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Mechanisms of Relaxation of Coronary Artery by Hypoxia

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This study was designed to clarify the dependency of hypoxic coronary vasodilatation (HCD) on the endothelium and the role of the K^+ channels on HCD in the rabbit coronary artery. HCD was investigated in an isolated left circumflex coronary artery precontracted with prostaglandin $F_2\alpha$. Vascular rings were suspended for isometric tension recording in an organ chamber filled with Krebs-Henseleit (KH) solution. Hypoxia was induced by gassing the chamber with 95% N_2 + 5% CO_2 and was maintained for 15~25 min. Hypoxia elicited a vasodilatation in the precontracted coronary artery with and without endothelium. There was no difference between the amplitude of the HCD induced by two consecutive hypoxic challenges and the effects of 20% O_2 + 5% CO_2 + 75% N_2 and 95% O_2 + 5% CO_2 control K-H solution on subsequent responses to hypoxia. Inhibition of the cyclooxygenase pathway by treatment with indomethacin had no effect on HCD. Blockades of the tetraethylammonium chloride-sensitive K^+ channel abolished HCD. Apamin, a blocker of the small conductance Ca^{2+}-activated K^+ (K_{Ca}) channel, and iberiotoxin, a blocker of the large conductance K_{Ca} channel had no effect on HCD, respectively. Glibenclamide, a blocker of the ATP-sensitive K^+ (K^+_{ATP}) channel, reduced HCD. Cromakalim, an opener of the K^+_{ATP} channel, relaxed the coronary artery precontracted with prostaglandin $F_2\alpha$. The degree of relaxation by cromakalim was similar to that by hypoxia while glibenclamide reduced both hypoxia- and cromakalim-induced vasodilatations. In conclusion, these results suggest that HCD is independent on endothelium and HCD is considered to be induced by activation of K^+_{ATP} channel.

Key Words: Hypoxic coronary vasodilatation, glibenclamide, K^+ channel, K^+_{ATP} channel

Reducing arterial P_{O2} to less than 40 mmHg produces vasodilatation in most beds, including the coronary artery. Hypoxic coronary vasodilatation (HCD) demonstrates a physiological regulatory process that maintains blood flow in response to ischemia or increased metabolic need (Wadsworth, 1994). Although HCD has been recognized by many researchers (Busse *et al.* 1984; Toda, 1984; Kwan *et al.* 1988), the effects of hypoxia on contractility are conflicting. In isolated coronary artery rings, hypoxia has been shown to produce a constrictor response in many studies of tissue from dog, calf, pig, sheep and monkey (Rubanyi and Vanhoutte, 1985; Kwan *et al.* 1989a & b; Graser and Vanhoutte, 1991; Muramatsu *et al.* 1992).

The proposed mechanisms underlying this dilatation of coronary arteries have been controversial. There are several routes by which a reduction in oxygen tension could cause coronary arteries to dilate. (i) The oxygen tension could act directly on smooth muscle cells of the coronary arteries, leading to relaxation (Daut *et al.* 1990). (ii) Reduced intra-

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vascular O_2 tension could cause the release of vasodilator substances such as nitric oxide or prostaglandins from endothelial cells lining the arteries (Graser and Vanhoutte, 1991). (iii) Cardiac myocytes could release vasodilator metabolites, such as adenosine, which in turn act on coronary smooth muscle or endothelium, leading to dilatation (Berne, 1980). Clearly, these routes are not mutually exclusive, and each could exert part or all of its dilator effect through membrane hyperpolarization caused by activation of K^+ channels.

Several reports suggest that hypoxic coronary arteries make use of the efflux of potassium through ATP-dependent potassium channels (K^+_{ATP}) to induce hyperpolarization, coronary smooth muscle cell relaxation (Standen *et al.* 1989; Daut *et al.* 1990; von Beckerath *et al.* 1991). However, no study in functionally intact coronary artery tissue, separated from myocardium, has up to now shown a role for these K^+ channels in response to hypoxia or in any other coronary contractile event.

The present study was designed to clarify the dependency of HCD on the endothelium and the role of the K^+ channel on HCD in the rabbit coronary artery.

MATERIALS AND METHODS

Preparation of artery rings and conditions of hypoxia

The experiments were performed on rings (about 3 mm in width) of the left circumflex coronary artery taken from rabbits of either sex. The animals were anesthetized with pentobarbital sodium (30mg/kg iv) and exsanguinated. After excision, the vessels were placed in Krebs-Henseleit (KH) solution of the following composition (in mM): NaCl 119, KCl 4.6, $CaCl_2$, 2.5, $NaHCO_3$ 25, $MgCl_2$ 1.2, KH_2PO_4 1.2, glucose 11. Endothelium was removed mechanically by rotating the ring gently on the tip of a forceps. The rings were then mounted in water-jacketed baths containing KH solution at 37°C, gassed with 95% O_2 + 5% CO_2 (hyperoxic gas) or 20% O_2 + 5% CO_2 + 75% N_2 (normoxic gas) and connected to force transducers to measure isometric tension. A resting tension of 0.2g was maintained throughout the experiments. Tissues were allowed to equilibrate for 90min before each experiment. The function of the endothelium was checked at the beginning of each experiment with acetylcholine ($5X10^{-7}$M) in rings contracted with prostaglandin $F_2\alpha$ ($PGF_2\alpha$; 1.5×10^{-6}M). Hypoxia of $PGF_2\alpha$-precontracted rings was induced by bubbling with 95% N_2 + 5% CO_2 gas (hypoxic gas) instead of hyperoxic or normoxic gas for 15~25min. To reduce the margin of error on the onset of the hypoxic effect, the exposure times to hypoxia between the control and drug-treated groups using the same preparations were kept constant. The P_{O2} of the KH solution in the tissue baths was determined with a blood gas analyzer (Radiometer, Westlake, Ohio, U.S.A) during each cycle of each experiment. The mean dissolved partial pressures of oxygen in the bath fluid during hyperoxia and normoxia cycles were 543 ± 8 mmHg and 134 ± 12 mmHg, respectively and during hypoxia, 30.1 ± 0.3 mmHg.

Influence of drugs on hypoxic responsiveness

Resting state (absence of vasoconstrictor agonist) rings were exposed to hypoxia for 15-25 min. After 45 min of recovery under hyperoxic gas the rings were contracted with $PGF_2\alpha$ (1.5×10^{-6}M) and exposed again to hypoxia. After an additional period of 15 min of hyperoxic gas, the following inhibitors and/or blockers were administered for at least 30 min: indomethacin [an inhibitor of cyclooxygenase pathways (Miller and Vanhoutte, 1985)], tetraethylammonium chloride [TEA; a blocker of non-specific K^+ channels (Post *et al.* 1992)], apamin [a blocker of small conductance Ca^{2+}-activated K^+ (K_{Ca}) channels (Wadsworth *et al.* 1996)], iberiotoxin [a blocker of large conductance K_{Ca} channels (Giangiacomo *et al.* 1995)] and glibenclamide [a blocker of K^+_{ATP} channels (Standen, 1992)]. In the presence of these drugs, the effects of hypoxia were tested under precontracted arteries.

Data analysis

Results were expressed as mean±SE. The amplitude of HCD was evaluated by maximum relaxation during hypoxia. The number of preparations taken from separate animals was indicated by n. The

tension development by hypoxia was expressed as percent peak amplitude of $PGF_2\alpha$ (1.5×10^{-6}M)-induced contraction. Significance tests were performed by Student's paired or unpaired t test. P values of less than 0.05 were considered significant.

RESULTS

Response to hypoxia

To record precisely the contractile effects of hypoxia over time on individual coronary artery rings, they were first contracted submaximally with a potent agonist in coronary arteries, $PGF_2\alpha$. After individual responses to 1.5×10^{-6}M had reached plateau values under conditions of hyperoxic gas, the contracted vessel segments were exposed to

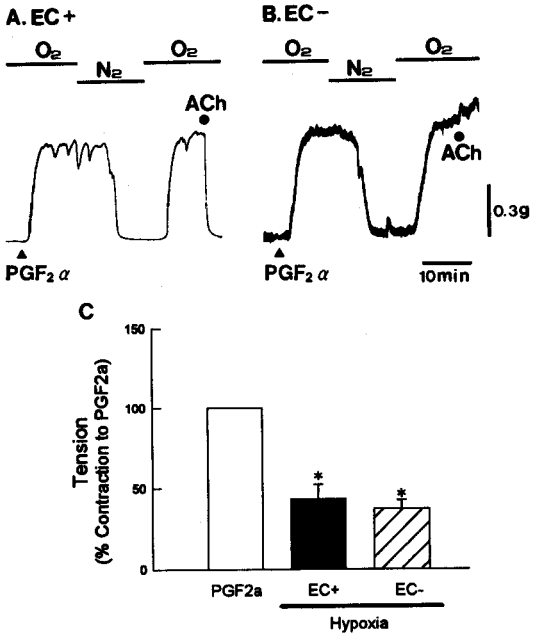

*Fig. 1. Effect of hypoxia on the contractile responses in rabbit coronary artery. A, B : shows typical response to hypoxia in rings of coronary artery with (A) and without (B) endothelium. C : shows mean response of coronary artery with (EC+; n=54) and without (EC-; n=11) endothelium under the same conditions. The preparations were contracted with prostaglandins $F_2\alpha$ ($PGF_2\alpha$, 1.5×10^{-6}M). Hypoxia was induced by switching from 95% O_2 + 5% CO_2 (O_2) to a 95% N_2 + 5% CO_2 gas mixture (N_2). Data are expressed as mean±SE. *: significant difference between $PGF_2\alpha$-induced contractility and hypoxia-induced contractility ($P<0.05$). EC+: ring with endothelium, EC-: ring without endothelium, ACh: acetylcholine.*

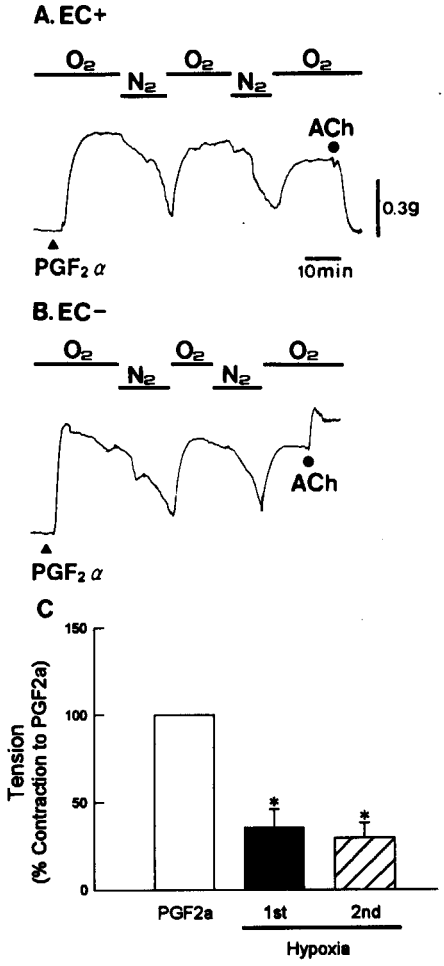

*Fig. 2. Reproducibility of hypoxic coronary vasodilation following two consecutive hypoxic challenges to coronary artery. A, B: shows typical response to two consecutive hypoxic challenges in precontracted (prostaglandins $F_2\alpha$: $PGF_2\alpha$, 1.5×10^{-6}M) coronary artery with (A) and without (B) endothelium. C: shows mean response of coronary artery without endothelium under the same conditions (n=9). Hypoxia was induced by switching from 95% O_2 + 5% CO_2 (O_2) to a 95% N_2 + 5% CO_2 gas mixture (N_2). Data are expressed as mean±SE. *: significant difference between PGF2α-induced contractility and hypoxia-induced contractility ($P<0.05$). EC+: ring with endothelium, EC-: ring without endothelium, ACh: acetylcholine.*

hypoxic gas for 15~25 min, followed by a return to hyperoxic gas. The preparations contracted with $PGF_2\alpha$ relaxed progressively during the hypoxia, and tone gradually returned to prehypoxia levels after switching back to the hyperoxic gas (Fig. 1). The amplitude of HCD in $PGF_2\alpha$-precontracted artery with and without endothelium was 62.7±10.1% (n=54) and 63±5.4% (n=11), respectively and HCD was independent on endothelium.

In $PGF_2\alpha$-precontracted artery with and without endothelium, HCD and the recovery of tone after return to hyperoxic gas were reproducible in individual tissues throughout the course of a given experiment (Fig. 2). The amplitude of HCD was 64.6±10.1% (1st episode; n=9) and 70.6±8.7% (2nd episode; n=9), respectively. As shown in Fig. 3, the amplitude of HCD did not differ between arteries equilibrated with normoxic gas and hyperoxic gas. The mean amplitude of HCD was 67.3±7% (normoxic gas; n=7) and 62.8±6.3% (hyperoxic gas; n=20).

Effects of indomethacin

An inhibitor of the cyclooxygenase pathway, indo-

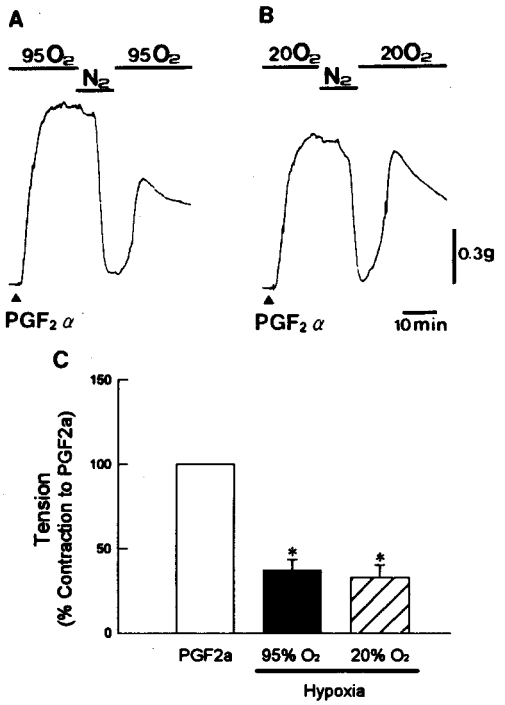

*Fig. 3. Effect of hyperoxic or normoxic control Krebs-Henseleit solution on subsequent response to hypoxia in coronary artery without endothelium. A, B: shows typical recording to effect of hyperoxic (A) or normoxic (B) control Krebs-Henseleit solution on subsequent response to hypoxia in precontracted (prostaglandins $F_2\alpha$: $PGF_2\alpha$, $1.5\times10^{-6}M$) coronary artery. C: shows mean response of coronary artery incubated with hyperoxic (95% O_2: n=20) and normoxic (20% O_2: n=7) control Krebs-Henseleit solution under the same conditions. Hypoxia was induced by switching from 95% O_2 + 5% CO_2 (O_2) to a 95% N_2 + 5% CO_2 gas mixture (N_2). Data are expressed as mean±SE. *: significant difference between $PGF2\alpha$-induced contractility and hypoxia-induced contractility ($P<0.05$).*

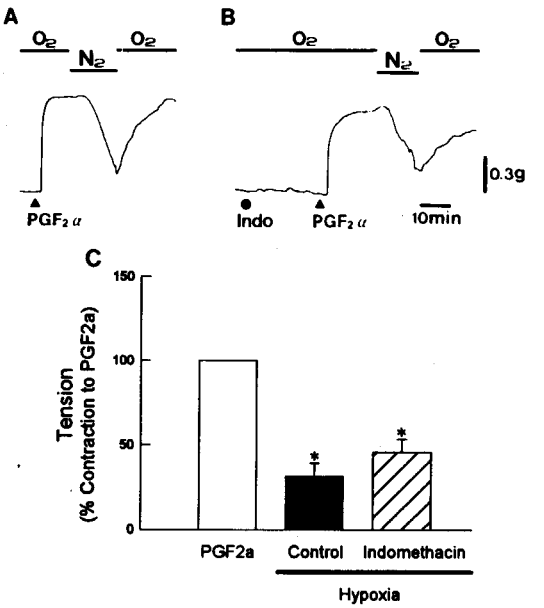

*Fig. 4. Effect of indomethacin on the response to hypoxia in coronary artery without endothelium. A, B: shows typical response to hypoxia in the precontracted (prostaglandins $F_2\alpha$: $PGF_2\alpha$, $1.5\times10^{-6}M$) coronary artery without (A) and with (B) indomethacin ($10^{-5}M$). C: shows mean response of coronary artery with (Indomethacin) and without (Control) indomethacin under the same conditions (n=6). Indomethacin was applied 20-30min before testing the effect of hypoxia efficacy. Hypoxia was induced by switching from 95% O_2 + 5% CO_2 (O_2) to a 95% N_2 + 5% CO_2 gas mixture (N_2). Data are expressed as mean±SE. *: significant difference between $PGF_2\alpha$-induced contractility and hypoxia-induced contractility ($P<0.05$). Indo: indomethacin.*

methacin, had no significant effect on HCD (Fig. 4). In endothelium denuded arteries, tissues contracted with $PGF_2\alpha$ relaxed during a 15-min exposure to hypoxic gas by 68.7±7.9% (n=6) in the absence, and by 54.3±7.5 (n=6) in the presence, of indomethacin (10^{-5}M).

Effects of K^+ channel blockade

To determine whether K^+ channels are involved in HCD, pretreatment of coronary artery rings with 1mM TEA, a non-specific blocker of K^+ channels, attenuated significantly ($p<0.05$) the relaxation response during hypoxia of preparations precontracted with $PGF_2\alpha$ (Fig. 5). The amplitude of HCD with and without TEA were 13.3±11.8% and 83±

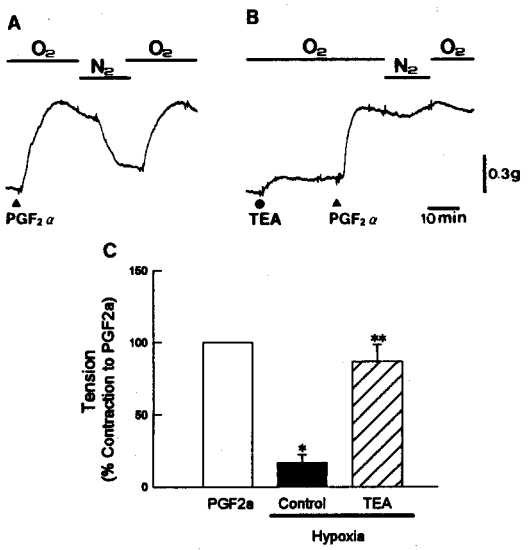

*Fig. 5. Effect of tetraethylammonium chloride on the response to hypoxia in coronary artery without endothelium. A, B: shows typical response to hypoxia in precontracted (prostaglandins $F_2\alpha$: $PGF_2\alpha$, 1.5×10^{-6}M) coronary artery without (A) and with (B) tetraethylammonium chloride (TEA, 10mM). C: shows mean response of coronary artery with (TEA) and without (Control) TEA under the same conditions (n=11). TEA was applied 25min before testing the effect of hypoxia. Hypoxia was induced by switching from 95% O_2 + 5% CO_2 (O_2) to a 95% N_2 + 5% CO_2 gas mixture (N_2). Data are expressed as mean±SE. *: significant difference between $PGF_2\alpha$-induced contractility and control ($P<0.05$). **: significant difference between control and TEA group ($P<0.05$).*

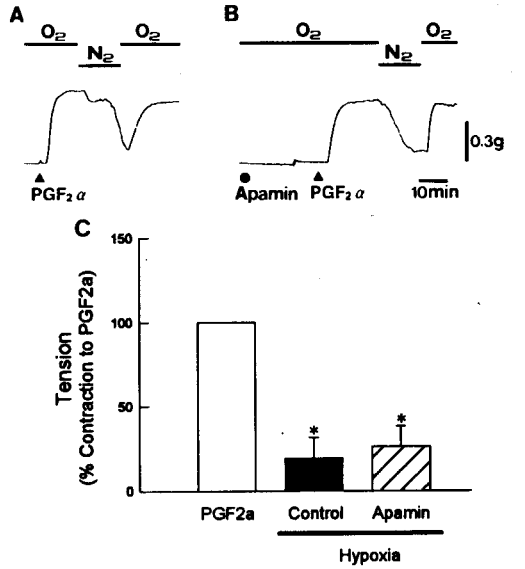

*Fig. 6. Effect of apamin on the response to hypoxia in coronary artery without endothelium. A, B: shows typical response to hypoxia in precontracted (prostaglandins $F_2\alpha$: $PGF_2\alpha$, 1.5×10^{-6}M) coronary artery without (A) and with (B) apamin (10^{-7}M). C: shows mean response of coronary artery with (Apamin) and without (Control) apamin under the same conditions (n=9). Apamin was applied 20 - 25min before testing the effect of hypoxia. Hypoxia was induced by switching from 95% O_2 + 5% CO_2 (O_2) to a 95% N_2 + 5% CO_2 gas mixture (N_2). Data are expressed as mean±SE. *: significant difference between $PGF_2\alpha$-induced contractility and hypoxia-induced contractility ($P<0.05$).*

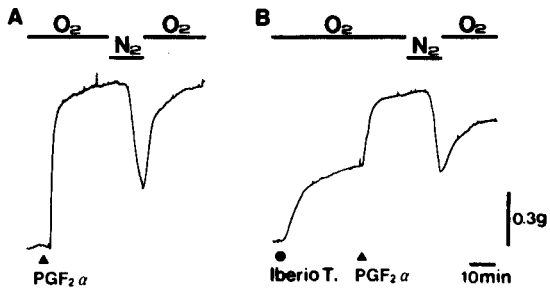

Fig. 7. Effect of iberiotoxin on the response to hypoxia in coronary artery without endothelium. A, B: shows typical response to hypoxia in precontracted (prostaglandins $F_2\alpha$: $PGF_2\alpha$, 1.5×10^{-6}M) coronary artery without (A) and with (B) iberiotoxin (Iberio T., 5×10^{-8}M). Iberiotoxin was applied 30min before testing the effect of hypoxia. Hypoxia was induced by switching from 95% O_2 + 5% CO_2 (O_2) to a 95% N_2 + 5% CO_2 gas mixture (N_2).

5.3% (n=11), respectively.

To determine whether K_{Ca} channels are involved in HCD, coronary artery rings were contracted with $PGF_2\alpha$ in the presence of apamin (10^{-7}M), a blocker of small conductance K_{Ca} channels (Fig. 6) and iberiotoxin (1.5×10^{-8}M), an antagonist of large conductance K_{Ca} channels (Fig. 7). Both drugs had no significant effect on the amplitude of HCD. The amplitude of HCD with and without apamin were $74\pm12.1\%$ and $80.6\pm11.9\%$ (n=9), respectively.

Pretreatment of coronary artery rings for 30 min with glibenclamide (10^{-6}M), the potent sulfonylurea blocker of K^+_{ATP} channels, attenuated significantly, but did not eliminate, the relaxation response during hypoxia of preparations contracted with $PGF_2\alpha$ (Fig. 8). The amplitude of HCD was reduced significantly ($p<0.05$) from a mean of $69\pm8.3\%$ (n=7) to a mean of $27.2\pm16.6\%$ (n=7) in glibenclamide treated rings. The opening of K^+_{ATP} chan-

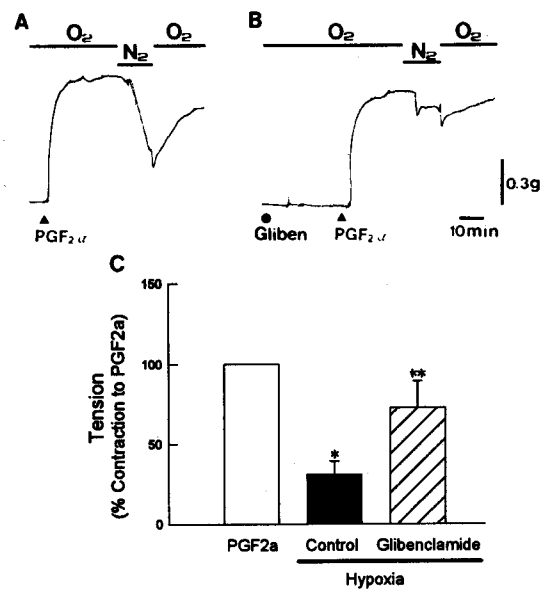

*Fig. 8. Effect of glibenclamide on the response to hypoxia in coronary artery without endothelium. A, B: shows typical response to hypoxia in precontracted (prostaglandins $F_2\alpha$: $PGF_2\alpha$, 1.5×10^{-6}M) coronary artery without (A) and with (B) glibenclamide (Gliben, 10^{-6}M). C: shows mean response of coronary artery with (Glibenclamide) and without (Control) glibenclamide under the same conditions (n=7). Glibenclamide was applied 25 min before testing the effect of hypoxia. Hypoxia was induced by switching from 95% O_2 + 5% CO_2 (O_2) to a 95% N_2 + 5% CO_2 gas mixture (N_2). Data are expressed as mean±SE. *: significant difference between $PGF_2\alpha$-induced contractility and control ($P<0.05$). **: significant difference between control and glibenclamide group ($P<0.05$).*

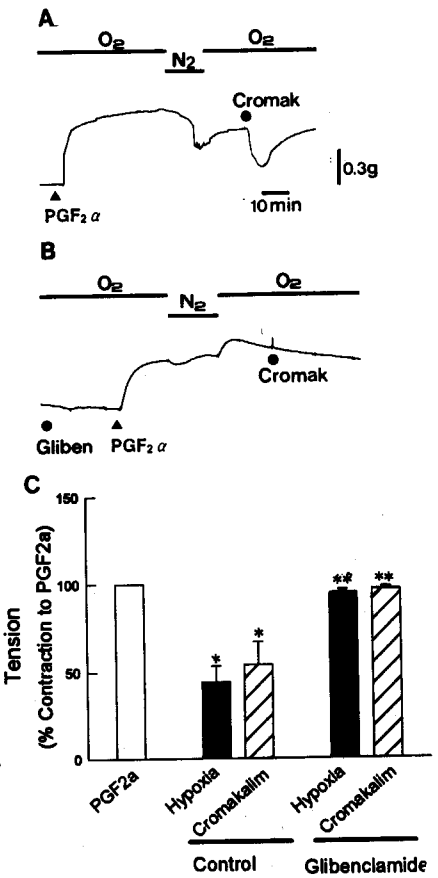

*Fig. 9. Effect of glibenclamide on the response to hypoxia and cromakalim in coronary artery without endothelium. A, B: shows typical response to hypoxia and cromakalim (Cromak, 5×10^{-6}M) in precontracted (prostaglandins $F_2\alpha$: $PGF_2\alpha$, 1.5×10^{-6}M) coronary artery without (A) and with (B) glibenclamide (Gliben, 10^{-6}M). C: shows mean response of coronary artery with (Glibenclamide) and without (Control) glibenclamide under the same conditions (n=7). Glibenclamide was applied 25min before testing the effect of hypoxia. Hypoxia was induced by switching from 95% O_2 + 5% CO_2 (O_2) to a 95% N_2 + 5% CO_2 gas mixture (N_2). Data are expressed as mean±SE. *: significant difference between $PGF_2\alpha$-induced contractility and hypoxia or cromakalim-induced contractility ($P<0.05$). **: significant difference between control and glibenclamide group ($P<0.05$).*

nels, as measured by the amount of relaxation to the K^+ channel opener cromakalim (1.5×10^{-6}M; Fig. 9), induced relaxation in the $PGF_2\alpha$-precontracted arteries aerated with hyperoxic gas and the amplitude of cromakalim-induced relaxation similar to that of HCD. Contractions to $PGF_2\alpha$ were reduced $55.9 \pm 9.1\%$ (n=7) by hypoxia and $46 \pm 13\%$ (n=7) by cromakalim. Glibenclamide antagonized relaxations to the K^+ channel opener cromakalim, but also to hypoxia. The amplitude of HCD and cromakalim-induced relaxations in the presence of glibenclamide were $12.5 \pm 2\%$ and $8.9 \pm 1.2\%$, respectively (n=7).

DISCUSSION

Several groups of researchers using data obtained with patch-clamp methodology have hypothesized that under ordinary or normoxic conditions, the concentration of ATP in vascular smooth muscle cells is sufficient to maintain K^+_{ATP} channels in the closed state, but that under hypoxic conditions, the reduction in ATP levels releases the channels from inhibition (Standen, 1992; Edwards and Weston, 1993; McPherson, 1993). Open channels allow K^+ efflux, hyperpolarization and presumably vascular relaxation (Standen *et al.* 1989). However, no study has so far demonstrated clearly a role for K^+, especially K^+_{ATP} channels in tone regulation in functionally-intact arterial smooth muscle preparations. It should be noted that a recent study examined hypoxic relaxation in segments of porcine coronary arteries in vitro (Mellemkjaer and Nielsen-Kudsk, 1994). Removal of the endothelium had no significant effect on the relaxation response during hypoxia. These workers further demonstrated a glibenclamide-sensitive component of hypoxic relaxation, but they could do so only in Krebs-Henseleit solution in which glucose was replaced with 2-deoxyglucose.

In the present study, hypoxia induced relaxation in a $PGF_2\alpha$-precontracted artery, but the patterns of HCD were different. Differences in the patterns of HCD may be due to different artery wall thicknesses and the diffusion velocity of hypoxic gas (Tucker *et al.* 1975).

The results of this study indicate that the hypoxic relaxations from contraction to $PGF_2\alpha$ occur in epicardial coronary arteries during exposure to hypoxic gas in KH solution and partially involve the K^+_{ATP} channels. The endothelium clearly was not involved in the HCD described here because the HCD dose was not affected in the endothelium-denuded preparation and most of the preparations were routinely denuded. Similarly, an inhibitor of the cyclooxygenase pathway, indomethacin, had no observable effects on HCD.

The primary evidence provided here for the involvement of smooth muscle K^+_{ATP} channels in HCD is the finding that the sulfonylurea compound glibenclamide significantly reduced relaxation of precontracted coronary artery rings under hypoxia. Glibenclamide has been described as "the most potent of the family of antidiabetic sulphonylurea compounds" and for muscle "the most effective blocker of K^+_{ATP} channels found so far," effective in the range of 10^{-6}M (Standen, 1992). In confirmation of the specificity of glibenclamide's action on rabbit coronary arteries, we found here that glibenclamide antagonized relaxations to the K^+ channel opener cromakalim, but also HCD.

The most obvious mechanism by which a reduced P_{O2} could lead to activation of K^+_{ATP} channels is by causing a change in the energy metabolism of coronary myocytes, so that K^+_{ATP} channels open in response to a fall in the submembrane concentration of ATP (Kajioka *et al.* 1991; Beech *et al.* 1993). Such a mechanism was suggested by von Beckerath *et al.* (1991) to explain the hypoxic vasodilatation of an isolated heart, where vasodilatation could also be produced by the inhibitor of glycolysis, 2-deoxyglucose, or the mitochodrial uncoupler dinitrophenol. Inhibition of cellular metabolism using metabolic poisons has been shown to activate glibenclamide-sensitive K^+ currents in cells isolated from the mesenteric artery and portal vein (Silberberg and van Breemen, 1992; Beech *et al.* 1993), while conventional whole-cell recording with low ATP concentrations in a pipette solution can also lead to activation of K^+_{ATP} currents (Clapp and Gurney, 1992; Quayle *et al.* 1994).

While it seems clear that metabolic inhibition or a fall in intracellular ATP concentration ($[ATP]_i$) can activate K^+_{ATP} channels, it is not certain that

hypoxia will lead to a fall in $[ATP]_i$. In aortic smooth muscle, for example, hypoxia has been reported to cause little change in ATP content (Namm and Zucker, 1976; Post and Jones, 1991). It is possible that the metabolism of small arteries may differ from that of large vessels, or it may be the case that submembrane changes in ATP concentration during hypoxia are greater than changes in bulk cytosol. Since K^+_{ATP} channels in other tissues have been shown to be regulated by intracellular factors other than nucleotides, for example pH (Davies *et al.* 1992), it is also possible that hypoxia activates K^+_{ATP} channels by changing one of these. It is clear that the mechanisms in addition to K^+_{ATP} channels participate in what appears to be a complex coronary artery response to hypoxia, because the HCD is not completely inhibited by glibenclamide. Although the mechanisms involved in this residual relaxation are unclear, they do not appear to involve the K_{Ca} channels because apamin and iberiotoxin had no inhibitory effect on relaxation. In conclusion, these results strongly suggest that HCD is independent on endothelium and HCD is considered to be induced by activation of the K_{ATP} channel.

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