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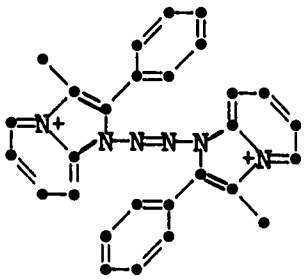
Kinetic Analysis of the AH8165–Receptor Interaction at the Mammalian Neuromuscular Junction

*B. E. Waud, M.D.**

The effect of a new neuromuscular blocking agent, AH8165, on carbachol-induced end-plate depolarization was measured in isolated guinea pig lumbrical muscles. The results showed that the kinetics were competitive (parallel shift of dose-response curves to the right and a slope of Schild plot of 1.09 ± 0.07) and the AH8165–receptor dissociation constant was estimated as 0.377 ± 0.008 μM. Once the dissociation constant was known, the fraction of receptors occupied by any given concentration of AH8165 could be determined. This fractional receptor occupancy was then compared with the indirect twitch response in an isolated guinea pig nerve–lumbrical muscle preparation. These measurements of the effect of AH8165 on the margin of safety of neuromuscular transmission gave values comparable to those obtained with *d*-tubocurarine, *i.e.*, the twitch remained normal until 75–80 per cent of the receptors were blocked and was abolished when 90–95 per cent of the receptors were occluded. Thus, the neuromuscular blocking action of AH8165 appears to be consistent with a simple postsynaptic competitive interaction with the transmitter. (Key words: Neuromuscular relaxants, AH8165.)

THE COMPOUND AH8165 1,1′-azo bis(3-methyl-2-phenyl-1H-imidazo(1,2-a pyridinium),



available as the dibromide, has two aromatic ammonium groups separated by an azo bridge designed to confer increased susceptibility to metabolic breakdown, and thus is of clinical interest as a short-acting neuromuscular blocking agent.

The drug is said to be competitive because the neuromuscular block produced is not preceded by potentiation of the twitch response or by fasciculation.[1–3] A tetanic response is not sustained and is followed by posttetanic facilitation.[3] The block can be reversed by neostigmine[1–3] and succinylcholine,[2] while competitive antagonists have additive blocking effects.[2] There are also conflicting opinions. In chick biventer cervicis muscle the agent has been found by some to cause flaccid paralysis,[2] and by others to cause contracture.[4] In the rat phrenic nerve–diaphragm preparation, AH8165 has been found by some to decrease[2] and by others to increase[4] the response to indirect stimulation.

In none of the above studies was depolarization or its absence measured directly. Only indirect criteria were presented. The present experiments provide a direct kinetic analysis of the action of AH8165 in a well-controlled experimental system.

Methods

Two types of study were done. In the first series of experiments, the effect of AH8165 on carbachol-induced end-plate depolarization was measured in isolated guinea pig lumbrical muscles. End-plate depolarization was measured by the moving fluid electrode technique.[5] After a control dose–response curve had been obtained, dose–response curves were measured in the presence of two concentrations of AH8165, the antagonist was washed out, and finally the control dose–response curve was repeated. The family of dose–response curves thus obtained was tested for parallelism and then used to estimate the slope of the Schild plot and the dissociation constant (K_B) of the AH8165–receptor interaction. The actual statistical estimates of the slope of the Schild plot and of K_B were obtained from an iterative nonlinear least-squares technique described previously.[6]

In the second set of experiments, the isometric twitch response to indirect stimulation was monitored in an isolated guinea pig nerve–lumbrical muscle preparation. The nerve lay in an epoxy tunnel containing two platinum electrodes, and was stimulated once every 10 seconds with shocks of 0.3 ms duration at twice maximal intensity. After 30–60 minutes, when the twitch response was stable, the effects of various concentrations of AH8165 on the response to indirect stimulation were determined. Concentrations of AH8165 were given either cumulatively or non-cumulatively, and the order of concentrations was varied. Fractional receptor occupancy corresponding to a given concentration of AH8165 was determined from the relation: $Y = [B]/([B] + K_B)$, where Y = fractional receptor occupancy, [B] = concentration of AH8165, K_B = dissociation constant of AH8165 obtained in the first series of experiments.

Both series of experiments were done in Krebs' solution of the composition (mM): Na^+, 138;

* Department of Anesthesia, Peter Bent Brigham Hospital, Boston, and Department of Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts.

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Address reprint requests to: B. E. Waud, M.D., Department of Anesthesia, University of Massachusetts Medical School, 55 Lake Avenue N., Worcester, Massachusetts 01605.

K^+ 5.9; Ca^{++}, 2.5; Mg^{++}, 1.22; Cl^-, 123; $H_2PO_4^-$, 1.2; $SO_4^=$, 1.22; HCO_3^-, 25; plus glucose, 20.8 g/l, bubbled with 95 per cent oxygen and 5 per cent carbon dioxide and kept at 36–37 C.

Results

In the first set of experiments, there were parallel shifts of dose–response curves to the right in the presence of AH8165. A representative example is shown in figure 1. The parallelism variance ratio was not significant, a result consistent with competitive kinetics. In this experiment, the slope of the Schild plot was 1.05 ± 0.28 (again a result consistent with competitive kinetics) and the AH8165–receptor dissociation constant was estimated to be 0.39 ± 0.05 μM. Although the results were analyzed by a somewhat more sophisticated statistical technique, the results of an experiment such as that in figure 1 can also be viewed in the framework of a Schild plot[7] in which the logarithm of the dose ratio minus one (*i.e.*, a measure of the extent of the shift of the dose–response curve to the right) is plotted against the logarithm of the concentration of the antagonist producing that shift. If the kinetics of the AH8165 are competitive, then that plot should have a slope of one. Figure 2 gives the Schild plot from experiment of figure 1. The results are consistent with unit slope and the K_B can be estimated from the intercept of the Schild plot to give 0.39

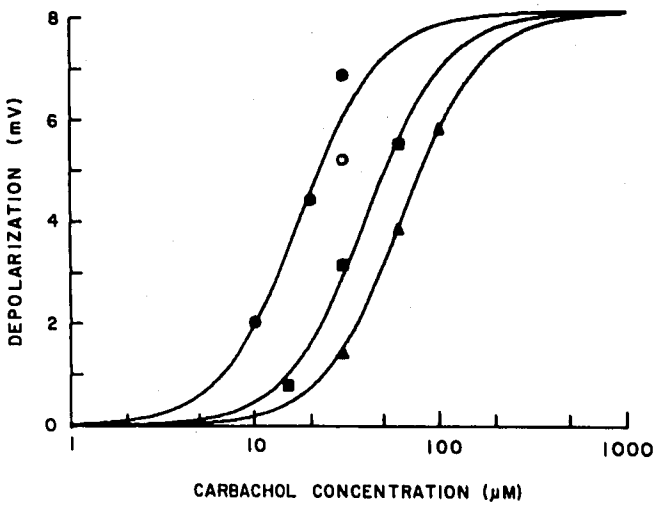

FIG. 1. Example of an experiment to measure K_B. Three carbachol dose–response curves. *Ordinates*: depolarization (mV) recorded extracellularly. *Abscissae*: Carbachol concentration (μM). *Circles*: control carbachol dose–response curve (open circles are recovery values). *Squares and triangles*: dose–response curves in the presence of 0.5 and 1 μM AH8165, respectively. Lines represent the function[6] Depolarization = $8\ A^P/(A^P + [K(1 + B^Q/K_B)]^P)$ with the parameters fitted by least squares. (A = carbachol concentration, B = AH8165 concentration, K = carbachol ED_{50}, K_B = AH8165-receptor dissociation constant, P determines the steepness of the dose–response curves, and Q corresponds to the slope of a Schild plot).

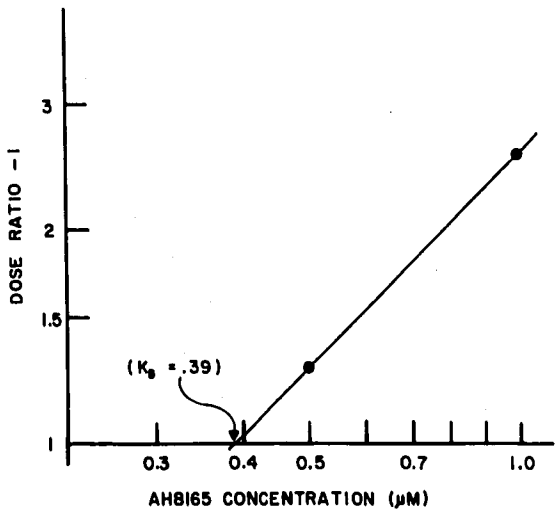

FIG. 2. Schild plot from values in figure 1. *Ordinates*: dose ratio −1. *Abscissae*: concentration (μM) of AH8165. The line with unit slope is consistent with the observations, and the axis intercept gives an estimate of 0.39 μM for the AH8165–receptor dissociation constant.

μM in this case. When the results of 12 such experiments were pooled, the mean values were 1.09 ± 0.07 and 0.377 ± 0.008 μM for the slope of the Schild plot and for K_B, respectively.

The second set of experiments is most conveniently summarized by a margin-of-safety plot, *i.e.*, a plot of the height of the indirect twitch response against the estimate of receptor occupancy associated with that response. Figure 3 gives the results. These may be summarized roughly by saying the indirect twitch response remained normal until 75–80 per cent of the receptors were blocked, and the twitch response was abolished when 90–95 per cent of the receptors were occluded. Figure 4 gives the results of figure 3 along with values obtained with *d*-tubocurarine for comparison. The overlap of values suggests the margin of safety is not different, but a more objective statistical comparison would be more reassuring. To this end, the values for *d*-tubocurarine and AH8165 were fitted with logistic functions of the form $Tw = 1 - y^p/(y^p + y_{50}{}^p)$. (This function was chosen empirically because it produces a sigmoid curve, has been convenient in the past when fitting sigmoid curves to observations, and is reasonably easy to handle mathematically. Tw is the twitch response; y_{50} corresponds to an ED_{50}, *i.e.*, the y_{50} is the occupancy of receptors by AH8165 that reduces the twitch response to half its normal value. p determines the slope and can also be used to test parallelism. Since the function $y^p/y^p + y_{50}{}^p$ is an increasing function of y, the form $1 - y^p/(y^p + y_{50}{}^p)$ was taken as the model for the observations). The observations were fitted with these curves by an iterative nonlinear least-squares technique analogous to that used in the first series of experiments.[6] The two sets of

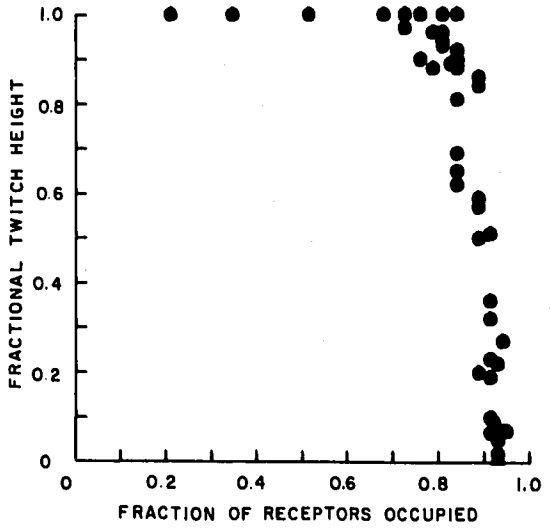

FIG. 3. Margin-of-safety plot. *Ordinates*: fractional twitch height of indirectly stimulated muscle. *Abscissae*: fractional receptor occlusion by AH8165.

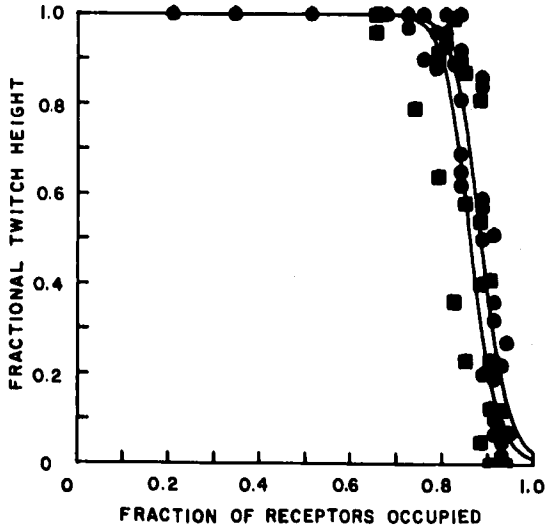

FIG. 4. Margin-of-safety plot comparing AH8165 with *d*-tubocurarine. Plot as in figure 3. *Squares*: *d*-tubocurarine. *Circles*: AH8165. The curves represents the function: $Tw = 1 - y^p/(y^p + y_{50}{}^p)$ fitted by least squares (Tw = fractional twitch height, y = fractional receptor occlusion; p determines the slope and y_{50} is the fractional receptor occlusion that halves the twitch response).

values were not found to be non-parallel, so curves with a common slope (p) were fitted and are superimposed on the values in figure 4. The estimates of the y_{50}'s were 0.857 and 0.886 for *d*-tubocurarine and AH8165, respectively. The least significant difference was estimated as 0.03, indicating no significant difference between drugs.

In no experiment did I observe that AH8165 caused either depolarization of the end-plate region or an increased twitch response.

Discussion

The results of the first series of experiments show that the kinetics of AH8165 are compatible with a simple competitive one-to-one reaction between the drug and the receptor. Thus, the usual heuristic model applicable to *d*-tubocurarine also applies to the newer compound. In the system examined, the K_B of 0.377 μM compared with a K_B of 0.105 μM for *d*-tubocurarine indicates a one-third lower potency for AH8165. While mechanism extrapolates well from species to species, relative potency generally can show variation. Thus, extrapolation of the present results to the clinic implies AH8165 can be viewed as a competitive antagonist to the transmitter. The potency appears to be slightly less than that of *d*-tubocurarine, but obviously a more precise statement can be made only from comparison of equieffective steady-state plasma concentrations in man.

The results of the second series of experiments indicate that occlusion of a given fraction of receptors by AH8165 produced an interference with the indirectly elicited twitch indistinguishable from that produced by occlusion of the same fraction of receptors by *d*-tubocurarine (the values for *d*-tubocurarine, in turn, agree with those found previously in this system,[8] *in vivo*,[9] and with other competitive blocking agents[9]). This means the neuromuscular blocking activity of AH8165 can be attributed completely to its ability to block postsynaptic receptors.

Thus, when examined kinetically at the neuromuscular junction, AH8165 can be summarized as a drug that occludes receptors competitively and produces neuromuscular block consonant with the extent of the receptor occlusion.

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