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Iontophoretic Study of Speed of Action of Various Muscle Relaxants

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The speed of action of nondepolarizing muscle relaxants is inversely related to potency. The hypothesis that this effect occurs at the end plate was tested in a frog (*Rana pipiens*) cutaneous pectoris muscle preparation. Brief acetylcholine pulses (10–100 ms) were applied iontophoretically from a central barrel of a triple-barrelled microelectrode located near an end plate. Long pulses (10–200 s) of muscle relaxant (gallamine, rocuronium, *d*-tubocurarine, atracurium, vecuronium, pancuronium, and doxacurium) were applied from one of two other barrels. The responses were a voltage change at the end plate, measured with an intracellular electrode. To evaluate potency, intracellular voltage changes following iontophoretic acetylcholine pulses were measured after application of various concentrations of muscle relaxants. The following were the equilibrium dissociation constants, which represent concentration of relaxant for 50% inhibition of response (mean plus or minus standard deviation): gallamine, 4.56 ± 0.44 μM (n = 5); rocuronium, 0.71 ± 0.09 μM (n = 6); *d*-tubocurarine, 0.59 ± 0.07 μM (n = 4); atracurium, 0.31 ± 0.03 μM (n = 4); vecuronium, 0.23 ± 0.02 μM (n = 5); pancuronium, 0.18 ± 0.03 μM (n = 3); doxacurium, 0.11 ± 0.03 μM (n = 5). Both onset and offset of effect of muscle relaxant proceeded with an exponential time course. The following were the onset time constants (seconds) for 46–56% blockade: gallamine, < 0.3 (n = 6); rocuronium, 0.91 ± 0.16 (n = 5); *d*-tubocurarine, 1.27 ± 0.24 (n = 7); atracurium, 2.10 ± 0.24 (n = 7); vecuronium, 2.60 ± 0.31 (n = 7); pancuronium, 3.01 ± 0.35 (n = 8); doxacurium, 3.65 ± 0.42 (n = 9). Offset time constants (seconds) were: gallamine, < 0.4 (n = 6); rocuronium, 1.85 ± 0.25 (n = 5); *d*-tubocurarine, 2.40 ± 0.29 (n = 7); atracurium, 4.31 ± 0.30 (n = 6); vecuronium, 5.36 ± 0.31 (n = 7); pancuronium, 5.99 ± 0.38 (n = 8); doxacurium, 7.10 ± 0.43 (n = 9). Both onset and offset times increased with decreasing equilibrium dissociation constants. It is concluded that a reverse relationship between onset time and offset time of muscle relaxants and potency is due to the events that occur at the end plate. (Key words: Neuromuscular junction. Neuromuscular relaxants: atracurium; *d*-tubocurarine; doxacurium; gallamine; pancuronium; potency; rocuronium; vecuronium.)

IT HAS BEEN SUGGESTED that speed of onset of muscle relaxants may be inversely related to potency. Bowman *et al.*[1] reported that, for steroid compounds given to cats, onset time increased as effective dose for 95% blockade (ED_{95}), decreased. Similarly, in humans given equipotent doses of gallamine, *d*-tubocurarine, and pancuronium, Kopman[2] observed that onset of action was fastest with the least potent drug (gallamine) and slowest with the most potent drug (pancuronium).

These findings could be explained by the high density of receptors at the neuromuscular junction[3] and the need for a large proportion of them (75–92%) to be blocked before blockade can be measured.[4] This implies that if a potent drug (*i.e.*, fewer molecules) is injected, the time required for a critical number of receptors to be occupied would be longer than if a less potent drug (*i.e.*, more molecules) is injected.[5] Many factors can affect the relationship between potency and onset in animals or humans, however, such as volume of distribution, degree of protein binding, and rate of elimination.

To determine whether the relationship between potency and onset is due to an interaction at the end plate, muscle relaxants were applied through an electrode located near the neuromuscular junction in the frog (*Rana pipiens*). To determine the potency of the same relaxants in separate experiments, the bath concentration was changed. In all cases, the response to iontophoretically applied acetylcholine was measured.

Materials and Methods

Preparations and Solutions

Experiments were performed on frog cutaneous pectoris preparation[6] at room temperature (19–22° C) in Ringer's solution of the following composition: NaCl (116 mM), KCl (2 mM), $CaCl_2$ (1.8 mM), and Tris-maleate buffer, *p*H adjusted to 7.0–7.3. Fifty-three end plates from 17 frogs were used for experiments. Localization of the neuromuscular junctions was made with a Zeiss water-immersion Nomarski microscope (×320).

Recording and Iontophoresis

To construct iontophoretic electrodes, single-barrel capillaries, each containing a glass fiber, were fused during

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the pulling procedure. They were filled with KCl (3 M) and were used to impale the muscle fibers at the end plate to record the membrane potentials (fig. 1). Only the results from muscles in whom resting membrane potentials ranged from −65 to −85 mV were used. Acetylcholine (1 M; 1–2000 nA, 10–100 ms; every 0.5–2.0 s) was applied iontophoretically from one barrel of a double-barrelled or from a central barrel of a triple-barrelled microelectrode. Only the responses with rising phases < 15 ms were used. Longer rising phases might indicate too long a distance between acetylcholine and recording electrodes. One muscle relaxant (gallamine, rocuronium, *d*-tubocurarine, atracurium, vecuronium, pancuronium, or doxacurium) was released iontophoretically (10–200 nA, 10–200 s) from one barrel. Another muscle relaxant was supplied to the same end plate from another barrel. The relaxant concentrations in the electrodes were gallamine (22.5 mM), rocuronium (1.6 mM), *d*-tubocurarine (4.4 mM), atracurium (8.1 mM), vecuronium (3.2 mM), pancuronium (1.4 or 2.8 mM), and doxacurium (0.9 mM). The current through each barrel was the sum of a steady braking current to prevent leakage between pulses and a command pulse whose amplitude and duration could be varied independently.

Data were recorded with an FM tape recorder (model 3964, Hewlett-Packard, Boston, MA; frequency response, DC to 1,250 Hz) and were subsequently replayed at one half or one quarter speed into a digital storage oscilloscope (model OS4020, Gould) and printed on brush chart recorder (model 2200, Gould) for analysis.

Changes in end-plate potentials produced by iontophoretically applied acetylcholine were measured before, during, and after iontophoretic application of relaxant (fig. 2A). Figure 2B gives acetylcholine responses in absence of an iontophoretic application of relaxant. To characterize onset, the prerelaxant response (V_o) and a steady-state response after application of the relaxant (V_{ss})

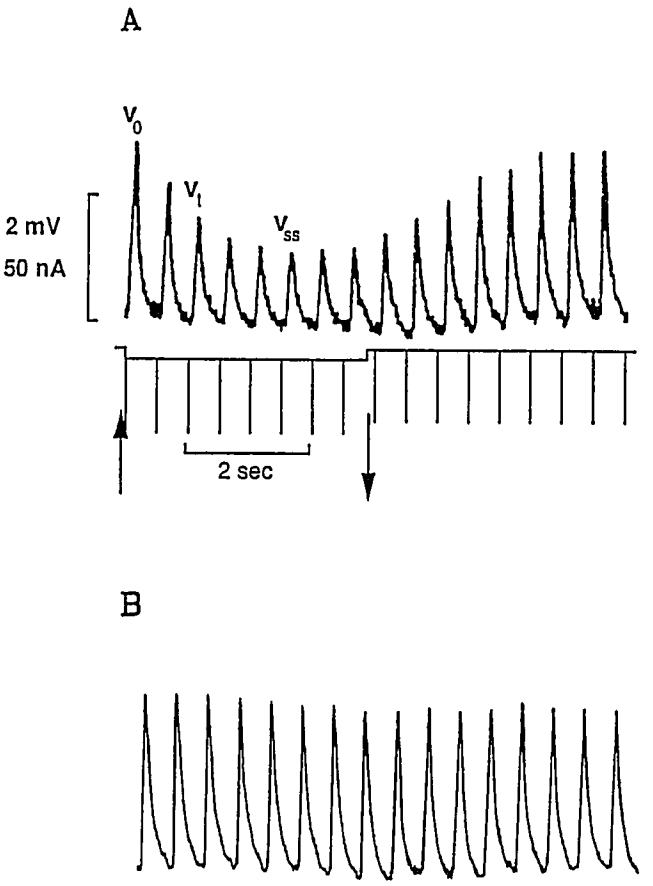

FIG. 2. *A:* Time course of inhibition. Upper trace shows a trace of acetylcholine (ACh) responses before, during, and after application of a nondepolarizing muscle relaxant (*d*-tubocurarine). Lower trace demonstrates a train of brief ACh pulses and a more prolonged iontophoretic application of a muscle relaxant. V_0 = the ACh response prior to the application of a muscle relaxant; V_t = the ACh response at a time t from the beginning of a steady state of inhibition; and V_{ss} = the ACh response during a steady state of inhibition. *B:* ACh responses in the absence of a nondepolarizing muscle relaxant application. Note that the responses are very stable.

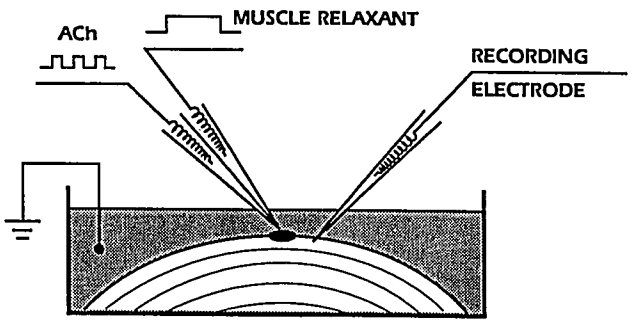

FIG. 1. A system for recording membrane potentials and iontophoretic application of acetylcholine (ACh) and various muscle relaxants to the neuromuscular junction. Note that the tip diameters of both intracellular electrode and of each barrel of iontophoretic electrode are typically less than 1 μm.

were measured. At any time t, the response (V_t) was measured and its difference to the steady-state response (V_{ss}) expressed as a fraction of total response, or $(V_t - V_{ss})/(V_o - V_{ss})$. Similarly, after the application of relaxant was stopped, the difference between V_o and V_t was expressed as a fraction of total response, or $(V_o - V_t)/(V_o - V_{ss})$. The logarithm of these values was plotted against time, and linear regressions were performed. The time constants for onset (τ_{on}) and offset (τ_{off}) were determined from these regressions.

To determine potency of the various muscle relaxants, the acetylcholine dose response curves were determined both before and after application of various concentrations of muscle relaxants. The muscle relaxants were applied either by replacement of solution in the whole bath

or by application of desired solution to the very limited area of several end plates through a short micropipette. The dose ratio, *i.e.*, the acetylcholine dose required to produce a response (typically 30–50% of a maximal response) in the presence of relaxant divided by acetylcholine dose required to produce the same response in the absence of relaxant, was plotted against relaxant dose. Linear regression was performed for each relaxant, and the dissociation constant (K_D) was defined as the x-intercept (fig. 3).[7]

Results are presented as mean plus or minus standard deviation. The constants τ_{on} and τ_{off} were related to potency (defined as $1/K_D$), and a linear regression was performed. Statistical significance was considered to be achieved at $P < 0.05$.

Results

For comparable extent of inhibition (46–55%), the time courses of onset were exponential and different for different muscle relaxants (fig. 4 and table 1). Offset also proceeded exponentially (fig. 5). Constants τ_{off}, although longer than for onset, were different for different relaxants, and the order was the same (fig. 5 and table 1). Constant τ_{on} was dependent on degree of depression because less time was required for intense blockade (fig. 6);

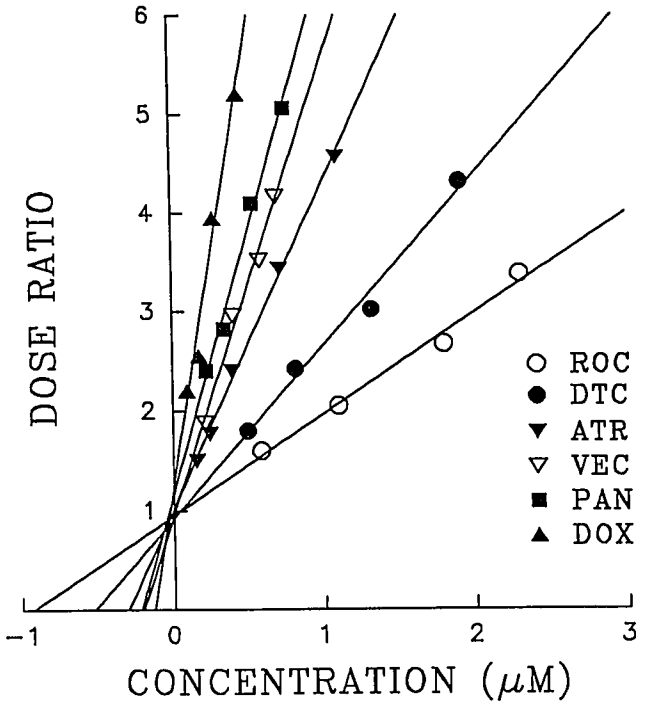

FIG. 3. Dose ratio test of inhibition of various muscle relaxants. The ratio of ACh doses (in picocoulombs) required to produce the same response with and without the muscle relaxant (rocuronium [ROC], *d*-tubocurarine [DTC], atracurium [ATR], vecuronium [VEC], pancuronium [PAN], doxacurium [DOX]) was measured at responses that ranged between 1 and 5 mV. The lines were fitted using the least-squares fitting procedure with the intercepts with the abscissa giving the equilibrium constants (K_Ds). K_Ds were (micromolar): 0.92, rocuronium; 0.52, *d*-tubocurarine; 0.31, atracurium; 0.22, vecuronium; 0.21, pancuronium; 0.13, doxacurium. Vertical bars indicate the standard deviations for dose ratios; number of end-plates in which dose ratios were determined (n) ranged from 3 to 5.

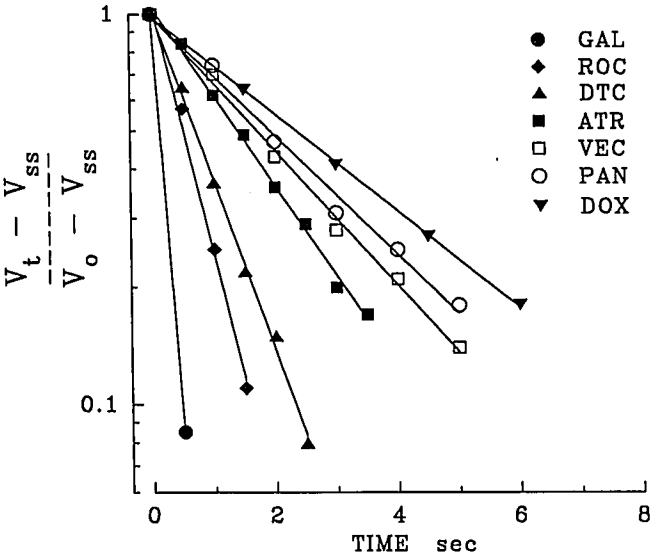

FIG. 4. Time course of the onset of inhibition for seven different nondepolarizing muscle relaxants (gallamine [GAL], rocuronium [ROC], *d*-tubocurarine [DTC], atracurium [ATR], vecuronium [VEC], pancuronium [PAN], and doxacurium [DOX]). Inhibition is plotted as the normalized fraction of the original response (see Materials and Methods) *versus* time after the beginning of the pulse of muscle relaxant. Vertical bars indicate standard deviations. Data for each relaxant are from the same locus at the end-plate. Number of observations (n) was 2 for gallamine, 3 for rocuronium, vecuronium, pancuronium, and doxacurium, and 4 for *d*-tubocurarine and atracurium. Note the linear nature of the plot, which indicates the exponential time course of inhibition (semilogarithmic plot). Inhibition ranged from 46 to 55%.

TABLE 1. Time Constants and Dissociation Constants

Drug	τ_{on} (s)	τ_{off} (s)	K_D (μM)
Gallamine	<0.3 (n = 6)	<0.4 (n = 6)	4.56 ± 0.44 (n = 5)
Rocuronium	0.91 ± 0.16 (n = 5)	1.85 ± 0.25 (n = 5)	0.71 ± 0.09 (n = 6)
d-Tubocurarine	1.27 ± 0.24 (n = 7)	2.40 ± 0.29 (n = 7)	0.59 ± 0.07 (n = 4)
Atracurium	2.10 ± 0.24 (n = 7)	4.31 ± 0.30 (n = 6)	0.31 ± 0.03 (n = 4)
Vecuronium	2.60 ± 0.31 (n = 7)	5.36 ± 0.31 (n = 7)	0.23 ± 0.02 (n = 5)
Pancuronium	3.01 ± 0.35 (n = 8)	5.99 ± 0.38 (n = 8)	0.18 ± 0.03 (n = 3)
Doxacurium	3.65 ± 0.42 (n = 9)	7.10 ± 0.43 (n = 9)	0.11 ± 0.03 (n = 5)

τ_{on} = time constant of onset; τ_{off} = time constant of recovery; K_D = equilibrium dissociation constant.

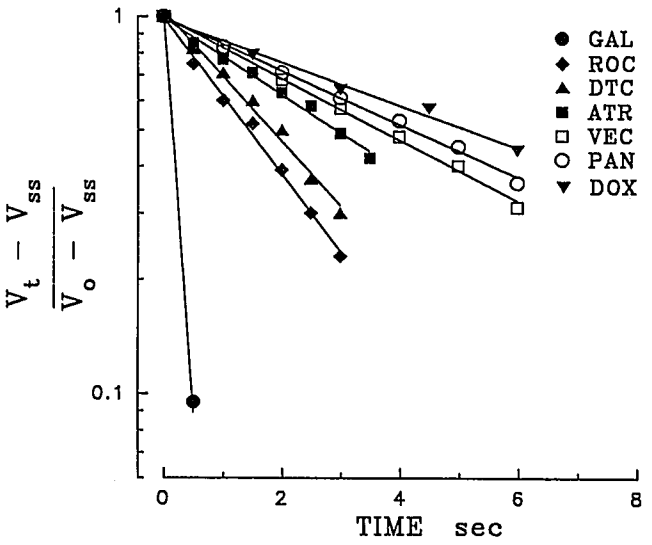

FIG. 5. Time course of recovery for seven different nondepolarizing muscle relaxants (gallamine [GAL], rocuronium [ROC], *d*-tubocurarine [DTC], atracurium [ATR], vecuronium [VEC], pancuronium [PAN], and doxacurium [DOX]) plotted as the remaining fraction of the steady-state inhibition after the end of the iontophoretically applied pulse of muscle relaxant (see Materials and Methods). Vertical bars indicate standard deviations. Data for each relaxant are from the same locus at the end-plate. Number of observations (n) was 2 for gallamine, 3 for rocuronium, vecuronium, pancuronium, and doxacurium, and 4 for *d*-tubocurarine and atracurium. Note the linear nature of recovery on a semilogarithmic scale for all muscle relaxants.

τ_{off} was independent of inhibition (fig. 7). The K_Ds of the various muscle relaxants are listed in table 1. With increasing K_D, τ_{on} and τ_{off} decreased (fig. 8). Both τ_{on} and τ_{off} were significantly correlated with potency, or $1/K_D$ ($P < 0.002$ in both cases).

Discussion

This study demonstrated that when muscle relaxants are applied directly to the end plate, their speed of both onset and offset of action are dependent on which drug is applied. Furthermore, these time constants were shown to be related inversely to the dissociation constant of the receptor. In other words, potent drugs have a slow onset and slow offset of action. In addition, for all neuromuscular relaxants tested, onset of action was faster if the degree of inhibition (or blockade) was intense, but offset was independent of the degree of inhibition.

The experiments were designed to deliver the muscle relaxant as close as possible to the end plate, and as quickly as possible. This was done to avoid many confounding factors present in the intact organism, such as circulation time, protein binding, elimination, distribution, and drug interactions. Application of the relaxant drug through the electrode was preferred to changing the medium bathing the preparation because, with the latter method, it is difficult to reach a new equilibrium in less than a few minutes with the latter method. The time constants obtained here are much shorter than this.

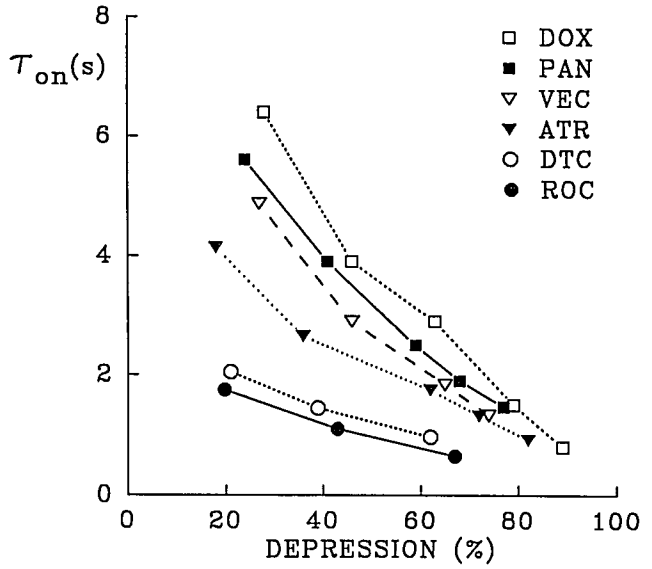

FIG. 6. Relationship between the time constant of onset (τ_{on}) and amount of inhibition. Rocuronium (ROC), *d*-tubocurarine (DTC), atracurium (ATR), vecuronium (VEC), pancuronium (PAN), and doxacurium (DOX) were tested. Note that τ_{on}s of all muscle relaxants clearly decrease as inhibition becomes more pronounced. Vertical bars indicate standard deviations. Data for each relaxant are from the same locus at the end-plate. Number of observations (n) was 3 for doxacurium, pancuronium, vecuronium, and rocuronium and 4 for atracurium and *d*-tubocurarine.

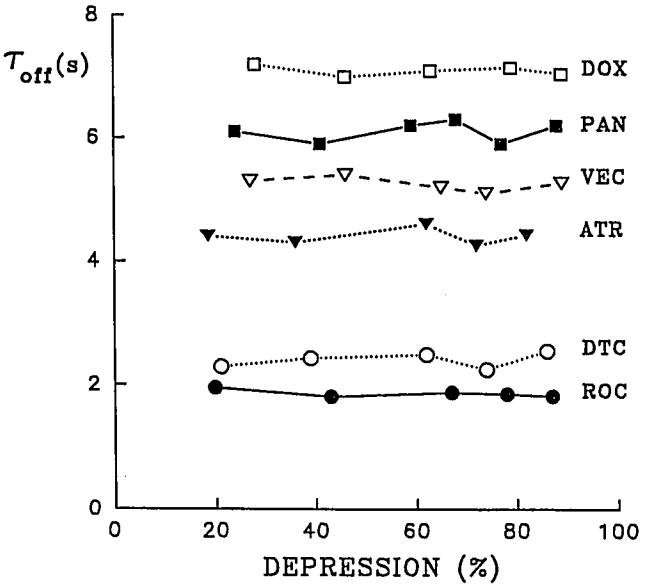

FIG. 7. Relationship between the time constant of recovery (τ_{off}) and the amount of inhibition. Note that for all muscle relaxants τ_{off} is essentially insensitive to the degree of inhibition. Vertical bars indicate standard deviations. Data for each relaxant are from the same locus at the end-plate. Number of observations (n) was 3 for doxacurium (DOX), pancuronium (PAN), vecuronium (VEC), and rocuronium (ROC) and 4 for atracurium (ATR) and *d*-tubocurarine (DTC).

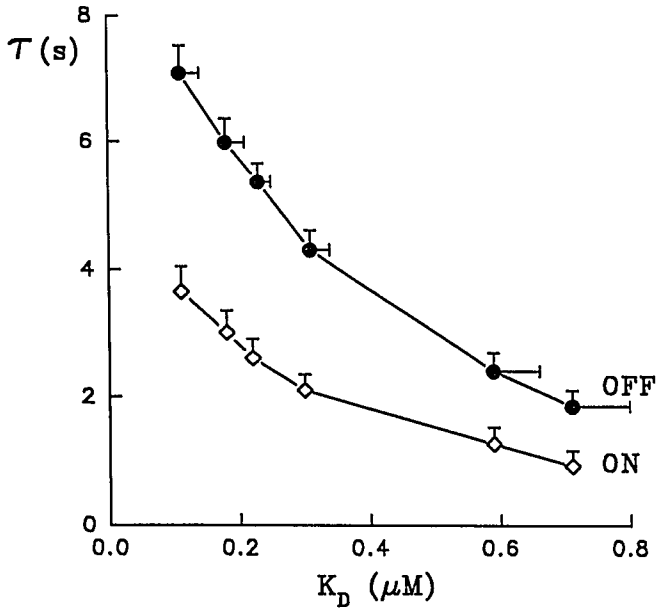

FIG. 8. Relationship between the time constants of onset of inhibition (τ_{on}s) or the time constants of recovery (τ_{off}s) and the equilibrium dissociation constant (K_D) for various muscle relaxants. (Inhibition ranged from 45 to 55%.) Both plots clearly indicate that the lower the K_Ds (greater potency), the longer the onset and the recovery times. Vertical bars indicate the standard deviations for τs and horizontal bars standard deviations for K_Ds. Number of endplates in which τs were determined (n) ranged from 4 to 9.

The desired pharmacologic effect of muscle relaxants is interruption of neurotransmission at the neuromuscular junction, and this can be measured by assessment of the muscle response to nerve stimulation. In this study, we chose to measure the change in end-plate potential produced by an acetylcholine pulse applied close to the end plate. This was chosen for two reasons: 1) the number of end plates stimulated was small, and the force of contraction of only a few muscle fibers is too small to be measurable; and 2) the acetylcholine delivered was adjusted not to produce a contraction; *i.e.*, it was a subthreshold response, thus avoiding dislodgement of electrodes with contraction. Clinically detectable blockade could be expected to occur over the range of 70–90% receptor occupancy (*i.e.*, 70–90% depression of response). In addition, the K_D derived here does not correspond to 50% twitch inhibition but to 50% receptor occupancy; however, because both variables are related, the results are compatible with the inverse relationship between onset time and potency.

When a muscle relaxant is delivered iontophoretically from a point source (electrode), the drug diffuses in all directions, resulting in a rapid decrease in concentration as distance away from the source increases. The concentrations in the electrode (0.9–2.25 mM) were many times greater than the concentration at the endplate (1 μM or less), based on the K_D values obtained. Such high concentration gradients were needed to counterbalance diffusion of the relaxant and establish sufficient concentrations at the end plate. In the intact human, however, the concentration gradient between drug source (intravascular space) and neuromuscular junction is much lower than in the experimental situation created in this study. After injection of a relaxant, maximum concentrations are 1–10 μM for most drugs, *i.e.*, three orders of magnitude less than in the study. Thus, the time constants are likely to be prolonged considerably, and differences of a few seconds probably translate into differences of a few minutes in a clinical setting.

After high-frequency nerve stimulation, the muscle response shows decrement, or fade, in the presence of nondepolarizing muscle relaxants. The response to a pulse of relaxant during onset, however, cannot be attributed to the mechanisms that produce fade. When acetylcholine is applied iontophoretically at a high frequency, as was the case here, no fade is seen, whereas fade is observed after nerve stimulation.[8] The frequency of acetylcholine pulses was rather limited by desensitization. The 0.5–2.0 Hz frequency chosen was a compromise between desensitization and accuracy of the onset and offset phases.

Offset of action was related to drug potency in the present experimental setting, but this simple relationship probably is offset by other factors in the clinical setting. On cessation of delivery of relaxant through the microelectrode, the concentration of the drug decreases to zero very rapidly. Thus, a concentration gradient, favoring exit of the drug from the neuromuscular junction, is established rapidly. In the clinical setting, the concentration gradient is smaller because the plasma concentration takes time to decrease to zero, and this slow process (minutes or hours) is likely to be the limiting factor.

The tendency of potent drugs to wear off slowly can be attributed to "buffered diffusion,"[9] which involves repetitive binding and unbinding from acetylcholine receptors.[10,11] If drug concentration decreases rapidly in the perijunctional area, a drug molecule that becomes unbound may either leave the junctional area, following the concentration gradient, or bind again to another (or the same) receptor. The probability of this last alternative is greater if the drug has a high affinity for the receptor. Thus, a potent drug molecule would have less chance of leaving the neuromuscular junction area, and the effect would take longer to wear off.

The relationship between onset and potency then can be explained in much the same way. When a drug is injected iontophoretically, some molecules reach the synaptic cleft and bind to acetylcholine receptors, reducing the drug concentration in the synaptic cleft. The same process is repeated in the surrounding area until a sufficient number of receptors is occupied. The speed of the process depends on two factors: 1) the concentration gradient between perijunctional area and synaptic cleft; and

2) the extent of repetitive binding of relaxant molecules to acetylcholine receptors. Concentration gradient needs to be greater for less potent drugs because these drugs produce an effect at higher concentrations. In addition, less potent drugs have less repetitive binding. Both factors would make less potent drug faster. The following example shows to what an extent the relaxant molecules are bound to acetylcholine receptors and to what an extent they are free when in equilibrium. If we assume that there are 26,000 receptors/μm^2 at the neuromuscular junction[3] and that the drug that has access to them lies in the synaptic cleft, which is 50 nm in width, it follows that the free drug molecules occupy 0.05 μm^3, or 5×10^{-17} l. When at equilibrium (50% of the receptors occupied), K_Ds are: gallamine (4.56 μM), rocuronium (0.71 μM), *d*-tubocurarine (0.59 μM), atracurium (0.31 μM), vecuronium (0.23 μM), pancuronium (0.18 μM), and doxacurium (0.11 μM). This means that at 50% inhibition, 13,000 molecules are bound, but on average the number of free molecules is only 137 for gallamine, 21 for rocuronium, 18 for *d*-tubocurarine, 9 for atracurium, 7 for vecuronium, 5 for pancuronium and 3 for doxacurium. Thus, the neuromuscular junction is an exceptionally powerful sink for muscle relaxants.

Nevertheless, because the time course of relaxant blockade may be determined by molecular rates of the relaxant's binding and unbinding to acetylcholine receptors, it is still to be determined whether buffered diffusion is the sole or most important factor that determines the speed of action of various muscle relaxants. The time course of relaxant blockade ought then to be as described previously.[12] In such a bimolecular ligand (L), binding reaction association with the receptor (R) is described by the following equation:

$$L + R \underset{k_-}{\overset{k_+}{\rightleftharpoons}} LR$$

On a step concentration in L, $\tau_{on} = 1/(k_+ *L + k_-)$, whereas the recovery time constant is simply $\tau_{off} = 1/k_-$. This model would describe well the fact that τ_{on} becomes shorter as relaxant dose increases and that τ_{off} is insensitive to such changes. In this model, however, one has to assume that molecular rates are rather low; more important, the model does not offer any explanation for the inverse relationship between the relaxant potency and the speed of action.

Several factors, such as tip size, distance between tip of electrode and idealized center of the acetylcholine receptor population, and transport number (number of relaxant molecules released per unit charge of the iontophoretic pulse), affect volume and diffusion of relaxant molecules. It thus may be argued that the apparent speed of action at the neuromuscular junction is due to their influence. It is not likely that the tip sizes or the distance between the tip and the junction is consistently different for different relaxants. Furthermore when the distance between the tip and the junction was known to be the same (in a triple-barrelled electrode where two barrels were filled with two different relaxants) the differences in speed of action of the different relaxants were clearly discernible. Finally, because our study clearly shows that the recovery times are insensitive to changes of the dose of muscle relaxants, the difference in their recovery times are very unlikely to be due to differences in their transport factors or volume.

The potencies for neuromuscular relaxants obtained here in the frog were in approximately the same order as that found in humans (gallamine < rocuronium < *d*-tubocurarine < atracurium < pancuronium < doxacurium). Onset times for equipotent doses have been found to follow this sequence,[2] and doxacurium has been found to have the longest onset times (10–15 min for subparalyzing doses) of all relaxants known.[13] As this study shows, this potency–onset relationship appears to be due to interaction between drug and end plate. A fast-onset, nondepolarizing relaxant then would be a drug with relatively little potency.

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