The Pre- and Postjunctional Components of the Neuromuscular Effect of Antibiotics

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The relative contributions of the pre- and postsynaptic components of the myoneural blocking effect of different antibiotics were studied using: (a) a radioactive method that measures selectively the Ca^{2+}-dependent, stimulation evoked, quantally released, ^{3}H-acetylcholine (^{3}H-ACh) from the mouse *in vitro* phrenic nerve-hemidiaphragm preparation without cholinesterase inhibition; (b) measurement of the force of contraction of the indirectly or directly stimulated muscle. The antibiotics studied (neomycin, polymyxin B and lincomycin), reduced the release of ^{3}H-ACh evoked by stimulation (18 trains of 40 shocks at 50 Hz) in a concentration dependent manner. While the inhibitory effect of neomycin was inversely related to $[Ca^{2+}]_o$, that of lincomycin was moderately and that of polymyxin B was not affected by increasing $[Ca^{2+}]_o$ from 0.75 to 5.0 mM. Similarly, the d-tubocurarine (d-Tc) -induced inhibition of the release of ^{3}H-ACh was independent of $[Ca^{2+}]_o$. The K-channel blocking agent, 4-aminopyridine (4-AP), enhanced the release of ACh in a concentration dependent manner and prevented the neuromuscular effect of neomycin. However, the neuromuscular effect of polymyxin B and of lincomycin was not affected by 4-AP. Atropine, enhanced the release of ^{3}H-ACh. Antibiotics, however, were still able to reduce the release of ACh when the negative muscarinic feedback mechanism of ACh release was eliminated by atropine. Our findings indicate that the antibiotics studied possess both pre - and postsynaptic effects. Presynaptically they reduce the evoked release of ACh; postsynaptically they inhibit muscle contractility. The rank order of presynaptic action is neomycin >polymyxin B >lincomycin. (Key words: neuromuscular junction, margin of safety, acetylcholine release, presynaptic effect, antibiotic, neomycin, diaphragm)

(Vizi, ES, Chaudhry IA, Goldiner PL, et al.: The pre- and postjunctional components of the neuromuscular effect of antibiotics. J Anesth 5: 1–9, 1991)

In experimental animals, antibiotics block neuromuscular transmission in a concentration dependent manner[1,2]. Furthermore, combinations of otherwise ineffective concentrations of muscle relaxants and antibiotics produce profound neuromuscular block[3,4]. In agreement with these findings severe respiratory depression was observed in surgical patients after combined administration of muscle relaxants and antibiotics[5–8,10]. The precise mechanism(s) and the site(s) of the neuromuscular effects of antibiotics are controversial.

It was reported that certain antibiotics inhibit the evoked release of acetyl-

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choline (ACh) from the motor nerve terminal[11,12]. In these experiments, however, anticholinesterases were used to prevent the hydrolysis of ACh and it is known that anticholinesterases themselves influence the release of ACh[13]. Since the neuromuscular effects of certain antibiotics can be reversed by increasing $[Ca^{2+}]_o$, it has been suggested that these antibiotics inhibit the voltage-dependent Ca-influx into the motor nerve terminal[14–16]. Consistent with these observations, it was reported[17] that neomycin, polymyxin B, oxytetracycline and clindamycin reduce the depolarization-dependent influx of Ca^{++} into rat forebrain synaptosomes. Measurement of the amplitude and decay kinetics of miniature endplate currents (mepc) and endplate currents (epc) indicated that antibiotics may also have postsynaptic effects[18]. Recently, a sensitive radioactive method was developed[19] that measures selectively the Ca_o^{2+}-dependent, stimulation-evoked, quantally released ACh from the *in vitro* mouse phrenic nerve-hemidiaphragm preparation without cholinesterase inhibition[19,20,22]. With this method, it was possible to determine the effect of different antibiotics on ACh release, which is a presynaptic event, and to investigate the Ca^{2+} dependence of this process. It appeared likely that comparison of the inhibitory effect of various antibiotics on presynaptic ACh release with their depressant effect on the development of the force of contraction during indirect and direct electrical stimulation would give useful information on the relative importance of the pre- and postsynaptic effects of these compounds. It has been recently suggested that the evoked release of ACh at the neuromuscular junction is modulated by a negative muscarinic feedback mechanism. Atropine[20,22,23] enhanced, and oxotremorine[21,22] reduced the evoked release of ACh. Therefore, the effect of antibiotics on ACh release was also measured in the presence of atropine, when the negative feedback modulation of ACh release mediated via muscarinic receptors was eliminated.

Methods and Materials

Male Swiss-Webster mice of 25–30 g body weight were exposed to halothane and after the loss of the righting reflex they were decapitated.

Effects of drugs on evoked release of ^{3}H-acetylcholine

The middle portions of both hemidiaphragms (about 2–3 mm to the right and to the left of the insertions of the phrenic nerves), together with the attached rib segments, were suspended in 1 ml organ baths. The rib end of the preparation was attached to the bottom of the bath and its tendon to an FT03 force displacement transducer. Since in human plasma the range of Ca^{++} is 0.94 to 1.33 mM[24] and its mean value is 1.11 ± 0.07 mM (mean ± SE)[25] the organ bath was filled with modified Krebs's solution (NaCl 113, KCl 4.7, $CaCl_2$ 1.4, $MgSO_4$ 0.9, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11.5 mmol·l^{-1}). In this solution Ca^{2+} was 1.1 mM. In some experiments, the concentration of $CaCl_2$ was varied from 0.75 to 5.0 mM. The bath temperature was maintained at 37°C. The pH of the solution when aerated with 95% O_2–5% CO_2 was 7.38–7.42.

Incubation

The preparations were loaded with ^{3}H-choline (5μCi·ml^{-1}) for 60 min. During this time the preparations were stimulated at 1 Hz to facilitate the incorporation of ^{3}H-choline into the ^{3}H-ACh pool of the motor nerve terminal. At the end of this period, the radioactivity of the muscles, consisting partly of ^{3}H-choline and partly of ^{3}H-ACh, was 7.54 ± 1.0 × 10^5 Bq·g^{-1}. To remove excess ^{3}H-choline the preparations were washed three times and then transferred to another bath and superfused with modified Krebs' solution at the rate of 2 ml·min^{-1} for 60 min. Subsequently, hemicholinium-3 (5 × 10^{-5} mol·litre^{-1}) was added to the solution and the perfusion rate was reduced to 0.5 ml·min^{-1}. Hemicholinium-3 was added to prevent reuptake of ^{3}H-choline.

Collection

During superfusion at the rate of 0.5 ml·min^{-1}, 3-min (1.5 ml) aliqouts of the

effluent were collected continuously with an automatic fraction collector into scintillation vials throughout the experiment. The preparations were stimulated for 3 min twice with 0.8s trains of 50 Hz, square wave, supramaximal impulses of 0.2 ms duration applied every 10s, starting at the 10th (S_1) and 52nd (S_2) min of the collection period.

In control experiments, no drug was added to the perfusing solution. In other experiments drugs were added to the perfusing solution between S_1 and S_2 at 34 min.

Calculation

In the mouse phrenic nerve – hemidiaphragm preparation, as in many other preparations[26,27] electrical stimulation increases only the release of ^{3}H-ACh while the output of ^{3}H-choline remains unchanged[21,28]. Accordingly, the evoked release of ^{3}H-ACh was calculated from the difference between the expected resting and the actual measured outflow of radioactivity caused by 3 min electrical stimulation. Resting release, assumed to follow first-order kinetics, was determined by "least square" fitting to an exponential curve of the $Bq \cdot g^{-1}$ concentrations of effluents collected before and after stimulation. The expected spontaneous outflow was obtained by interpolation between the fitted points.

The radioactivity of the samples was measured in a LKB Scintillation Beta Spectrometer. There was considerable variation from one preparation to the other in the amount of radioactivity released during corresponding stimulation periods. However, the ratios of the radioactivity, measured during the two consecutive stimulation periods, S_2/S_1, were relatively constant. For this reason the effect of antibiotics and other drugs on evoked release of ACh release was estimated from their effect on the S_2/S_1 ratio. Increase or decrease of this ratio indicates facilitation or inhibition of the evoked release of ACh, respectively.

Effect of drugs on force of contraction

In these experiments the preparations were placed in double jacketed, 100 ml organ baths, filled with modified Krebs' solution as described. The bath temperature was kept at 37°C and it was aerated with 95%O_2–5%CO_2. Under these conditions pH was 7.38–7.42. During indirect stimulation the phrenic nerves were placed on bipolar platinum electrodes immersed in the bath and the preparations were stimulated with 0.8s trains of 50 Hz supramaximal square wave impulses of 0.2 ms duration. These stimulation parameters were selected, because in mammals voluntary muscle movements are elicited by short trains of 16–60 Hz stimuli[29]. During direct stimulation, the "indirect component" of direct stimulation was eliminated by 5×10^{-6}M d-tubocurarine[30] and the hemidiaphragms were stimulated, through two platinium needle electrodes inserted 5 mm apart into the muscle. The stimulus duration was 2 ms. Otherwise the stimulation parameters were the same as during indirect stimulation. The optimal resting tension (1 to 2 g) was determined before each experiment. The force of contraction, quantitated with FTO3 transducers, was continuously recorded. The effect of antibiotics on the force of contraction during indirect and direct stimulation was determined with the cumulative dose-response method. The IC50 (concentration needed to reduce the size of contraction by 50%) and IC90 values were determined from the computer derived, best fit log dose-response regression lines.

Statistical analysis

The statistical significance of the results were determined by one-way analysis of variance of the logarithmically transformed data, followed by Dunn's test or by Student's t test as applicable. $P < 0.05$ was considered significant. Results are presented as mean values ± SEM.

Drugs

d-tubocurarine chloride, neomycin sulfate, polymyxin B, lincomycin, 4-aminopyridine and hemicholinium-3 were purchased from Sigma Chemical Co.

Results

Effect on evoked release of acetylcholine

When $[CaCl_2]_o$ was 1.4 mM the resting release of ACh was 2865 ± 332 $Bq \cdot g^{-1}$ 3 min^{-1} (n = 4). It was not affected by in-

Table 1. Effect of antibiotics on stimulation evoked ^{3}H-acetylcholine release from the neuromuscular junction

Drugs[3]/$[CaCl_2]_o$		S_2/S_1 Ratio[1] 0.75 mM$[CaCl_2]_o$	1.4 mM$[CaCl_2]_o$	5mM$[CaCl_2]_o$	Significance (p)[2] (5.0 vs 1.4)	(1.4 vs 0.75)
Control	—	0.70 ± 0.09 (10)[4]	0.78 ± 0.09 (16)	0.84 ± 0.05 (8)	> 0.05	> 0.05
Neomycin	0.1 μM	0.72 ± 0.11(4)	—	—		
	2 μM	0.36 ± 0.08* (4)	0.70 ± 0.14 (4)	—		< 0.01
	10 μM	—	0.61 ± 0.07 (4)	—		
	20 μM	0.11 ± 0.04* (4)	0.50 ± 0.05* (4)	—		< 0.01
	160 μM	—	0.18 ± 0.05* (4)	0.48 ± 0.06* (4)	< 0.01	
Lincomycin	200 μM	0.42 ± 0.08* (4)	0.62 ± 0.05 (4)	0.62 ± 0.06 (4)	> 0.05	< 0.05
	600 μM	—	0.50 ± 0.07* (3)	—		
Polymyxin B	20 μM	—	0.48 ± 0.11 (3)	—		
	50 μM	0.19 ± 0.08* (4)	0.29 ± 0.04* (4)	0.34 ± 0.08* (4)	> 0.05	> 0.05
	200 μM	—	0.17 ± 0.02* (4)	—		
d-Tubocurarine	10 μM	0.54 ± 0.04* (4)	0.41 ± 0.03* (4)	0.38 ± 0.04* (4)	> 0.05	< 0.05

[1]The release of ^{3}H-acetylcholine was measured as described in the Methods.

[2]Significant difference between the effect of antibiotics on ACh release at different $[CaCl]_o$ as indicated. One way of analysis of variance (ANOVA) was used; *indicates significant difference from control ($P > 0.05$).

[3]Drugs were added to the Krebs solution 18 min prior to second stimulation (S_2); 27 min elapsed between two stimulations. Intermittent stimulation, trains of 40 shocks (50 Hz), were applied every 10 sec, for 3 min (18 trains).

[4]Number of experiments is in bracketts.

creasing or decreasing $[Ca^{2+}]_o$. The evoked release of ^{3}H-ACh was also not influenced significantly by changing the $[CaCl_2]_o$ between 0.75 and 5.0 mM. However, when the concentration of $[CaCl_2]_o$ was reduced to 0.32 mM the release of ACh was inhibited by 76.5 ± 3.6% (n = 4). In the absence of $[CaCl_2]_o$ axonal stimulation did not release ACh. When $[CaCl_2]_o$ was 0.75 mM, concentrations of 2 to 160 μM neomycin, 50 μM polymyxin B, or 200 μM lincomycin, significantly inhibited the evoked release of ACh: S_2/S_1 ratio was reduced. When the $[CaCl_2]_o$ was increased to 1.4 or to 5.0 mM, 200 μM lincomycin did not inhibit the evoked release of ACh. Neomycin (160 μM) and polymyxin B (50 μM) significantly decreased S_2/S_1 ratio at all $[CaCl_2]_o$ studied (table 1). Although d-Tc also reduced the evoked release of ACh, its action was independent of $[CaCl_2]_o$ (table 1). 4-Aminopyridine (4-AP) significantly increased S_2/S_1 ratio of ^{3}H-ACh release. The corresponding S_2/S_1 ratios with 4,10 and 40 μM 4-AP were 0.93 ± 0.04 1.28 ± 0.08 and 1.85 ± 0.14, respectively (n = 4–4; $P < 0.01$). Whereas 4-AP antagonized the inhibition of the evoked release of ACh caused by neomycin (table 2), the effects of polymyxin B and lincomycin on ACh release were not antagonized even by 40 μM of 4-AP (data not shown).

As cholinergic nerve terminals of the neuromuscular junction are endowed with muscarinic autoreceptors[23] which inhibit the electrically-evoked release of ACh, experiments were performed to determine whether the effects of antibiotics were, at least in part, due to an activation of the autoinhibitory feedback mechanism. To achieve this, in some experiments, atropine was added to the perfusion fluid. Atropine alone, added to the perfusion fluid 18 min before the second stimulation (S_2), enhanced the evoked release of ACh as indicated by the significant increase of the S_2/S_1 ratio. The atropine-induced facilitation of the evoked release of ACh was also antagonized by antibiotics (table 3). This finding excludes the

Table 2. Effect of 4-aminopyridine (4-AP) on evoked release of ^{3}H-acetylcholine reduced by different antibiotics ($n = 4 - 4$)

Drugs[3]	S_2/S_1		Significance
	–	4 μM 4-AP	p[2]
Control	0.74 ± 0.05	0.93 ± 0.04	< 0.05
Neomycin, 40 μM	0.31 ± 0.08*	1.03 ± 0.08	< 0.01
Polymyxin, B, 50 μM	0.34 ± 0.07*	0.44 ± 0.04*	> 0.05
Lincomycin, 600 μM	0.41 ± 0.03*	0.40 ± 0.07*	> 0.05

[1]The release of ^{3}H-acetylcholine was measured as described in the Methods; $CaCl_2$ = 1.4 mM.

[2]Significant difference between the release in the presence and absence of 4-AP (4-aminopyridine).

One way of analysis of variance (ANOVA) was used. * indicates significant difference ($P < 0.05$) from control.

[3]Drugs were added to the Krebs solution 18 min prior to second stimulation (S_2).

Trains of 40 shocks (50 Hz) were delivered in every 10 sec for 3 min. Note that 4-AP (4-aminopyridine) only antagonized the neomycin induced depression of the evoked release of ACh.

Table 3. Presynaptic inhibitory effect of antibiotics on ^{3}H-acetylcholine release in the presence of atropine ($n = 4 - 4$)

Drugs[1]	S_2/S_1		Significance p[2]
1. Control	0.66 ± 0.12		
2. Atropine, 1 μM	2.53 ± 0.11	2:1	< 0.01
3. Atropine, 1 μM	0.39 ± 0.06	3:1	< 0.01
+ Neomycin, 10 μM		3:2	< 0.01
4. Atropine, 1 μM	0.86 ± 0.09	4:1	< 0.01
+ Polymyxin B, 50 μM		4:2	< 0.01
5. Atropine, 1 μM	1.15 ± 0.07	5:1	< 0.01
+ Lincomycin, 200 μM		5:2	< 0.01

[1]Drugs were added to the Krebs solution 18 min prior to second stimulation (S_2). 27 min elapsed between two stimulations. Intermittent stimulation (18 trains of 40 shocks were delivered at 50 Hz every 10 sec for 3 min).

[2]One way analysis of variance (ANOVA) was used for statistical analysis.

possibility that the effects of antibiotics are related to muscarinic feedback modulation.

Effects of antibiotics on force of contraction

All 3 antibiotics decreased the force of contraction of the preparations both during indirect and direct stimulation (table 4). The inhibitory effect was always greater during indirect than during direct stimulation. The ratio of IC50 values of the direct/indirect stimulation was higher with neomycin (2.34) than with polymyxin B (1.29) or lincomycin (1.35). The greater than 90% decrease of the force of contraction caused by neomycin

Table 4. Effect of antibiotics on the force of contraction of phrenic-nerve hemidiaphragm preparation with indirect or direct stimulation. ($n = 4 - 4$)

	IC50 (μM)				
	a Indirect[1]	b Indirect[2] (with reduced margin of safety)	c Direct[3]	c/a	a/b
Neomycin	478.2 ± 12.8	50.4 ± 2.4	1119.1 ± 18.1	2.34	9.5
Polymyxin B	32.8 ± 0.3	6.7 ± 0.2	42.5 ± 1.3	1.29	4.9
Lincomycin	1368.4 ± 896.0	332.9 ± 8.4	1846.4 ± 37.2	1.35	4.1

[1]phrenic nerve was stimulated with 50 Hz and 40 stimuli were applied in one train. 10 sec elapsed between the trains. Impulse duration, 0.2 ms.

[2]The margin of safety of neuromuscular transmission was reduced by 0.3 μM d-Tc.

[3]Stimulation parameters same as with indirect stimulation, except that the impulse duration was 2 ms and the electrodes were placed on the muscle. Neuromuscular transmission was inhibited by 10 μM d-Tc.

could not be antagonized by neostigmine but it could be completely reversed by 4 μM 4-AP. This concentration of 4-AP, however, only partially antagonized the effect of polymyxin B or lincomycin (data not shown). The depression of the force of contraction caused by the antibiotics could not be antagonized by neostigmine.

When the margin of safety of neuromuscular transmission was reduced with 0.3 μM of d-Tc, the IC_{50} of neomycin, polymyxin B and lincomycin decreased from 478.2 ± 12.8, 32.8 ± 0.28 and 1386.4 ± 89.6 to 50.4 ± 2.4, 6.7 ± 0.2 and 332.9 ± 8.4 μM, respectively (table 4).

Discussion

The relative contributions of the presynaptic and postsynaptic effects of antibiotics, which are known to possess depressant actions on neuromuscular transmission[4,8,18,31,32] have been studied.

It has been demonstrated by measurement of evoked release of ACh that all 3 antibiotics inhibit, to a variable extent, the evoked release of ACh. In previous studies, either intracellular recording techniques[18] or measurement of the contractile force of the striated muscle[4] were used to determine the site of action of antibiotics. In the neurochemical studies of the effects of antibiotics on ACh release from the neuromuscular junction[11,33], both quantally and non-quantally released ACh were measured together, and the latter, which is released in excess[34] might have masked the effect of antibiotics on the quantal portion of ACh release. In addition, in these studies physostigmine was used. This compounds causes antidromic firing, enhances the release of ACh and causes an accumulation of ACh in the synaptic cleft during repetitive stimulation of the nerve. Thus, in the presence of physostigmine it is very difficult to determine if a compound reduces the increased firing rate and subsequently the release of ACh or its effect is due to a direct effect on the motor nerve terminal. The recently developed method of measuring radiolabelled ACh release from the mouse hemidiaphragm preparation[20–22] in the absence of anticholinesterases seems to be a reliable method for the determination of presynaptic effects of antibiotics.

The findings presented (see table 1) indicate that the inhibitory effect of neomycin on the evoked release of ACh is significantly influenced by the $[Ca^{2+}]_o$. Increasing the $[Ca^{2+}]_o$, decreased the inhibitory effect of all neomycin concentrations investigated. These data are in agreement with those obtained by measuring mepcs and epcs[18]. 4-AP, a compound that blocks K^+ channels

and facilitates Ca^{2+} utilization, enhanced the release of ACh from the neuromuscular junction in a concentration-dependent manner. Similar effect of 4-AP was observed on Auerbach's plexus[34] and on the cerebral cortex[35]. Furthermore the inhibitory effect of neomycin on evoked release of ACh (table 2) and on the force of contraction can be antagonized by 4-AP[4]. Increasing $[Ca^{2+}]_o$ had little or no antagonistic effect on the polymyxin B-or lincomycin- induced inhibition of ACh release (table 3). Furthermore, 4-AP only partially antagonized the polymyxin B-induced depression of the force of contraction[4] and had no effect on the lincomycin-induced neuromuscular block.

In agreement with earlier observations[23] the release of ACh from the neuromuscular junction is also modulated by endogenous ACh via muscarinic receptors. Stimulation of these receptors by ACh present in the synaptic gap reduces subsequent release of ACh. When in this study, the negative feedback modulation was excluded with atropine, antibiotics were still able to reduce the release. This is an important observation, because patients undergoing surgery usually receive atropine. All three antibiotics, in concentrations higher than those required to depress the force of contraction, during indirect stimulation, also depressed this variable during direct stimulation of the completely curarized muscle. The ratio of the IC_{50} values of neomycin with direct and indirect stimulation was much higher than the corresponding ratios for polymyxin B or lincomycin (see table 4). This indicates that the effect of neomycin on neuromuscular transmission is relatively greater than on the inhibition of muscular contraction. This observation is substantiated by the findig that, neomycin, at all $[Ca^{2+}]_o$ is a more potent inhibitor of the evoked release of ^{3}H-ACh than the two other antibiotics (see table 1).

When d-Tc was present in the bath in an ineffective concentration and indirect stimulation was used, neomycin was 9.5 times more effective. This difference was likely due to reduction by d-Tc of the large margin of safety of ACh release at this transmission site[36,37]. This mechanism also explains why d-Tc, administered in concentration without effect on the contractile force, but occupying the majority of nicotinic receptors and thereby reducing the margin of safety to one or near to one, potentiated the effect of neomycin on responses to indirect stimulation (table 4).

Ms. Eva Gacsal is gratefully acknowledged for technical assistance and Ms. Agnes Muller for typing the manuscript.

(Received Jan. 16, 1990, accepted for publication Aug. 31, 1990)

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