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Effect of neomycin on post-tetanic twitch tension of the mouse diaphragm preparation

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1 The effect of calcium (0.5–6 mM) and neomycin (0.1–0.2 mM) on the maximum post-tetanic twitch tension (MTT) and post-tetanic depression (PTD) of the indirectly elicited twitch tension was studied on the mouse isolated phrenic nerve-diaphragm preparation. The effect of neomycin on MTT of directly stimulated twitch tension was also tested in (+)-tubocurarine pretreated preparations.

2 Three-dimensional plots between MTT and frequency and duration of indirect tetanic stimulation revealed that the frequencies and durations inducing maximal MTT were 500 Hz for 20 s in 0.5 mM $CaCl_2$, 100 Hz for 5 s in 2 mM $CaCl_2$ and 100 Hz for 10 s in 6 mM $CaCl_2$. The frequency and duration inducing maximal PTD was 100 Hz for 20 s in 0.5 mM $CaCl_2$, but there was no PTD in 2 mM or 6 mM $CaCl_2$.

3 Neomycin was associated with significantly greater MTT than in control if the duration of tetanic stimulation was 1 or 2 s, while it was associated with less MTT if the duration of tetanic stimulation was 10 or 20 s.

4 Neomycin caused PTD in 2 mM $CaCl_2$; sometimes the depressive effect was so severe that twitch tension was abolished. The maximal depression effect was found after 100 Hz tetanic stimulation for 20 s. Increasing the extracellular calcium concentration to 6 mM antagonized the effects of neomycin on MTT and PTD, whereas neostigmine (1.6 μM) antagonized the effect partially.

5 Neomycin had no effect on the MTT or PTD of the directly stimulated twitch tension.

6 It is concluded that neomycin alters the conditions of tetanic stimulation inducing MTT.

Introduction

The interest in the neuromuscular actions of neomycin, an aminoglycoside antibiotic, was stimulated by the report of episodes of apnoea following i.p. administration of neomycin in man (Pridgen & Tex, 1956). Neomycin possesses significant neuromuscular blocking actions (Elmqvist & Josefsson, 1962; Brazil & Prado-Franceschi, 1969; Wright & Collier, 1977; Singh *et al.*, 1980; Tsai, 1983; Fiekers, 1983a,b). It also induces a calcium-dependent post-tetanic exhaustion effect (Lee & De Silva, 1979) as it decreases twitch tension below its pre-tetanic level after a short period of post-tetanic potentiation. Hereafter, this exhaustion effect is referred to as post-tetanic depression (PTD). However, the relationships between the neomycin-induced PTD and the frequency and duration of tetanic stimulation remain unclear. In order to elucidate these relationships, the indirectly elicited twitch tension after various stimulation conditions was measured in the mouse isolated hemi-diaphragm preparation. The effects of different extracellular calcium or magnesium ion concentrations on neomycin-induced maximum post-tetanic twitch tension (MTT) were also determined because of the known competition between calcium ion and aminoglycoside antibiotics (Elmqvist & Josefsson, 1962; Singh *et al.*, 1978; Tsai, 1983; Wright & Collier, 1977).

Methods

Experiments were carried out *in vitro* on the isolated phrenic nerve hemi-diaphragm preparation (Bülbring, 1946) of I.C.R. mice of either sex. Tissues were immersed in a physiological solution containing (mM) NaCl 135.0, KCl 5.0, $CaCl_2$ 2.0, $MgCl_2$ 1.0, $NaHCO_3$ 15.0, NaH_2PO_4 1.0 and glucose 11.0. For low calcium medium, the calcium concentration was changed to 0.5 mM. The bath was maintained at 37 ± 1°C and continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2, maintaining the physiological solution at pH 7.1–7.3. The phrenic nerve of the diaphragm was stimulated by a pair of platinum electrodes with supramaximal square pulses of 0.05 ms duration delivered through a photoelectric isolation

unit (Digitimer D4030 with DS2 isolated stimulator). The stimulation frequency was 0.2 Hz (Tsai, 1985). For tetanic stimulation, frequency was varied while its duration was fixed, or the latter was varied while the frequency remained constant. After the tetanic stimulation, 0.2 Hz stimulation frequency was again used. For direct stimulation of the muscle, neuromuscular transmission was blocked by (+)-tubocurarine (0.14 mM) and nearly maximal rectangular pulses of 1.0–2.0 ms duration were applied at a rate of 0.2 Hz through a pair of platinum electrodes placed at the base of the diaphragm muscle near the intercostal muscles. The tetanic stimulation conditions were as described above. Tension was recorded by a Grass FT 03 force displacement transducer connected to a Grass model 5D polygraph or by a Gould UC2 transducer connected to a Gould 2200S recorder.

MTT was measured as the maximum twitch tension after tetanic stimulation and expressed as percentage of the pre-tetanic twitch tension. The post-tetanic depression induced by neomycin was measured as the minimum twitch tension after tetanic stimulation and expressed as percentage of the pre-tetanic twitch tension (PTD). The mean percentages of MTT and PTD were calculated. The relationships between frequency and duration of tetanic stimulation and the percentage of MTT or PTD are plotted on three dimensional graphs. The graphs were constructed by plotting the duration and the frequency of the tetanic stimulus expressed logarithmically on the x and z axes, respectively while the post-tetanic twitch tension (as a percentage of pre-tetanus control height) was plotted linearly on the y axis (Standaert, 1964).

The mean MTTs or PTDs after various tetanic stimulation conditions or neomycin treatment were compared by means of Student's t test, with $P < 0.05$ indicating significance.

Drugs

Neomycin and neostigmine were purchased from Sigma Chemical Co. (St. Louis, Mo.)

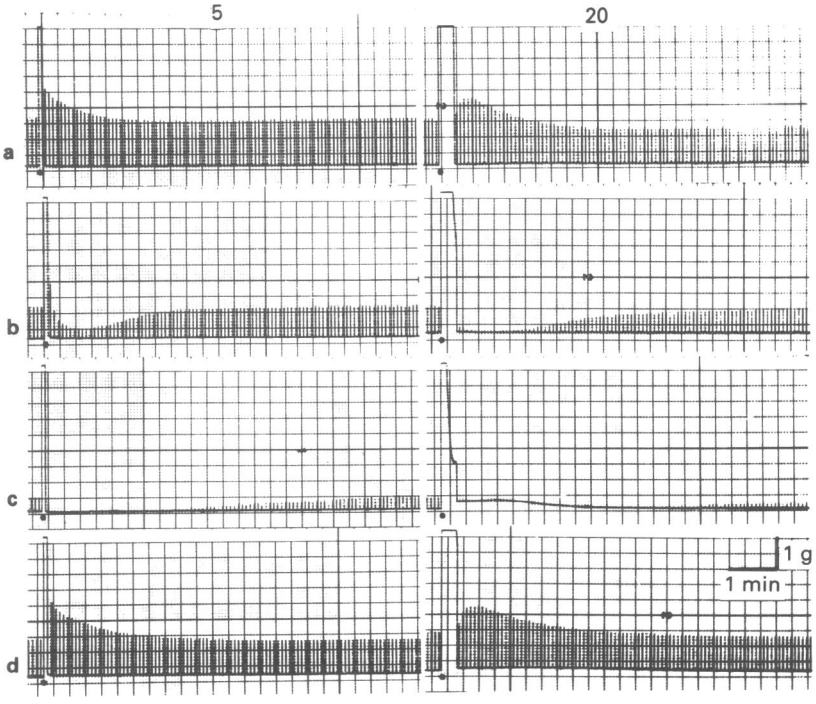

Figure 1 Effect of neomycin on the indirectly elicited twitch tension of mouse diaphragm preparations. The phrenic nerve was stimulated at 0.2 Hz. At the dots, the phrenic nerve was stimulated tetanically at 100 Hz. The numbers above the tracing denote the duration of tetanic stimulation. (a) Control; (b and c) preparations pretreated with neomycin, 0.1 and 0.2 mM, respectively; (d) the same preparation as shown in (c) but the extracellular calcium concentration was adjusted to 6 mM. Note that the indirectly elicited twitch tension in the presence of neomycin was abolished after 20 s of repetitive stimulation, and then gradually recovered.

Results

Post-tetanic maximum twitch tension (MTT)

Effect of frequency and duration of tetanic stimulation on MTT in normal physiological solution In the presence of 2 mM $CaCl_2$, the indirectly elicited twitch tension was greater than control immediately after tetanic stimulation, and then the twitch tension gradually recovered to the pre-tetanic level (Figure 1a). The magnitude of maximum post-tetanic twitch tension (MTT), i.e. the ratio of post-tetanic to pre-tetanic twitch tension, was dependent on the frequency and duration of tetanic stimulation. The mean MTTs (% of pre-tetanus control) after different tetanic stimulation are shown in Figure 2a.

MTT was dependent on the frequency and duration of tetanic stimulation. If tetanic stimulation frequency was set at 100 Hz, MTT was increased if tetanic stimulation duration was increased from 1 to 5 s. Further increasing the duration did not further increase MTT. If the tetanic stimulation duration was fixed at 5 s, MTT was also increased if tetanic stimulation frequency was increased from 10 to 100 Hz. Further increasing the stimulation frequency (200–500 Hz) did not further increase the MTT.

Effects of various calcium concentrations on MTT induced by indirect stimulation In the presence of 0.5 mM $CaCl_2$, the relationships between MTT elicited by indirect tetanic stimulation and frequency and duration of tetanic stimulation were changed from those in control. The mean MTT in low calcium medium with various tetanic stimulation conditions are shown in Figure 2c. If the duration of tetanic stimulation was fixed at a certain value while the frequency of stimulation was changed, MTT was increased significantly to that in control experiments at frequencies of 100–500 Hz.

The results obtained in high calcium medium (6 mM) are shown in Figure 2b. Compared with MTT in 2 mM $CaCl_2$, increasing the extracellular calcium concentration to 6 mM did not alter the relationships between MTT and duration and frequency of tetanic stimulation. However, the magnitude of MTT was significantly higher in 6 mM $CaCl_2$ following 5 and 10 s tetanic stimulation at 50 and 100 Hz.

Effects of various magnesium concentrations on MTT induced by indirect stimulation The indirectly elicited twitch tension was decreased if the extracellular magnesium ion concentration was increased from 1 to 6 mM. The decrease could be partially reversed if the extracellular calcium concentration was increased to 6 mM.

The relationships between MTT and frequency and duration of tetanic stimulation were tested in 5 mM $MgCl_2$ because the twitch tension decreased markedly in higher magnesium concentrations (6 mM) after several trains of tetanic stimulation. The results obtained in 5 mM magnesium are shown in Figure 2f. Compared with MTT in 1 mM $MgCl_2$ (Figure 2a), increasing the extracellular magnesium concentration to 5 mM altered the relationships between MTT and duration and frequency of tetanic stimulation. The magnitude of MTT was significantly higher in 5 mM $MgCl_2$ following 50–100 Hz tetanic stimulation for 5–10 s (Figure 2f).

MTT induced by direct stimulation To make sure that the effects of direct stimulation were not due to the concomitant release of acetylcholine, (+)-tubocurarine (0.14 mM) was added to the preparations for 30 min. After indirectly elicited twitch tension was abolished, the muscle was stimulated directly in the continued presence of (+)-tubocurarine. The relationships of MTT and the frequency and duration of direct tetanic stimulation are shown in Figure 3a. MTT was dependent on frequency and duration of tetanic stimulation.

Effect of neomycin on MTT induced by indirect stimulation The effects of various concentrations of neomycin on the MTT of indirectly elicited twitch tension are shown in Figure 1b and c. The effect of neomycin on MTT was dependent on the duration of tetanic stimulation. Compared with MTT in control preparations after similar tetanic stimulation conditions, MTT was significantly increased in neomycin-treated preparations which were tetanically stimulated for 1 or 2 s (Figure 2d). However, MTT in neomycin-treated preparations was significantly decreased following tetanic stimulation for 10 or 20 s. It seems that stimulation duration plays an essential role in the inhibition of MTT. The effect of neomycin on MTT was abolished if the extracellular calcium ion concentration was increased to 6 mM (Figure 1d).

Effect of neomycin on MTT induced by direct stimulation The effects of neomycin (0.2 mM) on the MTT of directly stimulated diaphragms are shown in Figure 3b. Compared with the MTT shown in Figure 3a, neomycin did not significantly alter the MTT induced by direct stimulation.

Post-tetanic depression (PTD)

Effect of tetanic stimulation frequencies and durations on post-tetanic depression (PTD) induced by indirect stimulation The relationships between PTD induced by indirect stimulation and frequency and duration of tetanic stimulation are shown in Figure 4a. It appears that no remarkable PTD of indirectly elicited twitch tension was found in normal physiological solution

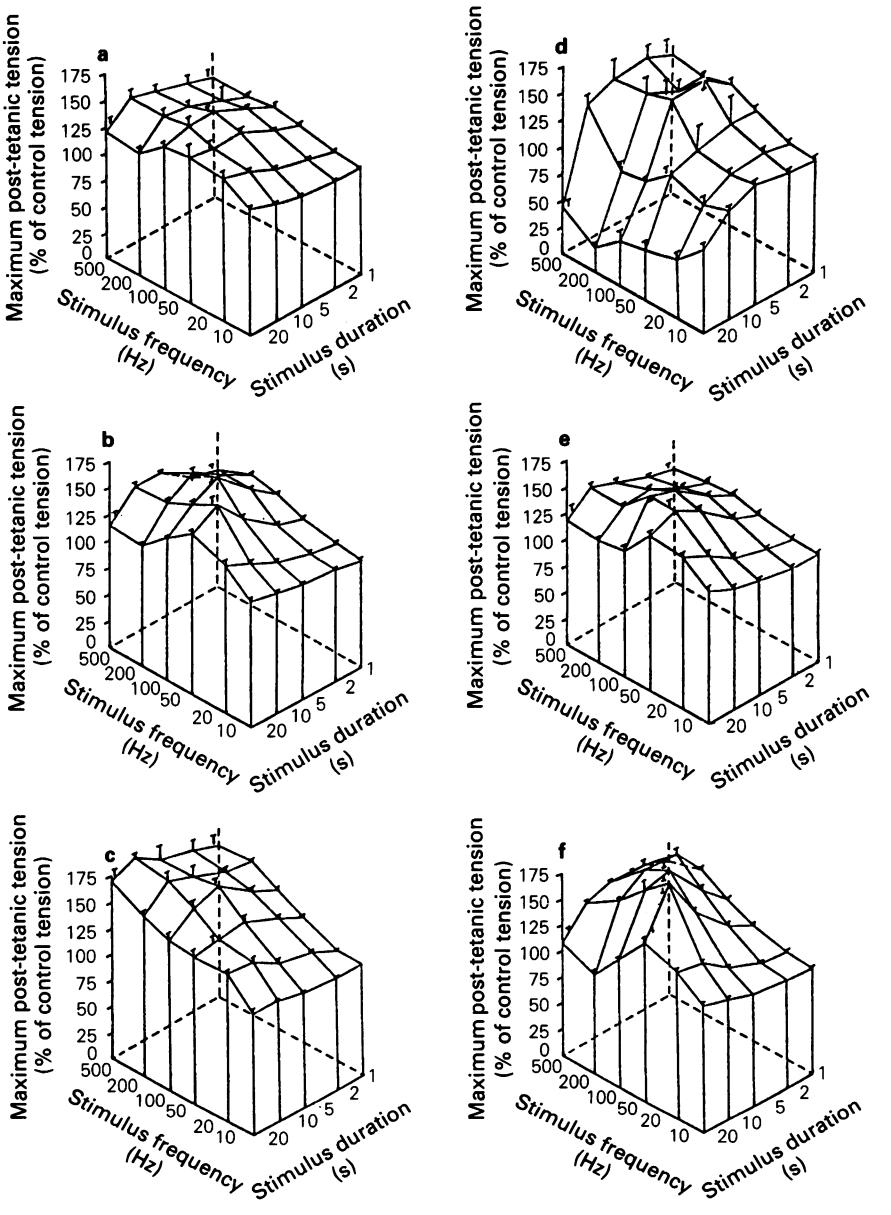

Figure 2 Maximum post-tetanic twitch tension (MTT) as a function of the frequency and duration of the tetanic stimulus. The duration and the frequency of the tetanic stimulus expressed logarithmically are plotted on the x and z axes, respectively, and the maximal post-tetanic twitch tension, expressed as percentage of pre-tetanic twitch tension (mean ± s.e.mean, $n = 4-12$) was plotted linearly on the y axis. Where no error bars are shown, the s.e.mean was smaller than the symbols. (a), (b) and (c) MTTs in 2 mM, 6 mM and 0.5 mM $CaCl_2$, respectively, (d) MIT in normal physiological solution containing 2 mM $CaCl_2$ and 0.1 mM neomycin; (e) MTT in 6 mM $CaCl_2$ containing 0.1 mM neomycin; (f) MTT in 2 mM $CaCl_2$ containing 5 mM $MgCl_2$. Note that MTT in neomycin is decreased after tetanic stimulation of long duration (d).

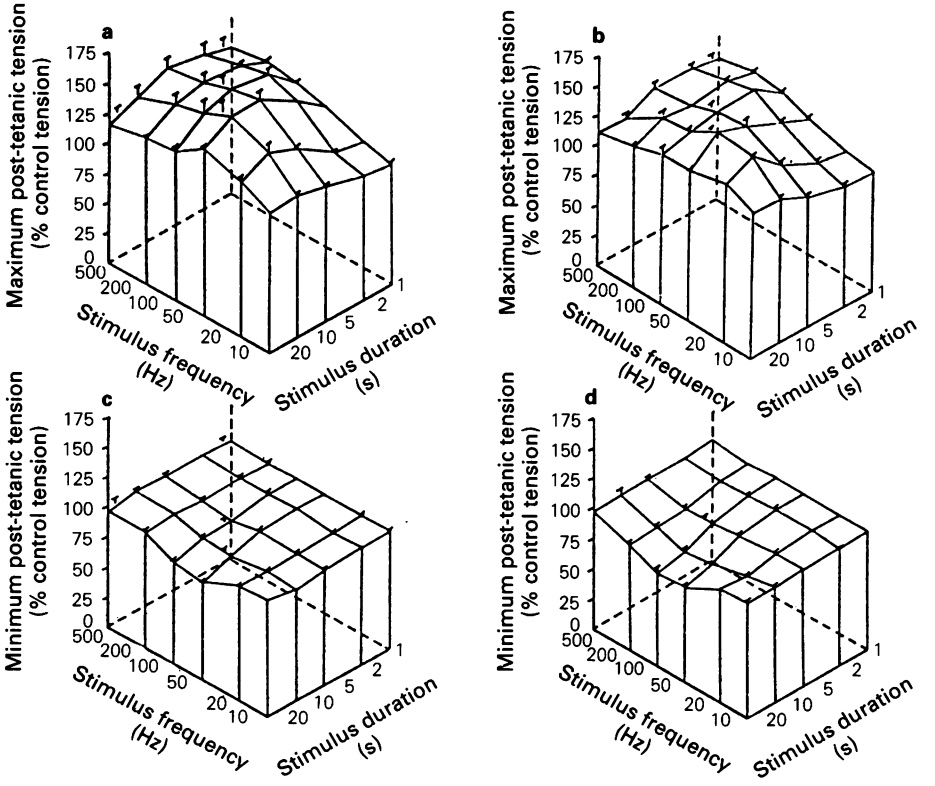

Figure 3 Effect of neomycin (0.2 mM) on maximum post-tetanic twitch tension (MTT) and post-tetanic depression (PTD) of directly elicited twitch tension. MTT or PTD was expressed as a function of the frequency and duration of the tetanic stimulus. The preparation was pretreated with (+)-tubocurarine (0.14 mM) for 30 min. The duration and the frequency of the tetanic stimulus expressed logarithmically were plotted on the x and z axes, respectively, and the MTT or PDT, expressed as percentage of pre-tetanic twitch tension (mean ± s.e.mean, $n = 4$), was plotted linearly on the y axis. Where no error bar is shown, the s.e.mean was smaller than the symbols. (a) and (c) were preparations in the presence of (+)-tubocurarine; (b) and (d) were preparations in the presence of (+)-tubocurarine and neomycin. (a) and (b): MTT; (c) and (d): PTD.

containing 2 mM $CaCl_2$.

Effects of low calcium or high magnesium concentrations on the PTD induced by indirect stimulation PTD of twitch tension was found if the extracellular calcium ion concentration was decreased to 0.5 mM (Figure 4c). PTD was dependent on both the frequency and duration of tetanic stimulation. If the frequency was fixed at 100 Hz, maximal depression was found after 20 s tetanic stimulation. If the duration of tetanic stimulation was fixed at 10 s, maximal depression was found after 100 Hz stimulation. The three dimensional graph revealed that there is a concave shape with maximal depression after 50–200 Hz for 5–20 s stimulation.

The effects of frequency and duration of tetanic stimulation on PTD in 5 mM $MgCl_2$ are shown in Figure 4f. Compared with PTD in 1 mM $MgCl_2$ (Figure 4a), increasing the extracellular magnesium concentration to 5 mM altered the relationships between PTD and duration and frequency of tetanic stimulation. PTD was significantly greater in 5 mM $MgCl_2$ following 100 Hz tetanic stimulation for 20 s. The PTD effect could be found in higher magnesium medium (6 mM); however, the pre-tetanic twitch tension was also significantly lowered. The pre-tetanic twitch tension was depressed after 1–2 periods of tetanic stimulation making measurement of PTD difficult.

PTD induced by direct stimulation After indirectly elicited twitch tension was abolished by (+)-

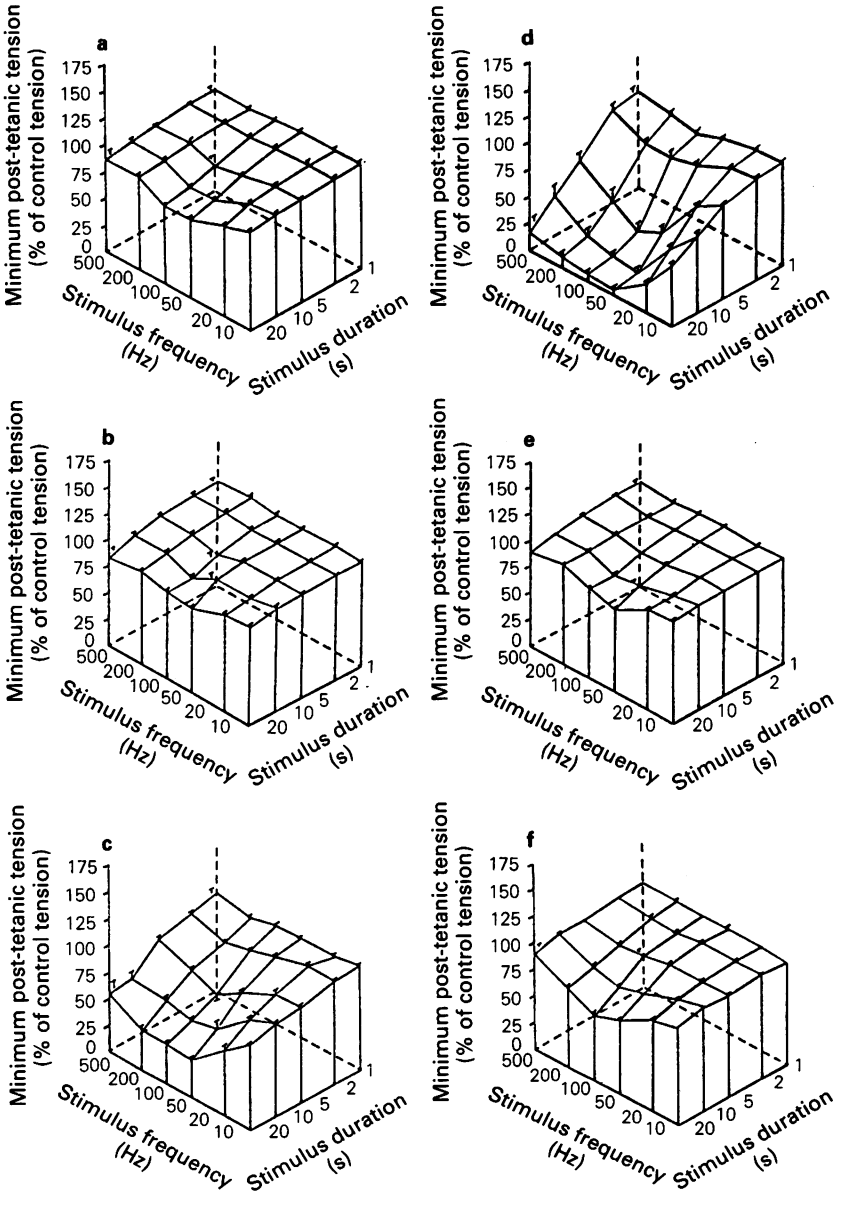

Figure 4 Post-tetanic depression (PTD) expressed as a function of the frequency and duration of the tetanic stimulus. The duration and the frequency of the tetanic stimulus expressed logarithmically are plotted on the x and z axes, respectively, and the post-tetanic depression, expressed as percentage of pre-tetanic twitch tension (mean ± s.e.mean, $n = 4-12$), is plotted linearly on the y axis. Where no error bar is shown, the s.e.mean was smaller than the symbols. (a), (b) and (c): PTDs in 2 mM, 6 mM and 0.5 mM $CaCl_2$, respectively;(d) PTD in preparations containing 2 mM $CaCl_2$ and 0.1 mM neomycin; (e) PTD in preparations containing 6 mM $CaCl_2$ and 0.1 mM neomycin; (f) PTD in preparations containing 2 mM $CaCl_2$ and 5 mM $MgCl_2$. Note the concave shape of the PTD in (c) and (d).

tubocurarine (0.14 mM), the muscle was stimulated directly in the continuous presence of (+)-tubocurarine. The relationships of PTD and the frequency and duration of direct tetanic stimulation are shown in Figure 3c. No significant PTD was found in directly stimulated preparations.

Effect of neomycin on PTD induced by indirect stimulation Neomycin produced PTD. The effect was so pronounced that indirectly elicited twitch tension could be abolished after tetanic stimulation for 20 s in the presence of 0.1–0.2 mM neomycin (Figure 1b and c). The effect of frequency and duration of tetanic stimulation of PTD in the presence of neomycin (0.1 mM) is shown in Figure 4d. The graph reveals that the effect of neomycin on PTD was dependent on the frequency and duration of the tetanic stimulation in a manner similar to that seen in 0.5 mM $CaCl_2$ solution; however, neomycin produced a greater depression.

On decreasing the extracellular calcium ion concentration to 0.1 or 0.25 mM, the indirectly elicited twitch tension was almost completely blocked, making the measurement of the twitch tension technically difficult. On adding neomycin (0.1 mM) to preparations containing 0.5 mM $CaCl_2$, the indirectly elicited twitch tension was abolished, although the same concentration of neomycin induced only partial block in 2 mM $CaCl_2$.

The depressive effect of neomycin on twitch tension and PTD was antagonized if the extracellular calcium ion concentration was increased to 6 mM (Figure 4e).

Effect of neostigmine on PTD induced by neomycin-

Neostigmine (1.6 μM) increased the indirectly elicited twitch tension of the mouse diaphragm. After tetanic stimulation at 100 Hz for 5 and 20 s, the indirectly elicited twitch tension was initially decreased and then it gradually recovered to the pre-tetanic level (Figure 5a). If neostigmine was added for 40 min to preparations pretreated with neomycin (0.1 mM), the PTD induced by neomycin was partially overcome (Figure 5c).

Neostigmine caused fade of tetanic contractions evoked by repetitive nerve stimulation. The tetanic fade was much less in the neomycin-treated preparations (Figure 5c).

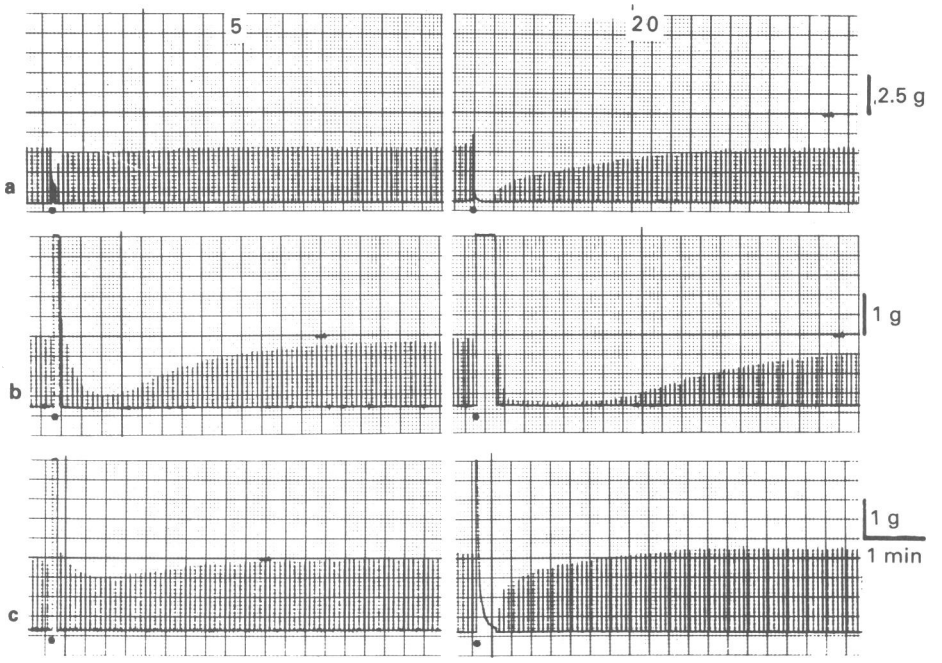

Figure 5 Effect of neostigmine (1.6 μM) on the indirectly elicited twitch tension of mouse diaphragm. The phrenic nerve was stimulated at 0.2 Hz. At the dots, the phrenic nerve was stimulated tetanically for 100 Hz for either 5 s (left-hand panels) or 20 s (right hand panels). (a) The preparation was treated with neostigmine; (b) the preparation was treated with neomycin (0.1 mM); (c) the same preparation as shown in (b), but neostigmine (1.6 μM) had been added for 40 min. Note that neostigmine partially decreases the PTD induced by neomycin.

Discussion

Neomycin reduces (1) the amount of acetylcholine released from motor nerve terminals, (2) the sensitivity of the postjunctional acetylcholine receptor by blockade of these recognition sites, (3) the endplate ionic conductance by blockade of the receptor activated ion channel (Singh *et al.*, 1979; 1982), and (4) intracellular calcium transients elicited by action potential stimulation in skeletal muscle fibres (Vergara *et al.*, 1985). The major neuromuscular blocking effect of neomycin is, however, due to its presynaptic effect (Wright & Collier, 1977; Tsai, 1983). Neomycin blockade can be rapidly reversed by an increase in calcium concentration (Singh *et al.*, 1978; Tsai, 1983).

The effects of neomycin on MTT and PTD are probably a consequency of its prejunctional actions. They are unlikely to be due to direct effects on muscle because, in the present study, neomycin did not alter the relationships between frequency and duration of tetanic stimulation and MTT or PTD induced by direct stimulation in preparations pretreated with (+)-tubocurarine. Also, the effects of neomycin are probably not due to its post-junctional inhibitory actions (Elmqvist & Josefsson, 1962; Tsai, 1983; Fiekers, 1983b) because its effects on MTT and PTD were different from those of high concentrations of (+)-tubocurarine. Thus, in preparations partially blocked by (+)-tubocurarine, MTT was dependent on the frequency of tetanic stimulation but independent of the duration of tetanic stimulation (Tsai *et al.*, 1986). In contrast, the effect of neomycin on MTT was dependent on both the frequency and the duration of tetanic stimulation. Also the PTD induced by neomycin was poorly reversed by the anticholinesterase, neostigmine.

Although the effect of neomycin on MTT and PTD may be ascribed to its prejunctional inhibitory effects, the relationships between changes in the amplitude of contraction and transmitter release is not simple. Variations in the amplitude of twitch tension in neomycin-treated preparations are determined by differences in the number of muscle fibres contributing to the response. Reduction of transmitter release, for example, will have no effect until it is sufficiently large to reduce the endplate potential in a fibre to a subthreshold level, and the effect in any one fibre will be all or none. Therefore, the extent of transmitter reduction and changes in contraction amplitude may not be linearly related.

Neomycin induces a failure of neuromuscular transmission that is quite similar to paralysis induced by high magnesium or low calcium medium (Elmqvist & Joseffson, 1962). In the present experiments, PTD was also found in high magnesium medium and in low calcium medium. However, there are differences between these effects and those in neomycin that indicate that the underlying mechanisms may be different. Further electrophysiological studies are needed to clarify these differences.

The PTD induced by neomycin was potentiated if longer tetanic stimulation was applied suggesting that the neomycin-induced PTD effect may be dependent on the duration of the tetanic stimulation.

With reference to the use of neomycin during anaesthesia, the following may be significant: because prolonging the duration of tetanic stimulation potentiates the PTD effect of neomycin, longer artificial respiration during neomycin treatment may worsen the respiratory arrest symptoms of the patient.

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References

BRAZIL, O.V. & PRADO-FRANCESCHI, J. (1969). The nature of neuromuscular block produced by neomycin and gentamicin. *Archs. int. Pharamcodyn. Thér.*, **179**, 78–85.

BULBRING, E. (1946). Observations on the isolated phrenic nerve diaphragm preparation of the rat. *Br. J. Pharmac. Chemother.*, **1**, 38–61.

ELMQVIST, D. & JOSEFSSON, J.O. (1962). The nature of the neuromuscular block produced by neomycin. *Acta physiol scand.*, **54**, 105–110.

FIEKERS, J.F. (1983a). Effects of the aminoglycoside antibiotics, streptomycin and neomycin on neuromuscular transmission. 1. pre-synaptic considerations. *J. Pharmac. exp. Ther.*, **225**, 487–495.

FIEKERS, J.F. (1983b). Effects of the aminoglycoside antibiotics, streptomycin and neomycin, on neuromuscular transmission. 11. post-synaptic considerations. *J. Pharmac. exp. Ther.*, **225**, 496–502.

LEE, C. & DE SILVA, A.J.C. (1979). Interaction of neuromuscular blocking effects of neomycin and polymycin B. *Anaesthesiology*, **50**, 218–220.

PRIDGEN, J.E. & TEX, S.A. (1956). Respiratory arrest thought to be due to intraperitoneal neomycin. *Surgery*, **40**, 571–574.

SINGH, Y.N., HARVEY, A.L. & MARSHALL, I.G. (1978). Antibiotic-induced paralysis of the mouse phrenic nerve-hemidiaphragm preparation, and reversibility by calcium and by neostigmine. *Anesthesiology*, **48**, 418–424.

SINGH, Y.N., MARSHALL, I.G. & HARVEY, A.L. (1979). Depression of transmitter release and post-junctional sensitivity during neuromuscular block produced by antibiotics. *Br. J. Anaesthesia*, **51**, 1027–1033.

SINGH, Y.N., MARSHALL, I.G. & HARVEY, A.L. (1980). The

mechanisms of the muscle paralysing actions of antibiotics, and their interaction with neuromuscular blocking agents. *Rev. Drug Metabolism Drug Interactions*, **3**, 129–153.

SINGH, Y.N., MARSHALL, I.G. & HARVEY, A.L. (1982). Pre- and postjunctional blocking effects of aminoglycoside, polymyxin, tetracycline and lincosamide antibiotics. *Br. J. Anaesth.*, **54**, 1295–1306.

STANDAERT, F.G. (1964). The mechanisms of post-tetanic potentiation in cat soleus and gastrocnemius muscles. *J. gen. Physiol.*, **47**, 987–1001.

TSAI, M.C. (1983). Mode of action of neomycin on the neuromuscular transmission. *J. Formosan Med. Assoc.*, **82**, 1–10.

TSAI, M.C. (1985). A pharmacological study of the effect of carbamazepine on neuromuscular transmission in the rat diaphragm. *Neuropharmacology*, **24**, 345–351.

TSAI, M.C., CHEN, M.L. & WANG, T.R. (1986). Effects of alpha-bungarotoxin and d-tubocurarine on the post-tetanic potentiations of the mouse diaphragm twitch tension. *Archs. int. Pharmacodyn*, **2255**, (in press).

VERGARA, J., TSIEN, R.Y. & DELAY, M. (1985). Inositol 1,4,5-triphosphate: a possible chemical link in excitation-contraction coupling in muscle. *Proc. natn. Acad. Sci. U.S.A.*, **82**, 6352–6356.

WRIGHT, J.M. & COLLIER, B. (1977). The effects of neomycin upon transmitter release and action. *J. Pharmac. exp. Ther.*, **200**, 576–587.

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