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The Influence of Antibiotics on Agonist Occupation and Functional States of the Nicotinic Acetylcholine Receptor

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SUMMARY

Four distinct classes of antibiotics, the aminoglycosides, tetracyclines, lincomycin-clindamycin, and peptides, were examined in intact BC3H-1 cells for their capacity to influence the relationship between agonist binding to the cholinergic receptor and the response elicited. The most potent inhibitors were the peptides of the polymyxin-colistin class, which at submicromolar concentrations noncompetitively blocked agonist-elicited Na^+ permeability. These agents at equivalent concentrations also enhanced the apparent affinity of agonists for the receptor. Comparisons of agonist occupation of the receptor and agonist promotion of desensitization showed that the noncompetitive inhibition by peptide antibiotics occurred largely by augmenting the conversion of the receptor to a high-affinity, desensitized state. Rates of receptor desensitization were also substantially enhanced in the presence of these antibiotics. Thus, an analysis of receptor occupation and response suggests that the polymyxin antibiotics act as heterotropic allosteric effectors by promoting the conversion of the cholinergic receptor to its desensitized state. By contrast, the other antibiotics studied inhibited permeability at far higher concentrations. Although a noncompetitive component of inhibition could be identified with certain antibiotics such as neomycin, they did not appear to enhance agonist affinity. Thus, the various antibiotics differ substantially in their potencies and basic mechanisms of inhibition of the acetylcholine receptor.

INTRODUCTION

When administered in therapeutic doses, a variety of antibiotics has been found to perturb neuromuscular transmission (1, 2). Acute effects of antibiotics manifest themselves as respiratory depression or as a more widespread drug-induced myasthenic syndrome. These transient effects may be reversed by withdrawal of drug therapy but are often compounded by administration of the antibiotic in combination with other drugs interfering with neuromuscular transmission, such as local anesthetics or depolarizing antagonists. In theory, antibiotics may be inhibitory at a number of anatomical loci involved in the junctional transmission process. For example, an antibiotic might act presynaptically by inhibiting the propagated nerve action potential or by blocking neurotransmitter release. Alternatively, an antibiotic could act postjunctionally to prevent acetylcholine occupation of the receptor, to interfere with channel opening, to inhibit the muscle-propagated action potential, or to disrupt activation of the muscle contractile apparatus.

To determine the site of action of the antibiotics investigators have examined antibiotic effects on electrical or neurotransmitter-evoked muscle contractile responses (3, 4). More recently, intracellular recordings of endplate potential changes have added further insight into inhibitory effects of antibiotics (5, 6). Previous studies have often examined antibiotic actions on the neuromuscular junction by comparison with agents of defined pharmacological activity. Such studies are important for assigning effects of antibiotics in a physiological context. However, the complexity of the intact neuromuscular junction often precludes direct quantitation of antibiotic effects on individual steps in the junctional transmission process. An alternative approach to delineating the inhibitory action of antibiotics is through use of specialized systems where specific events of neuromuscular transmission can be examined *in situ*.

In the present study, we have used the BC3H-1 clonal muscle cell line to evaluate the effects of representative antibiotics on functional responses of the nicotinic acetylcholine receptor. These cells grow in monolayer cultures, and elaborate high densities of uniformly distibuted surface AchR,[1] permitting simultaneous measurements of ligand occupation and receptor functional responsiveness in a homogeneous population of intact cells. Ligand occupation is measured by competition with the initial rate of cobra [^{125}I]α-toxin binding, and the ion permeability response is determined from the initial rate

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[1] The abbreviation used is: AchR, acetylcholine receptor.

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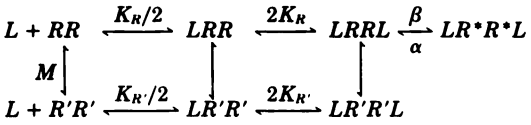
of agonist-stimulated $^{22}Na^+$ influx (7) by a modification of procedures described by Catterall (8).

The kinetic behavior of the nicotinic AchR has been extensively studied (7, 9, 10). As indicated in Scheme 1, AchR exists in equilibrium between at least three states: resting (R), activated (R^*), and desensitized (R').



SCHEME 1

It is the changes in the population distribution of receptor molecules between these states which govern the functional properties of the receptor. In the absence of prior exposure to agonist, the receptor exists predominantly in an activatable state (R) exhibiting a comparatively low agonist affinity. Addition of agonist to the receptor protein results in a rapid association and activation of receptor-mediated ion permeability (via the R^* state) followed by a slow decline in permeability response as receptor is converted to a desensitized state (R'). This state possesses increased agonist binding affinity but abolished responsiveness. Thus, agonists bind to receptor, activate an associated ion channel, and concomitantly but more slowly convert the receptor to the desensitized state. The preference of agonist for binding to desensitized receptor is the driving force underlying the transition to the desensitized state (7, 9).

Agents which perturb receptor functional responses may do so by competitive or noncompetitive mechanisms. Classical antagonists inhibit agonist-stimulated ion fluxes by direct competition at the agonist binding site, but exhibit little or no preference for R' versus R state, and do not effect transitions between receptor states. A large and heterogeneous class of inhibitors noncompetitively inhibits activation of AchR by agonists. Although they may act at multiple loci on or around the receptor complex, most of these noncompetitive blockers enhance the conversion of receptor to the desensitized state (10–12). Typical of such agents are the local anesthetics, general anesthetics, and certain toxins such as histrionicotoxin. In order to characterize postjunctional mechanisms of antibiotic inhibition of the receptor, we have employed BC3H-1 cells to examine the influence of antibiotics on agonist occupation of the receptor and the elicited permeability response. Some of these results have been presented previously in abstract form (13).

EXPERIMENTAL PROCEDURES

Materials. Dulbecco's modified Eagle's medium, Ham's nutrient mixture (F-12), fetal calf serum, and horse serum were obtained from GIBCO (Grand Island, N. Y.). Radionuclides $^{22}Na^+$ and ^{125}I were obtained carrier-free from New England Nuclear Corporation (Boston, Mass.). Cobra α-toxin was prepared from *Naja naja siamensis* venom (Miami Serpentarium, Miami, Fla.), ^{125}I-iodinated, and the mono-iodo conjugate purified as described previously (14). *d*-Tubocurarine chloride was a generous gift from Eli Lilly and Company (Indianapolis, Ind.). Octapeptin (EM 49) was kindly provided by Squibb Institute for Medical Research (Princeton, N. J.). Clindamycin HCl was a gift from The Upjohn Company (Kalamazoo, Mich.). Polymyxin B sulfate, polymyxin E (colistin)methanesulfonate, streptomycin sulfate, neomycin sulfate, gentamicin sulfate, tetracycline hydrochloride, lincomycin hydrochloride, and bovine serum albumin were obtained from Sigma Chemical Company (St. Louis, Mo.). All other chemicals were reagent-grade.

Cell culture. Propagation of the BC3H-1 cell line was accomplished as described (7). Briefly stated, experimental cultures grown in 35-mm Petri dishes were seeded at a density of 8.3×10^3 cells/ml in 2 ml of medium containing Dulbecco's modified Eagle's medium/F-12, 3:1, supplemented with 8% fetal calf serum and 2% horse serum. The growth medium was replaced at days 4 and 11, and experiments were usually performed at days 13–14.

Assays of agonist occupation and the agonist-stimulated permeability response. Kinetic assays to measure ligand competition with the initial rate of [^{125}I]α-toxin binding or with the initial rate of carbamylcholine-stimulated $^{22}Na^+$ influx were performed essentially as described (7, 15) under the following conditions: sets of 15–25 culture dishes were equilibrated at room temperature and atmosphere in growth medium for 30 min. Subsequent manipulations were performed in series at 22° so that each culture received equivalent handling. Sets of cultures were washed twice with 2-ml additions of depolarizing assay buffer and allowed to equilibrate with the second buffer addition for an additional 10 min. Following this initial regimen, cultures were exposed for 20–25 min with 750 μl of depolarizing buffer containing added ligands at indicated concentrations. Rate assays were initiated by aspirating the conditioning buffer and applying a fresh 750-μl aliquot of depolarizing buffer containing requisite ligands plus either [^{125}I]α-toxin (15–25 nM) or tracer quantities of $^{22}Na^+$ (0.5–1.0 μCi/ml). Reaction was allowed to proceed for 20 sec; the assay solution then was removed by aspiration and the monolayer was rapidly washed with either four 3-ml aliquots of depolarizing buffer ([^{125}I]α-toxin competition) or two 3-ml aliquots of depolarizing buffer containing 500 μM *d*-tubocurarine chloride followed by two 3-ml aliquots of depolarizing buffer with no further additions (agonist-stimulated $^{22}Na^+$ influx). Cultures were solubilized and counted, and the data were analyzed as previously described. The composition of depolarizing buffer was as follows: 5.4 mM NaCl, 140 mM KCl, 1.8 mM $CaCl_2$, 1.7 mM $MgSO_4$, 5.5 mM glucose, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), and bovine serum albumin (0.06 mg/ml) (7). For experiments involving tetracycline hydrochloride, the depolarizing buffer was modified by substitution of $MgCl_2$ for $MgSO_4$, and omission of NaH_2PO_4 in order to avoid precipitation. Curve fitting was performed on a Tektronix Model 4052 minicomputer according to the iterative nonlinear least-squares procedure of Marquardt (16).

RESULTS

Concentration dependence for antibiotic inhibition of initial rates of [^{125}I]α-toxin binding and agonist-stimulated $^{22}Na^+$ permeability. Since the classical agonists and

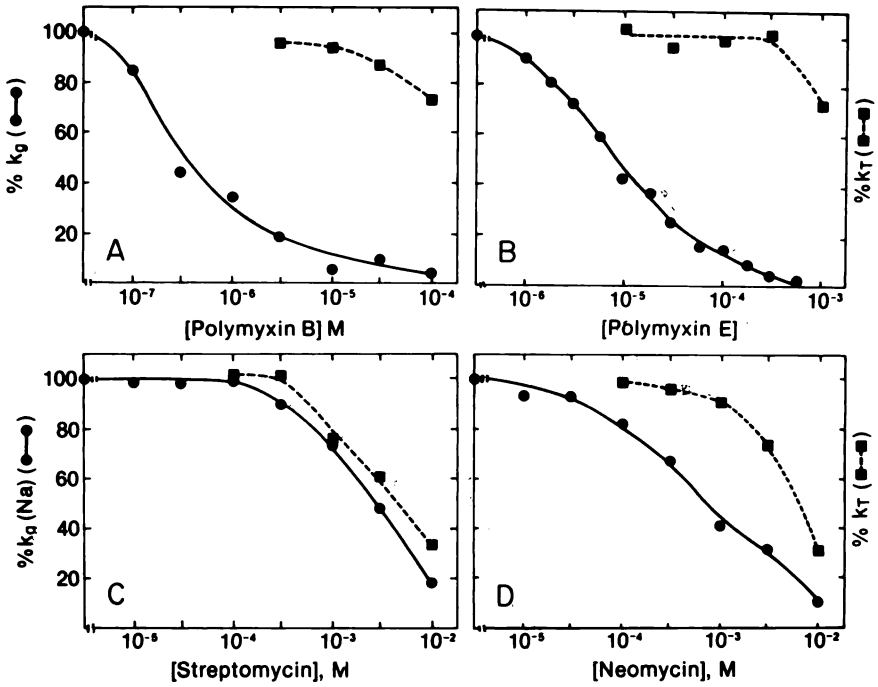


FIG. 1. *Concentration-dependent antibiotic inhibition of initial rates of cobra [^{125}I]α-toxin binding and carbamylcholine-stimulated $^{22}Na^+$ influx in BC3H-1 cells*

Monolayer cultures of BC3H-1 cells were equilibrated with specified concentrations of polymyxin B (A), polymyxin E (B), streptomycin (C), or neomycin (D) for 20 min. Following this interval, the conditioning solution was replaced by a solution containing the same concentration of antibiotic plus a fixed concentration of either [^{125}I]α-toxin (10–20 nM) or carbamylcholine plus $^{22}Na^+$ (100 μM carbamylcholine and $^{22}Na^+$, 0.5–1.0 μCi/ml). The initial rates of toxin binding or carbamylcholine-stimulated $^{22}Na^+$ influx were measured over a 20-sec interval. Results are expressed as percentage of control rate in the absence of antibiotic. ●—●, Antibiotic inhibition of carbamylcholine-stimulated $^{22}Na^+$ influx; ■- - -■, antibiotic inhibition of initial rate of [^{125}I]α-toxin binding.

antagonists competitively inhibit the initial rate of α-toxin binding to the receptor (14, 17), antibiotics were initially tested for their concentration-dependent inhibition of the initial rate of [^{125}I]α-toxin binding to the receptor. Second, we examined their concentration dependences for inhibition of agonist-stimulated $^{22}Na^+$ permeability. Data for four representative agents are shown in Fig. 1, and measured protection constants are summarized in Table 1. The observed rank order of potency for antibiotic block of agonist-elicited permeability was polymyxin B > octapeptin > polymyxin E > clindamycin ≫ tetracycline > neomycin > gentamicin > streptomycin > lincomycin. Comparison of antibiotic potency in inhibiting the initial rate of α-toxin binding versus the capacity to inhibit agonist-stimulated Na^+ permeability revealed several distinctions. The polymyxin derivatives showed the highest potencies in blocking agonist-stimulated $^{22}Na^+$ permeability, whereas direct blockade of the agonist/antagonist site monitored by α-toxin binding occurred at far higher concentrations (Fig. 1A and B). This separation of effective concentration ranges for inhibition of permeability response versus α-toxin binding implied that the polymyxins exerted their effects by a noncompetitive mechanism. Clindamycin exhibited rather similar behavior. On the other hand, the aminoglycosides streptomycin and gentamicin inhibited the initial rates of α-toxin binding and $^{22}Na^+$ permeability over very similar concentration ranges, consistent with a more direct competitive inhibition of receptor responses (Fig. 1C). Neomycin (Fig. 1D) typified an intermediate case, inhibiting binding and response over partially overlapping concentrations. Indeed, the inhibition of permeability by neomycin revealed two apparent affinities, with the lower affinity component corresponding to the concentration range for direct neomycin competition with α-toxin binding. Lincomycin and tetracycline also showed partially overlapping concentration dependencies for blockade of the permeability response and α-toxin binding. None of the antibiotics tested showed any stimulation of $^{22}Na^+$ influx in the absence of agonist.

Antibiotic inhibition of receptor activation as a func-

TABLE 1

Protection constants for antibiotic inhibition of initial rates of carbamylcholine-stimulated $^{22}Na^+$ influx and [^{125}I]α-toxin binding

Inhibition data were obtained as described under Experimental Procedures and in Fig. 1. Data were analyzed as described in ref. 7.

Compound	K_p ($^{22}Na^+$ influx)	K_p [^{125}I]α-toxin)
Polymyxin B	3.15×10^{-7}	$>10^{-4}$
EM 49 (octapeptin)	4.62×10^{-7}	$>10^{-4}$
Polymyxin E (colistin)	9.16×10^{-6}	$>10^{-3}$
Neomycin	7.93×10^{-4}	5.76×10^{-3}
Streptomycin	2.53×10^{-3}	4.72×10^{-3}
Gentamicin	2.03×10^{-3}	4.72×10^{-3}
Lincomycin	4.33×10^{-3}	$>10^{-2}$
Clindamycin	2.23×10^{-5}	$>10^{-2}$
Tetracycline	6.75×10^{-4}	$>10^{-3}$

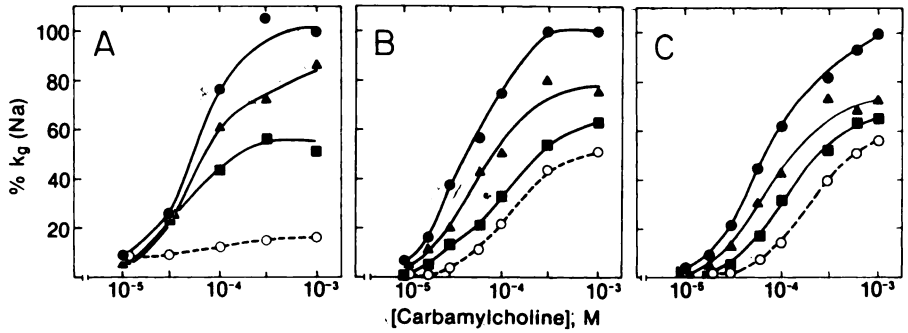


FIG. 2. *Antibiotic inhibition of concentration-dependent carbamylcholine activation of receptor permeability response*

Sets of cultures were equilibrated with fixed concentrations of antibiotics, following which the $^{22}Na^+$ permeability response to the specified concentrations of carbamylcholine in the presence of the conditioning concentrations of antibiotic was measured over a 20-sec interval. Results are expressed as percentage of maximal rate in the absence of antibiotic.

A. Polymyxin B: ●——●, no added polymyxin B; ▲——▲, 10^{-7} M; ■——■, 3×10^{-7} M; ○- - -○, 3×10^{-6} M.

B. Neomycin: ●——●, no added neomycin; ▲——▲, 10^{-4} M; ■——■, 6×10^{-4} M; ○- - -○, 3×10^{-3} M.

C. Streptomycin: ●——●, no added streptomycin; ▲——▲, 10^{-3} M; ■——■, 3×10^{-3} M; ○- - -○, 10^{-2} M.

tion of agonist concentration. To examine further the question of whether antibiotic inhibition of the receptor response was competitive or noncompetitive with agonist, the representative drugs polymyxin B, streptomycin, and neomycin were employed. The receptor was exposed to fixed concentrations of antibiotic, and the concentration dependence of carbamylcholine activation of $^{22}Na^+$ permeability was measured. The data shown in Fig. 2 were quantitatively fit to Eq. 1:

$$k_g = k_{g(\max)}\left(\frac{L}{L + K_{act}}\right)^2 \qquad (1)$$

where k_g is the observed rate of carbamylcholine-stimulated $^{22}Na^+$ influx, $k_{g(\max)}$ is the maximal $^{22}Na^+$ influx rate obtainable at saturating carbamylcholine concentrations, L is the carbamylcholine concentration, and K_{act} is the carbamylcholine concentration producing half-maximal $^{22}Na^+$ influx activation. The best-fit estimates for $k_{g(\max)}$ and K_{act} are shown in Table 2.

TABLE 2

Effects of antibiotics on activation parameters of carbamylcholine-stimulated $^{22}Na^+$ permeability

Data shown in Fig. 2 were fit according to Eq. 1 by nonlinear regression analysis (16). The more rapid desensitization seen at higher agonist concentrations may reduce the observed Hill coefficient, which will in turn introduce uncertainty into the values obtained for $k_{g(\max)}$ K_{act}. The values are means ± standard error.

Antibiotic	$k_{g(\max)}$	K_{act}
		μM
Polymyxin B		
Control	0.771 ± 0.054	25.6 ± 6.3
10^{-7} M	0.602 ± 0.024	24.7 ± 3.5
3×10^{-7} M	0.383 ± 0.021	15.4 ± 3.3
3×10^{-6} M	0.100 ± 0.007	4.2 ± 1.5
Neomycin		
Control	0.688 ± 0.026	23.6 ± 2.6
10^{-4} M	0.536 ± 0.028	28.4 ± 4.1
6×10^{-4} M	0.430 ± 0.012	45.0 ± 3.2
3×10^{-3} M	0.381 ± 0.022	70.0 ± 9.6
Streptomycin		
Control	0.639 ± 0.019	30.2 ± 2.9
10^{-3}	0.502 ± 0.025	34.9 ± 5.4
3×10^{-3} M	0.452 ± 0.019	49.9 ± 6.2
10^{-2} M	0.434 ± 0.013	107.5 ± 8.3

As seen in Fig. 2A and Table 2, the pattern of inhibition produced by polymyxin B was consistent with that expected for noncompetitive blockade; namely, increasing concentrations of polymyxin B progressively depressed the maximal agonist-elicited permeability response without increasing the agonist concentration required for half-maximal activation.

The aminoglycoside antibiotics streptomycin and neomycin displayed mixed competitive and noncompetitive inhibition, with the noncompetitive component appearing larger in the case of neomycin (Fig. 2B; Table 2). Low concentrations of neomycin depressed the maximal obtainable permeability response, whereas higher neomycin concentrations also displaced the concentration dependence of agonist-elicited permeability toward higher carbamylcholine concentrations. Similar analysis of streptomycin inhibition (Fig. 2C; Table 2) revealed predominantly competitive inhibition resulting in increased agonist concentrations required for half-maximal activation.

Influence of antibiotics on the affinity of agonist binding to the receptor. Prolonged exposure to agonist converts the AchR to a state possessing increased agonist binding affinity but abolished functional responsiveness. Most noncompetitive inhibitors such as the local and general anesthetics augment the rate and extent of agonist-induced conversion of receptor to its high-affinity state (11, 18, 19), and it has been possible to correlate the saturation functions for agonist binding with the state functions for receptor desensitization in the presence of noncompetitive inhibitors (12). It was therefore of interest to examine whether antibiotics previously shown to exhibit noncompetitive inhibition of receptor function could also convert receptor to the state of high affinity for agonists. BC3H-1 cultures were equilibrated with fixed concentrations of polymyxin B or neomycin plus increasing concentrations of agonist. Following this exposure, the initial rate of α-toxin binding was measured in the presence of the specified concentrations of antibiotic plus agonist. Results are shown in Fig. 3. Concentrations of polymyxin B which effectively blocked ion permeability responses produced an increase in the ap-

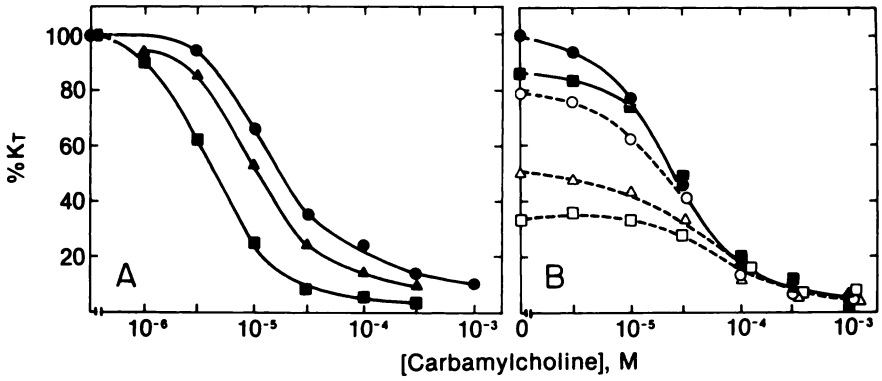


FIG. 3. *Effects of antibiotics on the concentration dependence of agonist-occupation of the receptor*

Sets of BC3H-1 cultures were equilibrated with fixed concentrations of antibiotic plus the specified concentrations of carbamylcholine for 20 min. Following this interval, the conditioning solution was replaced by a fresh solution containing the same concentrations of antibiotic and carbamylcholine, plus a fixed concentration of [^{125}I]α-toxin. The binding of toxin to AchR was measured over a 20-sec interval. The concentration dependence of carbamylcholine inhibition of toxin binding rate reflects the affinity of the agonist for AchR. Results are expressed as percentage of toxin binding rate in the absence of added carbamylcholine or antibiotic.

A. Polymyxin B: ●——●, no added polymyxin B; ▲——▲, 3×10^{-7} M; ■——■, 3×10^{-6} M.

B. Neomycin: ●——●, no added neomycin ■——■, 3×10^{-4} M; ○- - -○, 10^{-3} M; △- - -△, 3×10^{-3} M; □- - -□, 10^{-2} M.

parent affinity of agonist binding to receptor, as measured by carbamylcholine competition with initial rates of [^{125}I]α-toxin binding (Fig. 3A). The promotion of agonist binding to receptor occurred at polymyxin B concentrations which did not in themselves alter the initial rate of α-toxin binding. Virtually identical behavior was observed with the analogue polymyxin E, whereas no change in the concentration dependence of carbamylcholine occupation was seen upon exposure to neomycin (Fig. 3B). Thus, in concentrations which effectively blocked agonist-elicited permeability, neomycin produced no alteration in agonist binding affinity upon equilibrium exposure to AchR. Higher neomycin concentrations resulted in a diminution of the initial rate of α-toxin binding in the absence of agonist, reflecting direct competition between neomycin and α-toxin at these concentrations. Neomycin therefore did not enhance the conversion of receptor toward a state, R', of increased agonist binding affinity.

Influence of antibiotics on desensitization of the receptor. The influence of polymyxin B and neomycin on the concentration dependence of agonist-elicited desensitization of the permeability response was examined in a fashion similar to the corresponding binding experiments (Fig. 4). Receptor was exposed to the antibiotic plus specified agonist concentrations in order for desensitization to occur. After a sufficient exposure period to allow equilibration, functional responsiveness was then tested in the presence of the same antibiotic concentration plus carbamylcholine at a maximally effective concentration. Polymyxin B (Fig. 4A) enhanced carbamylcholine desensitization of the permeability response in a fashion similar to the enhancement of carbamylcholine binding to the receptor (cf. Fig. 3A). That is, the concentration dependences for conversion to the high-affinity state and for desensitization of the permeability response shifted by similar increments to lower carbamylcholine concentration. The conversions in state occurred at the polymyxin B concentrations effective in blocking the agonist-stimulated permeability response (Fig. 1). The effects of neomycin on desensitization were also consistent with those previously observed. Neomycin depressed

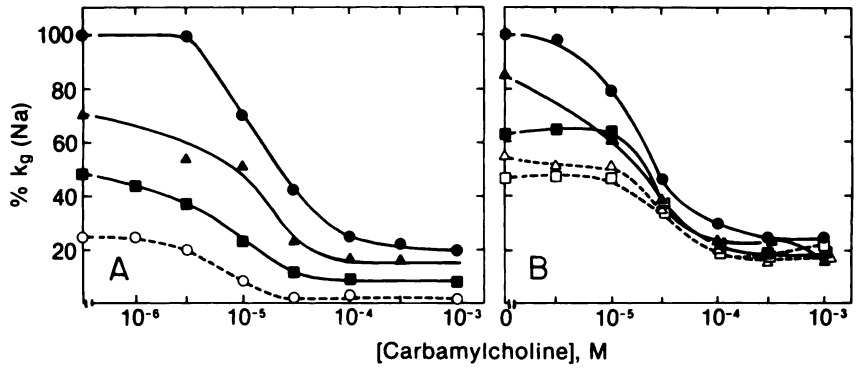


FIG. 4. *Effects of antibiotics on the concentration dependence of agonist-induced desensitization of receptor responsiveness*

BC3H-1 cultures were equilibrated with fixed concentrations of antibiotic plus the specified concentrations of carbamylcholine. Following the conditioning interval, the initial rate of $^{22}Na^+$ influx was measured in the presence of the conditioning concentration of antibiotic plus a maximally stimulating concentration of carbamylcholine (1 mM). Results are expressed as percentage of maximal rate of carbamylcholine-stimulated $^{22}Na^+$ influx in the absence of preconditioning carbamylcholine or antibiotic.

A. Polymyxin B: ●——●, no added polymyxin B; ▲——▲, 10^{-7} M; ■——■, 3×10^{-7} M; ○- - -○, 10^{-6} M.

B. Neomycin: ●——●, no added neomycin; ▲——▲, 10^{-4} M; ■——■, 3×10^{-4} M; △- - -△, 10^{-3} M; □- - -□, 3×10^{-3} M.

the agonist-stimulated permeability response in accordance with data shown in Fig. 1, but produced no effect on the concentration dependence of agonist desensitization of receptor (Fig. 4B).

Effect of polymyxin B on the rate of conversion of the receptor to a high-affinity state. Previous data have shown that polymyxin antibiotics enhance the net conversion of AchR to a state which possesses increased agonist binding affinity but is refractory to activation by agonists. The effects of polymyxin B on the onset kinetics of this state transition were also examined (Fig. 5). BC3H-1 cells were exposed to a subsaturating concentration of carbamylcholine (30 μM) in the presence or absence of polymyxin B (1.0 μM) for the indicated times. The initial rate of [^{125}I]α-toxin binding was then measured over a 15-sec interval in the presence of the same polymyxin and carbamylcholine concentrations used for the exposure period prior to adding α-toxin. Rates were expressed relative to the rate of toxin binding observed upon exposure of receptor to α-toxin plus 30 μM carbamylcholine. The *inset* to Fig. 5 shows the fractional approach of AchR to its equilibrium desensitized state plotted on an exponential scale. The biphasic character of desensitization onset has been noted previously in BC3H-1 cells (15). As seen in the *upper curve* (●), increasing the duration of prior exposure to carbamylcholine led to enhanced ability of the agonist to compete with the initial rate of toxin binding, indicating fractional agonist-induced conversion of AchR to its high-affinity, desensitized state. The inclusion of 1 μM polymyxin B in the conditioning solution (○) enhanced agonist competition with toxin binding rate at the earliest measurable exposure times. This result indicates that polymyxin B significantly accelerated the rate of conversion of AchR to the high-affinity, desensitized state. At this concentration, polymyxin B alone had no effect on the initial rate of α-toxin binding (Fig. 1). In accord with previous data (Fig. 3), the extent of equilibrium conversion of AchR to its desensitized state was also enhanced by polymyxin B.

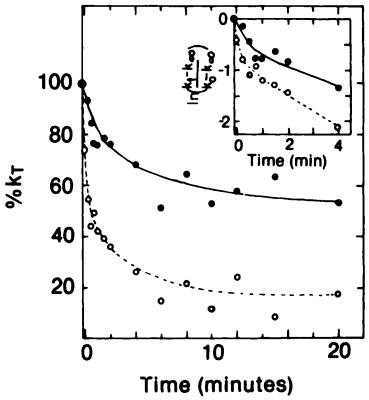


FIG. 5. *Effect of polymyxin B on the rate of conversion in state of AchR*

Cultures were exposed for the specified times to 30 μM carbamylcholine in the presence or absence of 10^{-6} M polymyxin B. Following conditioning, the initial rate of [^{125}I]α-toxin binding (15-sec interval) was measured in the presence of the conditioning concentrations of carbamylcholine plus or minus polymyxin B, as noted. Results are expressed as percentage of the rate of [^{125}I]α-toxin binding upon simultaneous addition of carbamylcholine plus α-toxin in the absence of polymyxin B. ●——● Exposure to 30 μM carbamylcholine; ○- - -○, exposure to 30 μM carbamylcholine plus 10^{-6} M polymyxin B.

Inset. Exponential approach to equilibrium desensitization of receptor. Data were expressed as indicated on the ordinate. k_0 was the initial rate of α-toxin binding obtained when carbamylcholine and α-toxin were added simultaneously, as described above. k_t was the rate of α-toxin binding observed following specified times of exposure of AchR to carbamylcholine in the absence (●——●) or presence (○- - -○) of polymyxin B. k_∞ was the rate of α-toxin binding obtained following equilibrium exposure of AchR to carbamylcholine in the absence or presence of polymyxin B, as appropriate.

DISCUSSION

Antibiotics known to inhibit neuromuscular transmission *in vivo* have been examined to ascertain how they influence the coupling between agonist occupation and functional state of the acetylcholine receptor in a cultured mammalian muscle cell line. This simplified system avoids many of the complexities of *in vitro* preparations, and provides a means to assess specific mechanisms of postjunctional antibiotic action in an intact muscle cell. The order of potency observed for antibiotic blockade of the functional response in this system (polymyxin B > polymyxin E > clindamycin ≫ tetracycline > neomycin > gentamicin > streptomycin > lincomycin) compares well with the order of antibiotic potency for inhibition of nerve-stimulated muscle twitch response in the phrenic nerve-hemidiaphragm preparation (polymyxin B > polymyxin E > neomycin > gentamicin > streptomycin > lincomycin > tetracycline) (3). Effective concentrations for blockade of AchR response by aminoglycosides, tetracycline, or lincomycin determined in the present study were generally similar to concentration ranges determined in previous studies, with half-maximal response inhibition occurring at 1–10 mM antibiotic (3, 4, 6). By contrast, the polymyxin and octapeptin derivatives exhibited increased potencies in perturbing AchR function in the BC3H-1 system as compared with potencies obtained by monitoring antibiotic inhibition of muscle responses to supramaximal presynaptic stimulation ($K_p \backsim$ 1 μM versus 70–100 μM, respectively) (3, 4). This difference likely reflects, in part, a safety factor or receptor reserve intrinsic to neuromuscular transmission *in situ*.

Mechanisms of receptor desensitization by representative peptide and aminoglycoside antibiotics were examined in further detail. The peptide antibiotics acted as potent heterotropic allosteric inhibitors, whereas the aminoglycosides were less potent and exerted multiple modes of inhibition.

The polymyxin and octapeptin antibiotics were noncompetitive inhibitors of agonist-mediated permeability responses and simultaneously enhanced the conversion of the receptor to its high-affinity desensitized state, R'. We have shown previously that the slow conversions of acetylcholine receptor state in BC3H-1 cells can be described in terms of a two-state cyclic scheme (7) (cf. Scheme 1).[2] In this scheme the allosteric constant, M, which describes the ratio of receptor in the two states (e.g., R'/R), is 1.0×10^{-4}, and the dissociation constants for the two states are $K_R = 6.0 \times 10^{-5}$ and $K_{R'} = 2 \times 10^{-7}$

[2] The low-affinity, activatable state likely reflects a composite of more rapidly equilibrating receptor states (7, 12). The open-channel state is likely to be a dominant species, since $\alpha/\beta < 1$. Also, desensitization often exhibits a rapid component which is followed by a slower step (20).

(12). Thus in the absence of agonist the receptor is predominantly in the low-affinity activatable state, and it is the 300-fold higher affinity of the R' state for agonist which promotes the state transition (7, 9). Both the saturation function for carbamylcholine binding to receptor and the state function for carbamylcholine desensitization of receptor can be described by the same three constants: K_R, $K_{R'}$, and M. For a receptor functioning as a cooperative dimer (Scheme 1), the saturation function, $\bar{Y}$, and the state function for desensitization, $\bar{R}$, are described by the following equations:

$$\bar{Y} = \frac{LRR + LRRL + LR'R' + LR'R'L}{\Sigma R} = \frac{L/K_R(1 + L/K_R) + ML/K_{R'}(1 + L/K_{R'})}{(1 + L/K_R)^2 + M(1 + L/K_{R'})^2} \quad (2)$$

$$\bar{R} = \frac{R'R' + LR'R' + LR'R'L}{\Sigma R} = \frac{(1 + L/K_R)^2}{(1+ L/K_R)^2 + M(1 + L/K_{R'})^2} \quad (3)$$

If polymyxin behaves as a heterotropic ligand affecting the allosteric constant, M, then the constant in the presence of local anesthetic, M', will be described by (cf. ref. 21)

$$M' = M\left(\frac{1 + [H]/K_{H'}}{1 + [H]/K_H}\right)^n \quad (4)$$

where $[H]$ is the concentration of heterotropic inhibitor, K_H and $K_{H'}$ are its dissociation constants for the R and R' states, and n represents the number of sites for antibiotic binding. An increase in M' caused by polymyxin, reflecting a redistribution of the receptor population toward the R' state, predicts a shift in the carbamylcholine binding function to lower agonist concentrations, and a similar shift in the state function for desensitization. Carbamylcholine binding functions obtained in the presence of increasing polymyxin B concentrations as described (cf. Fig. 3) were fit according to Eq. 2 by assigning $K_R = 6 \times 10^{-5}$ M, $K_{R'} = 2 \times 10^{-7}$ M, and allowing M to vary. As shown in Table 3, the shift in concentration dependence for carbamylcholine occupation of the receptor produced by polymyxin B can be accounted for solely by increasing M. A comparison of the concentration dependence for carbamylcholine occupation of the receptor with that of carbamylcholine production of desensitization emerges from the respective dissociation constants, K_p in Fig. 3 and K_{des} in Fig. 4. These constants are also compiled in Table 3. Both show comparable shifts to lower agonist concentration, as would be predicted if the antibiotic were to increase M. However, a complete quantitative description of Eq. 3 is not possible since polymyxin also enhances the rate of desensitization (Fig. 5) and considerable desensitization of the permeability response can be expected to occur in the 20-sec assay interval. Enhanced rates of desensitization would be reflected in the permeability estimates, k_{Go}, found when the receptor is not exposed to carbamylcholine prior to measuring carbamylcholine-elicited permeability in the presence of polymyxin. These values are given by the ordinate intercepts in Fig. 4. Also, a reduction in permeability without prior exposure to agonist, k_{Go}, will arise from other noncompetitive modes of inhibition; for example, direct interference of the channel conductance properties may be intrinsic to the action of some of the antibiotics. Measurements made in the time scale of manual application and removal of agonist cannot distinguish between these two possibilities.

The quantitative behavior of polymyxin in affecting both agonist occupation and desensitization resembles that found for representative local anesthetics and histrionicotoxin in BC3H-1 cells (12), and is consistent with polymyxin B acting as a potent and specific heterotropic allosteric inhibitor. A number of studies have shown previously that local anesthetics and histrionicotoxin will enhance the binding of agonists (18, 22–25) and promote desensitization (25, 26). The enhanced conversion of AchR to its desensitized state by polymyxin reflects preferential interaction of the antibiotic with the R' state of receptor. Local anesthetic-like actions for the polymyxin antibiotics in other systems have previously been suggested (27).

The capacity of polymyxin to function as an allosteric inhibitor in a manner similar to the local anesthetics is likely to be of importance to the understanding of transitions between functional states of the receptor. Polymyxin's potency as an allosteric inhibitor slightly exceeds that of histrionicotoxin and the more potent local anesthetics (11, 12). However, the physical properties of polymyxin differ substantially from those of the local anesthetics. The latter are small, nonpolar, tertiary or quaternary amines whereas the polymyxins are polar, basic compounds possessing a cationic peptide head esterified to a 7–8 carbon fatty acid tail. Studies on bacterial membranes have shown that these peptides act as general membrane perturbants (28) where the fatty acid tail is inserted into the bilayer and the polycationic cyclic peptide exists between the more polar entities of the phospholipid. Selectivity for membranes or membrane

TABLE 3

Effects of polymyxin B on equilibrium agonist occupation and desensitization parameters

$K_{p(equil)}$ was determined from data obtained in the equilibrium agonist binding experiment described in Fig. 3. Data were analyzed as described in ref. 7. K_{des} was determined from data obtained in the equilibrium agonist desensitization experiment described in Fig. 4. Data were analyzed as described in ref. 7. M was determined by fitting the data for agonist binding at equilibrium to Eq. 2 using $K_R = 6 \times 10^{-5}$ M and $K_{R'} = 2 \times 10^{-7}$ M (12). In this case, if $\bar{Y}$ is the equilibrium occupation function for agonist, then $(1 - \bar{Y})$ is the fractional inhibition of initial rate of [^{125}I]α-toxin binding observed experimentally.

[Polymyxin B]	$K_{p(equil)}$	K_{des}	M	M'/M_0
M	*M*	*M*		
0	1.69×10^{-5}	1.42×10^{-5}	1.11×10^{-4}	1.00
10^{-7}	1.77×10^{-5}	1.14×10^{-5}	1.30×10^{-4}	1.17
3×10^{-7}	1.14×10^{-5}	6.24×10^{-5}	1.65×10^{-4}	1.49
10^{-6}	6.82×10^{-6}	6.09×10^{-5}	4.27×10^{-4}	3.85
3×10^{-6}	3.75×10^{-6}	3.55×10^{-5}	1.26×10^{-3}	11.35

domains rich in anionic lipids has been noted (29). Reported effects of the polymyxins in perturbing neuronal membranes occur at concentrations 2–3 orders of magnitude higher than we observed in this study (30). The striking sensitivity of the receptor to polymyxin may reflect a more specific mode of interaction (perhaps at a lipid-receptor interface) and on the propensity of the receptor to convert to a high-affinity state with selective changes in local membrane structure.

The differences between peptide antibiotics and local anesthetics with respect to chemical structure and possible membrane interactions suggest that more than a single site of association may exist for allosteric effectors of receptor function. The sites of interaction might be examined by direct studies. For example, radiolabeled and fluorescent local anesthetics have been employed to uncover their sites of interaction (18, 31), and fluorescent polymyxin analogues which retain activity have been synthesized (32).

The aminoglycoside antibiotics revealed lower potencies and more heterogeneous actions on AchR than the peptide antibiotics. Streptomycin inhibited receptor activation in a largely competitive fashion, whereas apparent noncompetitive inhibition of receptor response dominated the effects of neomycin. Interestingly, although neomycin acted as a noncompetitive inhibitor of the permeability response, it did not affect the state transition behavior of AchR as measured by agonist occupation and desensitization functions. This result indicates that, unlike other noncompetitive inhibitors studied on the acetylcholine receptor in BC3H-1 cells, neomycin evinced no preference for receptor in the desensitized state.

Previous work has established that aminoglycosides act presynaptically to inhibit Ca^{2+}-mediated acetylcholine release as well as postsynaptically to reduce muscle responsiveness to nerve stimulation (3). The present study shows that postjunctional effects of these drugs may be due in part to inhibition of AchR responsiveness, and that individual antibiotics may display varying proportions of competitive versus noncompetitive inhibitory actions. The aminoglycosides are polybasic, highly polar molecules. They appear to act with rather weak, but nearly equivalent affinities at multiple loci in the neuromuscular junction, and their effects may be partly or totally reversed by elevated Ca^{2+} concentrations. Taken together, these results suggest that aminoglycosides act by a generalized ionic interaction with charged protein and phospholipid residues on the surfaces of junctional membranes. Effects on AchR presumably could also occur by this mode of action.

Clinically effective doses of aminoglycoside (33–35), tetracycline (36), and lincomycin-clindamycin (37) antibiotics typically produce plasma antibiotic concentrations in the range of 1–50 μM, whereas concentrations of these antibiotics in the millimolar range are required to inhibit neuromuscular transmission *in vivo* (3, 4, 6). Inhibition of AchR by these antibiotics in the BC3H-1 system also occurs at considerably higher concentrations except in the case of clindamycin. These results suggest that AchR inhibition by these agents *in vivo* would occur only under conditions of elevated plasma antibiotic concentrations or impaired junctional transmission. Polymyxin antibiotics are clinically effective at plasma concentrations of 1–5 μM (38–40). These concentrations are very similar to those observed for inhibition of AchR responses in BC3H-1 cells. Therefore, compromised neuromuscular transmission seen clinically could be a direct consequence of enhanced receptor desensitization. Since desensitization can be demonstrated with nerve-evoked release of acetylcholine at high frequencies of stimulation (41), it will be of interest to compare susceptibilities to desensitization in the presence of the peptide antibiotics and local anesthetics using current electrophysiological methodology.

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