Journal of Physiology (1992), **458**, *pp.* 487–499
With 5 figures
Printed in Great Britain

ADAPTATION OF QUANTAL CONTENT TO DECREASED POSTSYNAPTIC SENSITIVITY AT SINGLE ENDPLATES IN α-BUNGAROTOXIN-TREATED RATS

BY J. J. PLOMP, G. TH. H. VAN KEMPEN AND P. C. MOLENAAR

From the Division of Physiological Pharmacology, Department of Physiology, Sylvius Laboratory, Post Box 9503, 2300 RA Leiden, The Netherlands

(*Received 13 January 1992*)

SUMMARY

1. Rats were injected once every 48 h with α-bungarotoxin (αBTX) for periods up to 6 weeks. Injections caused weakness of facial muscles which lasted about 8 h. Hemidiaphragms were dissected for biochemical and electrophysiological measurements.

2. In muscles from animals treated for 2–3 weeks with toxin, the binding of ^{125}I-αBTX was reduced to 58 %, and the ACh content to 81 % of control values. Choline acetyltransferase activity was unchanged. ACh release evoked by 3 Hz nerve stimulation was increased to 175 % of control values.

3. The use of μ-conotoxin, which specifically blocks muscle action potentials, enabled the recording of full-sized endplate potentials (EPPs) and miniature endplate potentials (MEPPs) at normal muscle membrane potentials (−70 to −80 mV). The amplitude of MEPPs was decreased to 57 % in muscles from animals treated for 3 weeks with αBTX. The mean of the quantal contents, calculated from the ratio of the corrected EPPs and the MEPPs, was increased to 154 %.

4. Within individual muscles of both αBTX-treated and control rats, there was an inverse relationship between the quantal content of an endplate and its MEPP amplitude.

5. The MEPP frequency of endplates from control muscles was positively correlated with the quantal content. However, this correlation was not found in αBTX-affected muscles.

6. Three hours after a single injection of αBTX the amplitude of the MEPPs was reduced to about 60 % of control values but no increase of the quantal content was found. During the first few days of αBTX treatment the quantal content gradually increased; it reached a plateau between 20 and 30 days.

7. The results suggest the existence of an adaptive mechanism, operating at individual endplates, in which retrograde signals at the motor nerve terminals modulate ACh release when neuromuscular transmission is endangered by block of acetylcholine receptors.

MS 1036

INTRODUCTION

In myasthenia gravis (MG) the loss of acetylcholine receptors (AChRs) at the neuromuscular junction induced by auto-antibodies results in subthreshold endplate potentials (EPPs) in many fibres and thus in weakness of skeletal muscles (Vincent, 1980). In addition, there are also presynaptic changes in MG: the activity of choline acetyltransferase (ChAT) and the content of ACh are increased (Ito, Miledi, Molenaar, Vincent, Polak, Van Gelder & Newsom-Davis, 1976; Molenaar, Polak, Miledi, Alema, Vincent & Newsom-Davis, 1979; Molenaar, Newsom-Davis, Polak & Vincent, 1981). The stimulus-evoked ACh release, determined by biochemical and electrophysiological methods, is considerably increased in MG (Molenaar *et al.* 1979; Cull-Candy, Miledi, Trautmann & Uchitel, 1980) and also in an auto-immune model for MG in rats (Molenaar *et al.* 1979; Takamori, Sakato & Okumura, 1984). Increased transmitter release may be a secondary phenomenon, compensating for the loss of AChRs in MG and thereby alleviating weakness.

Using a chemical technique for measuring ACh we recently found that an increase of transmitter release also occurs when AChRs have been reduced under non-immunopathological conditions (Molenaar, Oen, Plomp, Van Kempen, Jennekens & Hesselmans, 1991). In these experiments, AChRs in rat muscles were chronically reduced by systemic injections of α-bungarotoxin (αBTX, a highly specific and irreversible blocker of AChRs of muscle, Chang & Lee, 1963), a procedure causing weakness especially of facial muscles (Molenaar, Plomp & Van Kempen, 1990).

A possible mechanism for increase of ACh release is enlargement of the endplate area with a concomitant increased number of active zones. It is known that in normal frog and mouse muscle, the fibre diameter is positively correlated with endplate area and quantal content (Kuno, Turkanis & Weakly, 1971; Harris & Ribchester, 1979). As a low input resistance is an important feature of thick muscle fibres, the miniature endplate potentials (MEPPs) in such fibres are relatively small (Katz & Thesleff, 1957). The high quantal content in thick muscle fibres has been considered as an attempt of the endplate to compensate for the relatively small depolarizing effect of the transmitter (Harris & Ribchester, 1979). It is uncertain whether such adaptation gradually emerges during development or that there exists a special mechanism which is turned on as soon as the neuromuscular transmission is endangered.

In the present electrophysiological experiments we investigated whether the low postsynaptic sensitivity in the αBTX model for MG also induces a high quantal content and whether this increase is correlated to the level of postsynaptic sensitivity, i.e. the extent of reduction in the number of functional AChRs. To prevent muscle action potentials we used μ-conotoxin (Cruz, Gray, Olivera, Zeikus, Kerr, Yoshikami & Moczydlowski, 1985; Yanagawa, Abe & Satake, 1987; Di Gregorio, Fesce, Cereser, Favaro & Fiori, 1989; Olivera, Rivier, Clark, Ramilo, Corpuz, Abogadie, Mena, Woodward, Hillyard & Cruz, 1990). The advantage of this method was that it allowed the 'direct' calculation of the quantal content even in 'myasthenic' endplates with very small MEPPs.

There was a striking dependence of the quantal content on the postsynaptic sensitivity in chronic 'myasthenic' muscle, the smaller the MEPPs of an endplate the higher was the quantal content. However, biochemical measurements suggested that the mean size of the presynaptic apparatus was unchanged.

METHODS

Animal model for MG

Male Wistar rats, weighing 70–90 g at the beginning of the experiment, received repeated injections of αBTX (Biotoxins Inc., St Cloud, FL, USA) for 3 weeks (one subcutaneous injection of 3–5 μg αBTX per 48 h); control rats received saline. In some experiments, in which the time course of the effect of αBTX was investigated, different groups of rats were killed either 3 h after a single intraperitoneal injection of αBTX (20 μg), or after 1–28 days treatment with αBTX (one intraperitoneal injection of 3–5 μg per 48 h). The initial weights of the rats were chosen so that the weights were all about 200 g on the day scheduled for the *in vitro* experiments.

As observed previously (Molenaar *et al.* 1990, 1991), the αBTX-treated rats showed signs of bulbar paralysis during the first 8 h after the injection of the toxin (e.g. drooping lower lip); breathing was not affected. Their growth was somewhat decreased compared to that of controls. This was due to reduced eating and drinking on the day of injection but not to muscle atrophy caused by impaired muscle activity. We refer to this condition as toxin-induced myasthenia gravis (TIMG).

The rats were killed by cervical dislocation under ether anaesthesia and the hemidiaphragms with their phrenic nerve were dissected rapidly and kept at room temperature (20–22 °C) in 95 % O_2–5 % CO_2-saturated Ringer solution containing (mM): NaCl, 116; KCl, 4·5; $NaHCO_3$, 23; NaH_2PO_4, 1; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 11.

Biochemical experiments

The experiments were performed at room temperature (20–22 °C). Binding of ^{125}I-αBTX was determined (Ito, Miledi, Vincent & Newsom-Davis, 1978) in hemidiaphragms of TIMG and control rats. For biochemical measurements of ACh release, the muscles were treated for 45 min with 1 μM soman (*O*-pinacolyl-methylphosphonyl-fluoridate; kindly given by Dr H. P. Benschop, Prins Maurits Laboratory, The Netherlands) in order to block completely the cholinesterase of the tissue so that all the released ACh in the muscle reached the incubation medium during stimulation of the nerve. ACh was purified from the incubation medium (Miledi, Molenaar & Polak, 1980) and measured using high-performance liquid chromatography (HPLC) with a method in which ACh was converted enzymically to H_2O_2 (Israël & Lesbats, 1981). The H_2O_2 was measured with a platinum electrode (Damsma, Lammerts van Bueren, Westerink & Horn, 1985). The activity of ChAT was estimated at 37 °C in homogenates of hemidiaphragms as described earlier (Molenaar *et al.* 1981).

Elimination of the muscle action potential

The muscles were pinned out on the silicon rubber-covered bottom of a glass dish and continuously superfused (except during actual recordings) with 95 % O_2–5 % CO_2-saturated Ringer solution. The temperature was kept at 26–28 °C. Hemidiaphragms were treated for 40 min with 2·3 μM μ-conotoxin, which abolishes muscle action potentials by blocking voltage-gated Na^+ channels of muscle but not those of nerve. μ-Conotoxin types GIIIB (Scientific Marketing Associates, Barnet, UK) and GIIIA were used during the experiments; they had the same potency. Control experiments on muscles of untreated rats were performed to exclude an effect of μ-conotoxin on the quantal content. The quantal content of cut-fibre preparations of the diaphragm (Barstad & Lilleheil, 1968) was not influenced by μ-conotoxin ($43 \pm 4{\cdot}2$ *vs* $43 \pm 4{\cdot}0$ (mean ± S.E.M.), $n = 5$ muscles).

After the incubation with μ-conotoxin no muscle contraction occurred when the nerve was stimulated, and uninfluenced EPPs could be recorded in the absence of muscle spikes for 1–2 h. After that period contraction in some fibres reappeared.

Electrophysiological recordings

Muscle fibres were impaled at the ending of fine branches from the phrenic nerve with a glass capillary microelectrode filled with 3 M KCl (15–25 MΩ resistance). The criterion for being near enough to the endplate to record MEPPs and EPPs at their maximum amplitude was a time-to-peak of the MEPPs of less than 1·5 ms. Otherwise the microelectrode was withdrawn and another fibre was impaled.

At each endplate about thirty EPPs were recorded during supramaximal stimulation of the

phrenic nerve at 0·3 Hz. At the same endplate about thirty MEPPs were recorded. The signal was preamplified × 10 with a high-input impedance DC preamplifier and amplified further with an AC-coupled instrumentation amplifier. The resting membrane potential during these recordings was monitored. The analog signal was converted to digital at 10 kHz sampling frequency using a 1401 system in combination with the SIGAVG program (Cambridge Electronic Design, Cambridge, UK) and was stored on the computer hard disk. Off-line analysis was done on computer to determine amplitudes of the EPPs and MEPPs. MEPPs and irregular shaped spontaneous potentials which had amplitudes of more than twice the population mean amplitude were discarded.

The amplitudes of the MEPPs and EPPs were normalized to −75 mV, assuming 0 mV as the reversal potential for the ACh-induced ion current (Magleby & Stevens, 1972). The normalized mean EPP amplitude was corrected for non-linear summation using the correction formula derived by McLachlan & Martin (1981): $EPP' = EPP/(1-f(EPP/E))$, in which EPP′ is the corrected EPP amplitude and E the difference between resting membrane potential and the reversal potential for ACh. The value of f (a constant dependent on the duration of transmitter action relative to the membrane time constant) was set to 0·8 (McLachlan & Martin, 1981). The quantal content was calculated for each endplate as the ratio between this corrected EPP amplitude and the mean normalized MEPP amplitude. Quantal contents in endplates with a mean MEPP amplitude less than 0·1 mV were discarded because a large portion of the MEPPs in these endplates must have been overshadowed by the noise, resulting in an overestimation of the mean MEPP amplitude and thus an underestimation of the calculated quantal content.

In every muscle at least ten endplates were sampled at random sites in the hemidiaphragm.

RESULTS

Biochemical experiments

The results of the biochemical experiments are summarized in Table 1. The number of AChRs in the hemidiaphragms, measured as ^{125}I-αBTX binding sites, was reduced to 58 % of controls after 2–3 weeks of αBTX treatment.

ACh release evoked by nerve stimulation at 3 Hz in muscles from TIMG rats was 75 % higher than that in control diaphragms. This result obtained with HPLC measurements of ACh confirmed previous findings obtained with gas chromatography/mass spectrometry (Molenaar *et al.* 1991).

The ACh content in hemidiaphragms of TIMG rats was 20 % less than found in controls ($P < 0·05$). On the other hand, the activity of ChAT was unchanged.

Electrophysiological experiments

The resting membrane potentials of the fibres were the same in TIMG and control muscles (Table 2). The overall mean amplitude of the MEPPs in the muscles of the animals treated for 3 weeks with αBTX was reduced to 57 % of that of controls. The mean of the amplitude of the EPPs, however, was only slightly reduced (to 92 %). Consequently, the calculated mean quantal content was increased in the TIMG muscles (by 54 %). This increase was in apparent agreement with the increase of ACh release as found by our chemical measurements. The overall mean of the frequency of the MEPPs was 25 % reduced in TIMG muscle.

As can be seen in Fig. 1, the MEPPs in control muscles showed a bell-shaped distribution. However, due to the reduced postsynaptic sensitivity of the endplate in TIMG the distribution of MEPPs was shifted to the left which resulted in a skewed profile. It is possible that the difference in overall mean MEPP frequency between TIMG and control muscle is due to the fact that relatively many endplates in TIMG muscle had a mean amplitude near the detection limit. Obviously, in such endplates

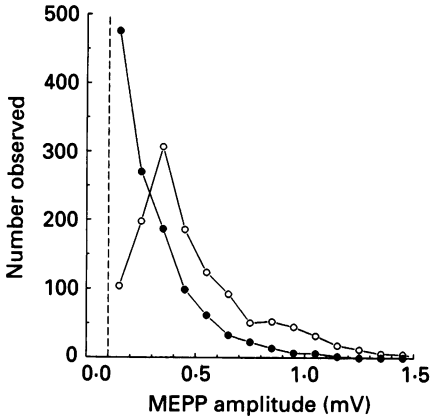

Fig. 1. Distribution of the MEPP amplitude in control (○) and TIMG (●) endplates. Each distribution profile was formed by the pooled MEPP amplitudes of four muscles. MEPPs with amplitudes smaller than 0·1 mV were considered as artifacts, being under the detection limit (dashed line), and discarded. The bin width chosen was 0·1 mV.

TABLE 1. Biochemical measurements in diaphragms of 2- to 3-week-treated TIMG rats

	TIMG	Control	TIMG/Control
^{125}I-αBTX binding (c.p.m.)	11800±1640 (6)	20200±2120 (6)	0·58*
ACh release (pmol min^{-1})	1·4±0·23 (8)	0·8±0·16 (8)	1·75*
Choline acetyltransferase activity (nmol h^{-1} g^{-1})	360±33 (12)	340±33 (12)	1·06
ACh content (pmol)	130±4 (6)	160±10 (6)	0·81*

The ACh release was evoked by supramaximal stimulation of the phrenic nerve at 3 Hz. The resting ACh release (about 0·6 pmol min^{-1}) has been subtracted. Means±S.E.M. with the number of rats in parentheses. *$P < 0·05$, Student's t test.

TABLE 2. Electrophysiological measurements in diaphragms of 3-week-treated TIMG rats

	TIMG	Control	TIMG/Control
Resting membrane potential (mV)	−74±0·6 (42)	−73±0·5 (42)	1·01
MEPP amplitude (mV)	0·27±0·02 (42)	0·47±0·04 (42)	0·57**
EPP amplitude (mV)	21·72±0·85 (42)	23·67±0·77 (42)	0·92
Quantal content	120±6·7 (42)	78±4·6 (42)	1·54**
MEPP frequency (s^{-1})†	0·94±0·08 (33)	1·25±0·09 (39)	0·75*

TIMG and control groups both consisted of four animals. The values in the table are the means±S.E.M. of the total number of endplates, which is indicated in parentheses. The mean amplitude of the MEPP of the individual endplates has been normalized to the standard resting membrane potential of −75 mV before the calculation of the overall mean. The amplitude of the EPP has also been normalized in this way, but has not yet been corrected for non-linear summation. The resting membrane potentials in this table are the means of the values which were measured during the recording of the MEPPs and EPPs.

† Values from 6-week-treated TIMG rats. *$P < 0·05$, **$P < 0·001$, Student's t test.

a loss occurs of MEPPs with amplitudes in the lower region of the normal variance of the MEPP amplitudes.

Correlation of MEPP amplitude and quantal content

In endplates where the amplitude of the MEPP was very small, very large quantal contents were calculated which could be as large as 2–3 times the mean value of

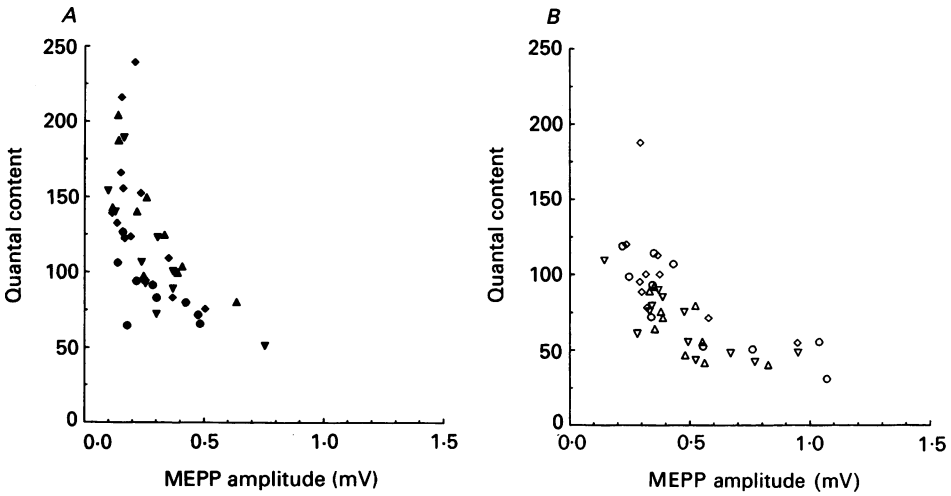

Fig. 2. Correlation between quantal content and MEPP amplitude in TIMG (*A*) and controls (*B*) determined in individual endplates of intact diaphragms. Values obtained from individual muscles are represented by different symbols. The amplitudes of MEPPs were normalized to the standard resting membrane potential of −75 mV. The means of these data points are given in Table 2.

control endplates. In Fig. 2*A* the quantal contents of forty-two TIMG endplates have been plotted against the mean of the MEPP amplitudes, recorded in the same endplates. The data points show that there was an inverse relationship between the amplitude of the MEPP of an endplate and the quantal content. Each muscle showed this tendency (see symbols in Fig. 2*A*).

In the control endplates a similar relationship between MEPP amplitude and quantal content was found (Fig. 2*B*). Of course, endplates with MEPP amplitudes smaller than 0·3 mV were relatively scarce in control muscles. Endplates with a mean MEPP amplitude in the TIMG–control overlapping range of about 0·2–0·4 mV had about the same quantal content, irrespective of whether it was a TIMG or a control endplate.

Correlation of MEPP amplitude and MEPP frequency

As mentioned above, the overall mean MEPP frequency in TIMG muscles might have been underestimated. In order to allow a fair comparison between MEPP frequencies of TIMG and control endplates a classification was made of the endplates with respect to their MEPP amplitudes. The width of the classes was set to 0·1 mV

and within these classes the mean MEPP frequency was calculated. These values were used to construct curves of the correlation between MEPP amplitude and MEPP frequency. Figure 3 shows a negative correlation between MEPP amplitude and MEPP frequency of control endplates, in agreement with the results of Harris

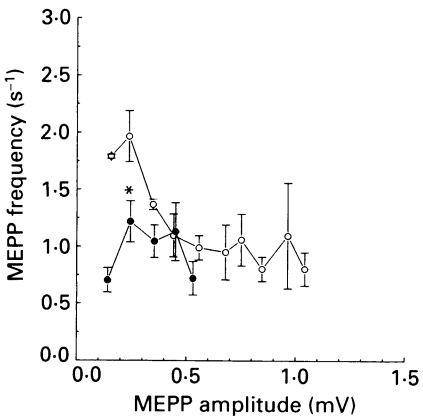

Fig. 3. Relation between the MEPP frequency of control (○) and TIMG (●) endplates and their MEPP amplitude which was normalized to the standard resting membrane potential of −75 mV. Endplates were classified with regard to their MEPP amplitudes in classes with a width of 0·1 mV. The mean MEPP frequency within these classes was plotted against the mean MEPP amplitude within a class. Each class consisted of two to twelve endplates. The bars represent the S.E.M. within a class. *Statistically significant difference from control endplate class ($P < 0·05$, Student's t test).

& Ribchester (1979). In contrast, such a correlation does not clearly exist in TIMG endplates. Both curves show a drop of frequency at the MEPP amplitude range of 0·1–0·2 mV. This was probably due to loss of MEPPs in the background noise. The MEPP frequency in TIMG endplates with MEPP amplitudes between 0·2 and 0·3 mV was statistically significantly lower than that of controls ($P < 0·05$). It is unlikely that a relatively high loss of MEPPs in the noise in TIMG endplates was responsible for this difference because the comparison was made in endplates of the same amplitude class and such loss would therefore have been roughly the same in both TIMG and control endplates.

In Fig. 4 the individual endplate data used was the same data used to calculate the overall mean MEPP frequency in Table 2. The MEPP frequency of a control endplate was positively correlated with its quantal content (Fig. 4*A*), in agreement with the results of Kuno *et al.* (1971). However, such a correlation was not apparent in the TIMG muscle (Fig. 4*B*).

Time-dependent increase of quantal content in TIMG

Quantal contents were determined in muscles of groups of rats who had received αBTX for varying periods of time. For these experiments it was desirable to achieve a relatively large reduction of the AChRs in order to maximize the effect on the quantal content. To this end αBTX was administered with an intraperitoneal instead

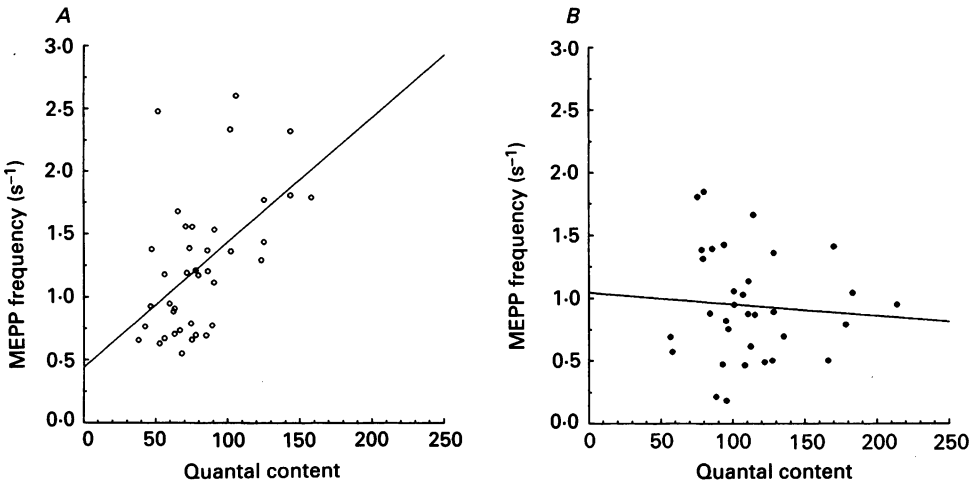

Fig. 4. Relation between the MEPP frequency of an endplate and its quantal content in control (*A*) and chronic (6-week-treated) TIMG (*B*) diaphragm. The control and TIMG groups consisted of thirty-nine and thirty-three endplates from four and three diaphragms, respectively (means of these data points are given in the bottom row of Table 2). The linear least-squares regression lines are shown (correlation coefficients were 0·53 (*A*) and 0·08 (*B*)).

TABLE 3. Increase of the mean quantal content during TIMG treatment

Treatment duration (days)	MEPP amplitude (mV)	Quantal content	Quantal content (%)
0*	0·32±0·02 (40)	67±3·3 (40)	100
1–3	0·24±0·02 (75)	95±5·0 (75)	141
6–8	0·22±0·02 (40)	115±7·5 (40)	171
14–15	0·18±0·01 (40)	142±7·8 (40)	212
21–28	0·16±0·01 (28)	160±8·0 (28)	239

Each time group consisted of at least four rats. In the 0-day treatment group quantal contents were measured 3 h after a single αBTX injection. The values in the table are the means±S.E.M. of the total number of endplates (indicated in parentheses). For the percentage increase in the last column, the quantal contents of the different time groups were compared to the quantal content at 0 days TIMG. *The mean amplitude of the MEPP in this treatment group has probably been overestimated; values of a considerable number of endplates had to be rejected because of the lack of detectable MEPPs (see Methods).

of a subcutaneous injection, because this route ensured predominance of the endplate classes with MEPPs of 0·1–0·3 mV. As shown in Table 3, the mean quantal content of hemidiaphragms 3 h after rats had received a single αBTX injection was not yet increased. It was similar to the control quantal content of Table 2, notwithstanding the fact that the mean MEPP amplitude was decreased to the level found in the chronic TIMG situation (cf. Table 2). Prolonged periods of TIMG treatment caused a gradual increase in the mean quantal content to 239 % after 3–4 weeks treatment. During this period there was some further decrease of the mean MEPP amplitude.

The values of individual endplates were transformed into curves as described above. As depicted in Fig. 5, all endplates had about equal quantal contents after the

single αBTX injection, independent of the class of MEPP amplitude. Even the class between 0·1 and 0·3 mV had a mean quantal content in the range of control endplates with MEPP amplitudes between 0·4 and 1·0 mV (about 60, cf. Fig. 2). The largest increase in quantal content during chronic TIMG treatment was found particularly

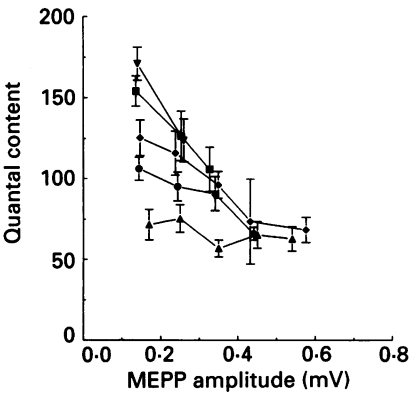

Fig. 5. Correlation between quantal content and MEPP amplitude after various durations of TIMG treatment: ▲, 0 days (actually 3 h after αBTX injection); ●, 1–3 days; ◆, 6–8 days; ■, 14–15 days; ▼, 21–28 days. Endplates were sorted into MEPP classes with a width of 0·1 mV. Data points, representing the mean quantal content ± S.E.M. of the endplates within a MEPP class, have been plotted against the mean of the MEPPs of these endplates. The means are based on three to forty-three endplates and each curve is composed of data derived from at least four rats.

in the endplate classes with very small MEPP amplitudes. The quantal contents in these classes reached plateau values after 3–4 weeks treatment. In the relatively scarce TIMG endplates with large MEPP amplitudes, i.e. larger than 0·3 mV, the increase in quantal content was less pronounced or absent.

DISCUSSION

Biochemistry

As mentioned in the Introduction a possible mechanism for an increase of evoked ACh release is enlargement of the presynaptic apparatus, with concomitant increase of active zones. The fact that the activity of choline acetyltransferase was not increased in the TIMG diaphragm suggests that there is no enlargement of the nerve terminals. This extends the earlier reported finding that the area of acetylcholinesterase staining in chronic TIMG tibialis anterior muscle was not increased (Molenaar *et al.* 1991). An unexpected finding was that the ACh content was decreased in freshly excised TIMG diaphragms. This might be caused by a higher *in vivo* release of ACh in TIMG rats than in controls. At any rate, this finding indicates that in TIMG the nerve terminals do not contain a relatively large store of ACh.

Miledi, Molenaar & Polak (1978) found that acute blockade of the total population of AChRs in the rat diaphragm by αBTX *in vitro* caused an increase of evoked

transmitter release as assayed by chemical means, 2–3 h after the introduction of αBTX to the medium. On the other hand, in the present experiments no increase of transmitter release could be demonstrated at an early point in time, 3 h after injection of the αBTX, notwithstanding the fact that many endplates showed the same reduction of MEPP amplitudes as found in the chronic TIMG diaphragm. Therefore, with respect to ACh release, the result of application of αBTX *in vivo* was different from that *in vitro*. It remains to be seen whether or not this difference results from differences in conditions, e.g. the extent of blockade of AChRs.

Electrophysiology

The present electrophysiological findings confirm earlier observations with biochemical methods that increased evoked ACh release can be brought about by the reduction of AChRs alone (Molenaar *et al.* 1991), indicating that the action of auto-immune immunoglobulin G *per se* is not required for the effect in human and experimental auto-immune MG.

In this earlier work, quantal contents in TIMG extensor digitorum longus muscles were determined in the presence of reduced extracellular Ca^{2+} to prevent muscle contraction. However, these quantal contents were not increased compared to controls. Recent experiments in our laboratory in which quantal contents were measured at various extracellular Ca^{2+} concentrations in the μ-conotoxin-blocked preparation indicate that the increase in quantal content in TIMG endplates is Ca^{2+} dependent and absent at low Ca^{2+} concentrations (J. J. Plomp, unpublished observations). Therefore, this apparent discrepancy between the present and earlier work is probably caused by Ca^{2+}.

The electrophysiological measurements in μ-conotoxin-blocked muscles enabled us to study transmitter release in greater detail than could be done with biochemical methods. Measurements with biochemical methods were at the level of the whole muscle and thus represented the average behaviour of thousands of endplates, whereas with our electrophysiological method the evoked ACh release in individual endplates could be determined. The great advantage of μ-conotoxin as a blocker of the muscle contraction compared to other methods, such as curarization or the cut fibre preparation, was that it did not reduce the MEPP amplitude. Thus, quantal contents could be determined directly from the EPP and MEPP values, even in endplates with very small MEPP amplitudes which, of course, were frequently found in TIMG muscles.

Two possible artifacts should be considered. Firstly, the calculated mean MEPP amplitude in TIMG muscles could have been overestimated in the endplates which had very small mean MEPP amplitudes, close to the detection limit, by the loss of MEPPs in the signal noise. However, if we indeed overestimated the mean MEPP amplitude in these endplates, the resulting quantal content in these endplates would consequently have been underestimated. Thus we certainly did not overestimate the quantal contents and this underscores the validity of our finding that in chronic TIMG and control endplates with small MEPP amplitudes the quantal content is much higher than in endplates with large MEPP amplitudes. Secondly, as mentioned in the Results, MEPP frequencies could have been underestimated in endplates with small MEPP amplitudes. This difficulty was eliminated by comparison of MEPP

frequencies of endplates of the same MEPP amplitude classes, so that both TIMG and control endplates had, if any, the same handicap.

From the work by Kuno *et al.* (1971) in the frog and Harris & Ribchester (1979) in normal and dystrophic mice, it emerges that relatively thick fibres in normal muscles have big endplates with small MEPPs, characterized by a high MEPP frequency and a large quantal content. The present results suggest that the same is true for the rat: endplates with small MEPPs (presumably located on thick muscle fibres) had a high MEPP frequency and a large quantal content. However, our results with TIMG muscles were different. Although the quantal content was inversely correlated with the MEPP amplitude of endplates, just as in control rat muscle, there was no apparent correlation with MEPP frequency, suggesting that endplates with high quantal content were not especially large. This result, in conjunction with the present biochemical data on ChAT and earlier histological findings on acetylcholinesterase, suggests that chronic reduction of AChRs by treatment with αBTX causes an increase of quantal content, probably without enlargement or outgrowth of the presynaptic terminals. If true, this would imply that the neuromuscular junction possesses at least two mechanisms for compensating low postsynaptic sensitivity with increased ACh output, one characterized by enlargement of the endplate and the other by some change of the release process within the confinement of the existing endplate. On the basis of our results and the reports cited in the Introduction we hypothesize that in control muscle there is a tuning mechanism which is used to respond to slowly occurring small postsynaptic changes in sensitivity during the life of an endplate. Furthermore, that in TIMG there is an emergency reaction to a sudden and large decrease in postsynaptic sensitivity, which otherwise would threaten normal neuromuscular transmission. It is possible that increased transmitter release in MG is due to the combined results of these two mechanisms.

Possible involvement of retrograde signals

The present results strongly indicate that αBTX indirectly influences a physiological regulatory system of motor function and the question arises as to the nature and localization of the different components of this system. On the basis of a teleological argument it seems plausible that the sensory component is localized at a postsynaptic site, which would imply the existence of retrograde signals from muscle to nerve. Retrograde action of muscle on nerve has been reported in embryonic amphibian tissue *in vitro*; in growth cones of nerves the process of spontaneous transmitter release is started within minutes when a myoball is placed at the cone (Xie & Poo, 1986; Sun & Poo, 1987). However, the action of myoballs on transmitter release from growth cones and the present effect of αBTX might represent different processes, among other things because the first is triggering and the second is modulating transmitter release.

Retrograde signals could act on the nerve terminal or be carried to the cell body of the motoneurone, for instance via sensory fibres. Although the time course of the effect of αBTX is compatible with fast anterograde axonal transport along peripheral cholinergic nerves (Grafstein & Forman, 1980) of a factor promoting transmitter release, our evidence is in favour of a local effect on the nerve terminal of the

endplate. If the effect were to involve the cell body of the motoneurone then all endplates of a motor unit would show increased transmitter release, including the endplates with relatively large MEPPs. This is unlikely in view of the good correlation between quantal contents and MEPP amplitudes of the endplates.

One possibility for local retrograde signalling is the release of a substance by the muscle cell in response to a decreased number of functional AChRs; the trigger for such a postsynaptic response could be the decreased endplate current. An alternative possibility for retrograde action is that presynaptic nicotinic AChRs are involved in an autoregulatory mechanism which inhibits ACh release under normal conditions. Blockade of these receptors by αBTX would then result in an increase of transmitter release. However, the existence of presynaptic nicotinic receptors on nerve terminals in the neuromuscular junction is uncertain (Matthews-Bellinger & Salpeter, 1978).

We are greatly indebted to Dr T. Abe of the University of Niigata for the generous gift of μ-conotoxin GIIIA. We gratefully acknowledge support by the Prinses Beatrix Fonds, the Association Française contre les Myopathies, and the Promeso foundation.

REFERENCES

BARSTAD, J. A. B. & LILLEHEIL, G. (1968). Transversely cut diaphragm preparation from rat. *Archives Internationales de pharmacodynamie et de Thérapie* **175**, 373–390.

CHANG, C. C. & LEE, C. Y. (1963). Isolation of neurotoxins from the venom of bungarus multicinctus and their modes of neuromuscular blocking action. *Archives Internationales de pharmacodynamie et de Thérapie* **144**, 241–257.

CRUZ, L. J., GRAY, W. R., OLIVERA, B. M., ZEIKUS, R. D., KERR, L., YOSHIKAMI, D. & MOCZYDLOWSKI, E. (1985). *Conus geographus* toxins that discriminate between neuronal and muscle sodium channels. *Journal of Biological Chemistry* **260**, 9280–9288.

CULL-CANDY, S. G., MILEDI, R., TRAUTMANN, A. & UCHITEL, O. D. (1980). On the release of transmitter at normal, myasthenia gravis and myasthenic syndrome affected human end-plates. *Journal of Physiology* **299**, 621–638.

DAMSMA, G., LAMMERTS VAN BUEREN, D., WESTERINK, B. H. C. & HORN, A. S. (1985). Determination of acetylcholine and choline in the femtomole range by means of HPLC, a post column enzyme reactor, and electrochemical detection. *Chromatographia* **24**, 827–831.

DI GREGORIO, F., FESCE, R., CERESER, S., FAVARO, G. & FIORI, M. G. (1989). Spontaneous and nerve-evoked quantal transmission in regenerated motor terminals. *Cell Biology International Reports* **13** (12), 1119–1126.

GRAFSTEIN, B. & FORMAN, D. S. (1980). Intracellular transport in neurons. *Physiological Reviews* **60**, 1167–1283.

HARRIS, J. B. & RIBCHESTER, R. R. (1979). The relationship between end-plate size and transmitter release in normal and dystrophic muscles of the mouse. *Journal of Physiology* **296**, 245–265.

ISRAËL, M. & LESBATS, B. (1981). Chemiluminescent determination of acetylcholine, and continuous detection of its release from torpedo electric organ synapses and synaptosomes. *Neurochemistry International* **3** (1), 81–90.

ITO, Y., MILEDI, R., MOLENAAR, P. C., VINCENT, A., POLAK, R. L., VAN GELDER, M. & NEWSOM-DAVIS, J. (1976). Acetylcholine in human muscle. *Proceedings of the Royal Society* B **192**, 475–480.

ITO, Y., MILEDI, R., VINCENT, A. & NEWSOM-DAVIS, J. (1978). Acetylcholine receptors and end-plate electrophysiology in myasthenia gravis. *Brain* **101**, 345–368.

KATZ, B. & THESLEFF, S. (1957). On the factors which determine the amplitude of the miniature end-plate potential. *Journal of Physiology* **137**, 267–278.

KUNO, M., TURKANIS, S. A. & WEAKLY, J. N. (1971). Correlation between nerve terminal size and transmitter release at the neuromuscular junction of the frog. *Journal of Physiology* **213**, 545–556.

McLACHLAN, E. M. & MARTIN, A. R. (1981). Non-linear summation of end-plate potentials in the frog and the mouse. *Journal of Physiology* **311**, 307–324.

MAGLEBY, K. L. & STEVENS, C. F. (1972). The effect of voltage on the time course of end-plate currents. *Journal of Physiology* **223**, 151–171.

MATTHEWS-BELLINGER, J. & SALPETER, M. M. (1978). Distribution of acetylcholine receptors at frog neuromuscular junctions with a discussion of some physiological implications. *Journal of Physiology* **279**, 197–213.

MILEDI, R., MOLENAAR, P. C. & POLAK, R. L. (1978). α-Bungarotoxin enhances transmitter 'released' at the neuromuscular junction. *Nature* **272**, 641–643.

MILEDI, R., MOLENAAR, P. C. & POLAK, R. L. (1980). The effect of lanthanum ions on acetylcholine in frog muscle. *Journal of Physiology* **309**, 199–214.

MOLENAAR, P. C., NEWSOM-DAVIS, J., POLAK, R. L. & VINCENT, A. (1981). Choline acetyltransferase in skeletal muscle from patients with myasthenia gravis. *Journal of Neurochemistry* **37**, 1081–1088.

MOLENAAR, P. C., OEN, B. S., PLOMP, J. J., VAN KEMPEN, G. TH. H., JENNEKENS, F. G. I. & HESSELMANS, L. F. G. M. (1991). A non-immunogenic myasthenia gravis model and its application in a study of transsynaptic regulation at the neuromuscular junction. *European Journal of Pharmacology* **196**, 93–101.

MOLENAAR, P. C., PLOMP, J. J. & VAN KEMPEN, G. TH. H. (1990). Differences between the dilator naris and diaphragm of 'myasthenic' rats. *Journal of Physiology* **420**, 25*P*.

MOLENAAR, P. C., POLAK, R. L., MILEDI, R., ALEMA, S., VINCENT, A. & NEWSOM-DAVIS, J. (1979). Acetylcholine in intercostal muscle from myasthenia gravis patients and in rat diaphragm after blockade of acetylcholine receptors. *Progress in Brain Research* **49**, 449–458

OLIVERA, B. M., RIVIER, J., CLARK, C., RAMILO, C. A., CORPUZ, G. P., ABOGADIE, F. C., MENA, E. E., WOODWARD, S. R., HILLYARD, D. R. & CRUZ, L. J. (1990). Diversity of conus neuropeptides. *Science* **249**, 257–263.

SUN, Y. & POO, M.-M. (1987). Evoked release of acetylcholine from the growing embryonic neuron. *Proceedings of the National Academy of Sciences of the USA* **84**, 2540–2544.

TAKAMORI, M., SAKATO, S. & OKUMURA, S. (1984). Presynaptic function modified by acetylcholine-receptor interaction in experimental autoimmune myasthenia gravis. *Journal of Neurological Sciences* **66**, 245–253.

VINCENT, A. (1980). Immunology of acetylcholine receptors in relation to myasthenia gravis. *Physiological Reviews* **60** (3), 756–824.

XIE, Z.-P. & POO, M.-M. (1986). Initial events in the formation of neuromuscular synapse: rapid induction of acetylcholine release from embryonic neuron. *Proceedings of the National Academy of Sciences of the USA* **83**, 7069–7073.

YANAGAWA, Y., ABE, T. & SATAKE, M. (1987). μ-Conotoxins share a common binding site with tetrodotoxin/saxitoxin on eel electroplax Na channels. *Journal of Neuroscience* **7** (5), 1498–1502.