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Activation of Ca^{2+}-Dependent K^+ Current by Nordihydroguaiaretic Acid in Porcine Coronary Arterial Smooth Muscle Cells[1]

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ABSTRACT

The effects of nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor and an antioxidant, on membrane currents were examined in single smooth muscle cells isolated from porcine coronary artery. Spontaneous transient outward currents (STOCs) recorded at −30 mV were markedly enhanced by NDGA (≥10 μM). Pretreatment with caffeine and ryanodine abolished STOCs and reduced NDGA-induced increase in outward current at −30 mV by ~60%. NDGA showed dual action on an outward current elicited by step depolarization from −60 to 0 mV: inhibition and enhancement at concentrations of 3 and ≥10 μM, respectively. In the presence of Cd^{2+}, the inhibition of outward current by NDGA disappeared and the enhancement remained. NDGA inhibited both the voltage-dependent Ca^{2+} channel current (IC_{50} = 2.5 μM) and the delayed rectifier K^+ current (IC_{50} = 9.8 μM). The NDGA-induced enhancement of STOCs and outward currents on depolarization was abolished by 100 nM iberiotoxin but was not affected by glibenclamide or apamin. Under current clamp mode, 30 μM NDGA significantly hyperpolarized myocytes. The application of lipoxygenase inhibitors (caffeic acid and esculetin), a cyclooxygenase inhibitor (indomethacin), antioxidants (ascorbic acid and erythorbic acid), and structural-related compounds of NDGA (catechol and dopamine) did not enhance K^+ currents. These results indicate that the opening of the large conductance Ca^{2+}-dependent K^+ channel by NDGA, which is independent of its lipoxygenase inhibition or antioxidant effect, results in membrane hyperpolarization.

Relatively low potassium conductance of the plasma membrane in vascular smooth muscle cells leads to the resting membrane potential around −50 mV, which is ~30 mV more positive than the K^+ equilibrium potential. The activation of an outward current by the opening of K^+ channels, therefore, effectively hyperpolarizes vascular myocytes (Kuriyama et al., 1995). This may result in the decrease in voltage-dependent Ca^{2+} channel activity and, therefore, Ca^{2+} influx through the channels (Nelson and Quayle, 1995). The vasodilatation induced by membrane hyperpolarization is supposed to be the major mechanism for the antihypertensive effect of ATP-sensitive K^+ channel openers (Edwards and Weston, 1993).

The large conductance Ca^{2+}-dependent K^+ channels (BK channels) are highly expressed in smooth muscle cells of various organs, including blood vessels (Carl et al., 1996). Spontaneous transient outward currents (STOCs) have been recorded at the resting or more depolarized membrane potentials in smooth muscle cells (Bolton and Imaizumi, 1996). STOCs are due to the activation of BK channels via spontaneous Ca^{2+} release from local storage sites, presumably through ryanodine receptor Ca^{2+}-releasing channels (Bolton and Imaizumi, 1996). It has been suggested that STOCs contribute partly to the resting membrane potential and vascular tone under normal conditions and, more importantly, under pathophysiological conditions (Asano et al., 1993; Nelson and Quayle, 1995; Karaki et al., 1997). Agents that enhance BK channel activity have been reported as a new class of K^+ channel opener (BK channel opener; Edwards and Weston, 1995). These agents include natural products (McManus et al., 1993; Singh et al., 1994), widely used anti-inflammatory drugs (Ottolia and Toro, 1994), and newly synthesized compounds (Sargent et al., 1993; Edwards et al., 1994). Nordihydroguaiaretic acid (NDGA), which is contained in *Creosote bush*, is widely used as a natural antioxidant for fats and oil in foods. It is also a selective lipoxygenase inhibitor (Beetens et al., 1986). We found that the

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ABBREVIATIONS: BK channel, large conductance Ca^{2+}-dependent K^+ channel; NDGA, nordihydroguaiaretic acid; $I_{K\text{-}Ca}$, Ca^{2+}-dependent K^+ current; I_{KD}, delayed rectifier K^+ current; SR, sarcoplasmic reticulum; STOCs, spontaneous transient outward currents; IbTx, iberiotoxin; TEA, tetraethylammonium; DMSO, dimethyl sulfoxide.

application of NDGA markedly enhanced single BK channel activity in excised and on-cell patches of porcine coronary arterial smooth muscle cells (Nagano et al., 1996). Similar effects of NDGA were confirmed in type I carotid body cells (Hatton and Peers, 1997).

The present study was undertaken to examine the selectivity of NDGA to BK channels over other ionic channels and the total effects of NDGA on whole-cell currents and membrane potential. In contrast to the inhibition of whole-cell K^+ currents by NDGA in type I carotid body cells (Hatton and Peers, 1997), a marked enhancement of total K^+ currents was observed in porcine coronary arterial smooth muscle cells. In addition, the possibility of whether the enhancement of BK channel activity by NDGA is due to its antioxidant or antilipoxygenase effect or the potentiation of Ca^{2+} release from sarcoplasmic reticulum (SR) was examined.

Materials and Methods

Cell Isolation. Whole hearts from young pigs (3–6 months old) were obtained at a local slaughterhouse and transported to the laboratory in ice-cold normal Krebs' solution. Segments of vessels were dissected from the left circumflex coronary arteries, cleaned of blood and surrounding tissues, and stored at 4°C in normal Krebs' solution. For cell isolation, a small piece of vessel, 5 mm in length, was dissected and immersed for 40 min in Ca^{2+}-free Krebs' solution containing 1% albumin (bovine fraction V, fatty acid free; Miles, Kankakee, IL), 0.2% collagenase (Amano, Aichi, Japan), 0.1% papain, and 0.2% trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) at 37°C in a test tube. After this incubation, the solution was replaced with Ca^{2+}- and collagenase-free Krebs' solution. Cells 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 bath, 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 HEPES solution at a flow rate of 5 ml/min. Spindle-shaped relaxed cells over 100 μm in length were used.

Solutions. The normal Krebs' solution had an ionic composition 112 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, and 14 mM glucose. The pH was adjusted to 7.4 by gassing with a mixture of 95% O_2/5% CO_2. The Ca^{2+}-free Krebs' solution was prepared by the removal of 2.2 mM $CaCl_2$ from the normal Krebs' solution. For electrophysiological recordings, a standard HEPES-buffered solution was used that consisted of 137 mM NaCl, 5.9 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 14 mM glucose, and 10 mM HEPES (Dojin, Kumamoto, Japan). The pH of the solution was adjusted to 7.4 with NaOH. The pipette solution, for the measurement of STOCs, Ca^{2+}-dependent K^+ current ($I_{K\text{-}Ca}$), and membrane potential, contained 140 mM KCl, 4 mM $MgCl_2$, 10 mM HEPES, 5 mM Na_2ATP, and 0.05 mM EGTA (Dojin). The pH was adjusted to 7.2 with KOH. For the recording of delayed rectifier K^+ current (I_{KD}), the concentration of EGTA in this solution was increased to 5 mM. When the Ca^{2+} current was recorded, the components of pipette solution were 140 mM CsCl, 4 mM $MgCl_2$, 10 mM HEPES, 5 mM Na_2ATP, and 5 mM EGTA. The pH was also adjusted to 7.2 with KOH.

Electrophysiological Recording and Data Analysis. The whole-cell patch-clamp technique was applied to single cells by the method originally introduced by Hamill et al. (1981) using EPC-7 (List, Darmstadt, Germany) and CEZ-2200 (Nihon Kohden, Tokyo, Japan) amplifiers. The procedures of electrophysiological recording and data analysis (by using Data-Acquisition software and Cell-Soft software developed in the laboratory of Dr. Wayne Giles) were performed as described previously (Imaizumi et al., 1989). All electrophysiological recordings were carried out at 30 ± 0.5°C.

Statistics. Pooled data were shown as mean ± S.E. Statistical significance between two and among multiple groups was determined by Student's t test and Scheffé's test after one-way ANOVA, respectively. Significance was expressed in figures as $^*p < .05$ and $^{**}p < .01$. Data regarding the relationships between concentrations of NDGA and the inhibition of Ca^{2+} current (Fig. 6) or I_{KD} (Fig. 7) were fitted by the following equation: relative amplitude $= 1 - (1 - C)/\{1 + (K_d/[A]^n)\}$, where C is the component resistant to NDGA, K_d is the apparent dissociation constant of NDGA, A is the concentration of NDGA, and n is the Hill coefficient.

Drugs. Iberiotoxin (IbTx) was obtained from Peptide Institute (Osaka, Japan). Caffeine was obtained from Wako Pure Chemical Industries (Osaka, Japan). Tetraethylammonium (TEA) chloride was obtained from Tokyo Kasei (Tokyo, Japan). All other pharmacological reagents were obtained from Sigma Chemical Co. NDGA was dissolved in dimethyl sulfoxide (DMSO) at the concentration of 10^{-1} M, stored as a stock solution, and used within 1 week. The final DMSO concentration of bathing solution was always prepared to 0.1% throughout the experiments, and it was confirmed that 0.1% DMSO did not affect the currents recorded.

Results

Effects of NDGA on STOCs. At a holding potential of −30 mV, STOCs were recorded in ~80% of single smooth muscle cells isolated from porcine coronary artery (n = ~75). The amplitude (20–200 pA) and frequency (0.5–15 Hz) of STOCs varied widely from cell to cell and with time. The application of 30 μM NDGA for 10 to 60 s markedly increased both the amplitude and the frequency of STOCs and, thereby, often elicited a sustained outward current that was superimposed by large STOCs (Fig. 1A). Whenever STOCs were enhanced by 10 μM NDGA, the enhancement was preceded by a slight inhibition for a short period (~3 min; not shown) and a longer exposure (~6 min) was necessary for significant enhancement in comparison with 30 μM NDGA, which enhanced STOCs within a few minutes. The enhanced STOCs by 30 μM NDGA were almost completely abolished by the application of 2 mM TEA (n = 6; Fig. 1A) or 100 nM IbTx (n = 4; Fig. 1B).

To evaluate quantitatively the effect of NDGA on STOCs, STOCs were integrated for 1 min before the application of NDGA and after the effect of NDGA reached the steady state. The integrated value before the application of NDGA was taken as 1.0. After the application of 10 or 30 μM NDGA, the relative integrated values of STOCs were increased to 7.4 ± 1.8 (n = 9, $p < .01$) or 17.5 ± 1.7 (n = 17, $p < .01$), respectively, and recovered with washout in most cells examined. The application of 0.1 mM Cd^{2+} to block voltage-dependent Ca^{2+} channels did not affect STOCs at −30 mV. In the presence of Cd^{2+}, an addition of NDGA significantly increased STOCs in a similar manner as in the absence of Cd^{2+} (10 μM NDGA: 7.5 ± 1.4, n = 5, $p < .01$; 30 μM NDGA: 17.6 ± 1.4, n = 21, $p < .01$). The relative integrated values of STOCs after the application of NDGA in the absence or presence of 0.1 mM Cd^{2+} are shown in Fig. 1C. The application of either 10 μM glibenclamide or 100 nM apamin, in the presence of 0.1 mM Cd^{2+}, did not significantly affect the enhancement of STOCs by NDGA (n = 9, respectively).

Effects of NDGA on Membrane Potential. Effects of NDGA on the resting membrane potential were recorded in the presence of 0.1 mM Cd^{2+} under current-clamp mode (Fig. 2). The averaged resting membrane potential was −41.4 ± 3.6 mV (n = 11). The application of 30 μM NDGA caused hyperpolarization of −14.1 ± 2.5 mV (n = 11, $p < .05$ versus control) in all cells examined. The NDGA-induced hyperpo-

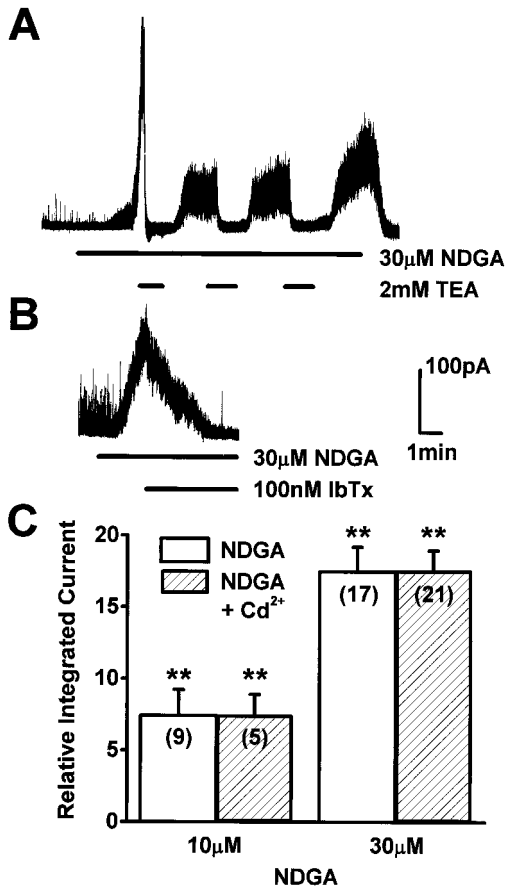

Fig. 1. Effects of NDGA on STOCs at a holding potential of −30 mV. A, application of 30 μM NDGA markedly enhanced STOCs. The enhancement of STOCs resulted in a sustained outward current on which STOCs were superimposed. The enhanced STOCs were blocked by 2 mM TEA and reversed by washout of TEA. B, the enhanced STOCs were also blocked by 100 nM IbTx. C, the results obtained from the application of NDGA in the absence (open column) or presence (hatched column) of 0.1 mM Cd^{2+} are summarized. STOCs before and after the application of NDGA were integrated for 1 min as integrated current. The integrated current before the application of NDGA was taken as 1.0. The number of experiments is given in parentheses, and the statistical significance of the difference versus control (before NDGA application; 1.0) is expressed as $^{**}p < .01$.

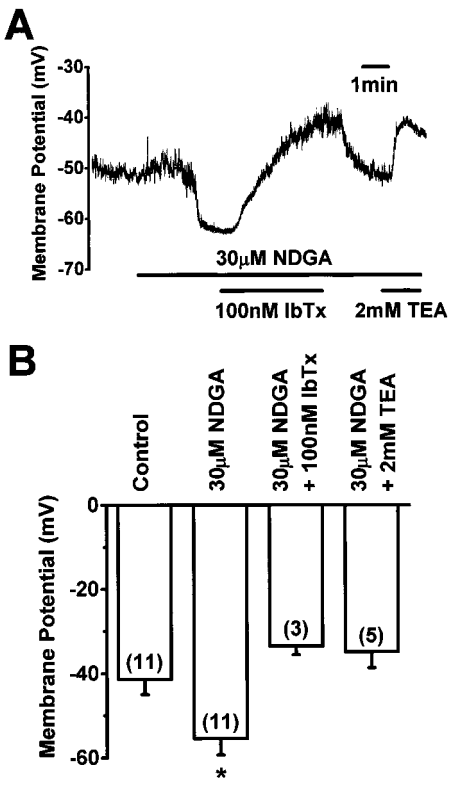

Fig. 2. Effects of NDGA on membrane potential under current clamp in the presence of 0.1 mM Cd^{2+}. A, application of 30 μM NDGA induced hyperpolarization that was reversed by 100 nM IbTx. The effect of IbTx was removed by washout. The application of 2 mM TEA also depolarized the cell. B, summarized results about the effects of 30 μM NDGA, 100 nM IbTx, and 2 mM TEA on membrane potential. The number of experiments is given in parentheses, and the statistical significance of the difference versus control is expressed as $^{*}p < .05$.

larization was removed by washout (not shown). The addition of 100 nM IbTx abolished NDGA-induced hyperpolarization and, moreover, depolarized the cell by ~5 mV over the initial resting potential ($n = 3$). The block of hyperpolarization by IbTx was partly removed by washout. The addition of 2 mM TEA also blocked the hyperpolarization and elicited further small depolarization ($n = 5$).

Effects of Caffeine and Ryanodine on NDGA-Induced Outward Current. STOCs are considered to be due to activation of BK channels by Ca^{2+} release from local SR through ryanodine-receptor Ca^{2+} releasing channels (Bolton and Imaizumi, 1996). Whether NDGA-induced enhancement of STOCs may include the increase in Ca^{2+} release was examined in the presence of 0.1 mM Cd^{2+}. The application of 10 mM caffeine markedly enhanced STOCs transiently for 10 to 30 s and suppressed them thereafter, indicating the exhaustion of stored calcium in SR. For irreversible calcium exhaustion in SR, caffeine was applied together with 10 μM ryanodine (Fig. 3A). After washout of caffeine and ryanodine, STOCs did not recover, and the second application of 10 mM caffeine did not change the holding current ($n = 7$, not shown). The addition of 30 μM NDGA in the presence of caffeine and ryanodine induced a slowly developing outward current (Fig. 3A). The NDGA-induced outward current was also blocked by 2 mM TEA or 100 nM IbTx (not shown, $n = 3$ for each). For quantitative evaluation, the integrated value of STOCs for 1 min before the application of drugs was taken as 1.0. The relative value of outward current integrated for 1 min after the application of caffeine and ryanodine was 8.4 ± 1.9 ($n = 7$, $p < .01$ versus 1.0). The relative value of NDGA-induced current in the presence of caffeine and ryanodine was 5.5 ± 0.7 ($n = 7$) and is significantly smaller than that obtained in the absence (13.6 ± 1.3, $n = 9$, $p < .01$; Fig. 3B).

Dual Action of NDGA on $I_{K\text{-}Ca}$. When coronary arterial myocytes were depolarized from −60 to 0 mV for 150 ms, a large transient and following sustained outward currents were recorded (Fig. 4A). The peak outward current was reduced to 30.1 ± 2.5% ($n = 9$, $p < .01$ versus the control) by the addition of 0.1 mM Cd^{2+}. The most prominent effect of Cd^{2+} on the outward current is to abolish the transient component (Fig. 4, A and B). The transient component in the absence of Cd^{2+} was also reduced to less than 20% of the control by 2 mM TEA or 100 nM IbTx, indicating that the major part of the transient component occurs through BK channels. When 30 μM NDGA was applied, the transient component of outward current disappeared just as when Cd^{2+} was applied (Fig. 4A). After 1 to 2 min, however, the remaining outward

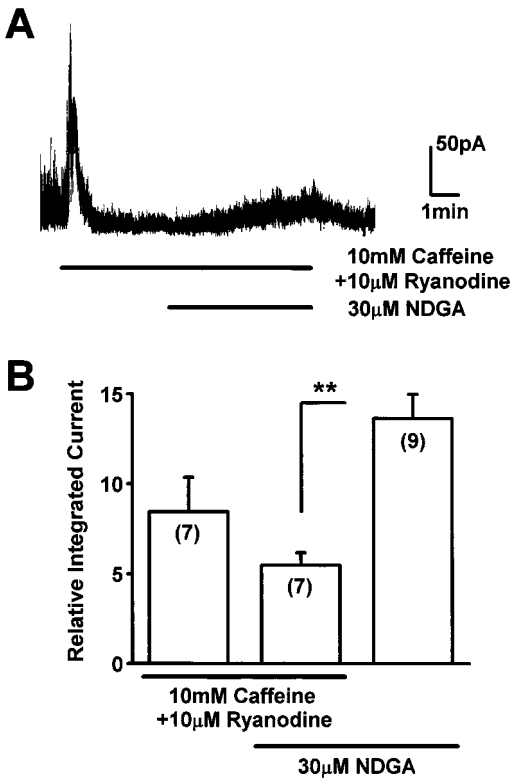

Fig. 3. Effects of caffeine and ryanodine on NDGA-induced outward current in the presence of 0.1 mM Cd^{2+}. A, membrane potential was held at −30 mV. The application of 10 mM caffeine and 10 μM ryanodine enhanced STOCs and induced a large phasic outward current, which was followed by suppression of STOCs. The addition of 30 μM NDGA induced a slow outward current. B, summarized results about the integrated outward current induced by the application of caffeine and ryanodine and/or NDGA. Left, 10 mM caffeine and 10 μM ryanodine. Middle, caffeine and ryanodine plus 30 μM NDGA. Right, only NDGA. The number of experiments is given in parentheses, and the statistical significance of the difference versus control is expressed as $^{**}p < .01$.

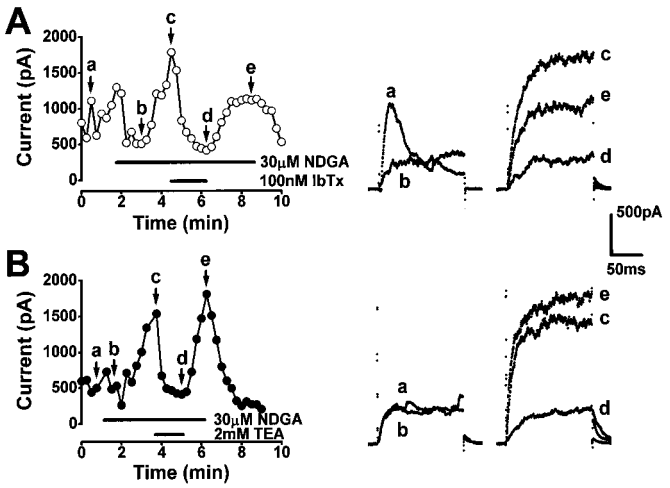

Fig. 4. Effects of NDGA on outward current activated by depolarization in the absence (A) and presence (B) of Cd^{2+}. Cells were depolarized for 150 ms from the holding potential of −60 to 0 mV every 15 s. A, peak amplitude of outward current was plotted against time. The original traces (a–e) were obtained at the time indicated correspondingly in the time course. The applications of 30 μM NDGA and 100 nM IbTx are indicated by horizontal lines in the time course. B, an experiment was performed in a similar protocol shown in A, except for the presence of 0.1 mM Cd^{2+}. TEA (2 mM) was applied instead of IbTx.

current was markedly enhanced and reached to a steady level within 3 min. The enhanced outward current did not show the transient component but a relatively slow activation, which reached 50% of the peak at ~20 ms from the start of depolarization to 0 mV (Fig. 4A). The enhancement by NDGA was almost completely blocked by 100 nM IbTx (n = 3) or 2 mM TEA (n = 4). In the presence of 0.1 mM Cd^{2+}, not the dual effect but only an enhancing effect was observed at 30 μM and higher concentrations of NDGA (Fig. 4B). The enhanced current was blocked by 2 mM TEA (n = 5) or 100 nM IbTx (n = 3).

Figure 5A shows the enhancement by 30 μM NDGA of outward currents at various potentials in the presence of 0.1 mM Cd^{2+}. The current-voltage relationships of the peak outward currents in the absence (open circles) and presence (triangles) of 30 μM NDGA indicate that the enhancement was observed at any potentials positive to −30 mV (Fig. 5B). The effect of NDGA was almost completely removed by washout (closed circles). Figure 5C summarized results about the effects of 1 to 100 μM NDGA on the amplitude of peak outward current at 0 mV. The amplitude of outward current was 619.4 ± 71.2 (n = 37) and 276.7 ± 25.2 (n = 34) pA in the absence and presence of Cd^{2+}, respectively. In the absence of Cd^{2+}, the application of 3 μM NDGA abolished the transient component and reduced the amplitude of outward current to 68.3 ± 4.6% (n = 4, $p < .01$ versus 100% by Student's t test) of that before the application (Fig. 5C). The relative amplitude after the enhancement in the presence of 1, 10, 30, and 100 μM NDGA was shown in Fig. 5C.

Ca^{2+} Channel Inhibition by NDGA. Effects of NDGA on voltage-dependent Ca^{2+} channel current (I_{Ca}) were examined. K^+ currents were blocked by the replacement of K^+ in the pipette solution with Cs^+. When myocytes were depolarized from the holding potential of −60 mV, the maximum peak amplitude of inward current was obtained at 0 mV (27.7 ± 5.0 pA, n = 6). The addition of 0.1 mM Cd^{2+} completely blocked the inward current. The Cd^{2+}-sensitive inward current was taken as I_{Ca}. NDGA inhibited I_{Ca} at 0 mV in a concentration-dependent manner with the IC_{50} value of 2.5 μM and Hill coefficient of 1.5 (Fig. 6, A and B). I_{Ca} was completely blocked by 100 μM NDGA. The I_{Ca} block by NDGA was removed, at least in part, by washout.

Effects of NDGA on I_{KD}. The outward current activated by depolarization in the presence of 0.1 mM Cd^{2+} (Fig. 4B) had relatively a slow activation time course, which was similar to that in the presence of 100 nM IbTx. The current in the presence of Cd^{2+} was only slightly reduced by 100 nM IbTx (to ~90%) but markedly reduced by 10 mM TEA (to ~20%) or 5 mM 4-aminopyridine (to ~30%). These results indicate that an I_{KD} is the major component of the outward current remained in the presence of Cd^{2+}. To remove the $I_{K\text{-}Ca}$ component completely, 5 mM EGTA and 100 nM IbTx were added to the pipette and bathing solutions, respectively, as well as Cd^{2+} in the bathing solution. Under these conditions, the application of 30 μM NDGA markedly reduced the I_{KD} activated by depolarization from −60 to +30 mV (Fig. 7). The block of I_{KD} by NDGA was almost complete removed by washout. Although the block of I_{KD} by NDGA was concentration dependent, there remained NDGA-insensitive component of 22% even in the presence of 100 μM NDGA. The half-inhibition of total I_{KD} was obtained by 9.8 μM NDGA.

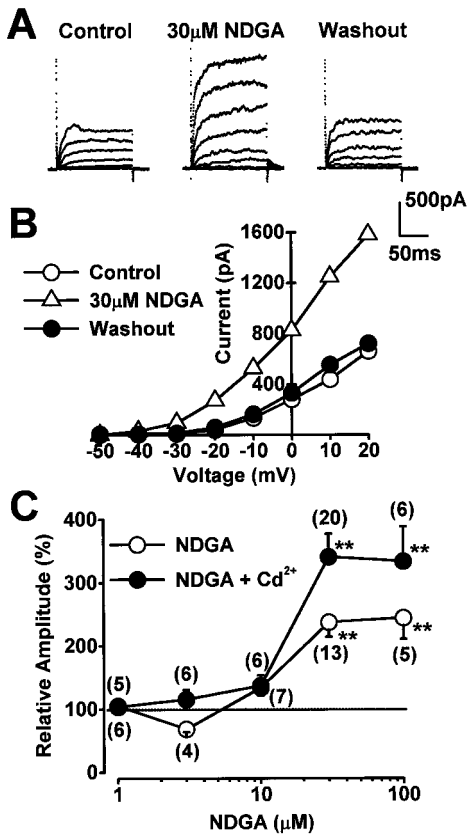

Fig. 5. Effects of NDGA on current-voltage relationship of outward current and the concentration dependence of the effects. A, effects of 30 μM NDGA on outward current activated by depolarization from -60 mV to various potentials in the presence of 0.1 mM Cd^{2+}. B, effects of 30 μM NDGA on the current-voltage relationship of peak outward current in the presence of 0.1 mM Cd^{2+}. The amplitude of peak outward currents before the application of 30 μM NDGA (○) and after washout of NDGA (●). Triangles indicate the amplitude in the presence of NDGA. C, the relationships between the peak amplitude of outward currents and concentrations of NDGA in the absence (○) and presence (●) of 0.1 mM Cd^{2+}. Outward currents were elicited by depolarization from -60 to 0 mV. The amplitude after the application of NDGA was normalized by that before the application in each cell. The dotted line indicates 100% (before the application of NDGA). The number of experiments is given in parentheses, and the statistical significance of the difference versus 100% is expressed as $^{**}p < .01$.

Effects of NDGA-Related Compounds. NDGA is a lipoxygenase inhibitor and an antioxidant, whereas at high concentrations, it also inhibits cyclooxygenase. It was examined whether the BK channel opening action was shared by other inhibitors of lipoxygenase, cyclooxygenase, or antioxidant agents. The effects of several compounds at 100 μM on the outward current elicited by depolarization from -60 to 0 mV were tested in the absence or presence of 0.1 mM Cd^{2+}. The peak outward current amplitude before the application of tested compounds was taken as 100%. The relative values after exposure to the compounds for 10 min in the absence and presence of 0.1 mM Cd^{2+} were shown in Fig. 8; lipoxygenase inhibitors were caffeic acid and esculetin; a cyclooxygenase inhibitor was indomethacin; antioxidants were ascorbic acid and erythorbic acid; and structurally related compounds of NDGA were catechol and dopamine. The outward current was markedly enhanced by 100 μM NDGA regardless of the presence of Cd^{2+}, as has been shown in Figs. 4 and 5. The effects of all other compounds tested were not statistically significant ($p > .05$ versus 100%).

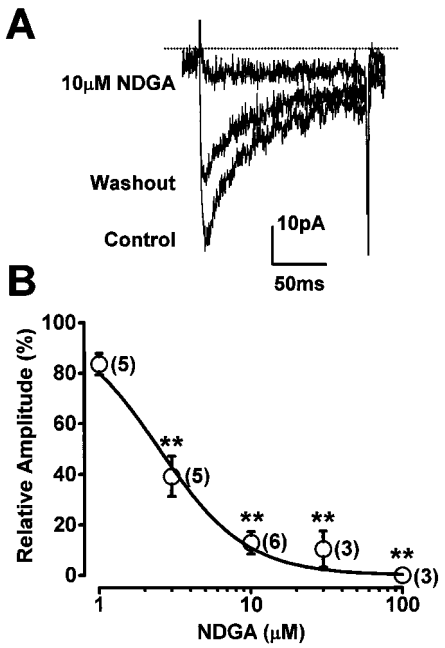

Fig. 6. Effects of NDGA on Ca^{2+} current (I_{Ca}) elicited by depolarization for 150 ms from the holding potential of -60 to 0 mV every 15 s. K^+ currents were blocked by Cs^+ added in the pipette solution. A, current traces before the application were superimposed in the presence of and after washout of 10 μM NDGA. The dotted line indicates zero I_{Ca} level at 0 mV, which was detected by an addition of 0.1 mM Cd^{2+}. B, concentration-response curve of NDGA for the inhibition of I_{Ca}. The peak I_{Ca} amplitude was obtained from the zero I_{Ca} level. The current amplitude in the presence of NDGA was normalized by that before the application (100%). The K_d and Hill coefficient were 2.5 and 1.5 μM, respectively. I_{Ca} was completely blocked by 100 μM NDGA. The number of experiments is given in parentheses, and the statistical significance of the difference versus 100% is expressed as $^{**}p < .01$.

Discussion

The present study clearly shows that NDGA ($\geq$10 μM) possesses a potentiating effect on whole-cell K^+ current in porcine coronary arterial smooth muscle cells. The enhancement of STOCs and outward currents on depolarization is due to activation of BK channels because the application of BK channel blockers (100 nM IbTx and 2 mM TEA; Kuriyama et al., 1995) or the addition of 5 mM EGTA to the pipette solution abolished the enhancement. This finding is consistent with our previous findings in single BK channel recordings (Nagano et al., 1996). Other two membrane currents examined in this study, I_{Ca} and I_{KD}, were both inhibited by NDGA; IC_{50} values are 2.5 and 9.8 μM, respectively. The currents enhanced by NDGA was not affected by glibenclamide or apamin; therefore, it can be strongly suggested that NDGA, as an ionic channel opener, acts on BK channels selectively.

NDGA showed dual action on outward currents activated by depolarization; an inhibition of the initial transient component and a strong enhancement of the remaining slowly activating K^+ current. The inhibition of the transient component indicates the decrease in $I_{K\text{-}Ca}$, which may be resulted from the block of Ca^{2+} entry through voltage-dependent Ca^{2+} channels. A similar decrease in the $I_{K\text{-}Ca}$ component of the outward current was observed when I_{Ca} was blocked by Cd^{2+} (Imaizumi et al., 1996, 1998). The finding in the present study that the total outward K^+ current on depolarization was markedly enhanced by NDGA ($\geq$30 μM) is in clear

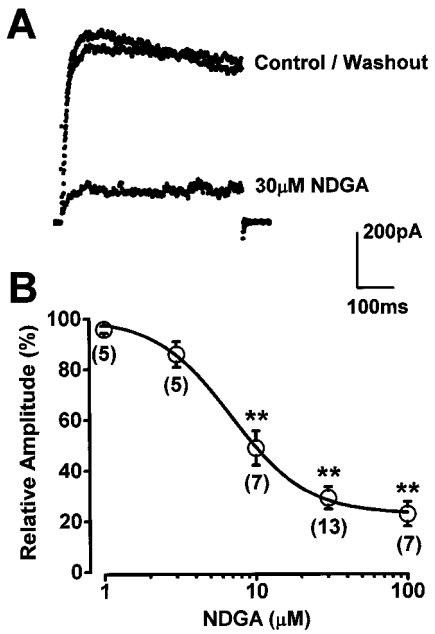

Fig. 7. Effects of NDGA on I_{KD} activated by depolarization from −60 to +30 mV for 500 ms every 15 s. The pipette and bathing solutions contained 5 mM EGTA and 0.1 mM Cd^{2+} plus 100 nM IbTx, respectively. A, I_{KD} was markedly reduced by the application of 30 μM NDGA and recovered by washout. B, concentration-response relationship of NDGA for the inhibition of I_{KD}. The current amplitude in the presence of NDGA was normalized by that before the application (100%). The K_d and the Hill coefficient were 6.9 μM and 1.7, respectively. The NDGA-resistant component in the outward current was 22%. The concentration of NDGA required for 50% inhibition (IC_{50}) of outward was 9.8 μM. The number of experiments is given in parentheses, and the statistical significance of the difference versus 100% is expressed as $^{**}p < .01$.

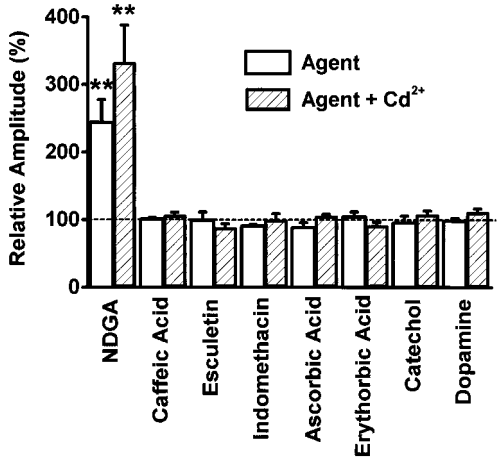

Fig. 8. Effects of several lipoxygenase inhibitors, cyclooxygenase inhibitor, antioxidants, and structural-related compounds of NDGA on the amplitude of outward current at the concentration of 100 μM. The outward current was elicited by depolarization from −60 to 0 mV for 150 ms every 15 s. The current amplitude before the application of drugs was taken as 100% in each preparation. Open and hatched columns indicate the relative amplitude in the absence and presence of 0.1 mM Cd^{2+}. The number of experiments was three to six for each. The statistical significance of the difference versus control (100%) is expressed as $^{**}p < .01$.

contrast with those reported in type I carotid body cells, where NDGA simply reduced whole-cell K^+ current activated by depolarization (Hatton and Peers, 1997). Our previous results that the open probability of BK channels in excised patches (Nagano et al., 1996) is greatly enhanced by NDGA have been confirmed in carotid body cells (Hatton and Peers, 1997). The reason for the discrepancy may be mainly due to the difference in the extent of the contribution of BK channel current to the whole-cell K^+ current, with the larger contributed in coronary artery than in carotid body cells (Hatton and Peers, 1997).

The most important finding in the present study is that NDGA markedly enhanced STOCs and, correspondingly, hyperpolarized the cell under the current clamp conditions. Spontaneous quantal Ca^{2+} release from local SR has been detected in cardiac myocytes as a Ca^{2+} "spark," which is considered to be the elementary event of excitation-contraction coupling (Cheng et al., 1993, 1996). Similar random Ca^{2+} sparks have been detected in smooth muscle cells to presumably be the cause of STOCs (Nelson et al., 1995; Mironneau et al., 1996; for a review, see Bolton and Imaizumi, 1996). A STOC may be due to the activation of BK channels in a small area near Ca^{2+} spark. It has been suggested that STOCs partly contribute to the resting membrane potential (Nelson and Quayle, 1995). The open probability of L-type voltage-dependent Ca^{2+} channels in an arterial smooth muscle cells depends strongly on the changes in the resting membrane potential (Nelson and Quayle, 1995). In arteries that have inherent tone, the resting membrane potential of smooth muscle cells is one of the important factors regulating the muscle tone. The block of BK channels results in the membrane depolarization by several millivolts and the increase in tone, whereas some other K^+ channels may also take part in the mechanisms regulating the resting membrane potential and the muscle tone (Leblanc et al., 1994). A larger contribution of BK channel activity to the arterial muscle tone at resting conditions has been presented in spontaneously hypertensive rats (Asano et al., 1993).

These observations indicate that BK channel opener may have a substantial potency for the treatment of angina, hypertension, bronchial asthma, hypersensitive urinary bladder, and some other diseases (Edwards and Weston, 1993). BK channel openers such as NS-004 (Sargent et al., 1993; Olesen et al., 1994; Xu et al., 1994) and NS-1619 (Edwards et al., 1994; Holland et al., 1996) have been tested and are expected as a new type of K^+ channel modulators after ATP-sensitive K^+ channel openers. Arachidonic acid and/or its metabolites have been suggested as modulators of ionic channel activity in various types of cells (Meves, 1994), including smooth muscle (Nagano et al., 1995, 1997). Modulation of BK channel activity by arachidonic acid, its metabolites, and related fatty acids has been extensively studies (Ordway et al., 1991) but the exact mechanism has not been clarified yet. Some cytochrome P-450 metabolites of arachidonic acid and/or endocannabinoids may possibly be endogenous BK channel openers (Hu and Kim, 1993; Randall and Kendall, 1998). Niflumic acid, a potent cyclooxygenase inhibitor, acts as a BK channel opener (Ottolia and Toro, 1994).

NDGA has been widely used as a natural antioxidant for fats and oil in foods. NDGA at low concentrations (<5 μM) acts as a relatively selective inhibitor of lipoxygenase (Beetens et al., 1986), whereas NDGA at higher concentrations inhibits also cyclooxygenase and phospholipase A_2 (Billah et al., 1985). Based on the present results, it is unlikely that the enhancement of BK channel activity by NDGA is due to its effects as an antioxidant, a lipoxygenase inhibitor, or a cyclooxygenase inhibitor. NDGA markedly activates BK

channels also in inside-out patches (Nagano et al., 1996). The result suggests that NDGA-induced enhancement of BK channel activity may not be mediated by the inhibition of cytosolic enzymes or by second messenger-mediated mechanisms but rather by a direct effect on ion channel itself or on phospholipids of cell membrane. The application of high concentrations of NDGA ($\geq$30 μM) often elicited the disruption of gigaohm seal that has also been reported in other preparations (Korn and Horn, 1990). This may suggest the interaction of NDGA with cell membrane phospholipids.

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 Ca^{2+} release probably from mitochondria itself and results in the activation of BK channels (Yuan et al., 1996). Because high concentrations of NDGA inhibit electron transport in mitochondria and deplete ATP (Pardini et al., 1970), the activation of STOCs may be due to Ca^{2+} release that results from mitochondria poisoning. In the present study, BK channel activation induced by NDGA at holding potential of -30 mV was reduced by $\sim$60%, when calcium store in SR was depleted by pretreatment with caffeine and ryanodine. These results strongly suggest that NDGA enhances Ca^{2+} release from SR but not from mitochondria. Because application of NDGA did not induce contraction (H.Y., unpublished observation), it is unlikely that the global Ca^{2+} concentration in myocytes was substantially increased by NDGA. NDGA may enhance the spontaneous Ca^{2+} release through ryanodine receptor in SR, especially available for activation of STOCs.

In conclusion, NDGA enhances STOCs under whole-cell voltage-clamp conditions and thereby hyperpolarizes myocytes under current clamp via the marked effect as a BK channel opener in porcine coronary arterial smooth muscle cells. The enhancement is selective to BK channels. Voltage-dependent Ca^{2+} channels and delayed rectifier type K^+ channels are blocked by NDGA. The enhancement of BK channel activity by NDGA is mediated by at least two different mechanisms: the direct activation of BK channels and the enhancement of Ca^{2+} release from caffeine/ryanodine-sensitive store sites.

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