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Nitric oxide mediated endothelium-dependent relaxation induced by glibenclamide in rat isolated aorta

Wai-Kei Chan, Xiaoqiang Yao, Wing-Hung Ko, Yu Huang*

Department of Physiology, Faculty of Medicine, Chinese University of Hong Kong, Shatin, NT, Hong Kong, People's Republic of China

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Abstract

Objectives: Glibenclamide was found to act as both a selective ATP-sensitive K^+ channel blocker and a vasorelaxant. The exact mechanisms underlying the relaxant effect of glibenclamide are unknown. The present study was designed to examine the role of endothelium/nitric oxide in glibenclamide-induced relaxation in rat isolated aortic rings. **Methods:** A combination of experimental approaches including isometric force measurement, cell culture, Ca^{2+} fluorescence measurement and radioimmunoassay were used to examine the vascular effect of glibenclamide. **Results:** Glibenclamide induced a concentration-dependent relaxation more effectively in rings with endothelium (IC_{50} of 32±4 μM) than those without endothelium (IC_{50} of 365±29 μM). Incubation with N^G-nitro-L-arginine methyl ester (L-NAME) or methylene blue significantly reduced and L-arginine (3 mM) potentiated the glibenclamide-induced relaxation. L-Arginine (3 mM) partially antagonized the effect of L-NAME. Glibenclamide (100 μM) increased the cyclic GMP content of endothelium-intact tissues. Pretreatment with N^G-nitro-L-arginine (100 μM) or removal of endothelium significantly suppressed the effect of glibenclamide on cyclic GMP production. Glibenclamide elevated the intracellular Ca^{2+} levels in cultured rat aortic endothelial cells. Glibenclamide also inhibited the endothelium-independent contractile response to 60 mM K^+ (IC_{50} of 137±21 μM) and caused a rightward shift in the concentration–contraction curve for $CaCl_2$. Besides, glibenclamide inhibited phorbol-12,13-diacetate (1 μM)-induced contraction in Ca^{2+}-free Krebs solution. **Conclusion:** These results indicate that glibenclamide-induced endothelium-dependent relaxation involves nitric oxide release and this effect may be related to its stimulatory effect on endothelial Ca^{2+} levels. However, the glibenclamide-induced endothelium-independent relaxation may be associated with its inhibitory effect on Ca^{2+} influx through Ca^{2+} channels and on the protein kinase C-mediated contractile mechanism.

Keywords: Arteries; Ca-channel; Endothelial function; K-ATP channel; Nitric oxide; Vasoconstriction/dilation

1. Introduction

Sulfonylurea drugs such as glibenclamide, long used in the treatment of non-insulin-dependent diabetes mellitus, are known to promote insulin secretion through inhibition of ATP-sensitive K^+ (K_{ATP}) channels in pancreatic cells [1]. Sulfonylureas selectively inhibited K_{ATP} channels in cardiac myocytes [2], vascular smooth muscle [3,4], skeletal muscle [5] and central neurons [6]. Glibenclamide blocked K_{ATP} channels without an effect on Ca^{2+}-activated K^+ (K_{Ca}) channels in arterial smooth muscle [3,7] and glibenclamide-sensitive K_{ATP} current underlies a large component of hyperpolarization and relaxation induced by endogenous dilators, such as acetylcholine and calcitonin gene-related peptide [3,4]. K_{ATP} channel-independent effects of glibenclamide on smooth muscle were reported by several research groups. Glibenclamide appears to involve a competitive interaction at the prostaglandin receptors since glibenclamide reduced contraction induced by vasoconstricting prostanoids such as $PGF_{2\alpha}$ [8,9] and U46619 [10,11], but not by other constrictors such as noradrenaline, 5-hydroxytryptamine or endothelin-I [10,12,13]. An interspecies difference in the inhibitory effect of glibenclamide on muscle tension was also reported, for

*Corresponding author. Tel.: +852-2-609-6787; fax: +852-2-603-5022.

E-mail address: yu-huang@cuhk.edu.hk (Y. Huang)

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instance, glibenclamide failed to affect the U46619-induced contraction in human subcutaneous arteries [14]. However, other studies show that glibenclamide may have a direct relaxant activity that was independent of receptor interaction. Glibenclamide suppressed the contractile response to high extracellular K^+, endothelin-I and phenylephrine in some blood vessels [11,15] and in tracheal smooth muscle [16]. Endothelium also plays a role in the relaxant response to glibenclamide in rat mesenteric artery in our previous study [15], whilst removal of endothelium did not affect glibenclamide-induced relaxation in canine blood vessels [9], indicating that the endothelium-related component may be tissue-dependent.

Here we have further examined the endothelium-dependent and -independent relaxant action of glibenclamide in rat isolated aortic rings, its effect on Ca^{2+} concentrations in cultured rat aortic endothelial cells, and on cyclic GMP production in endothelium-intact aortic rings. In addition, we also examined whether glibenclamide could interfere with protein kinase C-mediated endothelium-independent contractile response in rat aorta.

2. Methods

2.1. Vessel preparation

This investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). After approval from Animal Ethical Committee of the Chinese University of Hong Kong was obtained, male Sprague–Dawley rats (250–300 g) were killed by cervical dislocation. The thoracic segment of aorta was dissected free from surrounding tissues and cut into rings of 3 mm in length. The preparation was then transferred into 10-ml organ baths containing Krebs solution bubbled with a mixture of 95% O_2 and 5% CO_2. Each aortic ring was mounted between two L-shaped stainless steel hooks. One of the hook was mounted at the bottom of the bath while the other was connected to FT03 force-displacement transducer (Grass Instruments Co). Krebs solution contained (mM): 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$,1.2 KH_2PO_4, 11 D-glucose. One and half gram basal tension was applied to all experiments. All experiments were performed at 37°C. In some arterial rings, the endothelial layer was mechanically removed by gently rubbing the luminal surface of the artery back and forth several times with plastic tubing. Endothelium integrity or functional removal was verified by the presence or absence, respectively, of the relaxant response (over 80% relaxation) to 3 μM acetylcholine at the start of each experiment. The removal of endothelium was also evaluated by light microscopy of the histological section of the artery. Each experiment was performed on the rings prepared from different rats.

2.2. Endothelial cell culture

The procedures used to culture endothelial cells were described elsewhere [17]. Briefly, the thoracic artery was cut longitudinally into stripes and they were washed twice in physiological saline solution, and then placed in a 25-cm^2 culture flask (Coster, Cambridge, MA, USA) with 0.2% collagenase in PBS (w/v) in a shaking bath at 37°C for 15 min. The tissues plus medium were transferred to a 15-ml conical tube and centrifuged at $800\times g$ for 5 min. The cell pellet was resuspended in 5 ml RPMI (10% FBS+2× PS) in a 25-cm^2 culture flask. Cells were incubated at 37°C with 5% CO_2 for 1 h and RPMI was replaced with a fresh medium. Culture medium was changed twice a week until a confluent layer of endothelial cells was obtained. Endothelial cells after 3-day culture were confirmed by their positive reactivity (over 99%) to antibody against Von Willebrand factor (DAKO A/S, Denmark).

2.3. Contraction experiments

Cumulative concentration–response relationships for the relaxant effect of glibenclamide were determined in aortic rings following steady contraction with phenylephrine (0.3 μM in rings with endothelium and 0.1 μM in rings without endothelium or in the presence of nitric oxide inhibitors). The role of endothelium/nitric oxide was evaluated following incubation of endothelium-intact rings with the nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, 3–100 μM) or with methylene blue (3 μM) for 30 min prior to application of phenylephrine. Once the sustained tension was established, glibenclamide was added cumulatively to the bath. In order to test whether the substrate for nitric oxide synthase, L-arginine, would counteract with L-NAME, the rings were incubated with L-arginine (3 mM) for 10 min before the addition of L-NAME (100 μM). The vasodilator effects of A23187 and sodium nitroprusside were also examined in the absence and presence of L-NAME.

In another set of experiments, a possible inhibitory effect of glibenclamide on Ca^{2+} influx was examined in endothelium-denuded vessels. The aortic rings were stimulated first with 1 μM phenylephrine in Ca^{2+}-free Krebs solution containing 0.5 mM Na_2-EGTA to deplete intracellular Ca^{2+} stores and then rinsed with Ca^{2+}-free 60 mM K^+ solution without EGTA several times until the resting tension was maintained at 15 mN. The control cumulative concentration–response curve for $CaCl_2$ (0.1–10 mM) was obtained and the ring was washed first in Ca^{2+}-free solution and then in Ca^{2+}-free 60 mM K^+ solution every 20 min until the basal tone was restored. The second concentration–response curve for $CaCl_2$ was repeated in the absence and presence of 300 μM glibenclamide (30-min contact time). Contractions were presented as mN. The

effect of the L-type voltage-sensitive Ca^{2+} channel inhibitor, nifedipine (10 nM) was also tested. In some experiments, the endothelium-independent relaxation induced by glibenclamide was determined in endothelium-denuded rings following stable tension induced by 60 mM extracellular K^+. In these experiments, Na^+ was replaced with an equimolar mount of K^+ to maintain the ionic strength.

In the last group of experiments, a sustained contraction of the endothelium-denuded aorta to 1 μM phorbol-12,13-acetate was induced in Ca^{2+}-free Krebs solution containing 1 mM Na_2-EGTA and glibenclamide was applied cumulatively. The effect of staurosporine (30 nM) was also examined.

2.4. Cyclic GMP assay

After a 60-min period of equilibration in an oxygenated Krebs solution at 37°C, the arterial rings were first incubated with 0.3 μM phenylephrine for 10 min, then with 100 μM glibenclamide in the absence and presence of 100 μM L-NNA. At the end of the reaction, arterial rings were rapidly frozen in liquid nitrogen and stored at −70°C until homogenized in 0.5 ml of ice-cold 6% trichloroacetic acid using a glass homogenizer. The homogenate was centrifuged at 2000×*g* for 10 min at 4°C. The supernatant was extracted three times with three volumes of diethyl ether before lyophylization. The amount of cyclic GMP was assayed by radioimmunoassay with a [^{125}I]cyclic GMP RIA kit (DuPont, Wilmington, Delaware, USA). The tissue residue was dissolved in 2 M NaOH and protein content was determined using a protein assay kit (Sigma, St. Louis, MO, USA) with bovine serum albumin as the standard. The concentration of cyclic GMP was presented as pmol/mg protein.

2.5. Measurement of intracellular Ca^{2+}

Cultured rat aortic endothelial cells were grown on a coverslip overnight at a density of 10^4 cells. The cells were then loaded with Fura-2 (Molecular probes Inc., Eugene, OR, USA) for 1 h in the dark at room temperature in normal physiological saline solution (PSS). After Fura-2 loading, cells were rinsed with PSS to remove excessive external Fura-2. The coverslip was then transferred into a chamber which was used for $[Ca^{2+}]_i$ measurement on a PTI RatioMaster Fluorescence System (Photon Technology Internal Inc., NJ, USA).

2.6. Drugs

The following drugs were used: glibenclamide, phenylephrine hydrochloride, N^G-nitro-L-arginine methyl ester, N^G-nitro-L-arginine, methylene blue, indomethacin, L-arginine, sodium nitroprusside, acetylcholine hydrochloride, phorbol-12,13-acetate, nifedipine, A23187, staurosporine (Sigma, St. Louis, MO, USA). Glibenclamide, indomethacin, methylene blue, phorbol-12,13-acetate, A23187, staurosporine and nifedipine were dissolved in dimethyl sulfoxide (DMSO) and others in distilled water. DMSO at 0.2–0.6% (v/v) did not affect the sustained contraction induced by phenylephrine, 60 mM K^+ or phorbol-12,13-acetate.

2.7. Statistical analysis

The relaxant effect of glibenclamide was expressed as percentage relaxation of the agonist-constricted arterial rings. IC_{50} values were calculated as the glibenclamide concentration inducing 50% of the maximum inhibition (E_{max}). Data were presented as means±S.E.M. of *n* experiments. Statistical significance was analyzed by Student's *t*-test or by one-way ANOVA followed by Student–Newman–Keuls test when more than two treatments were compared. A *P* value less than 0.05 was considered significant.

3. Results

3.1. Endothelium-dependent relaxant effect of glibenclamide

Phenylephrine at submaximal concentration of 0.3 μM induced a steady tone in rat endothelium-intact aortic rings (7.1±0.4 mN, $n=27$) while it induced a slightly greater tone in the endothelium-denuded rings at 0.1 μM (9.4±1.3 mN, $n=14$).

Traces in Fig. 1 show that glibenclamide caused a concentration-dependent relaxation of aortic rings constricted with phenylephrine. The IC_{50} value for the relaxant response to glibenclamide following sustained contraction was significantly less in rings with a functional endothelium (Fig. 1a) than those without endothelium (Fig. 1b) (31.8±3.7 μM, $n=9$ with endothelium vs. 365.3±29.1 μM, $n=6$ without endothelium, $P<0.05$, Fig. 1c). The effects of inhibitors of nitric oxide-mediated relaxation were examined only in endothelium-intact aortic rings. Pretreatment with L-NAME at three concentrations caused significant shifts to the right of the concentration response curve for the relaxant effect of glibenclamide (Fig. 2a, Table 1). L-Arginine at 3 mM potentiated the glibenclamide-induced relaxation, but it partially antagonized the effect of 100 μM L-NAME (Fig. 2b, Table 1). Methylene blue at 3 μM also inhibited glibenclamide-induced relaxation (Fig. 2c, Table 1). In contrast, indomethacin at 3 μM did not alter glibenclamide-induced relaxation (Fig. 2c, Table 1).

L-NAME at 10 μM significantly inhibited the endothelium-dependent relaxation induced by A23187 (Table 2), but this concentration of L-NAME did not affect the endothelium-independent relaxation induced by sodium nitroprusside (Table 2), while 3 μM methylene blue

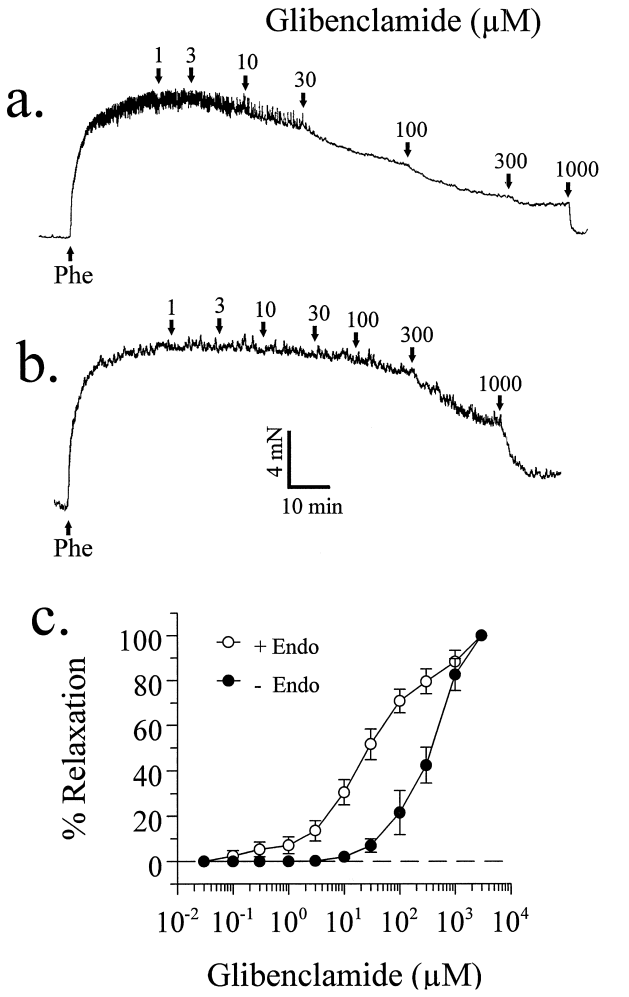

Fig. 1. Representative traces showing the relaxant response to glibenclamide in phenylephrine (Phe)-contracted rat isolated aortic rings with endothelium (a) and without endothelium (b). (c) Concentration–effect curves for the relaxant effect of glibenclamide in aortic rings precontracted with phenylephrine (○, $n=8$ with endothelium; ●, $n=14$, without endothelium). Results are means±S.E.M. of n experiments.

significantly reduced sodium nitroprusside-induced relaxation (Table 2), further suggesting the involvement of nitric oxide in glibenclamide-induced relaxation.

3.2. Effect of glibenclamide on tissue cyclic GMP content

To determine whether glibenclamide-induced endothelium-dependent relaxation was associated with an increase of cyclic GMP level in smooth muscle cells, we investigated the effect of glibenclamide on the tissue content of cyclic GMP. As shown in Fig. 3, glibenclamide at 100 μM induced approximately two-fold increase in cyclic GMP levels in endothelium-intact aortic rings. Acetylcholine (1 μM), an endothelium-dependent dilator, also caused about three-fold increase of cyclic GMP ($n=4$, data not shown) and sodium nitroprusside (1 μM), an endothelium-independent dilator, induced four-fold increase in cyclic GMP levels ($n=4$). Pretreatment with 100 μM L-NNA abolished the stimulatory effect of glibenclamide on cyclic GMP production ($n=5$, Fig. 3). In addition, glibenclamide did not affect cyclic GMP contents in endothelium-denuded tissues (Fig. 3). DMSO (0.1%, v/v) did not affect the cyclic GMP content in the presence

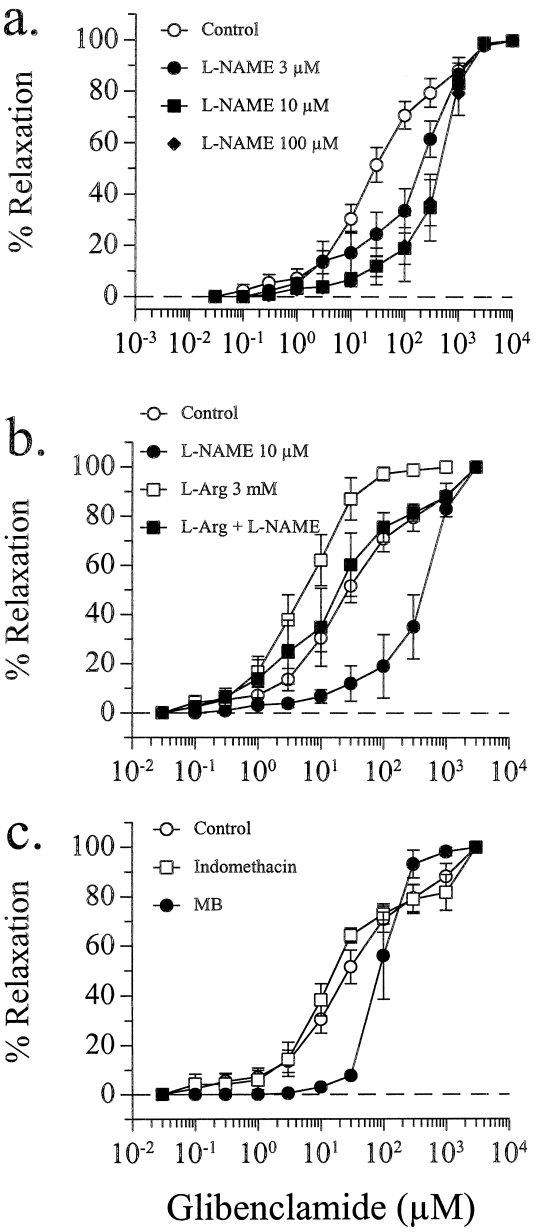

Fig. 2. Effects of inhibitors of nitric oxide activity on the relaxant response to glibenclamide. (a) The concentration–response curves for glibenclamide-induced relaxation in control (○, $n=9$) and in the presence of L-NAME (●, 3 μM, $n=5$; ■, 10 μM, $n=5$; ♦, 100 μM, $n=5$). (b) The concentration–response curves under different treatments (○, $n=7$ in the presence of endothelium; ●, $n=5$ for 100 μM L-NAME; □, $n=5$ in 3 mM L-arginine; ■, $n=5$ in L-arginine plus L-NAME). (c) The concentration–response curves for the relaxant effect of glibenclamide in the absence (○, $n=9$) and presence of 3 μM methylene blue (●, $n=4$) or 3 μM indomethacin (□, $n=4$). Results are means±S.E.M. of n experiments.

Table 1
Effects of various inhibitors on glibenclamide-induced relaxation[a]

	IC_{50} (μM)	E_{max} (%)	*n*
Control	31.8±3.7	100	9
L-NAME (3 μM)	231.3±66.4*	100	5
L-NAME (10 μM)	441.4±60.5*	99±2.5	4
L-NAME (100 μM)	448.8±61.0*	98±1.7	5
L-Arg (3 mM)	5.59±9.42*	100	6
L-Arg (3 mM)+ L-NAME (100 μM)	19.8±4* **	100	5
Methylene blue (3 μM)	89.5±1.0*	100	4
Indomethacin (3 μM)	22.8±4.3	100	4

[a] The IC_{50} values and the maximum relaxation (E_{max}) induced by glibenclamide in the absence and presence of inhibitors of endothelium-dependent relaxation in rat aorta. L-NAME, N^G-nitro-L-arginine methyl ester; L-Arg, L-arginine. Significant differences between the control and treatment groups (* $P<0.05$) and between L-NAME-treated and L-Arg+ L-NAME-treated groups (** $P<0.05$) are indicated. Results are means±S.E.M. of *n* experiments.

of 0.3 μM phenylephrine (2.844±0.149 pmol/mg protein, $n=5$, for phenylephrine control and 3.012±0.328 pmol/mg protein, $n=3$, for DMSO, $P>0.05$).

3.3. Effect of glibenclamide on $[Ca^{2+}]_i$ in endothelium

The trace in Fig. 4a shows that in cultured rat aortic endothelial cells bathed with normal Krebs solution, glibenclamide at 100 μM elevated $[Ca^{2+}]_i$ ($n=3$). Glibenclamide at 100 μM did not affect the basal level of intracellular $[Ca^{2+}]$ in Ca^{2+}-free Krebs solution, perfusion of normal Krebs solution containing 2.5 mM Ca^{2+} induced a relatively sustained rise of $[Ca^{2+}]$ in the presence of glibenclamide ($n=4$, Fig. 4b). DMSO at 0.1% did not affect the basal level of $[Ca^{2+}]_i$ in the endothelial cells response ($n=3$).

3.4. Endothelium-independent relaxant effect of glibenclamide

Removal of a functional endothelium effectively reduced potency of glibenclamide to relax phenylephrine-contracted arteries, suggesting that glibenclamide may have a direct relaxant effect on aortic smooth muscle. To study this possibility, its effect on 60 mM K^+-induced contraction was examined. Fig. 5a and b show that glibenclamide caused a concentration-dependent relaxation with an IC_{50} of 137.4±21.1 μM ($n=5$). In aortic rings in the absence of extracellular Ca^{2+}, and stimulated with 60 mM K^+, $CaCl_2$ (0.1–10 mM) induced a concentration-dependent increase of muscle tone. Fig. 5c shows that prior incubation of rings for 30 min with 300 μM glibenclamide caused a significant rightward shift in the $CaCl_2$ concentration–response curve ($n=5$). Nifedipine at 10 nM inhibited the response to $CaCl_2$ ($n=4$, Fig. 5c). In addition, we also examined the effect of glibenclamide on the contractile response of endothelium-denuded rings to phorbol-12,13-diacetate (PDA, 1 μM) in Ca^{2+}-free Krebs solution (zero Ca^{2+} plus 1 mM Na_2-EGTA). The PDA-induced contraction was significantly inhibited by glibenclamide with an IC_{50} of 123.3±22.1 μM ($n=6$, Fig. 6). Staurosporine at 30 nM completely reversed the PDA-induced contraction ($n=3$, data not shown).

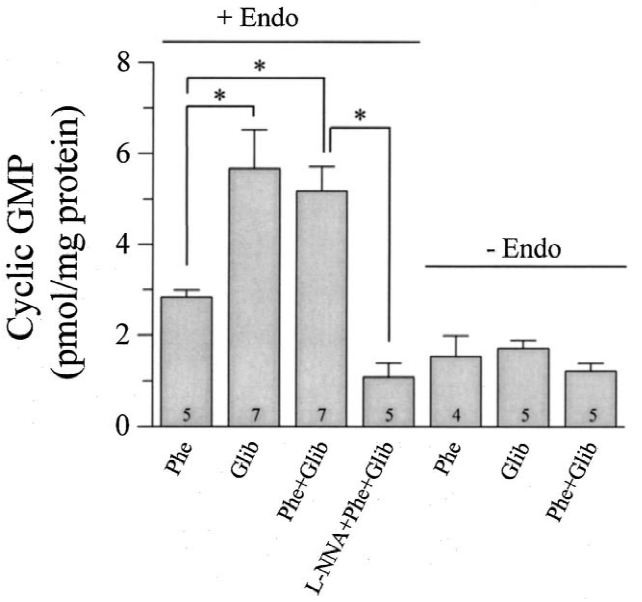

Fig. 3. The effects of glibenclamide on tissue contents of cyclic GMP in aortic rings (0.3 μM phenylephrine; 100 μM glibenclamide). The results are mean±S.E.M. of four to six experiments. * $P<0.05$ calculated by one-way ANOVA followed by Student–Newman–Keuls test.

Table 2
Effects of various inhibitors on A23187- or nitroprusside-induced relaxation[a]

	IC_{50} (nM)	E_{max} (%)	*n*
A23187	8.08±0.88	100±5.8	4
+L-NAME (10 μM)	87.1±3.7*	31.2±10.9*	5
Sodium nitroprusside	4.86±0.25	100	5
+L-NAME (10 μM)	4.96±0.44	100	6
+methylene blue (3 μM)	38.5±3.8*	100	7

[a] The IC_{50} values and the maximum relaxation (E_{max}) induced by A23187 or sodium nitroprusside in the absence and presence of inhibitors of endothelium-dependent relaxation in rat aorta. L-NAME, N^G-nitro-L-arginine methyl ester. A significant difference between the control and treatment groups (* $P<0.05$) is indicated. Results are means±S.E.M. of *n* experiments.

4. Discussion

Sulfonylurea drugs such as glibenclamide are well known for their blocking effects on K_{ATP} channels in a diversity of cells including vascular smooth muscle [3,18], but glibenclamide at higher concentrations was found to be a potent inhibitor of thromboxane A_2-induced contraction

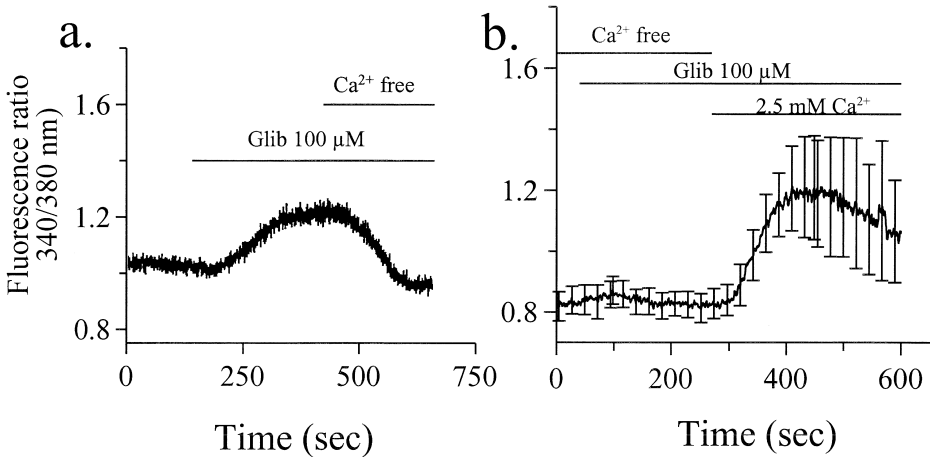

Fig. 4. (a) The trace showing the effect of 100 μM glibenclamide on intracellular Ca^{2+} concentration in cultured rat aortic endothelial cells in normal Krebs solution. (b) The effect of glibenclamide on $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} and after readdition of 2.5 mM $CaCl_2$ to superfusion solution. Results are means±S.E.M. of four experiments.

in mammalian arteries [8–11]. Increasing evidence shows that glibenclamide may not act only as a competitive antagonist of vasoconstricting prostanoids as originally suggested by Cocks et al. [10] since glibenclamide also inhibits contractile responses of some vascular tissues to a number of constrictors such as phenylephrine, endothelin-I, and high K^+ [11,15]. This indicates that glibenclamide may have multiple sites of action. It is evident that the observed muscle relaxant effect of glibenclamide is a property additional to its pharmacological action as a selective blocker of K_{ATP} channels. Blockade of K_{ATP} channels would be expected to result in membrane depolarization of smooth muscle cells, which promotes Ca^{2+} influx through voltage-dependent Ca^{2+} channels and thus elevates vascular tension.

The results of the present investigation show that glibenclamide induced both endothelium-dependent and -independent relaxation in rat isolated aortic rings. Removal of the functional endothelium partially, but significantly, attenuated glibenclamide-induced relaxation. Pretreatment of endothelium-intact rings with L-NAME or methylene blue, which are inhibitors of the endothelial nitric oxide-induced relaxation, reduced glibenclamide-induced relaxation to the same as that in endothelium-denuded tissues. Exposure of the endothelium-intact rings to L-arginine, the precursor of nitric oxide, enhanced the relaxant response to glibenclamide, and it also partially antagonized the effect of L-NAME on glibenclamide-induced relaxation. In contrast, indomethacin, an inhibitor of endothelial prostacyclin formation, had no effect, indicating that the relaxing prostanoids are not involved. These findings suggest that glibenclamide may act on the endothelium to release nitric oxide which primarily accounts for an endothelium-dependent relaxation. Once released from the endothelium, nitric oxide readily diffuses into the adjacent smooth muscle cells to stimulate guanylate cyclase and subsequent cGMP formation which produces a nitric oxide-mediated relaxation [19]. The present results show that exposure of endothelium-intact rings to glibenclamide raised the tissue content of cGMP by approximately two-fold, and this rise was abolished in the presence of the nitric oxide synthase inhibitor, L-NNA, or in the endothelium-denuded rings. It can be ruled out that the effects of L-NAME or L-NNA were non-specific since L-NAME inhibited the relaxations induced not only by glibenclamide but also by the endothelium-dependent dilator, A23187, while it was ineffective against the relaxation induced by the endothelium-independent dilator, sodium nitroprusside. Besides, L-NNA also inhibited acetylcholine-induced increase in the cGMP content in the same preparations [20]. These results support a significant role of endothelium/nitric oxide in glibenclamide-induced relaxation of rat aorta.

Our results contrast the earlier observation with canine middle cerebral arteries in which glibenclamide-induced relaxation was unaltered by methylene blue or in the endothelium-removed tissues [9]. This discrepancy may be caused by the use of different arteries from two species. It is at present unknown how glibenclamide would stimulate release of nitric oxide from the endothelium in isolated rat arteries. However, we found that glibenclamide could induce a relatively sustained increase of intracellular Ca^{2+} levels in cultured rat aortic endothelial cells. It is therefore possible that glibenclamide acts on endothelium to raise $[Ca^{2+}]_i$, which then stimulates nitric oxide synthase to enhance nitric oxide production and release. The present study shows that glibenclamide-induced rise of endothelial $[Ca^{2+}]$ depends on the presence of extracellular Ca^{2+}, this raises a possibility that glibenclamide induces Ca^{2+} influx probably through changing the cell membrane potential. In epithelial cells, glibenclamide was found to inhibit Cl^- outflow currents or Cl^- secretion [21,22]. If glibenclamide has the same blocking effect on vascular endothelial Cl^-

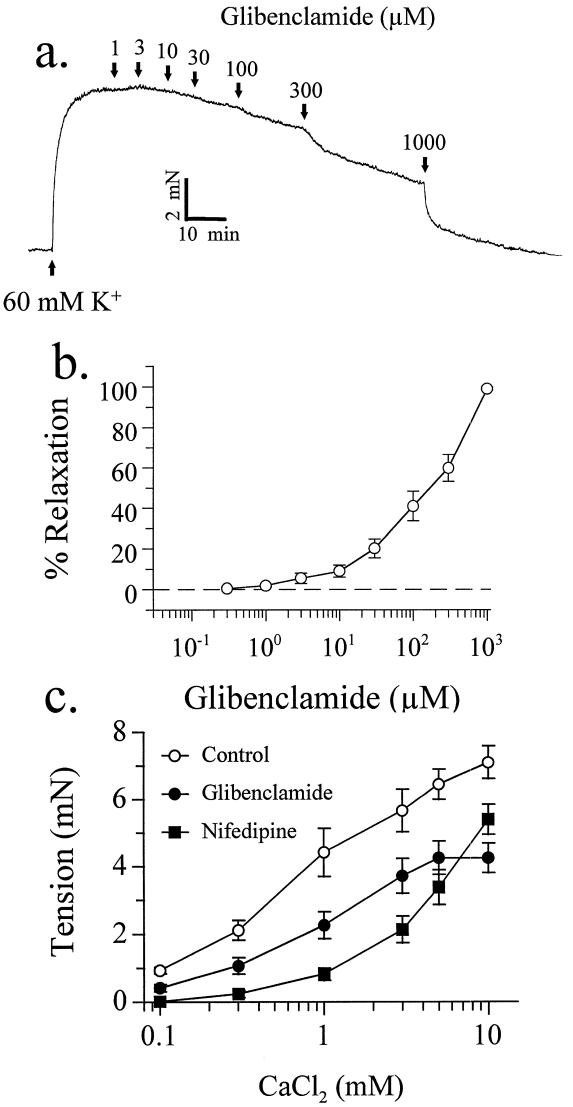

Fig. 5. (a) The trace showing the relaxant effect of glibenclamide on 60 mM K^+-induced aortic contraction. (b) The concentration–response curve for glibenclamide-induced relaxation in 60 mM K^+-contracted rings (n=5). (c) Concentration–response curve for $CaCl_2$-induced contraction of 60 mM K^+-stimulated rat aortic rings (○, n=6 for control; ●, n=5 for 300 μM glibenclamide; ■, n=4, for 10 nM nifedipine). All vessels were removed of endothelium. Results are means±S.E.M. of n experiments.

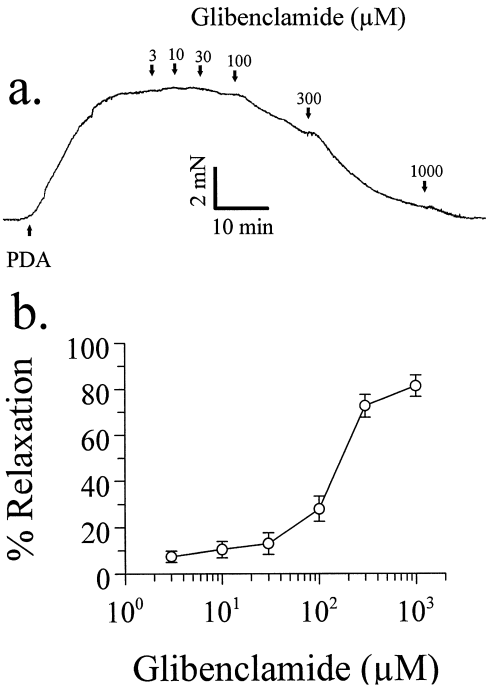

Fig. 6. (a) The trace showing glibenclamide-induced relaxation of an endothelium-denuded ring precontracted with 1 μM phorbol-12,13-acetate (PDA) in Ca^{2+}-free solution containing 1 mM Na_2-EGTA. (b) The concentration–response curve for the relaxant effect of glibenclamide. Results are means±S.E.M. of six experiments.

channels, it would likely cause hyperpolarization and thus increases the driving force for Ca^{2+} entry in non-excitable endothelial cells. This hypothesis needs to be determined with use of electrophysiological techniques.

The present results also show that glibenclamide induced a concentration-dependent relaxation of the high-K^+-contracted endothelium-denuded rings. This finding is consistent with previous observations with rabbit aorta [23] and rat mesenteric artery [15]. Glibenclamide was demonstrated to reduce intracellular Ca^{2+} levels and force without affecting the $[Ca^{2+}]_i$–force relationship [23], implying that the relaxant action of glibenclamide in arterial tissues does not result from a reduced Ca^{2+} sensitivity of contractile proteins, but may involve the initial steps along the excitation–contraction pathway. Nifedipine (10 nM), the L-type voltage-dependent Ca^{2+} channel blocker, also inhibited Ca^{2+}-induced contraction in the high K^+ solution.

Apart from the stimulatory effect on plasma membrane Ca^{2+} channels through depolarization, a high K^+ condition would likely exert Ca^{2+} channel-independent actions such as activation of protein kinase C [24], which can also cause muscle contraction even with normal resting $[Ca^{2+}]_i$ [25]. The present results show that glibenclamide produced a concentration-dependent reduction of the sustained contractile response to the protein kinase C activator in the absence of extracellular Ca^{2+}, suggesting that the protein kinase C-mediated steps in excitation–contraction coupling could be another site of action for glibenclamide when it is used at higher concentrations.

Taken together, the present results demonstrate that glibenclamide exerts K_{ATP} channel-independent effects on the rat isolated aorta. It induces both endothelium-dependent and -independent relaxation. Nitric oxide but not other endothelium-derived factors is primarily responsible for the glibenclamide-induced endothelium-dependent relaxation. Interference with Ca^{2+} influx through voltage-sensitive Ca^{2+} channels or with the protein kinase C-mediated contractile mechanism is likely involved in the endothelium-independent relaxation induced by glibenclamide at high concentrations. These data also suggest

that glibenclamide may have two sites of pharmacological actions, antagonism of the vasodilator effects to K^+ channel activators [3,15] and vasorelaxation. Since there is an overlap in the concentrations of glibenclamide that induce aforementioned two distinct vascular responses [15], it is possible that use of glibenclamide at higher concentrations (>10 μM) as a selective K_{ATP} channel blocker may have led to an underestimation of the role of K_{ATP} channels in control of smooth muscle contractility. It remains to be evaluated whether chronic use of glibenclamide may have a beneficial effect on the vascular system in type II diabetic patients.

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