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The in vivo effect of lipopolysaccharide on neuromuscular transmission in the mouse

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Abstract

The in vivo effect of lipopolysaccharide (endotoxin) on nerve-evoked muscle contractions and neuromuscular transmission was studied in the mouse phrenic nerve-diaphragm preparations. In lipopolysaccharide-treated mouse diaphragms it was observed that indirectly induced twitch tension was unchanged while tetanic tension significantly decreased. Neostigmine (50 nM) increased the amplitude of nerve evoked muscle contractions, while it caused partial fade of tetanic contractions (Wedensky inhibition) and accelerated the run-down of end-plate potentials (e.p.ps) evoked by repetitive nerve stimulation, in the diaphragm of saline-control mice, but not of lipopolysaccharide-treated mice. These effects of neostigmine could be abolished by ouabain (5 μM). Measurement of the quantal contents of e.p.ps revealed that ouabain (5 μM) significantly increased it in the diaphragm of saline-control mice to an extent similar to that in diaphragm of lipopolysaccharide-treated mice. Moreover, ouabain-sensitive Na^+, K^+-ATPase activity in the sciatic nerve of lipopolysaccharide-treated mice was markedly decreased. The alterations in neuromuscular transmission of the diaphragm during endotoxemia could be reversed by the administration of polymyxin B (a lipopolysaccharide neutralizer) and N^G-nitro-L-arginine (a nitric oxide (NO) synthase inhibitor), suggesting that NO is involved in these lipopolysaccharide-induced alterations of neuromuscular transmission mediated by an impairment of ouabain-sensitive Na^+, K^+-ATPase activity in mouse motor nerves. © 1997 Elsevier Science B.V.

Keywords: Endotoxin; Neostigmine; Neurotransmission; Na^+, K^+-ATPase; N^G-nitro-L-arginine

1. Introduction

The induction of cytokine release from mononuclear phagocytes by lipopolysaccharide (endotoxin), is probably the central event in the pathophysiology of Gram-negative bacterial septicemia (Nathan, 1987; Pruzanski and Vadas, 1991), while in clinical practice, ventilatory failure in septic shock invariably occurs. This had been thought to be due to respiratory muscle fatigue (Hussain et al., 1985), however, the major cause of respiratory muscle fatigue is still unclear.

Na^+, K^+-ATPase generates a transmembrane Na^+–K^+ gradient and is essential for the specific properties of muscle and nerve tissue such as contractility and excitability (Skou, 1965). The alteration of Na^+, K^+-ATPase activity is related to some abnormal disease states. For example, the diabetic sciatic nerve has lower Na^+, K^+-ATPase activity (Das et al., 1976; Greene and Lattimer, 1984). Moreover, Nishimura et al. (1989) have shown that ouabain could abolish the twitch potentiation by neostigmine and suggested that ionic gradients maintained by Na^+, K^+-ATPase activity were important for the mechanisms of potentiation of twitches in the presence of anticholinesterases.

Previously, we found that the spontaneous transmitter release from motor nerve terminals was increased during endotoxemia and considered that this alteration might be related to impairment of the Na^+-pump (Liu et al., 1995). The present study was carried out to further investigate the influence of lipopolysaccharide on evoked transmitter release occurring during endotoxicity and to study the relationship between altered neuromuscular transmission and the Na^+-pump involved and the role of nitric oxide (NO) in these effects of lipopolysaccharide. The results show

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that lipopolysaccharide alters the evoked neurotransmission and Na^+-pump activity in the motor nerve. The L-arginine:NO pathway was involved in these in vivo effects of lipopolysaccharide.

2. Materials and methods

2.1. Mouse phrenic nerve-diaphragm preparation

Adult mice (20–25 g, ICR strain) were intraperitoneally pretreated with lipopolysaccharide (7.5 mg/kg) for 24 h to induce endotoxemia or with normal saline before the isolation of the phrenic nerve-diaphragm preparation. A modified Krebs' solution was used that had the following composition (mM): NaCl 130.6, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 12.5 and glucose 11.1. The diaphragm preparation was suspended in 10 ml of Krebs' solution maintained at 37.0 + 0.5°C and oxygenated with 95% O_2 and 5% CO_2. The pH of the solution was 7.2–7.4. The phrenic nerve was stimulated with supermaximal pulses of 0.05 ms duration at 0.2 Hz (single twitch) or trains of pulses at 50 Hz for 1 s (tetanic contraction). Contractions were recorded isometrically with a force displacement transducer (Grass FT.03) on a Grass 7D polygraph.

2.2. Intracellular recording

Conventional intracellular recording techniques were used. Glass microelectrodes, which were used for intracellular recording of superficial fibers of muscle, were filled with 3 M KCl and had resistances in the range of 3–10 MΩ. An Axoclamp-2A preamplifier and Tektronix 2221A oscilloscope were used to record intracellular responses. A DATA 6100 waveform analyzer (Data Precision, USA) was used to store and analyze waveforms. Muscle fibre action potentials were elicited by stimulation of the phrenic nerve with rectangular pulses of 0.05 ms duration. For recordings of train endplate potentials (e.p.ps) at 50 Hz, cut muscle preparations (Barstad and Lillehell, 1968) with resting membrane potential at about −45 mV were used. The amplitudes of e.p.ps were corrected for non-linear summation to −45 mV assuming reversal potential of 0 mV as previously described (Chang et al., 1986). For measurement of the quantal content of e.p.ps, e.p.ps were evoked by stimulation of the phrenic nerve with supramaximal pulses of 0.05 ms duration at 1 Hz in the low Ca^{2+} bathing solution. The quantal content (m) was estimated by the method of failures from

$$m = \log_e(N/N_0),$$

where N = number of trials; N_0 = number of failures (Crawford, 1974).

2.3. Na^+, K^+-ATPase assay

For the ATPase assay, sciatic nerves were surgically exposed at mid-thigh and dissected out from the sciatic notch to the popliteal fossa. The nerves were then weighed and homogenized in a chilled solution containing 0.25 M sucrose, 1.25 mM EGTA and 10 mM Tris, pH 7.5. Aliquots were frozen and stored at −70°C until assayed. Total (composite), ouabain-sensitive (Na^+, K^+-) and ouabain-insensitive (Mg^{2+}-)ATPase activity were determined spectrophotometrically by the coupled-enzyme assay of Scharschmidt et al. (1979). Na^+, K^+-ATPase activity was calculated by subtracting the activity assayed with ouabain from that assayed without. Proteins were determined according to Lowry et al. (1951) with bovine serum albumin as standard.

2.4. Drugs

Lipopolysaccharide (*E. coli*, 055:B5, lyophilized powder prepared by trichloro acetic acid extraction procedure), neostigmine bromide, ouabain, polymyxin B, N^G-nitro-L-arginine and sodium nitroprusside were purchased from Sigma (USA).

2.5. Statistics

The values given are means ± S.E. The significance of differences from the respective controls for each experimental test condition was assessed by using one-way analysis of variance (ANOVA) followed by Dunnett's test for each paired experiment. P values < 0.05 were regarded as indicating significant differences.

3. Results

As shown in Table 1, phrenic nerve-diaphragm preparations from lipopolysaccharide-treated mice were examined after 24 h treatment with lipopolysaccharide. The twitch force in these muscles was unchanged, but significant reductions in tetanic tension were seen when the latter was compared with those of the saline controls. In lipopolysaccharide-treated mouse preparations, increases in average twitch/tetanus ratio, which were significantly greater than those of saline controls, were also seen. Moreover, the post-tetanic facilitation of the indirect twitch tension was increased in lipopolysaccharide-treated mouse preparations (control, 135.98 ± 5.44% of control, $n = 5$; lipopolysaccharide, 153.70 ± 5.62% of control, $n = 5$).

Neostigmine (50 nM) increased the amplitudes of nerve-evoked contractions and caused partial fade of tetanic contractions in the diaphragm of saline control mice, but not, or only slightly, increased those of lipopolysaccharide-treated mice (Fig. 1; twitch amplitudes: saline control,

Table 1
Altered nerve-evoked muscle contractions of mouse diaphragm during endotoxemia

Treatment	*N*	Tension (g)		
		Twitch	Tetanus	Twitch/Tetanus
Control	30	0.89 ± 0.02	1.63 ± 0.06	0.55 ± 0.02
Lipopolysaccharide	16	0.84 ± 0.05	1.30 ± 0.05 [a]	0.65 ± 0.03 [a]
Lipopolysaccharide + Polymyxin B	8	0.91 ± 0.06	1.60 ± 0.04 [b]	0.57 ± 0.03 [b]
Lipopolysaccharide + N^G-nitro-L-arginine	8	0.83 ± 0.08	1.50 ± 0.05 [b]	0.56 ± 0.03 [b]

The mice were treated with either lipopolysaccharide (7.5 mg/kg) or normal saline (control) for 24 h before the isolation of phrenic nerve-diaphragm preparations. The diaphragm contractions were indirectly evoked by either single (0.05 ms duration, 0.2 Hz) or train (50 Hz) to induce twitch and tetanic tensions. Some mice were pretreated with polymyxin B (7.5 mg/kg) or N^G-nitro-L-arginine (100 mg/kg) for 15 min before the administration of lipopolysaccharide.
Data are presented as means ± S.E.
N denotes the experiment numbers.
[a] $P < 0.05$ as compared with control.
[b] $P < 0.05$ as compared with those treated with LPS for 24 h.

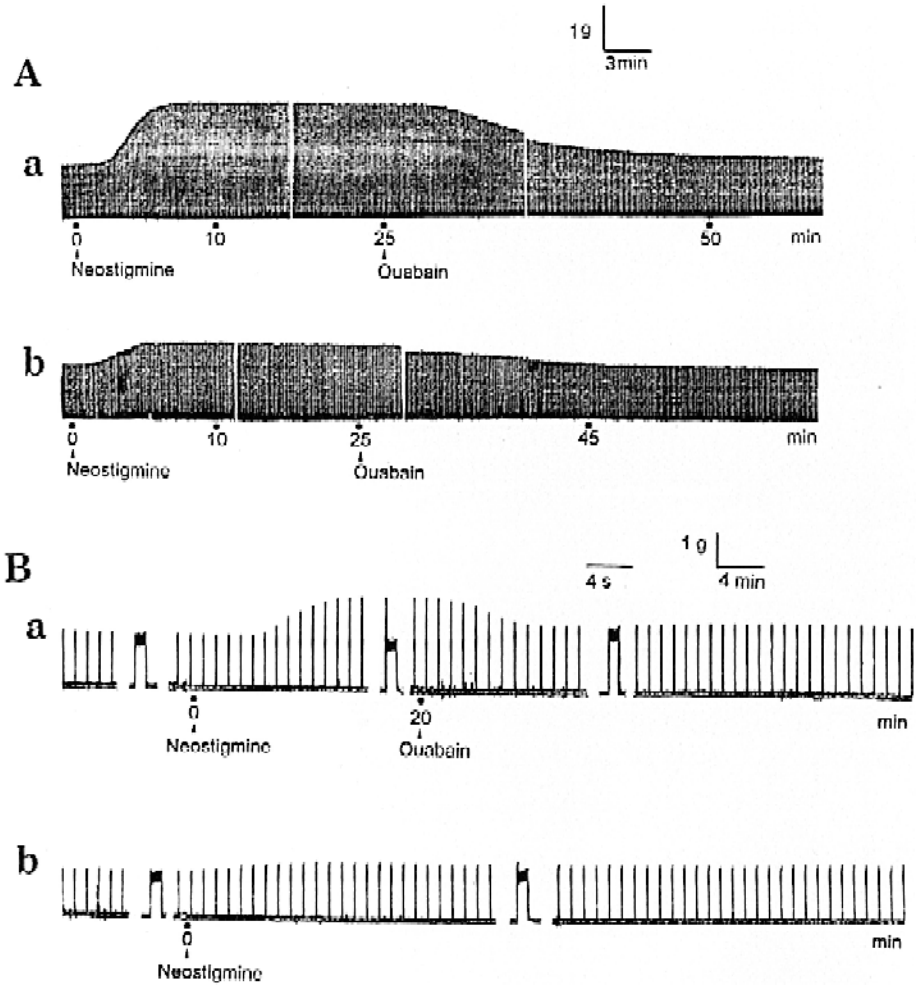

Fig. 1. Interaction between neostigmine and ouabain on the nerve-evoked twitches and tetanic tension in the diaphragm of saline control and lipopolysaccharide-treated mice. The phrenic nerve was electrically stimulated with 0.05 ms pulses at 0.2 Hz to induce twitch tension (A) or with 1 s trains of pulses every 60 s at 50 Hz to induce tetanic tension (B). Contractions of the diaphragm of saline control (a) and lipopolysaccharide-treated (b) mice were recorded isometrically on a Grass polygraph. Note that neostigmine (50 nM) markedly increased the twitch and tetanic tensions and induced tetanic fade in the diaphragm of saline control mice, but not in that of lipopolysaccharide-treated mice. Ouabain (5 μM) was capable of antagonizing the effect of neostigmine.

Table 2
Alterations of the resting membrane potential (RMP), action potential and quantal content in the diaphragm of endotoxemic mice

Treatment	RMP (-mV)	Action potential		Quantal content
		Amplitude (mV)	dv/dt (kV/s)	
Control	74.59 + 0.54	87.48 + 1.25	0.42 + 0.01	0.44 + 0.02
+ Ouabain	66.45 + 0.81 [a]	72.14 + 1.37 [a]	0.25 + 0.01 [a]	0.58 + 0.03 [a]
Lipopolysaccharide	69.50 + 0.58 [a]	81.64 + 2.10 [a]	0.33 + 0.02 [a]	0.51 + 0.03 [a]
+ Ouabain	67.58 + 0.46 [a]	74.29 + 2.46 [a]	0.28 + 0.02 [a]	0.50 + 0.04
Lipopolysaccharide + polymyxin B	73.63 + 0.89 [b]	91.68 + 1.28 [b]	0.39 + 0.02 [b]	0.44 + 0.04 [b]
Lipopolysaccharide + N^G-nitro-L-arginine	72.67 + 0.49 [b]	84.96 + 2.06 [b]	0.36 + 0.03	0.45 + 0.04

The mice were treated with either lipopolysaccharide (7.5 mg/kg) or normal saline (control) for 24 h before isolation of the diaphragm preparations. In other experiments, the mice were pretreated with either polymyxin B (7.5 mg/kg) or N^G-nitro-L-arginine (100 mg/kg) for 15 min prior to the administration of lipopolysaccharide. Ouabain (0.1 mM) was applied to diaphragm isolated from the control and the lipopolysaccharide-treated groups. Data are presented as means ± S.E. from 3–6 preparations, for each preparation 10–15 muscle fibres (RMP and action potential) or 8–14 endplates (quantal content) were studied.

[a] $P < 0.05$ as compared with control.

[b] $P < 0.05$ as compared with those of the lipopolysaccharide group.

224.70 ± 15.10% of control, $n = 5$; lipopolysaccharide: 147.30 ± 10.67% of control, $n = 5$). Ouabain (5 μM) application whether after or before neostigmine abolished these effects of neostigmine (Fig. 1). Moreover, ouabain (5 μM) itself increased the amplitude of tetanic contractions in the diaphragm of saline control mice, but not in that of lipopolysaccharide-treated mice (saline control: 141.08 ± 4.21% of control, $n = 5$; lipopolysaccharide: 114.70 ± 3.66% of control, $n = 5$).

In electrophysiological studies, the resting membrane potentials and nerve-evoked action potential amplitude and rate of rise (dv/dt) were decreased compared with the control (Table 2). Measurement of quantal contents of e.p.ps revealed a significant increase in diaphragm of lipopolysaccharide-treated mice (Table 2). Moreover, ouabain itself also increased the quantal contents of e.p.ps in the diaphragm of saline-control mice to an extent similar to that in the diaphragm of lipopolysaccharide-treated mice (Table 2). On the other hand, neostigmine (50 nM) accelerated the run-down of e.p.ps in the diaphragm of saline-control mice (amplitude being approximately from 80% to 60% of 1st e.p.ps, Fig. 2A), but not that of lipopolysaccharide-treated mice (Fig. 2B). Ouabain was still capable of antagonizing the neostigmine acceleration of e.p.ps run-down (Fig. 2A). Moreover, the increased amplitude and prolonged duration of endplate potentials induced by neostigmine (50 nM) were not altered by bath-applied ouabain or lipopolysaccharide in vivo treatment (data not shown).

For studying the possible role of the Na^+-pump in neuropathy induced by lipopolysaccharide, we measured the ouabain-sensitive Na^+, K^+-ATPase activity of sciatic nerve in mouse. The result showed that ouabain-sensitive Na^+, K^+-ATPase activity was markedly decreased during endotoxemia (Fig. 3).

The in vivo effects of lipopolysaccharide on neuromuscular transmission and ouabain-sensitive Na^+, K^+-ATPase activity could be reversed by the intraperitoneal injection with polymyxin B and N^G-nitro-L-arginine administered 15 min before the treatment with lipopolysaccharide for 24

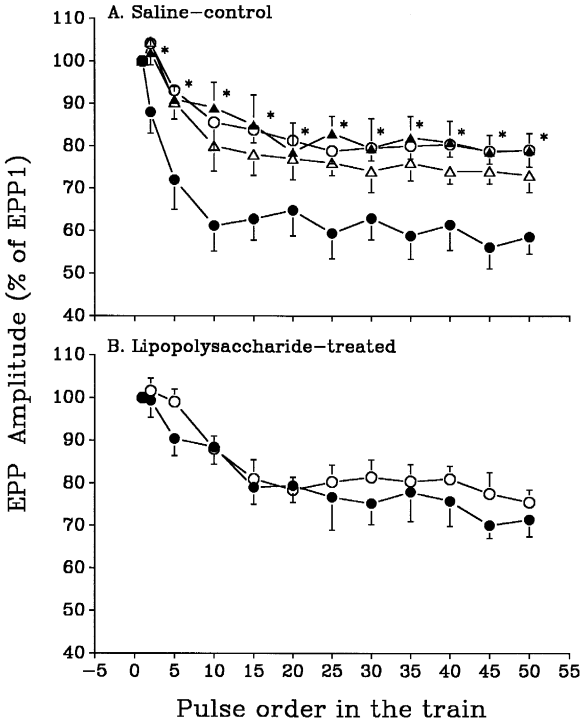

Fig. 2. Antagonism by ouabain of the accelerated run-down of endplate potentials induced by neostigmine in cut diaphragm of saline control and lipopolysaccharide-treated mice. Fifty endplate potentials (e.p.ps) were evoked at a frequency of 50 Hz for 1 s in the cut diaphragm of saline control (A) and lipopolysaccharide-treated (B) mice. – ○ –, control; – △ –, 5 μM ouabain; – ● –, 50 nM neostigmine; – ▲ –, ouabain plus neostigmine. Note that neostigmine accelerated the e.p.ps run-down in the diaphragm of saline control mice, but not in that of lipopolysaccharide-treated mice. Ouabain could antagonize the effect of neostigmine. E.p.ps amplitudes were expressed as percentage of the first e.p.ps in the same train. Data are presented as means ± S.E. ($n = 3$–6 preparations). * $P <$ 0.05 as compared with neostigmine alone.

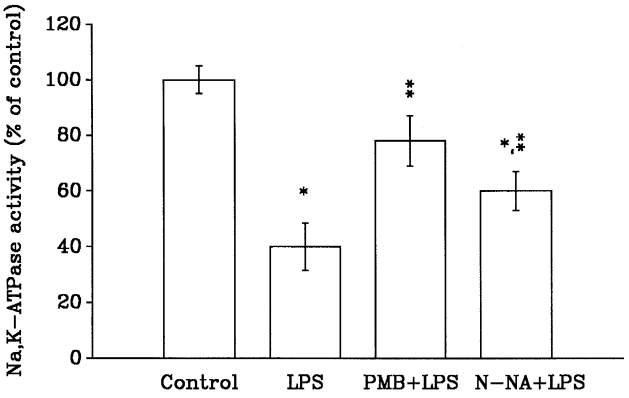

Fig. 3. The in vivo effect of lipopolysaccharide on the Na^+, K^+-ATPase activity of mouse sciatic nerve. The mice were treated with either lipopolysaccharide (LPS, 7.5 mg/kg) or normal saline (control) for 24 h before isolation of the sciatic nerves. In other experiments, the mice were pretreated with either polymyxin B (PMB, 7.5 mg/kg) or N^G-nitro-L-arginine (N-NA, 100 mg/kg) for 15 min prior to the administration of lipopolysaccharide. Data are presented as means ± S.E. of quadruplicate samples from representative experiments replicated on average two or three times. * $P < 0.05$ as compared with control. ** $P < 0.05$ as compared with those of the lipopolysaccharide group.

h (Tables 1 and 2 and Fig. 3; the twitch amplitude increased by 50 nM neostigmine in lipopolysaccharide, polymyxin B + lipopolysaccharide and N^G-nitro-L-arginine + lipopolysaccharide groups are 147.30 ± 10.67, 198.42 ± 5.86 and 181.24 ± 7.89% of control, $n = 5$, respectively; the tetanic amplitude increased by 5 μM ouabain in lipopolysaccharide, polymyxin B + lipopolysaccharide and N^G-nitro-L-arginine + lipopolysaccharide groups are 114.70 ± 3.66, 140.53 ± 2.89 and 131.43 ± 2.19% of control, $n = 5$, respectively).

On the other hand, sodium nitroprusside (0.5 mM) increased the post-tetanic facilitation of twitch tension (control, 133.83 ± 4.53% of control, $n = 6$; sodium nitroprusside, 157.40 ± 7.12% of control, $n = 5$). Moreover, sodium nitroprusside (0.5 mM) decreased the Na^+, K^+-ATPase activity of mouse sciatic nerve (73.64 ± 5.89% of control, $n = 6$), while Mg^{2+}-ATPase activity was not affected (101.62 ± 2.65% of control, $n = 6$).

4. Discussion

This study concerned the alterations of neuromuscular transmission and muscle contractions and pharmacological characteristics of neostigmine and ouabain in the mouse diaphragm during endotoxemia.

Leon et al. (1992) have reported that diaphragm strength was decreased at 50 and 100 Hz phrenic stimulations in rats during endotoxic shock and suggested that this diaphragmatic dysfunction was mainly related to an impaired neuromuscular transmission secondary to a decreased resting membrane potential. In accordance with this report, we have found in the present investigation that the nerve evoked tetanic contraction (50 Hz stimulation) and resting membrane potential of diaphragm, 24 h after lipopolysaccharide administration, was markedly reduced compared with that of the control group. Moreover, the quantal content of e.p.ps was slightly but significantly increased in the diaphragm of lipopolysaccharide-treated mice. On the other hand, neostigmine (50 nM) increased the indirect contractions and induced a Wedensky inhibition and accelerated the run-down of e.p.ps in diaphragm of control mice, but not in that of lipopolysaccharide-treated mice. However, the increased amplitude and prolonged duration of endplate potentials induced by neostigmine was not altered in the diaphragm of lipopolysaccharide-treated mice. These phenomena induced by neostigmine have been suggested to be due to an accumulation of acetylcholine in the synaptic cleft (Hobbiger, 1976). It has also been demonstrated that the fade of tetanic tension, which was congruent with the acceleration of e.p.ps run-down, caused by inhibition of acetylcholinesterase was induced by the inactivation of Na^+-channels in the area surrounding the endplates and that the sustained fade was due to a decrease of transmitter release (Chang et al., 1986). Thus, these findings indicate that neuromuscular transmission of mouse diaphragm during endotoxemia may be impaired.

Na^+, K^+-ATPase is involved in various important cell functions like cationic equilibrium and recovery of resting membrane potential in neurons (Hernandez, 1992). Ouabain has been shown to inhibit Na^+, K^+-ATPase and could accelerate transmitter release (Vizi, 1972; Baker and Crawford, 1975; Vizi and Oberfrank, 1992). In our previous studies, we have found that the spontaneous release of transmitter from the mouse motor nerve terminal was increased during endotoxemia, and suggested that the impairment of Na^+, K^+-ATPase of the end-plate area might be involved in the effect of endotoxin (Liu et al., 1995). Nishimura et al. (1989) have also shown that ouabain could abolish the twitch potentiation by neostigmine and suggested that ionic gradients maintained by Na^+, K^+-ATPase activity were important for the mechanisms of potentiation of twitch in the presence of anticholinesterases. We now found that ouabain by itself increased the tetanic contraction and quantal content of e.p.ps and antagonized the neostigmine-induced twitch potentiation and acceleration of the e.p.ps run-down in the diaphragm of saline-control mice, but not in that of lipopolysaccharide-treated mice. Similarly, the phenomena of increased quantal content of e.p.ps and decreased neostigmine-induced twitch potentiation and acceleration of the e.p.ps run-down were also shown in the diaphragm of lipopolysaccharide-treated mice. Moreover, we have also found that the Na^+, K^+-ATPase activity was decreased in the sciatic nerve of lipopolysaccharide-treated mice. Thus, from these findings, we infer that suppression of Na^+, K^+-ATPase activity of the end-plate area contributes to the

alteration of neuromuscular transmission during endotoxemia.

Polymyxin B, a polycationic antibiotic, has been reported to directly bind to the anionic lipid A portion of lipopolysaccharide, presumably accounting for its ability to neutralize lipopolysaccharide (Morrison and Jacobs, 1976). Therefore, the antagonistic action of polymyxin B on the alteration of neuromuscular transmission in diaphragm during endotoxemia, implies a pathophysiological role of lipopolysaccharide in the end-plate area. On the other hand, because lipopolysaccharide (20 μM) applied directly to the mouse diaphragm did not affect the nerve-evoked contractions and contraction potentiation by either neostigmine or ouabain (data not shown), we supposed that lipopolysaccharide may affect the function of the end-plate area through a secondary mediator, likely some cytokine.

NO, induced by bacterial lipopolysaccharide or cytokines, is known to play an important role in macrophage killing of cells (Stuehr and Nathan, 1989). NO release has been described in several neuronal types in response to various stimuli (Bredt et al., 1990; Bult et al., 1990; Garthwaite, 1991). It has been also shown that certain cytokines such as tumor necrosis factor-α and interferon-r were capable of inducing neuroblastoma cell differentiation and NO may be an important mediator (Munoz-Fernandez et al., 1994). We now found that the decreased tetanic contraction, decreased resting membrane potential and the increased quantal content of e.p.ps in the diaphragm of lipopolysaccharide-treated mice could be reversed by pretreatment with N^G-nitro-L-arginine, a NO synthase inhibitor. We also found that the NO donor, sodium nitroprusside, could mimic some effects of lipopolysaccharide. These results indicate that NO may be a mediator in the action of lipopolysaccharide on the neuromuscular transmission.

In conclusion, the results obtained indicate that the tetanic contraction and an ouabain-sensitive component of the contraction potentiation induced by neostigmine was altered in the endotoxemic diaphragm. These alterations in neuromuscular transmission of the diaphragm during endotoxemia could be reversed by the administration of polymyxin B and N^G-nitro-L-arginine, suggesting that lipopolysaccharide is indeed responsible for this altered neuromuscular transmission mediated by an impairment in ouabain-sensitive Na^+, K^+-ATPase activity of the mouse motor nerves and NO may be involved in the effects of lipopolysaccharide.

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