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Direct Stimulation of K_{ATP} Channels by Exogenous and Endogenous Hydrogen Sulfide in Vascular Smooth Muscle Cells

Guanghua Tang, Lingyun Wu, Wenbin Liang, and Rui Wang

Departments of Physiology (G.T., W.L., R.W.) and Pharmacology (L.W.), College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

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ABSTRACT

ATP-sensitive K^+ (K_{ATP}) channels in vascular smooth muscle cells (VSMC) are important targets for endogenous metabolic regulation and exogenous drug therapy. H_2S, as a novel gasotransmitter, has been shown to relax rat aortic tissues via opening of K_{ATP} channels. However, interaction of H_2S, exogenous-applied or endogenous-produced, with K_{ATP} channels in resistance artery VSMC has not been delineated. In the present study, using the whole-cell and single-channel patch-clamp technique, we demonstrated that exogenous H_2S activated K_{ATP} channels and hyperpolarized cell membrane in rat mesenteric artery VSMC. H_2S enhanced the amplitude of whole-cell K_{ATP} currents with an EC_{50} value of 116 ± 8.3 μM and increased the open probability of single K_{ATP} channels. H_2S hyperpolarized membrane potentials by −12 mV in nystatin-perforated VSMC. Furthermore, inhibition of endogenous H_2S production with D,L-propargylglycine (PPG) reduced whole-cell K_{ATP} currents. PPG alone had no effect on unitary K_{ATP} channel currents in cell-free membrane patches. In addition, effects of H_2S on K_{ATP} channels and membrane potentials were independent of cGMP-mediated phosphorylation. This study demonstrated modulation of K_{ATP} channel activity by exogenous and endogenous H_2S in resistance artery VSMC, thus helping elucidate cardiovascular functions of this endogenous gas.

ATP-sensitive K^+ (K_{ATP}) channels couple cellular electrical activity to metabolism in a variety of tissues (Quayle et al., 1997). In vascular smooth muscle cells (VSMC), opening of K_{ATP} channels hyperpolarizes cell membrane and inactivates voltage-dependent L-type Ca^{2+} channels, leading to cell relaxation and blood vessel dilation by reducing intracellular free Ca^{2+} concentration (Nelson and Quayle, 1995). Under physiological conditions, metabolic regulation of K_{ATP} channel is achieved through changes in cytosolic levels of nucleotides like ATP and MgADP. Under pathophysiological conditions such as hypertension and diabetes, K_{ATP} channels are important therapeutic targets of drugs such as K^+ channel openers. Therefore, K_{ATP} channels in VSMC play important roles in the fine regulation of vascular tone.

H_2S not only exists as an environmental pollutant, but is also generated from L-cysteine metabolism catalyzed by two pyridoxal-5′-phosphate-dependent enzymes: cystathionine-γ-lyase (CSE) and cystathionine β-synthase (CBS) in mammalian tissues (Zhao et al., 2001; Wang, 2002; Zhao and Wang, 2002). Having an established toxicological profile for decades (Reiffenstein et al., 1992; Guidotti, 1996), H_2S is physiologically important, which has not been realized until recently. In vascular tissues, H_2S, like other gasotransmitters (Wang, 2002), may serve as a modulator of VSMC contractility. Endogenous production of H_2S has been measured in different vascular tissues like aorta, tail, and mesenteric arteries (Cheng et al., 2004). Physiological level of H_2S in rat serum is approximately 45 μM (Zhao et al., 2001). H_2S at physiologically relevant concentrations has been demonstrated to induce relaxation of rat aortic tissue and transient reduction of blood pressure (Zhao et al., 2001; Zhao and Wang, 2002). Vascular effect of H_2S might be mediated by a direct stimulation of K_{ATP} channels and subsequent hyperpolarization of rat aortic VSMC (Zhao et al., 2001). All of these observations suggest an important physiological role of H_2S in cardiovascular system.

Substantial differences exist between conduit and resis-

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ABBREVIATIONS: K_{ATP}, ATP-sensitive K^+ channels; 8-Br-cGMP, 8-bromo-cGMP; VSMC, vascular smooth muscle cell; CSE, cystathionine γ-lyase; CBS, cystathionine β-synthase; PPG, D,L-propargylglycine; HP, holding potential; TP, testing potential; MP, membrane potential; PSS, physiological salt solution; KCO, ATP-sensitive K^+ channel opener; βCAN, β-cyano-L-alanine; I-V, current-voltage; KT5823, (8*R*,9*S*,11*S*)-(−)-9-methoxy-carbamyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*,11*a*-trizadibenzo-(*a*,*g*)-cycloocta-(*c*,*d*,*e*)-trinden-1-one.

tance arterial VSMC in many functional properties, such as resting membrane potential, ionic channel currents, role of endothelium-derived hyperpolarization factor, and endothelium-dependent relaxation (Shimokawa et al., 1996; Takamura et al., 1999). H_2S action on conduit artery aorta (Zhao et al., 2001) cannot be simply extrapolated to that on peripheral resistance vessels, like mesenteric artery. Therefore, the first objective of this study was to investigate actions of exogenous H_2S on K_{ATP} currents and membrane potentials in single VSMC from rat mesenteric artery. Second, exogenous H_2S has been used, to date, to study the interaction of this gasotransmitter with K_{ATP} channels, and it becomes a critical issue to determine effect of endogenous H_2S on K_{ATP} channels. Third, important information has been collected previously using the whole-cell patch-clamp technique regarding H_2S effect on K_{ATP} channels, and no analysis on changes in single-channel behavior of K_{ATP} channels in the presence of H_2S has been conducted. In the present study, therefore, the whole-cell and single-channel patch-clamp recording technique was used to examine the effects of H_2S on K_{ATP} channels in isolated VSMC from rat mesenteric artery. Endogenous production of H_2S in VSMC was modulated using CSE inhibitors, and thereafter, change in K_{ATP} channel currents was monitored.

Materials and Methods

Preparation of Single VSMC. Single mesenteric artery VSMCs were isolated according to our previously published method with modification (Lu et al., 2001; Zhao et al., 2001). In brief, male Sprague-Dawley rats (120–150g) were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight). Small mesenteric arteries below the second branch off the main mesenteric artery were dissected and kept in ice-cold physiological salt solution (PSS) that contained 137 mM NaCl, 5.6 mM KCl, 0.44 mM NaH_2PO_4, 0.42 mM Na_2HPO_4, 4.17 mM $NaHCO_3$, 1 mM $MgCl_2$, 2.6 mM $CaCl_2$, 10 mM HEPES, and 5 mM glucose (pH adjusted to 7.4 with NaOH). Connective tissues were gently removed under a dissecting microscope with surgical tweezers. Freshly isolated tissues were cut into 5-mm long pieces and then incubated for 40 min at 37°C in Ca^{2+}-free PSS containing 1 mg/ml albumin, 0.5 mg/ml papain, and 1 mg/ml dithiothreitol, and for another 30 min in the nominally Ca^{2+}-free PSS including 1 mg/ml albumin, 0.8 mg/ml collagenase, and 0.8 mg/ml hyaluronidase. Single cells released by gentle triturating through a Pasteur pipette exhibited a long and spindle shape under a microscope. Cells were stored in Ca^{2+}-free PSS at 4°C and were used within the same day of isolation.

Whole-Cell Recording of Membrane Potential and K_{ATP} Channel Currents. The whole-cell patch-clamp technique was used to record K_{ATP} channel currents (Tang and Wang, 2001; Zhao et al., 2001; Wu et al., 2002). In brief, two or three drops of cell suspension were added to the perfusion chamber inside a Petri dish that was mounted on the stage of an inverted phase-contrast microscope (Olympus IX70; Olympus, Tokyo, Japan) for 5 to 10 min before an experiment was started. Pipettes were pulled from soft microhematocrit capillary tubes (Fisher, Nepean, ON, Canada) with tip resistance of 2 to 4 MΩ when filled with the pipette solution. Currents were recorded with an Axopatch 200-B amplifier (Molecular Devices (Sunnyvale, CA), controlled by a Digidata 1200 interface and pCLAMP software (version 7; Molecular Devices). Membrane currents were filtered at 1 kHz with a four-pole Bessel filter, digitized, and stored. At the beginning of each experiment, junction potential between pipette and bath solutions was electronically adjusted to 0. In the current-clamp mode, membrane potential of single VSMC was measured using the nystatin-perforated patch-recording technique while holding the membrane current at 0 pA (Zhao et al., 2001). A stable recording of membrane potential was achieved at least 2 min after nystatin penetrated the cell membrane. The bath solution contained 140 mM NaCl, 5.4 mM KCl, 1.2 mM $MgCl_2$, 10 mM HEPES, 2 mM EGTA, and 10 mM glucose, with pH adjusted to 7.4 with NaOH. The pipette solution comprised 140 mM KCl, 1 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, 5 mM glucose, and 250 μg/ml nystatin. Because nystatin may destabilize the cell, the appearance of nystatin at the tip of the electrode was avoided by dipping the pipette tip into a nystatin-free solution and backfilling the remainder of the pipette with a nystatin-containing solution. In the voltage-clamp mode, K_{ATP} channel currents of single VSMC were recorded using the conventional whole-cell patch-clamp technique. In most experiments, K_{ATP} currents were recorded at a holding potential of −60 mV in symmetrical 140 mM K^+ solutions. The absence of Ca^{2+} and the presence of EGTA in bath and pipette solutions and the recording made at −60 mV would minimize K_{Ca} and K_V currents. The bath solution for recording whole-cell K_{ATP} current contained 140 mM NaCl, 5.4 mM KCl, 1.2 mM $MgCl_2$, 10 mM HEPES, 1 mM EGTA, and 10 mM glucose, with pH adjusted to 7.4 with NaOH. The pipette solution was composed of 140 mM KCl, 1 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, 5 mM glucose, 0.3 mM Na_2ATP, and 0.5 mM MgGDP, with pH adjusted to 7.2 with KOH. Cells were superfused continuously with the bath solution at a rate of approximately 2 ml/min. A complete solution change in the recording chamber was accomplished within 30 s.

Single-Channel Recording of K_{ATP} Currents. Inside-out configuration of the patch-clamp technique was used to record single K_{ATP} channel currents. Pipettes with a tip resistance of 4 to 8 MΩ were used, and the seal resistance was usually greater than 10 GΩ. Membrane patches with no more than three channels were used for experiments. Single-channel currents were filtered at 2 kHz (8-pole Bessel, −3 dB), recorded with a 100-μs sampling interval in a gap-free mode, and performed using an Axopatch 200A amplifier (Molecular Devices). For each concentration of a tested agent, such as H_2S, glibenclamide, pinacidil, or diazoxide, at least 60 s of channel activity was recorded directly on the hard disk of a computer. NP_0 and unitary current amplitude of K_{ATP} channels were determined from all-point histograms using FETCHAN and pSTAT of pCLAMP 6.0 Software (Molecular Devices). NP_0 is the product of N (the number of single channels in one patch) and P_o (the mean channel open probability) and calculated by the equation (Kajioka et al., 1991) $NP_0 = (A_1 + 2A_2 + 3A_3 + \ldots nA_n)/(A_0 + A_1 + A_2 + A_3 + \ldots + A_n)$. A_0, A_1, A_2, A_3, and A_n are areas under each histogram peak when channels are closed, one channel open, and simultaneous openings of 2 to n channels, respectively, assuming that all channel in the patch have the same open probability under given conditions and that they behave independently. A current level greater than 50% of unitary channel current was considered to reflect a channel opening.

Unitary current amplitude was determined from an amplitude histogram of 15 to 20 s of recorded data. The histogram was fitted to a sum of Gaussian distributions. The difference between two adjacent Gaussian peaks was taken as a measure of unitary current amplitude. Because most recordings contained more than a single K_{ATP} channel, no attempts were made to study the distribution of channel dwell times. Holding potential was defined as pipette potential with reference to the ground. Single-channel currents were recorded, whereas holding potentials were varied from −100 to +100 mV in steps of 30 mV. To establish current-voltage curves of single K_{ATP} channels, VSMC was exposed to symmetrical 140 mM K^+ solutions. Bath solution (for intracellular side of membrane) included 120 mM KCl, 20 mM KOH, 1 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, 5 mM glucose, 0.3 mM Na_2ATP, and 0.5 mM MgADP, pH 7.2; whereas pipette solution (for extracellular side of membrane) contained 140 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 10 mM glucose, and 10 mM HEPES, pH 7.4. All electrophysiological experiments were conducted at room temperature (20–22°C).

Chemicals and Data Analysis. H_2S solution was made by bubbling continuously pure H_2S gas (>99.99%) into bath solution or distilled water (50 ml) at 30°C at 100 kPa for 40 min. Final concentration of H_2S in this stock solution is 90 mM (Zhao et al., 2001). H_2S stock solution was prepared freshly on the day of the experiment and then immediately diluted to the desired concentration with bath solution. Effects of H_2S on membrane potentials or K_{ATP} channel currents were recorded continuously before and after perfusing cells with H_2S-containing bath solution. A stable effect of H_2S was usually observed within 1 to 3 min of H_2S application and recorded correspondingly.

Pinacidil, nystatin, GDP, ATP, PPG, β-cyano-L-alanine, aminooxyacetate, ammonium chloride, and sodium pyruvate were purchased from Sigma Chemical (St. Louis, MO); glibenclamide was from Sigma/RBI (Natick, MA); and iberiotoxin was from Alomone Labs (Jerusalem, Israel). Stock solutions of pinacidil and glibenclamide were made in dimethyl sulfoxide and diluted to desired concentrations immediately before use. dimethyl sulfoxide alone was without effect at the concentration used (up to 0.3%). Na_2ATP, GDP, and nystatin were directly dissolved in pipette solution to achieve the desired concentrations on the day of experiments.

All data were expressed as means ± S.E.M. from at least three independent experiments performed in duplicate unless otherwise stated. Statistical analyses were done using paired and unpaired Student's t test, analyses of variance in conjunction with Newman-Keuls test where appropriate. Group differences at level of $p < 0.05$ were considered statistically significant.

Results

K_{ATP} Currents in Rat Mesenteric Artery VSMC. With symmetrical 140 mM K^+ concentration across cell membrane, basal whole-cell K_{ATP} currents were increased by 10 μM pinacidil (from −156 ± 19 to −286 ± 37 pA, $n = 5$, $p < 0.05$) and then attenuated by 10 μM glibenclamide (to −76 ± 15 pA, $n = 5$, $p < 0.01$) (Fig. 1, A and B). Unitary K_{ATP} channel currents (at −100 mV) in inside-out patches were activated by diazoxide and inhibited by glibenclamide (Fig. 1C). To exclude potential K_{Ca} current contamination, 100 nM iberiotoxin, a selective K_{Ca} channel blocker, was used. Increase in K_{ATP} currents in response to switching from 5.4 mM external K^+ to 140 mM K^+ was not sensitive to iberiotoxin. With 5.4 mM $[K^+]_o$, resting membrane potential in nystatin-perforated cells was depolarized from −48 ± 7 mV to −36 ± 4 mV ($n = 5$, $p < 0.01$) by 10 μM glibenclamide.

Effects of Exogenous H_2S on K_{ATP} Currents and Membrane Potentials. Under conditions with symmetrical 140 mM K^+ in Ca^{2+}-free solutions at negative membrane potential, recorded K_{ATP} currents were increased by 300 μM H_2S from −108 ± 17 to −222 ± 33 pA ($n = 5$, $p < 0.01$) and then inhibited by 10 μM glibenclamide to −74 ± 11 pA ($n = 5$, $p < 0.05$) (Fig. 2, A and B). H_2S increased inward K_{ATP} currents in a concentration-dependent fashion with EC_{50}

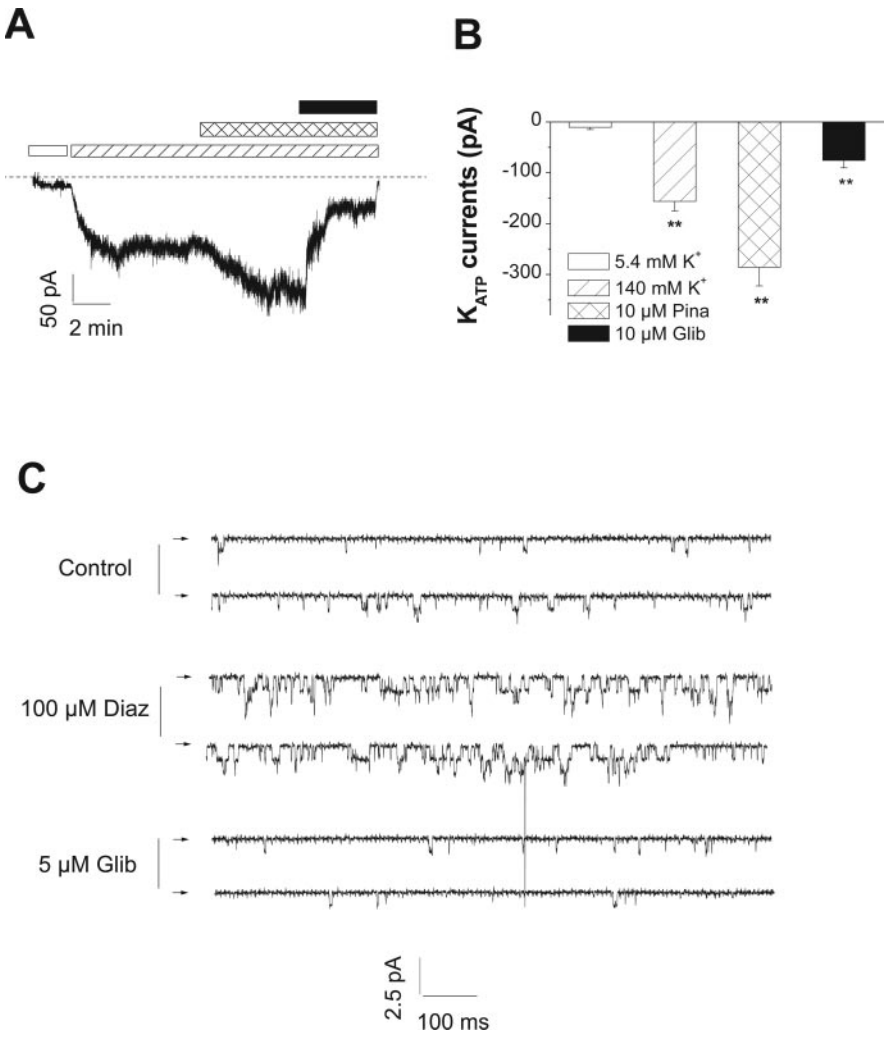

Fig. 1. The pharmacological properties of K_{ATP} current and the resting membrane potential in VSMC. A, the original recording of basal whole-cell K_{ATP} currents activated by 10 μM pinacidil (Pina) and inhibited by 10 μM glibenclamide (Glib) with symmetrical 140 mM K^+. Membrane potential (MP), − 60 mV. The current amplitudes were measured when they became stable at the maximal or minimal levels with different treatments for 0.5 to 1 min. The broken line indicates zero current. B, summary of the changes in whole-cell K_{ATP} currents activated by Pina and inhibited by Glib. MP = −60 mV, $n = 5$ for each group, **, $p < 0.01$ (140 versus 5.4 mM K^+, 10 μM Pina versus 140 mM K^+, 10 μM Glib versus 10 μM Pina). C, the original current traces of single K_{ATP} channels in inside-out patch activated by 100 μM diazoxide (Diaz) and inhibited by 5 μM Glib. The pipette potential was set at −100 mV. The arrows indicate the close state of the channels.

value of 116 ± 8.3 μM (Fig. 2C). In nystatin-perforated cells, H_2S hyperpolarized membrane from −46 ± 4 to −58 ± 3 mV ($n = 8$, $p < 0.01$). H_2S-induced hyperpolarization was reversed to −42 ± 3 mV ($n = 8$, $p < 0.05$) by the removal of H_2S from the bath solution. In the same cell, glibenclamide (10 μM) further depolarized membrane to −23 ± 2.4 mV ($n = 5$, $p < 0.01$). In inside-out membrane patches, K_{ATP} channel activity was hardly detectable with ATP-free bath solution ($n = 8$), but increased significantly by superfusing with 0.3 mM ATP and 0.5 mM GDP ($n = 10$). Exogenous H_2S increased unitary K_{ATP} currents at different concentrations (Fig. 3B). Glibenclamide (5 μM) abolished the activation of K_{ATP} channels by diazoxide and H_2S (Fig. 3A). H_2S significantly increased the open probability of single K_{ATP} channels. H_2S at 200 μM increased NP_0 of K_{ATP} channels from 0.53 to 2.67 (Fig. 3A) and from 0.31 to 1.55 (Fig. 3B). The I-V relationship of single K_{ATP} channels showed that unitary K_{ATP} channel conductance was 12.9 ± 0.6 pS ($n = 6$) in the absence of H_2S, which is similar to vascular K_{NDP} channel conductance (Zhang and Bolton, 1995; Quayle et al., 1997). In the presence of H_2S, K_{ATP} channel conductance was 14.8 ± 1.0 pS ($n = 5$) (Fig. 4, A and B). H_2S seemed not to affect channel conductance ($p > 0.05$).

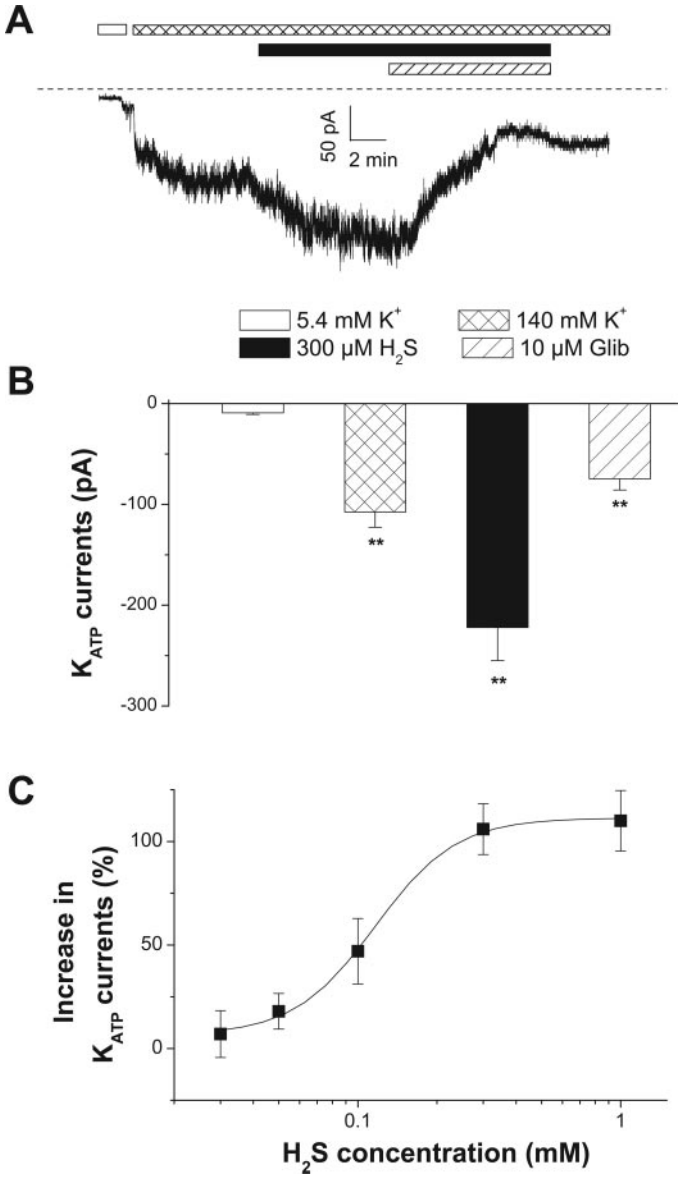

Fig. 2. The stimulatory effects of H_2S on K_{ATP} currents recorded with 140 mM symmetrical K^+. A, the original current trace of K_{ATP} currents activated by 300 μM H_2S and inhibited by 10 μM glibenclamide (Glib). MP, − 60 mV. The current amplitudes were measured when they became stable at the maximal or minimal levels with different treatments for 0.5 to 1 min. The broken line indicates zero current. B, summary of the change of K_{ATP} currents activated by H_2S and inhibited by 10 μM Glib. MP = −60 mV, $n = 5$ for each group. **, $p < 0.01$ (140 versus 5.4 mM K^+, 300 μM H_2S versus 140 mM K^+, 10 μM Glib versus 300 μM H_2S). C, the concentration-effect curve of the stimulatory effects of H_2S on K_{ATP} currents; MP = −60 mV, $n = 5$–8.

Effects of Endogenous H_2S on K_{ATP} Currents in VSMC. To determine the effects of endogenous H_2S on K_{ATP} currents, various inhibitors of H_2S-generating enzymes (CSE or CBS) were used. Single cells dialyzed with 3 mM PPG exhibited a time-dependent inhibition of whole-cell K_{ATP} currents (+40 mV) by 31.3, 49.8, 59.6, and 64.8% at 5, 10, 15, and 20 min, respectively (Fig. 5A). β-Cyano-L-alanine (βCAN), another inhibitor of CSE, similarly inhibited K_{ATP} currents by 12.7 ± 1.1%, 30.5 ± 0.9%, and 55.8 ± 1.3% at 6, 12, and 18 min, respectively, after dialyzing the cells ($n = 6$) (Fig. 5B). To examine possible involvement of CBS in vascular tissue (Zhao et al., 2001), the effect of aminooxy-acetate, a CBS inhibitor, was examined. Intracellularly applied aminooxy-acetate for 10 min had no effect on K_{ATP} currents ($n = 5$, $p > 0.05$) (Fig. 5C). Two coproducts of H_2S generation in L-cysteine metabolism, ammonium chloride and sodium pyruvate, also had no effects on K_{ATP} currents ($n = 6$, $p > 0.05$) when dialyzed with the pipette solution for at least 10 min (data not shown). To expel the possibility that the decrease of whole-cell K_{ATP} currents by PPG was caused by nonspecific effect of PPG unrelated to CSE inhibition, activity of single K_{ATP} channels was directly measured in the presence of PPG. In inside-out patches, 3 mM PPG did not change channel open probability ($n = 4$).

Effects of H_2S on K_{ATP} Currents and Membrane Potentials Are Independent of cGMP Signaling Pathway. The cGMP signaling pathway plays an important role in mediating NO- and CO-induced vasorelaxation (Ignarro, 1989; Furchgott and Jothianandan, 1991; Wang et al., 1997; Wang, 1998). To determine whether H_2S-induced increase in K_{ATP} currents was mediated by the cGMP pathway, we examined the effect of a membrane-permeable analog of cGMP, 8-Br-cGMP, on K_{ATP} currents. Either basal K_{ATP} currents or H_2S-increased K_{ATP} currents were not affected by 0.5 mM 8-Br-cGMP. With symmetrical 140 mM K^+, H_2S-stimulated K_{ATP} currents were not affected by 8-Br-cGMP (from −243 ± 32 to −229 ± 26 pA at −60 mV, $n = 6$, $p > 0.05$). Even after the application of 8-Br-cGMP was prolonged to 30 min or the accumulated concentration of 8-Br-cGMP was increased to 2 mM, no significant increase in K_{ATP} currents appeared. Furthermore, 8-Br-cGMP did not change H_2S-induced membrane hyperpolarization (−52 ± 4 versus −50 ± 3 mV) ($n = 4$, $p > 0.05$).

Discussion

Novel findings of this study are summarized as follows: 1) exogenous H_2S increased whole-cell K_{ATP} currents with EC_{50} values of 116 ± 8.3 μM and hyperpolarized membrane potentials of rat mesenteric artery smooth muscle cells. H_2S also increased single-channel activity of K_{ATP} channels by

increasing open probability of single K_{ATP} channels without altering single-channel conductance; 2) by reducing endogenous H_2S production, CSE inhibitors suppressed K_{ATP} currents; and 3) direct effects of H_2S on K_{ATP} channels and membrane potentials were not mediated by cGMP signal pathway.

Contribution of K_{ATP} Channels to Basal Activity of Mesenteric Artery VSMC. K_{ATP} channels in certain types of VSMC are active at the resting state with physiological concentration of intracellular nucleotides, thus playing an important role in maintaining resting membrane potentials (Clapp and Gurney, 1992; Miyoshi et al., 1992; Kubo et al., 1994). In our study, glibenclamide inhibited K_{ATP} currents and depolarized the resting membrane of rat mesenteric artery VSMC by approximately 12 mV. This result confirmed other observations that glibenclamide was able to cause significant membrane depolarization (5~9 mV) at the resting state of different vascular tissues and species (Clapp and Gurney, 1992; Mishra and Aaronson, 1999), and K_{ATP} channel was a contributor to background K^+ conductance in resistance vascular beds (Nelson et al., 1990; Quayle et al., 1997). Membrane potential of VSMC is an important regulator of vascular tone by controlling voltage-dependent Ca^{2+} entry. Thus, K_{ATP} channels might participate in modulating mesenteric artery contractility and contributed to basal activity of VSMC from resistance vessels.

Characteristics of K_{ATP} Channels in Rat Mesenteric Artery VSMC. Although inward whole-cell K_{ATP} currents were stimulated by KCOs, including pinacidil and diazoxide, and were inhibited by glibenclamide in rat mesenteric artery VSMC, single-channel data reflect more convincingly electrophysiological features of K_{ATP} channels. Our single K_{ATP} channel data in rat mesenteric artery VSMC demonstrated that 1) K_{ATP} channels were not opened in ATP-free bath solution in inside-out patches. GDP addition was required to evoke single-channel activity. Thus, our bath recording solu-

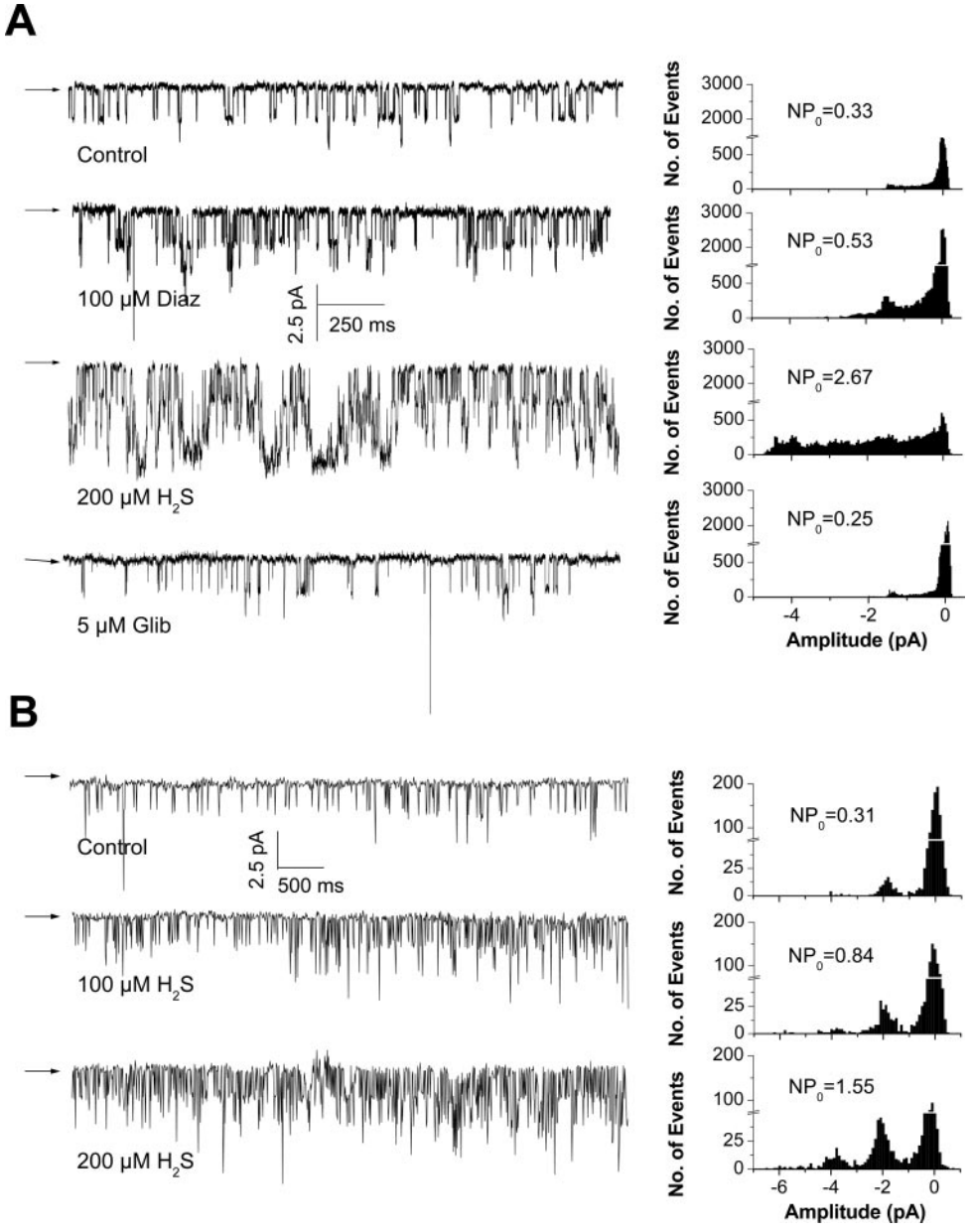

Fig. 3. H_2S stimulated unitary K_{ATP} channel currents in VSMC. A, the original recording traces of unitary K_{ATP} current activated by diazoxide (Diaz) and H_2S and inhibited by glibenclamide (Glib) in an inside-out patch with the pipette potential of −100 mV. The arrows indicate the close state of the channels. The corresponding all-points amplitude histograms are plotted on the right with the values of NP_0. B, the original recording traces of unitary K_{ATP} currents activated by H_2S in two different concentrations under the same recording condition as in A. The arrows indicate the close state of the channels. The corresponding all-points amplitude histograms are plotted on the right with the values of NP_0.

tion included 0.3 mM ATP plus 0.5 mM GDP; 2) single-channel conductance is 13 pS in symmetrical 140 mM K^+ recording solution; 3) H_2S stimulated K_{ATP} channel activity through increasing open probability, not single-channel conductance; 4) K_{ATP} channel activation is independent of membrane potential and with a linear I-V relationship; and 5) KCOs opened and glibenclamide blocked basal K_{ATP} channels and H_2S-increased K_{ATP} channels. All of these results were well consistent with the observation that small-conductance K_{NDP} channels (20 pS at 60:130 K^+ gradient) open in rat mesenteric artery VSMC in response to GDP, KCOs, and metabolic inhibitors (Zhang and Bolton, 1995).

In terms of molecular compositions, K_{ATP} channels are heterogenous in rat mesenteric artery, in which four channel subunits (Kir6.1, Kir6.2, SUR1, and SUR2B) have been cloned and identified at mRNA levels (Cao et al., 2002). This diversity of molecular entities of K_{ATP} channels in native VSMC is exemplified in its single-channel conductance, ranging 15 to 50 pS (Kajioka et al., 1991; Zhang and Bolton, 1995; Davie et al., 1998; Wang et al., 2003) to 111 to 135 pS (Standen et al., 1989; Liu and Zhao, 2000). A small unitary conductance (13 pS) of K_{ATP} channels was found in rat mesenteric artery smooth muscle cells in our study. In the same rat mesenteric artery and under almost identical conditions (60:120 mM K^+ gradient and negative holding potentials), two different K_{ATP} channels were found with unitary conductance of 135 pS (Standen et al., 1989) and 20 pS (Zhang and Bolton, 1995). Still, for the same rat portal vein VSMC, two types of K_{ATP} channels were recorded with different unitary

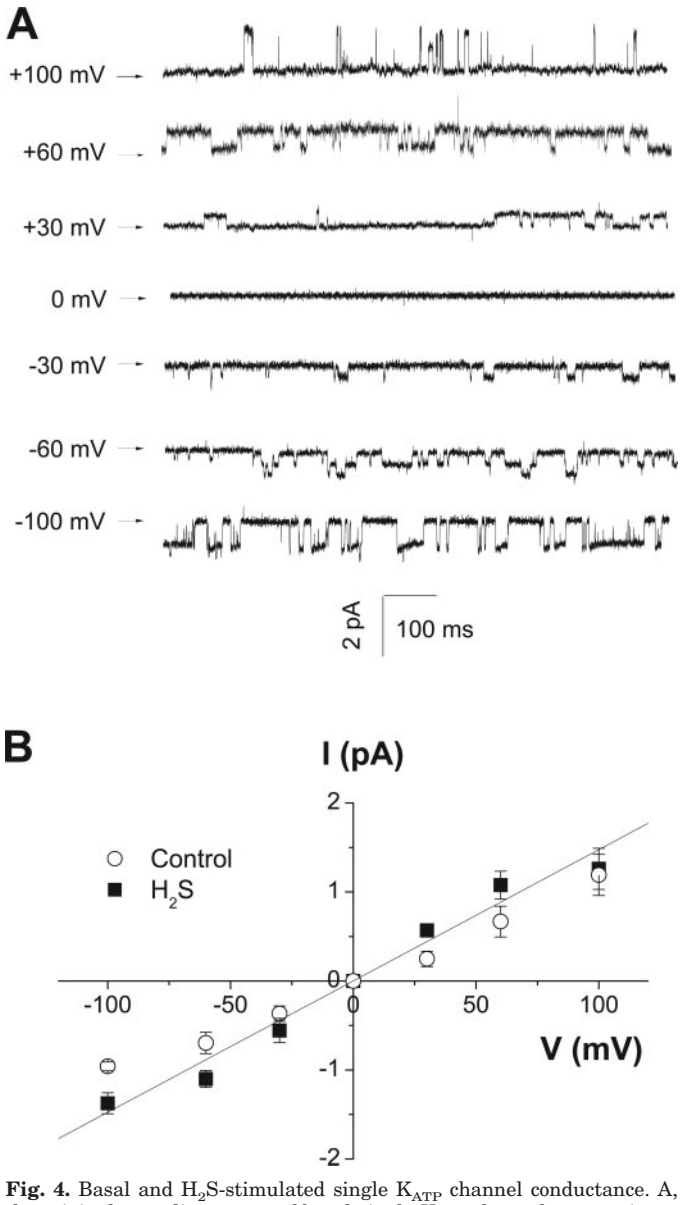

Fig. 4. Basal and H_2S-stimulated single K_{ATP} channel conductance. A, the original recording traces of basal single K_{ATP} channel current in an inside-out patch at different pipette potentials (−100 to +100 mV). The arrows indicate the close state of the channels. B, the I-V relationships of single K_{ATP} channels with or without H_2S stimulation.

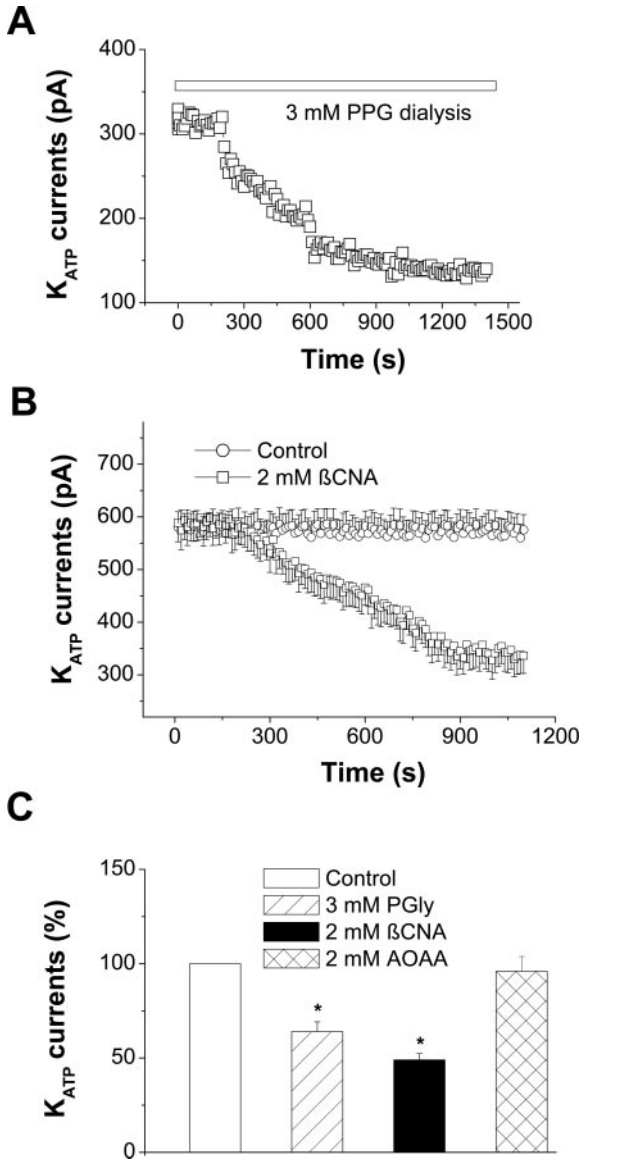

Fig. 5. The inhibitory effects of K_{ATP} channels by endogenous H_2S production inhibitors with extracellular 5.4 mM K^+ in VSMC. A, representative time course of the inhibitory effect on K_{ATP} currents of 3 mM PPG, an inhibitor of CSE, used to dialyze the cells. Testing potential (TP), +40 mV; holding potential (HP), −60 mV. B, mean time course of the inhibitory effect on K_{ATP} currents of 2 mM βCAN, another inhibitor of CSE, used to dialyze the cells. TP, +40 mV; HP, −60 mV, $n = 6$. C, summary of the inhibitory effects on K_{ATP} currents of different inhibitors of H_2S-generating enzymes (CSE and CBS) in the pipette solution 10 min after the dialysis of cells. TP, +40 mV; HP, −60 mV; *, $p < 0.05$ (3 mM PPG versus control; 2 mM βCAN versus control); $n = 5$–12.

conductance (50 and 22 pS) and various sensitivities to ATP inhibition and NDP activation (Zhang and Bolton, 1996). K_{ATP} channel conductance in our study (13 pS) and another study (20 pS) (Zhang and Bolton, 1995) belong to small-conductance range of K_{ATP} channels in rat mesenteric artery VSMC. A slight difference in single-channel conductance between these two studies (approximately 7 pS) can be explained by different experimental conditions in these studies. First, single VSMC in our study was dispersed from Sprague-Dawley rat mesenteric arteries in Ca^{2+}-free cell isolation solution by the digestion of collagenase and papain, whereas Zhang and Bolton (1995) used mice mesenteric arteries to isolate VSMC in low Ca^{2+} solution (10 μM) with the digestion of collagenase and pronase. Second, symmetrical 140 mM K^+ was used in our study, whereas quasiphysiological K^+ gradient ($[K^+]_{o/i}$ = 60/130) was used in the experiment of Zhang and Bolton (1995). These distinct conditions in individual laboratories may explain the differences in single-channel conductance. Furthermore, unitary channel conductance was affected by analyzing methods such as direct measurement from the isolated patch recordings and indirect calculation from amplitude of current noise generated by KCOs in the whole-cell recordings (Criddle et al., 1994).

Effects of H_2S on K_{ATP} Currents in VSMC. In mammalian tissues, CSE and/or CBS cleave L-cysteine to produce H_2S, ammonium, and pyruvate. CBS is the predominant H_2S-generating enzyme in brain and nervous system (Kimura, 2000), whereas CSE is mainly expressed in vascular smooth muscle (Hosoki et al., 1997; Zhao et al., 2001; Wang, 2002). Our results for the first time demonstrated that when CSE was inhibited by its specific inhibitors like PPG and βCAN, whole-cell K_{ATP} currents were reduced in VSMC. However, PPG did not affect unitary K_{ATP} currents in inside-out patches. These results indicate that PPG inhibited whole-cell K_{ATP} currents via inhibiting endogenous H_2S production because CSE only exists inside cytosol of intact cells, not in excised patches. The notion that inhibition of K_{ATP} currents by PPG and βCAN resulted from reduced generation of endogenous H_2S caused by CSE inhibition was also supported from our previous observation that the generation of endogenous H_2S from vascular tissues was completely abolished by PPG (Zhao et al., 2001).

H_2S at low concentration exerts a range of biological effects as a vasodilator (Wang, 2002) and neurotransmitter (Kimura, 2000), whereas at a high concentration or administered in the short term, H_2S becomes toxic via blocking mitochondrial oxidative phosphorylation (Reiffenstein et al., 1992; Dorman et al., 2002). A delicate mechanism in vivo exists to maintain H_2S levels within physiological range, because rapid oxidation of H_2S in mitochondria (Wang, 2002) may prevent the intoxication of cells from accumulation of endogenously generated H_2S under physiological conditions. H_2S relaxes rat aortic tissues with IC_{50} values of 124.7 $\pm$ 14.4 μM. In single VSMCs from rat mesenteric artery, H_2S stimulated K_{ATP} channel activity with EC_{50} values of 116 $\pm$ 8.3 μM. Endogenous levels of H_2S are 50$\sim$160 μM in rat brain (Hosoki et al., 1997), $\sim$46 μM in Sprague-Dawley rat plasma (Zhao et al., 2001), 10 to 100 μM in human blood (Richardson et al., 2000), and 300 μM in rat pulmonary artery VSMC (Zhang et al., 2003). Thus, the H_2S concentration (100$\sim$300 μM) used in the present study is within the spectrum of physiological concentration of H_2S. On the other hand, H_2S at a physiological concentration (50 μM) was reported to inhibit cytochrome *c* oxidase, an enzyme critical for oxidative phosphorylation of mitochondrial respiration, and led to the depletion of $[ATP]_i$ (Evans, 1967; Guidotti, 1996; Dorman et al., 2002). It is troublesome that the activation of K_{ATP} channels by H_2S in this study was caused by the indirect depletion of $[ATP]_i$. In our experiments, $[ATP]_i$ was clamped to 0.3 mM, and there were 5 or 10 mM glucose in pipette and bath solutions, respectively. These manipulations were sufficient to avoid the possible decrease of $[ATP]_i$ levels. Thus, activation of K_{ATP} channels by H_2S and subsequent hyperpolarization of VSMC are unlikely to result from the reduction of $[ATP]_i$.

H_2S Effects on K_{ATP} Currents and Membrane Potentials Are Independent of cGMP-Mediated Signaling Pathway. The relaxation of vascular smooth muscle is governed by multiple mechanisms. Different vasodilators have diverse signal transduction pathways. For example, the cGMP-protein kinase G pathway is involved in NO- and CO-induced vasorelaxations (Furchgott and Jothianandan, 1991; Wang et al., 1997; Wang, 1998). Our previous studies showed that H_2S-induced relaxation of rat aortic tissues was not mediated by cGMP pathway (Zhao et al., 2001, 2003; Zhao and Wang, 2002), but endogenous H_2S production was up-regulated by NO in a cGMP-dependent fashion (Zhao et al., 2003). Whether H_2S-induced K_{ATP} channel activation in rat mesenteric artery VSMC is mediated by cGMP signal pathway has not been defined. In the present study, extracellularly applied 8-Br-cGMP did not affect either basal K_{ATP} currents or H_2S-stimulated K_{ATP} currents when membrane currents were recorded at -60 mV under symmetrical 140 mM K^+ condition. This result was consistent with other observations that NO donor SNP and protein kinase G inhibitor KT5823 had no effect on K_{ATP} currents with the same recording condition as ours (Quayle et al., 1994; Wellman et al., 1998). In addition, low dose ($<$100 μM) of 8-Br-cGMP and short exposure ($<$5 min) were reported not to cause hyperpolarization of VSMC from rabbit mesenteric arteries (Murphy and Brayden, 1995). Increase in K_{ATP} currents by 8-Br-cGMP was found in cell-attached single-channel recording in cultured VSMC from rat thoracic aorta (Kubo et al., 1994) but not in freshly isolated VSMC from resistance mesenteric artery in our study. In our experiments, we used a high dose (0.5$\sim$2 mM) of 8-Br-cGMP to treat cells for more than 15 min. This manipulation should rule out the possibility of insufficient increases in intracellular concentration of 8-Br-cGMP. Thus, modulation of activity of K_{ATP} channels in VSMC by H_2S is probably independent of cGMP-mediated pathway.

References

Cao K, Tang G, Hu D, and Wang R (2002) Molecular basis of ATP-sensitive K^+ channels in rat vascular smooth muscles. *Biochem Biophys Res Commun* **296:**463–469.

Cheng Y, Ndisang JF, Tang G, Cao K, and Wang R (2004) Hydrogen sulfide induced relaxation of resistance mesenteric artery beds of rats. *Am J Physiol* **287:**H2316–H2323.

Clapp LH and Gurney AM (1992) ATP-sensitive K^+ channels regulate resting potential of pulmonary arterial smooth muscle cells. *Am J Physiol* **262:**H916–H920.

Criddle DN, Greenwood IA, and Weston AH (1994) Levcromakalim-induced modulation of membrane potassium currents, intracellular calcium and mechanical activity in rat mesenteric artery. *Naunyn-Schmiedeberg's Arch Pharmacol* **349:**422–430.

Davie CS, Kubo M, and Standen NB (1998) Potassium channel activation and relaxation by nicorandil in rat small mesenteric arteries. *Br J Pharmacol* **125:**1715–1725.

Dorman DC, Moulin FJ, McManus BE, Mahle KC, James RA, and Struve MF (2002)

Cytochrome oxidase inhibition induced by acute hydrogen sulfide inhalation: correction with tissue sulfide concentrations in the rat brain, liver, lung and nasal epithelium, *Toxicol Sci* **65:**18–25.
Evans CL (1967) The toxicity of hydrogen sulphide and other sulphides. *Q J Exp Physiol Cogn Med Sci* **52:**231–248.
Furchgott RF and Jothianandan D (1991) Endothelium-dependent and -independent vasodilation involving cyclic GMP: relaxation induced by nitric oxide, carbon monoxide and light. *Blood Vessels* **28:**52–61.
Guidotti TL (1996) Hydrogen sulfide. *Occup Med* **46:**367–371.
Hosoki R, Matsuki N, and Kimura H (1997) The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochem Biophys Res Commun* **237:**527–531.
Ignarro LJ (1989) Biological actions and properties of endothelium-derived nitric oxide formed and released from artery and vein. *Circ Res* **65:**1–21.
Kajioka S, Kitamura K, and Kuriyama H (1991) Guanosine diphosphate activates an adenosine 5′-triphosphate-sensitive K^+ channel in the rabbit portal vein. *J Physiol* **444:**397–418.
Kimura H (2000) Hydrogen sulfide induces cyclic AMP and modulates the NMDA receptor. *Biochem Biophy Res Commun* **267:**129–133.
Kubo M, Nakaya Y, Matsuoka S, Saito K, and Kuroda Y (1994) Atrial natriuretic factor and isosorbide dinitrate modulate the gating of ATP-sensitive K^+ channels in cultured vascular smooth muscle cells. *Circ Res* **74:**471–476.
Liu J and Zhao K (2000) The ATP-sensitive K^+ channel and membrane potential in the pathogenesis of vascular hyporeactivity in severe hemorrhagic shock. *Chin J Traumatol* **3:**39–44.
Lu Y, Zhang J, Tang G, and Wang R (2001) Modulation of voltage-dependent K^+ channel currents in vascular smooth muscle cells from rat mesenteric arteries. *J Membr Biol* **180:**163–175.
Mishra S and Aaronson PI (1999) A role for a glibenclamide-sensitive, relatively ATP-insensitive K^+ current in regulating membrane potential and current in rat aorta. *Cardiovasc Res* **44:**429–435.
Miyoshi Y, Nakaya Y, Wakatsuki T, Nakaya S, Fujino K, Saito K, and Inoue I (1992) Endothelin blocks ATP-sensitive K^+ channels and depolarizes smooth muscle cells of porcine coronary artery. *Circ Res* **70:**612–616.
Murphy M and Brayden JE (1995) Nitric oxide hyperpolarizes rabbit mesenteric arteries via ATP-sensitive potassium channels. *J Physiol* **186:**47–58.
Nelson MT, Huang Y, Brayden JE, Hescheler JK, and Standen NB (1990) Arterial dilations in response to calcitonin gene-related peptide involve activation of K^+ channels. *Nature (Lond)* **344:**770–773.
Nelson MT and Quayle JM (1995) Physiological roles and properties of potassium channels in arterial smooth muscle. *Am J Physiol* **268:**C799–C822.
Quayle JM, Bonev AD, Brayden JE, and Nelson MT (1994) Calcitonin gene-related peptide activated ATP-sensitive K^+ currents in rabbit arterial smooth muscle via protein kinase A. *J Physiol* **475:**9–13.
Quayle JM, Nelson MT, and Standen NB (1997) ATP-sensitive and inwardly rectifying potassium channels in smooth muscle. *Physiol Rev* **77:**1165–1232.
Reiffenstein RJ, Hulbert WC, and Roth SH (1992) Toxicology of hydrogen sulfide. *Annu Rev Pharmacol Toxicol* **32:**109–134.
Richardson CJ, Magee EA, and Cummings JH (2000) A new method for the determination of sulphide in gastrointestinal contents and whole blood by microdistillation and ion chromatography. *Clin Chim Acta* **293:**115–125.
Shimokawa H, Yasutake H, Fujii K, Owada MK, Nakaike R, Fukumoto Y, Takayanagi T, Nagao T, Egashira K, Fujishima M, et al. (1996) The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium dependent relaxations in rat mesenteric circulation. *J Cardiovasc Pharmacol* **28:**703–711.
Standen NB, Quayle JM, Davies NW, Brayden JE, Huang Y, and Nelson MT (1989) Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science (Wash DC)* **245:**177–180.
Takamura Y, Shimokawa H, Zhao H, Hirohito I, Egashira K, and Takeshita A (1999) Important role of endothelium-derived hyperpolarizing factor in shear stress-induced endothelium-dependent relaxation in the rat mesenteric artery. *J Cardiovasc Pharmacol* **34:**381–387.
Tang G and Wang R (2001) Differential expression of K_V and K_{Ca} channels in vascular smooth muscle cells during 1-day culture. *Pflueg Arch Eur J Physiol* **442:**124–135.
Wang R (1998) Resurgence of carbon monoxide: an endogenous gaseous vasorelaxing factor. *Can J Physiol Pharmacol* **76:**1–15.
Wang R (2002) Two's company, three's a crowd: can H_2S be the third endogenous gaseous transmitter? *FASEB J* **16:**1792–1798.
Wang R, Wang Z, and Wu L (1997) Carbon monoxide-induced vasorelaxation and the underlying mechanisms. *Br J Pharmacol* **121:**927–934.
Wang X, Wu J, Li L, Chen F, Wang R, and Jiang C (2003) Hypercapnic acidosis activates K_{ATP} channels in vascular smooth muscles. *Circ Res* **92:**1225–1232.
Wellman GC, Quayle JM, and Standen NB (1998) ATP-sensitive K^+ channel activation by calcitonin gene-related peptide and protein kinase A in pig coronary arterial smooth muscle. *J Physiol* **507:**117–129.
Wu L, Cao K, Lu Y, and Wang R (2002) Different mechanisms underlying the stimulation of KCa channels by nitric oxide and carbon monoxide. *J Clin Investig* **110:**691–700.
Zhang C, Du J, Bu D, Yan H, Tang X, Si Q, and Tang C (2003) The regulatory effect of endogenous hydrogen sulfide on hypoxic pulmonary hypertension. *Beijing Da Xue Xue Bao* **35:**488–493.
Zhang H and Bolton TB (1995) Activation by intracellular GDP, metabolic inhibition and pinacidil of a glibenclamide-sensitive K^+ channel in smooth muscle cells of rat mesenteric artery. *Br J Pharmacol* **114:**662–672.
Zhang HL and Bolton TB (1996) Two types of ATP-sensitive potassium channels in rat portal vein smooth muscle cells. *Br J Pharmacol* **118:**105–114.
Zhao W, Ndisang JF, and Wang R (2003) Modulation of endogenous production of H_2S in rat tissues. *Can J Physiol Pharmacol* **81:**848–853.
Zhao W and Wang R (2002) H_2S-induced vasorelaxation and underlying cellular and molecular mechanisms, *Am J Physiol* **283:**H474–H480.
Zhao W, Zhang J, Lu Y, and Wang R (2001) The vasorelaxant effects of H_2S as a novel endogenous gaseous K_{ATP} channel opener. *EMBO (Eur Mol Biol Organ) J* **20:**6008–6016.

Address correspondence to: Dr. Rui Wang, FAHA, Department of Physiology, College of Medicine, University of Saskatchewan, 107 Wiggins Road, Saskatoon, SK S7N 5E5, Canada. E-mail: rwang@lakeheadu.ca