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Electrophysiological Effects of Chronic Lead Treatment on Synaptic Transmission in Murine Dorsiflexor Muscle

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Lead poisoning is a potential health hazard that affects various systems including nerves and muscles. Several investigators demonstrated that lead exposure, during the prenatal period, exerts adverse effects on development and function of the nervous system (Kumar and Desiraju 1992; Salanki et al. 1993; Shao and Suszkiw 1991). Additionally, it was reported that lead treatment modifies skeletal muscle functions and neuromuscular junctions (Al Dhaheri et al. 1996). Various mechanisms could explain lead's actions on nerves and muscles (Audesirk 1985; Bressler and Goldstein 1991). Like other heavy metals such as cadmium (Guan et al. 1987) lead exhibited specific effects on neurotransmitters release potentially causing muscle weakness (Hirata and Kosaka 1993; Oortigiesen et al. 1993; Shao and Suszkiw 1991; Struzynska and Rafalowska 1994). Muscle palsy occurred in fatigued frog muscles (Zacharova et al. 1993) may be a result of lead effects on cholinergic nerve terminals (Silbergeld et al. 1974). Evidence presented earlier indicated that lead reduces acetylcholine release from nerve terminals and exerts its influence on the neuromuscular junction rather than a direct effect on the muscle (Kostial and Vouk 1957). For instance, when the presynaptic trunk of superior cervical ganglia of cats was stimulated, after lead nitrate, the force of membrane contraction was reduced. However, similar results were not achieved when the postganglionic trunk was stimulated. These data suggested lead's activity on synaptic function (Kostial and Vouk 1957). Additional support for the presynaptic site for lead action came when the same authors demonstrated that the postsynaptic response to applied acetylcholine was not influenced by lead. Furthermore, lead was found to decrease the force of contraction and to increase the latency between nerve action potential and muscle action potential (Silbergeld et al. 1974). At the neuromuscular junction, lead produced a blocking effect on rat diaphragm (Atchison and Narahashi 1984). The notion that lead dose dependently inhibited Ca^{2+} induced transmitter release in mouse diaphragm (Wang and Quastel 1991; Wiegand et al. 1994) could further explain a presynaptic site of action. Despite extensive work, most experiments dealing with the effects of lead on transmitter release have been performed under acute *in vitro* conditions (Anwyl et al. 1982; Atchison and Narahashi 1984; Cooper et al., 1984; Manalis et al. 1984). The present study was conducted to investigate the effects of chronic lead treatment *in vivo* on resting membrane potential, spontaneous and evoked transmitter release, synaptic delay and muscle twitch tension at *dorsiflexor* muscle of male mice.

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MATERIALS AND METHODS

The experiments were performed on male C57 BL mice. Animals were housed in standard mouse cages (groups of 5), exposed to a 12 hr light / dark cycle at 25°C and were allowed food and water *ad Libitum.* Twenty mice were assigned randomly to 2 groups (10 mice each): one control (vehicle treated) and another chronically lead treated group. Chronic lead treatment experiments were carried out on mice given daily subcutaneous injections of 1.0 mg/kg lead acetate in 5% glucose solution for 4 weeks rabbits (Al Dhaheri et al. 1996; Falke and Zwennis 1990). Except for their intended treatment both groups were handled in the same manner until the 29th day, when electrophysiological protocols were performed, after weighing the animals. Animals were housed and treated according to welfare procedures and were continuously observed for safety by the institution. The study was granted by the institutional research committee. Control and lead treated mice were anaesthetised using urethane (2 mg/g, i.p.). The dorsiflexor muscle was exposed, dissected out and special care was taken to ensure minimum compromise to blood supply. Because transmitter release is affected by muscle stretching, the excised muscle was pinned at 1.1 times the resting length for electrophysiological experiments. Freshly oxygenated solution (95% O_2-5% CO_2) was circulated at a rate of IO-15 ml/min by a gas-lifting device without agitating the recording chamber.

A combination of oblique and transillumination was used in conjunction with a Leitz-Wild microscope to locate endplate regions. Glass capillary microelectrodes, filled with 3 mol/l KCI and drawn to a tip yielding 8-15 mega-ohm resistance (measured in Krebs solution), were inserted into muscle fibers at the endplate region. Conventional intracellular recordings of endplate potentials (EPPs) and miniature endplate potentials (MEPPs) were conducted. Quantal content was also calculated using the direct method (EPP amplitude / MEPP amplitude). Because lead precipitates bicarbonate / phosphate buffered solutions, experiments were conducted in solutions buffered with 4-(2-hydroxyethyl)-I-pipeazineethanesulphonic acid (HEPES) and bubbled with 95% O_2. Exposed muscles were bathed in Krebs solution which had the following composition in mM:. NaCl 135, KCI 5, Ca Gluconate 2.5, $MgS0_4$1, HEPES 3, glucose 11 and pH = 7.2. Only focal MEPPs and EPPs with rise time <1ms were accepted. In order to record evoked EPPs a low Ca^{2+} (0.5 mM) - high Mg^{2+} (2.75 m*M)* Krebs saline was used and the nerve was stimulated supramaximally at 0.5 Hz.

Isometric twitch tension, evoked either directly by muscle stimulation or indirectly by nerve stimulation, was measured after the tendons insertion was attached to a force-displacement transducer (Grass Model FT-03C). The output was differentially amplified and displayed on a chart recorder for later analysis. Following a temperature equilibration of 37°C twitch responses to supramaximal stimuli delivered to the *dorsiflexor* nerve at 1 Hz were recorded in *dorsiflexor* muscle. Direct muscle stimulation was accomplished by placing two wide platinum wires underneath the muscle. Twitches were evoked either directly or indirectly using a Grass (S44) stimulator delivering 5 V, 0.5 ms duration DC square wave pulses. The muscle was lengthened until a maximum twitch

response was elicited. This was achieved usually when the muscle was stretched by 1.1 times its resting length. Frequency - tension relationship was then tested using a train of frequencies of stimulation ranging from 2 to 25 Hz for IO seconds each separated by 5 minutes of rest.

For hematological studies, the carotid artery was exposed, severed and blood was collected in EDTA vaccutainers. Red blood cell (RBC), white blood cell (WBC) and platelets counts plus hemoglobin content and mean cell volume were determined by an automated hematology analyser (Coulter STKS). For measuring lead level, blood was placed in capped plastic vials and allowed to clot, then spun at 11,800 rpm/5min. The resultant serum was collected and lead determination was carried out by a Perkin-Elmer inductively coupled plasma spectrophotometer (Model ICP - Plasma 400) equipped with an autosampler that detected lead content against blank and standard solutions.

Results were statistically treated using one-way analysis of variance and a significance level of 0.05 was used as a cut off for all analyses. The results of two groups comparison are expressed as means ± SD.

RESULTS AND DISCUSSION

Mice chronically treated with lead did not differ significantly in their weights from control groups (34.1 ± 0.8 g for control vs. 32.3 ± 0.5 g for lead treated). The *dorsiflexor* muscle to body weight ratio also remained unchanged. Furthermore, treated mice did not exhibit signs of lead induced neuromuscular pathology such as ataxia or splayed gait. Treated animals showed significantly higher lead level in plasma (0.10 ± 0.02 in control vs. 0.76 ± 0.01 in lead treated, $P < 0.01$). Lead did not modify WBC, RBC and platelet counts. However, hemoglobin content and mean cell volume were significantly lower in lead treated animals (Table 1).

Table 1. Effects of chronic lead treatment on hematological parameters in mice (means ± SD of 10 animals)

Hematological parameters	Vehicle controlled	Lead treated	P value
WBC $10^3/mm^3$	006.5 ± 0.30	007.2 ± 0.20	NS
RBC $10^6/mm^3$	009.1 ± 0.60	008.9 ± 0.60	NS
Platelet $10^3/mm^3$	797.3 ± 110.00	865.0 ± 98.20	NS
Hemoglobin g/dl	015.5 ± 0.04	012.0 ± 0.60	$P < 0.05$
Mean cell volume	049.3 ± 0.40	043.8 ± 0.70	$P < 0.05$

There was no differences in resting membrane potential between control and lead treated mice (77.9 ± 3.2 mV for control and 78.1 ± 4.0 mV for lead treated). Although there were different modes of MEPPs amplitude, the rise time was the same (<1ms) for all. MEPP frequencies were deferentially altered by lead treatment (Table 2). Unimodal and bimodal were significantly lower while small and large modes were unaffected.

Table 2. Effects of chronic lead treatment on spontaneous transmitter release in mice dorsiflexor muscle (means ± SD of 10 animals, 20 muscle fibers from each mouse).

Characteristics	Vehicle controlled	Lead treated	P value
Unimodal (Hz)	3.4 ± 0.20	2.0 ± 0.30	*P < 0.05*
Bimodal (Hz)	2.6 ± 0.40	1.5 ± 0.30	*P < 0.05*
Small mode (Hz)	0.3 ± 0.10	0.4 ± 0.10	NS
Large mode (Hz)	0.3 ± 0.20	0.4 ± 0.10	NS

Compared with control, chronic lead treatment significantly reduced both evoked transmitter release and quantal content (Table 3).

Table 3. Effects of chronic lead treatment on evoked transmitter release in mice dorsiflexor muscle (means ± SD of 10 animals, 130 muscle fibers from control and 186 from treated mice).

Characteristics	Vehicle controlled	Lead treated	P value
EPP amplitude (mV)	1.3 ± 0.20	0.8 ± 0.10	*P < 0.01*
Quantal content	1.2 ± 0.20	0.6 ± 0.10	*P < 0.01*

Isometric force of contraction in response to indirect supramaximal nerve and direct muscle stimulation were reduced in chronically lead treated mice. The reduction was proportional to the size of the muscle. Muscles from chronically lead treated mice generated a significantly smaller force of contraction upon stimulation. Lead treatment had no effect on contractile speed (Table 4).

Table 4. Effects of chronic lead treatment (1mg/kg) on dorsiflexor muscle contraction in mice (means ± SD of 10 animals)

Muscle characteristics	Vehicle controlled	Lead treated	P value
Nerve Stimulation:			
Rise time (ms)	0.40 + 0.05	0.37 ± 0.06	NS
½ Decay time (ms)	0.41 + 0.08	0.41 ± 0.06	NS
Twitch tension (s)	2.82 + 0.10	1.04 ± 0.20	*P < 0.01*
muscle stimulation:			
Rise time (ms)	0.42 + 0.06	0.39 ± 0.05	NS
½ Decay time (ms)	0.42 ± 0.09	0.44 ± 0.07	NS
Twitch tension (s)	3.22 ± 0.70	1.56 ± 0.30	*P < 0.01*

Understanding features of heavy metals poisoning and corresponding cellular and molecular mechanisms will certainly facilitate developing treatments for specific neurotoxic conditions. Lead poisoning represents a common example of heavy metals exposure and can be considered a worthy phenomenon for investigation (Winder et al. 1982). Since most experiments dealing with the

physiological effects of lead ions on transmitter release had been performed under acute conditions we have designed the current study to investigate the effects of chronic lead treatment on spontaneous and evoked transmitter release in mouse *dorsiflexor* muscle. This muscle was chosen because it contains predominantly fast twitch fibres and using the same muscle we have previously reported significant reduction of twitch tension after seven days of lead treatment (Al Dhaheri et al. 1996). In the present study, treated animals showed significantly higher plasma lead levels and serological screening were differentially modified by lead exposure. While lead did not alter WBC, RBC and platelet counts, hemoglobin content and mean cell volume were significantly lower in lead treated animals. These results are in accordance with previously reported effects of chronic subcutaneous lead injection (1mg/kg) in female rabbits (Falke and Zwennis 1990).

According to the data presented here, miniature endplate potential (MEPP) frequencies were found to be differentially modified after chronic lead exposure. MEPP amplitude distributions have been described as unimodal, bimodal, small mode and large mode. All these multimodal types were recorded throughout the experiment and majority of amplitudes observed constituted of unimodal ones. Chronic lead treatment had a selective effect on decreasing the frequencies of only unimodal and bimodal without significantly modulating those of small and large modes. Additionally, chronic lead-treatment did not change resting membrane potential significantly. Based on these findings and the reported changes in MEPP frequencies, lead's activity on skeletal muscle is not solely explained by the depolarization action. The mechanism for different sensitivities of multimodal MEPPs to chronic lead treatment is not adequately understood. Various MEPP amplitude distributions have been previously recognised at the developing phase of the regenerating neuromuscular junctions and at the junctions exposed to botulinum toxin (Bevan 1976; Dennis and Miledi 1971; Harris and Miledi 1971, Kriebel et al. 1976). The presence of small mode MEPPs is possibly ascribed to the release of partially filled vesicles (Glavinovic 1988, Lupa 1988). Researchers studying the action of L-vesamicol, an inhibitor of vesicular acetylcholine uptake, had suggested that small mode MEPPs are generated from a separate highly active and readily releasable pool of vesicles (Searl et al. 1990). It appears that the release mechanism for this pool is different and therefore its sensitivity to bivalent metal ions such as lead varies. The existence of divers release mechanisms was also discussed by other investigators who were able to eliminate, selectively, large mode MEPPs with botulinum toxin (Kriebel et al. 1976 and Cull-Candy et al. 1976).

Chronic lead treatment exerted significant effects on evoked transmitter release and reduced both EPP amplitude and calculated quantal content. Reduced evoked transmitter release in lead treated mice significantly diminished dorsiflexor twitch tension when compared to control. Nerve stimulation and muscle stimulation elicited similar responses and tension decrement was observed in both cases. The inhibitory effects of lead on transmitter release resembled that shown by cadmium (Guan et al. 1988) and zinc (Nishimura 1987; Wang and Quastel 1990). Lead, in contrast to zinc, may have much greater potency and effectiveness on transmitter release (Wang and Quastel 1991). This suggests that lead exerts some action on the process of depolarisation but

possibly through modification of transmitter release. The phenomenon that no changes were noticed in contractile speed after lead treatment further points towards the effect of lead on synaptic transmission rather than muscle.

The present results are in agreement with previously suggested presynaptic effects of lead on synaptic transmission (Manalis et al. 1984; Pickett and Bornstein 1984). Much evidence had been accumulated to support the notion that lead interferes with the process of evoked release of neurotransmitter from nerve terminals. For instance, there were no significant changes in endplate response to ionophoretically applied acetylcholine, input resistance of the muscle membrane and nerve terminal action potential (Zacharova et al., 1993). Effects of chronic lead treatment could also result from blocking entry of calcium into nerve terminals via voltage sensitive channels. The presynaptic site of action of lead is possibly attributed either to affecting the Ca^{2+} receptors located on the surface of nerve terminal (Oortigiesen et al. 1993; Zacharova et al.,1993) and / or disrupting the processes involved in Ca^{2+} buffering inside the nerve terminal (Manalis et al. 1984). Several mechanisms could be implicated such as modulating the number or properties of voltage dependent Ca^{2+} channels in the nerve terminal membrane where lead could compete with Ca^{2+} for its binding sites (Hess and Tsien 1984). Further explanations may revolve around the available Ca^{2+} ions inside the terminal that are usually utilised in transmitter release (Quastel and Saint 1988). Lead may have an influence on surface charge affecting the amount of Ca^{2+} available to its channels in the terminal membrane (Shao and Suszkiw 1991) or it may have an action on the internal Ca^{2+} binding sites and consequently the level of Ca^{2+} sequestering activity (Oortigiesen et al. 1993).

Evidence was also presented to demonstrate direct effect of lead on increasing Ca^{2+} binding by mitochondria (Ooritigiesen et al. 1993). The reduction of spontaneous transmitter release presented here may endorse such an idea. Lead imposed modifications in the regulation of the myoplasmic transient could form an essential part of explaining altered twitch contractile properties (Zacharova et al. 1993). In summary although presynaptic component seems to be more likely in lead's mechanism of action further research is required on the postsynaptic part. Whichever site is involved, the end result would be induction of neuromuscular impairment, that in turn causes muscle weakness, ataxia and paraplegia in animals. Humans exposed to lead are also at much higher risk for neuromuscular impairments (Matsumoto et al. 1993), particularly in oil producing countries.

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