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ELECTROPHYSIOLOGICAL STUDY OF NEUROMUSCULAR BLOCKING ACTION OF COBRA NEUROTOXIN*

BY

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It is well established that the respiratory failure caused by cobra venom in dogs and rabbits is due to its peripheral curare-like action (Kellaway, Cherry & Williams, 1932; Lee & Peng, 1961; Vick, Ciuchta & Polley, 1965). Su (1960) has concluded that the neuromuscular blocking effect of the Formosan cobra (*Naja naja atra*) venom is mainly due to non-depolarizing curare-like action, but is complicated with a direct musculotropic effect, probably due to other components than its neurotoxin. Recently, Su, Chang & Lee (1966) have analysed this problem again with a purified neurotoxin isolated electrophoretically from this venom. In contrast to the crude venom, the purified neurotoxin blocks neuromuscular transmission by competing with acetylcholine (ACh), without affecting the release of ACh on nerve stimulation in the rat phrenic nerve-diaphragm preparation or causing contracture of the chick biventer cervicis or frog rectus abdominis muscle. These findings led them to conclude that cobra neurotoxin behaves just like d-tubocurarine, although the former agent acts much more slowly and less reversibly than the latter.

On the other hand, Meldrum (1965a) isolated component(s) by electrophoresis from Indian cobra venom (*Naja naja*), which possessed most of the toxicity of the original venom and also depolarized the frog sartorius muscle. It was of interest therefore to study neurotoxin, the major toxic component of Formosan cobra venom, with electrophysiological techniques, in order to clarify the exact mechanism of action. The results so obtained are in good agreement with our previous conclusion that cobra neurotoxin acts quite similarly with d-tubocurarine and indicate that depolarization induced by Meldrum's neurotoxic fraction might be due to incomplete separation from other depolarizing component(s), such as cardiotoxin.

METHODS

Cobra neurotoxin

This was prepared by either ammonium sulphate precipitation (70–95% saturation) or starch zone electrophoresis as described by Su, Chang & Lee (1966). For the convenience of comparison, the pattern of protein distribution on electrophoresis is shown in Fig. 1. As reported previously, the neurotoxin was located on Peak II while phospholipase A activity and the cardiotoxic component

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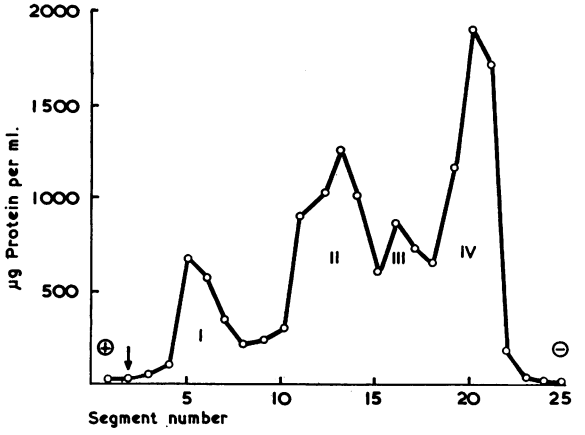

Fig. 1. Cobra venom, 100 mg, was charged at segment No. 2, indicated by an arrow in the figure, of potato starch (Kowng Young Dispensary Ltd.) packed into a semicylindrical glass trough, 40×4 cm. An equi-volume mixture of acetate buffer (pH 5.0, ionic strength 0.1) and sodium chloride (ionic strength 0.1) was used. An average potential difference of 180 V was applied between two ends of trough for 24 hr at 4° C.

were on Peaks I and IV respectively. The subcutaneous LD50 of the neurotoxin in mice was 0.095 μg/g, its toxicity being increased to about seven times that of the crude venom.

Intracellular recording

The conventional micro-electrode recording technique (Fatt & Katz, 1951) was followed, using Grass P6 DC preamplifier and Tektronix 502A oscilloscope. The microelectrodes were filled with 3M KCl and had a resistance of 6–10 MΩ. No capacity compensation for the microelectrode was incorporated. For the rat phrenic nerve-diaphragm preparation, Tyrode solution, oxygenated with 95% O_2+5% CO_2, was used. The temperature was kept at 32–35±0.5° C. For the frog nerve-sartorius muscle, the preparation was suspended in a Ringers solution containing NaCl 117 mM, KCl 2.0 mM, $CaCl_2$ 1.8 mM and $NaHCO_3$ 6 mM, at room temperature (20–24° C).

The end-plate focus was localized with the aid of the time-course of the miniature end-plate potential (EPP) or evoked EPP. Indirect stimulation was applied through a pair of electrodes on the nerve with supramaximal rectangular pulses of 0.2 msec duration, and direct stimulation through electrodes, one on the muscle-tendon junction and the other in the bath fluid, also with 0.2 msec rectagular pulses.

Terminal nerve spike

Extracellular recording of the terminal nerve spike with a microelectrode having resistance of about 5 MΩ was performed on the frog sartorius muscle, according to the technique described by Hubbard & Schmidt (1963); Katz & Miledi (1965). The muscle was immobilized by adding 11 mM $MgCl_2$ to Ringers solution. Under this condition, the terminal nerve spike potential could be recorded together with an EPP.

Antidromic activity

The method described by Randić & Straughan (1964) for the recording of antidromic activity of the isolated rat phrenic nerve was followed. To 30 ml. of modified Tyrode solution, which contained 3.6 mM $CaCl_2$ and 0.12 mM $MgCl_2$, neostigmine methylsulphate was added to a final concentration of 0.3 μg/ml. The temperature was kept at 22±0.5° C. In these conditions it was possible to record the antidromic repetitive discharges of the nerve after single nerve volleys for more than 2 hr if the stimulus frequency was kept at 0.05/sec or less.

RESULTS

Effects on resting membrane potential

Muscle fibres of either the rat diaphragm or frog sartorius were penetrated at random with microelectrodes at either end-plate or non-end-plate zone and the resting membrane potentials recorded. As shown in Table 1, the crude venom severely depolarized both the diaphragm and sartorius muscles. This effect usually occurred almost immediately

TABLE I

EFFECTS ON RESTING MEMBRANE POTENTIALS OF CRUDE COBRA VENOM AND ISOLATED COMPONENTS

Membrane potentials (mV ± S.D.) were recorded from both endplate and non-endplate zones of muscle fibres at the indicated periods after addition of 10 μg/ml. of each agent

	Control	0–5 min.	5–10 min.	10–15 min.	15–20 min.
		Rat diaphragm			
Crude venom	83·0±3·7 (n=30)	49·3±8·3 (n=9)	34·0±17·2 (n=11)	29·0±6·2 (n=9)	23·0±6·8 (n=8)
Neurotoxin	78·1±4·4 (n=28)	81·4±4·4 (n=11)	77·0±3·9 (n=5)	78·4±4·2 (n=10)	76·0±4·8 (n=7)
Cardiotoxin	81·4±4·1 (n=30)	73·7±8·5 (n=11)	54·5±9·7 (n=10)	45·3±14·2 (n=9)	28·9±12·8 (n=12)
Phospholipase A	70·2±6·5 (n=20)	67·4±2·8 (n=9)	69·8±5·9 (n=12)	—	69·7±5·4 (n=22)
Phosphalipase A +Cardiotoxin	66·0±8·1 (n=26)	34·5±9·9 (n=12)	27·6±12·3 (n=18)	18·9±10·4 (n=22)	13·7±3·5 (n=4)
		Frog sartorius			
Crude venom	92·9±5·4 (n=23)	78·9±14·2 (n=10)	54·2±26·3 (n=12)	29·2±12·2 (n=13)	22·8±11·3 (n=17)
Neurotoxin	92·0±2·6 (n=33)	88·3±5·5 (n=11)	87·0±4·9 (n=10)	85·6±5·3 (n=10)	86·9±6·5 (n=11)
Cardiotoxin	88·6±1·2 (n=22)	70·9±15·1 (n=13)	36·0±3·2 (n=18)	23·8±7·0 (n=13)	17·0±2·7 (n=9)

n = number of observations.

after addition of the venom and was accompanied with muscular contracture if a higher concentration such as 50 μg/ml. was used. With 10 μg/ml., however, the depolarization occurred only at the superficial fibres and the contracture was not prominent. On the other hand, cobra neurotoxin did not show any effect on the resting potential of either the diaphragm or sartorius muscle at a concentration of 10 μg/ml. ; this almost completely blocked the neuromuscular transmission within 20 min and was about 100 times higher than the threshold concentration for inducing a complete paralysis of the rat diaphragm (Su *et al.*, 1966). This result is at variance with that of Meldrum (1965a). It was found, however, that the cardiotoxic fraction caused depolarization of both muscles and was even more prominent than the crude venom in this respect. The fraction with phospholipase A activity did not by itself depolarize the rat diaphragm but enhanced the depolarizing effect of the cardiotoxic fraction.

Effect on action potentials

Figure 2 shows the action potentials elicited by direct stimulation of the preparations in which neuromuscular transmission was blocked with the neurotoxin. The amplitude and time-course were apparently not changed. This is in agreement with our previous

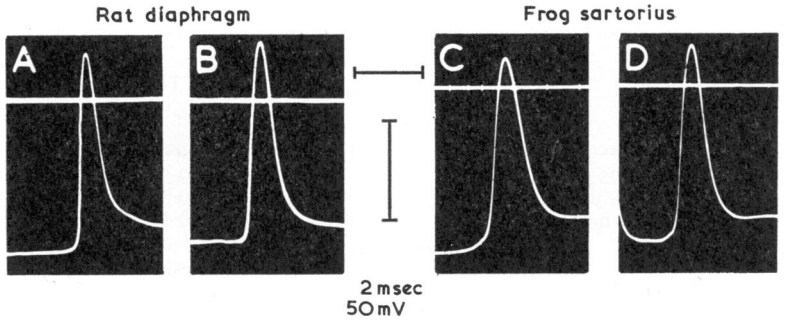

Fig. 2. Effect on action potentials. A and B are from rat diaphragm and C and D from frog sartorius. A and C are control action potentials initiated by indirect stimulation. B and D are action potentials obtained by direct stimulation 40 min after addition of 10 μg/ml. cobra neurotoxin. Calibrations are 2 msec and 50 mV respectively.

finding that the neurotoxin, in contrast to the crude venom, has no effect on the muscle itself.

Effect on miniature EPP and EPP

The spontaneous miniature EPP decreased rapidly in size on addition of the neurotoxin and disappeared completely before the transmission block took place in both the frog sartorius and the rat diaphragm preparations.

No EPP could be recorded from the superficial fibres of the rat diaphragm when the muscle was paralysed by the neurotoxin in concentrations from 0.3 μg/ml. to 10 μg/ml. In order to record the EPP the rat diaphragm preparation was, therefore, first immersed in a concentration of 1 μg/ml. of the neurotoxin for about 1 hr to block the N-M transmission, then washed for 1 hr, and finally switched to a lower concentration, such as 0.03–0.05 μg/ml., which was just enough to prevent the reappearance of mechanical response on nerve stimulation. The time-course of the EPP thus obtained was compared with that of the EPP in the preparation treated with either 0.8 μg/ml. dimethyltubocurarine (DMTC) or 12 mM $MgCl_2$. As shown in Fig. 3 and Table 2, both the

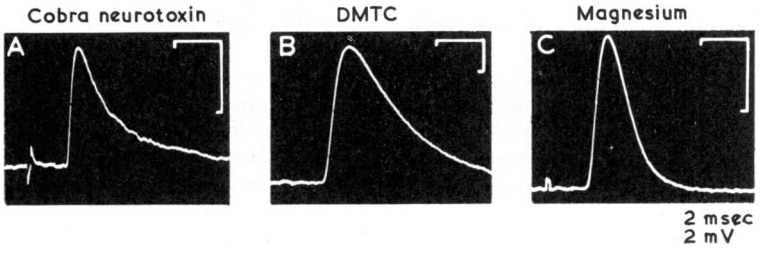

Fig. 3. EPPs as affected by cobra neurotoxin, dimethyltubocurarine or magnesium. EPPs were obtained from rat phrenic diaphragm preparations whose neuromuscular transmission was blocked: A by 0.05 μg/ml. of cobra neurotoxin (see text for detail); B by 0.8 μg/ml. of dimethyltubocurarine; C by 12 mM of $MgCl_2$. Calibrations are 2 msec and 2 mV respectively.

TABLE 2

TIME-COURSES OF EPPs AS AFFECTED BY COBRA NEUROTOXIN AND OTHER NEUROMUSCULAR BLOCKING AGENTS

Agent	Concentration	n	Amplitude (mV±S.D.)	Time from onset to peak (msec±S.D.)	Time from peak to ½ decay (msec±S.D.)
Cobra neurotoxin	0·05 μg/ml.	68	3·14±1·29	0·59±0·13*†	1·02±0·29*†
Dimethyltubocurarine	0·8 μg/ml.	31	3·43±0·94	0·82±0·20†	1·54±0·12†
Magnesium chloride	12 mM	45	3·04±0·60	0·41±0·03	0·71±0·09

n=number of observations.
* $p<0.01$ vs dimethyltubocurarine. † $p<0.01$ vs Magnesium chloride

time for rise to the peak and that for half decay of the EPP in the neurotoxin-treated preparation are shorter than those in the DMTC-treated muscle but longer in duration than those in the magnesium-paralysed one. The more prolonged EPP obtained in the muscle treated with curare as compared with that obtained in the magnesium- or neurotoxin-treated muscle appears to be due to a weak anti-cholinesterase activity of curare (Cogni, 1950).

Antagonism with neostigmine

The EPPs, recorded in either the frog sartorius or the rat diaphragm preparation treated with the neurotoxin, were increased in amplitude and prolonged in time-course by neostigmine (Fig. 4). Some of them became large enough to generate action potentials. Such an antagonistic effect of neostigmine to the neurotoxin is exactly the same as that to d-tubocurarine, as described by Fatt & Katz (1951).

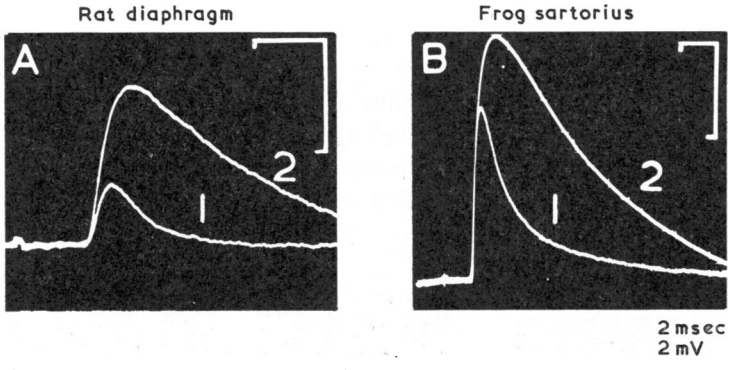

Fig. 4. Effect of neostigmine on EPPs in cobra neurotoxin treated preparations. A=Rat diaphragm preparation treated with 0.05 μg/ml. of cobra neurotoxin. B=Frog sartorius preparation treated with 10 μg/ml. of cobra neurotoxin. 1 in each figure shows EPP before, and 2, 20 min after addition of 1 μg/ml. of neostigmine. Calibrations are 2 msec and 2 mV respectively.

Effect on the train of EPPs

The rapid decline of the successive EPPs in response to repetitive stimulation in the curarized muscle has been considered as the cause of failure to get a sustained contraction. As shown in Fig. 5 and Table 3, in the rat diaphragm treated with the neurotoxin the

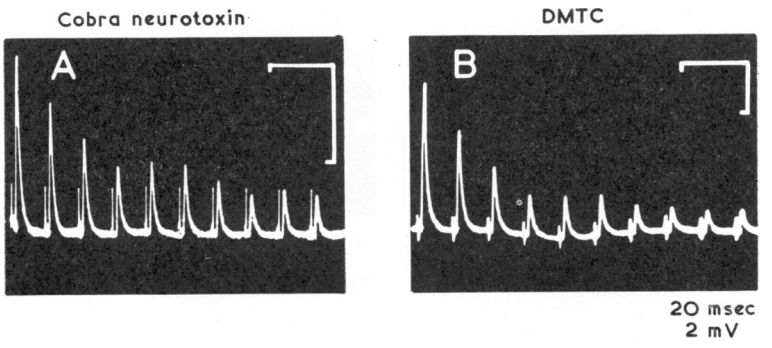

Fig. 5. Pattern of trains of EPPs. Rat diaphragm preparations were treated in same way as described in legend to Fig. 3. Phrenic nerve was stimulated with train of 10 pulses at 100/sec. A=Cobra neurotoxin. B=Dimethyltubocurarine. Calibrations are 20 msec and 2 mV respectively.

TABLE 3

EFFECT OF COBRA NEUROTOXIN AND DIMETHYLTUBOCURARINE ON A TRAIN OF EPPs

The phrenic nerve was stimulated with a train of supramaximal pulses for 100 msec at various pulse intervals. Amplitude of second, fifth and tenth EPPs was expressed as per cent of that of the first EPP respectively

Agent	Pulse intervals (msec)	n	Amplitude of EPP 1st (mV±S.D.)	2nd (%±S.D.)	5th (%±S.D.)	10th (%±S.D.)
Dimethyltubocurarine 0·5–0·8 μg/ml.	50	27	4·95±2·04	73·9±16·4		
	20	24	4·50±2·08	79·2±19·7	45·6±15·5	
	10	37	4·38±1·56	83·2±19·7	48·1±19·6	28·5±10·3
	5	24	3·72±1·22	92·4±22·9	50·8±22·2	30·0±13·1
Cobra neurotoxin 1 μg/ml. for 1 hr then reduced to 0·03–0·05 μg/ml.	50	76	2·36±1·22	74·1±15·7		
	20	72	2·28±1·10	84·2±21·2	50·6±18·7	
	10	75	2·34±1·38	89·2±26·8	56·9±25·1	39·1±22·5
	5	48	2·22±0·97	100·9±24·9	61·6±25·6	41·8±18·7

n=number of observations.

amplitude of the successive EPPs on repetitive stimulation declined almost as rapidly as that in the curarized muscle.

Effect on antidromic activities

It has been shown that curare abolishes repetitive antidromic discharges of motor nerve fibres, evoked by orthodromic nerve impulse under the influence of anti-cholinesterase agents, at a dose much lower than that required for blocking N-M transmission (Feng & Li, 1941 ; Riker, Werner, Roberts & Kuperman, 1959). As shown in Fig. 6, the repetitive discharges of the rat phrenic nerve evoked by a single volley in the presence of neostigmine (0.3 μg/ml.) were progressively diminished and finally abolished by the neurotoxin as well as by d-tubocurarine before the complete N-M block took place.

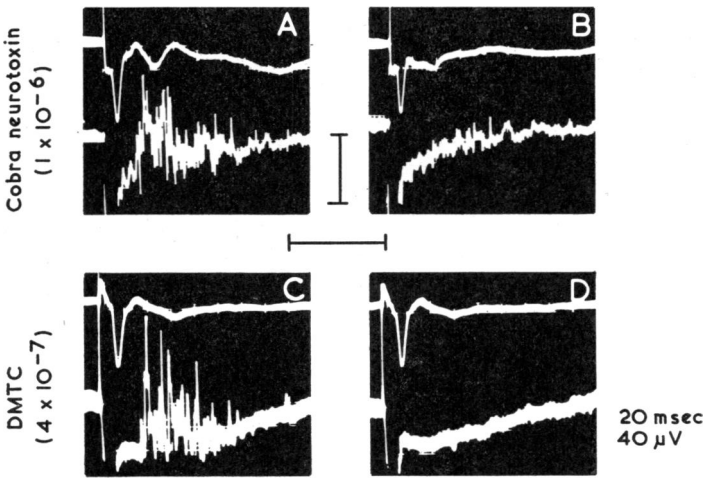

Fig. 6. Effect of cobra neurotoxin on antidromic discharges. A and C=Control antidromic discharges evoked by a orthodromic impulse. (See "Method" for experimental procedures.) B=60 min after addition of 1 μg/ml. of cobra neurotoxin. D=15 min after addition of 0.4 μg/ml. of dimethyltubocurarine. Calibrations are 40 μv and 20 msec respectively. Upper tracing in each figure shows a monitored muscle action potential with extracellular microelectrode. Note that action potential remained when antidromic discharges were abolished.

Effect on terminal nerve spikes

While the terminal nerve spike recorded extracellularly in the frog nerve-sartorius muscle preparation was abolished by the cardiotoxic component, it remained unaffected after the EPP, simultaneously recorded with the same electrode, had been abolished by the neurotoxin (Fig. 7). This finding indicates that the neurotoxin does not interfere with

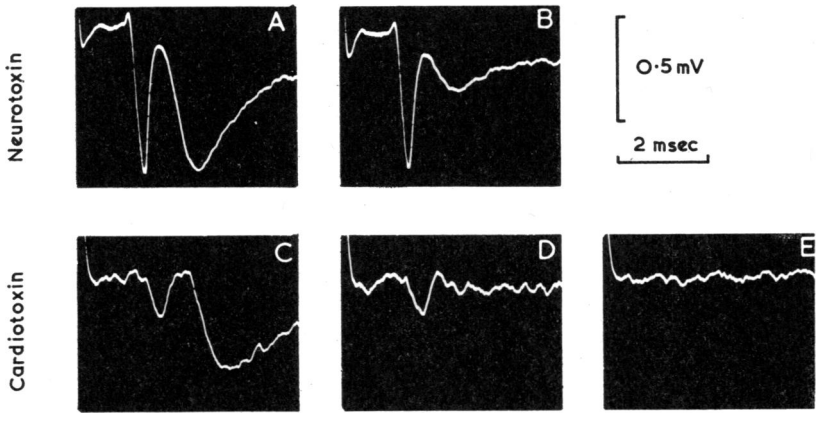

Fig. 7. Effects of cobra neurotoxin and cardiotoxin on terminal nerve spikes and EPPs. Frog nerve sartorius preparations immobilized by 11 mM $MgCl_2$. A and C=Control terminal nerve spike and EPP. B=15 min after addition of 10 μg/ml. of cobra neurotoxin. D and E=10 and 15 min after addition of 10 μg/ml. of cardiotoxin respectively.

the conduction of the nerve axons up to the nerve terminals, whereas cardiotoxin blocks nerve conduction probably by its depolarizing effect. This effect of cardiotoxin may explain the inhibition of ACh output by the crude cobra venom (Su *et al.*, 1966).

DISCUSSION

It is evident from the present experiments that the Formosan cobra (*Naja naja atra*) venom contains component(s) which depolarizes muscle fibres and induces a contracture of the muscle. Unlike the toxic component(s) isolated by Meldrum (1965a), our neurotoxin, isolated by either ammonium sulphate preciptation or starch zone electrophoresis, is devoid of such a depolarizing action even at a concentration nearly 100 times higher than that required to cause neuromuscular blockade. The neurotoxin is also devoid of many other actions, originally present in the crude venom, such as liberation of histamine (Chiang, Ho & Lee, 1964), local irritation, anticoagulant, hypotensive and cardiotoxic actions (Su *et al.*, 1966). It is also free from various enzymic activities, such as protease, cholinesterase, phospholipase A, and hyaluronidase (unpublished observations).

The major component which depolarizes the muscle was found in the cardiotoxic fraction, which migrated toward the cathode most rapidly (Fig. 1) and possessed various actions on the myocardium, blood vessels, intestinal smooth muscle, and nerve axon (Lee, Chang, Chiu, Tseng & Lee, 1966). The toxicity of this fraction in mice (LD50: 2 μg/g), however, is only one-twentieth that of neurotoxin (LD50: 0.095 μg/g) and contributes less than 10% to the toxicity of whole venom. It is probable therefore that, as pointed out by Meldrum (1965b), the toxic component(s) isolated by Meldrum (1965a) may contain both neurotoxic and cardiotoxic fractions as judged from its toxicity and the behaviour on electrophoresis. The direct effect on skeletal and cardiac muscles of the toxin isolated by Detrait & Boquet (1958) may also be due to contamination with cardiotoxic component. On the other hand, the fraction with phospholipase A activity did not depolarize the rat diaphragm by itself, confirming the finding of Meldrum (1965a), although it enhanced the depolarizing effect of the cardiotoxic fraction.

Our previous conclusion (Su *et al.*, 1966) that cobra neurotoxin blocks neuromuscular transmission by an antidepolarizing action, just like d-tubocurarine, was based on the following findings: (1) no inhibition of ACh output after neuromuscular blockade; (2) unchanged response of the muscle to direct stimulation; (3) antagonism by neostigmine; (4) competitive inhibition of ACh response in the frog's rectus abdominis; (5) absence of contracture in the frog's rectus abdominis and sartorius, chick's biventer cervicis and rat's diaphragm; and (6) protection by pretreatment with d-tubocurarine against the paralytic effect of cobra neurotoxin on the chick biventer cervicis muscle. This conclusion was further substantiated by the present electrophysiological findings: (1) absence of effect on the action potential of muscle and on the terminal nerve spikes; (2) depression of the amplitude of both spontaneous miniature EPP and induced EPP; (3) increase in the amplitude and prolongation of the time-course of the EPP in the neurotoxin-treated muscle by neostigmine; (4) rapid decline of the successive EPPs on repetitive stimulation in the rat diaphragm (Wedensky inhibition); and (5) no effect on the resting potentials of end-plates.

The similarity between the actions of cobra neurotoxin and those of d-tubocurarine was further observed in their inhibitory effect on the antidromic activities of the rat phrenic nerve in the presence of neostigmine. Since the antidromic activities of motor nerve fibres have been shown not to be induced by muscle activities but by a presynaptic effect of the neurotransmitter (Barstad, 1962; Randić & Straughan, 1964; Hubbard, Schmidt & Yokota, 1965) it follows that cobra neurotoxin also has an effect on some presynaptic site.

Cobra neurotoxin and d-tubocurarine also share similar species differences of sensitivity. Thus, both are highly active to rats but much less active to cats. The main cause of death in cats treated with cobra venom is not respiratory paralysis due to neuromuscular blockade but circulatory failure (Epstein, 1930; Lee & Peng, 1961).

It is interesting from the pharmacological point of view that d-tubocurarine and cobra neurotoxin have so many actions in common although their chemical structures are quite different from each other. The latter agent is a basic polypeptide, having a molecular weight of about 6,000 (Sasaki, 1957) or 11,000 (Yang, 1965). The slower onset, and less reversible action, of this neurotoxin when compared with d-tubocurarine can be accounted for by its large molecular size. It has several disulphide linkages which are essential for the polypeptide to be active (Lee, Chang & Su, 1960). High contents of arginine in the molecule (Sasaki, 1957) and loss of activity after masking the guanidine group (Lee *et al.*, 1960) are suggestive of the participation of this basic group in the neuromuscular blocking action of cobra neurotoxin.

SUMMARY

1. The effect of purified neurotoxin from Formosan cobra (*Naja naja atra*) venom on neuromuscular transmission was studied electrophysiologically in the rat phrenic nerve-diaphragm and frog sciatic-sartorius preparations.

2. While end-plate potentials were depressed markedly by the neurotoxin, no effect was found on the terminal nerve spikes, action potentials of muscles, and resting membrane potentials.

3. The spontaneous miniature end-plate potentials were diminished rapidly before transmission block occurred.

4. End-plate potentials of the neurotoxin-paralysed muscle showed a shorter time-course than those obtained in the muscle treated with dimethyl-tubocurarine. The former were increased in their size and prolonged in their time-course by neostigmine as were the latter.

5. Antidromic activities in the phrenic nerve in the presence of neostigmine were abolished by the neurotoxin as well as by d-tubocurarine.

6. The amplitude of successive end-plate potentials on repetitive stimulation declined markedly as in the curarized muscle.

7. A component which depolarizes the muscle was found in the cardiotoxic fraction. The terminal nerve spike was abolished by this fraction.

8. It is concluded that cobra neurotoxin acts both post- and pre-synaptically just like d-tubocurarine does.

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