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Vasorelaxant and antiproliferative effects of berberine

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Abstract

The present study was intended to examine the relaxant effects of berberine in rat isolated mesenteric arteries. Berberine produced a rightward shift of the concentration–response curve to phenylephrine and significantly reduced the maximal contractile response to phenylephrine. Berberine (10^{-7}–3×10^{-5} M) also relaxed the phenylephrine- and 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$-precontracted arteries with respective IC_{50} values of $1.48\pm0.16\times10^{-6}$ and $2.23\pm0.22\times10^{-6}$ M. Removal of a functional endothelium significantly attenuated the berberine-induced relaxation (IC_{50}: $4.73\pm0.32\times10^{-6}$ M) without affecting the maximum relaxant response. Pretreatment with N^G-nitro-L-arginine methyl ester (L-NAME) or methylene blue reduced the relaxant effect of berberine, and L-arginine (10^{-3} M) partially antagonized the effect of L-NAME. In contrast, pretreatment with 10^{-6} M glibenclamide or 10^{-5} M indomethacin had no effect. Berberine (10^{-5} M) reduced over by 50% the transient contraction induced by caffeine or phenylephrine in endothelium-denuded rings bathed in Ca^{2+}-free Krebs solution. Pretreatment with putative K^+ channel blockers, such as tetrapentylammonium ions ($1–3\times10^{-6}$ M), 4-aminopyridine (10^{-3} M), or Ba^{2+} (3×10^{-4} M), significantly attenuated the berberine-induced relaxation in endothelium-denuded arteries. In contrast, tetraethylammonium ions (3×10^{-3} M), charybdotoxin (10^{-7} M) or glibenclamide (10^{-6} M) were without effect. Berberine reduced the high-K^+-induced sustained contraction and the relaxant response to berberine was greater in rings with endothelium (IC_{50}: $4.41\pm0.47\times10^{-6}$ M) than in those without endothelium (IC_{50}: $8.73\pm0.74\times10^{-6}$ M). However, berberine (10^{-6}–10^{-4} M) did not affect the high-K^+-induced increase of intracellular [Ca^{2+}] in cultured aortic smooth muscle cells. Berberine did not affect active phorbol ester-induced contraction in Ca^{2+}-free Krebs solution. In addition, berberine inhibited proliferation of cultured rat aortic smooth muscle cells with an IC_{50} of $2.3\pm0.43\times10^{-5}$ M. These findings suggest that berberine could act at both endothelium and the underlying vascular smooth muscle to induce relaxation. Nitric oxide from endothelium may account primarily for the berberine-induced endothelium-dependent relaxation, while activation of tetrapentylammonium-, 4-aminopyridine- and Ba^{2+}-sensitive K^+ channels, inhibition of intracellular Ca^{2+} release from caffeine-sensitive pools, or a direct relaxant effect, is likely responsible for the berberine-induced endothelium-independent relaxation. Mechanisms related to either Ca^{2+} influx or protein kinase C activation may not be involved. Both vasorelaxant and antiproliferative effects may contribute to a long-term benefit of berberine in the vascular system.

Keywords: Berberine; Nitric oxide (NO); K^+ channel; Ca^{2+} channel; Vasorelaxation; Proliferation; Mesenteric artery; (Rat)

1. Introduction

Berberine, an isoquinoline alkaloid, derived from the Chinese herb Huanglian and many other plants, such as the *Berberidaceae* family, has a varied pharmacology, including antibiotic activity (Hahn and Cuak, 1975), antitumor action (Nishino et al., 1986) and antimotility properties (Yamamoto et al., 1993). Extracts of berberine-containing plants have been used for many centuries in the treatment of diarrhea (Dutta et al., 1972; Sack and Froehlich, 1982; Chang and But, 1987), probably through inhibition of mucosal Cl^- secretion (Taylor et al., 1999). Several studies indicate important cardiovascular effects of berberine. Berberine exerts both positive ionotropic and negative chronotropic effects in isolated guinea-pig atria (Shaffer, 1985), and an antifibrillatory effect (Pang et al., 1986). Berberine was also used in the treatment of congestive heart failure (Martin-Neto et al., 1988). In vivo, berberine lowers blood pressure in mammals (Sabir and Bhide, 1971; Chun et al., 1979). In isolated vascular preparations, berberine causes relaxation (Chiou et al., 1991). More

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recently, Chiou et al. (1998) have reported that berberine possessed a relaxant effect on rabbit corpus cavernosal tissues. Activation of some K^+ channels may contribute to the endothelium-independent relaxation induced by berberine (Chiou et al., 1998). Besides, a phospholipase C-mediated contractile mechanism may be partially involved in the berberine-induced vascular response (Bova et al., 1992). Nevertheless, the vascular sites for a hypotensive activity of berberine are not clear. It is possible that berberine acts on both endothelium and the underlying vascular smooth muscle to induce vasorelaxation via multiple cellular mechanisms. In this study, we attempted to examine the roles of endothelium-derived vasoactive factors, K^+ channel activation, Ca^{2+} influx, intracellular Ca^{2+} release and protein kinase C-mediated pathway in the berberine-induced relaxation of rat isolated mesenteric artery, and to examine a possible antiproliferative effect of berberine on cultured aortic smooth muscle cells, which could account for its long-term beneficial effect in the cardiovascular system.

2. Methods and materials

2.1. Tissue preparation

Male Sprague–Dawley rats (supplied by Animal Services Center, Chinese University of Hong Kong, Hong Kong) weighing ~250–300 g were killed by cervical dislocation and bled. The main branch of the superior mesenteric artery was dissected out and cut into three 3-mm wide ring segments. The ring was mounted between two stainless wire hooks in a 10-ml organ bath filled with Krebs solution. The upper wire was connected to a force-displacement transducer (Grass Instruments, USA) and the lower one fixed at the bottom of the organ bath. Krebs solution contained (mM): 119 NaCl, 4.7 KCl, 25 $NaHCO_3$, 2.5 $CaCl_2$, 1 $MgCl_2$, 1.2 KH_2PO_4, and 11 D-glucose. The bath solution was continuously gassed with a mixture of 95% O_2 and 5% CO_2, and maintained at 37°C to give a pH of approximately 7.4. The rings were placed under an optimal resting tension of 0.5 g, which had been determined in length–tension relationship experiments. The tissues were allowed to equilibrate for 90 min during which time the bath solution was replaced every 20 min with pre-warmed and oxygenated Krebs solution. The resting tension was readjusted to 0.5 g when necessary. 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 to 10^{-6} M acetylcholine. Removal of the endothelium was also evaluated by light microscopy of the histological section of the artery. Each experiment was performed on rings prepared from different rats.

2.2. Force measurement

In the first set of experiments, an endothelium-intact ring was contracted with phenylephrine applied cumulatively (ranging from 3×10^{-8} to 3×10^{-5} M) to obtain the first (control) concentration–response curve. Once the maximal response to phenylephrine had been obtained, the preparation was washed every 20 min with Krebs solution until the tension returned to the basal level. The ring was then exposed to berberine for 30 min and another cumulative concentration–response curve to phenylephrine was done.

In the second group of experiments, after a 60-min equilibration period, a steady contraction of the arterial rings with intact endothelium, in response to 3×10^{-6} M phenylephrine and to 3×10^{-8} M 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$, or of rings without endothelium in response to 10^{-6} M phenylephrine was induced, and berberine was then added cumulatively to evoke concentration-dependent relaxation. In experiments examining the role of endothelium-derived vasoactive factors, arterial rings were first exposed for 30 min to various inhibitors (3×10^{-5}–10^{-4} M N^G-nitro-L-arginine methyl ester (L-NAME), 3×10^{-6}–10^{-5} M methylene blue, 10^{-5} M indomethacin and 10^{-6} M glibenclamide) before they were contracted with phenylephrine to establish a sustained tone, berberine was then applied cumulatively. The effect of the vehicle was also tested. In the second group of experiments, endothelium-denuded rings were incubated for 30 min with putative K^+ channel inhibitors ($1–3 \times 10^{-6}$ M tetrapentylammonium, 3×10^{-3} M tetraethylammonium, 10^{-7} M charybdotoxin, 3×10^{-4} M Ba^{2+} and 10^{-3} M 4-aminopyridine) before application of phenylephrine, berberine was then added cumulatively to the bath solution. In some experiments, the phenylephrine-contracted arterial rings were first relaxed with 10^{-5} M berberine, and then re-contracted by application of 3×10^{-4} M Ba^{2+}, 10^{-3} M 4-aminopyridine or 3×10^{-6} M tetrapentylammonium.

In the third group of experiments using Ca^{2+}-free Krebs solution, the rings were exposed to Ca^{2+}-free solution containing 3×10^{-4} M Na_2-EGTA, washed with this solution twice and left for 15 min before application of 10^{-6} M phenylephrine or 10^{-2} M caffeine to induce the first transient contraction (T_1). The rings were thereafter rinsed twice with normal Krebs solution (30 min contact time for refilling of the intracellular stores) and twice with Ca^{2+}-free Krebs solution (15 min contact time). The second contraction (T_2) was then induced by phenylephrine or caffeine in the absence and presence of 10^{-5} M berberine (10 min contact time). The ratio of the second contraction to the first contraction (T_2/T_1) in Ca^{2+}-free solution was calculated.

In the fourth set of experiments, sustained contraction of both endothelium-intact and denuded arteries in response to 6×10^{-2} M K^+ was induced, and berberine was

then applied cumulatively. In these experiments, Na^+ ions were replaced with an equimolar concentration of K^+ to maintain the same ionic strength. In addition, the effect of berberine was tested on a contraction induced by 10^{-6} M phorbol 12,13-diacetate in a Ca^{2+}-free Krebs solution containing 5×10^{-4} M Na_2-EGTA.

Since pretreatment with some inhibitors of nitric oxide activity or K^+ channel blockers could enhance the level of the phenylephrine-induced tone, 10^{-6} M phenylephrine was used to produce a similar amplitude of muscle contraction in these experiments.

2.3. Measurement of $[Ca^{2+}]_i$

A7r5 cells (ATCC, Bethesda, MD, USA) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10^{-3} M penicillin–streptomycin (Gibco) in a humidified atmosphere (5% CO_2) at 37°C. Cells were cultured on 25-mm glass coverslips at a density of 5×10^4/coverslip, grown for 4–5 days, and serum-starved (i.e. 0.4% fetal bovine serum) for 24 h before the experiment. Cells were loaded with Fura-2 (Molecular Probes, USA) by incubation in medium containing the dye's membrane permeant, acetoxymethyl ester (AM) form (3×10^{-6} M) together with 1.6×10^{-6} M pluronic F127 for 60 min at 37°C. The cells were then rinsed and incubated for another 30 min in fresh serum-free medium to allow intracellular esterases to hydrolyze Fura-2/AM to Fura-2. The cell-containing coverslip was fixed to the bottom of an enclosed chamber (Warner Instrument, England) with Apiezon grease (M&I Materials, Manchester, England), whereas the top of the chamber was enclosed by another coverslip. The chamber, with an inlet port for perfusion and an outlet to drain away excess solution, was placed on an inverted microscope stage equipped with fluorescent optics (Nikon, Japan). Cells were continuously perfused with Krebs solution, which was maintained at exactly 37°C by a temperature controller and an inline solution heater (Warner Instrument). Changes of perfusate were achieved by manual operation of three-way valves and a perfusion pump system. Changes in $[Ca^{2+}]_i$ were monitored by Fura-2 ratiometric fluorescence measurement using a PTI RatioMaster Fluorescence System (Photon Technology International, NJ, USA). The cells were excited at 340 and 380 nm, and emission was monitored at 510 nm. The change in the 340/380 nm fluorescence ratios was used as an index of changes in $[Ca^{2+}]_i$.

2.4. Cell proliferation

Rat aortic smooth muscle cells (A7r5) were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 u ml^{-1} penicillin and 10^{-6} g ml^{-1} streptomycin. The proliferative response of vascular smooth muscle cells was determined by the uptake of [^{3}H]thymidine. A7r5 cells (10^4 cells/well) were cultured in a 96-well plate. After 24 h, the confluent smooth muscle cells were rendered quiescent by culturing them for 48 h in 0.4% (v/v) fetal bovine serum, together with berberine added to the culture medium at the desired concentrations. Subsequently, the cells were stimulated with 2% fetal bovine serum and treated with berberine for 3 days, and then rendered quiescent again in 0.4% fetal bovine serum for another 24 h, together with berberine. The cells were finally incubated in medium containing 5% fetal bovine serum and berberine for 24 h before the addition of [^{3}H]thymidine (1 μCi/well; Sigma, St. Louis, MO, USA). After 6 h, the cells were collected by cell harvester (Cambridge Technology, USA). [^{3}H]Thymidine incorporation into the DNA of A7r5 cells was counted in a scintillation counter. The results were expressed as counts per minute per well and the antiproliferative effect of berberine was expressed as a percentage of the control.

2.5. Drugs

The following chemicals and drugs were used: phenylephrine hydrochloride, acetylcholine hydrochloride, berberine, L-NAME, L-arginine, methylene blue, 4-aminopyridine, glibenclamide, tetraethylammonium chloride, tetrapentylammonium bromide, indomethacin, charybdotoxin, phorbol 12,13-diacetate, staurosporine, nifedipine, calcium ionophore A23187, caffeine, 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$ (Sigma). Berberine, glibenclamide, indomethacin nifedipine, staurosporine and phorbol 12,13-diacetate were dissolved in dimethyl sulfoxide (DMSO). Other drugs were dissolved in distilled water and further dilution was made with Krebs solution. DMSO at 0.2% (v/v) did not affect the berberine-induced relaxation.

2.6. Data analysis

The relaxant response to berberine in the absence and presence of various inhibitors was expressed as a percentage of the agonist-induced contraction. IC_{50} values (the concentration producing a 50% maximum relaxation, E_{max}) were calculated by non-linear regression analysis of the concentration–response curves using all the data point. The results are presented as means ± S.E.M. of *n* experiments. Statistical analysis of the results was performed with Student's two-tailed *t*-test. A probability value less than 0.05 was considered as significant.

3. Results

3.1. Vasorelaxant effect of berberine

Phenylephrine contracted the rat isolated mesenteric artery rings with an EC_{50} of $6.0 \pm 0.07 \times 10^{-7}$ M and a

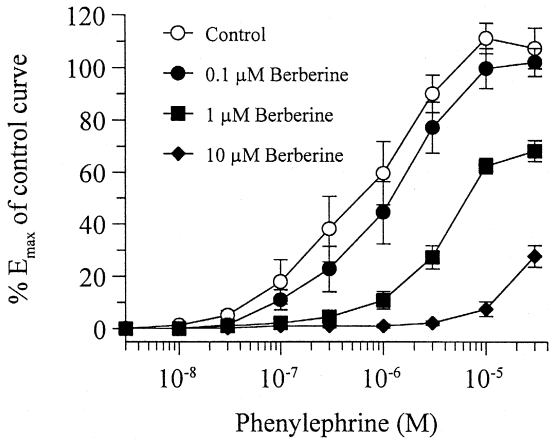

Fig. 1. Logarithmic concentration–response curves of contractile responses of rat mesenteric artery rings to phenylephrine in the absence (○, $n = 5$) and presence of berberine (●, 10^{-7} M, $n = 5$; ■, 10^{-6} M, $n = 5$; ◆, 10^{-5} M, $n = 5$). Berberine was applied 30 min before the second concentration–response curve was repeated. Data are expressed as percentages of the maximum contraction obtained in the first (control) concentration–response curve. Curves were drawn by connection of the adjacent points. Results are means ± S.E.M. of n experiments.

maximum increase in tension of 10.1×0.6 mN ($n = 5$). After a control curve for the phenylephrine-induced contractile response, the rings were incubated for 30 min with different concentrations of berberine and a concentration–response curve for phenylephrine was again obtained. A low concentration of berberine (10^{-7} M) caused an approximately parallel shift of the phenylephrine concentration–response curve to the right (IC_{50}: $1.01 \pm 0.09 \times 10^{-6}$ M, $n = 5$, $P < 0.05$ compared with control, Fig. 1). At higher concentrations, berberine (10^{-6}–10^{-5} M) exerted an insurmountable inhibition, reducing the magnitude of the maximum contraction (E_{max}: $107.6 \pm 7.8\%$, $68.5 \pm 5.4\%$, and $27.9 \pm 4.1\%$, respectively, for control, 10^{-6} and 10^{-5} M berberine, $n = 5$ in each case, $P < 0.05$ compared with control).

Phenylephrine produced a steady contraction in the rat isolated mesenteric artery rings (4.9 ± 0.4 mN, $n = 17$, by 3×10^{-6} M phenylephrine, with endothelium; 6.6 ± 0.4 mN, $n = 19$, by 10^{-6} M phenylephrine, without endothelium). Tracings in Fig. 2 show that berberine (10^{-7}–3×10^{-5} M) induced a concentration-dependent relaxation in phenylephrine-precontracted artery rings. Berberine caused complete relaxation of endothelium-intact (Fig. 2a) and -denuded (Fig. 2b) artery rings. In endothelium-intact rings, berberine induced relaxation with an IC_{50} of $1.48 \pm 0.16 \times 10^{-6}$ M ($n = 17$, Fig. 2c). Functional removal of the endothelium attenuated the berberine-induced relaxation without an effect on the maximum response (IC_{50}: $4.73 \pm 0.32 \times 10^{-6}$ M, $n = 19$, $P < 0.05$ compared with the value obtained with endothelium, Fig. 2c). Berberine (3×10^{-5} M) did not affect the basal tone ($n = 6$, with endothelium; $n = 5$, without endothelium). Similarly, berberine (3×10^{-7}–3×10^{-5} M) also reduced 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$ (3×10^{-8} M)-induced sustained contraction with an IC_{50} of $2.23 \pm 0.22 \times 10^{-6}$ M and a $80.1 \pm 4.8\%$ maximum relaxation ($n = 5$, with endothelium). In either phenylephrine- or 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$-contracted rings, contractility was totally restored after berberine washout ($n = 4$–6).

3.2. Effect of berberine on high K^+ response and A23187 response

Berberine induced relaxation of artery rings contracted with 6×10^{-2} M K^+ (Fig. 3a). The relaxant effect of berberine was greater in rings with intact endothelium (high-K^+ contraction: 8.3 ± 1.0 mN, IC_{50}: $4.41 \pm 0.47 \times 10^{-6}$ M, E_{max}: $60.5 \pm 3.7\%$, $n = 8$) than in those without endothelium (high-K^+ contraction: 8.6 ± 1.0 mN, IC_{50}: $8.73 \pm 0.74 \times 10^{-6}$ M, E_{max}: $54.6 \pm 3.5\%$, $n = 6$, Fig. 3b). It is apparent that berberine was less effective against the high-K^+ response. Berberine at 3×10^{-5} M induced approximately 60% ($n = 8$) inhibition of the high-K^+-induced contraction, while this concentration completely re-

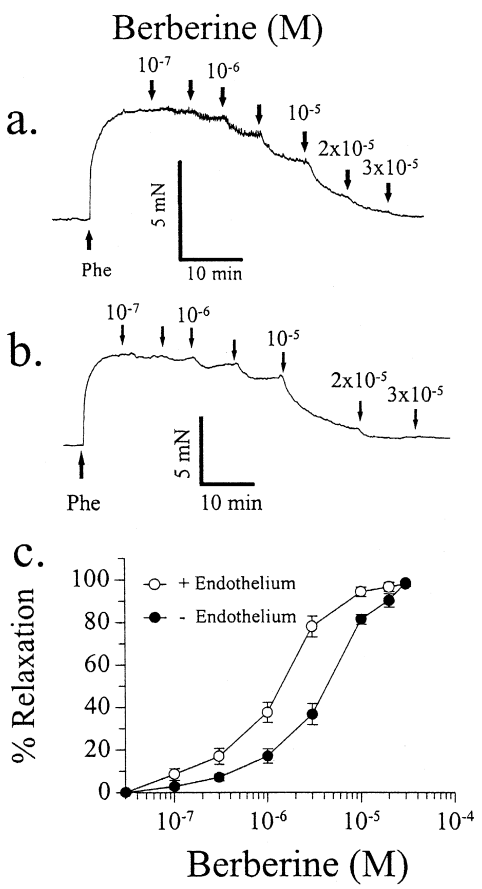

Fig. 2. Representative traces showing the relaxant effect of berberine on the phenylephrine-contracted arterial rings with (a) and without endothelium (b). (c) Logarithmic concentration–response curves for the relaxant effect of berberine (○, with endothelium, $n = 17$; ●, without endothelium, $n = 19$). Results are presented as means ± S.E.M. of n experiments.

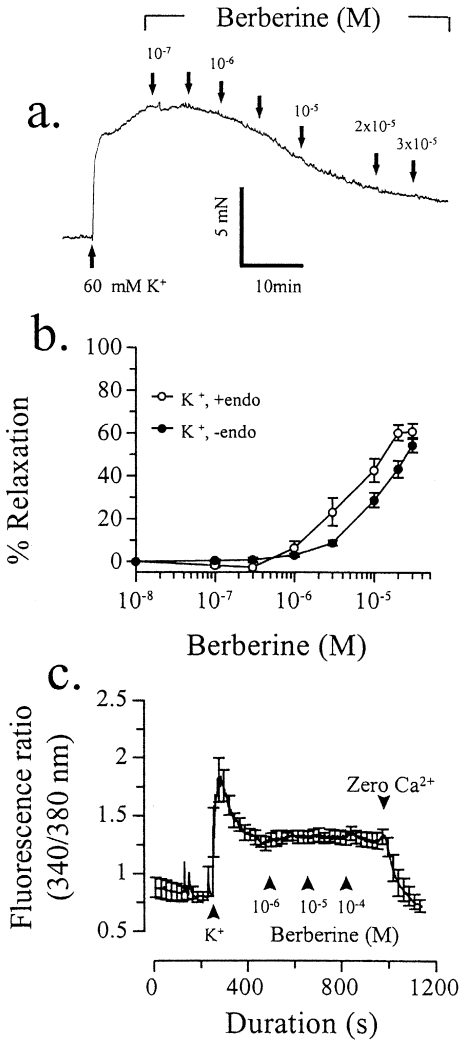

Fig. 3. (a) Representative record showing concentration-dependent relaxation induced by berberine in high K^+-contracted endothelium-intact artery. (b) Logarithmic concentration–response curves for the relaxant effect of berberine in high-K^+-contracted arteries (○, $n=8$, with endothelium; ●, $n=6$, without endothelium). (c) The lack of effect of berberine on the 6×10^{-2} M K^+-induced increase of $[Ca^{2+}]_i$ in cultured rat aortic smooth muscle cells. Berberine (10^{-6}–10^{-4} M) was applied through a perfusion system at a flow rate of 4 ml/min. Change in $[Ca^{2+}]_i$ was expressed as 340/380 nm fluorescence ratio. The results are means ± S.E.M. of five independent experiments.

laxed the phenylephrine-contracted rings. Both nifedipine (5×10^{-9} M) and staurosporine (10^{-7} M) abolished the high-K^+ response ($n=5$, in each case).

A23187 at 10^{-5} M induced a small and steady contraction (3.3 ± 0.5 mN, $n=4$) in endothelium-denuded rings and application of 5×10^{-5} M berberine reduced the A23187-evoked contraction by $74.4\pm4.8\%$ ($n=4$).

3.3. Effect of berberine on transient contractions in $Ca2^+$-free solution

Fig. 4 shows that in endothelium-denuded rings, a transient contractile response in Ca^{2+}-free Krebs solution was induced by 10^{-2} M caffeine (T_1: 1.41 ± 0.3 mN; T_2: 1.40 ± 0.3 mN, $n=4$) or by 10^{-6} M phenylephrine (T_1: 2.2 ± 0.23 mN; T_2: 2.1 ± 0.24 mN, $n=5$). Pretreatment with 10^{-5} M berberine (10 min contact time) reduced the caffeine- or phenylephrine-induced contraction by $74\pm3.6\%$ and $47\pm6.5\%$, respectively ($P<0.05$, Fig. 4).

3.4. Effects of L-NAME and methylene blue on berberine-induced relaxation

Since berberine induced both endothelium-dependent and -independent relaxation in rat isolated mesenteric arteries, an attempt was made to investigate what endothelium-derived vasoactive factors contributed to the berberine-induced relaxation. Fig. 5a shows that pretreatment of the endothelium-intact ring with L-NAME attenuated the berberine-induced relaxation without affecting the maximum relaxation, while L-arginine at 10^{-3} M partially antagonized the effect of 10^{-4} M L-NAME. L-NAME at 10^{-4} M also inhibited the endothelium-dependent relaxation induced by acetylcholine (IC_{50}: $2.0\pm0.2\times10^{-8}$ M, E_{max}: 100%, $n=4$ for control; IC_{50}: $1.2\pm0.9\times10^{-7}$ M, E_{max}: $60.3\pm2.6\%$, $n=4$ for L-NAME, $P<0.05$). In addition, methylene blue caused a significant rightward shift of the concentration–response curve for berberine in endothelium-intact rings without affecting the maximum response (Fig. 5b). In contrast, glibenclamide (10^{-6} M) and indomethacin (10^{-5} M) had no effect (Table 1). Table 1 summarizes the IC_{50} and E_{max} values for the relaxant effect of berberine with various treatments.

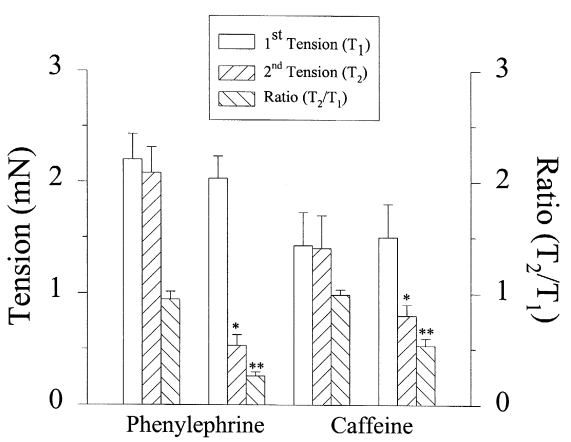

Fig. 4. Two transient contractile responses of endothelium-denuded rings induced by 10^{-6} M phenylephrine or 10^{-2} M caffeine in Ca^{2+}-free Krebs solution. The peak amplitude in mN for the first (T_1, open bar) and second (T_2, left diagonal) contractions in the absence and presence of 10^{-5} M berberine (10 min contract time), and the ratio (T_2/T_1) of the second contraction to the first contraction (right diagonal) are presented. Difference in the ratios is indicated between the first and second contraction (*) and between control and treatment group (**). Values are means ± S.E.M. from 4–5 separate experiments.

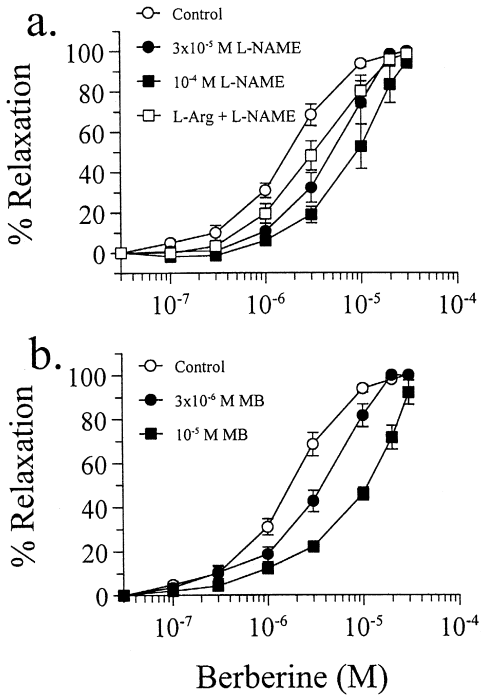

Fig. 5. (a) Logarithmic concentration–response curves for the relaxant effect of berberine in endothelium-intact arteries (○, $n=8$ for control; ●, $n=6$ for 3×10^{-5} M L-NAME; ■, $n=5$ for 10^{-4} M L-NAME; □, $n=6$ for 10^{-3} M L-arginine plus 10^{-4} M L-NAME). (b) Concentration–response curves for the relaxant effect of berberine in control (○, $n=8$), in the presence of 3×10^{-6} M MB (●, $n=6$) and 10^{-5} M MB (■, $n=4$). Tissues were exposed to L-NAME or MB for 30 min prior to application of phenylephrine. L-Arginine was added 10 min before the application of L-NAME. Results are means ± S.E.M. of n experiments.

3.5. Effect of K^+ channel blockers on berberine-induced relaxation

The reduced potency of berberine to relax the high K^+-precontracted arteries suggested that activation of K^+ channels may also make a contribution. Various K^+ channel blockers were therefore tested for their possible inhibitory effects on berberine-induced relaxation. Pretreatment of endothelium-denuded rings with tetrapentylammonium ions ($1–3\times10^{-6}$ M) significantly shifted to the right the concentration–relaxation curve for berberine without affecting the maximum response (Fig. 6a, Table 1). Pretreatment with 3×10^{-4} M Ba^{2+} or 3×10^{-3} M 4-aminopyridine also attenuated the berberine-induced relaxation (Fig. 6b, Table 1). In contrast, pretreatment with 3×10^{-3} M tetraethylammonium ions or 10^{-6} M glibenclamide had no effect (Fig. 6c, Table 1). Charybdotoxin slightly potentiated the berberine-induced relaxation (Fig. 6c, Table 1).

In phenylephrine-precontracted endothelium-denuded rings, berberine at 10^{-5} M caused a 79.3 ± 4.2% relaxation ($n=12$). Subsequent application of three putative K^+ channel blockers, Ba^{2+} (3×10^{-4} M), 4-aminopyridine (3×10^{-3} M) or tetrapentylammonium (3×10^{-6} M), partially reversed the berberine-induced relaxation by 46.5 ± 5.8%, 45.8 ± 10.5% and 42.2 ± 6.1%, respectively ($n=4$ in each case).

3.6. Effect of berberine on $[Ca^{2+}]_i$ in cultured aortic smooth muscle cells

The relaxant effect on the high K^+-contracted arteries (Fig. 3a and b) indicates that berberine may inhibit Ca^{2+} influx; therefore, a possible inhibitory effect of berberine was tested on $[Ca^{2+}]_i$ in a confluent layer of aortic myocytes. In cultured A7r5 cells, the basal $[Ca^{2+}]_i$ measured as 340/380 nm fluorescence ratio was 0.84 ± 0.05 ($n=5$). Perfusion of 6×10^{-2} M K^+ induced a biphasic increase in $[Ca^{2+}]_i$, a rapid rise (1.85 ± 0.17, $n=5$) followed by a sustained component (1.31 ± 0.05, $n=5$, Fig. 3c). Fig. 3c shows that berberine (10^{-6}–10^{-4} M) did not affect the sustained increase of $[Ca^{2+}]_i$ in response to 6×10^{-2} M K^+. The high-K^+-induced response was completely reversed when the perfusion solution was switched to Ca^{2+}-free solution. In some experiments, a transient rise of $[Ca^{2+}]_i$ was induced twice (S_1 and S_2) by brief exposure to 6×10^{-2} M K^+ of an interval of about 7 min. Berberine at 10^{-4} M, when added before the second high-K^+ stimulation, was found to be ineffective against the high-K^+ response (the ratio of S_2/S_1: 101 ± 8% for vehicle DMSO and 91 ± 5% for berberine, $P>0.05$, $n=4$ in each case). Berberine at a concentration higher than 3×10^{-5} M was autofluorescent in cell-free Krebs solution, therefore, the effect of berberine on the high-K^+-induced rise of $[Ca^{2+}]_i$ (Fig. 3c) was revealed after subtraction of

Table 1
Effects of nitric oxide inhibitors on berberine-induced relaxation

	IC_{50} (10^{-6} M)	E_{max} (%)	n
Nitric oxide inhibitors			
Control (with endothelium)	1.48 ± 0.16	100	8
+ 3×10^{-5} M L-NAME	5.94 ± 0.61[a]	100	6
+ 10^{-4} M L-NAME	7.45 ± 0.71[a]	94.9 ± 3.4	5
+ 10^{-3} M L-arginine + L-NAME	3.62 ± 0.27[a,b]	98.8 ± 0.9	6
+ 3×10^{-6} M MB	4.89 ± 0.90[a]	100	6
+ 10^{-5} M MB	7.61 ± 0.87[a]	92.2 ± 5.5	4
+ 10^{-6} M glibenclamide	1.83 ± 0.18	100	7
+ 10^{-5} M indomethacin	1.58 ± 0.2	98 ± 3.2	5
K^+ channel inhibitors			
Control (without endothelium)	3.42 ± 0.42	100	18
+ 10^{-6} M TPA^+	4.60 ± 0.39[a]	95.7 ± 1.2	8
+ 3×10^{-6} M TPA^+	10.3 ± 0.15[a]	90.7 ± 3.5	8
+ 3×10^{-4} M Ba^{2+}	6.09 ± 0.59[a]	100	5
+ 3×10^{-3} M 4-Aminopyridine	7.80 ± 0.76[a]	89.9 ± 9.8	5
+ 3×10^{-3} M TEA^+	4.13 ± 0.46	100	5
+ 10^{-7} M CTX	2.32 ± 0.18[a]	97.6 ± 1.1	4
+ 10^{-6} M Glibenclamide	3.54 ± 0.37	100	4

Values are means ± S.E.M. of n experiments. IC_{50} was the drug concentration causing 50% maximum relaxation (E_{max}).

[a] $P<0.05$ between the control and treatment group.

[b] $P<0.05$ between L-NAME group and L-arginine + L-NAME group is indicated (10^{-4} M L-NAME, 10^{-3} M L-arginine).

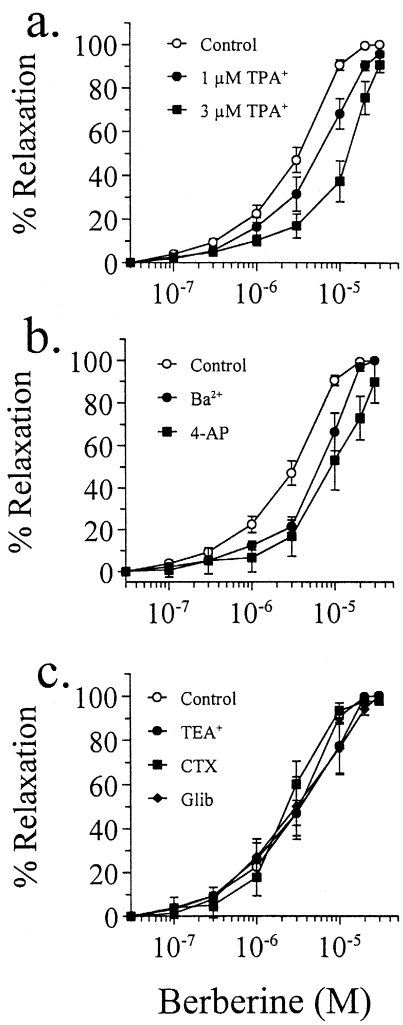

Fig. 6. (a) Logarithmic concentration–response curves for the relaxant effect of berberine on endothelium-denuded arteries (○, $n=18$ for control; ●, $n=8$ and ■, $n=8$ in the presence of 10^{-6} and 3×10^{-6} M TPA^+, respectively). (b) Logarithmic concentration–response curves for the relaxant effect of berberine on endothelium-denuded arteries (○, $n=18$ for control; ●, $n=5$ for 3×10^{-4} M Ba^{2+}; ■, $n=5$ for 3×10^{-3} M 4-AP). (c) Logarithmic concentration–response curves for the relaxant effect of berberine in endothelium-denuded arteries (○, $n=18$ for control; ●, $n=5$ for 3×10^{-3} M TEA^+; ■, $n=4$ for 10^{-8} M CTX; ♦, $n=4$ for 10^{-6} M glibenclamide). Tissues were exposed to each putative K^+ channel blocker for 30 min prior to the application of phenylephrine. Results are presented as means ± S.E.M. of n experiments. 4-AP, TEA^+, tetraethylammonium; TPA^+, tetrapentylammonium; 4-aminopyridine; CTX, charybdotoxin.

this autofluorescent intensity in time-matched control experiments.

3.7. Effect of berberine on phorbol ester-induced contraction

Since berberine inhibited the high-K^+-induced relaxation in rat mesenteric arteries but failed to reduce the 6×10^{-2} M K^+-induced increase of $[Ca^{2+}]_i$ in cultured rat aortic smooth muscle cells, it is possible that berberine

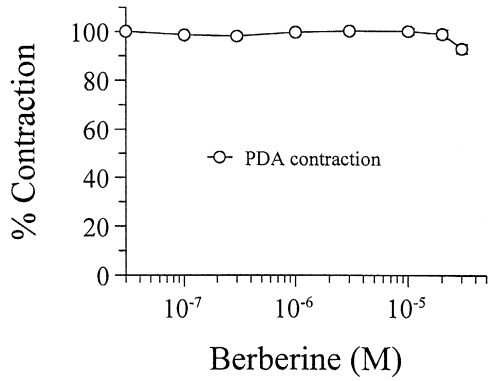

Fig. 7. Lack of effect of berberine on the PDA (10^{-6} M)-contracted endothelium-denuded arteries in the absence of extracellular Ca^{2+}. The results are means ± S.E.M. of five experiments.

relaxed the artery rings through Ca^{2+}-independent pathways. In order to test this possibility, phorbol 12,13-diacetate, a protein kinase C activator, was used to evoke a sustained contraction in the absence of extracellular Ca^{2+} (zero Ca^{2+} plus 5×10^{-4} M Na_2-EGTA). Phorbol 12,13-diacetate at 10^{-6} M induced a slowly developing contraction of endothelium-denuded rings and the maximum sustained tension was 11.6 ± 1.3 mN ($n=5$). Fig. 7 shows that berberine (10^{-7}–3×10^{-5} M) did not affect phorbol 12,13-diacetate-induced contraction ($n=4$). In contrast, staurosporine, a protein kinase C inhibitor, at 10^{-7} M completely abolished the contraction induced by 10^{-6} M phorbol 12,13-diacetate in Ca^{2+}-free solution ($n=4$).

3.8. Effect of berberine on cell proliferation

In cultured rat aortic smooth muscle cells, berberine (3×10^{-7}–10^{-4} M) inhibited [^{3}H]thymidine incorporation into DNA in a concentration-dependent manner (IC_{50}: $2.29\pm0.04\times10^{-5}$ M, $n=6$, Fig. 8). The control value for [^{3}H]thymidine incorporation is presented as a percent-

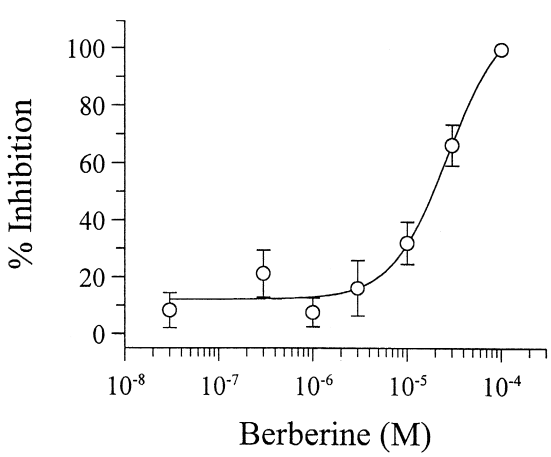

Fig. 8. Inhibitory effect of berberine (3×10^{-7} $\times10^{-4}$ M) on [^{3}H]thymidine incorporation into rat aortic cells (A7r5). The results are means ± S.E.M. of six experiments.

age of control in the presence of vehicle (0.2% DMSO) was 38686 ± 3061 cpm/well (10^4 cells) ($n = 6$). Berberine at 10^{-4} M almost totally suppressed cell proliferation (242 ± 50 cpm/well, $n = 6$).

4. Discussion

Berberine was found to exert a hypotensive action in rats (Chun et al., 1979) and to raise intracavernous pressure in rabbits (Chiou et al., 1998), however, the mechanism responsible for its vasodilator effect is not clear. The present study showed a relaxant action of berberine in rat isolated mesenteric arteries. Berberine at concentrations higher than 10^{-7} M showed a non-competitive antagonism against the phenylephrine-induced contraction. It was reported before that berberine derivatives may have an α_1-adrenoceptor antagonist effect (Olmez and Ilhan, 1992). Although berberine produced similar degrees of relaxation in endothelium-intact rings contracted by phenylephrine and by 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$, and the maximal contractile response to phenylephrine was reduced by berberine at concentrations of 1 and 10 μM, the present results cannot rule out the possibilities that either berberine was an irreversible competitive antagonist for α_1-adrenoceptors (with receptor reserve in the tissue) or berberine competitively antagonized α_1-adrenoceptors at the same time as activating the K^+ channels and inhibiting intracellular Ca^{2+} release.

Removal of the endothelium partially, but significantly, attenuated the relaxation induced by berberine at submaximal concentrations. L-NAME and methylene blue, inhibitors of nitric oxide-mediated vasodilation, reduced the berberine-induced relaxation to a similar extent. On the other hand, pretreatment of endothelium-intact rings with the nitric oxide precursor, L-arginine, partially antagonized the effect of L-NAME. However, this did not affect the endothelium-independent relaxation induced by sodium nitroprusside, an exogenous nitric oxide donor (Huang et al., 1998). In response to neurohumoral mediators, the intact endothelium of small arteries also secretes other vasodilator agents, such as an as-yet-unidentified endothelium-derived hyperpolarizing factor (Félétou and Vanhoutte, 1988; Chen and Suzuki, 1990) and prostanoids. In addition, nitric oxide was found to hyperpolarize rabbit mesenteric arteries via ATP-sensitive K^+ (K_{ATP}) channels (Murphy and Brayden, 1995). Neither glibenclamide, an inhibitor of arterial K_{ATP} channels (Standen et al., 1989), nor indomethacin, an inhibitor of endothelial prostacyclin biosynthesis, influenced the relaxant response to berberine. These results suggest that endothelial nitric oxide is the major factor responsible for the berberine-induced relaxation in rat mesenteric artery rings. These data are consistent with previous reports on the isolated arteries from rats and rabbits (Chiou et al., 1991, 1998). However, it remains to be examined how berberine would stimulate nitric oxide release in the endothelial cells.

K^+ channels play an important role in the regulation of muscle contractility and vascular tone (Nelson and Quayle, 1995). In many instances, the vasodilation mediated by membrane hyperpolarization is attributed to a rise in K^+ permeability. Direct activation of K^+ channels on arterial smooth muscle cells normally hyperpolarizes the cell membrane and thus inhibits Ca^{2+} influx through voltage-sensitive Ca^{2+} channels. There are several types of K^+ conductance present in vascular smooth muscle and they are subject to modulation by various factors (Nelson and Quayle, 1995). For example, the activity of charybdotoxin-sensitive Ca^{2+}-activated K^+ (K_{Ca}) channels is related to changes in myogenic tone in pressurized cerebral arteries (Brayden and Nelson, 1992), while glibenclamide-sensitive K_{ATP} channels are associated with the metabolic state in vasculature (Nelson and Quayle, 1995). The present results show that when contractions of similar size are induced by phenylephrine or by high-K^+, the latter were less strongly reduced by berberine. It is generally thought that one consequence of raising the extracellular K^+ concentration would be a reduction in the electrochemical gradient for K^+ efflux and, thus, the effect of a vasodilator, depending on a K^+ channel activation mechanism (Adeagbo and Triggle, 1993). The berberine-induced relaxation in endothelium-denuded rings was significantly attenuated by putative K^+ channel blockers, such as tetrapentylammonium, Ba^{2+} and 4-aminopyridine. Our previous study showed that tetrapentylammonium inhibited K_{Ca} channels in single arterial myocytes isolated from rabbit mesenteric arteries with a K_d value of 1.49 mM (Langton et al., 1991), while tetrapentylammonium at low concentrations (1–3 μM) reduced the relaxant effect of berberine. Besides, more potent blockers of K_{Ca} channels in vascular smooth muscle, such as charybdotoxin and tetraethylammonium (10^{-3} M), did not inhibit berberine-induced relaxation, suggesting that K_{Ca} channels are not involved. These results contrast with a recent report on rabbit corpus cavernosal tissue where charybdotoxin significantly inhibited berberine-induced relaxation (Chiou et al., 1998). This discrepancy may be caused by the use of different vascular preparations or different species. Berberine induced complete relaxation in rat mesenteric artery rings as observed in our study, while it only produced an approximately 60% maximum relaxation in endothelium-denuded rabbit corpus cavernosum (Chiou et al., 1998).

K_{ATP} channels also were not involved since glibenclamide did not inhibit the berberine-induced relaxant effect. Similarly, glibenclamide had no effect on the berberine-induced relaxation in rabbit corpus cavernosum (Chiou et al., 1998). Consistent with the results reported by Chiou et al. (1998), the berberine-induced relaxation was effectively inhibited by 4-aminopyridine. 4-Aminopyridine is an inhibitor of voltage-gated K^+ channels in smooth muscle (Okabe et al., 1987; Robertson and Nelson, 1994). In addition, we showed that the berberine-induced relaxation was also inhibited by Ba^{2+} ions that inhibit the inwardly

rectifying K^+ channels and other K^+ channels in vascular smooth muscle (Standen et al., 1989; Nelson and Quayle, 1995). It is probable that berberine activates some non-selective K^+ conductance in rat endothelium-denuded mesenteric arteries, which contributes in part to the endothelium-independent relaxation. Nevertheless, further electrophysiological investigation is needed to confirm the stimulatory effect of berberine on K^+ channels in arterial smooth muscle cells.

The present results demonstrated that berberine relaxed the high-K^+-contracted rings in a concentration-dependent manner, but could not induce a full relaxant response. In contrast, berberine in the same concentration range failed to influence the high-K^+ response of rat mesenteric artery (Chiou et al. , 1991), guinea-pig aorta (Bova et al., 1992) and rabbit corpus cavernosum (Chiou et al., 1998). It is not known what causes this difference. Conflicting data were also reported for the possible effect of berberine on the intracellular Ca^{2+}-mediated contractile response in different arterial tissues (Chiou et al., 1991; Bova et al., 1992). The high-K^+-induced contraction is usually caused by an increased Ca^{2+} influx through voltage-sensitive Ca^{2+} channels since the dihydropyridine antagonist, nifedipine at 5×10^{-9} M, completely abolished the high-K^+ response in the present study. In order to test whether berberine may inhibit Ca^{2+} influx, its effect was examined on the high-K^+-induced increase of $[Ca^{2+}]_i$ in cultured aortic smooth muscle cells (A7r5). It was surprising to note that berberine did not affect the $[Ca^{2+}]_i$ that had been raised by 6×10^{-2} M external K^+. This indicates that mechanisms, other than inhibition of Ca^{2+} channels may underlie the endothelium-independent relaxation induced by berberine. However, berberine was reported to inhibit both L- and T-type voltage-gated Ca^{2+} currents in guinea-pig ventricular myocytes (Xu et al., 1997). Alternatively, the effect of berberine on Ca^{2+} channels may be tissue dependent. However, we did observe an inhibitory action of berberine on the contraction induced by calcium ionophore A23187, implying that the relaxant effect of berberine on the high K^+ response may be mediated by intracellular mechanisms. Berberine was reported to abolish the caffeine-induced transient contraction in the rat artery (Chiou et al., 1991), but had no effect in the guinea-pig aorta (Bova et al., 1992). The present study also showed that berberine reduced the transient contractile response to either phenylephrine or caffeine in Ca^{2+}-free bath solution. However, Ca^{2+} from intracellular stores normally plays a minor role in the agonist-induced sustained contraction in arteries, therefore, a possible inhibition of internal Ca^{2+} release may account in only a small part for the berberine-induced relaxation.

Since the α_1-adrenoceptor-mediated vasoconstriction was inhibited by a protein kinase C inhibitor, staurosporine (Huang, 1996), it was worthwhile testing whether berberine could antagonize the protein kinase C-mediated contractile response. The present study showed that phorbol 12,13-acetate, an active phorbol ester, produced a slow tonic contraction in the absence of extracellular Ca^{2+}, indicating that protein kinase C activation could interact with contractile filaments at the resting $[Ca^{2+}]_i$ (Rasmussen et al., 1987). However, berberine did not affect the phorbol-induced contraction within a concentration range that relaxed the agonist-contracted arteries, suggesting that berberine did not influence protein kinase C-mediated contractile mechanisms in rat mesenteric arteries. It cannot be excluded that berberine may affect other intracellular second messenger pathways leading to endothelium-independent vasorelaxation in addition to its possible activator effect on arterial K^+ channels.

Many factors may contribute to a long-term beneficial effect of berberine on the cardiovascular system. For example, vascular smooth muscle proliferation is an essential factor involving the formation of atherosclerotic plaques (Ross, 1993). In the present study, we also examined the possible inhibitory effect of berberine on aortic smooth muscle cell proliferation. We found that berberine significantly suppressed cell proliferation within a concentration range that induced relaxation in rat mesenteric arteries. This study represents the first attempt to describe the antiproliferative action of berberine in vascular smooth muscle cells.

In summary, the present results showed that berberine induced both endothelium-dependent and -independent relaxation in rat isolated mesenteric arteries. Nitric oxide, but not other endothelium-derived factors, is likely involved in the endothelium-dependent relaxation, while the stimulatory effect of berberine on tetrapentylammonium-, Ba^{2+}- and 4-aminopyridine-sensitive arterial K^+ channels and inhibition of intracellular Ca^{2+} release contribute in part to the endothelium-independent relaxation. It appears that berberine does not interfere with either Ca^{2+} influx or protein kinase C-mediated contractile mechanisms. It has yet to be determined whether or not berberine relaxed blood vessels partially by inhibiting vasoconstrictor-induced phospholipase C-mediated inositol triphosphate production that would reduce intracellular Ca^{2+} release. Both vasodilator and antiproliferative activity of berberine would contribute to its long-term beneficial effect in the cardiovascular system.

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