Mechanisms Underlying the Activation of Large Conductance
Ca\(^{2+}\)-Activated K\(^{+}\) Channels by Nordihydroguaiaretic Acid

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ABSTRACT—The mechanisms underlying the activation of large conductance Ca\(^{2+}\)-activated K\(^{+}\) (BK) channel by nordihydroguaiaretic acid (NDGA) were examined in human embryonic kidney (HEK293) cells, where BK channel \(\alpha\) (BK\(\alpha\)) or \(\alpha\) plus \(\beta1\) subunit (BK\(\alpha\)\(\beta1\)) was heterologously expressed, and also in freshly isolated porcine coronary arterial smooth muscle cells (PCASMCs). The activity of both BK\(\alpha\) and BK\(\alpha\)\(\beta1\) channels was increased by 10 \(\mu\)M NDGA in similar manners, indicating the selective action on the \(\alpha\) subunit to increase Ca\(^{2+}\) sensitivity. The application of NDGA to PCASMCs induced outward current and hyperpolarization under voltage and current clamp, respectively, in a concentration-dependent manner (\(\geq 3 \mu\)M). These effects were blocked by 100 nM iberiotoxin. Electrical events induced by NDGA (\(\geq 10 \mu\)M) were, unexpectedly, associated with the increase in [Ca\(^{2+}\)]\(_i\). After the treatment with caffeine and ryanodine, the [Ca\(^{2+}\)]\(_i\) increase by NDGA was markedly reduced and the hyperpolarization by NDGA was attenuated. The Ca\(^{2+}\) release by 10 \(\mu\)M NDGA was preceded by membrane depolarization of mitochondria. These results indicate that BK channel opening by NDGA in PCASMCs is due to the direct action on \(\alpha\) subunit and also to Ca\(^{2+}\) release from sarcoplasmic reticulum, presumably via, at least in part, the inhibition of mitochondria respiration.

Keywords: Nordihydroguaiaretic acid, Ca\(^{2+}\)-activated K\(^{+}\) channel, Coronary artery smooth muscle, K\(^{+}\) channel opener, Mitochondria

Large conductance Ca\(^{2+}\)-activated K\(^{+}\) (BK) channels are highly expressed in smooth muscle cells of various organs, including blood vessels (1). 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 (2). Several compounds have been reported to be BK channel openers: dehydrosoyasaponin-I, maxikdiol, NS-004, NS-1608, NS-1619, niflumic acid, BMS-204352, NS-8, CGS-7181 and CGS-7184 (for a review, see ref. 3). BK channels consist of the pore forming \(\alpha\) subunit and the accessory \(\beta\) subunit; the latter regulates the sensitivity to Ca\(^{2+}\) and voltage and/or channel kinetics such as inactivation (4, 5).

In contrast to a major type of \(\alpha\) subunit including splice variants, several subtypes of \(\beta\) subunit have been determined and suggested to be responsible for the differential characteristics of BK channels in various tissues (5, 6).

The \(\beta\) subunit of BK channels in coronary arterial smooth muscle is suggested to be \(\beta1\) as in other smooth muscles (7).

Nordihydroguaiaretic acid (NDGA), which is a natural product contained in Creosote bush, has been widely used as a natural antioxidant for fats and oil in foods. NDGA at low concentrations (<5 \(\mu\)M) acts as a relatively selective inhibitor of lipoxygenase (8), whereas NDGA at higher concentrations also inhibits cyclooxygenase and phospholipase A\(_2\) (9). The inhibition of mitochondria respiration by high concentrations of NDGA has been also reported (10, 11). We found that the application of NDGA directly enhanced single BK channel activity in excised and on-cell patches of porcine coronary arterial smooth muscle cells (PCASMCs) (12). Moreover, the opening of BK channels by NDGA, which is independent of its lipoxygenase inhibition or antioxidant effect, results in membrane hyperpolarization in PCASMCs (13). Similar effects of NDGA have been observed in type I carotid body cells (14). A possibility has been suggested that BK channel openers may have a substantial potency for the treatment of angina, hypertension, bronchial asthma, hyper reactivity of urinary tract...
bladder and some other diseases characterized by the increased tonus of smooth muscles (15) as well as ischemic stroke (16). The molecular mechanism underlying the BK channel activation by these openers is an issue of special importance with respect to the development of tissue selective BK channel openers.

Our previous studies indicate that the potentiation of BK channel activity by NDGA is mediated by at least two different mechanisms; the direct activation of BK channels (12) and the enhancement of Ca$^{2+}$ release from intracellular storage sites (13). In the present study, whether NDGA acted on the α or β subunit of BK channel was examined using human embryonic kidney (HEK293) cell, where BK channel α subunit (BKα) or both α and β1 subunits (BKαβ1) were as expressed by cDNA transfection. To obtain the direct evidence supporting the suggestion that Ca$^{2+}$ release is involved in the mechanisms of the BK channel activation by NDGA, Ca$^{2+}$ images were obtained together with the measurement of either membrane potential or current in freshly isolated single PCASMCs. In addition, effects of NDGA on mitochondria respiration were also examined to elucidate the mechanism of Ca$^{2+}$ mobilization.

MATERIALS AND METHODS

Cell isolation from porcine coronary artery

Single PCASMCs were prepared as described previously (13). In brief, whole hearts from young pigs (6-month-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 Ca$^{2+}$-free Krebs’ solution containing 1% albumin (bovine fraction V, fatty acid free; Miles, Kankakee, IL, USA), 0.2% collagenase (Amano Pharmaceutical Co., Nagoya, 0.1% papain and 0.2% trypsin inhibitor (Sigma Chemical Co., St. Louis, MO, USA) at 37°C. After the incubation, the solution was replaced with Ca$^{2+}$- 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 (Diaphot TMD 300; Nikon, Tokyo). After these cells were settled, the bath was continuously perfused with the HEPES-buffered solution at a flow rate of 5 mL/min. Spindle-shaped relaxed cells over 100 μm in length were used.

Vector constructs and transfection of HEK293 cell with BK channel cDNA

The vector constructs and following transfection of HEK293 cells with cDNAs encoding rat BKα and rat BK channel β1 subunit (BKβ1) were performed as shown previously (17). In brief, restriction enzyme-digested DNA fragments of BKα (Kpn I/Xba I-double digested) and BKβ1 (EcoR I/Xba I-double digested) were ligated into the mammalian expression vector, pcDNA3.1(+) and pcDNA3.1/Zeo(+)(Invitrogen Co., Carlsbad, CA, USA) using TaKaRa ligation kit Ver. 1 (Takara, Tokyo), respectively. HEK293 cell lines were obtained from Health Science Research Resources Bank (Osaka) and maintained in minimum essential medium (MEM; Gibco BRL, Life Technologies, Inc., Rockville, MD, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; JRS Biosciences, Lenexa, KS, USA), penicillin (100 units/mL; Wako Pure Chemical Industries, Osaka) and streptomycin (100 μg/mL; Meiji Seika, Tokyo). Stable expression of BKα and BKβ1 was performed by the calcium phosphate co-precipitation technique. G418 (1 mg/mL, Gibco BRL)- and G418/Zeo (0.25 mg/mL, Invitrogen Co.)-resistant cells were selected as BKα-expressed and BKα/BKβ1 co-expressed ones, respectively. Expression of BKα and BKβ1 transcripts was confirmed by RT-PCR. Transfected cell lines were maintained in MEM supplemented with 10% FCS and G418 (0.5 mg/mL). The expression levels of BKα by over 90% and BKαβ1 by over 80% were confirmed by inside-out patch clamp based on the existence of BK channels and also higher Ca$^{2+}$ sensitivity of BKαβ1 than that of BKα (see Results) in approximately 30 cells in separate 5 culture dishes out of 10 prepared at the same time.

Solutions

The normal Krebs’ solution had an ionic composition of 112 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgCl₂, 25 mM NaHCO₃, 1.2 mM KH₂PO₄ and 14 mM glucose. The pH was adjusted to 7.4 by gassing with a mixture of 95% O₂ and 5% CO₂. The Ca$^{2+}$-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 137 mM NaCl, 5.9 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgCl₂, 14 mM glucose and 10 mM HEPES. The pH of the solution was adjusted to 7.4 with NaOH. The pipette solution contained 140 mM KCl, 1 mM MgCl₂, 10 mM HEPES and 2 mM Na₂ATP. The pH was adjusted to 7.2 with KOH. For recording of single BK channel current under the inside-out patch clamp mode, the pipette solution contained the HEPES-buffered solution in which KC1 concentration was increased to 40 mM by replacement with equimolar NaCl, and the bathing solution contained 140 mM KCl, 1.2 mM MgCl₂, 14 mM glucose, 10 mM HEPES and 5 mM EGTA. Each pCa of the bathing solution was obtained by adding Ca$^{2+}$, and the pH was adjusted to 7.2 with NaOH.
**Electrophysiological experiments**

The whole-cell and inside-out patch clamp were applied to single cells by means of the techniques originally introduced by Hamill et al. (18) using a CEZ-2400 amplifier (Nihon Kohden, Tokyo) and EPC-7 amplifier (List, Darmstadt, Germany), respectively. The procedures of electrophysiological recordings and data analyses for whole-cell recording were performed as described previously by using programs, Data-Acquisition and Cell-Soft, developed at the University of Calgary (Calgary, Canada) (19). Single channel current analyses were done using the software PAT V7.0C developed at the University of Strathclyde (Glasgow, UK) (Dr. J. Dempster). The resistance of the pipette was 2 to 5 MΩ for whole-cell and 10 to 15 MΩ for inside-out configurations when filled with the pipette solutions. The series resistance was partly compensated electrically under whole-cell voltage clamp. Whole-cell solutions. The series resistance was partly compensated for inside-out configurations when filled with the pipette pipette was 2 to 5 MΩ (Glasgow, UK) (Dr. J. Dempster). The resistance of the pipette was 2 to 5 MΩ for whole-cell and 10 to 15 MΩ for inside-out configurations when filled with the pipette solutions. The series resistance was partly compensated electrically under whole-cell voltage clamp. Whole-cell and single channel recordings were carried out at 30 ± 1°C and at room temperature (24 ± 1°C), respectively.

**[Ca²⁺]i measurement**

Two dimensional Ca²⁺ images were obtained by a fast scanning confocal fluorescent microscope (Nikon RCM-8000, Nikon) equipped with objective lens (Fluor 40 x 1.15 NA, water immersion; Nikon) and Ratio3 software (Nikon), as described previously (20, 21). Without electrophysiological recording, 5 μM indo-1 acetoxymethyl ester (indo-1/AM) was loaded for 10 min and removed thoroughly for 10 min at room temperature. When simultaneous measurement of intracellular Ca²⁺ concentration ([Ca²⁺]) and electrophysiological recording was performed, the recording was 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. The excitation wavelength from the argon ion laser was 531 nm and the emission wavelengths were 405 and 485 nm. The Ca²⁺ image was scanned over a full frame (512 x 512 pixels) every 10 to 30 s. The data analyses were performed as described previously (20, 21).

**Simultaneous measurements of [Ca²⁺]i and membrane potential of mitochondria**

In order to monitor changes in [Ca²⁺]i, and mitochondrial membrane potential (∆Ψm) simultaneously, isolated PCASMCs were co-incubated with 10 μM fluo-4 acetoxymethyl ester (fluo-4/AM) and 100 nM tetramethylrhodamine ethylester (TMRE) for approximately 10 min at room temperature, and then the excessive indicators were thoroughly washed away from intra/extracellular spaces for over 10 min. The potential dependent accumulation of TMRE within mitochondria produces a quench of fluorescence, which is relieved by redistribution of the dye when the ∆Ψm is depolarized: thus, an increase in TMRE fluorescence is indicative of mitochondrial depolarization (22, 23). Confirmation of this effect was provided by application of a mitochondria uncoupler, 1 μM carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) in PCASMCs, which caused ∆Ψm increase to 229 ± 20% (n = 5). The excitation wavelength from an argon ion laser for fluo-4 was 488 nm and the emission wavelength was 505–535 nm. The excitation and emission wavelengths from a helium neon laser for TMRE were 543.5 and >570 nm, respectively. Fluorescent signals are described as F/Fo, where F is the fluorescence intensity during a test procedure and F₀ is that before the procedure. The resolution of the microscope is approximately 0.33 × 0.27 µm (1 pixel) and approximately 1.2 µm to Z-axis direction. Images of fluo-4 and TMRE were obtained alternatively every 33 ms and eight images per each were averaged as one measurement. This measurement protocol was repeated every 5 s. The data analyses were performed as described previously (20, 21).

**Statistics**

Pooled data were shown as the mean ± S.E.M. Statistical significance between two groups and among multi groups was determined by Student’s t-test and Scheffé’s test after one-way ANOVA, respectively. Significant difference was expressed in the figures as *P<0.05 or **P<0.01. Data regarding the relationships between concentrations of NDGA and the responses were fitted by the following equation:

\[
\Delta[NDGA] = \Delta[NDGA(max)] / \{1 + (K_d / [NDGA]^n)\} + C,
\]

where \(\Delta[NDGA(max)]\) is the maximum amplitude of the NDGA-induced response, \(K_d\) is the apparent dissociation constant of NDGA, [NDGA] is the concentration of NDGA, \(n\) is the Hill coefficient, and \(C\) is the constant (Figs. 2B and 3B). In Fig. 2A, the equation was modified as

\[
\Delta[NDGA] = -\Delta[NDGA(max)] / \{1 + (K_d / [NDGA]^n)\}
\]

**Drugs**

Pharmacological reagents were obtained from Sigma Chemical Co. except for iberiotoxin (IbTx; Peptide Institute Inc., Osaka); cadmium chloride and caffeine (Wako Pure Chemical Industries); EGTA, HEPES, indo-1 and indo-1 AM (Dojin Laboratories, Kumamoto); fluo-4, fluo-4/AM, fura-2 and TMRE (Molecular Probes, Inc., Eugene, OR, USA). NDGA was dissolved in dimethyl sulfoxide (DMSO) at the concentration of 10⁻¹ M as a stock solution and used within 1 week. The external solutions always contained 0.1% DMSO throughout the experiments, regardless of the presence/absence of NDGA. It was confirmed that 0.1% DMSO did not affect the membrane potential, current, [Ca²⁺], and ∆Ψm.
RESULTS

Activation of BKα and BKαβ1 channels expressed in HEK293 cells by NDGA

It has been clearly determined that the BK channel in coronary arterial smooth muscle mainly consists of the combination of α and β1 subunits (7). To determine whether NDGA acts on the BKα or BKβ1 subunit, effects of NDGA on recombinant BK channels were examined by single channel recording in HEK293 cells, where BKα or BKαβ1 channels were highly expressed (Fig. 1). The activity of the BKα channel was recorded in inside-out

Fig. 1. Activation of single BKα and BKαβ1 channels expressed in HEK293 cells by NDGA. Effects of 10 μM NDGA on BKα or BKαβ1 channels were examined in inside-out patches from HEK293 cells. The K+ concentrations were 40 and 140 mM in the outside and inside phases, respectively. The pCa in the solution facing to the inside was kept at 6.5. A: Original recordings obtained at +20 mV from the patches including the BKα (a) and BKαβ1 channel (b). The activity of the BKαβ1 channel was higher than that of BKα channel under the same conditions. The activity was markedly increased by application of 10 μM NDGA in both BKα and BKαβ1 channels. The arrowheads indicate the closed state of the BK channel. B: The current-voltage relationships of BKα (a) and BKαβ1 channels (b) obtained under the conditions described in “A”. The single channel conductance of the BKα channel (181.5 ± 3.8 pS) was identical to that of the BKαβ1 channel (181.0 ± 5.1 pS). NDGA did not change the conductance (open vs closed circles). C and D: The open probabilities (P_o) of BKα and BKαβ1 channels in the absence (open column) and presence of 10 μM NDGA (closed column). Each P_o was measured from continuous recording for 60 s. In the absence of NDGA, P_o of BKα and BKαβ1 channels were 0.00084 ± 0.00017 and 0.00439 ± 0.00093, respectively. The application of NDGA significantly increased P_o in BKα (open column) and BKαβ1 channels (closed column) by 69.3 ± 16.3 and 43.7 ± 8.5 fold, respectively. Data were obtained from 6 cells for each. The statistical significance of the difference between two groups is expressed as *P<0.05 or **P<0.01.
patches under the experimental conditions, where pCa of the bathing solution was 6.5, the holding potential was +20 mV and K+ concentrations in the pipette and the bathing solution were 40 and 140 mM, respectively. The single channel conductance was 181.5 ± 3.8 pS (n = 6). The open probability (P_o) was measured from the event histogram versus current amplitude obtained from the continuous recording for 60 s. The number of channels in a patch was determined by elevating Ca2+ concentration from pCa 7.0 to 4.0. Data from patches including less than five BK channels were analyzed. The P_o at pCa 6.5 and +20 mV was 0.00084 ± 0.00017 (n = 6) under these conditions. The P_o was increased by NDGA at concentrations of 3 μM and higher. The application of 10 μM NDGA increased the P_o to 0.188 ± 0.053 (n = 6, P<0.05 vs absence of NDGA) but did not change the conductance (182.2 ± 5.4 pS, n = 6, P>0.05). When the Ca2+ concentration in the cytosolic phase was very low (pCa>8.5), the activity of neither BKα nor BKαβ1 was observed even at very positive potentials (+80 mV) and the addition of 30 μM NDGA did not induce the channel activity (not shown). Taken together, it can be suggested that NDGA acts on BKα in a Ca2+-dependent manner.

**Activation of BK channel by NDGA in PCASMCs**

When the membrane potential of single PCASMCs was recorded under the current clamp mode (Fig. 2A), the averaged resting membrane potential was −42.2 ± 2.3 mV (n = 19). The application of 30 μM NDGA induced hyperpolarization (14.1 ± 2.5 mV, n = 11, P<0.01 vs control), which was abolished by the addition of 100 nM IbTx (n = 7), a selective BK channel inhibitor, as has been reported previously (13). Moreover, the application of IbTx depolarized the cell by 4.8 ± 1.7 mV from the initial resting potential (n = 7). NDGA produced the membrane hyperpolarization in a concentration-dependent manner with the K_d value of 16 μM and Hill coefficient of 1.9. Significant hyperpolarization was observed at concentrations of 10 μM and higher.

Under voltage clamp mode, spontaneous transient out-
ward currents (STOCs) were recorded in approximately 60% of myocytes at a holding potential of −40 mV (13). The amplitude (20–200 pA) and frequency (0.5–15 Hz) of STOCs varied widely from cell to cell and also with time. The application of 30 μM NDGA markedly increased both the amplitude and frequency of STOCs and, thereby, often elicited a sustained outward current that was superimposed by large STOCs (Fig. 2Ba). The outward currents evoked by NDGA were almost completely abolished by the application of 100 nM IbTx (n = 6) or by washout of NDGA. The peak amplitude of sustained outward current induced by 30 μM NDGA was 159 ± 9 pA (n = 18, P < 0.01 vs control). The sustained outward current component evoked by NDGA at −40 mV was concentration-dependent with the Kd value of 22 μM and Hill coefficient of 1.6 (Fig. 2Bb). The significant outward current was observed at concentrations of 10 μM and higher.

\[ [\text{Ca}^{2+}]_{i}, \text{increase by NDGA in PCASMCs} \]

Without electrophysiological recording, PCASMCs were loaded with a cell permeable Ca\(^{2+}\)-sensitive fluorescent indicator, 5 μM indo-1/AM, for [Ca\(^{2+}\)]_i measurement (Fig. 3). The mean resting [Ca\(^{2+}\)]_i as the average from whole cell area was 109 ± 8 nM (n = 18). The application of 30 μM NDGA significantly increased [Ca\(^{2+}\)]_i to 445 ± 33 nM (n = 11, P < 0.01 vs control). The increase in [Ca\(^{2+}\)]_i was mostly removed by washout of NDGA. The increase in [Ca\(^{2+}\)]_i was concentration-dependent with the Kd value of 22 μM and Hill coefficient of 2.6. The increase in [Ca\(^{2+}\)]_i was observed at concentrations of 10 μM and higher, while the statistical significance versus the control was detected at 30 and 100 μM (by Scheffé’s test).

**Simultaneous measurements of [Ca\(^{2+}\)]_i and membrane current in PCASMCs**

The simultaneous measurements of [Ca\(^{2+}\)]_i and membrane current were performed under the voltage clamp mode in PCASMCs (Fig. 4). At a holding potential of −40 mV, the application of 30 μM NDGA enhanced outward current by 14.5 pA (n = 8) and markedly increased [Ca\(^{2+}\)]_i to 509 ± 56 nM (n = 8, P < 0.01 vs 132 ± 7 nM at rest). To elucidate the relationship between [Ca\(^{2+}\)]_i, increase and BK channel current in the presence of 30 μM NDGA, the responses observed in eight cells examined were plotted as the relation of [Ca\(^{2+}\)]_i versus amplitude of sustained outward current (Fig. 4B). The amplitude of NDGA-induced outward current was positively correlated with [Ca\(^{2+}\)]_i increment, so as the [Ca\(^{2+}\)]_i increase became larger, so did the outward current (correlation coefficient of 0.84, \( \chi^2 < 0.05 \)). The intersection (66 pA) presumably corresponds to the activation of BK channel current independent of [Ca\(^{2+}\)]_i.

Effects of extracellular Ca\(^{2+}\) removal and caffeine/ryanodine-pretreatment on NDGA-induced responses in PCASMCs

The simultaneous measurements of [Ca\(^{2+}\)]_i and membrane potential were performed under current clamp mode in PCASMCs (Fig. 5). The averaged resting membrane potential and [Ca\(^{2+}\)]_i were −41.2 ± 1.9 mV and 123 ± 8 nM, respectively (n = 6). The application of 30 μM NDGA caused membrane hyperpolarization by 14.9 ± 0.9 mV (n = 6, P < 0.01 vs control) and also significant increase in [Ca\(^{2+}\)]_i to 463 ± 62 nM (n = 6, P < 0.01 vs control; Fig. 5A and C). To determine the source of the increased [Ca\(^{2+}\)]_i, by NDGA, the effects of NDGA were examined after the removal of extracellular Ca\(^{2+}\). When Ca\(^{2+}\) was removed from and 0.5 mM Cd\(^{2+}\) was added to the external solution, the application of 30 μM NDGA significantly increased [Ca\(^{2+}\)]_i by 339 ± 36 nM during the membrane hyperpolarization by 14.3 ± 1.3 mV in a similar manner as in the presence of Ca\(^{2+}\) (n = 4, P > 0.05 vs in normal external solution,
respectively; Fig. 5C).

In the next series of experiments, effects of NDGA were examined after intracellular Ca$^{2+}$ storage sites were depleted by the treatment with caffeine and ryanodine. As shown in Fig. 5B, the simultaneous application of 10 mM caffeine and 10 μM ryanodine caused a transient rise of [Ca$^{2+}$] to 482 ± 39 nM and a concomitantly transient membrane hyperpolarization by 14.5 ± 2.4 mV at the peak (n = 8). In the presence of caffeine and ryanodine for approximately 5 min, the membrane potential and [Ca$^{2+}$] were changed to stable values of ~40.5 ± 1.7 mV (n = 8, P < 0.05 vs ~43.1 ± 2.1 mV before the treatment with caffeine and ryanodine) and 237 ± 32 nM (n = 8, P < 0.05 vs 115 ± 9 nM before caffeine and ryanodine), respectively. Under these conditions, the addition of 30 μM NDGA induced significant membrane hyperpolarization by 9.8 ± 0.4 mV (n = 8, P < 0.01). The hyperpolarization was slightly but significantly smaller than that in the absence of caffeine and ryanodine (P < 0.01 vs in the absence, 14.9 ± 0.9 mV; Fig. 5C). On the other hand, the increase in [Ca$^{2+}$] by NDGA was reduced by ~70% in the presence of caffeine and ryanodine (P < 0.01 vs in the absence, Fig. 5C). These results suggest that only a part of the membrane hyperpolarization by 30 μM NDGA is due to BK channel activation by released Ca$^{2+}$.
Effects of NDGA on membrane potential of mitochondria and Ca\(^{2+}\) release in PCASMCs

It has been reported that NDGA inhibits mitochondrial respiration and ATP production at relatively high concentrations (>30 \(\mu M\)) (10, 11). To examine the relation between the Ca\(^{2+}\) release from SR and the inhibition of mitochondria by NDGA, membrane potential change in mitochondria and changes in [Ca\(^{2+}\)] were simultaneously measured by use of TMRE and fluo-4/AM, respectively, in PCASMCs (Fig. 6). Figure 6A shows the pseudo color images of fluo-4 and TMRE fluorescence obtained from the same myocyte. Under control conditions, fluo-4 fluorescence was almost uniform in a PCASMC, whereas TMRE signals were fragmental or punctuated, indicating the distribution of mitochondria in the myocytes. The ratio of fluorescence was almost uniform in a PCASMC, whereas the same myocyte. Under control conditions, fluo-4 fluorescence from a PCASMC.

**DISCUSSION**

Our previous study showed that 10 \(\mu M\) NDGA increased \(P_o\) of BK channels in inside-out patches of PCASMCs at pCa 7.0 and holding potential of 0 mV by about 30 times, indicating that NDGA directly opened the BK channel (12). Since the opening of BK channel by NDGA depended on the presence of Ca\(^{2+}\) at concentrations higher than pCa 8.5, the effect might be due to the increase in Ca\(^{2+}\) sensitivity of BK channels. In the present study, the site of action for NDGA was determined by use of recombinant BK channels in HEK293 cells. It has been reported that the \(\alpha\) subunit encoded by KCNMA1 and \(\beta\)1 subunit encoded by KCNB1 are the combination expressed in coronary arterial smooth muscle (7), while these clones used here were those of the rat. The accessory \(\beta\)1 subunit increases Ca\(^{2+}\) and voltage-sensitivity of the \(\alpha\) subunit, which possesses the basic features of a channel itself (4, 24). It has been revealed in the last few years that the tissue-dependent diversity of membrane current characteristics via BK channel activation is mainly due to the differential expression...
of β subunit subfamily (5, 25). The β1 subunit is highly expressed in smooth muscle tissues but not in brain, and the knockout of the β1 subunit in mice results in the increased tone of arteries and, thereby, hypertension (26). The β4 subunit is highly expressed in brain and is responsible for the low sensitivity of brain BK channels to IbTx (27). The inactivation of BK channel in some tissues such as adrenal chromaffin cells has been explained by the specific nature of β2 or β3 subunit subtypes (5, 6). The molecular sites of action for some BK channel openers have been determined; dehydroisosapogenin-I, 17β-estradiol and tamoxifen act on the β subunit, and then NS-004, NS-1608 and NS-1619 act on the α subunit of the BK channel (3, 28, 29). The present results clearly indicate that NDGA activates the α subunit but may not the β1 subunit, since the potency of NDGA to activate the channels appeared to be not much different between the α subunit and α+β1 subunits.

Effects of NDGA on membrane potentials and currents in PCASMCs shown in this study are consistent with those in our previous investigation (13), and it is demonstrated that the effect of NDGA was concentration-dependent with EC50 value of approximately 20 μM. The enhancement of BK channel activity by NDGA has been suggested to be mediated by at least two different mechanisms; the direct activation of BK channels and the enhancement of Ca2+ release from storage sites (13). The present study gave clear evidence supporting the existence of the latter mechanism that NDGA induced Ca2+ release mainly from caffeine /ryanodine sensitive storage sites. The increase in [Ca2+]i by NDGA was not any artifacts due to the optical measurement, since it was also observed using other Ca2+ indicators, fluo-4 and fura-2, and was markedly reduced by pretreatment with caffeine and ryanodine. The concentration of NDGA required for the significant Ca2+ release (>30 μM) was apparently higher than that for the direct activation of BK channels (>3 μM) (12). Application of 30 μM NDGA induced the hyperpolarization by 15 mV or the outward current of 150 pA and the increase in [Ca2+]i by 300–400 nM. Among the outward current induced by 30 μM NDGA, 66 pA (45%) was independent from the increase in [Ca2+]i, and was, therefore, due to the direct activation of the BKα subunit. Interestingly, similar Ca2+ release has been also observed when NS-1619, the most widely used BK channel opener for research, is applied to PCASMCs (21).

Although the mechanism of Ca2+ release by NDGA was not fully elucidated in this study, a line of new information was obtained. It is unlikely that the direct activation of BK channel induces Ca2+ release, since Evans’ blue, another BK channel opener (30) did not change [Ca2+], under voltage clamp (H. Yamamura et al., unpublished data). Moreover, the increase in [Ca2+]i by NDGA was also observed in native HEK293 cells as well as those expressing the BKαβ1 channel (H. Yamamura, unpublished observations). It has been reported that the application of NDGA inhibits sarco/endoplasm reticulum (SR/ER) Ca2+-ATPase in skeletal muscle and blood platelets (31) and induces the [Ca2+]i increment via Ca2+ influx through La2+-sensitive pathway to activate capacitative Ca2+ entry in canine kidney cells (32). The inhibition of Ca2+-ATPase in SR/ER by NDGA may be, at least in part, responsible for the NDGA-induced [Ca2+]i increase. The present result that the [Ca2+]i increase by NDGA was independent of Ca2+ influx from extracellular spaces was in clear contrast with those reported in kidney cells, where the NDGA-induced [Ca2+]i increase was reduced in Ca2+-free medium (32). Moreover, the [Ca2+]i increase by NDGA was depressed in PCASMCs when the intracellular Ca2+ store was depleted. These observations were not consistent with the interpretation in kidney cells that the NDGA-induced [Ca2+]i elevation was due to enhancement of capacitative Ca2+ entry following the depletion of stored Ca2+ (32). The major reason for the discrepancy may be the different contribution of the capacitative Ca2+ entry pathway to the regulation of [Ca2+]i.

It has been reported that the application of FCCP, a mitochondrial uncoupler, to single smooth muscle cells of rat pulmonary artery induces Ca2+ release probably from mitochondria and results in the activation of BK channels (33). Because high concentrations of NDGA inhibit electron transport in mitochondria and deplete ATP (10, 11), the Ca2+ release may also result from mitochondria poisoning. The finding that depolarization of mitochondria preceded the elevation of [Ca2+]i in the presence of 10 μM NDGA is consistent with the idea that poisoning of mitochondria is the cause of Ca2+ release. Since the Ca2+ release by NDGA was markedly reduced by the pretreatment with caffeine and ryanodine, it could be suggested that NDGA enhanced Ca2+ release from SR but not from mitochondria. The close functional coupling between ER and neighboring mitochondria has been suggested (34). It is, therefore, possible that poisoning of mitochondria reduces ATP supply to Ca2+-ATPase in neighboring SR and enhances the net amount of Ca2+ leak from the SR.

NDGA may enhance the spontaneous Ca2+ release preferentially from superficial SR fragments and effectively activate BK channels by the concerted action of both direct and indirect effects. This assumption is based on the observation that the global [Ca2+]i of approximately 500 nM in the presence of 30 μM NDGA at holding potential of –40 mV can not explain the sustained BK channel current as large as 150 pA. The P0 of BK channel in the inside-out patch under these conditions (at 500 nM [Ca2+], and –40 mV) was as low as 0.0001 even when increased by 30 times in the presence of 30 μM NDGA (ref. 12
and unpublished observation by K. Sakamoto). Assuming similar BK channel density in PCASMCs to that in human coronary myocytes (4 \( \mu \text{m}^{-2} \)) (7), cell surface area of 4000 \( \mu \text{m}^2 \) (from cell capacitance of 40 pF), and unitary current amplitude of 2 pA at \(-40 \text{ mV}\), the \( P_\text{o} \) can be calculated as 0.0047. The NDGA-induced \([\text{Ca}^{2+}]_i\) elevation in the junctional areas between the peripheral SR fragment and plasma membrane may be higher than 500 nM and, thereby, may induce a large current as observed. A similar assumption was proved for the \(\text{Ca}^{2+}\) release from the peripheral SR fragment by NS-1619 (21). Spontaneous \(\text{Ca}^{2+}\) release from local storage sites through ryanodine receptor \(\text{Ca}^{2+}\)-releasing channels has been detected as a “\(\text{Ca}^{2+}\) spark” in many types of smooth muscle cells including coronary artery (35 – 38). It has been clarified that \(\text{Ca}^{2+}\) sparks from storage sites located in peripheral regions of myocytes elicit STOCs via BK channel activation and correspondingly, transient membrane hyperpolarization, but are not related to cell contraction (36 – 38). The specific activation of ryanodine receptor in superficial SR fragments by a selective \(\text{Ca}^{2+}\) releaser, 9-methyl-7-bromoeudistomin D, very effectively enhances STOCs (39).

The peak \([\text{Ca}^{2+}]_i\) of PCASMCs in the presence of 30 \(\mu\text{M}\) NDGA under the current clamp mode was approximately 300 nM, which may be the threshold concentration to induce contraction of the cell. In the preliminary experiments, however, 30 \(\mu\text{M}\) NDGA did not induce contraction in a tissue preparation of porcine coronary artery (H. Yamamura, unpublished observation). It has been reported that NDGA inhibits the voltage-dependent \(\text{Ca}^{2+}\) channel (13, 14, 40), the voltage-dependent K’ channel (13, 14), swelling-activated Cl– channel (41), and swelling-activated, ATP-sensitive taurine channel (11). Information, including data on NS-1619 (21), about the effect on \(\text{Ca}^{2+}\) release is beneficial for the drug development of BK channel openers. In conclusion, the application of NDGA to PCASMCs increases the \(\text{Ca}^{2+}\) sensitivity of BK\(\alpha\) and also releases \(\text{Ca}^{2+}\) from caffeine/ryanodine-sensitive \(\text{Ca}^{2+}\) storage sites. The BK/\(\beta\)1 subunit may not be the target of NDGA. When NDGA was applied at the concentrations of 10 \(\mu\text{M}\) or higher, the latter mechanism presumably via poisoning of mitochondria also contributed to the BK channel activation and resulting membrane hyperpolarization in PCASMCs.

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References

BK Channel Activation by NDGA


