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Use of geographutoxin II (μ-conotoxin) for the study of neuromuscular transmission in mouse

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1 Endplate potentials (e.p.ps) were investigated in the presence of geographutoxin II (GTXII) in the mouse phrenic nerve diaphragm preparation. This toxin preferentially blocks muscle Na^+ channels which allows the study of e.p.ps in the absence of nicotinic receptor antagonists or substances to depress acetylcholine release.

2 GTXII abolished muscle action potentials and antagonized the depolarization of the muscle membrane produced by the crotamine-induced opening of Na^+ channels.

3 E.p.ps as large as 19–25 mV were observed after 2–4 $\mu g\,ml^{-1}$ GTXII. These concentrations of GTXII did not cause discernible changes of resting membrane potential and frequency and amplitude of miniature e.p.ps.

4 Lower concentrations (1–2 $\mu g\,ml^{-1}$) of GTXII caused incomplete blockade of the muscle Na^+ channel resulting in exaggerated 'e.p.ps', while higher concentrations of GTXII (8 $\mu g\,ml^{-1}$) abolished e.p.ps by a prejunctional effect.

5 Trains of e.p.ps on repetitive stimulation after GTXII neither ran down, as in tubocurarine-treated preparations, nor facilitated, as in low Ca^{2+} and/or high Mg^{2+}-treated preparations, and were indistinguishable from those of untreated cut muscle preparation.

6 In cut muscle preparations, GTXII did not affect the rise and decay times, amplitude or run-down of e.p.ps.

7 It is concluded that GTXII is a useful agent for studying neuromuscular transmission. This method provides e.p.ps which are neither attenuated nor modified because manipulations that alter transmitter release and postjunctional receptor responses are avoided.

Introduction

Studies of skeletal neuromuscular transmission have relied very much on recordings of endplate potentials (e.p.ps) or endplate current. The information obtained by electrophysiological methods is unrivalled by biochemical or classical pharmacological methods with respect to time resolution especially when pulse to pulse changes of acetylcholine (ACh) release and receptor response are studied. It has been impossible, however, to record the unattenuated e.p.p. in normal preparations because of the ensuing muscle action potential. In order to abolish the muscle action potential and contraction, it is necessary to reduce the amplitude of the e.p.p. pharmacologically, either by decreasing the evoked ACh release in low Ca^{2+} and/or high Mg^{2+} media or by diminishing the postjunctional endplate response with tubocurarine. Another method is to inactivate muscle Na^+ channels by cutting the muscle (Barstad & Lillehel, 1968). These manipulations impose serious limitations on the interpretation of data from such experiments.

Uncontaminated e.p.ps or endplate currents might be revealed if a selective blockade of Na^+ channels of the muscle (and not nerve) can be achieved. Geographutoxin II (GTXII), a novel 22 amino acid peptide toxin isolated from the venom of *Conus geographus* (Nakamura *et al.*, 1983; Sato *et al.*, 1983; Cruz *et al.*, 1985) inhibits the contraction and abolishes the action potential of skeletal muscles at concentrations that scarcely affect conduction in neurones. Binding experiments indicate that GTXII interacts competitively with saxitoxin at neurotoxin receptor site 1 on the Na^+ channel in a tissue-specific manner (Cruz *et al.*, 1985; Moczydlowski *et al.*, 1986; Ohizumi *et al.*, 1986b). The present experiments studied the effect of GTXII on e.p.ps in cut and intact mouse diaphragm preparations, in order to explore the potential of GTXII for obtaining an

unattenuated e.p.p. in the investigation of neuromuscular transmission. It was found that large e.p.ps free from undesirable pre- or postjunctional interferences could be disclosed by treatment with an appropriate concentration of GTXII.

Methods

Nerve-muscle preparations

Phrenic nerve diaphragm preparations were isolated from 20–25 g mice (ICR strain) and bathed in 12 ml Tyrode solution (composition mM: NaCl 137, KCl 2.8, $CaCl_2$ 1.8, $MgCl_2$ 1.1, NaH_2PO_4 0.33, $NaHCO_3$ 11.9 and dextrose 11.2) maintained at 37 ± 0.5°C and aerated with 95% O_2 + 5% CO_2.

For preparing cut muscle preparations, muscles of the diaphragm were dissected at the costal end and crushed at the junction to central tendon. The cut preparations were equilibrated with Tyrode solution for 2–2.5 h, unless otherwise indicated, until the resting membrane potential declined to −44 to −36 mV at which values the Na^+ channels were completely inactivated.

Electrophysiological studies

Intracellular membrane potentials and e.p.ps of superficial muscle fibres were recorded with a d.c.-coupled waveform recorder by using glass microelectrodes (3–10 MΩ when filled with 3 M KCl). E.p.ps were evoked by delivering supramaximal single pulses (width 0.05 ms) to the phrenic nerve, either at 0.66 Hz or by trains of stimulation at 100 Hz for 400–700 ms, at intervals not less than 100 s to minimize post-tetanic potentiation. Square pulses of 0.5 ms duration and of suitable voltage were applied for direct stimulation of diaphragm muscles.

A single electrode current clamp/bridge amplifier (Dagan, Minneapolis) was used to inject current (2.5–60 nA, 5 ms) into muscle fibres.

For each endplate, means of amplitudes, rise times (from 10 to 90%), decay times (to 1/e) and rates of rise of e.p.ps or miniature e.p.ps were calculated from 30–50 successive events. The quantal content of e.p.ps was estimated either by the method of variance or by the direct method (del Castillo & Katz, 1954). E.p.ps were corrected for non-linear summation according to Chang *et al.* (1986) for this purpose. Toxins were added cumulatively because of the very limited amount available and 8–15 endplates were tested for each concentration applied in each preparation.

Statistics

The results are given as mean ± s.e.mean; number of experiments is indicated by *n*. The significance of differences was evaluated by Student's *t* test.

Drugs

Geographutoxin II (GTXII) was a generous gift from Dr C.H. Wu (Northwestern University Medical School, Chicago) and was the same batch as used by Kobayashi *et al.* (1986). Crotamine was kindly supplied by Dr C. Bon (Pasteur Institute, Paris). Tetrodotoxin and tubocurarine were purchased from Calbiochem (San Diego, California).

Results

Effects of GTXII on e.p.ps

Intact diaphragm preparation In uncut mouse diaphragm twitch responses elicited by stimulation of the phrenic nerve were suppressed markedly by GTXII at concentrations greater than 0.7 μg ml^{-1}. For intracellular recordings of e.p.ps to be successful, the concentration of GTXII had to be raised to 1 μg ml^{-1} to avoid dislocation of the microelectrode

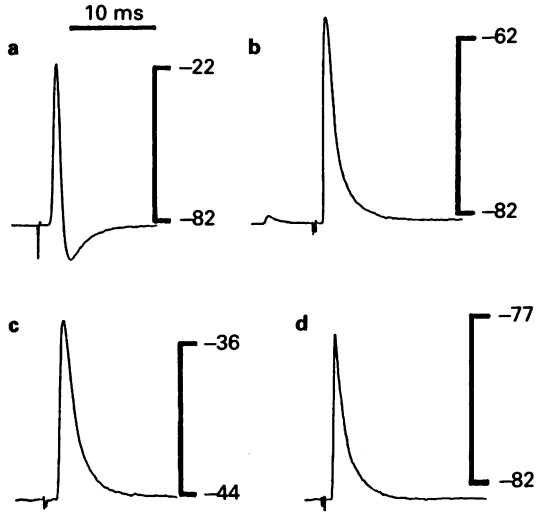

Figure 1 Effects of geographutoxin II (GTXII) or tubocurarine on the e.p.ps of mouse phrenic nerve diaphragm preparations. E.p.ps were recorded in uncut preparations treated with 1 μg ml^{-1} GTXII (a), 2 μg ml^{-1} GTXII (b) or 2 μM tubocurarine (d). The e.p.p. in (c) was obtained from a cut muscle preparation without GTXII or tubocurarine. Note that, in preparations treated with 1 μg ml^{-1} GTXII, about 15% of junctions showed a response similar to that in (a) (the 'e.p.p' was overwhelmed by the active membrane response) and the others showed responses similar to (b). Also note the miniature e.p.p. in (b).

Table 1 Effects of geographutoxin II (GTXII) on the amplitude, decay time and rate of rise of e.p.ps and the amplitude of miniature e.p.ps (M.e.p.ps) in the mouse phrenic nerve diaphragm preparations

		Uncut preparation[a]			*Cut muscle preparation*[a]		
	M.e.p.ps	*E.p.ps*			*E.p.ps*		
GTXII[b] ($\mu g\,ml^{-1}$)	*Amplitude* (mV)	*Amplitude* (mV)	*Decay time* (ms)	*Rate of rise* ($V\,s^{-1}$)	*Amplitude* (mV)	*Decay time* (ms)	*Rate of rise* ($V\,s^{-1}$)
0	1.4 ± 0.3	—	—	—	8.3 ± 0.9	1.26 ± 0.02	27 ± 3
1	1.3 ± 0.2	28.3 ± 2.8	1.14 ± 0.01	97 ± 8	8.9 ± 0.8	1.28 ± 0.07	30 ± 4
2	1.2 ± 0.3	22.3 ± 1.1*	1.15 ± 0.03	75 ± 5*	7.5 ± 0.6	1.20 ± 0.02	22 ± 3
4	1.3 ± 0.3	20.9 ± 1.2*	1.11 ± 0.08	73 ± 5*	7.3 ± 0.6	1.26 ± 0.1	21 ± 3
8	1.3 ± 0.3	7.8 ± 2.1*,**	1.22 ± 0.14	37 ± 11*,**	—	—	—

[a] The number of endplates was at least 24 and 16, respectively, for uncut ($n = 3$) and cut ($n = 2$) phrenic nerve diaphragm preparations except that treated with 8 $\mu g\,ml^{-1}$ GTXII for which values of e.p.ps were obtained from 5 junctions in one preparation before complete block. The resting membrane potentials of uncut and cut muscle preparations were −84.4 ± 2.5 and −42.1 ± 1.2 mV (before GTXII) and −81.9 ± 1.7 and −42.4 ± 1.8 mV (after 4 $\mu g\,ml^{-1}$ GTXII), respectively. The frequencies of miniature e.p.ps of uncut preparations before and after 4 $\mu g\,ml^{-1}$ GTXII were 0.93 ± 0.14 and 1.21 ± 0.27 s^{-1}, respectively.

[b] GTXII was added cumulatively and the incubation time was more than 30 min for each concentration added except that treated with 8 $\mu g\,ml^{-1}$ (7–15 min).

* $P < 0.05$, compared with those after 1 $\mu g\,ml^{-1}$ GTXII.

** $P < 0.05$, compared with those after 2 $\mu g\,ml^{-1}$ GTXII.

by movement of the diaphragm. At this concentration, about 15% of muscle fibres still generated propagating Na^+-spikes in response to nerve stimulations, albeit the amplitudes were reduced (Figure 1a). The amplitude of the spikes measured at the endplate area was 57 ± 11 mV ($n = 3$) with pronounced after-hyperpolarization, while that taken at the ends of the muscle fibre (about 5 mm distal to the endplate) was 25–80% lower. In the rest (85%) of the junctions, stimulation of the nerve produced e.p.ps of 28.3 ± 2.8 mV without triggering propagative potentials. Table 1 shows a comparison of the effects of GTXII on the amplitude, rate of rise and decay time of e.p.ps and on the amplitude of miniature e.p.ps. It should be noted that GTXII affected neither the resting membrane potential of the muscle nor the frequency and amplitude of miniature e.p.ps to any significant extent (cf. Table 1). However, the depolarization of muscle membrane induced by crotamine, a rattlesnake toxin acting preferentially and irreversibly on muscle Na^+ channels at neurotoxin receptor site 3 (Hong & Chang, 1983), was antagonized by GTXII (Figure 2). The membrane potential depolarized again after washout of both toxins, indicating that the binding of GTXII is reversible and the site is distinct from that of crotamine binding.

When the concentration of GTXII was raised to 2 $\mu g\,ml^{-1}$, the diaphragm was paralysed completely and e.p.ps as large as 19–25 mV (Figure 1b) could be recorded only at the endplate area, indicating that the propagative capability of the muscle membrane was lost whereas that of the nerve membrane was still functioning. The amplitude and rate of rise of e.p.ps were 20% smaller than those after 1 $\mu g\,ml^{-1}$ GTXII (Table 1). The rise time was slightly increased from 0.52 ± 0.06 to 0.64 ± 0.05 ms.

Further increase of the concentration of GTXII to 4 $\mu g\,ml^{-1}$, however, had no greater inhibitory effect

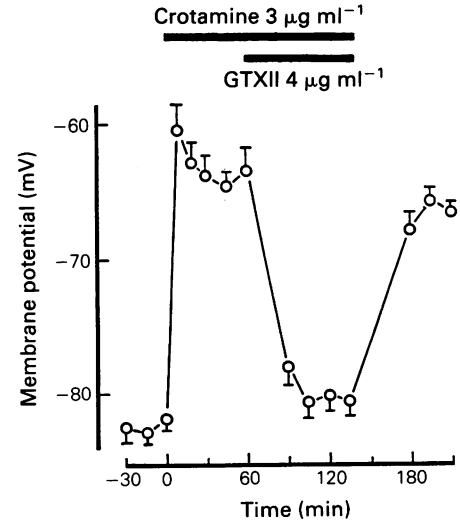

Figure 2 Antagonism of crotamine-induced membrane depolarization by geographutoxin II (GTXII) in uncut mouse diaphragms. Diaphragms ($n = 2$) were treated firstly with 3 $\mu g\,ml^{-1}$ crotamine at 0 min and, 60 min later, co-incubated with 4 $\mu g\,ml^{-1}$ GTXII. Both toxins were removed at 140 min.

so that the amplitudes as well as the rate of rise of e.p.ps were not reduced. Finally, as the concentration of GTXII was increased to $8\,\mu g\,ml^{-1}$, e.p.ps were abolished within 15 min. Before total failure, e.p.p. amplitudes were reduced progressively as the time of toxin incubation increased. The e.p.p. amplitude recorded from 5 junctions before complete failure was 7.8 ± 2.1 mV. The rate of rise of e.p.p. was also depressed. By contrast, the rise and decay times of e.p.ps and the amplitudes of miniature e.p.ps remained unaffected even after treatment with this high concentration of GTXII (Table 1). Washout of GTXII caused e.p.ps to reappear and eventually muscles began to twitch in response to nerve stimulation.

Quantal contents of e.p.ps obtained after GTXII at 2 and $4\,\mu g\,ml^{-1}$ were calculated, by the direct method, to be 21.9 and 18.71 respectively.

Cut muscle preparation GTXII seemed to depress e.p.ps in the uncut diaphragm at two ranges of concentrations, up to $2\,\mu g\,ml^{-1}$ and over $4\,\mu g\,ml^{-1}$. The effect of GTXII was therefore studied in the cut muscle preparation. The e.p.p. amplitude, rise time and rate of rise of the untreated cut muscle at resting membrane potentials of -44 to -36 mV were 8.3 ± 0.9 mV, 0.66 ± 0.07 ms and $27 \pm 3\,V\,s^{-1}$, respectively (cf. Figure 1c). The amplitude of miniature e.p.ps was 0.3–0.5 mV. These values, except the rise time, were about 60–70% less than those obtained in the uncut muscle treated with 2–$4\,\mu g\,ml^{-1}$ GTXII. Unlike in the intact muscle, GTXII up to $4\,\mu g\,ml^{-1}$ caused no statistically significant reduction of these values (Table 1), indicating that GTXII up to $4\,\mu g\,ml^{-1}$ does not affect the quantal release of neurotransmitter. The quantal contents of e.p.ps evoked at 0.66 Hz, calculated by the direct method, were 23.2 and 21.5 respectively before and after GTXII.

In a separate experiment, the effect of GTXII on e.p.p. amplitude was studied in a more depolarized (-32 to -24 mV) cut muscle preparation which would ensure a complete inactivation of muscle Na^+ channels. GTXII $4\,\mu g\,ml^{-1}$ had no apparent depressant effect on the e.p.p. amplitude (control 4.2 ± 0.7 mV, after toxin 3.8 ± 0.6 mV).

Effects of GTXII on the run-down of e.p.ps

The effect of GTXII on e.p.ps evoked at 100 Hz was studied in the uncut diaphragm preparation at higher toxin concentrations which produced a satisfactory immobilization of muscle. In the presence of 2–$4\,\mu g\,ml^{-1}$ GTXII, e.p.ps exhibited an initial facilitation for the first 2–3 e.p.ps, followed by a gradual decline from the 5th onwards and finally levelled off after about 20 pulses; the amplitude being approximately 75% of that of the 1st e.p.p. of the train stimulation (Figures 3c and 4). This profile of run-down of e.p.ps on repetitive stimulation was indistinguishable from that of cut muscle in the absence of drugs. In the cut muscle preparation, GTXII up to $4\,\mu g\,ml^{-1}$ did not alter the pattern of e.p.p. run-down (Figures 3a,b and 4).

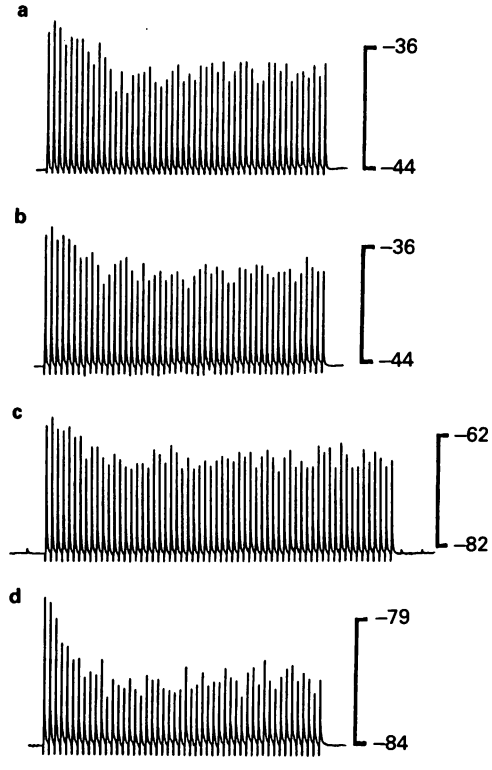

Figure 3 Effects of geographutoxin II (GTXII) or tubocurarine on the run-down of e.p.ps in the mouse phrenic nerve diaphragm preparations. E.p.ps were evoked by trains of stimulation of the phrenic nerve at 100 Hz for 500–630 ms. (a) Taken from an untreated cut muscle preparation. (b) The same endplate as in (a) but after 60 min treatment with $4\,\mu g\,ml^{-1}$ GTXII. (c and d) Different uncut preparations after 60 min incubation with $4\,\mu g\,ml^{-1}$ GTXII and $2\,\mu M$ tubocurarine, respectively.

Figures 3d and 4 compare the run-down of e.p.ps of uncut preparations paralysed with tubocurarine 1.5–$2\,\mu M$, which is the minimum concentration to immobilize the diaphragm. After 60 min incubation, miniature e.p.ps were undetectable and the amplitude of e.p.ps was only 4.9 ± 0.8 mV (cf. Figure 1d) despite the high resting membrane potential (-84.4 ± 1.38 mV, $n = 3$). Noticeably, the run-down of e.p.ps was twice as great as those obtained without tubocurarine. In cut muscle preparations,

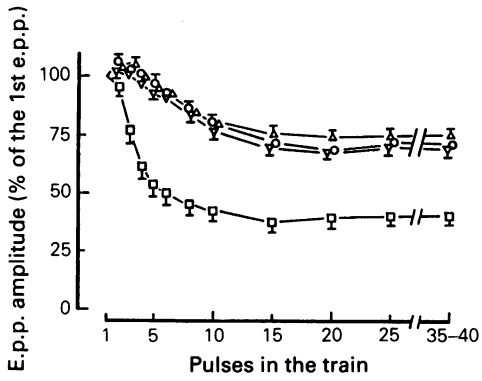

Figure 4 Effects of geographutoxin II (GTXII) on the run-down of e.p.ps in the mouse phrenic nerve diaphragm preparations. E.p.ps were evoked by train stimulations of the phrenic nerve at 100 Hz on uncut (△, □, $n = 3$) or cut (○, ▽, $n = 2$) diaphragms. The uncut preparations were treated with either 4 $\mu g\,ml^{-1}$ GTXII (△) or 1.5–2 μM tubocurarine (□) for 60 min. The cut preparations were either not treated (○) or treated with 4 $\mu g\,ml^{-1}$ GTXII (▽).

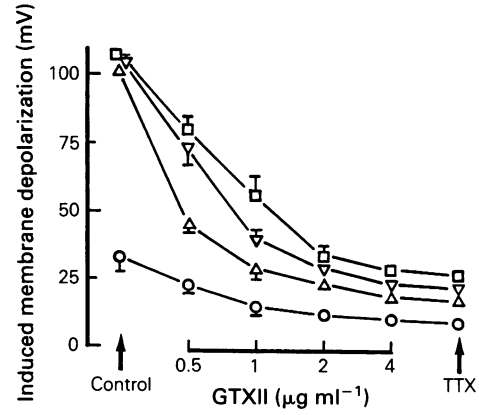

Figure 5 Effects of geographutoxin II (GTXII) and tetrodotoxin (TTX) on the muscle membrane depolarization induced by current injection in the uncut mouse diaphragm. Depolarizing currents of 5 (○), 10 (△), 20 (▽) and 40 (□) nA were injected into the endplate area ($n = 3$). The electrotonic components of the induced membrane depolarization was indicated by the membrane potential shift after treatment with 120–350 $ng\,ml^{-1}$ TTX for 60 min. The resting membrane potentials of control, after 4 $\mu g\,ml^{-1}$ GTXII and after tetrodotoxin were -83.5 ± 1.1, -82.3 ± 1.8 and -85.6 ± 2.2 mV, respectively.

tubocurarine accelerated the run-down of e.p.ps as previously observed (Glavinovic, 1979; Chang *et al.*, 1988).

Effects of GTXII on potential changes induced by current injection

In the uncut muscle, the muscle resting membrane potential is as high as −80 mV and most Na^+ channels should be available for opening on depolarization provided that channels are not plugged. The question then arises as to whether the large e.p.ps observed in the uncut preparation after GTXII treatment resulted from a superimposed activation of residual unblocked Na^+ channels in addition to opening of ACh channels. To assess the contribution of Na^+ channels to the large e.p.ps, the inhibitory effects of GTXII on depolarization induced by current injection were investigated at the endplate area where the density of Na^+ channels is high (Beam *et al.*, 1985). Sufficient current was injected to simulate the depolarizing effects caused by the opening of ACh channels. Depending on the amount of current injected, muscle membranes generated either a simple sustained electrotonic potential, or a brief local depolarization on top of the electrotonic potential due to a local opening of Na^+ channel or, as the threshold was reached, a spike which overshot zero potential by 15–25 mV. Tetrodotoxin 120 $ng\,ml^{-1}$ completely suppressed these responses except the electrotonic potential. The concentration of tetrodotoxin producing half inhibition of the active response was about 20 $ng\,ml^{-1}$. Similar to the effect of tetrodotoxin, GTXII 0.5–4 $\mu g\,ml^{-1}$ inhibited the local depolarization and the spike dose-dependently (Figure 5). The concentration of GTXII producing half inhibition was about 0.5 $\mu g\,ml^{-1}$. With this concentration of GTXII, action potentials, though reduced in amplitude, could be obtained when large amounts of current (20–40 nA) were applied. The action potentials were completely abolished and local active depolarizations were largely suppressed as the concentration of GTXII was raised to 2 $\mu g\,ml^{-1}$. As the concentration of GTXII reached 4 $\mu g\,ml^{-1}$ the local depolarizations were negligible and the total membrane depolarization was suppressed to the same extent as in 120 $ng\,ml^{-1}$ tetrodotoxin (Figure 5). With regard to the inhibitory action on muscle Na^+ channels, the effect of 2 $\mu g\,ml^{-1}$ GTXII was approximately equal to that produced by 60 $ng\,ml^{-1}$ tetrodotoxin.

Discussion

Ligand binding studies have revealed that GTXII binds to the Na^+ channel competitively against tetrodotoxin and saxitoxin, but, in contrast to the latter two toxins, GTXII had higher affinity for skeletal muscle than nerve (Moczydlowski *et al.*, 1986; Ohizumi *et al.*, 1986b; Gonoi *et al.*, 1987; Yanagawa

et al., 1987). Electrophysiological studies confirmed the preferential blocking action of GTXII on Na^+ channel of muscle (Cruz *et al.*, 1985; Kobayashi *et al.*, 1986; Ohizumi *et al.*, 1986a). The present results showed that GTXII, at concentrations that did not appear to impair nerve action potential, abolished the muscle action potential and effectively reversed the membrane depolarization caused by crotamine, a peptide toxin which opens the Na^+ channel of mammalian skeletal muscle (Chang *et al.*, 1983). With low concentrations of GTXII an appreciable fraction of Na^+ channels in the diaphragm seemed unaffected and could be opened by large step depolarizations (Figure 5). It is likely, in this respect, that the end-plate depolarization caused by excitation of the ACh receptor may activate some of the residual unblocked Na^+ channels and result in an overestimate of e.p.p. amplitude. Indeed, in the uncut muscle preparations, the apparent e.p.p. amplitude was reduced by about 20% when GTXII was increased from 1 to 2–4 $\mu g\,ml^{-1}$, while no change of e.p.p. amplitude was observed up to 4 $\mu g\,ml^{-1}$ GTXII in the cut muscle preparations in which the muscle Na^+ channel was already inactivated due to depolarization (Table 1). This contamination from Na^+ channel activation should become greatly reduced on increasing the GTXII concentration to 2–4 $\mu g\,ml^{-1}$, since the membrane depolarization due to Na^+ channel activation was negligible as far as the amplitude of step depolarization was within the range of e.p.ps. In the rat cultured myoball, these concentrations of GTXII were sufficient to block the tetrodotoxin-sensitive binding or Na^+ current (Gonoi *et al.*, 1987).

The e.p.p. amplitude and rise time appeared constant in 2–4 $\mu g\,ml^{-1}$ GTXII, either in the cut or in the uncut preparations, indicating that the release of neurotransmitter was not affected. E.p.ps were finally abolished after incubation with 8 $\mu g\,ml^{-1}$ GTXII. Since amplitudes of miniature e.p.ps were unchanged, the blockade of e.p.ps must derive from a prejunctional failure of transmitter release, probably due to an inhibitory action on the nerve Na^+ channel.

In the present experiments with mouse phrenic nerve diaphragm preparations, GTXII seems not to discriminate in favour of muscle against neuronal Na^+ channels as effectively as previously observed (Nakamura *et al.*, 1983; Cruz *et al.*, 1985; Ohizumi *et al.*, 1986a). Part of the reason for the discrepancy in the relative potency of GTXII with respect to muscle and nerve may be that we looked at the effects on e.p.p. amplitude instead of muscle action potential or axonal conduction. Yet, a careful treatment of vertebrate neuromuscular preparations with a proper concentration (2–4 $\mu g\,ml^{-1}$) of GTXII may be the best known procedure to study uncontaminated e.p.ps and thus to study the prejunctional as well as the postjunctional events with unblocked ACh receptors and unimpaired ACh release. The resting membrane potential is unchanged, acetylcholinesterase activity is not inhibited, the e.p.ps are large and can be recorded together with the miniature e.p.ps. Tetrodotoxin and saxitoxin cannot substitute for GTXII for these purposes because muscle Na^+ channels are not preferentially blocked.

To date, with normal Ca^{2+} and Mg^{2+} concentrations, the quantal content of e.p.ps in uncut preparations can be measured in the presence of tubocurarine either by the method of variance, which deviates from poisson statistics when the release is high (Ginsborg & Jenkinson, 1976), or by the 'semi-direct' method, which relies on the determination of tubocurarine's dissociation constant (Ceccarelli & Hurlbut, 1975). It is now possible to estimate the normal quantal release by directly comparing the amplitudes of e.p.ps with those of miniature e.p.ps. After correcting for non-linear summation of e.p.ps, the quantal content of the intact and cut mouse diaphragms is estimated to be only 20–25 in contrast to 127 ± 19 estimated by the method of variance from the same set of data. The lesser extent of e.p.p. run-down on high frequency stimulation in GTXII-treated or cut muscle preparations than in tubocurarine-treated preparations may further support the view that the marked e.p.p. run-down after tubocurarine is due to a prejunctional effect (Glavinovic, 1979; Bowman *et al.*, 1986; Chang *et al.*, 1988).

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