Br. J. Pharmac. (1986), **88**, 25–31 bjp031a

The effect of chronic neostigmine treatment on channel properties at the rat skeletal neuromuscular junction

M. Gwilt[1] & D. Wray[2]

Pharmacology Department, Royal Free Hospital School of Medicine, Rowland Hill Street, London, NW3 2PF

1 We have studied the effects of chronic neostigmine treatment on single channel properties at the rat skeletal neuromuscular junction. Rats received 0.86 mg kg^{-1} neostigmine (s.c.) daily for 9–11 days. Microelectrode recordings were then made from the extensor digitorum longus muscle.

2 The amplitude of miniature endplate potentials was significantly reduced in muscles from neostigmine-treated rats as compared with controls.

3 Acetylcholine (2–5 μM) applied in the bath produced a depolarization and associated channel opening frequency (from voltage noise analysis) which were significantly reduced in neostigmine-treated muscles with respect to controls.

4 The depolarization resulting from the opening of a single channel (from voltage noise analysis) and single channel open time and conductance (from current noise analysis) were not significantly changed by chronic neostigmine treatment.

5 It is concluded that chronic neostigmine treatment causes an adaptive reduction in the number of functional acetylcholine receptors at the endplate without otherwise affecting single channel properties themselves.

Introduction

Anticholinesterase drugs prolong the action of acetylcholine (ACh) in the synaptic cleft (Katz & Miledi, 1973), thereby affecting neuromuscular transmission. During prolonged treatment with an anticholinesterase, endplate acetylcholine receptors (AChRs) may be exposed to chronically elevated concentrations of ACh (see Discussion). It might be expected that adaptive changes at the endplate would occur in response to possible increased synaptic ACh levels. Muscles isolated from rats chronically treated with neostigmine have been shown to possess reduced miniature endplate potential (m.e.p.p.) amplitudes (Roberts & Thesleff, 1969; Engel *et al.*, 1973; Ward *et al.*, 1975) and reduced postsynaptic sensitivity to iontophoretically applied ACh (Tiedt *et al.*, 1978). These reports have led to the suggestion that chronically elevated endplate ACh levels might reduce postsynaptic sensitivity at the neuromuscular junction. A decrease in the number of AChRs (measured using α-bungarotoxin binding) in rat muscle has been seen following chronic neostigmine treatment (Chang *et al.*, 1973).

[1]Present address: Medicinal Biology Department, Pfizer Central Research, Sandwich, Kent, CT13 9NJ.
[2]Author for correspondence.

Another way in which endplate ACh concentration can be altered is by denervation, producing a chronic deficit of ACh. In this case an adaptive increase in AChR number occurs (Axelsson & Thesleff, 1959; Miledi, 1960). This increase in postsynaptic ACh sensitivity is further augmented by a three to five times increase in channel open time (for review see e.g. Wray, 1980). This results in an increased ion flow through such channels causing an increase in the depolarization produced by the opening of a single channel (Katz & Miledi, 1972). These changes operate in the direction which tends to minimise the effect of the deficit of ACh.

Conversely, a chronic excess of ACh, as might be expected in chronic neostigmine treatment, may cause an adaptive *reduction* in the charge passed by a single channel, along with a reduction in the number of AChRs at the endplate.

In this paper, possible changes in single channel properties have been studied in isolated extensor digitorum longus (EDL) muscles from control rats and from rats treated with neostigmine at doses and duration of treatment similar to those studies described above. The frequency of channel opening, and depolarization produced by a single channel, were measured by voltage noise analysis. The mean single

channel open time and conductance were found by current noise analysis in voltage clamped fibres.

A preliminary abstract of this work has been presented at the IUPHAR IXth Congress of Pharmacology, London (Gwilt & Wray, 1984).

Methods

Pretreatment of animals

Rats (Sprague-Dawley 230–240 g) were injected with neostigmine methylsulphate (0.43 mg kg^{-1} s.c. in the scruff of the neck) twice daily for 9–11 days. Injections were given at intervals of approximately 12 h. For the first six treatments, rats were also given atropine (1 mg s.c.) 30 min before neostigmine to reduce undesirable muscarinic effects. After this period, rats tolerated neostigmine treatment well, although pronounced muscular fasciculation was seen after all injections. The last injection of neostigmine was given at least 12 h before the start of an experiment. Control rats were sham-treated (atropine followed by neostigmine injections replaced by an equal volume of isotonic saline) or untreated. Drugs were dissolved in isotonic saline and the volume of each injection was 0.2 ml.

Electrophysiological recording

Rats were killed and the extensor digitorum longus muscle was quickly removed into well-oxygenated (95% O_2/5% CO_2) Krebs solution of the following composition (mM): Na^+ 143.0, K^+ 5.9, Cl^- 127.7, Mg^{2+} 1.2, Ca^{2+} 2.5, HCO_3^- 25.0, $H_2PO_4^-$ 1.2, SO_4^{2-} 1.2 and glucose 11.1. Intracellular microelectrode recordings were made as previously described (Wray, 1981) under continuous perfusion. M.e.p.ps and voltage noise were recorded at 37°C while voltage clamped current noise was recorded at 23–24°C. For the noise experiments, ACh was applied in the perfusing solution in the presence of physostigmine (3 μM) (to prevent inactivation of ACh) and tetrodotoxin (250 nM). All recordings were stored on magnetic tape and analysed by computer. Voltage noise recordings were low pass filtered at 400 Hz, digitized at 1 kHz and the depolarization produced by a single channel (a), the channel opening frequency (n) and the membrane time constant (τ_m) were calculated as previously described (Wray, 1981). Briefly, single channel depolarization, a, was obtained from a = (voltage noise variance)/(depolarization). The time constant, τ_m, was obtained from the half power frequency, f_c, of the power spectrum using $\tau_m = 1/(2\pi f_C)$. This time constant is expected to be the same as the membrane time constant, τ_m (see e.g. Wray, 1981). Channel opening frequency was calculated from n = (noise variance)/($a^2\tau_m$). Corrections were made for non-linear summation (Katz & Miledi, 1972). At each endplate, the time course of changes in channel opening frequency was followed throughout each ACh exposure and the maximum channel opening frequency (n_{max}) and maximum depolarization attained (V_{max}) were found. Current noise in voltage clamped fibres was low pass filtered at 800 Hz and digitized at 2 kHz to obtain the single channel open time (τ_o) and the single channel conductance (γ) (see e.g. Wray, 1980). The channel open time was obtained from the half power frequency of the current noise power spectrum using $\tau_o = 1/(2\pi fc)$. The conductance was obtained from $\gamma = \bar{I}^2/(I.E)$ where I^2 is the current noise variance, I the mean current passed and E the clamp potential. The reversal potential was taken as 0 mV (Colquhoun *et al.*, 1977; Sellin & Thesleff, 1981; Alema *et al.*, 1981). Corrections were made throughout to n, a and γ for the effects of the low pass filters used (Colquhoun *et al.*, 1977). Recordings of m.e.p.ps were digitized at 10 kHz and their amplitudes found. At least 25 m.e.p.ps. were analysed at each endplate to obtain the mean m.e.p.p. amplitude.

All results in this paper are presented as mean ± s.e.mean (one value per endplate). Significance was determined by Students' t test (2-tailed) and the level for non-significant results was set at $P > 0.05$.

Results

Voltage recordings

Voltage recordings were made at 37°C at endplates of EDL muscles in control and neostigmine-treated rats. The mean membrane potential in neostigmine-treated animals was 70 ± 1 mV (30 endplates from 7 rats). This was not significantly different from the membrane potential in controls (72 ± 1 mV, 25 endplates from 9 rats), indicating lack of effect of neostigmine treatment on membrane potential.

The mean m.e.p.p. amplitude was 0.50 ± 0.04 mV (14 endplates from 2 rats) in controls and 0.34 ± 0.02 mV (24 endplates from 4 rats) in muscles from neostigmine-treated rats. This represents a significant ($P < 0.02$) reduction in m.e.p.p. amplitudes in muscles from neostigmine-treated rats as compared with controls. M.e.p.p. frequency was also measured for the same muscle fibres. There was no significant reduction in m.e.p.p. frequency in neostigmine-treated animals (6.40 ± 0.51 s^{-1}) as compared with controls (7.3 ± 1.2 s^{-1}).

M.e.p.p. amplitudes are reduced by a postsynaptic mechanism (Tiedt *et al.*, 1978), and the mechanism of this effect was investigated here by noise analysis. ACh was applied to EDL muscles in the bath and examples of records obtained during exposures to ACh are

shown in Figure 1. When ACh 2 μM was applied to muscles the endplate showed well-maintained depolarization. Meanwhile, channel opening frequency rose quickly to a maximum and then declined slightly towards a plateau, indicating little desensitization at this concentration. When the ACh was washed off, both membrane potential and channel opening frequency returned to near their original values. During exposure to ACh 5 μM the channel opening frequency could be seen to fall markedly from an initial maximum, indicating greater desensitization at this higher concentration. The slight desensitization at ACh 2 μM and greater desensitization at 5 μM were typical features of these experiments. Values of maximum depolarization reached (V_{max}) and maximum channel opening frequency (n_{max}) in similar experiments were calculated for each endplate and the mean values are shown in Table 1A. Chronic neostigmine treatment significantly reduced V_{max} ($P < 0.001$) and n_{max} ($P < 0.01$) during exposures to 2 μM ACh. At 5 μM ACh, n_{max} was significantly reduced ($P < 0.05$), while V_{max} was reduced although not significantly. The small reduction in V_{max} at this concentration is probably due to an indirect effect of the more rapid desensitization which occurred at this concentration (Figure 1). Because of chloride shifts during ACh exposures (Jenkinson & Terrar, 1973), the depolarization is not a sensitive indicator of postsynaptic function during desensitization. Chloride shifts retard the attainment of maximum depolarization until marked desensitization has occurred. On the other hand, the measurement of channel opening frequency, n, allows the effect on channels underlying the depolarization to be seen.

Analysis of the voltage noise also yields values at each endplate for the depolarization produced by a single channel opening, a, and for the membrane time constant, τ_m. Mean values of these parameters for all endplates are shown in Table 1B. No significant difference was seen for a between control and neostigmine-treated muscles. This strongly suggests that the channel open time and conductance of endplate channels were not altered by chronic neostigmine treatment. In addition, no significant difference in τ_m was found between control and treated muscles, indicating that the passive electrical properties of the muscle fibre membrane were not markedly changed by chronic neostigmine treatment. The grand mean values for a and τ_m when averaged over test and control endplates was 0.063 μV and 1.25 ms respectively.

In summary, neostigmine treatment caused a decrease in depolarization produced by ACh. This

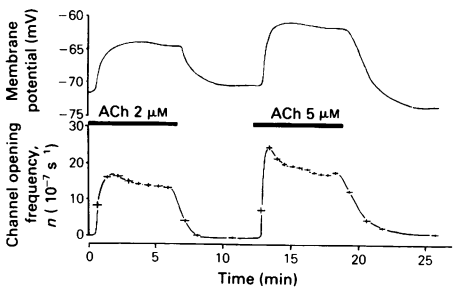

Figure 1 Time course of changes in membrane potential and channel opening frequency in control rat EDL muscle (37°C) during exposure to acetylcholine (ACh) as shown. Vertical bars represent s.e.mean, horizontal bars represent the period of analysis. Channel opening frequency was determined from mean values of a (0.089 μV) and τ_m (0.85 ms) obtained by averaging between the two exposures.

Table 1 Effect of chronic neostigmine treatment on channel properties in rat EDL muscle measured by voltage noise analysis

	ACh 2 μM		*ACh* 5 μM	
A	*Control* (5, 5)	*Neostigmine* (6, 3)	*Control* (8, 6)	*Neostigmine* (4, 2)
V_{max}(mV)	7.4 ± 0.3	4.4 ± 0.4***	9.9 ± 1.1	7.6 ± 0.5
n_{max}($10^7 s^{-1}$)	17.4 ± 3.0	6.9 ± 1.7**	27.7 ± 4.8	11.2 ± 3.0*
B	*Control* (11, 7)		*Neostigmine* (6, 3)	
a (μV)	0.058 ± 0.008		0.071 ± 0.005	
τ_m (ms)	1.16 ± 0.09		1.41 ± 0.10	

Means ± s.e.mean are shown (using one value for each endplate). Muscles were exposed to acetylcholine (ACh) 2 μM, 5 μM or both (though rarely at the same endplate). In **B**, when the same endplate was exposed to both 2 μM and 5 μM ACh, values of a and τ_m were averaged between the two exposures and one value per endplate again used in the calculation of mean values. The first figure in parentheses shows the number of endplates used, the second figure shows the number of animals. Significant difference from control is denoted by asterisks: *$0.05 > P > 0.01$; **$0.01 > P > 0.001$; ***$P < 0.001$.

occurred when ACh was applied in the bath (decreased V_{max}) or when ACh was released spontaneously (decreased m.e.p.p. amplitude). The decrease in postsynaptic depolarization was in turn caused by a reduction in channel opening frequency without any change in the depolarization produced by single channels. The apparent lack of effect of neostigmine treatment on single channel properties was further investigated by use of current noise analysis.

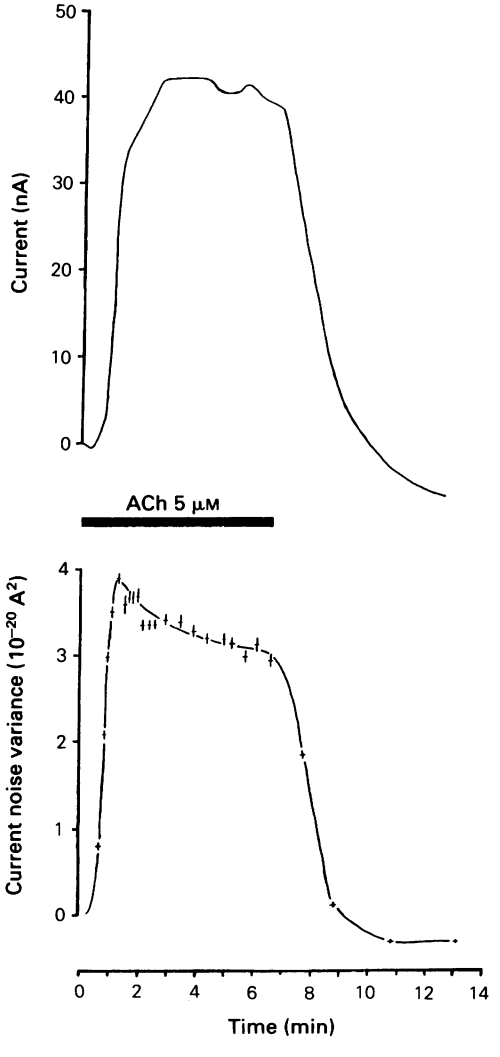

Figure 2 Time course of changes in endplate clamp current and associated current noise variance during exposure of a control EDL muscle to acetylcholine (ACh, 5 μM). Vertical bars represent s.e.mean, horizontal bars represent the period of analysis. Clamp potential − 70 mV, temperature 23°C.

Current recordings

Current recordings at endplates of voltage-clamped EDL muscles were made at 23–24°C. Input resistance (R_{in}) in neostigmine-treated animals was 0.42 ± 0.03 MΩ (5 endplates from 2 rats), which was not significantly different from controls (0.40 ± 0.07 MΩ, 4 endplates from 2 rats).

The analysis of agonist-induced current fluctuations in voltage clamped fibres allows the estimation of channel open time and conductance. Endplates were clamped at − 70 mV and Figure 2 shows an example of a voltage clamp record from one of these experiments. Bath-applied ACh (2–5 μM) caused an increase in clamp current together with an increase in current noise. Current and its associated noise returned to near baseline after the ACh was washed off. These records show similar features to those in unclamped fibres except that onset and recovery were faster than for depolarization because of the absence of chloride currents in clamped fibres (Jenkinson & Terrar, 1973).

Current noise power spectra were always fitted well with a single component Lorentzian curve, and an example from a neostigmine-treated animal is shown in Figure 3. The mean channel open time, obtained from such power spectra, was not significantly affected by neostigmine treatment: the open time was 1.04 ± 0.10 ms (7 endplates from 3 muscles) in controls and 1.07 ± 0.09 ms (5 endplates from 2 muscles)

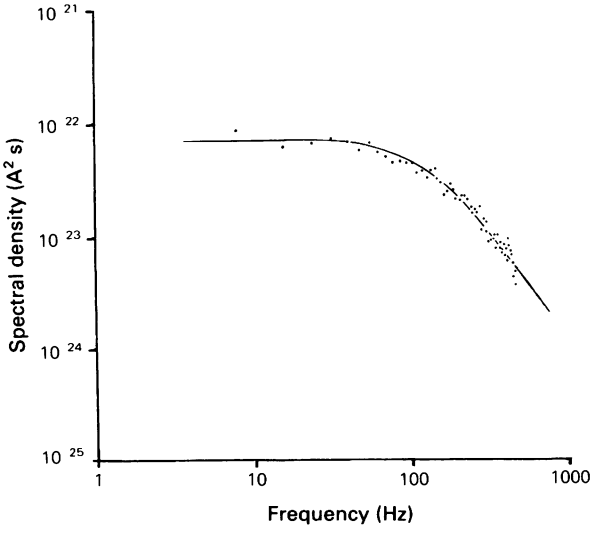

Figure 3 Power spectrum of current noise. EDL muscle from a neostigmine-treated rat was exposed to acetylcholine (ACh, 5 μM) at 24°C. The half power frequency of this spectrum is 134 Hz, which gives a value for the time constant, τ_o of 1.19 ms. Clamp potential = − 70 mV.

in neostigmine-treated animals. The mean single channel conductance for these endplates was also not significantly changed by neostigmine treatment (22.7 ± 3.5 pS in controls and 18.8 ± 3.8 pS for neostigmine-treated animals). This lack of change in γ and τ_0 together with lack of change in passive membrane properties (τ_m and R_{in}) would lead to lack of change in a, the depolarization produced by a single channel. This latter result for a was obtained directly in our experiments involving voltage noise analysis in the previous section. Grand mean values of γ and τ_0 when averaged over test and control endplates were 21.1 pS and 1.05 ms respectively.

Discussion

In this study we have shown that chronic neostigmine treatment of rats for 9–11 days reduced m.e.p.p. amplitudes as well as the depolarization produced by bath-applied ACh. The frequency of opening of channels by applied ACh was reduced in neostigmine-treated muscles as compared with controls. On the other hand, the properties of open single channels (single channel depolarization, open time and conductance) were all unchanged by chronic neostigmine treatment.

The reduction in m.e.p.p. amplitudes found here by neostigmine treatment was similar in extent to that seen in previous studies (Roberts & Thesleff, 1969; Engel *et al.*, 1973; Ward *et al.*, 1975; Tiedt *et al.*, 1978). This reduction in m.e.p.p. amplitude correlated in our study, and in that of Tiedt *et al.* (1978), with the reduction in postsynaptic sensitivity to either bath-applied or iontophoretically applied ACh. Hence the reduction in m.e.p.p. amplitude produced by neostigmine treatment is of postsynaptic rather than presynaptic origin. Furthermore, our study showed that neostigmine treatment for 9–11 days did not lead to marked passive changes in membrane properties: resting potential, R_{in} and τ_m. Thus passive changes do not account for the reduction in m.e.p.p. amplitude.

The postsynaptic reduction in sensitivity to ACh by chronic neostigmine treatment is entirely accounted for by the decrease in channel opening frequency, without any other change in single channel properties. Current noise power spectra were always fitted well with single Lorentzian curves, indicating the absence of any other obvious changes in channel kinetics.

Neostigmine may produce its chronic effect on channel opening frequency by simply decreasing receptor number, by decreasing receptor affinity for ACh, or by reducing the probability of channel opening once ACh has bound to the receptor. Chang *et al.* 1973, demonstrated a 42% reduction in α-bungarotoxin binding to endplates in rat diaphragm muscle by neostigmine treatment similar to that used here. This correlates fairly well with the reduction in channel opening frequency (by 60%) seen in our study. These observations taken together, therefore, suggest that chronic neostigmine treatment causes the loss of acetylcholine receptor from endplates without otherwise affecting channel properties. Associated with these effects are characteristic morphological changes (Hudson *et al.*, 1978).

The mechanism of the chronic effect of neostigmine is not known. It has been argued (Tiedt *et al.*, 1978) that the changes are produced by a chronic excess of ACh in the synaptic region, via inhibition of acetylcholinesterase. Half an hour after injection of neostigmine, m.e.p.p. amplitudes and m.e.p.p. rise and decay times are increased, indicative of acetylcholinesterase inhibition (Tiedt *et al.*, 1978). The effects of neostigmine might be expected to wear off on a time scale of the order of hours (e.g. about 43% of an i.m. dose in rats is excreted within 1 h, Roberts *et al.*, 1965). Thus cholinesterases may not be inhibited *continuously* throughout the period of chronic treatment. Indeed, the m.e.p.p. time course is not prolonged 5–17 h after the last injection of neostigmine, showing that acetylcholinesterase is no longer inhibited at such a time after injection (Roberts & Thesleff, 1969; Tiedt *et al.*, 1978). Clearly, then, during chronic neostigmine treatment, cholinesterases are inhibited intermittently for periods of a few hours following each injection which was given approximately every 12 h. During these periods of enzyme inhibition, is there an excess of synaptic ACh? Nerve-stimulated ACh release is reduced by chronic neostigmine treatment to around 55% of control values (Roberts & Thesleff, 1969). Thus for stimulated release this reduction in ACh may counter any increase in synaptic ACh levels produced by acetylcholinesterase inhibition. On the other hand, our results show that the frequency of spontaneous release is not markedly reduced by chronic neostigmine treatment. This is in agreement with Roberts & Thesleff (1969) although Tiedt *et al.* (1978) found a small reduction. For each packet of transmitter spontaneously released, during acetylcholinesterase inhibition, ACh molecules are present in the cleft for an increased time before diffusing away from the synaptic cleft (Katz & Miledi, 1973). Therefore, spontaneous release may lead to transient excess ACh in the synaptic cleft during periods of enzyme inhibition by neostigmine. Perhaps more importantly, non-quantal leakage of ACh also occurs spontaneously and continuously (Katz & Miledi, 1977), and this mechanism may also lead to continuously increased ACh levels in the synaptic cleft during enzyme inhibition.

Although the chronic effects of neostigmine treatment may be produced by increased ACh levels, an alternative mechanism is by direct action of neostigmine on the receptor. It is known that anticholinesterase drugs at high concentrations interact directly

with the acetylcholine receptor/channel complex to cause a depression of endplate potential amplitudes (Eccles & MacFarlane, 1949; Kuba *et al.*, 1973; Kordas *et al.*, 1975; Albuquerque *et al.*, 1984). It might be thought that such direct actions on the receptor by neostigmine would contribute in some way to the chronic effects. Such a mechanism appears unlikely because the direct action of neostigmine is weak or absent at the micromolar concentrations encountered in our injected rats (Eccles & McFarlane, 1949; Kordas *et al.*, 1975; Sherby *et al.*, 1985). Furthermore neostigmine ($< 10\,\mu M$) applied in the bath does not change the open time channel kinetics (Katz & Miledi, 1973; Fiekers, 1985). At the much higher concentrations of $60-90\,\mu M$, neostigmine itself causes a depolarization of only 0.3 mV (Katz & Miledi, 1977). Moreover, after chronic treatment with the anticholinesterase paraoxon, characteristic morphological changes normally appear but these are eliminated by pretreatment with a reactivator of acetylcholinesterase (Laskowski *et al.*, 1975). This again suggests the lack of involvement of a direct action of the anticholinesterase drug on the acetylcholine receptor in producing these chronic changes. In support of this conclusion, denervation before chronic treatment with neostigmine prevented the occurrence of morphological changes (Hudson *et al.*, 1978). However, other direct actions of neostigmine (e.g. to increase receptor desensitization) may be important (Sherby *et al.*, 1985).

In summary, therefore, the elevation of synaptic ACh levels apparently induced during chronic neostigmine treatment might be the mechanism producing an adaptive reduction in the number of functional AChRs at the endplate with no change in single channel properties themselves. For denervated muscle (chronic deficit of ACh), there is a corresponding increase in the number of receptors. However, this increase for devervated muscles is accompanied by an increase in channel open time (see Introduction). Therefore, it appears that while chronically decreased ACh levels tend towards being compensated for by an increase in both AChR number and the amount of ionic charge passed per channel, chronically increased ACh levels might be adapted to by a decrease in AChR number with no compensating decrease in channel open time or conductance.

M.G. was supported by an S.E.R.C. studentship.

References

ALBUQUERQUE, E.X., AKAIKE, A., SHAW, K-P. & RICKETT, D.L. (1984). The interaction of anticholinesterase agents with the acetylcholine receptor-ionic channel complex. *Fund. Appl. Tox.*, **4**, S27–S33.

ALEMA, S., CULL-CANDY, S.G., MILEDI, R. & TRAUTMANN, A. (1981). Properties of end-plate channels in rats immunised against acetylcholine receptors. *J. Physiol.*, **311**, 251–256.

AXELSSON, J. & THESLEFF, S. (1959). A study of supersensitivity in denervated mammalian skeletal muscle. *J. Physiol.*, **147**, 178–193.

CHANG, C.C., CHEN, T.F. & CHUANG, S-T. (1973). Influence of chronic neostigmine treatment on the number of acetylcholine receptors and the release of acetylcholine from the rat diaphragm. *J. Physiol.*, **230**, 613–618.

COLQUHOUN, D., LARGE, W.A. & RANG, H.P. (1977). An analysis of the action of a false transmitter at the neuromuscular junction. *J. Physiol.*, **266**, 361–395.

ECCLES, J.C. & MACFARLANE, W.V. (1949). Actions of anticholinesterases on endplate potential of frog muscle. *J. Neurophysiol.*, **12**, 59–80.

ENGEL, A.G., LAMBERT, E.H. & SANTA, T. (1973). Study of long-term anticholinesterase therapy. Effects on neuromuscular transmission and on motor end-plate fine structure. *Neurology*, **23**, 1273–1281.

FIEKERS, J.F. (1985). Concentration-dependent effects of neostigmine on the endplate acetylcholine receptor channel complex. *J. Neurosci.*, **5**, 502-514.

GWILT, M. & WRAY, D. (1984). Chronic neostigmine treatment and channel properties at the rat neuromuscular junction. *IUPHAR 9th International Congress of Pharmacology*, London, 1984, Abstracts of Communications.

HUDSON, C.S., RASH, J.E., TIEDT, T.N. & ALBUQUERQUE, E.X. (1978). Neostigmine-induced alterations at the mammalian neuromuscular junction. II. Ultrastructure. *J. Pharmac. exp. Ther.*, **205**, 340–356.

JENKINSON, D.H. & TERRAR, D.A. (1973). Influence of chloride ions on changes in membrane potential during prolonged application of carbachol to frog skeletal muscle. *Br. J. Pharmac.*, **47**, 363–376.

KATZ, B. & MILEDI, R. (1972). The statistical nature of the acetylcholine potential and its molecular components. *J. Physiol.*, **224**, 665–699.

KATZ, B. & MILEDI, R. (1973). The binding of acetylcholine to receptors and its removal from the synaptic cleft. *J. Physiol.*, **231**, 549–574.

KATZ, B. & MILEDI, R. (1977). Transmitter leakage from motor nerve terminals. *Proc. R. Soc. B.*, **196**, 59–72.

KORDAS, M., BRZIN, M. & MAJCEN, Z. (1975). A comparison of the effect of cholinesterase inhibitors on end-plate current and on cholinesterase activity in frog muscle. *Neuropharmac.*, **14**, 791–800.

KUBA, K., ALBUQUERQUE, E.X. & BARNARD, E.A. (1973). Diisopropylfluorophosphate: suppression of ionic conductance of the cholinergic receptor. *Science*, **181**, 853–855.

LASKOWSKI, M.B., OLSON, W.H. & DETTBARN, W-D. (1975). Ultrastructural changes at the motor end-plate produced by an irreversible cholinesterase inhibitor. *Exp. Neurol.*, **47**, 290–306.

MILEDI, R. (1960). The acetylcholine sensitivity of frog muscle fibres after complete or partial denervation. *J. Physiol.*, **151**, 1–23.

ROBERTS, J.B., THOMAS, B.H. & WILSON, A. (1965). Distribution and excretion of [^{14}C]-neostigmine in the rat and hen. *Br. J. Pharmac. Chemother.*, **25**, 234–242.

ROBERTS, D.V. & THESLEFF, S. (1969). Acetylcholine release from motor-nerve endings in rats treated with neostigmine. *Eur. J. Pharmac.*, **6**, 281–285.

SELLIN, L.C. & THESLEFF, S. (1981). Pre- and post-synaptic actions of botulinum toxin at the rat neuromuscular junction. *J. Physiol.*, **317**, 487–495.

SHERBY, S.M., ELDEFRAWI, A.T., ALBUQUERQUE, E.X. & ELDEFRAWI, M.E. (1985). Comparison of the actions of carbamate anticholinesterases on the nicotinic acetylcholine receptor. *Molec. Pharmac.*, **27**, 343–348.

TIEDT, T.N., ALBUQUERQUE, E.X., HUDSON, C.S. & RASH, J.E. (1978). Neostigmine-induced alterations at the mammalian neuromuscular junction. I. Muscle contraction and electrophysiology. *J. Pharmac. exp. Ther.*, **205**, 326–339.

WARD, M.D., FORBES, M.S. & JOHNS, T.R. (1975). Neostigmine methylsulfate. Does it have a chronic effect as well as a transient one? *Arch. Neurol.*, **32**, 808–814.

WRAY, D. (1980). Noise analysis and channels at the postsynaptic membrane of skeletal muscle. *Prog. Drug. Res.*, **24**, 9–56.

WRAY, D. (1981). Prolonged exposure to acetylcholine: noise analysis and channel inactivation in cat tenuissimus muscle. *J. Physiol.*, **310**, 37–56.

(Received July 22, 1985.
Revised November 20, 1985.
Accepted January 6, 1986)