Sulphydryl Modification Affects Coronary Artery Tension by Changing Activity of Delayed Rectifier K⁺ Current

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Abstract

It has been reported that a change in the cellular redox state may be involved in the regulation of vascular tone, but the underlying mechanism is not fully understood. The present study was designed to investigate the cellular effect of sulphydryl modifying agents in the coronary artery of rabbit using the tension measurement and whole cell clamping method. The application of diamide, a sulphydryl oxidizing agent, relaxed the endothelium denuded coronary arteries in a dose dependent manner. The fact that this diamide-induced relaxation was significantly attenuated by a pretreatment of 4-AP, and the coronary arteries precontracted with 100 mM K⁺ instead of histamine, suggests the involvement of 4-AP sensitive K⁺ channels in the diamide-induced relaxation of coronary arteries. Whole cell patch clamp studies revealed that the 4-AP sensitive I_{K} was significantly enhanced by the membrane permeant oxidizing agents, diamide and DTDP, and were reversed by subsequent exposure to the reducing agent, DTT. Neither the membrane impermeant oxidizing or reducing agents, GSSG or GSH, had any effect on the activity of I_{K}, indicating that intracellular sulphydryl modification is critical for modulating I_{K} activity. The Diamide failed to significantly alter the voltage dependence of the activation and inactivation parameters, and did not change the inactivation process, suggesting that diamide increases the number of functional channels without altering their gating properties. Since I_{K} has been believed to play an important role in regulating membrane potential and arterial tone, our results about the effect of sulphydryl modifying agents on coronary arterial tone and I_{K} activity should help understand the pathophysiology of the diseases, where oxidative damage has been implicated.

Key Words: Coronary artery, delayed rectifier K⁺ current, diamide, dithiothreitol, glutathione, redox

INTRODUCTION

One of the major biological targets of free radical oxidation is the cardiovascular system. In particular, the coronary smooth muscle cells are directly exposed to reactive oxygen species during ischemia-reperfusion, atherosclerosis, and hypertension.1-3 It has been reported that reactive oxidant species markedly affect the vascular tone by changing the transsarcolemmal Ca²⁺ influx or Ca²⁺ release from intracellular stores.4-6 However, the mechanism for the oxidant induced change of vascular contractility has not been fully characterized.

There is increasing evidence that many cellular effects of reactive oxygen species are mediated by changes in the membrane ionic conductance. Oxidation of sulphydryl groups on or in an ion channel protein might directly affect the ion channel activity or a change in cellular redox status might modulate the ion channel function.7,8 There is substantial evidence that the K⁺ current activity is regulated by an oxidation/reduction reaction, and the changes in the K⁺ current activity can regulate the vascular tone by changing the membrane potential of the vascular smooth muscle cells.9-11

At least four different types of macroscopic K⁺ currents have been identified in the coronary smooth muscle cells, including the delayed rectifier K⁺ current (I_{K}), Ca-activated K⁺ current (I_{KCa}), inward rectifier K⁺ current (I_{K,i}), and ATP-sensitive K⁺ current (I_{KATP}).12-15 Among these currents, I_{K} is a major determinant of the resting membrane potential and has been shown to be inhibited during atherosclerosis.16,17 These studies, therefore, suggest that I_{K} might be the principal target that is affected by reac-
tive oxygen species. However, there have been no reports on the oxidant-induced change of \( I_{\text{K}} \) in the coronary smooth muscle cells, or whether this change in \( I_{\text{K}} \) is responsible for the change in coronary tone during oxidative stress.

In light of these uncertainties, the objective of the present study was to determine whether \( I_{\text{K}} \) is responsible for oxidant-induced change in the coronary vascular tone using sulfhydryl oxidizing and reducing agents, and to examine the mechanism of how sulfhydryl modifying agents affect \( I_{\text{K}} \) using the whole cell patch clamp technique in coronary smooth muscle cells. We present direct evidence that sulfhydryl oxidant increases the activity of \( I_{\text{K}} \) in coronary smooth muscle cells, and this enhancement of \( I_{\text{K}} \) is responsible for the oxidant-induced relaxation of the coronary artery.

**MATERIALS AND METHODS**

**Measurement of tension**

Albino rabbits (2.0–2.5 Kg) of either sex were anaesthetized by injecting 60 mg/Kg sodium pentobarbital into the ear vein, and they were exsanguinated by cutting a femoral artery. The left anterior descending coronary artery was excised and placed into Krebs-Henseleit (KH) solution with the following composition: 136 mM NaCl, 5.4 mM KCl, 2.5 mM CaCl\(_2\), 1.2 mM MgCl\(_2\), 24 mM NaHCO\(_3\), 0.01 mM EDTA and 5.5 mM Glucose. The pH of the solution was maintained at 7.4 after bubbling with 95% \( \text{O}_2 \) and 5% \( \text{CO}_2 \) gas mixture. Arterial rings with a width of 2–3 mm were prepared for isometric contractile force recordings as described previously.\(^{13}\)

To eliminate any possible involvement of endothelium-derived vasoactive factors, the endothelium was removed by gently rubbing the intimal surface, and its functional integrity was checked by the method of Furchgott and Zawadzki.\(^{18}\)

The coronary rings were mounted using two triangular stainless wires, with one fixed to the bottom of the tissue bath and the other attached to a force transducer, and the force was continuously recorded using a chart recorder. The KH solution was used as a tissue bath solution, which was continuously oxygenated (95% \( \text{O}_2 \) and 5% \( \text{CO}_2 \)) and heated to 37°C. The coronary rings were equilibrated for 120 minutes under a 0.5 g of resting tension before each experiment. During equilibration, rings were exposed to 100 mM K\(^+\) solution, which maximally contracted the arteries and ensured the stabilization of the muscle. The 100 mM K\(^+\) solution was prepared by equimolar replacement of NaCl with KCl in a normal KH solution to maintain normal osmolarity and Cl\(^-\) concentration. The effect of the sulfhydryl modifying agent was measured after the coronary rings reached a stable maximum contraction with agonist (histamine 10\(^{-5}\) M) or 100 mM K\(^+\).

**Measurement of \( I_{\text{K}} \)**

**Preparation of the coronary smooth muscle cells:** After removing the surrounding connective tissues, the coronary artery was exposed longitudinally and bathed in a Ca\(^{2+}\)/Mg\(^{2+}\) free Tyrode solution for five The coronary artery was incubated for an additional 60 minutes in a temperature controlled (37°C) water bath after changing with a solution containing 0.1% collagenase. After this incubation, the coronary strips was transferred to a Ca\(^{2+}\)/Mg\(^{2+}\) free Tyrode solution, and agitated with fine glass rod. Single coronary smooth muscle cells were stocked in the Tyrode solution at 4°C until the experiment.

**Patch clamp method:** An aliquot of single coronary smooth muscle cells in suspension was added to a recording chamber mounted on a Nikon diaphot inverted microscope. The solutions were superfused at a rate of 1 ml/min, and the experiments were performed at room temperature (23–25°C). The standard whole cell patch clamp technique was used to voltage-clamp the single coronary smooth muscle cells.\(^{19}\) Borosilicate patch pipettes were pulled on a two-stage horizontal puller and heat polished using a microforge. The tip diameter of each patch pipette was 1–2 \( \mu \text{m} \) and the pipette resistance ranged from 2–3 M\(\Omega \) when the pipette was filled with internal pipette solution.

Voltage command pulses were generated and the resulting membrane currents were measured with an Axopatch 1-D patch clamp amplifier using pClamp software (Version 6.0, Axon Instruments, Foster City, CA, USA). The membrane currents and voltage commands were monitored on a storage oscilloscope and simultaneously stored on a computer hard disk for later analysis. The data were low pass filtered at 5 kHz with an 8-pole Bessel filter and digitized at 25 kHz using a 12 bit A/D converter.
Solutions and chemicals

The tyrode solution for cell preparation contains (mM): NaCl, 140; KCl, 5.4; CaCl₂, 1.8; MgCl₂, 1.0; HEPES, 10; Glucose 5.5, and the pH was adjusted with Tris to 7.4. The standard micropipette solution for the whole-cell experiments contained 100 mM potassium gluconate, 30 mM KCl, 5.7 mM MgSO₄, 5 mM K₂ATP, 1 mM Na₃GTP, 10 mM BAPTA, and 10 mM HEPES, and the pH was adjusted with Tris to 7.2. The standard external solution for the whole-cell experiments contained 135 mM NaCl, 5.6 mM KCl, 1.2 mM MgCl₂, 2.5 mM MnCl₂, 10 mM glucose, and 10 mM HEPES, and pH was adjusted with Tris to 7.4. All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA).

RESULTS

Characterization of vasorelaxation to sulfhydryl oxidizing agent

As shown in Fig. 1, diamide (sulfhydryl oxidizing agent) produced concentration dependent relaxation of the endothelium-denuded coronary ring preparations precontracted with histamine (10⁻⁵ M). On average, 0.3 mM diamide induced a 78.3±4.6% relaxation (n=10). The relationship between the concentration response and diamide-induced relaxation was determined, and the EC₅₀ was calculated to be 62.3±0.2 μM (n=7). This diamide induced relaxation was completely reversed by applying DL-dithiothreitol (DTT, 1 mM), a sulfhydryl reducing agent (n=10).

To elucidate the underlying mechanism of the diamide-induced relaxation, we first tried to compare the relaxing effect of diamide under different external K⁺ concentration conditions. The coronary rings exhibited a contraction after exposure to 100 mM K⁺ solution due to an increased Ca²⁺ influx via depolarization of the membrane, and this depolarization reduced the driving force for the K⁺ efflux. In the coronary rings precontracted with 100 mM K⁺, the relaxation responses of diamide were significantly attenuated relative to the case of a normal external K⁺ concentration (5.6 mM). The relaxing effect of 0.3 mM diamide in 80 mM K⁺-induced contraction was 23.1±10.2%, which was significantly attenuated compared to the effects of diamide in the histamine-induced contraction (p<0.01, n=10, Fig. 2A and C).

These results suggest the involvement of the K⁺ channel in the diamide-induced relaxation response. In rabbit coronary smooth muscle cells, I₆K plays an important role in determining the resting membrane potential and vascular tension, so we measured the effect of pretreating with 4-aminopyridine (4-AP, a blocker of I₆K) on the diamide-induced relaxation response. The 4-AP pretreatment significantly attenuated relaxation response induced by 0.3 mM diamide (78.3±4.6% vs. 24.1±3.2% in the control vs. in the 4-AP, n=10, p<0.01, Fig. 2B and C).

Sulfhydryl oxidizing agents increase I₆K

The result obtained from the tension measurement
strongly suggest the involvement of \( I_{\text{BK}} \) in the diamide-induced relaxation responses in the coronary rings, so a whole cell patch clamp was applied to determine whether \( I_{\text{BK}} \) increased due to sulfhydryl oxidizing agent. For the whole-cell experiments, \( I_{\text{BK}} \) was isolated from \( I_{\text{K-Ca}} \) with 10 mM BAPTA in the pipette solution to buffer the intracellular \( \text{Ca}^{2+} \) and also by replacing the external \( \text{Ca}^{2+} \) with \( \text{Mn}^{2+} \). Under these experimental conditions, the contribution of \( I_{\text{K-Ca}} \) to the total outward current was minimal or absent, especially at potentials less than +30 mV, as assessed using iberiotoxin, a specific blocker of \( I_{\text{K-Ca}} \) channels.\(^{21}\) The contribution of \( I_{\text{K-ATP}} \) to the macroscopic outward currents was minimized by adding 5 mM ATP in the internal solution.\(^{22}\) These experimental conditions were used for all whole-cell experiments reported in this study.

Fig. 3A shows the representative whole-cell \( I_{\text{BK}} \) currents elicited by a series of depolarizing voltage-clamp steps over the range −50 to +25 mV from a holding potential of −80 mV during the control, and 10 minutes after superfusion with the membrane permeant oxidizing agent, 1 mM diamide.\(^{23}\) During exposure to diamide, the amplitudes of \( I_{\text{BK}} \) were higher over the entire range of potentials. In most of the cells that we examined, the diamide increased \( I_{\text{BK}} \) at the concentration greater than 10 \( \mu \text{M} \), and reached to a maximum effect at 1 mM. When we used 1 mM diamide, the \( I_{\text{BK}} \) typically started to increase within one minute of its application, and washing the bath with a diamide free solution for up to ten minutes did not reverse the effect of diamide on enhancing the \( I_{\text{BK}} \) amplitude. This diamide-induced enhancement of \( I_{\text{BK}} \) was completely reversed by applying DTT (Fig. 3B). It is interesting to note that DTT completely reversed the effects of diamide with the \( I_{\text{BK}} \) currents amplitudes returning to nearly the same as in the control, suggesting that these effects can be attributed to alterations in redox state of \( I_{\text{BK}} \) channel. Fig. 3C shows the relationship between the mean current and applied voltage of \( I_{\text{BK}} \) obtained from six different cells under control conditions, after ten minutes of superfusion with diamide, followed by superfusion with DTT (\( n=6 \)).

We also tested the effect of another sulfhydryl modifying agent on the amplitude of \( I_{\text{BK}} \). When we used a 2,2-dithiodipyridine (DTDP), a membrane permeable oxidizing agent,\(^{24}\) produced a similar enhancement of \( I_{\text{BK}} \) as with the diamide treatment. However, applying the oxidized form of glutathione (GSGG), a membrane impermeable oxidizing agent or the reduced form of glutathione (GSH), a membrane impermeable reducing agent showed no apparent effect on \( I_{\text{BK}} \). These results suggest that a modifica-
Fig. 3. The Effect of diamide on \( I_{\text{dK}} \) in rabbit coronary arterial smooth muscle cells. \( K^+ \) currents were evoked by stepping the membrane potential from a holding potential of \(-80\) mV to potentials ranging from \(-50\) to \(+25\) mV in \(5\) mV increments. (A) A family of \( K^+ \) currents was recorded under control, \(1\) mM diamide, and after \(5\) mM DTT. (B) A plot of \( K^+ \) current amplitude, evoked by repetitive step depolarizations from \(-80\) mV to \(0\) mV versus time. The horizontal bars show the time during which the cells were exposed to diamide (\(1\) mM) or DTT (\(5\) mM). The inset shows the representative current traces during control, \(1\) mM diamide, and \(5\) mM DTT. (C) A summary of diamide and DTT effects on the current-voltage relations of \( I_{\text{dK}} \) (\(n=6\)). The current amplitudes were normalized with cell capacitance.

Effects of oxidizing agents on gating properties of \( I_{\text{dK}} \)

Several possible mechanisms exist that explain the diamide-induced enhancement of \( I_{\text{dK}} \). It is possible that diamide enhances the \( I_{\text{dK}} \) by altering the voltage dependence of the steady state activation or inactivation so that more channels are open at a lesser negative voltage or more channels are available to open on depolarization.\(^{25}\) To test these possibilities, we examined the effects of diamide on the voltage-dependence of the steady-state activation and inactivation of \( I_{\text{dK}} \). The midpoint of the activation curve was not affected statistically by diamide (\( -16.2 \pm 0.5 \) mV in the control group and \( -18.1 \pm 0.5 \) mV in the diamide groups; \( p > 0.05, n = 6 \)). The midpoint of the inactivation curve before and after oxidation was also statistically insignificant (\( -32.8 \pm 0.4 \) and \( -31.8 \pm 0.4 \) mV in the control and diamide groups, respectively; \( p > 0.05, n = 7 \)). These results suggest that
diamide enhanced the I_{K} without altering the steady-state voltage dependency (Fig. 5).

It has been reported that a slowed inactivation may enhance the peak current amplitude, so we examined the kinetics of inactivation before and after applying the diamide. Fig. 6A shows typical current traces obtained in the control and after the diamide. The diamide treatment did not affect the voltage dependence of the activation and inactivation parameters of I_{K} (p > 0.05).

DISCUSSION

Our findings clearly indicate that I_{K} in rabbit coronary smooth muscle cells can be regulated by the cellular redox status, and this change in I_{K} activity might be responsible for the oxidant-induced change.
of coronary arterial tone. The redox regulation of K+ channels in vascular smooth muscle cells has previously been demonstrated and is suggested to play an important potential role in the modulation of K+ channels and resting membrane potential during oxidative stress. However, such effects have not been demonstrated for I_{K} in coronary smooth muscle cell, even though this current is known to be an important determinant of the resting membrane potential and tension in the coronary artery of rabbits.

**Involvement of K⁺ channel activation in diamide-induced relaxation of coronary artery**

In the present study, diamide relaxed a coronary artery precontracted with agonist, a histamine. In contrast, diamide-induced relaxation was significantly attenuated in the coronary arteries precontracted by high extracellular K⁺. Raising the extracellular K⁺ decreases the driving force for K⁺ efflux because the electromotive force for K⁺ flux (E_m–E_K) becomes smaller in high external K⁺ than in normal external K⁺. In this case, Activating or opening the K⁺ channels had a lesser effect on the membrane potential and tension. The decreased ability of diamide to relax arteries precontracted with high external K⁺ suggested that the diamide-induced relaxation involved the activation of the K⁺ channels.

At least four different types of macroscopic K⁺ currents have been identified in the coronary smooth muscle cells. Among these currents, I_{K} is a major determinant of the resting membrane potential and important effector systems that mediate changes in the agonist induced vascular tone by altering the membrane potential in the coronary artery of rabbits. As shown in Fig. 2B, a pretreatment of 4-AP (a effective blocker of I_{K}) significantly attenuated the diamide induced coronary relaxation. Moreover, pretreatment with a low dose of 4-AP (a sensitive blocker of I_{K-Ca}) had only a small effect on diamide-induced coronary relaxation (data not shown). These results best fit the hypothesis that diamide relaxed the coronary artery by activating the I_{K} in the coronary smooth muscle cells.

**Mechanism of diamide-induced activation of I_{K}**

Several precautions were taken in the present study to insure effective isolation of I_{K} from contamination by I_{K-Ca}, I_{Cl-Ca}, or I_{Cl-ATP}. We included 5 mM ATP in the pipette solution to inhibit I_{Cl-ATP} and the activity of I_{K-Ca}, I_{Cl-Ca} was minimized by using 10 mM BAPTA to buffer the intracellular Ca\(^{2+}\) and replacing the external Ca\(^{2+}\) with Mn\(^{2+}\). Under these conditions, we were able to record a time and voltage dependent outward current that represented the properties of I_{K}. Furthermore, this current was markedly inhibited by 4-AP, a effective blocker of I_{K}. The contribution of I_{K} on the outward current also seemed negligible, since the density of I_{K} in the coronary artery was very low compared to the I_{K} and the voltage we used to record the I_{K} was much more positive from E_{K}, where the activity of I_{K} was minimal.

The I_{K} was enhanced by the membrane permeable oxidizing agents, diamide and DTDP, and this enhancement was completely reversed by the reducing agent, DTT. These effects are consisten with the results of earlier studies, which demonstrated that a variety of voltage dependent K⁺ channels have a critical cysteine residue, and that the changing of the redox status of these cysteine residues affect the channel function. We assumed in our experiments the intracellular cysteine residues were responsible for the diamide or DTDP-induced increase in I_{K}, since GSSG and GSH (two membrane impermeable oxidizing and reducing agents) had a very small effect on the I_{K} activity.

It is still uncertain whether diamide or DTDP directly interact with the cysteine residues of a channel protein. Previous studies have proposed a variety of molecular pathways that can be stimulated by oxidizing agents. For example, applying H\(_2\)O\(_2\) can activate guanylate cyclase, protein kinase C, and phospholipase A\(_2\) in the vascular smooth muscle cells, which might be related to the oxidant-induced change of vascular tone. However, the involvement of these intracellular messenger systems in our diamide-induced coronary relaxation or activation of I_{K} seems unlikely, since pretreatment of indomethacin or eicosatrinonic acid did not inhibit the diamide-induced relaxation in our preparation (unpresented results). Furthermore, in the canine pulmonary artery, diamide could activate I_{K} in the cell-free, inside-out patches, even in the presence of protein kinase C or protein kinase G inhibitors.

In many voltage dependent channels, oxidizing agents alter the gating properties by changing the redox status of the cysteine residue of the channel.
protein, which may be related to the change in channel activity. For example, oxidizing the methionine or cysteine residue of the transient outward K⁺ current enhanced the current amplitude with concomitant prolongation of inactivation.²⁶,²⁷ It is possible that the diamide-induced enhancement of Iₖᵢ in our preparation was due to prolonged inactivation process or negative shift of the steady state voltage dependency. However, the diamide failed to significantly alter the voltage dependence of activation or inactivation parameters, and it did not change the inactivation process.

There are some possibilities that explain these discrepancies between the enhancement of Iₖᵢ and failure of alteration of gating properties. Firstly, The macroscopic Iₖᵢ current in the smooth muscle cells have been attributed to multiple types of delayed rectifier K⁺ channels,²⁸ and it is conceivable that the possible differential effects on these multiple channels might prevent the detection of clear-cut alterations in the composite channel gating properties at the macroscopic current level. Secondly, oxidation of the cysteine residues by diamide might increase the number of functional channels in the cell without altering the gating properties. However, the precise biophysical mechanism responsible for the redox alteration of Iₖᵢ requires substantially more information than is currently available on the molecular identity of the delayed rectifier K⁺ channels in coronary smooth muscle cells.

In summary, these data suggest that the delayed rectifier K Channels in coronary smooth muscle cells contain at least one cysteine residue on the cytoplasmic side of the membrane, which is readily available for redox modulation. Oxidation of this site(s) is capable of increasing the magnitude of Iₖᵢ, and inducing the vasorelaxation by hyperpolarizing the membrane potential in rabbit coronary smooth muscle cells. Understanding the mechanism of oxidant-induced relaxation might help understand the pathophysiologic progress of those diseases in which oxidative damage has been implicated.

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