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The effects of the antibiotic, primycin, on spontaneous transmitter release at the neuromuscular junction

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1 The effects of primycin, a potent ionophore in biological membranes, have been studied at the neuromuscular junction of the garter snake.

2 Primycin in concentrations greater than 2×10^{-7}M produced a time- and concentration-dependent depolarization of twitch muscle fibres.

3 Primycin (10^{-7}–5×10^{-7}M) produced an increased rate of quantal release of acetylcholine, which was not maintained, and a slight reduction in quantal size. Time to onset and to peak effect of primycin were concentration-dependent whereas maximum frequency was not.

4 Absence of extracellular Ca^{2+} produced a significant delay in the time to onset and to peak effect of primycin, but did not affect the peak miniature endplate potential (m.e.p.p.) frequency.

5 Following 60 min exposure to primycin (5×10^{-7}M), introduction of a high concentration of potassium (20 mM) produced no further increase in spontaneous release.

6 In cut muscle preparations, exposure to primycin (10^{-7}–5×10^{-7}M) reduced peak endplate current (e.p.c.) amplitude until nerve stimulation resulted in failures or the release of one or two quanta. E.p.c. amplitude was not restored with prolonged washing.

7 The effects of primycin on the nerve terminal are considered to be consistent with its ability to increase the permeability of membranes to calcium ions resulting in an influx of extracellular calcium, an efflux of mitochondrial calcium and eventual depletion of synaptic vesicles.

Introduction

The antibiotic primycin was first isolated in 1954 from cultures of *S. primycini* found in the intestinal contents of the larvae of the wax moth *Galeria melonella* (Valyi-Nagy, Uri & Szilagyi, 1954). The antibiotic is toxic when administered systemically, but is effective in the treatment of gram-positive skin infections when applied topically (Valyi-Nagy *et al.*, 1954). The bactericidal action of primycin is thought to be due to an increase in the ionic permeability of the bacterial cell membrane (Horvath, Kramer, Bauer & Buki, 1979).

In frog skeletal muscle primycin produces a rapid time- and concentration- dependent decrease in resting membrane potential (Kover, Gesztelyi, Konya & Daroczi, 1976). This has been ascribed to a reduction of resting potassium conductance (Kover *et al.*, 1976), although in other membrane systems, primycin has been shown to increase potassium permeability (Blasko, Gyorgyi & Horvath, 1979; Meszaros, Konig, Paroczai, Nahm & Horvath, 1979). In addition, primycin has been shown to increase the permeability of rat liver mitochondrial inner membranes to calcium (Meszaros, Hoffmann, Paroczai, Konig & Horvath, 1980). Subsequently, by using bioassay techniques, primycin was shown to increase spontaneous but not evoked output of acetylcholine from the guinea-pig ileum and rat cortical brain slices (Adam-Vizi, Horvath & Vizi, 1980). As the increase in spontaneous release was blocked by tetrodotoxin (TTX) and by elimination of calcium from the physiological solution, this effect was attributed to a depolarization of nerve terminals resulting in spontaneous firing.

We have carried out further studies on the mechanism of action of primycin on transmitter release by studying its effects with intracellular recording techniques at the neuromuscular junction.

Methods

Snake costocutaneous nerve-muscle preparation

All experiments were carried out on twitch muscle

fibres of the costocutaneous nerve-muscle preparation of the garter snake (*Thamnophis sirtalis*). This preparation is especially suitable for intracellular voltage clamp recording as it possesses large muscle fibres with easily identifiable endplate areas and has a large space constant relative to the size of the endplate (Ridge, 1971), allowing good voltage control of the endplate region (Dionne & Parsons, 1981).

Preparations were bathed unless otherwise stated in snake physiological solution containing (mM): NaCl 159, KCl 2.15, $MgCl_2$ 4.2, $CaCl_2$ 2.0, HEPES(N-2-hydroxy-ethylpiperazine-N^1-2-ethanesulphonic acid) 1.0, at pH 7.1–7.2 and at room temperature (20–24° C). In experiments performed in calcium-free physiological solution the concentration of $MgCl_2$ was increased to 8.4 mM and EGTA (ethyleneglycol-bis-tetraacetic acid) 0.5 mM was included in the solution. Drug solutions were added to the muscle by complete exchange of the bathing solution at a rate of 5 ml min^{-1}.

In experiments in which evoked release was measured, muscle contractions were eliminated by using a transected preparation as previously described (Fiekers, Henderson, Marshall & Parsons, 1983). Endplate currents (e.p.cs) were elicited by rectangular pulses of 0.05 ms duration and of strength greater than that required to produce evoked responses, at a frequency of 0.5 Hz.

Intracellular recording

Microelectrodes were filled with 3M KCl and had resistances between 4 and 10 MΩ. Standard microelectrode recording techniques were used for potential recording and the two microelectrode voltage clamp technique was used for current recording. Potentials were filtered at 5 kHz and currents at 3 kHz with 6 d.b. per octave roll off. Under voltage clamp conditions the voltage deviation during e.p.c. recording was always less than 1% of the driving force (clamp potential-reversal potential).

For measurements of miniature endplate potential (m.e.p.p.) or current (m.e.p.c.) frequency, 30 s samples were recorded on FM tape (Racal 4DS) every 5 min. Frequency was measured either by counting m.e.p.cs directly from the screen of a storage oscilloscope or by filming m.e.p.ps and calculating their frequency from

$$\frac{\text{number of m.e.p.ps} \times 1000}{\text{number of oscilloscope sweeps} \times \text{sweep time (ms)}}$$

E.p.c. quantal content in cut muscle preparations was assessed by the direct method of

$$m = \frac{\text{e.p.c. amplitude}}{\text{m.e.p.c. amplitude}}$$

where m is the quantal content, e.p.c. is the average peak endplate current amplitude of 100 to 120 e.p.cs, m.e.p.c. is the average peak miniature endplate current amplitude of 50 to 60 m.e.p.cs. All currents were amplified, captured by a PDP 11/23 laboratory computer at a digitization rate of 25 kHz and averaged by alignment at the point of 50% rise. E.p.cs and m.e.p.cs decayed as a single exponential function according to the following relationship:

$$I(t) = I(0)e^{-t/\tau}$$

where I(t) is the current amplitude at time t after the peak, I(0) is the peak current amplitude and τ is the decay time constant. The decay time constant (τ) was estimated from the reciprocal of the slope of the least squares regression line fitted to the semilogarithmic plot of amplitude between 5 and 97% of the peak against time.

Drugs and solutions

The drugs used were primycin sulphate (supplied by Professor I. Horvath, Semmelweiss University, Budapest, Hungary), tetrodotoxin, tetraphenylboron sodium, EGTA (all Sigma). Primycin sulphate was dissolved in dimethylsulphoxide (DMSO) and diluted with snake physiological solution. The final concentration of DMSO was 0.5% and all control measurements were made in solutions containing this concentration of DMSO.

Statistics

Results are expressed as mean ± s.e. Student's paired or unpaired *t* test was used to determine differences between groups. Differences were considered significant when $P < 0.05$.

Results

Effects of primycin on resting membrane potential

In concentrations greater than 2×10^{-7}M, primycin produced a concentration- and time-dependent decrease in resting membrane potential of the twitch fibres (Figure 1). The highest concentration studied (10^{-6}M) caused vigorous twitching of the muscle fibres and it was possible to record muscle action potentials from the twitch fibres.

Effects of primycin on miniature endplate potential and current frequency

Primycin (10^{-7} to 5×10^{-7}M) produced a marked time-dependent increase in spontaneous release that

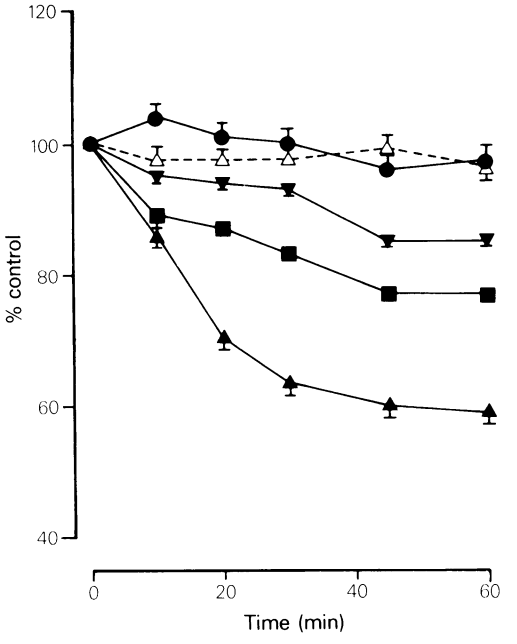

Figure 1 The effect of primycin on the resting membrane potential of twitch fibres. Resting membrane potential was recorded at each time point in at least eight fibres from three preparations. Each muscle was exposed to only one concentration of primycin and the results are expressed as the mean with s.e. (unless smaller than the symbol): (△) DMSO 0.5%; (●) primycin 10^{-7}M, (▼) 3×10^{-7}M, (■) 5×10^{-7}M, (▲) 10^{-6}M.

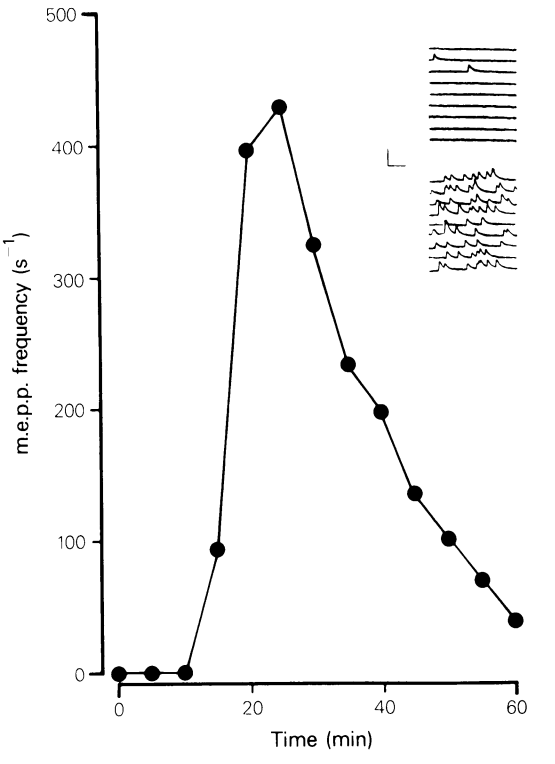

Figure 2 The time course of the increase in m.e.p.p. frequency produced by 3×10^{-7}M primycin followed in a single endplate for 60 min. Control m.e.p.p. frequency at time zero was $0.47\,s^{-1}$. Insert: control m.e.p.p. frequency and frequency after 15 min exposure to primycin 3×10^{-7}M. Calibration 10 ms, 1 mV.

was unaffected by the presence of TTX (10^{-7}M). After a short latent period which decreased with increasing concentrations of primycin, bursts of m.e.p.ps or m.e.p.cs were seen, followed by a steady increase in frequency from the control level of $0.41 \pm 0.12\,s^{-1}$ to several hundred per s (Figure 2). The increased frequency was not maintained and fell eventually to levels below control with prolonged exposure to primycin (greater than about 60 min). Although increasing the concentration of primycin reduced the time to peak effect, there was no evidence that the maximum effect was concentration-dependent. However, the accurate quantification of m.e.p.p. or m.e.p.c. frequency at rates greater than $500\,s^{-1}$ was difficult due to summation of the responses. Furthermore, concentrations of primycin greater then 5×10^{-7}M produced spontaneous movements of the slow fibres of the preparation leading to unstable microelectrode penetrations. The slow fibres contract via an action potential-independent mechanism (Ridge, 1971) and therefore were unaffected by TTX. Thus the concentration range of primycin that could be studied was limited.

When spontaneous release had decreased to a low level following treatment with 5×10^{-7}M primycin, challenge with 20 mM potassium solution produced no further increase in frequency. For example in 3 endplates from 3 muscles, m.e.p.c. frequency after 60 min exposure to primycin (5×10^{-7}M) was $7.8 \pm 3.6\,s^{-1}$ and after exposure to 20 mM potassium for 10 min was $4.8 \pm 2.0\,s^{-1}$ in the same endplates.

Effects of reduced extracellular calcium

To assess the role of nerve terminal depolarization in the increase in m.e.p.p. frequency, experiments were performed in the presence and absence of extracellular calcium. For these experiments a concentration of primycin (3×10^{-7}M) was used that would produce a rapid increase in m.e.p.p. frequency in normal snake physiological solution without the complication of slow fibre movement. The effects of this concentration of primycin were then re-tested in fresh preparations bathed in calcium-free solutions containing 0.5 mM EGTA.

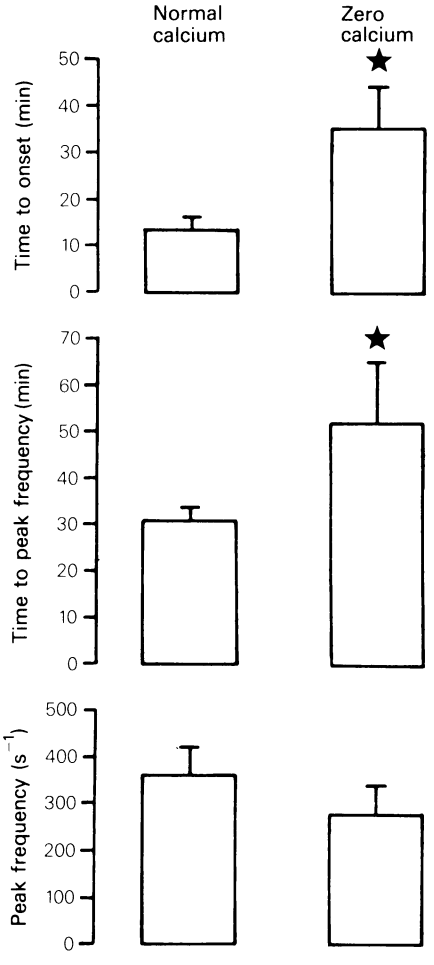

Figure 3 The effect of normal (2 mM calcium, $n = 10$) and calcium-free physiological solution containing EGTA 0.5 mM ($n = 6$) on the time course and magnitude of the increase in m.e.p.p. or m.e.p.c. frequency produced by 3×10^{-7}M primycin. * $P < 0.05$, unpaired *t* test.

In solutions from which extracellular calcium was omitted, a significant delay in both the time to onset of effect and the time to peak frequency ($P < 0.05$) was observed although there was no effect on peak m.e.p.p. frequency ($P > 0.05$; Figure 3). To investigate the possibility that the increased magnesium concentration in the calcium-free solution was responsible for the delayed effect of primycin, experiments were carried out in physiological solutions containing 2 mM calcium and 8.4 mM magnesium. There was no evidence for a delay in the time course of the effect of primycin (3×10^{-7}M) in this solution.

Effects of elevated extracellular potassium

The effects of increased extracellular potassium concentration on m.e.p.p. frequency were assessed in the presence and absence of extracellular calcium. High potassium solutions (20 mM) produced an immediate fall in resting membrane potential from 89 ± 2 mV to 47 ± 4 mV ($n = 8$). M.e.p.p. frequency in the presence of extracellular calcium increased rapidly (within 5 min) with the increased potassium concentration (Figure 4). The peak effect was less than that produced by primycin, and m.e.p.p. frequency remained elevated over a 60 min period of exposure to high potassium [$21 \pm 2.2\ s^{-1}$ at 60 min ($n = 4$) compared to $96 \pm 27\ s^{-1}$ following 60 min exposure to primycin 3×10^{-7}M ($n = 6$)].

In the absence of extracellular calcium, and in the presence of EGTA 0.5 mM, high potassium solutions produced no increase in m.e.p.p. frequency (Figure 4).

Effects of primycin on miniature endplate current amplitude

To assess the effects of primycin on quantal size, m.e.p.cs were recorded in muscle fibres voltage clamped at −80 mV, to eliminate the necessity to correct m.e.p.p. amplitude for changes in membrane potential produced by the drug. Control m.e.p.c. amplitude was 2.77 ± 0.21 nA ($n = 5$ fibres from 5 muscles). After 60 min exposure to primycin (5×10^{-7}M), m.e.p.c. frequency rose rapidly and then fell to levels still greater than control. During the period of elevated release many small amplitude m.e.p.cs and several giant m.e.p.cs were observed.

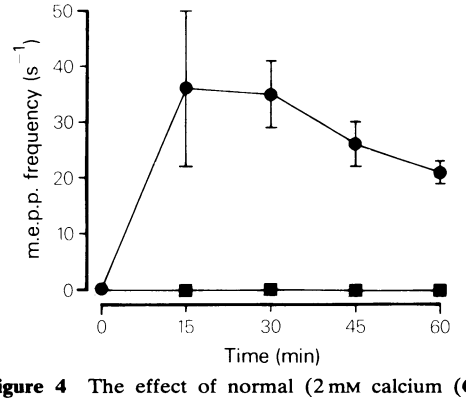

Figure 4 The effect of normal (2 mM calcium (●); $n = 4$) and calcium-free physiological solution containing EGTA 0.5 mM (■; $n = 4$) on m.e.p.p. frequency recorded from twitch fibres depolarized by 20 mM potassium. Control m.e.p.p. frequency in the presence of calcium was $0.40 \pm 0.20\ s^{-1}$ and in the absence of calcium was $0.09 \pm 0.01\ s^{-1}$.

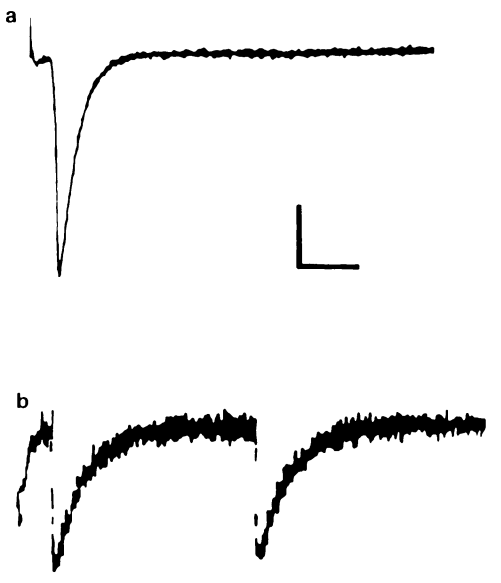

Figure 5 (a) A single e.p.c. recorded from a cut muscle preparation, voltage clamped at −90mV. (b) A single e.p.c. recorded from the same endplate as in (a) after 60 min exposure to 3×10^{-7}M primycin. E.p.c. time course is prolonged and quantal content in this instance is reduced to one quantum. An m.e.p.c. follows the e.p.c. Calibration: horizontal 5 ms; vertical (a) 150 nA; (b) 1 nA.

However, after 60 min exposure to primycin the m.e.p.c. amplitude (2.65 ± 0.06 nA) was not significantly different from that of control m.e.p.cs recorded from the same endplates ($P > 0.05$). In these experiments, the time constant of decay ($\tau_{m.e.p.c.}$) was significantly increased from 1.01 ± 0.07 ms to 1.26 ± 0.06 ms ($P < 0.05$).

Effects of primycin on endplate current quantal content and time course

In cut fibre preparations, primycin reduced quantal content from control levels of 149 ± 22 ($n = 10$ fibres) to one or two quanta shortly after the peak effect on m.e.p.c. frequency (Figure 5). Eventually motor nerve stimulation resulted in a complete failure of evoked release at a time when m.e.p.c. frequency was still elevated. Prolonged washing of the preparations with drug-free physiological solution produced no recovery of the e.p.c.

There was no significant difference between the time constant of decay of e.p.cs measured after 30 min exposure to primycin (3×10^{-7}M) and those measured in the solvent DMSO (0.5%) alone.

Effects of dimethylsulphoxide

DMSO (0.5%) produced no effect on the frequency of m.e.p.cs, nor on the resting membrane potential, but produced a time-dependent increase in e.p.c. and m.e.p.c. amplitude and a time-dependent prolongation of e.p.c. and m.e.p.c. time course. Figure 6 illustrates the effect of 30 min exposure to DMSO (0.5%) on e.p.c. time course. The voltage-dependence of $\tau_{e.p.c.}$ measured over a range of membrane potentials between −30 and −110 mV was not affected by exposure to DMSO; the value of H, i.e. the change in membrane potential required to produce an e-fold change in $\tau_{e.p.c.}$, calculated by linear regression of the cumulated results from three endplates was 113 mV compared to 116 mV in the presence of DMSO, measured in the same endplates (Figure 6).

Discussion

As found in previous studies, primycin depolarized muscle membranes (Kover *et al.*, 1976) and increased spontaneous release of acetylcholine (Adam-Vizi *et al.*, 1980). However, in the present study, whereas the depolarizing action of the compound was seen in similar concentrations to those previously reported in frog muscle, much lower concentrations than those reported by Adam-Vizi *et al.* (1980) were shown to increase spontaneous transmitter release. Thus concentrations of 10^{-7}M and above increased m.e.p.p. frequency at the neuromuscular junction. In contrast, concentrations of 4.4×10^{-5}M and 1.7×10^{-5}M were required to in-

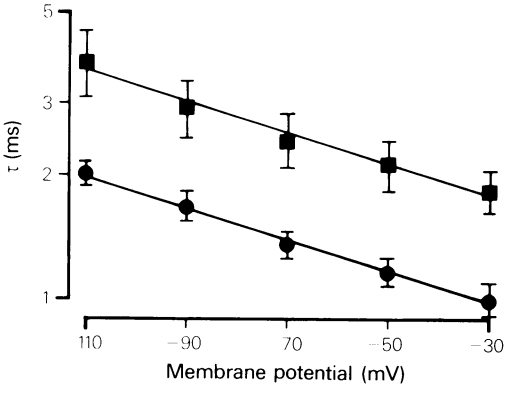

Figure 6 Semilogarithmic plot of $\tau_{e.p.c.}$ versus membrane potential in the absence (●) and following 30 min exposure to dimethylsulphoxide (DMSO) 0.5% (■) in the same endplates ($n = 3$). Best fit lines were calculated by linear regression analysis of the cumulated values from the three fibres.

crease acetylcholine output in the guinea-pig ileum and rat brain slices respectively (Adam-Vizi *et al.*, 1980). Despite the fact that the more sensitive technique of intracellular recording was used in the present study compared to transmitter collection and bioassay techniques in the study of Adam-Vizi *et al.* (1980), it is probable that different mechanisms are responsible for producing the increase of acetylcholine release in the two studies. Further evidence for this is that in the study of Adam-Vizi *et al.* (1980), the increased spontaneous transmitter release produced by primycin was completely absent in preparations treated with TTX or in the absence of extracellular calcium. This led Adam-Vizi *et al.* (1980) to propose that primycin acted by inhibiting resting potassium conductance (Kover *et al.*, 1976) to produce depolarization of nerve terminals which would in turn elicit action potentials and hence transmitter release. Although we were able to measure muscle action potentials in the presence of depolarizing concentrations of primycin, we could also measure an increase in spontaneous release at lower concentrations, that was not abolished by TTX nor by omission of calcium from the physiological solution.

As expected from the observations of previous workers (del Castillo & Katz, 1954; Liley, 1956), the effect of increased extracellular potassium concentration on m.e.p.p. frequency at the snake neuromuscular junction was completely abolished by removal of extracellular calcium. The delay in onset of the effect of primycin in calcium-free solutions suggests that nerve terminal depolarization may play a role in the initial increase in spontaneous transmitter release. However, as the peak effect of primycin on m.e.p.p. frequency was independent of the presence of external calcium, then a secondary effect of primycin in promoting resting acetylcholine release must also be involved.

In addition to its ability to inhibit resting potassium conductance (Kover *et al.*, 1976) primycin is a potent ionophore for monovalent and divalent cations in various biological membranes and lipid bilayers (Blasko *et al.*, 1979; Meszaros *et al.*, 1979) and such actions may be involved in its depolarizing action. In addition, primycin has been shown to increase the permeability of rat liver mitochondrial inner membranes to calcium (Meszaros *et al.*, 1980). As mitochondria play an important role in regulating intraterminal calcium levels and hence m.e.p.p. frequency (Rahamimoff & Alnaes, 1973) it is possible that an efflux of calcium from the mitochondria may explain the increase in m.e.p.p. frequency induced by primycin. Further evidence in support of an intracellular site of action is the concentration-dependent delay in the onset of the effect of the antibiotic. As primycin may act as an ionophore in many membrane systems, it is plausible that it may render the nerve terminal permeable to calcium ions via a voltage-independent mechanism. Thus the initial increase in spontaneous release may then be explained in terms of extracellular calcium entry through channels in the nerve terminal induced by primycin.

In addition to the increase in the spontaneous transmitter release, primycin subsequently reduced both spontaneous and evoked release to below control levels. At this time postjunctional sensitivity, as assessed by the quantal size, was unaffected. However, as the solvent DMSO 0.5% produced an increase in peak m.e.p.c. and e.p.c., then it can be deduced that primycin actually produced a small reduction in quantal size. These effects of DMSO on current amplitude and time course were probably a consequence of its reported anticholinesterase activity (Sams, Carroll & Crantz, 1966) and were not considered to have contributed to the observed effects of primycin.

Other agents that increase and then decrease spontaneous release at the neuromuscular junction have variable effects on quantal size. Thus latratoxin, which like primycin has a concentration-dependent action on the time to onset of effect but produces a constant peak effect, does not reduce m.e.p.p. amplitude, (Longenecker, Hurlbut, Mauro & Clark, 1970). In contrast, and as previously reported for m.e.p.ps in the frog (Marshall & Parsons, 1975; Marshall, Parsons & Paull, 1976) in the costocutaneous muscle, tetraphenylboron (2×10^{-5}M) produces a reduction of m.e.p.c. amplitude to below the level of baseline noise after an initial increase in m.e.p.c. frequency is observed (Henderson & Marshall, unpublished observations). Botulinum toxin also reduces m.e.p.p. amplitude (Boroff, del Castillo, Evoy & Steinhardt, 1974) and it has been suggested that both botulinum toxin and tetraphenylboron interfere with transmitter packaging resulting in reduced quantal size (Boroff *et al.*, 1974; Marshall & Parsons, 1975).

The lack of reversibility of the reduction of e.p.c. quantal content by washing, coupled with the inability of elevated potassium ion concentration to increase m.e.p.p. frequency after prolonged exposure to primycin, suggests that exposure to the antibiotic may result in depletion of nerve terminal vesicles of acetylcholine.

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