Volume 224, number 2, 277–282 November 1987

Multiple effects of α-toxins on the nicotinic acetylcholine receptor

R.J. Bradley, M.K. Pagala and M.T. Edge

The Neuropsychiatry Research Program, School of Medicine, University of Alabama at Birmingham, PO Box 190, University Station, Birmingham, AL 35294, USA

Received 13 August 1987; revised version received 20 September 1987

Very low concentrations (5 nM) of α-toxin from the venom of *Naja naja atra* produced a characteristic fade in muscle compound action potential and tetanus induced by repetitive nerve stimulation which was identical to the effects of curare. High concentrations of α-toxin and all concentrations of α-bungarotoxin reduced the response but produced very little fade in comparison to curare. These results suggest that α-toxins have more than one effect at the neuromuscular junction.

Desensitization; Curare; α-Toxin; α-Bungarotoxin; (Rat diaphragm, Fade)

1. INTRODUCTION

Curare-like alkaloids and α-toxins from snake venom are competitive antagonists at the muscle nicotinic acetylcholine receptor (AChR). Curare induces a waning or fade in muscle compound action potential (CAP) and tetanus during repetitive nerve stimulation [1]. It has been proposed that fade is caused in part by a presynaptic effect of curare [2–8]. Reports that α-toxins do not cause fade as does curare [5,6,9,10] and do not bind to the presynaptic nerve terminal [11] have been cited as support for this theory [2,5,9]. It has been reported that unlike curare, the α-toxin from *Laticauda semifasciata* does not cause a fade in end-plate currents [5], nor does it bring about tetanic fade in the isolated rat diaphragm [6]. Similarly, it has been reported that α-bungarotoxin from *Bungarus multicinctus* does not cause tetanic fade in the tibialis anterior muscle of the anaesthetized cat [9,10]. In contrast, it was reported that the α-toxin from *Naja naja atra* produced fade in surface recordings from the triceps surae muscle in anaesthetized rats [12]. In the past it has been assumed that there is a normal run-down in the release of ACh during high-frequency nerve stimulation and that the addition of AChR blockade by curare induces failure of neurotransmission [13]. Another theory [3] suggests that in addition to postsynaptic AChR block, curare blocks a presynaptic AChR which normally functions to facilitate ACh release during repetitive stimulation (positive feedback). This pre-synaptic effect could account for curare-induced fade in end-plate potentials during repetitive stimulation. A related theory [8] suggests that a presynaptic AChR normally functions to reduce ACh release during repetitive stimulation (negative feedback). The action of curare on this putative AChR would be initially to increase the quantal content followed by a decrease which is a direct consequence of the initial increase. The decrease in ACh release would result in fade because of the additional postsynaptic AChR block by curare. Here, we provide evidence that the α-toxin from *N. naja atra* does not produce much fade at high concentrations, which supports

Correspondence address: R.J. Bradley, The Neuropsychiatry Research Program, School of Medicine, University of Alabama at Birmingham, PO Box 190, University Station, Birmingham, AL 35294, USA

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

the findings of others [5,6,9,10]. However, it was found that very low concentrations of α-toxin (< 10 nM) did produce a dramatic fade which was identical to the effects of curare. This curare-like fade was not produced by α-bungarotoxin at any concentration.

2. MATERIALS AND METHODS

A special apparatus was used to measure the CAP and tetanus simultaneously in the isolated rat diaphragm [14]. The diaphragm was dissected from 200 g male rats to give a 2–3 mm strip of muscle with the phrenic nerve intact. The muscle was attached vertically to a strain gauge in a glass chamber containing oxygenated buffer. The temperature was maintained at 30°C. The nerve was stimulated supramaximally at 100 Hz with a 1 s train of square-wave pulses of 0.2 ms duration. The muscle CAP was measured with a platinum electrode which made contact with the whole muscle 2 mm below the top tendon. Before stimulating the nerve, the bathing solution was rapidly withdrawn from the chamber to a level below the electrode and below the nerve insertion. The solution in the bath acted as a fluid electrode in the measurement of the CAP. The fluid level was adjusted until a maximal biphasic CAP was obtained and the muscle was stretched until it generated maximal twitch tension to a single nerve stimulation. The fluid level was quickly returned to the normal level after each measurement. Control measurements could be made over a 6 h period without significant change in the amplitude of the CAP and tetanus. The venom from *N. naja atra* was obtained from the Miami Serpentarium and the α-toxin was purified to homogeneity by ion-exchange and gel chromatography [15]. Purified α-bungarotoxin was obtained from Sigma.

3. RESULTS

Curare induced a characteristic fade in the muscle CAP and contraction at 100 Hz stimulation frequency (fig.1). The fade became steeper as the curare concentration was increased. In addition, the fade increased as the stimulation frequency was raised as reported in [16]. The fade consisted of several time constants. The CAP first decreased to a minimum at about 100 ms and then increased

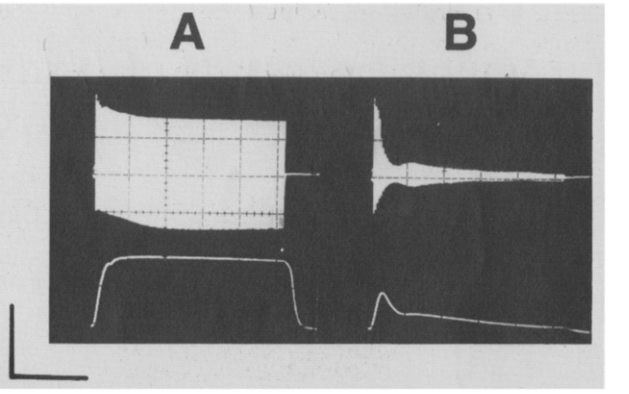

Fig.1. The effects of 400 nM d-tubocurarine on the compound action potential (top) and tetanus tension (bottom) of rat diaphragm after phrenic nerve stimulation for 1 s at 100 Hz. The bath solution was composed of 135 mM NaCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 15 mM $NaHCO_3$, 11 mM glucose (pH 7.4) and bubbled with 95% O_2 and 5% CO_2. The temperature was controlled at 30°C. (A) Control stimulation. (B) After 400 nM curare for 20 min. Calibration bars: compound action potential, 40 mV/400 ms. Tetanus tension, 100 g/400 ms.

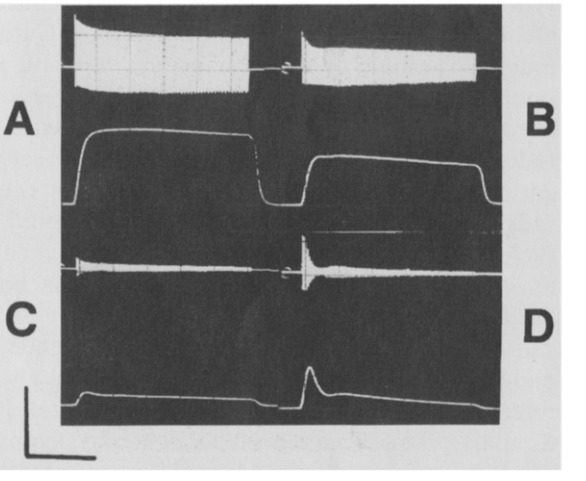

Fig.2. The effects of 100 nM α-toxin on compound action potential (top) and tetanus tension (bottom) of rat diaphragm after phrenic nerve stimulation for 1 s at 100 Hz. (A) Control stimulation. (B) After 100 nM α-toxin for 20 min. (C) After 100 nM toxin for 40 min. (D) After 2 h washout. Calibration bars: compound action potential, 40 mV/400 ms. Tetanus tension, 100 g/400 ms.

briefly again before fading to its final value. The fade was mirrored in the decline of the muscle tetanus recording. However, 100 nM α-toxin had a completely different effect on the CAP at the same stimulation frequency. The CAP and tetanus were measured every 5 min after the addition of 100 nM α-toxin to the bath and at all times there was only slight evidence of fade as the CAP and tension approached zero (fig.2B,C). When the α-toxin was washed out of the bath the response recovered very slowly and as the initial CAP increased in amplitude, the total response began to exhibit exactly the same type of fade which had been observed in the case of curare (fig.2D). Similar examples of fade could be produced by incubating the muscle with very low concentrations of α-toxin (5 nM) for 4–5 h (fig.3). In fact, the fade produced by 5 nM α-toxin after 4 h incubation (fig.3B) was virtually identical to that produced by 400 nM curare (fig.1B). The slow onset of fade after low doses of α-toxin may be due in part to slow diffusion of the high-M_r α-toxin into the end-plate region. The same results were observed when the stimulation frequency was reduced to 50 and 30 Hz but the fade became more pronounced as the nerve stimulation frequency was increased [16]. The average results for the fade produced by both curare and α-toxin before and after washout are summarized in fig.4.

Similar experiments using α-bungarotoxin at a wide range of concentrations produced only the lesser degree of fade which was observed at high concentrations of α-toxin. A curare-like fade was never observed at any concentration of α-bungarotoxin. Fig.5 shows the effects of 5 nM α-bungarotoxin after 5 and 6 h incubation. At all

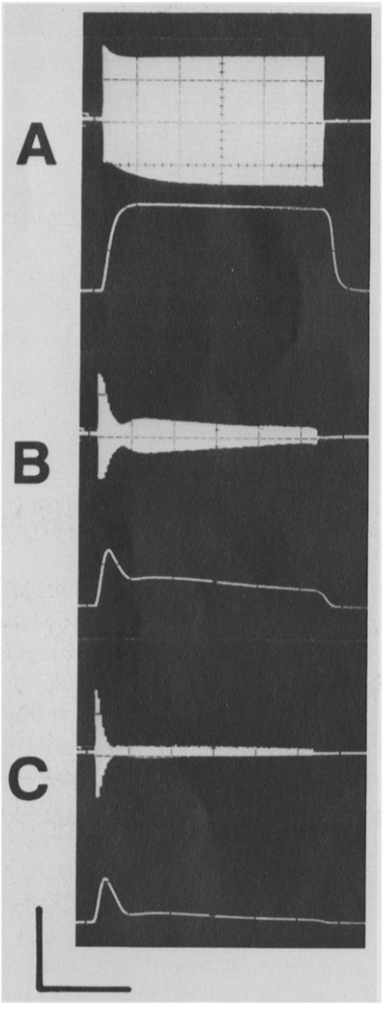

Fig.3. The effects of 5 nM α-toxin from *N. naja atra* on compound action potential (top) and tetanus tension (bottom) of rat diaphragm after phrenic nerve stimulation for 1 s at 100 Hz. (A) Control stimulation. (B) After 5 nM α-toxin for 4 h. (C) After 5 nM α-toxin for 5 h. Calibration bars: compound action potential, 40 mV/400 ms. Tetanus tension, 100 g/400 ms.

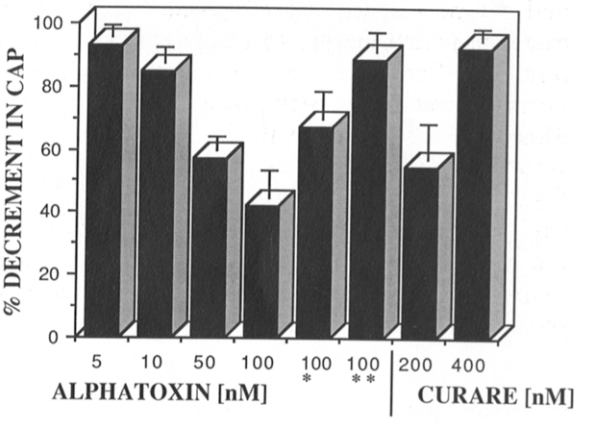

Fig.4. The effects of different concentrations of α-toxin and d-tubocurarine on the CAP. The nerve was stimulated for 1 s at 100 Hz. The average maximum decrement of the 10th CAP of the train as compared to the first CAP and the standard error of the mean are shown (N = 4). In the case of 100 nM α-toxin, the average decrement is also shown after the toxin has been washed out for 1 h* and 2 h**.

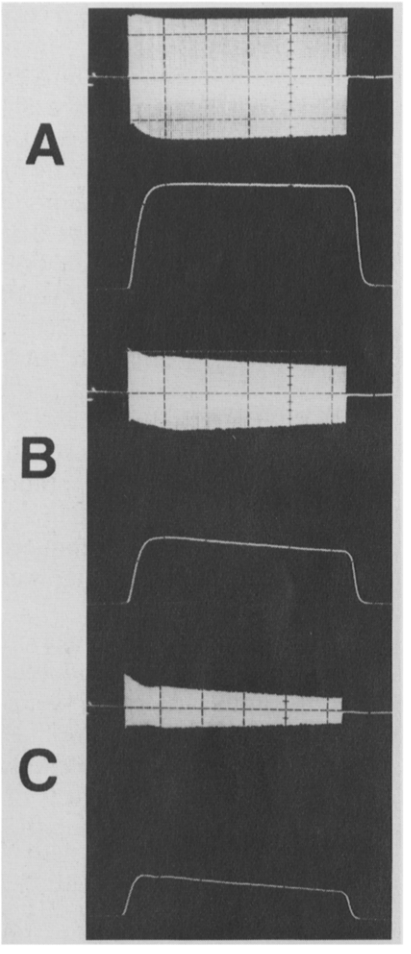

Fig.5. The effects of 5 nM α-bungarotoxin from *Bungarus multicinctus* on compound action potential (top) and tetanus tension (bottom) of rat diaphragm after phrenic nerve stimulation for 1 s at 100 Hz. (A) Control stimulation. (B) After 5 nM α-bungarotoxin for 5 h. (C) After 5 nM α-bungarotoxin for 6 h. Calibration bars: compound action potential, 40 mV/400 ms. Tetanus tension, 100 g/400 ms.

concentrations and incubation times α-bungarotoxin never produced a decrement greater than 50% even when the first CAP of the train was reduced by more than 80%. The effects were almost completely irreversible. The decrement produced by different concentrations of α-bungarotoxin and washout times are summarized in fig.6. The average maximum decrement after treatment with 100 nM α-bungarotoxin was increased after 3 h washout (43 vs 36%) but this change was not statistically significant.

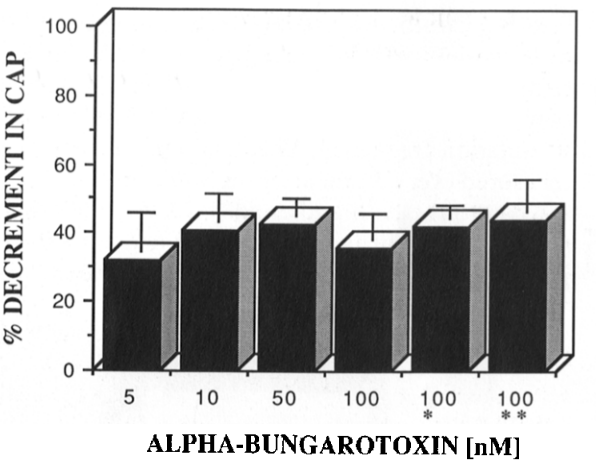

Fig.6. The effects of different concentrations of α-bungarotoxin on the CAP. The nerve was stimulated for 1 s at 100 Hz. The average maximum decrement of the 10th CAP of the train as compared to the first CAP and the standard error of the mean are shown ($N = 5$). In the case of 100 nM α-bungarotoxin, the average decrement is also shown after the toxin has been washed out for 2 h* and 3 h**.

4. DISCUSSION

The results show that curare and very low concentrations of α-toxin produce a similar type of fade at the neuromuscular junction. At high concentrations of α-toxin and at all concentrations of α-bungarotoxin, very little fade was observed. The fade increased significantly when a high concentration of α-toxin was rapidly reduced by washing out but the effects of α-bungarotoxin were essentially unchanged after washout. These results confirm reports in the literature that α-toxins do cause a fade [12] and do not cause a fade [5,6]. Our results show that these conflicting results can now be explained as a concentration-dependent effect. Low concentrations of α-toxin cause a curare-like fade but high concentrations do not. The lack of fade in comparison to curare which we observed in the presence of a range of α-bungarotoxin concentrations confirms other reports in the literature [9,10].

α-Toxins appears to have an additional effect, not shared by curare, therefore it is possible that they may act at an additional binding site. In fact,

binding studies with the reversibly acting α-toxin from *N. naja siamensis* have revealed two separate rates of dissociation from *Electrophorus* AChR [17]. There is evidence that the agonist site on the AChR can be converted to a high-affinity desensitized state [18] or that these sites exist independently [17,18]. Raftery and colleagues [19,20] claim that a low-affinity site for ACh opens the ion channel and binding to a separate high-affinity site appears to block ion flux or induce desensitization [19,20]. This group has claimed that agonists can still bind to the low-affinity sites after the high-affinity sites on the AChR have been blocked by curare or covalently labeled with bromoacetylcholine [21]. It has been reported that although the α-toxin from *N. naja siamensis* has two binding sites on the AChR (one on each of the two α-subunits), the α-toxin from *Dendroaspis viridis* has four binding sites on the AChR [22]. The possibility of more than two ACh activation sites per AChR is supported by reports that the Hill coefficient is between 2 and 3 for AChR activation of frog [23] and rat [24] AChR. Hess and colleagues [25] have recently presented evidence for a new site on the AChR which causes complete inactivation by ACh (isosteric regulation) in the 1 ms time range. This site, which they call the isosteric regulation site, was found to be distinct from the ACh-binding sites for channel activation and for desensitization. The binding process (1 ms) was also much faster than desensitization, which occurred in the 100–300 ms range. α-Toxin, like curare and many other drugs, may have a non-competitive blocking activity, leading to enhanced stabilization of desensitized states of the AChR. However, high concentrations of α-toxin and all concentrations of α-bungarotoxin do not cause such fade. One possibility is that there might be an additional toxin site which is bound irreversibly by α-bungarotoxin and also by high concentrations of α-toxin. Binding to this site might completely inactivate the AChR thus reducing the response but masking the use-dependent fade caused by binding to the desensitization site. Further binding studies will be required in order to determine which of the binding sites for ACh are involved in these complex effects of the α-toxins.

Open channel blocking by curare has also been suggested as a use-dependent process which might help to explain the fade in response to high-frequency nerve stimulation [26]. Given the above results, if channel blocking by curare causes fade, then α-toxin from *N. naja atra* must also be a channel blocker. However, there is no evidence that α-toxin is a channel blocker.

Others have suggested that the binding site involved in the production of fade could be a presynaptic AChR [2–8]. In evaluation of our data it is important to underscore the fact that all efforts to detect interaction or binding of α-toxins to putative presynaptic ACh receptors of any type have been unsuccessful [11]. The inevitable conclusion is that low concentrations of α-toxin and curare have identical effects and therefore must produce fade by acting at the postsynaptic AChR. They may convert the AChR to a desensitizable state thus promoting desensitization by ACh during a stimulation train and thereby causing fade.

ACKNOWLEDGEMENTS

This work was supported in part by US Army Medical Research and Development Command, contract no. DAMD 17-84-C-4182 and the Alabama Chapter of the Myasthenia Gravis Foundation.

REFERENCES

[1] Paton, W.D.M. and Zaimis, E.J. (1952) Pharmacol. Rev. 4, 219–253.
[2] Bowman, W.C. (1980) Anesth. Analg. 59, 935–943.
[3] Bowman, W.C. and Webb, S.N. (1976) Clin. Exp. Pharmacol. Physiol. 3, 545–555.
[4] Galindo, J. (1971) J. Neurophysiol. 34, 289–301.
[5] Gibb, A.J. and Marshall, I.G. (1984) J. Physiol. 351, 275–957.
[6] Gibb, A.J. and Marshall, I.G. (1986) Br. J. Pharmacol. 89, 619–624.
[7] Magleby, K.L., Pallotta, B.S. and Terrar, D.A. (1981) J. Physiol. 312, 97–113.
[8] Wilson, D.F. (1982) Am. J. Physiol. 242, 366–372.
[9] Lee, C., Chen, D. and Katz, R.L. (1977) Can. Anaesth. Soc. J. 24, 212–219.
[10] Alves-do-Prado, W., Corrado, P. and Prado, W.A. (1987) Anesth. Analg. 66, 492–496.
[11] Jones, S.W. and Salpeter, M.M. (1983) J. Neurosci. 3, 326–331.
[12] Satyamurti, S., Drachman, D.B. and Slone, F. (1975) Science 187, 955–957.
[13] Hutter, O.F. (1952) J. Physiol. 118, 216–222.

[14] Pagala, M.K. (1983) J. Electrophysiol. Tech. 10, 111–118.
[15] Yang, C.C., Yang, H.J. and Huang, J.S. (1969) Biochim. Biophys. Acta 188, 65–77.
[16] Bradley, R.J., Pagala, M.K. and Edge, M.T. (1986) Brain Res. 377, 194–198.
[17] Klett, R.P., Fulpius, B.W., Cooper, D., Smith, M., Reich, E. and Possani, L.D. (1973) J. Biol. Chem. 19, 6841–6853.
[18] Hess, G.P., Cash, D.J. and Aoshima, H. (1983) Annu. Rev. Biophys. Bioeng. 12, 443–473.
[19] Dunn, S.M.J. and Raftery, M.A. (1982) Biochemistry 21, 6264–6272.
[20] Dunn, S.M.J. and Raftery, M.A. (1982) Proc. Natl. Acad. Sci. USA 79, 6757–6761.
[21] Dunn, S.M.J., Conti-Tronconi, B.M. and Raftery, M.A. (1983) Biochemistry 22, 2512–2518.
[22] Conti-Tronconi, B.M. and Raftery, M.A. (1986) Proc. Natl. Acad. Sci. USA 83, 6646–6650.
[23] Dreyer, F., Peper, K. and Sterz, R. (1978) J. Physiol. 281, 395–419.
[24] Peper, K., Sterz, R. and Bradley, R.J. (1982) Ann. NY Acad. Sci. 377, 519–543.
[25] Udgaonkar, J.B. and Hess, G.P. (1987) Trends Pharmacol. Sci. 8, 190–192.
[26] Colquhoun, D., Dreyer, F. and Sheridan, R.E. (1979) J. Physiol. 293, 247–284.