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Abnormal activation of K^+ channels in aortic smooth muscle of rats with endotoxic shock: electrophysiological and functional evidence

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1 This study examined the role of K^+ channels in vascular hyporeactivity of rats with endotoxic shock *ex vivo*.

2 At the end of the *in vivo* experiments, thoracic aortas were removed from endotoxaemic and control rats. After removal of the endothelium, aortic segments were mounted in myographs for recording of isometric tension and smooth muscle membrane potential.

3 Membrane potentials recorded from endotoxaemic rats were hyperpolarized compared to those of the controls. This hyperpolarization was partially reversed by tetraethylammonium, charybdotoxin or glibenclamide, but not significantly affected by apamin. The hyperpolarization was also partially attenuated by N^{ω}-nitro-L-arginine methyl ester (L-NAME) or 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-l-one (ODQ).

4 In phenylephrine-contracted aortic rings, both agonists of K^+ channels, NS1619 and pinacidil, induced greater relaxations and re-polarizations in the preparations obtained from endotoxaemic rats. The NS1619-induced relaxation and re-polarization in arteries from endotoxaemic rats were partially inhibited by tetraethylammonium and completely inhibited by charybdotoxin, L-NAME or ODQ, but not significantly affected by apamin. Similarly, the greater relaxation and re-polarization induced by pinacidil in arteries from endotoxaemic rats were also inhibited by glibenclamide, L-NAME or ODQ. However, these inhibitors had no significant effect on relaxations and re-polarizations induced by NS1619 and pinacidil in arteries from controls.

5 This study provides the electrophysiological and functional evidence showing an abnormal activation of K^+ channels in vascular smooth muscle in animals with endotoxic shock. Our observations suggest that overproduction of nitric oxide causes an activation of large conductance Ca^{2+}-activated K^+ channels and ATP-sensitive K^+ channels which contributes to endotoxin-mediated vascular hyporeactivity.

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Keywords: K^+ channels; nitric oxide; membrane potential; relaxation; endotoxin

Abbreviations: BK channels, large conductance Ca^{2+}-activated K^+ channels; cyclic GMP, guanosine 3′5′ cyclic monophosphate; GC, guanylyl cyclase; HR, heart rate; K_{ATP} channels, ATP-sensitive K^+ channels; L-NAME, N^{ω}-nitro-L-arginine methyl ester; MAP, mean arterial pressure; NO, nitric oxide; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-l-one

Introduction

In the clinic, septic shock is a major cause of death among patients in intensive care units and ranks high in the overall causes of death. Septic shock is characterized by a decrease in systemic vascular resistance. In laboratory studies, the administration of endotoxin (lipopolysaccharide) or cytokines to animals produces a shock-like syndrome, characterized by low blood pressure and vascular hyporeactivity to vasoconstrictor agents such as noradrenaline (Fink & Heard, 1990; Bone, 1991). In addition, isolated blood vessels exposed to endotoxin *in vitro* or obtained from endotoxin-treated animals *ex vivo* also show diminished responses to vasoconstrictor agents (Rees *et al*., 1990; Fleming *et al*., 1991; Joly *et al*., 1994; Wu *et al*., 1995).

Recent evidence indicates that an enhanced formation of nitric oxide (NO), principally by the calcium-independent inducible isoform of NO synthase, contributes to hypotension and hyporeactivity to endogenous and exogenous vasoconstrictor agents in septic shock (see Moncada *et al*., 1991; Thiemermann, 1994). The mechanism of NO-induced relaxation in blood vessels appears to be associated with activation of soluble guanylyl cyclase (GC) and elevation of guanosine 3′5′ cyclic monophosphate (cyclic GMP) (see Moncada *et al*., 1991; McDonald & Murad, 1996). However, the molecular mechanism by which cyclic GMP decreases arterial tone still remains uncertain. There is good evidence that cyclic GMP reduces agonist-induced increases in intracellular calcium, at least in part, through membrane hyperpolarization (see McDonald & Murad, 1996). This conclusion is consistent with those of previous studies showing that NO and cyclic GMP-elevating agents could hyperpolarize some vascular beds (Ito *et al*., 1978; Tare *et al*., 1990; Garland & McPherson, 1992; Krippeit-Drews *et al*., 1992). Permeability to K^+ is one of the most important factors controlling the membrane potential, and is also important in controlling vascular tone (see Nelson *et al*., 1990). Indeed, Williams *et al*. (1988) and Fujino *et al*. (1991) reported that nitrovasodilators and exogenous cyclic GMP

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activated large conductance Ca^{2+}-activated K^+ (BK) channels. It has been shown that subsequent binding of NO to cyclic GMP-dependent protein kinase (see Schmidt *et al*., 1993) results in the phosphorylation and activation of BK channels in vascular smooth muscle cells (Taniguchi *et al*., 1993; Archer *et al*., 1994; Bolotina *et al*., 1994). These channels are associated with the autoregulation of blood flow in the brain (Asano *et al*., 1993). In addition, ATP-sensitive K^+ (K_{ATP}) channels have been also identified as regulators of membrane potential of vascular smooth muscle (Standen *et al*., 1989). The opening of BK and K_{ATP} channels in the cell membrane of the microcirculation arterioles increases K^+ efflux, which causes membrane potential hyperpolarization. Thus, these channels are believed to control myogenic tone in blood vessels (see Nelson & Quayle, 1995).

Our previous study demonstrated that altered K^+ channels function could occur in septic shock since glibenclamide reversed much of the hypotension associated with animal models of endotoxic shock (Wu *et al*., 1995). Recent studies also revealed that abnormal activation of K^+-channels, possibly BK and K_{ATP} channels, could be responsible for endotoxin-induced hypotension and hyporeactivity to vasoconstrictor agents in vascular smooth muscle (Hall *et al*., 1996; Sorrentino *et al*., 1999). However, no direct membrane potential recording evidence has been reported in isolated blood vessels obtained from endotoxaemic animals. Therefore, we examined the role of K^+ channels in aortas from controls and lipopolysaccharide-treated rats by simultaneously measuring the membrane potential and the basal tension as well as the relaxation induced by two types of K^+ channels agonists (i.e. NS1619 for BK channels and pinacidil for K_{ATP} channels).

Methods

In vivo *experiments*

Ten-week-old male Wistar-Kyoto (WKY) rats, whose stock originated from the Charles River Breeding Laboratories in Japan, were purchased from the Department of Laboratory Animal Science of the National Defense Medical Centre. This study was approved by the local Institutional Review Board according to the recommendations from Helsinki and the internationally accepted principles in the care and the use of experimental animals. Rats were anaesthetized by intraperitoneal injection of a combination of thiobutabarbital (80 mg kg^{-1}) with urethane (0.4 g kg^{-1}). The trachea was cannulated to facilitate respiration and environmental temperature was maintained at 24°C with an air-conditioning system. The right carotid artery was cannulated and connected to a pressure transducer (P23ID, Statham, Oxnard, CA, U.S.A.) for the measurement of phasic blood pressure and mean arterial blood pressure (MAP) as well as heart rate (HR) which were displayed on a Gould model TA5000 polygraph recorder (Gould Inc., Valley View, OH, U.S.A.). The left jugular vein was cannulated for the administration of drugs.

Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 20 min. After recording baseline haemodynamic parameters, animals were given noradrenaline (1 μg kg^{-1}, i.v.), and 10 min later animals received vehicle (saline) or *Escherichia coli* lipopolysaccharide (5 mg kg^{-1}, i.v.) and were monitored for 6 h. The pressor responses to noradrenaline were reassessed every hour after vehicle or lipopolysaccharide injection.

Membrane potential recording and tension measurement

At 6 h after the injection of lipopolysaccharide, thoracic aortas were obtained from sham-treated controls as well as from endotoxaemic rats. The vessels were cleared of adhering periadventitial fat and the thoracic aortas were cut into 3 mm in length, and opened longitudinally. The endothelium was removed by gently rubbing the intimal surface of the vessel with a moistened cotton ball. Later, the lack of a relaxation to acetylcholine (1 μM) of aortic segments precontracted with noradrenaline (0.1–0.3 μM) was considered as evidence that the endothelium had been removed. The tissue was pinned down, intimal side upward, on the bottom of an organ chamber (capacity 3 ml), and superfused at a constant flow rate of 3 ml min^{-1} with warmed (37°C), oxygenated (95% O_2 and 5% CO_2) physiological salt solution (pH 7.4) consisting of (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11. Indomethacin (5.6 μM) was added to prevent the production of prostanoids. One end of the segment was fixed to the organ bath chamber while the other end was connected to Grass FT03 transducer (Grass Instrument Co., Quincy, MA, U.S.A.). After the preparations had equilibrated for at least 60 min, glass microelectrodes filled with 3 M KCl (tip resistance 20–60 MΩ) were inserted into the aortic smooth muscle from the intimal side. Electrical signals were monitored continuously on an oscilloscope (Gould ES 2000, Ballainvilliers, France) and recorded on a chart recorder (Gould TA11, Gould Instruments, Valley View, OH, U.S.A.). The effects of the following inhibitors were examined on the membrane potential and the vascular tension simultaneously: (i) NO synthase (N^{ω}-nitro-L-arginine methyl ester, L-NAME, 0.3 mM), (ii) soluble GC (1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-l-one, ODQ, 1 μM), and (iii) K^+-channels (tetraethylammonium 10 mM; charybdotoxin, 0.1 μM; glibenclamide, 10 μM or apamin, 0.1 μM).

Each preparation was used to test only one inhibitor. An impalement was considered to be successful only if it was maintained continuously before, during and after the drug application. This continuous recording took 1 h.

Organ bath relaxation experiments

At the end of *in vivo* experiments, thoracic aortas were obtained from both groups as described above and prepared as rings of 3–4 mm width. Similarly, the endothelium was removed by gently rubbing and its absence was confirmed as described earlier. The rings were mounted in 20 ml organ baths filled with physiological salt solution which contained indomethacin (5.6 μM) and was kept warmed (37°C) and oxygenated (95% O_2 and 5% CO_2). Isometric force was measured with Grass FT03 type transducers (Grass Instruments, Quincy, MA, U.S.A.) and recorded on a MacLab Recording and Analysis System (ADInstruments Pty Ltd., Castle Hill, Australia). A tension of 2 g was applied and the rings were equilibrated for 60 min, changing the physiological salt solution every 15 min. The relaxation responses to NS1619 and pinacidil were examined in the absence or presence of the following inhibitors: (i) NO synthase (L-NAME, 0.3 mM), (ii) soluble GC (ODQ, 1 μM), and (iii) K^+-channels (tetraethylammonium 10 mM; charybdotoxin, 0.1 μM; glibenclamide, 10 μM or apamin, 0.1 μM) for 15 min.

Statistical analysis

All values in the figures and text are expressed as mean ± s.e.mean of *n* observations, where *n* represents the number of animals studied. Statistical evaluation of membrane potential recording was performed by using paired and unpaired Student's *t*-test, whereas that of the relaxations induced by NS1619 and pinacidil was analysed by using analysis of variance (ANOVA) followed by a multiple comparison test (Scheffe's test). A P value of less than 0.05 was considered to be statistically significant.

Results

Lipopolysaccharide induced hypotension and vascular hyporeactivity

The mean baseline values for MAP, HR and pressor responses to noradrenaline were not significantly different between lipopolysaccharide-treated and sham-treated (i.e. control) rats studied (Table 1). The injection of lipopolysaccharide caused a significant fall of MAP in 6 h, and at this time there was a severe vascular hyporeactivity to noradrenaline *in vivo*. In addition, lipopolysaccharide induced a significant increase in HR during the experimental period.

NS1619 induced greater re-polarization and relaxation in arteries from endotoxaemic rats

The membrane potential recording showed that aortic tissues obtained from rats treated with lipopolysaccharide for 6 h (Figure 1b,c) were more hyperpolarized when compared to those from control rats (Figure 1a,c). Arteries obtained from both endotoxaemic rats and control rats were precontracted with phenylephrine 0.3 and 0.1 μM respectively, to similar degree of depolarization and contraction. The addition of NS1619 to arteries from both groups induced re-polarization and relaxation, and the re-polarization and relaxation were greater in preparations from the endotoxaemic group than those from the control group (Figure 1c and Table 2).

Effects of tetraethylammonium, charybdotoxin and apamin on the NS1619-induced changes of membrane potential and relaxation

Tetraethylammonium, a non-selective inhibitor of K^+ channels, partially inhibited the greater hyperpolarization seen in the arteries obtained from endotoxaemic rats (Figure 2a). This was concomitant with an increase of tension in these preparations (Table 2). In addition, in phenylephrine-contracted rings, the NS1619-induced relaxation was in a concentration-dependent manner in both groups and this relaxation was also greater in aortic rings obtained from the endotoxaemic group. Tetraethylammonium partially suppressed the greater re-polarization and relaxation induced by NS1619 in arteries obtained from endotoxaemic rats (Figure 2a,d). However, tetraethylammonium had no significant effect on the NS1619-induced re-polarization and relaxation in arteries obtained from control rats (Figure 2a,d).

In the presence of charybdotoxin, a selective inhibitor of BK channels, the greater hyperpolarization seen in the arteries obtained from endotoxaemic rats was inhibited partially (Figure 2b) with a concomitant increase in the basal tension

Table 1 Haemodynamic changes in rats treated with *E. coli* lipopolysaccharide (LPS; 5 mg kg^{-1} i.v.) or saline (control) for 6 h

	Control (n = 24)		*LPS* (n = 30)	
	0 h	*6 h*	*0 h*	*6 h*
MAP (mmHg)	122 ± 3	113 ± 5	121 ± 2	79 ± 6*†
HR (beats min^{-1})	343 ± 14	322 ± 16	338 ± 11	378 ± 12*†
Noradrenaline (mmHg × min)	33 ± 2	35 ± 3	34 ± 2	19 ± 4*†

*P < 0.05 vs C at the same time point. †P < 0.05 vs time 0 h in the LPS group. MAP, mean arterial pressure; HR, heart rate; Noradrenaline, pressor responses to noradrenaline (1 μg kg^{-1}, i.v.). Note that the pressor responses to noradrenaline are calculated as the area under the curve, i.e mmHg × min. Data are expressed as mean ± s.e.mean of *n* animals studied.

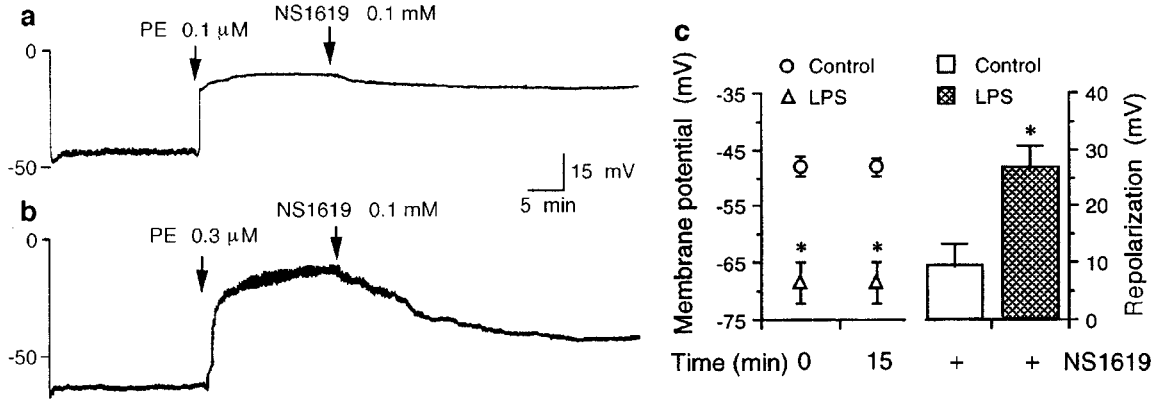

Figure 1 Membrane potential and NS1619-induced re-polarization in endothelium-denuded aortic segments from rats treated with *E. coli* lipopolysaccharide (LPS) or saline. This figure depicts a typical traces illustrating measurements of smooth muscle membrane potential with phenylephrine (PE) and stimulated with NS1619 (0.1 mM) in aortas from (a) saline-treated and (b) LPS (5 mg kg^{-1} i.v.)-treated rats. (c) Depicted is the statistical analysis of changes of membrane potential during time control and stimulation with NS1619 in endothelium-denuded aortic segments obtained from rats treated with saline (Control, n = 6) or LPS (n = 9) for 6 h. Data are expressed as mean ± s.e.mean of *n* animals studied. *P < 0.05 represents significant differences when compared to the control group.

Table 2 Effects of tetraethylammonium, charybdotoxin, apamin, L-NAME and ODQ on the tension before and after phenylephrine and NS1619 treatment in aortas from sham- and lipopolysaccharide-treated rats

	Basal		*Phenylephrine*		*NS1619*		n	
Treatment	*Control*	*LPS*	*Control*	*LPS*	*Control*	*LPS*	*Control*	*LPS*
None	0±0	0±0	710±87	669±72	561±36	667±11*	6	9
Tetraethylammonium, 10 mM	2±2	14±3*†	722±79	638±56	528±41	498±36†	6	9
Charybdotoxin, 0.1 μM	0±0	8±2*†	644±89	663±80	496±44	507±26†	6	8
Apamin, 0.1 μM	0±0	0±0	699±73	641±66	572±28	646±14*	6	8
L-NAME, 0.3 mM	1±1	10±2*†	690±79	647±50	594±32	545±24†	6	9
ODQ, 1 μM	3±3	21±5*†	664±54	689±75	576±34	560±24†	6	8

Tension before (i.e. basal) and after phenylephrine (0.1–0.3 μM) and NS1619 (0.1 mM) in aortic segments obtained from sham-treated (control) rats and lipopolysaccharide-treated (LPS) rats *in vitro* challenged with or without (i.e. none) tetraethylammonium, charybdotoxin. apamin, N^{ω}-nitro-L-arginine methyl ester (L-NAME) and 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) were recorded in electrophysiological recording experiments. *$P<0.05$ vs control at the same condition. †$P<0.05$ vs none in the same group. The unit of tension is mg. Note that values under NS1619 are expressed as mg of relaxation. Data are expressed as mean±s.e.mean of *n* animals studied.

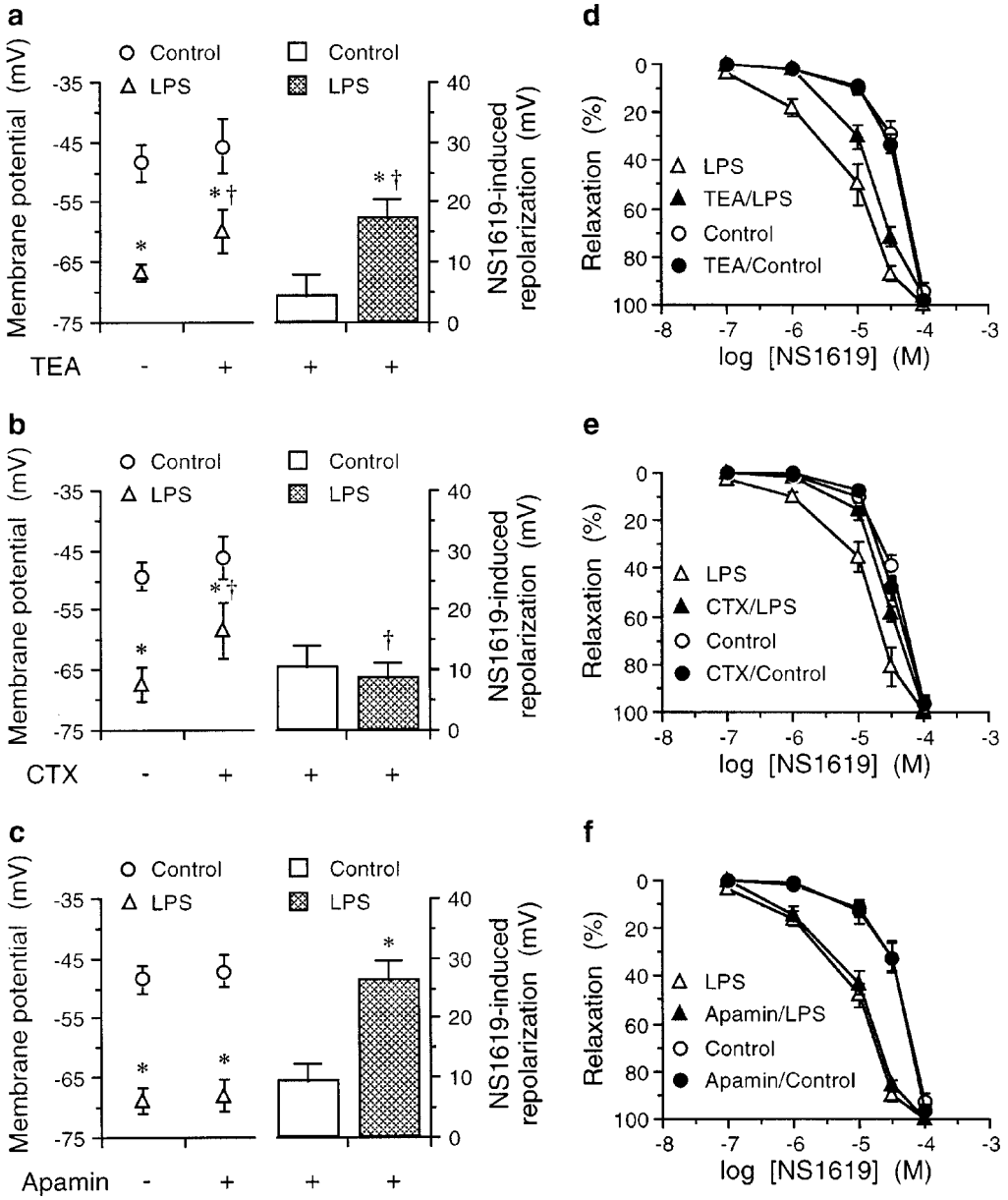

Figure 2 Effects of (a,d) tetraethylammonium (TEA: 10 mM), (b,e) charybdotoxin (CTX: 0.1 μM) and (c,f) apamin (0.1 μM) on (left panels) membrane potential and NS1619-induced re-polarization, and (right panels) NS1619-induced relaxation in endothelium-denuded aortic preparations obtained from saline (Control, $n=6$) rats or rats treated with lipopolysaccharide (LPS, $n=8-9$) for 6 h. Data are expressed as mean±s.e.mean of *n* animals studied. *$P<0.05$ represents significant differences when compared to the control group. †$P<0.05$ represents significant differences between with and without inhibitors in the LPS-treated group. Note that there is no significant difference in NS1619-induced relaxation between with and without TEA. CTX or apamin in the control group, whereas apamin has no significant inhibition on NS1619-induced relaxation in the LPS-treated group.

(Table 2). The greater re-polarization elicited by NS1619 in the arteries of endotoxaemic rats was abolished by charybdotoxin completely (Figure 2b) and the NS1619 induced greater relaxations in rings from endotoxaemic rats were restored by charybdotoxin to the values seen in the control group (Figure 2e). In addition, a more specific inhibitor of BK channels iberiotoxin exerted similar inhibition as charybdotoxin did on NS1619-induced relaxation and re-polarization in rings from both rats (unpublished data). However, charybdotoxin had no significant effect on the NS1619-induced re-polarization and relaxation in arteries obtained from control rats (Figure 2b,e).

Apamin, a selective inhibitor of the small conductance Ca^{2+}-activated K^+ channels, had no significant effect on the hyperpolarization elicited with or without NS1619 in arteries obtained from both endotoxaemic and control rats. In other words, re-polarization with NS1619 was still greater in lipopolysaccharide-treated group after apamin (Figure 2c). Apamin had no significant effect on the relaxation elicited by NS1619 in aortic rings obtained from either groups of rats (Figure 2f). In addition, apamin had no significant effect on the basal tension in arteries obtained from either endotoxaemic or control groups (Table 2).

Effects of L-NAME and ODQ on the NS1619-induced changes of membrane potential and relaxation

In arteries from lipopolysaccharide-treated rats, but not from sham-treated rats, incubated with the inhibitor of NO synthase (i.e. L-NAME) or NO-sensitive soluble GC (i.e. ODQ), the hyperpolarization induced by lipopolysaccharide was attenu-

Table 3 Effects of glibenclamide, L-NAME and ODQ on the tension before and after phenylephrine and pinacidil treatment in aortas from sham- and lipopolysaccharide-treated rats

	Basal		*Phenylephrine*		*Pinacidil*		n	
Treatment	*Control*	*LPS*	*Control*	*LPS*	*Control*	*LPS*	*Control*	*LPS*
None	0±0	0±0	697±34	691±44	636±16	684±6*	6	9
Glibenclamide, 10 μM	0±0	6±1*†	666±51	632±37	353±46†	357±42†	6	9
L-NAME, 0.3 μM	1±1	9±2*†	774±88	720±31	678±26	642±18†	6	9
ODQ, 1 μM	2±2	19±4*†	743±55	680±34	667±18	554±16*†	6	9

Tensions before (i.e. basal) and after phenylephrine (0.1–0.3 μM) and pinacidil (10 μM) in aortic segments obtained from sham-treated (control) rats and lipopolysaccharide-treated (LPS) rats *in vitro* challenged with or without (i.e. none) glibenclamide, N$^\omega$-nitro-L-arginine methyl ester (L-NAME) and 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) were recorded in electrophysiological recording experiments. *$P<0.05$ vs control at the same condition. †$P<0.05$ vs none in the same group. The unit of tension is mg. Note that the values under pinacidil are expressed as mg of relaxation. Data are expressed as mean±s.e.mean of n animals studied.

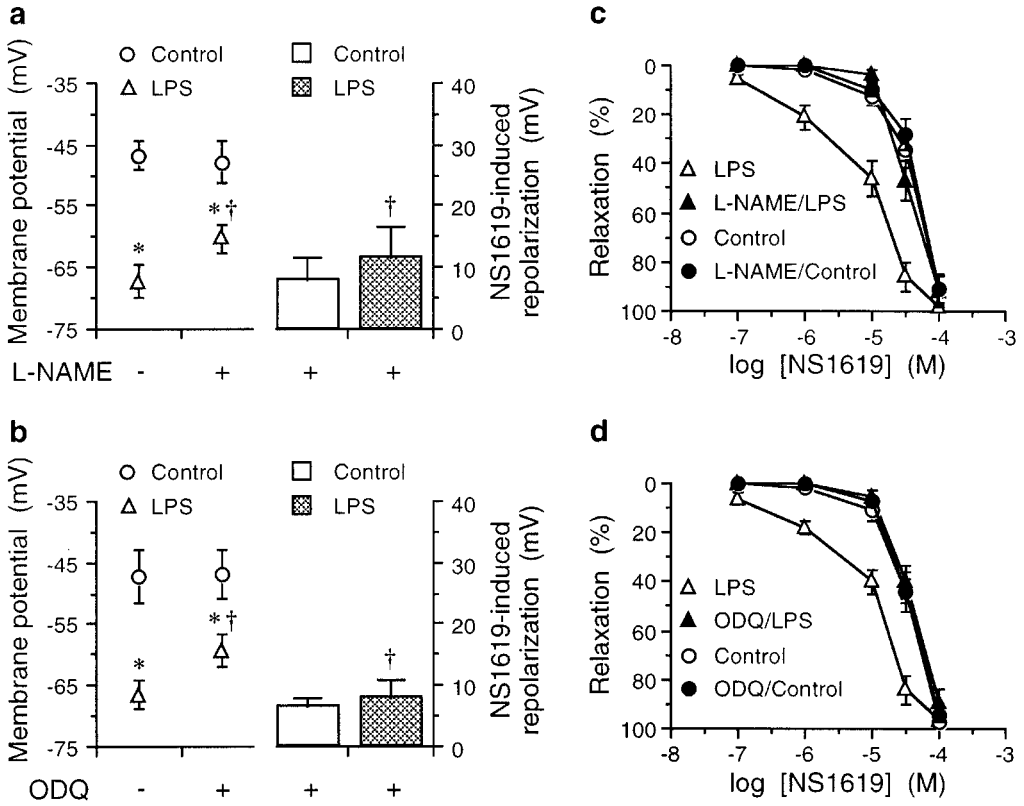

Figure 3 Effects of (a,c) N$^\omega$-nitro-L-arginine methyl ester (L-NAME; 0.3 mM) and (b,d) 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-l-one (ODQ; 1 μM) on (left panels) membrane potential and NS1619-induced re-polarization, and (right panels) NS1619-induced relaxation in endothelium-denuded aortic preparations obtained from saline (Control, $n=6$) rats or rats treated with lipopolysaccharide (LPS, $n=8-9$) for 6 h. Data are expressed as mean±s.e.mean of n animals studied. *$P<0.05$ represents significant differences when compared to the control group. †$P<0.05$ represents significant differences between with and without inhibitors in the LPS-treated group. Note that there is no significant difference in NS1619-induced relaxation between with and without L-NAME or ODQ in the control group.

ated (Figure 3a,b) and the basal tension was increased by these inhibitors (Table 2). In addition, the greater re-polarization and relaxation induced by NS1619 in arteries from endotoxaemic rats was abolished by L-NAME (Figure 3a,c) or ODQ (Figure 3b,d). However, the application of L-NAME or ODQ to arteries from control rats showed no significant effect on the NS1619-induced re-polarization (Figure 3a,b) and relaxation (Figure 3c,d).

Pinacidil induced greater re-polarization and relaxation in arteries from endotoxaemic rats

Similar to results shown in Figure 1, the membrane potential recording showed that aortic tissues obtained from rats treated with lipopolysaccharide for 6 h (Figure 4b,c) were more hyperpolarized when compared to those from control rats (Figure 4a,c). Arteries obtained from both endotoxaemic rats and control rats were precontracted with phenylephrine 0.3 and 0.1 μM, respectively, to similar degree of depolarization and contraction. The addition of pinacidil to arteries from both groups induced re-polarization and relaxation, and the re-polarization and relaxation were greater in preparations from the endotoxaemic group than those from the control group (Figure 4c and Table 3).

Effect of glibenclamide on the pinacidil-induced changes of membrane potential and relaxation

Glibenclamide, a selective inhibitor of K_{ATP} channels, partially reversed the hyperpolarization (Figure 5a) and increased the basal tension (Table 3) in arteries obtained from endotoxaemic rats, but not from controls. In addition, in phenylephrine-contracted rings, pinacidil induced relaxation in a concentration-dependent manner in both groups and this relaxation was also greater in aortic rings obtained from the endotoxaemic group. However, the pinacidil-induced re-polarization (Figure 5a) and relaxation (Figure 5b) were inhibited by glibenclamide in both endotoxaemic and control groups. In addition, after treatment of both arteries with glibenclamide, there was no significant difference in the pinacidil-induced re-polarization and relaxation between the endotoxaemic group and the control group (Figure 5a,b).

Effects of L-NAME and ODQ on the pinacidil-induced changes of membrane potential and relaxation

Similar to results shown in Figure 3a,b and Table 2, the hyperpolarization induced by lipopolysaccharide was attenuated by L-NAME or ODQ (Figure 6a,b) and the basal

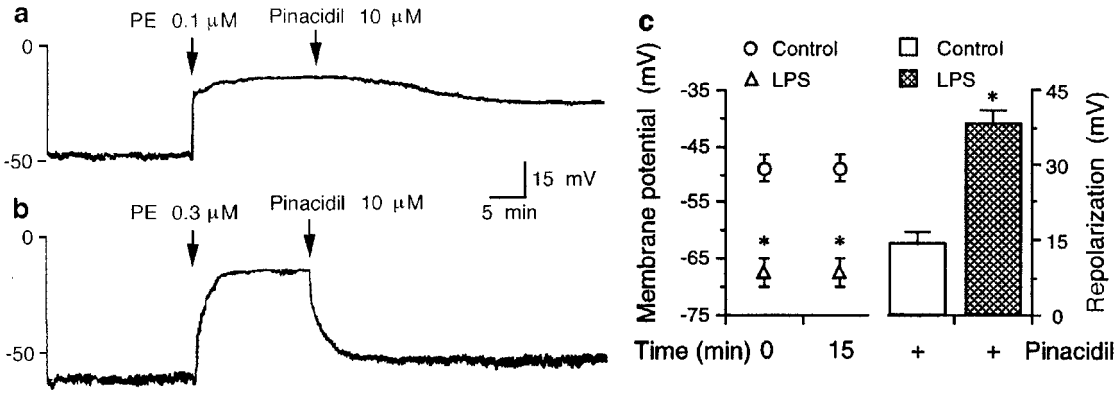

Figure 4 Membrane potential and pinacidil-induced re-polarization in endothelium-denuded aortic segments from rats treated with *E. coli* lipopolysaccharide (LPS) or saline. This figure depicts a typical traces illustrating measurements of smooth muscle membrane potential with phenylephrine (PE) and stimulated with pinacidil (10 μM) in aortas from (a) saline-treated and (b) LPS (5 mg kg^{-1} i.v.)-treated rats. (c) Depicted is the statistical analysis of changes of membrane potential during time control and stimulation with pinacidil in endothelium-denuded aortic segments obtained from rats treated with saline (Control, $n=6$) or LPS ($n=9$) for 6 h. Data are expressed as mean$\pm$s.e.mean of n animals studied. *$P<0.05$ represents significant differences when compared to the control group.

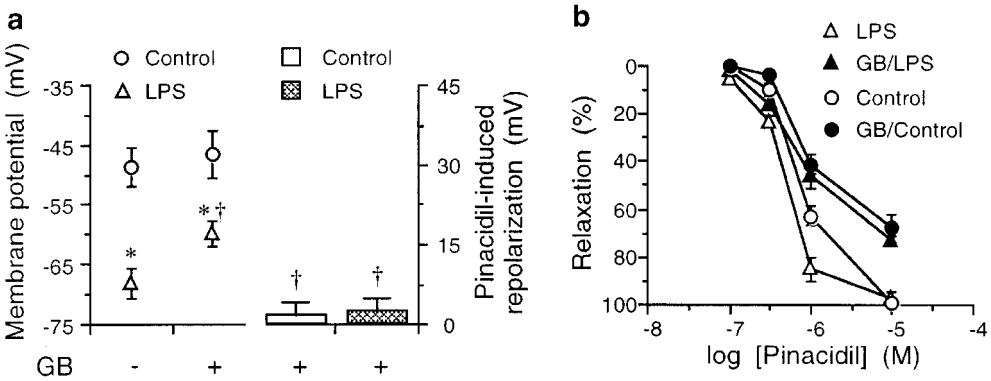

Figure 5 Effects of glibenclamide (GB: 10 μM) on (a) membrane potential and pinacidil-induced re-polarization and (b) pinacidil-induced relaxation in endothelium-denuded aortic preparations obtained from saline (Control, $n=6$) rats or rats treated with lipopolysaccharide (LPS, $n=9$) for 6 h. Data are expressed as mean$\pm$s.e.mean of n animals studied. *$P<0.05$ represents significant differences when compared to the control group. †$P<0.05$ represents significant differences between with and without inhibitors in the LPS-treated group. Note that glibenclamide significantly inhibited pinacidil-induced relaxation in both groups.

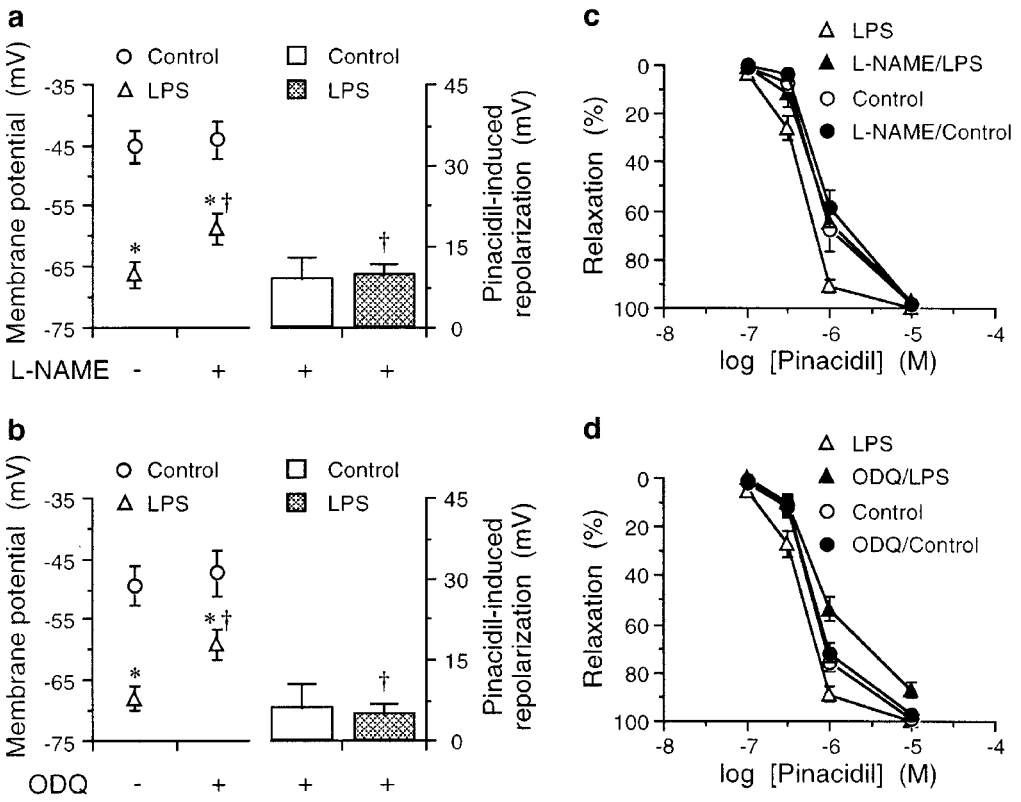

Figure 6 Effects of (a,c) N^{ω}-nitro-L-arginine methyl ester (L-NAME: 0.3 mM) and (b,d) 1*H*-[1,2,4]oxadiazolo[4,3-alquinoxalin-l-one (ODQ; 1 μM) on (left panels) membrane potential and pinacidil-induced re-polarization, and (right panels) pinacidil-induced relaxation in endothelium-denuded aortic preparations obtained from saline (Control, $n=6$) rats or rats treated with lipopolysaccharide (LPS, $n=9$) for 6 h. Data are expressed as mean$\pm$s.e.mean of n animals studied. $*P<0.05$ represents significant differences when compared to the control group. $\dagger P<0.05$ represents significant differences between with and without inhibitors in the LPS-treated group. Note that there is no significant difference in pinacidil-induced relaxation between with and without L-NAME or ODQ in the control group.

tension was increased by these inhibitors in arteries from endotoxaemic rats, but not from controls (Table 3). In addition, the greater re-polarization induced by pinacidil in arteries from endotoxaemic rats was abolished by L-NAME (Figure 6a) or ODQ (Figure 6b). The greater relaxations induced by pinacidil in the endotoxaemic group were inhibited by L-NAME to the values observed in the control group (Figure 6c) or by ODQ to a greater extent than those in controls (Figure 6d). However, the application of L-NAME or ODQ to arteries from control rats showed no significant effect on the pinacidil-induced re-polarization (Figure 6a,b) and relaxation (Figure 6c,d).

Discussion

Although our previous studies and others have indicated that abnormal activation of K^+ channels is associated with the vascular hyporeactivity to vasoconstrictor agents seen in septic shock (Wu *et al*., 1995; Hall *et al*., 1996; Chen *et al*., 1999; Sorrentino *et al*., 1999), no direct evidence has shown that the activities of these K^+ channels have been changed in vascular tissues obtained from endotoxaemic animals. In the current study we demonstrated that a greater hyperpolarization (by membrane potential recording) occurred in aortic segment from rats treated with endotoxin for 6 h. These rats showed a significant fall in blood pressure and a severe vascular hyporeactivity to noradrenaline. These results further support the hypothesis that abnormal activation of K^+ channels can counteract the contraction induced by vasoconstrictor agents (e.g. noradrenaline in this study) in animals with endotoxic shock.

There is good evidence showing that overproduction of NO, mainly by the calcium-independent inducible isoform of NO synthase, is associated with the hypotension and vascular hyporeactivity to vasoconstrictor agents in endotoxic shock (see Thiemermann, 1994). The mechanism by which NO causes relaxation in the vascular smooth muscle is attributed to the activation of soluble GC in the smooth muscle cell (see Schmidt *et al*., 1993; McDonald & Murad, 1996) and/or the activation of BK channels (Khan *et al*., 1993; Bolotina *et al*., 1994; Bialecki & Stinson-Fisher, 1995). However, this activation of BK channels by NO and NO-donors could be mediated by cyclic GMP (Taniguchi *et al*., 1993; Archer *et al*., 1994; Miyoshi & Nakaya, 1994). Indeed, Yao *et al*. (1995) have pointed out that a gene encoding a K^+ channel (*Kcn*l) isolated from rabbits is specifically regulated by cyclic GMP and mediates the effect of NO which increases intracellular cyclic GMP. In addition, Hall *et al*. (1996) further suggested that abnormal activation of K^+ channels (possibly BK channels) underlies relaxation to endotoxin in the rat aorta, whereas Taguchi *et al*. (1996) also showed that vascular expression of inducible NO synthase activates BK channels. Thus, the activation of BK by NO or cyclic GMP may contribute to impaired vasocontractile responses to vasoconstrictor agents in septic shock. Indeed, our results showed that aortas from animals with endotoxic shock were more hyperpolarized and more sensitive to NS1619-induced re-polarization than those from control animals. This observation suggests that BK is involved in this vascular hyporeactivity induced by endotoxin

since NS1619 is a putative activator of BK channels. On the other hand, in the presence of inhibitors of BK (charybdotoxin in this study), the hyperpolarization induced by endotoxin was partially inhibited, whereas the NS1619-induced greater re-polarization and relaxation were completely restored to the values seen in controls. In contrast, the inhibitor of small conductance Ca^{2+}-activated K^+ channels apamin had no effect on hyperpolarization caused by endotoxin and the NS1619-induced re-polarization and relaxation in arteries from endotoxaemic animals. Although tetraethylammonium is non-selective inhibitor of K^+ channels, it also partially reversed that hyperpolarization caused by endotoxin and the NS1619-induced re-polarization and relaxation in tissues from endotoxaemic rats. In addition, both L-NAME (an inhibitor of NO synthase) and ODQ (an inhibitor of NO-sensitive soluble GC) partially attenuated the hyperpolarization caused by endotoxin and completely abolished the NS1619-induced re-polarization and relaxation in arteries from animals with endotoxic shock, suggesting that the activation of BK by NO or cyclic GMP is associated with the impairment of vascular contraction. However, none of these inhibitors affected the NS1619-induced relaxation in arteries from control animals. This is consistent with a previous study showing that block of BK by specific inhibitors did not inhibit NS1619-induced relaxation in blood vessels from the normal rat, indicating that relaxation induced by NS1619 does not involve the opening of K^+ channels (Edwards *et al*., 1994). Therefore, our results, obtained in aortas from endotoxaemic animals, suggest that while K^+ channels opening is not necessary for NS1619-induced relaxation, endotoxin may make it act to increase the relaxant potency of the compound. This result fits well with the functional study showing that inhibitors (selective as charybdotoxin or non-selective as tetraethylammonium) of BK appear to be more potent as blockers of NS1619-induced relaxation in these tissues than in controls.

NO also causes hyperpolarization *via* the activation of K_{ATP} channels in mesenteric arteries, which is blocked by glibenclamide (Garland & McPherson, 1992; Murphy & Brayden, 1995). In addition, patch-clamp studies of cultured coronary arterial cells treated with lipopolysaccharide demonstrated that activation of inducible NO synthase with L-arginine caused persistent activation of K_{ATP} channels (Miyoshi *et al*., 1994). As mentioned above, the mechanism by which NO causes vasodilatation is linked to the activation of soluble GC leading to an increase of intracellular cyclic GMP levels in the vascular smooth muscle cell (see Schmidt *et al*., 1993; McDonald & Murad, 1996), which may alter K^+ channels activity by phosphorylation. A recent study demonstrated that 8-bromo-cyclic GMP increased the glibenclamide-sensitive K^+ current induced by a selective K_{ATP} channel opener cromakalim by 40–60%, whereas methylene blue (an inhibitor of soluble GC) irreversibly blocked that current by 20–30% in oocytes (Sakuta *et al*., 1993). This was confirmed by Kubo *et al*. (1994) and our studies which demonstrated that activators of soluble GC or cyclic GMP analogues could modulate activity of K_{ATP} channels in cultured aortic smooth muscle cells or in the rat aorta (Wu *et al*., 1999). It appears that hypotension and reduction in peripheral vascular resistance, which occur in septic shock, are at least in part due to activation of K_{ATP} channels in vascular smooth muscle. This is based on the fact that administration of glibenclamide to dogs at maximum peak of lipopolysaccharide-induced hypotension (about 30 min after lipopolysaccharide injection) (Landry & Oliver, 1992) or to rats at 60 min after lipopolysaccharide treatment (Wu *et al*., 1995) was able to revert the endotoxic shock instantaneously. A more recent study also showed that K_{ATP} channels were involved in a delayed vascular hyporesponsiveness induced by lipopolysaccharide in rats (Sorrentino *et al*., 1999). Here, we provide further direct evidence showing that the hyperpolarization in arteries from endotoxaemic animals was partially attenuated by glibenclamide. In addition, these arteries were more re-polarized and relaxed to pinacidil, which is another selective K_{ATP} channel opener, and this greater re-polarization and relaxation was restored by glibenclamide to the levels seen in the control animal, suggesting that K_{ATP} channels also contribute to impaired vasoconstrictor responses induced by endotoxin. This is consistent with a previous study showing that cromakalim had a greater relaxation, which was also blocked by glibenclamide, in arteries from the endotoxin-treated rat (Sorrentino *et al*., 1999). Our results may also explain why cromakalim produced a greater reduction of MAP in endotoxin-treated rats than in controls. L-NAME had similar inhibitory effect as that of glibenclamide on the re-polarization and relaxation induced by pinacidil in tissues from endotoxaemic animals and controls. However, ODQ had a greater inhibition on the pinacidil-induced relaxation in arteries from the endotoxin-treated rat than those from the control animal. This further indicates that accumulation of cGMP in the vascular smooth muscle cells by NO/NO donors or endotoxin may modulate activity of K_{ATP} channels, which leads to hyperpolarization and relaxation to K_{ATP} channel openers (pinacidil in this study; cromakalim in Sorrentino *et al*., 1999; Wu *et al*., 1999).

Although here we show good evidence that both BK and K_{ATP} channels are involved in the impaired vasoconstrictor responses manifested in animals with endotoxic shock, we cannot exclude possibilities of an increase of the number of BK and K_{ATP} channels by gene expression activation, and/or the kinetic changes of potassium currents in these channels. Indeed, recent studies showed that intracellular application of lipopolysaccharide altered kinetics of BK channels in rat cerebrovascular smooth muscle cells (Hoang *et al*., 1997; Hoang & Mathers, 1998a) since lipopolysaccharide was able to be taken up into mammalian cells by receptor mediated endocytosis (Ghermay *et al*., 1996). Their results suggest that bacterial endotoxin can alter the functional properties of BK channels when applied to the intracellular face of the vascular smooth muscle. Later, they also demonstrated that the activation of BK channels by lipopolysaccharide was suppressed by the competitive NO synthase inhibitors L-NAME and L-NOARG, suggesting that lipopolysaccharide action is mediated by a NO synthase-like enzyme present in rat cerebrovascular smooth muscle cells (Hoang & Mathers, 1998b). Thus, lipopolysaccharide may directly or indirectly cause the activation of K^+ channels in our present study.

In conclusion, plentiful evidence has shown that an enhanced formation of NO (principally by inducible NO synthase) contributes to hypotension and vascular hyporeactivity to endogenous and exogenous vasoconstrictor agents in septic shock (see Thiemermann, 1994). The current study further demonstrates that hyperpolarization by bacterial endotoxin produces relaxation (which counteracts vasoconstriction) of vascular smooth muscle through activation of K^+ channels and these K^+ channels are most likely both BK and K_{ATP} channels. Thus, our results provide another approach of potential therapeutic agents which inhibit K^+ channels in sepsis or septic shock. However, the adverse effects caused by the administration of K^+ channels inhibitors to animals with septic shock must be considered and evaluated cautiously.

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