



BK channel activation by NS-1619 is partially mediated by intracellular Ca^{2+} release in smooth muscle cells of porcine coronary artery

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1 Effects of NS-1619, an opener of large conductance Ca^{2+} -activated K^+ (BK) channel, on intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and membrane potential were examined in single myocytes freshly isolated from porcine coronary artery.

2 Under current clamp mode, the application of 1–30 μM NS-1619 hyperpolarized the membrane in concentration-dependent manner. The NS-1619-induced hyperpolarization was abolished by the presence of 100 nM iberiotoxin.

3 Application of 1–10 μM NS-1619 hyperpolarized the membrane by approximately 6 mV or less but did not change significantly the $[\text{Ca}^{2+}]_i$. When membrane hyperpolarization of 12 mV or so was caused by 30 μM NS-1619, $[\text{Ca}^{2+}]_i$ was unexpectedly increased by approximately 200 nM. This increase in $[\text{Ca}^{2+}]_i$ and the concomitant outward current activation were also observed under voltage-clamp at holding potential of -40 mV.

4 The increase in $[\text{Ca}^{2+}]_i$ by 30 μM NS-1619 occurred mainly in peripheral regions than in the centre of the myocytes. The removal of extracellular Ca^{2+} affected neither the membrane hyperpolarization nor the increase in $[\text{Ca}^{2+}]_i$.

5 In the presence of 10 mM caffeine and 10 μM ryanodine, the increase in $[\text{Ca}^{2+}]_i$ by 30 μM NS-1619 was not observed and the membrane hyperpolarization was reduced to approximately 67% of the control.

6 These results indicate that the opening of BK channels by NS-1619 at 30 μM , which is the most frequently used concentration of this agent, is partly due to Ca^{2+} release from caffeine/ryanodine-sensitive intracellular storage sites but is mainly due to the direct activation of the channels.

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Abbreviations: BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; BK channel, large conductance Ca^{2+} -activated K^+ channel; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; DMSO, dimethyl sulphoxide; IbTx, iberiotoxin; K_{ATP} channel, ATP-sensitive K^+ channel; SR, sarcoplasmic reticulum

Introduction

Large conductance Ca^{2+} -activated K^+ (BK) channels are highly expressed in smooth muscle cells of various organs, including blood vessels (Kuriyama *et al.*, 1998). In some smooth muscles, the blockade of BK channels results in the membrane depolarization by several millivolts and the rise of muscle tone, so that the channels may contribute to the regulation of the resting membrane potential and the muscle tone (Leblanc *et al.*, 1994; Nelson & Quayle, 1995; Carl *et al.*, 1996). Several compounds have been reported to be BK channel openers; dehydrosoyasaponin-I (McManus *et al.*, 1993; Giangiacomo *et al.*, 1998), maxikdiol (Singh *et al.*, 1994), niflumic acid (Ottolia & Toro, 1994), NS-004 (Sargent *et al.*, 1993; McKay *et al.*, 1994; Xu *et al.*, 1994), NS-1619 (Olesen *et al.*, 1994; Edwards *et al.*, 1994; Holland *et al.*,

1996), Evans blue (Hollywood *et al.*, 1998; Wu *et al.*, 1999) and nordihydroguaiaretic acid (Nagano *et al.*, 1996; Yamamura, *et al.*, 1999). A possibility has been suggested that BK channel openers may have a substantial potency for the treatment of angina, hypertension, bronchial asthma, hypersensitive urinary bladder and some other diseases characterized by the increased tonus of smooth muscles (Edwards & Weston, 1995). These are expected to be a new category of K^+ channel openers following those acting on ATP-sensitive K^+ (K_{ATP}) channel. The major mechanism underlying the vasodilating effect of K_{ATP} channel openers is supposed to be the depression of Ca^{2+} influx through the voltage dependent Ca^{2+} channel *via* the membrane hyperpolarization and the subsequent decrease in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in vascular myocytes (Nelson & Quayle, 1995). The modulation of Ca^{2+} mobilization by BK channel openers remains, however, unclear.

The present study was undertaken to examine the possibility that the regulation of intracellular Ca^{2+} mobiliza-

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tion could be involved in the mechanisms underlying the activation of BK channel by the most popular synthetic BK channel opener, NS-1619, in porcine coronary arterial smooth muscle cells. Based on the input resistance of single smooth muscle cells as high as approximately 2–4 G Ω , the measurement of membrane hyperpolarization under current clamp mode is often more sensitive to detect BK channel opening than the direct measurement of whole cell BK channel current under voltage clamp. In the present study, the confocal Ca²⁺ images and either membrane potentials or currents were simultaneously recorded from single coronary arterial smooth muscle cells in the absence or presence of NS-1619.

Methods

Cell isolation

Single smooth muscle cells of porcine coronary artery were prepared as described previously (Yamamura *et al.*, 1999). In brief, whole hearts from young pigs (6 months old) were obtained at a local slaughterhouse and transported to the laboratory in ice-cold normal Krebs' solution. A small piece of left circumflex coronary artery was dissected, cleaned of blood and surrounding tissues and immersed for 40 min in Ca²⁺-free Krebs' solution containing 1% albumin, 0.2% collagenase, 0.1% papain and 0.2% trypsin inhibitor at 37°C. After the incubation, the solution was replaced with Ca²⁺- and collagenase-free Krebs' solution. Myocytes were isolated by gentle agitation with a glass pipette and stored at 4°C until use. A few drops of cell suspension were placed in a recording chamber, which was mounted on the stage of a phase contrast microscope (Nikon TMD, Tokyo, Japan). After these cells were settled, the bath was continuously perfused with the HEPES-buffered solution at a flow rate of 5 ml min⁻¹.

Solutions

The normal Krebs' solution had an ionic composition of (in mM): NaCl 112, KCl 4.7, CaCl₂ 2.2, MgCl₂ 1.2, NaHCO₃ 25, KH₂PO₄ 1.2 and glucose 14. The pH was adjusted to 7.4 by gassing with a mixture of 95% O₂ and 5% CO₂. The Ca²⁺-free Krebs' solution was prepared by the removal of 2.2 mM CaCl₂ from the normal Krebs' solution. The HEPES-buffered solution for electrophysiological recording had an ionic composition of (in mM): NaCl 137, KCl 5.9, CaCl₂ 2.2, MgCl₂ 1.2, glucose 14 and HEPES 10. The pH of the solution was adjusted to 7.4 with NaOH. The pipette solution contained (in mM): KCl 140, MgCl₂ 1, HEPES 10 and Na₂ATP 2. The pH was adjusted to 7.2 with KOH.

Electrophysiological experiments

The whole cell patch clamp technique was applied to single cells by means of the techniques originally introduced by Hamill *et al.* (1981) using CEZ-2400 amplifier (Nihon Kohden, Tokyo, Japan). The procedures of electrophysiological recordings and data analyses were performed as described previously by use of programs, Data-Acquisition and Cell-Soft, which were developed in University of Calgary

(Imaizumi *et al.*, 1989). All electrophysiological recordings were carried out at 30 ± 1°C.

[Ca²⁺]_i measurements

Two dimensional Ca²⁺ images were obtained by a fast scanning confocal fluorescent microscope (Nikon RCM-8000; Nikon, Tokyo, Japan) equipped with objective lens (Fluor 40 × 1.15 NA, water immersion, Nikon) and Ratio3 software (Nikon). Recordings were started at least 3 min after rupturing the patch membrane to make indo-1 diffuse into the cell from the pipette, which was filled with the solution containing 100 μM indo-1. Excitation wavelength from an argon ion laser was 351 nm and the emission wavelengths were 405 and 485 nm. The resolution of the microscope is approximately 0.33 × 0.27 μm (1 pixel) and approximately 1.5 μm to Z-axis direction. The Ca²⁺ image was scanned over a full frame (512 × 512 pixels) every 20 or 30 s. The calibration of indo-1 signal was performed by the method reported by Kawanishi *et al.* (1994). The data analyses were performed as described previously (Imaizumi *et al.*, 1998).

Statistics

Pooled data were shown as mean ± s.e.mean. Statistical significance between two and multi groups was determined by Student's *t*-test and Scheffé's test after one-way ANOVA, respectively. Significant difference was expressed in figures as ** or ### *P* < 0.01.

Drugs

Pharmacological reagents were obtained from Sigma (St. Louis, U.S.A.) except for NS-1619 (1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl) phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one; Research Biochemicals International, Natick, U.S.A.), iberiotoxin (IbTx; Peptide Institute, Osaka, Japan), caffeine (Wako, Osaka, Japan), collagenase (Amano, Nagoya, Japan), HEPES, 1,2-bis(2-aminophenoxy)ethane-N,N',N'-tetraacetic acid (BAPTA), indo-1, fluo-3 (Dojin, Kumamoto, Japan) and fura-2 (Molecular Probes, Eugene, U.S.A.). NS-1619 was dissolved in dimethyl sulphoxide (DMSO) at the concentration of 10 mM as a stock solution. The external solutions always contained 0.3% DMSO throughout the experiments, regardless of the presence/absence of NS-1619. It was confirmed that 0.3% DMSO did not affect the membrane potential, current and [Ca²⁺]_i.

Results

Membrane hyperpolarization by NS-1619

When the membrane potential of single coronary smooth muscle cells was recorded under current clamp mode, the averaged resting membrane potential was -39.3 ± 1.1 mV (*n* = 10). The application of 30 μM NS-1619 hyperpolarized the membrane by 11.7 ± 1.1 mV (*n* = 10, *P* < 0.01 vs control; Figure 1) in all myocytes examined. The membrane potential was mostly recovered by washout of NS-1619 (-40.2 ± 1.1 mV, *n* = 10). The hyperpolarization induced by 30 μM NS-1619 was not affected significantly by either 10 μM

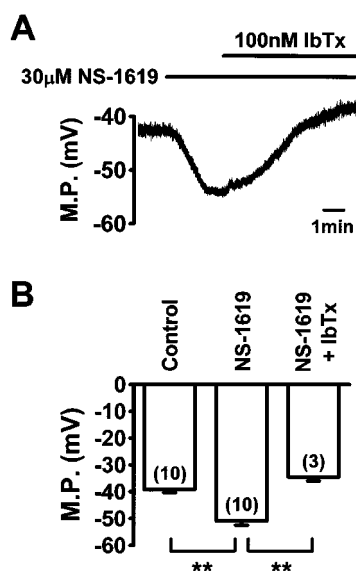


Figure 1 Effects of NS-1619 on membrane potential (M.P.) in single smooth muscle cells of porcine coronary artery under current clamp mode. (A) Application of $30 \mu\text{M}$ NS-1619 hyperpolarized the myocyte by 12 mV. The NS-1619-induced hyperpolarization was abolished by 100 nM iberiotoxin (IbTx). (B) Summarized data obtained from experiments typically shown in (A). The number of myocytes used is given in parentheses. The statistical significance of the difference between three groups is indicated by $**P < 0.01$.

glibenclamide, a K_{ATP} channel blocker, or 100 nM apamin, a small conductance Ca^{2+} -activated K^{+} channel blocker ($P > 0.05$, $n = 5$ for each). In contrast, the addition of 100 nM IbTx, a selective BK channel inhibitor, abolished the NS-1619-induced hyperpolarization and, moreover, depolarized the myocytes by approximately 5 mV (5.0 ± 0.6 mV depolarization from the initial resting potential, $n = 3$; Figure 1). Application of 100 nM IbTx in the absence of NS-1619 depolarized the membrane from -38.8 ± 1.2 to -34.3 ± 1.2 mV ($n = 3$, $P < 0.05$) and the subsequent addition of $30 \mu\text{M}$ NS-1619 did not induce further change in the membrane potential (-33.8 ± 0.4 mV, $P > 0.05$ vs only IbTx).

Effects of NS-1619 on $[\text{Ca}^{2+}]_i$ and membrane potential or current

The simultaneous measurements of $[\text{Ca}^{2+}]_i$ and membrane potential were performed using a confocal microscope and $100 \mu\text{M}$ indo-1 under current clamp mode. The averaged resting membrane potential and $[\text{Ca}^{2+}]_i$ as the average from whole cell area were -40.0 ± 1.0 mV and 114 ± 6 nM, respectively ($n = 21$). As shown in Figure 2, the application of $30 \mu\text{M}$ NS-1619 caused the membrane hyperpolarization by 12.5 ± 1.0 mV ($n = 11$, $P < 0.01$ vs control). Unexpectedly, a small but significant increase in $[\text{Ca}^{2+}]_i$ to 308 ± 20 nM ($n = 11$, $P < 0.01$ vs control) was observed during the membrane hyperpolarization (Figure 2). These effects of $30 \mu\text{M}$ NS-1619 were removed by washout and reproduced at least three times in each myocyte. Of importance is that the membrane hyperpolarization was caused by NS-1619 in a concentration-dependent manner in the range of 1 and $30 \mu\text{M}$, but the significant increase in $[\text{Ca}^{2+}]_i$ was elicited

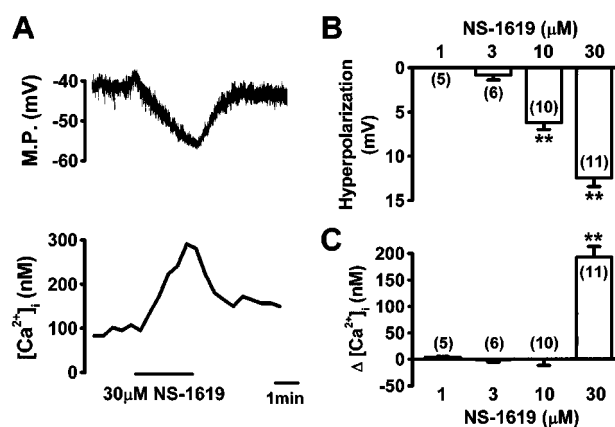


Figure 2 Effects of NS-1619 on membrane potential (M.P.) and $[\text{Ca}^{2+}]_i$. M.P. and $[\text{Ca}^{2+}]_i$ were recorded simultaneously using a fast scanning confocal fluorescent microscope with a Ca^{2+} fluorescent indicator $100 \mu\text{M}$ indo-1 under whole cell current clamp mode. (A) The changes in M.P. and $[\text{Ca}^{2+}]_i$ as the average from whole cell area are shown against time. Application of $30 \mu\text{M}$ NS-1619 produced a membrane hyperpolarization and, simultaneously, the $[\text{Ca}^{2+}]_i$ increase, which were removed by washout. (B) and (C) The summarized data of the relationships between concentrations of NS-1619 and either the membrane hyperpolarizations (B) or the changes in $[\text{Ca}^{2+}]_i$ (C). The number of myocytes used is given in parentheses. The statistical significance of the difference vs control is indicated by $**P < 0.01$.

only by $30 \mu\text{M}$ NS-1619 in all or none manner. Effects of $100 \mu\text{M}$ NS-1619 were similar to those of $30 \mu\text{M}$ but had faster onset and were removed only partly by washout (not shown). Therefore, most experiments were performed using NS-1619 in the range of 1 and $30 \mu\text{M}$. The addition of 100 nM IbTx abolished the hyperpolarization induced by $30 \mu\text{M}$ NS-1619, but did not affect significantly the $[\text{Ca}^{2+}]_i$ increase (299 ± 21 nM, $n = 4$, $P > 0.05$ vs 308 ± 20 nM). A change in cell shape was not detected during the response to $30 \mu\text{M}$ NS-1619 in any myocytes examined ($n = \sim 50$).

Figure 3 shows effects of $30 \mu\text{M}$ NS-1619 on $[\text{Ca}^{2+}]_i$ and membrane current measured under voltage clamp at holding potential of -40 mV. Application of NS-1619 first increased the frequency of spontaneous transient outward current with a small increase in $[\text{Ca}^{2+}]_i$ and then induced a sustained outward current with a larger increase in $[\text{Ca}^{2+}]_i$. The averaged sustained component of the outward current and the concomitantly measured $[\text{Ca}^{2+}]_i$ were 21.7 ± 6.4 pA and 295 ± 32 nM ($n = 7$, $P < 0.01$ vs control), respectively, indicating that the $[\text{Ca}^{2+}]_i$ increase under voltage-clamp at -40 mV was comparable to that measured under current-clamp conditions (308 ± 20 nM, $P > 0.05$).

Localized increase in $[\text{Ca}^{2+}]_i$ by NS-1619

The confocal Ca^{2+} images were obtained in the absence (Figure 4A,a) and presence of $30 \mu\text{M}$ NS-1619 (A,b) and after the removal of NS-1619 (A,c), respectively. The profiles of $[\text{Ca}^{2+}]_i$ along a cross-section of short axis of the myocyte ($3 \mu\text{m}$ in thickness) as indicated by the bars in Figure 4A were measured and plotted against the section in Figure 4B. It is clear from the analysis that the increase in $[\text{Ca}^{2+}]_i$ occurred mainly in the peripheral regions within $1 \mu\text{m}$ from

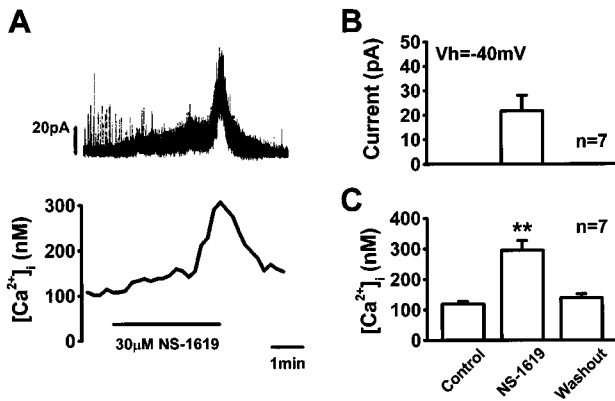


Figure 3 Effects of NS-1619 on membrane current and $[\text{Ca}^{2+}]_i$. (A) Membrane current was measured under voltage-clamp at a holding potential of -40 mV. $[\text{Ca}^{2+}]_i$ was simultaneously measured in the same manner as in Figure 2. Application of $30 \mu\text{M}$ NS-1619 induced an outward current and increased $[\text{Ca}^{2+}]_i$. (B) The amplitude of the sustained component of outward current induced by $30 \mu\text{M}$ NS-1619 at holding potential of -40 mV was measured in seven myocytes. (C) The $[\text{Ca}^{2+}]_i$ before and during the application and after washout of $30 \mu\text{M}$ NS-1619 under voltage-clamp at -40 mV were summarized from the results obtained in the same cells shown in (B). The statistical significance of the difference vs the control group is indicated by $**P < 0.01$.

both edges of the myocyte. To examine quantitatively the localized $[\text{Ca}^{2+}]_i$ increase by NS-1619, the averaged $[\text{Ca}^{2+}]_i$ in square areas ($3.0 \times 1.0 \mu\text{m}$) along the long axis of a myocyte was measured in peripheral and central regions (five for each). The initial $[\text{Ca}^{2+}]_i$ in central and peripheral regions was 102 ± 9 and 109 ± 14 nM, respectively ($n = 11$, $P > 0.05$; Figure 4C). Approximately 3 min after the application of $30 \mu\text{M}$ NS-1619, the $[\text{Ca}^{2+}]_i$ in central and peripheral regions was 181 ± 9 and 438 ± 52 nM, respectively ($n = 11$, $P < 0.01$; Figure 4C). These effects of NS-1619 were removed by washout.

Source of Ca^{2+} increased by NS-1619

To determine the source of Ca^{2+} increased by NS-1619, the effects of NS-1619 were examined after the removal of extracellular Ca^{2+} . When 2.2 mM Ca^{2+} in the external solution was replaced by 0.5 mM Cd^{2+} , the application of $30 \mu\text{M}$ NS-1619 significantly increased $[\text{Ca}^{2+}]_i$ to 333 ± 10 nM ($n = 4$, $P > 0.05$ vs under the control conditions) during the membrane hyperpolarization of 11.5 ± 1.0 mV ($n = 4$, $P > 0.05$; Figure 5).

For further experiments to determine the source of Ca^{2+} increased by NS-1619, the effects of NS-1619 were examined after intracellular Ca^{2+} storage sites were depleted by the treatment with caffeine and ryanodine. The simultaneous application of 10 mM caffeine and $10 \mu\text{M}$ ryanodine caused a transient rise of $[\text{Ca}^{2+}]_i$ to 401 ± 50 nM and, concomitantly, a transient membrane hyperpolarization of 8.2 ± 2.0 mV at the peak ($n = 10$; Figure 6A). Slight shortening of myocytes in the longitudinal direction was occasionally observed when caffeine and ryanodine were applied. In the presence of caffeine and ryanodine for 5 min, the membrane potential and $[\text{Ca}^{2+}]_i$ were changed to a stable value of approximately -38 mV (-38.4 ± 1.8 mV, $n = 10$, $P < 0.05$ vs -41.5 ± 1.1 mV before the treatment with caffeine and

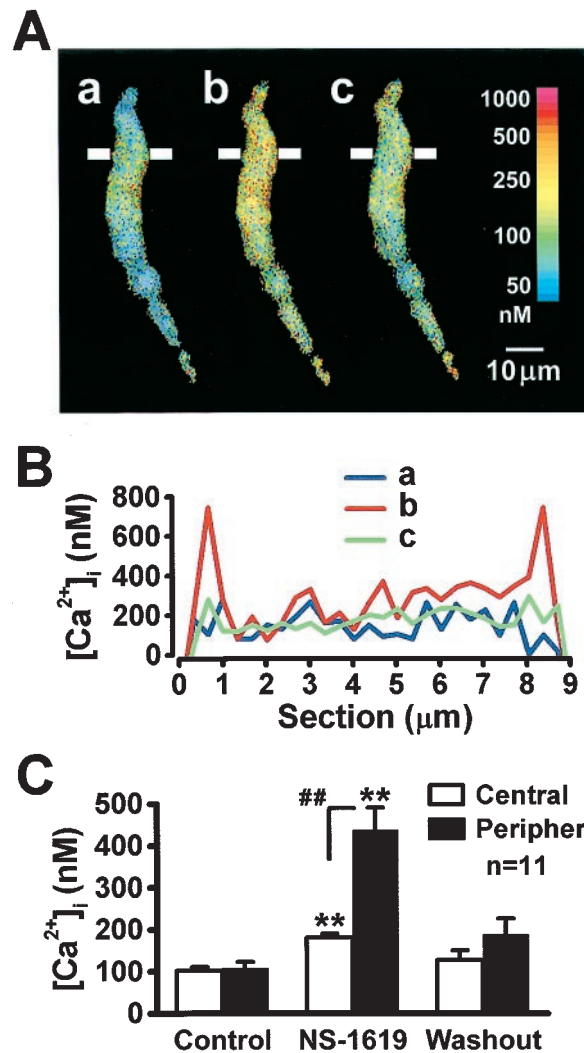


Figure 4 Analyses of confocal Ca^{2+} images. (A) Three images show the distribution of intracellular Ca^{2+} in the absence (a), in the presence of $30 \mu\text{M}$ NS-1619 for 3 min (b) and after washout (c). (B) The profile of $[\text{Ca}^{2+}]_i$ along the cross-section indicated by the line in (A) ($3 \mu\text{m}$ in thickness), are plotted along the section. The colour lines indicated by a, b and c were $[\text{Ca}^{2+}]_i$ profiles obtained in the corresponding images in (A). (C) $[\text{Ca}^{2+}]_i$ in squares ($3.0 \times 1.0 \mu\text{m}$) along the long axis of the cells was measured in the central and peripheral regions. The average from five squares for each region was obtained in a myocyte and the results from 11 myocytes were summarized. The statistical significance of the difference vs control and vs central region is indicated by $**$ and $##P < 0.01$, respectively.

ryanodine) and 223 ± 33 nM ($n = 10$, $P < 0.05$ vs 121 ± 10 nM before caffeine and ryanodine), respectively (Figure 6A). Under these conditions, the addition of $30 \mu\text{M}$ NS-1619 induced significant membrane hyperpolarization of 8.3 ± 0.6 mV ($n = 10$, $P < 0.01$ before application of NS-1619). The hyperpolarization was a slightly but significantly smaller than that in the absence of caffeine and ryanodine ($P < 0.01$ vs value in the absence of caffeine and ryanodine of 12.5 ± 1.0 mV; Figure 6B). On the other hand, the increase in $[\text{Ca}^{2+}]_i$ by NS-1619 was almost abolished in the presence of caffeine and ryanodine ($P < 0.01$ vs in the absence; Figure 6C).

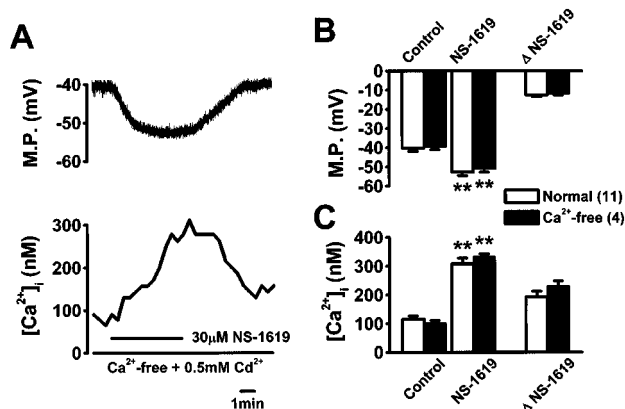


Figure 5 Effects of the removal of extracellular Ca^{2+} on NS-1619-induced responses of membrane potential (M.P.) and $[\text{Ca}^{2+}]_i$. The 2.2 mM Ca^{2+} in the external solution was replaced by 0.5 mM Cd^{2+} . (A) The change in M.P. and that in $[\text{Ca}^{2+}]_i$ as the average from whole cell area induced by application of 30 μM NS-1619 in the absence of external Ca^{2+} . (B) and (C) Summarized results of the changes in M.P. (B) and $[\text{Ca}^{2+}]_i$ (C) by 30 μM NS-1619 under normal and Ca^{2+} -free conditions are illustrated. The number of experiments is given in parentheses. The statistical significance of the difference vs control is indicated by $**P < 0.01$.

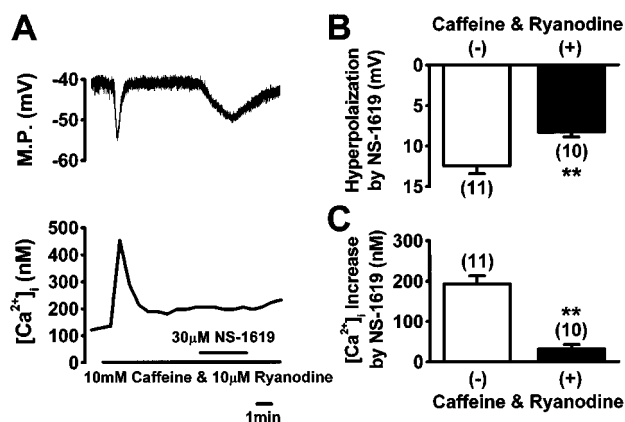


Figure 6 Effects of NS-1619 in the co-presence of 10 mM caffeine and 10 μM ryanodine on membrane potential (M.P.) and $[\text{Ca}^{2+}]_i$. (A) The changes in M.P. and $[\text{Ca}^{2+}]_i$ as the average from whole cell area in the presence of caffeine and ryanodine, are shown against time. (B) and (C) The data about the extent of membrane hyperpolarization (B) and $[\text{Ca}^{2+}]_i$ increase (C) by 30 μM NS-1619 in the absence and presence of caffeine/ryanodine are summarized. The number of experiments is given in parentheses. The statistical significance of the difference vs the corresponding control is indicated by $**P < 0.01$.

Discussion

The present study clearly shows that 30 μM NS-1619 releases Ca^{2+} from caffeine/ryanodine-sensitive Ca^{2+} storage sites and that the Ca^{2+} release may partially contribute to the membrane hyperpolarization *via* the activation of BK channels in porcine coronary artery smooth muscle cells.

The membrane hyperpolarization induced by NS-1619 was completely inhibited by IbTx, indicating that it was due to the activation of BK channels. The major mechanism underlying vasodilation induced by the enhancement of BK

channel activity has been suggested to be similar to that *via* the opening of K_{ATP} channel, which may be due to the decrease in Ca^{2+} influx through voltage dependent Ca^{2+} channels by the membrane hyperpolarization in smooth muscle cells (Nelson & Quayle, 1995). A decrease in $[\text{Ca}^{2+}]_i$ was, therefore, expected to be elicited by the application of NS-1619 in porcine coronary artery smooth muscle cells. On the contrary, the results showed the increase in $[\text{Ca}^{2+}]_i$ during the membrane hyperpolarization induced by 30 μM NS-1619. Since the $[\text{Ca}^{2+}]_i$ increase was also observed under voltage-clamp at holding potential of -40 mV, it can be neglected that NS-1619-evoked Ca^{2+} release is triggered by the membrane hyperpolarization. The possibility that the increase in $[\text{Ca}^{2+}]_i$ by NS-1619 is attributable to optical artifacts can be also ruled out for the following reasons: (1) The $[\text{Ca}^{2+}]_i$ increase by NS-1619 was minimized after the treatment with caffeine and ryanodine (Figure 6) or in the presence of 10 mM BAPTA in the pipette solution (data not shown). (2) The fluorescence intensity of indo-1 at emission wavelengths of 405 and 485 nm was increased and decreased, respectively, by the application of NS-1619. (3) The NS-1619-induced $[\text{Ca}^{2+}]_i$ increase was also observed when fluo-3 (excitation wavelength of 488 nm; emission wavelength of 515 nm) was used as a fluorescence indicator in the same equipment. (4) It was also confirmed using fura-2 (excitation wavelengths of 340 and 380 nm; emission wavelength of ≥ 520 nm) in a different system for the Ca^{2+} image measurements (Argus/HiSCA, Hamamatsu Photonics, Hamamatsu, Japan).

The membrane hyperpolarization induced by 30 μM NS-1619 was reduced to approximately 67%, when the increase in $[\text{Ca}^{2+}]_i$ was mostly abolished by the pretreatment with caffeine and ryanodine. This result indicates that the activation of BK channel by NS-1619 is partly attributable to the increase in $[\text{Ca}^{2+}]_i$, in addition to the direct action on the channel itself or associated sites, which has been reported by use of single channel recording (Sellers & Ashford, 1994; Holland *et al.*, 1996). Similar possibility has been speculated for one of the mechanisms underlying the activation of BK channel current by nordihydroguaiaretic acid in porcine coronary artery myocytes (Yamamura *et al.*, 1999). The increase in $[\text{Ca}^{2+}]_i$ elicited by NS-1619 occurred mainly in peripheral regions of the myocytes based on the analyses of confocal Ca^{2+} images. Although this result might fit with an idea that Ca^{2+} influx was enhanced by NS-1619, the fact that the $[\text{Ca}^{2+}]_i$ increase was not affected by the removal of external Ca^{2+} strongly suggests the Ca^{2+} release from intracellular storage sites. The reason why the Ca^{2+} release occurred mainly in peripheral storage sites and did not diffuse over whole cell area was not clear in the present study. It is likely that NS-1619 was highly soluble to lipids and, therefore, may preferentially distribute to the plasmalemma and subplasmalemma membranes including superficial storage sites.

It has been suggested that there are, at least, two spatially and functionally distinctive types of Ca^{2+} storage sites in smooth muscle cells with respect to intracellular Ca^{2+} mobilization coupled with the cellular functions. One type of storage sites is localized in the superficial areas just beneath the cell membrane and does not directly contribute to the activation of contractile system; 'non-contractile compartment' (van Breemen *et al.*, 1995; Karaki *et al.*, 1997). The other type is located in relatively centre of the cell

and surrounded with contractile elements; 'contractile compartment'. Spontaneous Ca²⁺ release from local storage sites in the superficial areas through ryanodine receptor Ca²⁺ releasing channels has been detected as a Ca²⁺ spark in smooth muscle cells (Nelson *et al.*, 1995). It has been clarified that Ca²⁺ sparks in superficial areas elicit spontaneous transient outward currents *via* BK channel activation (Zhuge *et al.*, 1999; Pérez *et al.*, 1999) but are not related to cell contraction (Bolton & Imaizumi, 1996; Imaizumi *et al.*, 1999; Jaggar *et al.*, 2000). The reason why NS-1619-induced [Ca²⁺]_i increase activated BK channels but did not induce cell shortening may probably be due to the localized increase in [Ca²⁺]_i in peripheral regions. Actually, 30 µM NS-1619 induces relaxation rather than contraction in smooth muscle tissues (Edwards *et al.*, 1994; Holland *et al.*, 1996).

The potentiation of BK channels by NS-1619 has been demonstrated in smooth muscles of vascular tissues (Olesen *et al.*, 1994; Edwards *et al.*, 1994; Holland *et al.*, 1996), trachea (Macmillan *et al.*, 1995), vas deferens (Huang *et al.*, 1997), urinary bladder (Sheldon *et al.*, 1997), and also neurons (Sellers & Ashford, 1994; Lee *et al.*, 1995). It has been reported that the BK channel activation by NS-1619 appears to be independent from ligand receptors, G-proteins or channel phosphorylation (Olesen *et al.*, 1994). NS-1619 has been found to be a highly effective relaxant with an EC₅₀ of about 10–30 µM in several smooth muscles of blood vessels (Edwards *et al.*, 1994; Holland *et al.*, 1996; Cadorette *et al.*, 2000) and other tissues (Huang *et al.*, 1997; Sheldon *et al.*, 1997). NS-1619, however, also inhibits the voltage dependent Ca²⁺ channel (Edwards *et al.*, 1994; Holland *et al.*, 1996; Sheldon *et al.*, 1997), the voltage dependent K⁺ channel (Edwards *et al.*, 1994; Holland *et al.*, 1996), K_{ATP}

channel (Edwards *et al.*, 1994), and even the cholinergic neurotransmission (Patel *et al.*, 1998). In addition to these, it was found in the present study that NS-1619 at the concentration of 30 µM or higher releases Ca²⁺ from peripheral storage sites.

It has been reported that the application of carbonyl cyanide p-trifluoromethoxyphenylhydrazone, a mitochondrial uncoupler, to single smooth muscle cells of the rat pulmonary artery induces Ca²⁺ release probably from mitochondria and results in the activation of BK channels (Yuan *et al.*, 1996). In the present study, the depletion of Ca²⁺ stored in sarcoplasmic reticulum (SR) by the treatment with caffeine and ryanodine almost abolished NS-1619-induced [Ca²⁺]_i increase. This strongly suggests that the Ca²⁺ release was mainly from SR rather than mitochondria. The mechanisms underlying the Ca²⁺ release by NS-1619 from SR remain to be determined.

In conclusion, the application of 30 µM NS-1619 to porcine coronary arterial smooth muscle cells releases Ca²⁺ from peripheral storage sites, which were sensitive to caffeine/ryanodine and, therefore, selectively activated BK channels. The Ca²⁺ release by NS-1619 is partly responsible for the activation of BK channels.

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