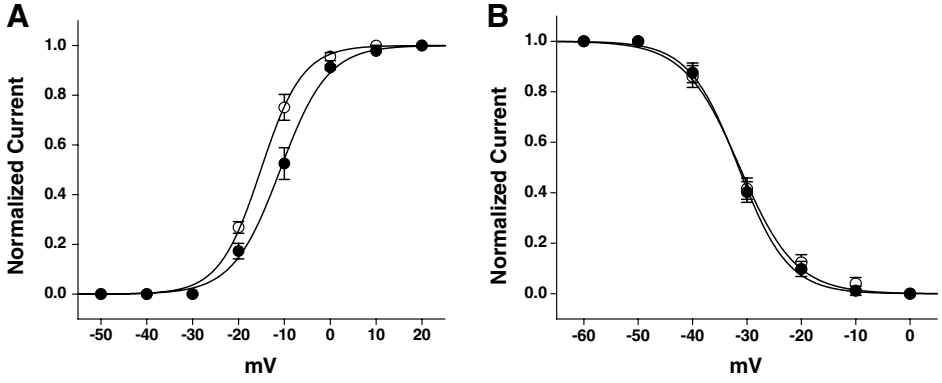

Fig. 2. Effects of NS 1619 on the activation and steady-state inactivation of Ca^{2+} currents. (A) The activation curves in the absence (○) and presence (●) of 10 μM NS 1619 ($n = 5$). (B) Steady-state inactivation curves under control conditions (○) and in the presence (●) of 10 μM NS 1619 ($n = 4$). The currents were activated by a test step to 0 mV after a 2-s conditioning pre-pulse at different voltages. The steady-state current amplitude in the test pulse was normalized to the peak amplitude after a pre-pulse potential. Data were fit to the Boltzmann equation.

by NS 1619 was due to activation of the membBK$_{Ca}$ channel. As shown in Fig. 3A, pretreatment with iberiotoxin (IbTX, 100 nM), a membBK$_{Ca}$ channel inhibitor, alone had little effect on the Ca^{2+} current, and did not significantly alter the effect of 10 μM NS 1619 on the Ca^{2+} current (Control: 32.73 ± 2.12, IbTX: 31.58 ± 1.96, IbTX + NS 1619: 12.16 ± 1.62 pA/pF, Fig. 3B). Similarly, pretreatment with the mitoBK$_{Ca}$ channel inhibitor paxilline (10 μM) did not significantly alter the effect of NS 1619 on the Ca^{2+} current and did not affect the Ca^{2+} current when applied alone (Control: 29.98 ± 2.42, paxilline: 29.20 ± 2.23, paxilline + NS 1619: 9.49 ± 1.73 pA/pF, Fig. 3C and D).

Inhibition of Ca^{2+} currents by NS 1619 does not involve PKC-, PKA-, and PKG-dependent pathways

To investigate whether the inhibition of the Ca^{2+} current by NS 1619 was due to changes in PKC, PKA, and PKG, we tested the effects of NS 1619 on the Ca^{2+} current on preincubation with PKC, PKA, and PKG inhibitors. Fig. 4A shows the effect of the PKC inhibitor GF 109203X (GFX) on the Ca^{2+} current. A 10-min exposure to 1 μM GFX did not inhibit the Ca^{2+} current, while the application of additional 10 μM NS 1619 inhibited the Ca^{2+} current by 62%, which was not significantly different from the inhibition induced by NS 1619 alone. Similarly, the PKA inhibitor RP-8-CPT-cAMPs (RP-cAMPs, 10 μM) and the PKG inhibitor RP-8-Br-PET-cGMPs (RP-cGMPs, 10 μM) alone had little effect on the Ca^{2+} current, and did not significantly change the effects of NS 1619 on the Ca^{2+} current (Fig. 4B and C). We summarized these results in Fig. 4D.

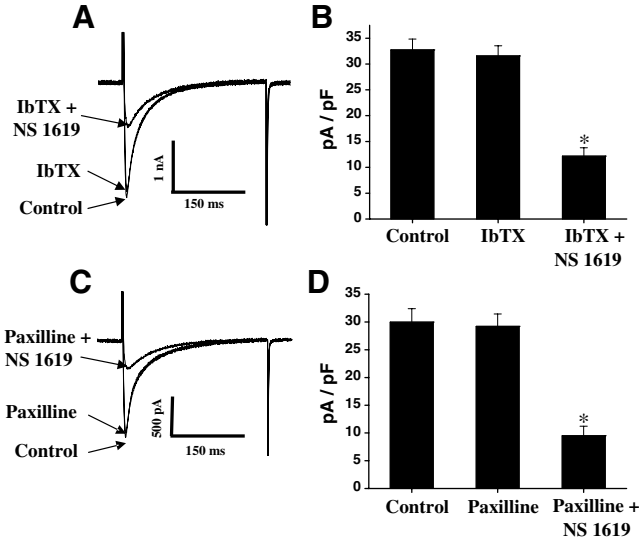

Fig. 3. Effects of membBK$_{Ca}$ or mitoBK$_{Ca}$ channel inhibitors on the NS 1619-induced inhibition of Ca^{2+} currents. (A) The traces of the currents obtained in the absence and presence of 100 nM iberiotoxin (IbTX), after the application of additional NS 1619 (10 μM), with a depolarization pulse to 0 mV from a holding potential of −50 mV. (B) Summary of the effects of IbTX on Ca^{2+} currents without and with NS 1619 ($n = 6$). (C) Similar current traces under control conditions, with 10 μM paxilline, and with the additional application of 10 μM NS 1619. (D) Summary of the effects of paxilline on Ca^{2+} currents of paxilline without and with NS 1619 ($n = 5$). $^{*}P < 0.05$.

The effect of NS 1619 on the Ca^{2+} current was also studied in myocytes pretreated with 1 μM isoproterenol (Iso), a selective β-adrenoreceptor agonist (Fig. 4E). It is well established that β-adrenergic stimulation activates adenylyl cyclase, leading to increased cAMP production, which increases the Ca^{2+} channel activities in various mammalian cardiac preparations [9,17,18]. Bath application of Iso increased the Ca^{2+} current approximately 113.20%. The application of additional NS 1619 in the presence of Iso reduced the Ca^{2+} current to approximately the same level as a single application of NS 1619 (Control: 30.23 ± 3.02, Iso: 64.33 ± 2.84, Iso + NS 1619: 9.98 ± 2.31 pA/pF, Fig. 4F).

We also tested the effect of NS 1619 on the Ca^{2+} current in the absence of pipette ATP to demonstrate that these effects occurred in a phosphorylation-independent manner. As shown in Fig. 4G and H, the presence or absence of ATP inside the pipette did not influence the blocking effect of NS 1619 on Ca^{2+} channels. These results suggest that the effect of NS 1619 occurs in a phosphorylation-independent manner.

Discussion

In this study, we investigated the effects of NS 1619 on L-type Ca^{2+} channels in rat ventricular myocytes. The inhibition of Ca^{2+} currents does not seem to occur through the intracellular signal transduction mechanism due for the following reasons. First, Ca^{2+} channels in cardiac myocytes are strongly regulated by protein kinases, especially PKC, PKA, and PKG [13,19–21]. However, pretreatment with PKC, PKA, and PKG inhibitors did not significantly change the effects of NS 1619 on the Ca^{2+} current. Moreover, the presence or absence of ATP inside the pipette did not influence the blocking effect of NS 1619 on the Ca^{2+} channels. Second, it was reported that cAMP stimulates the myocardial Ca^{2+} channels and any agent that increased the cAMP level would potentiate Ca^{2+} channels [22]. However, after the Ca^{2+} channel activities were elevated by β-adrenoreceptor agonist (Iso), the addition of NS 1619 reduced the Ca^{2+} current to approximately the same level as a single application of NS 1619 as shown in Fig. 4. Third, the effect of NS 1619 occurred rapidly (within 2 min) compared to the expected time course via protein kinases. The short exposure time required to reach steady-state inhibition is not explained simply by the inhibition of protein kinases.

In this study, we clearly demonstrated that NS 1619 inhibited the cardiac Ca^{2+} current independent of mitoBK$_{Ca}$ channel activation. Recent studies have also suggested that some chemicals inhibit the cardiac Ca^{2+} channel independent of their own role. For example, selective PKC inhibitors such as calphostin C and chelethyrine

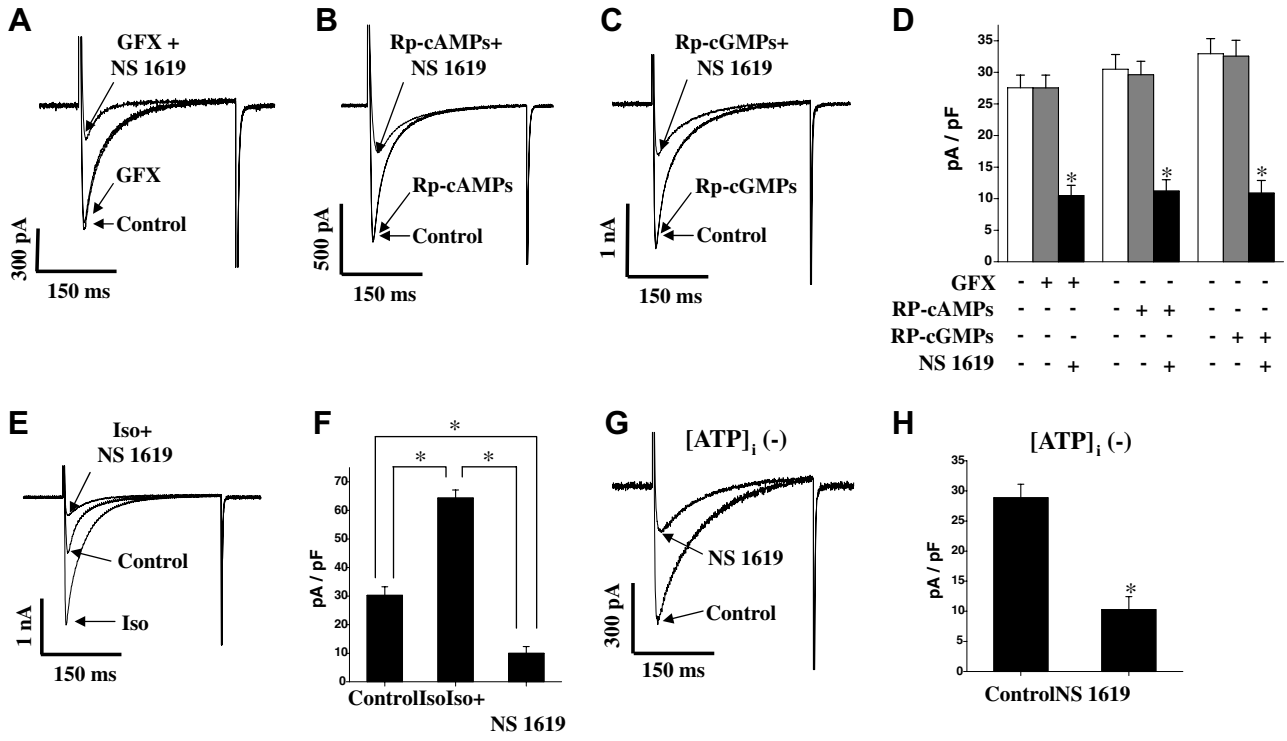

Fig. 4. Effect of PKC, PKA, and PKG inhibitors on the response to NS 1619. Current traces under control conditions, and in the presence of GFX (A, $n = 4$), RP-8-CPT-cAMPs (B, $n = 5$), RP-8-Br-PET-cGMPs (C, $n = 4$), and after the application of additional NS 1619 (10 μM) with a depolarization pulse to 0 mV from a holding potential of −50 mV. These results are summarized in (D). (E) The effect of 10 μM NS 1619 on the Ca^{2+} current previously stimulated by 1 μM Iso ($n = 5$). These results are summarized in (F). (G) The effect of pipette ATP on the NS 1619-induced inhibition of the Ca^{2+} current ($n = 7$). These results are summarized in (H). $^{*}P < 0.05$.

have been shown to directly block L-type Ca^{2+} channels in frog ventricular cells [23]. Another PKC inhibitor, staurosporine, also blocked rabbit ventricular L-type Ca^{2+} channels directly, without mediation through PKC or PKA inhibition [9]. Saxitoxin, which is a well-known sodium channel blocker, partially blocked the L-type Ca^{2+} channels in mouse ventricular myocytes and L-type Ca^{2+} channels heterologously expressed in tsA201 cells [24].

Although we could not inspect the binding structure of the L-type Ca^{2+} channel and NS 1619 exactly because no information on the three-dimensional structure of Ca^{2+} channel is available, we considered the binding action of NS 1619 based on the similar simulation of the Ca^{2+} channel and Ca^{2+} channel blockers. Most Ca^{2+} channel inhibitors and NS 1619 have oxygen atoms, electrophilic nitrogen atoms, and aromatic rings with a nitrogen atom, such as dihydropyridine or benzimidazole. According to a previous report that calculated the interaction between the L-type Ca^{2+} channel and the channel blocker, the plane of the dihydropyridine ring is parallel to the pore axis, the ligand NH group faces the IIIS5 segment, the starboard side of the heterocyclic ring points upward and the plane of the 4-aryl substituent is perpendicular to the pore axis [25]. Based on the three-dimensional construction of NS 1619, the benzimidazole of NS 1619 has to be perpendicular to the dihydropyridine according to the above results and considering the relative position of the two nitrogen atoms and the oxygen atom. On calculating the molecular dynamics (Chem3DUltra, CambridgeSoft, USA), the distances between the two nitrogen atoms and the ketone oxygen atom connected by another carbon atom were 2.5 ± 0.3 nm and 2.6 ± 0.2 nm, respectively, and 2.9 ± 0.4 nm and 4.3 ± 0.2 nm between the nitrogen atoms and hydroxyl oxygen atom, respectively. For the dihydropyridine blocker, the distances were 3.2 ± 0.8 nm between the nearest nitrogen atom and oxygen atom and 4.0 ± 0.4 nm between the nitrogen atom of dihydropyridine and the oxygen atom. These results suggest that the NS 1619 could occupy the position of dihydropyridine in a Ca^{2+} channel blocker.

Mibefradil is reported to inhibits L-type Ca^{2+} channels in a use-dependent manner without affecting the inactivation curve [26]. NS 1619 has a structure similar to that of mibefradil. Indeed, the benzimidazole, oxygen atoms of the hydroxyl and ketone groups, and fluoromethyl in NS 1619 correspond to the benzimidazole, methoxyacetate, and fluoroethyl in mibefradil, respectively. From the similarity of NS 1619 and mibefradil, NS 1619 likely inhibits the L-type Ca^{2+} channel in a manner similar to mibefradil.

In conclusion, we found that NS 1619 inhibited the Ca^{2+} current in a mitoBK$_{Ca}^{-}$, PKC-, PKA-, and PKG-independent manner. Therefore, caution is required when using NS

1619 in functional studies on the modulation of mitoBK_{Ca} channels.

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