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Endplate blocking actions of lophotoxin

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1 Effects of lophotoxin (LTX), a neurotoxin isolated from Pacific sea whips *Lophogorgia rigida* and *Lophogorgia chelensis*, on neuromuscular transmission were assessed in the rat phrenic nerve-hemidiaphragm preparation using conventional microelectrode recording techniques, and in the frog cutaneous pectoris preparation using two microelectode voltage clamp techniques.

2 LTX (2–25 μM) produced a progressive, irreversible block of miniature endplate potential (m.e.p.p.) and endplate potential (e.p.p.) amplitude. M.e.p.p. amplitude histograms were shifted markedly in the direction of lower amplitude by LTX. These effects occurred following a latency of 25–40 min. The latency to onset of block was decreased with increasing LTX concentrations.

3 In some preparations, LTX produced a transient increase in m.e.p.p. frequency during the first 5 min of application; however, m.e.p.p. frequency then declined to complete block.

4 The depressant effect of LTX on m.e.p.p. and e.p.p. amplitude progressed to complete block irrespective of the LTX concentration.

5 LTX also blocked the endplate depolarization produced by iontophoretic application of acetylcholine (ACh). The resting membrane potential of skeletal muscle fibres was unaffected by LTX.

6 In voltage clamp experiments, LTX (15 μM) depressed the peak amplitude of the endplate current (e.p.c.) nearly uniformly at potentials between − 120 and + 60 mV. LTX did not affect the e.p.c. reversal potential or the kinetics of e.p.c. decay suggesting that LTX does not block open ACh channels. E.p.c. block by LTX was also progressive and irreversible.

7 The results indicate that LTX blocks neuromuscular transmission by a postjunctional action. The binding site of LTX may be different from that of ACh.

Introduction

Lophotoxin (LTX, Figure 1) is a substituted diterpenoid isolated from Pacific sea whips of the genus *Lophogorgia* (Fenical *et al.*, 1981). LTX is a potent neuromuscular blocker, which blocks the nerve-evoked twitch in a progressive and irreversible fashion in concentrations as low as 80 nM (Fenical *et al.*, 1981; Culver & Jacobs, 1981a). Reports from other investigators suggest that LTX has postsynaptic blocking actions; LTX blocks the nerve-evoked endplate potential but does not affect mean quantal content (Langdon & Jacobs, 1983). LTX also blocks the effects of bath applied acetylcholine (ACh) or carbamylcholine on the frog rectus abdominis and sartorious muscles (Culver & Jacobs, 1981a; Langdon & Jacobs, 1983) yet does not compete effectively with α-bungarotoxin for binding to skeletal muscle nicotinic ACh receptors (Culver & Jacobs, 1982). This raised the possibility that LTX might interact

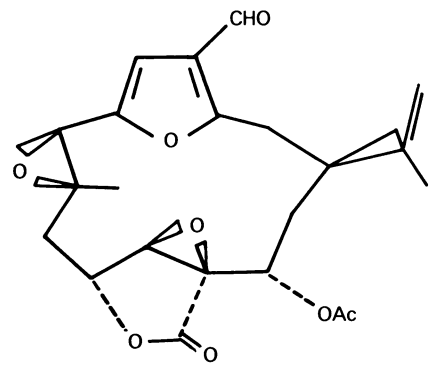

Figure 1 Structure of lophotoxin (LTX).

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irreversibly with the ACh-activated ionic channel, or with the ACh receptor at a site distinct from the cholinoceptive site.

The purpose of the present study was to test effects of LTX on the vertebrate neuromuscular junction using conventional microelectrode, iontophoretic and voltage clamp techniques. We were interested in determining conclusively whether LTX causes block of the postsynaptic membrane, and if so, whether the block was due to occlusion of the ACh-activated ionic channel by LTX.

A preliminary account of this study was presented to the Society for Neuroscience, Minneapolis, MN, U.S.A., October 31–November 5, 1982.

Methods

Experimental techniques

All experiments were conducted at room temperature of 23°–25°C on the isolated phrenic nerve-hemidiaphragm (Bülbring, 1946) of male Harlan Sprague-Dawley rats (180–250 g) or the cutaneous pectoris muscle from frogs (*Rana pipiens*).

Intracellular recordings were made using conventional techniques with borosilicate glass microelectrodes (5–15 MΩ) filled with 3 M KCl. A total of ten conventional microelectrode experiments, three iontophoretic experiments, and six voltage clamp experiments were performed. Miniature endplate potentials (m.e.p.ps) and endplate potentials (e.p.ps) were amplified and recorded on magnetic tape using a Tandberg 115D FM tape recorder. M.e.p.p. frequency and amplitude and e.p.p. amplitude were analysed off-line using a DEC LSI 11-2 computer. The phrenic nerve was stimulated supramaximally at a frequency of 0.3 Hz for 70 μs duration using a suction electrode.

Iontophoretic application of ACh to the endplate was essentially according to the technique of Nastuk (1951). An iontophoretic microelectrode (20–30 MΩ resistance) was positioned close to the endplate as determined by the presence of m.e.p.ps with rise time < 1.5 ms, and current (20 nA, 10 ms duration, 0.1 Hz) was passed across the pipette to eject a small amount of ACh. Iontophoretic current was passed across a 1 gigohm series resistance to provide a constant current pulse. The ACh-induced depolarizations were recorded using a microelectrode filled with 3 M KCl inserted into the endplate region.

Voltage clamp experiments were conducted on cutaneous pectoris muscles by the two microelectrode voltage clamp technique (Takeuchi & Takeuchi, 1959). Muscle contractions were abolished using the formamide method of excitation-contraction uncoupling (del Castillo & de Motta, 1978). Voltage recording and current passing electrodes had a resistance of 2–5 MΩ and were filled with 3 M KCl. Clamp steps were made from a holding potential of −40 mV, and endplate currents (e.p.cs) were elicited by nerve stimuli (0.1 Hz, 50 μs duration) applied through a suction electrode. E.p.cs were recorded before and at various intervals after LTX addition in a single fibre. In some experiments, LTX was omitted and the e.p.c. recorded at 'zero time' and 45 min later as a control procedure. The e.p.cs were digitized and the e.p.c. decay time constant was obtained from a least squares regression fit to the falling phase of the e.p.c. between 20% and 80% of the e.p.c. amplitude.

Chemicals and solutions

Purified lophotoxin (LTX) was the generous gift of Dr William Fenical, Scripps Institute of Oceanography, La Jolla, CA, U.S.A. LTX was dissolved in dimethylsulphoxide (DMSO) to produce a stock solution of 50 mM. Dilutions of this stock to 2–30 μM were made for experiments. Control physiological

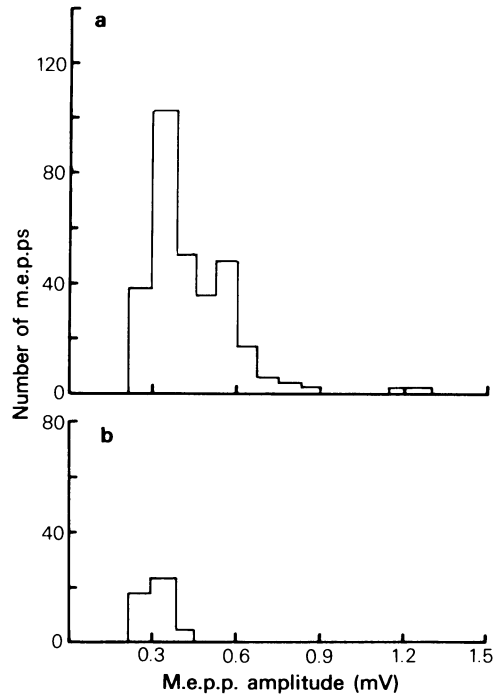

Figure 2 Miniature endplate potential (m.e.p.p.) amplitude histograms before and after lophotoxin (LTX) (25 μM). Data were collected from a single representative rat hemidiaphragm endplate during a control period (a) and during a 5 min experimental period which commenced after 25 min of LTX application (b).

saline (NaCl 0.9% W/V) solutions had a comparable amount of DMSO added. Acetylcholine chloride and formamide were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Mammalian preparations were superfused with a modified Liley's solution (Liley, 1956) which contained (mM): NaCl 135, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, $NaHCO_3$ 12, Na_2HPO_4 1, glucose 11. Nerve-evoked muscle contractions were abolished by lowering the Ca^{2+} concentration and raising the Mg^{2+} concentration to 1 mM and 8 mM, respectively. Isotonicity was maintained by equimolar substitutions of NaCl for $MgCl_2$. Solutions were aerated continuously with 95% O_2 and 5% CO_2 to a pH of 7.3–7–4.

Frog Ringer solution had the following composition (mM): NaCl 111, KCl 4.7, $CaCl_2$ 2, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) 2. The pH of frog Ringer solution was adjusted to 7.35.

Statistical analysis

Effects of LTX on mean m.e.p.p. frequency as a function of time were compared using one-way analysis of variance (Steel & Torrie, 1960). Differences between means were compared using Duncan's Multiple Range Test (Steel & Torrie, 1960).

Results

Effects of lophotoxim (LTX) on nerve-evoked endplate potential

In this and the next section, our purpose was to confirm the earlier work of Langdon & Jacobs (1983) which demonstrated that LTX depresses the e.p.p. and m.e.p.p. at the amphibian motor endplate and to extend this to the mammalian neuromuscular junction. Bath application of LTX (2, 15 and 25 μM) produced a gradual decrease in e.p.p. amplitude which progressed to complete endplate bock (not illustrated). In the five preparations tested, the time of onset of LTX-block of the e.p.p. was somewhat variable; typically 10–15 min were required at 25 μM, while 20–45 min were necessary at the lower concentrations. Although the time required to produce complete block of the e.p.p. was dependent upon the concentration of LTX in the bath solution, the magnitude of block achieved was not concentration-dependent within the range tested. That is, block of e.p.p. progressed to completion at all LTX concentrations tested. Moreover, even if LTX was washed out of the bath before complete e.p.p. block, e.p.p. amplitude still continued to decline until the e.p.p. was abolished. This confirms the previous findings of Langdon & Jacobs (1983) on effects of LTX on e.p.p. amplitude and those of Culver & Jacobs (1981a) on nerve-evoked contractions observed in twitch preparations. Because LTX block of the e.p.p. was progressive, a steady-state effect short of complete block could not be produced. Hence, complete concentration-response studies were not attempted. Once the e.p.p. was blocked, washing of the preparation for up to 45 min with LTX-free solution did not reverse the effect. Muscle fibre resting membrane potential was not affected by LTX.

Effect of LTX on spontaneous ACh release

M.e.p.p. amplitude was decreased progressively by LTX. For example, mean m.e.p.p. amplitude was decreased to approximately 45–55% of control (n= 3) after 20–30 min of 25 μM LTX. As shown for a representative preparation in Figure 2, m.e.p.p. amplitude histograms were shifted to lower amplitudes by LTX, suggesting a decrease in endplate sensitivity to ACh in the presence of LTX. As with effects on the e.p.p., decreased m.e.p.p. amplitude was not reversed by washing with LTX-free solutions for up to 45 min.

Application of LTX (25 μM) produced an initial increase in m.e.p.p. frequency (not illustrated). Increased m.e.p.p. frequency occurred within the first two min and then declined to pre-LTX control values. This increase in m.e.p.p. frequency was observed in all four preparations but was variable in magnitude and time course. Increased m.e.p.p. frequencies ranged from 138% to 255% of pre-LTX control values. Similarly, a slight transient increase in m.e.p.p. frequency was occasionally observed at lower concentrations of LTX, but the % increase over control was lower than that seen at 25 μM. Following the initial stimulation by 25 μM LTX, m.e.p.p. frequency slowly decreased until by 25 min no further m.e.p.ps were observed. This reflects the fact that m.e.p.ps were blocked by LTX. Lower concentrations of LTX (2 μM, 15 μM) also depressed m.e.p.p. frequency, although the time course was somewhat slower.

Effects of lophotoxin (LTX) on acetylcholine (ACh) potential

While LTX block of both e.p.ps and m.e.p.ps suggested a postsynaptic action, the results were not definitive because spontaneous and evoked release are dependent upon both pre- and postsynaptic processes. Therefore, effects of LTX on endplate depolarizations produced by ACh were determined. Because of the long latency between application of LTX and endplate block, it was difficult to maintain constant position of the iontophoretic pipette. However, in two experiments in which stable recordings

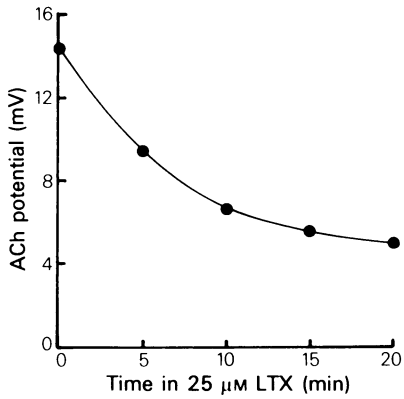

Figure 3 Inhibition by lophotoxin (LTX) of the iontophoretically-induced acetylcholine (ACh) potential. Amplitude of ACh potentials recorded 0, 5, 10, 15 and 20 min following application of LTX (25 μM). Data taken from a representative rat hemidiaphragm in the presence of LTX (25 μM) which was applied at zero time. Responses were elicited by a 27 nA, 10 ms current pulse passed through the iontophoretic micropipette. The time to the peak of the ACh potential remained constant in this experiment suggesting that the position of the iontophoretic pipette tip was essentially constant.

were maintained for 45 min or more, LTX produced a progressive decrease in amplitude of the ACh potential (Figure 3). Exposure to LTX for 20 min decreased the response to ACh to 30–40% of the control amplitude. These experiments were not carried out to complete block of the ACh response. Washout for 10 min with LTX-free solution did not restore the ACh potential, which actually declined even further. Thus LTX blocks the postsynaptic membrane in a progressive and apparently irreversible manner.

Voltage clamp studies

The postjunctional effects of LTX could be exerted either at the ACh receptor, or the receptor-activated ionic channel. In as much as no irreversible channel blocker has yet been discovered, we were particularly interested in determining whether LTX produced irreversible ACh channel block. Effects of LTX on endplate currents (e.p.cs) from a representative voltage-clamped frog endplate are shown in Figure 4. After 45 min, LTX (15 μM) inhibited the peak e.p.c. to a uniform extent (40–45%) at all holding potentials tested between −120 and +60 mV. Control records taken for a similar period of time showed no evidence of inhibition of e.p.c. (not illustrated), so the decreased e.p.c. was not due to deterioration of the preparation. Lower concentrations of (3–5 μM) LTX inhibited the e.p.c. to a similar extent, although the time of exposure necessary to produce a comparable effect was longer. Block of e.p.c. was also progressive and irreversible upon washing with LTX-free medium.

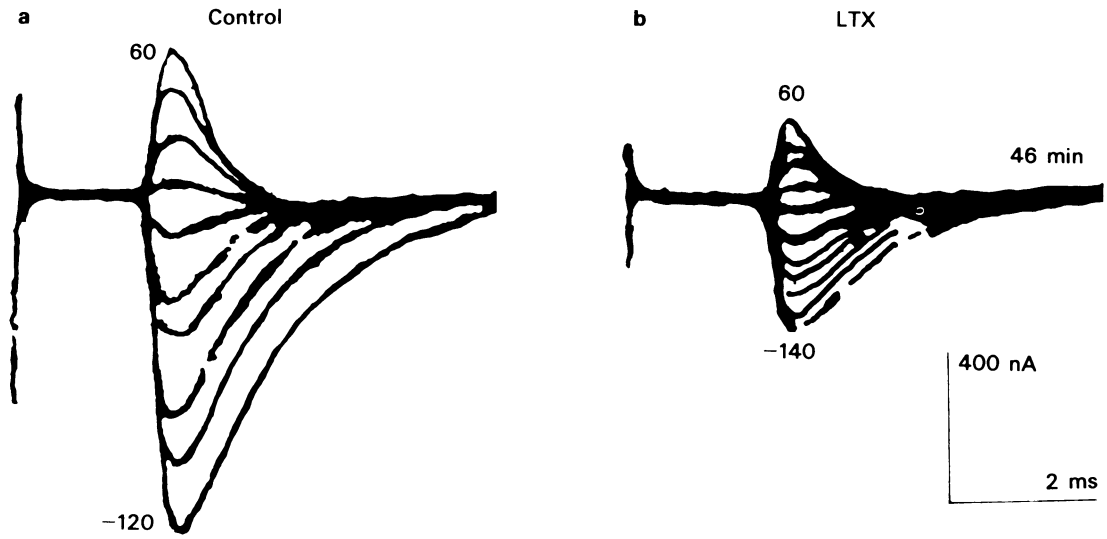

Figure 4 Effects of lophotoxin (LTX) on families of endplate currents (e.p.cs) recorded at various membrane holding potentials in single muscle fibres of a frog cutaneous pectoris preparation. E.p.cs recorded from a single endplate before (a) and 46 min after application of LTX (15 μM) (b). All e.p.cs were elicited 2 s after a step change of the membrane potential which was varied by 20 mV increments in the potential range indicated in each panel. Holding potential was −40 mV. E.p.cs in each family were evoked by nerve stimuli at 0.1 Hz.

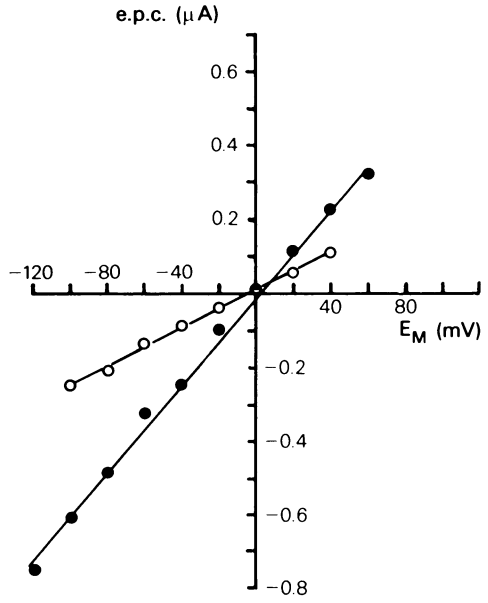

Figure 5 Effects of lophotoxin (LTX) on peak current/voltage relationship at the frog endplate. Results were obtained before (control) (●) and 45 min after addition of LTX (15 μM). (○). Data are from a single endplate of a representative preparation.

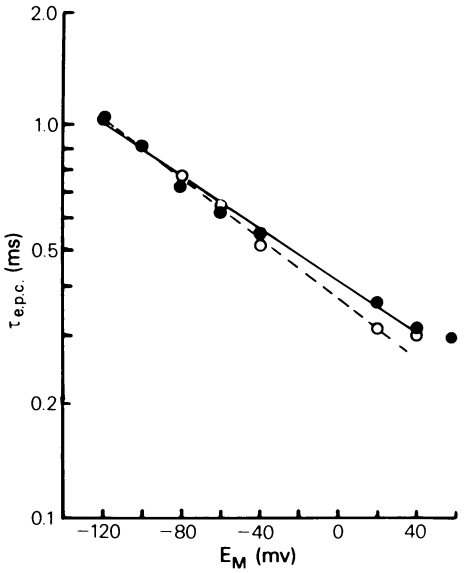

Figure 6 Effects of lophotoxin (LTX) on endplate current (e.p.c.) decay at the frog endplate. Logarithm of the time constant of e.p.c. decay plotted as a function of the membrane potential (E_M) before (●) and 45 min after administration of LTX (15 μM) (○). Slopes were −0.0072 (control) and −0.0081 (LTX), and correlation coefficients (r) were 0.99 (control) and 0.99 (LTX).

Current-voltage (*IV*) relationship for a representative preparation are shown in Figure 5. As can be seen, the reversal potential for the e.p.c. was not altered by LTX. Thus LTX did not appear to alter ionic selectivity of the channel. LTX block did not induce rectification in the peak IV curve. Thus, LTX block appears to be voltage independent.

LTX did not significantly alter the kinetics of decay of the e.p.c. as depicted in Figure 6. The results shown in Figures 5 and 6 are consistent with a voltage-independent block of the closed ACh channel and/or block of the ACh receptor.

Discussion

The results of the present study indicate that the neuromuscular effects of LTX are predominantly postjunctional. Effects on the prejunctional membrane cannot be ruled out definitively because LTX produced a transient increase in m.e.p.p. frequency. However, the fact that the amplitude of the iontophoretically-evoked ACh potential was markedly suppressed by LTX indicates conclusively that LTX blocks the postsynaptic receptor channel complex. This result confirms previous findings using bath applied cholinoceptor agonists in the rectus abdominis, a tonic muscle (Culver & Jacobs, 1981a, b) and sartorious (Langdon & Jacobs, 1983), and extends them to fast and slow twitch fibers employed in this study.

Voltage clamp studies indicated that LTX block of the e.p.c. occurred in a voltage-independent manner, that is, the degree of inhibition was the same at all potentials tested. In addition, LTX did not affect the kinetics of decay of the e.p.c.. In both of these respects, LTX differs from those agents known to interact with the ACh-activated ionic channel such as local anaesthetics (Kordas, 1970; Deguchi & Narahashi, 1971; Ogden *et al.*, 1981), histrionicotoxin (Albuquerque *et al.*, 1974), *n*-alkyl derivatives of guanidine (Farley *et al.*, 1981), and amantadine (Tsai *et al.*, 1978), all of which induce voltage dependence in the peak endplate *IV* relationship and have pronounced effects on the decay kinetics of the e.p.c. The present results are consistent with the notion that LTX reduces the number of available ACh receptor-channels and/or the unit conductance of the channels. Patch clamp analysis of single ACh channels will be needed to resolve this issue. Whether LTX blocks the ACh receptor in a manner analogous to α-bungarotoxin or acts by an alternative mechanism, as for example, a potential-independent block of the closed ACh-activated channel, remains to be clarified. Binding studies have suggested that the site of action of LTX may be distinct from that of curare, since LTX was ineffective in displacing [^{125}I]-α-

bungarotoxin from its specific binding site (Culver & Jacobs, 1982) and (±)-tubocurarine (10^{-3} M) could not protect against LTX block of the ACh-induced contracture (Culver & Jacobs, 1981b). One could retain the view that LTX binds to the ACh receptor by supposing that the binding site is destinct from that occupied by (±)-tubocurarine or χ-bungarotoxin. The furanoaldehyde and α, β-epoxy - γ - lactone functional groups on LTX could react with nucleophilic groups in the ACh receptor to alkylate the receptor (Fenical *et al.*, 1981) in a manner similar to that seen with phenoxybenzamine on α-adrenoceptrors (Nickerson, 1957; 1962). This explains the relatively slow onset and lack of reversibility of block observed with LTX in this and other studies (Culver & Jacobs, 1981; 1982a; Langdon & Jacobs, 1983).

The possibility that LTX produces additional presynaptic actions cannot be dismissed given the transient increase in spontaneous release observed in this study. Thus, LTX may produce both presynaptic stimulation and postsynaptic inhibition. A similar response pattern is seen with certain derivatives of the *n*-alkyl-guanidine group (Farley *et al.*, 1979). However, unlike the guanidine derivatives, the time course for this presynaptic effect of LTX is extremely rapid compared to the slowly developing postsynaptic block. Superimposition of postsynaptic effects by LTX makes studies of potential presynaptic mechanisms difficult. There may be some relationship between our observation *in vitro* that LTX produces a transient stimulation of neuromuscular transmission and *in vivo* observation (Culver & Jacob, 1981a) that subcutaneous administration of LTX to mice produced marked hyperexcitability before development of motor depression. However, we have no explanation at present for the mechanism of any potential prejunctional effects of LTX.

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References

ALBUQUERQUE, E.X., KUBA, K. & DALY, J. (1974). Effects of histrionicotoxin on the ionic conductance modulator of the cholinergic receptor. A quantitative analysis of the end-plate current. *J. Pharmac. exp. Ther.*, **189,** 513–524.

BÜLBRING, E. (1946). Observations on the isolated phrenic nerve diaphragm preparation of the rat. *Br. J. Pharmac.*, **1,** 38–61.

CULVER, P. & JACOBS, R.S. (1981a). Lophotoxin: A neuromuscular-acting toxin from the sea whip (*Lophogorgia rigida*). *Toxicon.*, **19,** 825–830.

CULVER, P. & JACOBS, R.S. (1981b). Postsynaptic inhibition of neuromuscular transmission by a toxin from coral. *The Pharmacologist*, **23,** 131.

CULVER, P. & JACOBS, R.S. (1982). Inhibition of (^{125}I)-α-bungarotoxin binding to skeletal muscle nicotinic receptors by lophotoxin. *Fedn. Proc.*, **41,** 1300.

DEGUCHI, T. & NARAHASHI, T. (1971). Effects of procaine on ionic conductances of end-plate membranes. *J. Pharmac. exp. Ther.*, **176,** 423–433.

DEL CASTILLO, J. & DE MOTTA, G.E. (1978). A new method for excitation-contraction uncoupling in frog skeletal muscle. *J. cell Biol.*, **78,** 782–784.

FARLEY, J.M., GLAVINOVIC, M.I., WATANABE, S. & NARAHASHI, T. (1979). Stimulation of transmitter release by guanidine derivatives. *Neuroscience*, **4,** 1511–1519.

FARLEY, J.M., WATANABE, S., YEH, J.Z. & NARAHASHI, T. (1981). Endplate channel block by guanidine derivatives. *J. Gen. Physiol.*, **77,** 273–293.

FENICAL, W.F., OKUDA, R.K., BANDURRANGA, M.M., CULVER, P. & JACOBS, R.S. (1981). Lophotoxin: A novel neuromuscular toxin from Pacific sea whips of the genus *Lophogorgia*. *Science*, **212,** 1512–1514.

KORDAS, K. (1970). The effect of procaine on neuromuscular transmission. *J. Physiol.*, **209,** 689–699.

LANGDON, R.B. & JACOBS, R.S. (1983). Quantal analysis indicates a curarimetic block by lophotoxin, a non-ionic marine natural product. *Life Sci.*, **32,** 1223–1228.

LILEY, A.W. (1956). An investigation of spontaneous activity at the neuromuscular junction of the rat. *J. Physiol.*, **132,** 650–666.

NASTUK, W.L. (1951). Membrane potential changes at a single muscle end-plate produced by acetylcholine. *Fedn. Proc.*, **10,** 96.

NICKERSON, M. (1957). Nonequllibrium drug antagonism. *Pharmac. Rev.*, **9,** 246–259.

NICKERSON, M. (1962). Mechanism of the prolonged adrenergic blockade produced by haloalkylamines. *Arch. Int. Pharmacodyn.*, **140,** 237–250.

OGDEN, D.C., SIEGELBAUM, S.A. & COLQUHOUN, D. (1981). Block of acetylcholine-activated ion channels by an uncharged local anesthetic. *Nature*, **289,** 596–598.

STEEL, R.G.D. & TORRIE, J.H. (1960), *Principles and Procedures of Statistics*, pp. 99–160. New York: McGraw-Hill Publishers.

TAKEUCHI, A. & TAKEUCHI, N. (1959). Active phase of frog's end-plate potential. *J. Neurophysiol.*, **22,** 395–411.

TSAI, M.C., MANSOUR, N.A., ELDEFRAWI, A.T., ELDEFRAWI, M.E. & ALBUQUERQUE, E.X. (1978). Mechanism of action of amantadine on neuromuscular transmission. *Molec. Pharmac.*, **14,** 787–803.

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