Laboratory Investigations

Relationships between block-of-twitch and train-of-four fade in the mouse phrenic nerve-diaphragm preparation

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The relationships between the block-of-twitch and train-of-four fade in the presence of nondepolarizing neuromuscular blocking drugs (d-tubocurarine, vecuronium and pancuronium) were examined in vitro by measuring the contractile tension from mouse phrenic nerve-diaphragm preparations. The slope of the block/fade relationship differed between onset of and recovery from neuromuscular block following single doses of d-tubocurarine, vecuronium or pancuronium. Decreasing the dose of d-tubocurarine or using a divided dose technique to accelerate onset (i.e., priming) increased the amount of fade for a given amount of block. In addition, the block/fade relationships for cumulative dosing and sequential dilution were the same when measurements were made at steady-state for several doses. It is concluded that the block/fade relationship in the mouse phrenic nerve-diaphragm preparation is variable, and is related to the time course of the neuromuscular block. In addition, the block/fade relationships for d-tubocurarine, vecuronium and pancuronium did not differ when determined at steady-state.

Les relations entre le blocage du twitch et de l'évanescence fade de l'ondée-de-quatre (train-of-four) en présence d'un bloqueur

Key words

MEASUREMENT TECHNIQUES: neuromuscular blockade;
NEUROMUSCULAR RELAXANTS: pancuronium,
d'tubocurarine, vecuronium; measurement of
response, train-of-four.

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neuromusculaire nondépolarisant (d-tubocurarine, vécuronium et pancuronium) ont été examinées in vitro en mesurant la tension contractile de préparations de diaphragmes nerf phrénique de rats. La pente de la relation du bloc/fade était différente entre le début d'installation et la récupération du bloc neuromusculaire après des doses uniques de d-tubocurarine, vécuronium ou pancuronium. En diminuant la dose de d-tubocurarine ou en utilisant la technique de dose subdivisée afin d'accélérer le début d'installation (i.e., amorçage), on a augmenté la quantité du fade pour la même quantité de bloc. En plus, les relations bloc/fade pour des doses cumulatives et de dilution séquentielle furent les mêmes que lorsque les mesures ont été faites en période d'équilibre stable (steady-state) pour des doses multiples. On conclut que la relation bloc/fade dans les préparations de diaphragme-nerf phrénique de rat est variable, et en relation avec l'évolution temporelle du bloc neuromusculaire. En plus, les relations bloc/fade pour le d-tubocurarine, vécuronium et pancuronium n'étaient pas différentes lorsque mesurées à l'état d'équilibre stable (steady-state).

Train-of-four (TOF) fade is a clinically useful measure of the degree of neuromuscular block, particularly with regard to the adequacy of reversal of residual block.¹ Recent clinical studies have shown that the relationships between the block-of-twitch and the TOF fade (block/fade relationship) differ during the onset of and recovery from neuromuscular block.²⁻⁴ This difference suggests that the TOF fade is not a fixed index of neuromuscular block. In addition, the differences in the block/fade relationships between the onset and recovery of neuromuscular block and between the different neuromuscular blocking drugs^{5,6} have been interpreted as supporting the contention that these drugs act at both pre- and postjunctional acetylcholine receptors.⁷

To date, researchers have not determined if changes observed in the block/fade relationship occur in the absence of anaesthetics or perianaesthetic drugs and if they are affected by neuromuscular fatigue or other time factors. It has also not been determined if the changes observed in the block/fade relationship are produced in the *in vitro* preparations used classically to study the blocking actions of neuromuscular drugs. In addition, the block/fade relationship at equilibrium has not been examined; pharmacokinetic analyses² have been complicated by the redistribution and the elimination of neuromuscular blocking drugs. In order to elucidate the above-mentioned issues, we re-examined the clinical findings on the block/fade relationship by using the mouse phrenic nerve-diaphragm preparation.

Methods

Female Swiss Albino mice (18–24 g) were killed by cervical dislocation, and the phrenic nerve-diaphragm of each one isolated. We dissected the phrenic nerve-diaphragm tissue of each mouse so that the left phrenic nerve inserted into the middle of a 6–8 mm wide portion of the left hemidiaphragm. An assemblage was hooked into a narrow region of the central tendon and the right diaphragm, and was also connected to a Grass FTO3 isometric tension transducer by a metal chain. Each mouse phrenic nerve-diaphragm tissue was then suspended in a glass organ bath containing 30 ml physiological saline solution (PSS). The costal margin was fixed in position by metal hooks attached to a rod. The PSS used to bathe the mouse phrenic nerve diaphragm tissue (hereafter referred to as the preparation) contained 118 mM NaCl, 25 mM NaCHO₃, 3.3 mM KCl, 1.1 mM KH₂PO₄, 2.5 mM CaCl₂, 0.9 mM MgCl₂, 11.1 mM glucose, and 0.01 mM choline chloride. It was aerated with 95% O₂/5% CO₂ and maintained at 37° C.

The nerve was stimulated supramaximally with 0.2 msec square wave pulses at 0.2 Hz. The resting length of the muscle was adjusted to yield the maximum isometric contractile responses (i.e., maximum twitch tension).

The twitch tension was allowed to stabilize for 10 to 20 min, and the nerve was subjected to a TOF stimulation consisting of four pulses at 2.0 Hz every 11.5 sec. One of the three nondepolarizing neuromuscular blocking drugs (d-tubocurarine, pancuronium or vecuronium) used for this study was added to the preparation. Neuromuscular block was monitored by measuring block-of-twitch and TOF fade which were calculated, as follows:

$$\text{Block} = 1 - (T_1/C)$$

$$\text{Fade} = 1 - (T_4/T_1)$$

Where T_1 is the tension of the first twitch in the TOF, C is

the control tension before the drug is added and T_4 is the tension of the last twitch in the TOF.

The block/fade relationship was examined using three experimental paradigms. In the first, the block/fade relationship was measured during the onset of and the recovery from neuromuscular block. Measurements were not taken at a steady-state. A single dose (i.e., one concentration) of d-tubocurarine (dTC), vecuronium or pancuronium was administered. In the second, the block/fade relationship was measured during the onset of and the recovery from a divided dose of dTC (a priming scheme used to accelerate neuromuscular block). Measurement was not taken at a steady-state. In the third, cumulative doses or sequential dilutions of dTC, vecuronium and pancuronium were used and the block/fade relationship was measured at a state of equilibrium.

In the first experimental paradigm, a small (<30 μ l) bolus of either dTC, vecuronium or pancuronium was added to 30 ml PSS. Except where noted, concentrations of $1.5\text{--}2 \times \text{ED}_{95}$ of these nondepolarizing neuromuscular blocking drugs (hereafter referred to as blocking drugs) were used. The onset of block-of-twitch was monitored for 10–15 min. The PSS was then replaced with 30 ml PSS that did not contain any blocking drugs (drug-free PSS). The recovery from neuromuscular block using the drug-free PSS was monitored.

Since recovery from neuromuscular block is always subsequent to its onset, differences in the block/fade relationships observed between the onset and recovery might be a consequence of preparation fatigue. To test this possibility, we conducted six experiments in which dTC was re-administered to a phrenic nerve preparation ten minutes after recovery from the neuromuscular block had been achieved. The onset of and the recovery from the neuromuscular block was monitored for a second time.

In the second paradigm, five primed preparations were used to test the effects of priming on the block/fade relationship, as follows. Each preparation was first exposed to 0.3 μ M of dTC for 15 min. More dTC was then added to the 30 ml bath bringing the final concentration to 2.0 μ M. The recovery from neuromuscular block was initiated 30 min after achieving 2.0 μ M of dTC by replacing the PSS with fresh, drug-free PSS. Five preparations were used as controls. They were exposed to a single dose of 2.0 μ M of dTC for 15 min. Then recovery was begun by replacing the bath with drug-free PSS.

Note that in the two experimental paradigms described above, several measurements of the block/fade relationship were made following the administration of a single blocking drug in the PSS. The measurements were made before neuromuscular block reached a steady-state.

In the third paradigm, the block-of-twitch and the TOF

fade were measured at steady-state then minimal change occurred during ten minutes. This suggests that the distribution of blocking drug had reached equilibrium. When "onset" and "recovery" with dTC were compared, onset was studied by replacing the bathing PSS with PSS containing an increased concentration of dTC. The bath was changed quickly so that neuromuscular block did not decrease and essentially cumulative dosing was performed. When TOF fade was $>90\%$, the PSS was brought to $2.0\ \mu\text{M}$ which resulted in 100% neuromuscular block and remained at this level for 15 min. Recovery from neuromuscular block was achieved by progressively decreasing the amount of dTC in the PSS. This was done by replacing the PSS with doses of increasingly diluted amounts of dTC. When the steady-state relationships for dTC, vecuronium and pancuronium were compared, a cumulative dosing was performed by adding additional blocking drug to that already in the bath. Note that in the third experimental paradigm, block-of-twitch and TOF fade were each measured only once for a given dose of blocking drug. Several doses of a given blocking drug were tested in each preparation.

Least squares fit lines were used to describe the relationship between the block-of-twitch and TOF fade ($10\% < \text{block} < 90\%$). Except where noted, data from all the preparations within each experimental group were pooled and each line was determined with more than 25 points. The slopes (m), X-intercepts ($X\text{-int}$), and means are presented $\pm$ SE, and they are compared by t-tests, with $P < 0.05$ considered significant.

Results

The relationship between the block-of-twitch and fade can differ between the onset of and the recovery from blocking drugs. Figure 1 illustrates this point using a single dose of dTC in a single phrenic nerve-diaphragm preparation. Note that over the range of 10–90% block-of-twitch, least squares fit lines reasonably represent the relationship between block-of-twitch and TOF fade (onset $r = 0.94$, recovery $r = 0.94$). As shown in the Table, the slopes and X-intercepts of the least squares fit lines for onset of and recovery from neuromuscular block differed significantly for each blocking drug examined.

There were no significant changes in the block/fade relationships between the first and the second dose of $2.0\ \mu\text{M}$ dTC (onset of first dose: $m = 1.0 \pm 0.048$, $X\text{-int} = 9.6 \pm 2.4$; onset of second dose: $m = 1.0 \pm 0.050$, $X\text{-int} = 6.9 \pm 2.5$ recovery from first dose: $m = 0.45 \pm 0.036$, $X\text{-int} = -25 \pm 5.6$; recovery from second dose: $m = 0.37 \pm 0.049$, $X\text{-int} = -42 \pm 11$). The block/fade relationships for a first and a second dose of pancuronium were also similar (data not shown).

FIGURE 1 Data from a single experiment in which neuromuscular block was monitored by observing the block-of-twitch and TOF fade. Onset is represented by open circles and dashed line, $r = 0.94$. Recovery is represented by filled squares and solid line, $r = 0.94$.

The dose of dTC affects the relationship between the onset of and the recovery from neuromuscular block. Both the onset of and the recovery from neuromuscular block were compared by using two doses of dTC. The $2.0\ \mu\text{M}$ dTC ($n = 5$) always caused 100% neuromuscular block while the $0.75\ \mu\text{M}$ ($n = 4$) always caused less than 100% neuromuscular block. Both the onset and recovery lines differed significantly between the two doses of dTC (Figure 2). In general, the lower dose exhibited greater fade for a given level of block.

TABLE Relationship between block and fade

Relaxant	Slope	X-intercept
2.0 μM d-tubocurarine		
6 preparations		
onset	1.00 ± 0.048	9.6 ± 2.4
89 points, $r = 0.91$		
recovery	$0.45 \pm 0.036^*$	$-25 \pm 5.6^*$
83 points, $r = 0.81$		
0.75 μM pancuronium		
11 preparations		
onset	1.1 ± 0.038	2.5 ± 2.0
150 points, $r = 0.92$		
recovery	$0.49 \pm 0.054^*$	$-27 \pm 8.3^*$
67 points, $r = 0.77$		
0.4 μM vecuronium		
9 preparations		
onset	1.0 ± 0.046	12 ± 2.3
116 points, $r = 0.90$		
recovery	$0.50 \pm 0.046^*$	$-31 \pm 7.2^*$
63 points, $r = 0.81$		

*Recovery significantly different from onset.

FIGURE 2 Onset and recovery of neuromuscular block for two doses of dTC are compared. Lines for the 0.75 μ M d-Tc preparations do not extend to 90% block, as this dose does not produce complete block-of-twitch. Onset lines are dashed (high dose: $m = 0.86 \pm 0.10$, $X\text{-int} = 6.4 \pm 6.4$, $r = 0.83$; low dose: $m = 1.2 \pm 0.083$, $X\text{-int} = -15 \pm 3$, $r = 0.90$). Recovery lines are solid (high dose: $m = 0.39 \pm 0.069$, $X\text{-int} = -38 \pm 15$, $r = 0.64$; low dose: $m = 1.2 \pm 0.48$, $X\text{-int} = -2.4 \pm 8.7$, $r = 0.66$, $n = 10$). The X-intercepts for both the onset and the recovery of blocking drugs and the slopes for the onset of these same drugs differed significantly between their doses.

In the second experimental paradigm ten preparations (five using single and five using divided doses) were used to monitor the onset of neuromuscular block following a total dose of 2.0 μ M dTC. Priming increased the amount of TOF fade for the same level of block-of-twitch, significantly shifting the block/fade relationship to the left. There was no significant change in slope (Figure 3). However, priming did not greatly affect the block/fade relationship during recovery (i.e., no significant difference in slope or X-intercept), or the time to 80% recovery from block-of-twitch (Figures 3 and 4).

The block/fade relationship was the same at steady-state (Figure 5) when dTC was administered as cumulative doses or sequential dilutions in seven preparations. There was little difference between the block/fade relationships of dTC ($n = 4$), vecuronium ($n = 5$) and pancuronium ($n = 4$) measured at steady-state following cumulative dosing (Figure 6). When each line was tested against each other by t tests, no significant differences were found.

In seven preparations, steady-state neuromuscular block was achieved by adding 0.6–0.75 μ M dTC. In all preparations, both block-of-twitch and TOF fade were greater than 0% and less than 100%. A second dose of dTC was then added to increase block-of-twitch and TOF fade while keeping them still less than 100%. For this second dose, the times to 90% of steady-state block-of-

FIGURE 3 Effect of priming on the block/fade relationship. There is a large and significant shift (X-intercept) in the block/fade relationship due to priming during onset (dashed lines; non-primed: $m = 1.1 \pm 0.063$, $X\text{-int} = 9.0 \pm 2.4$, $r = 0.94$; primed: $m = 1.0 \pm 0.089$, $X\text{-int} = -4.7 \pm 5.0$, $r = 0.93$). Priming did not significantly affect the slope or X-intercept of recovery (solid lines; non-primed: $m = 0.31 \pm 0.052$, $X\text{-int} = -49 \pm 16$, $r = 0.57$; primed: $m = 0.39 \pm 0.032$, $X\text{-int} = -27 \pm 6.4$, $r = 0.76$).

FIGURE 4 Priming has a much greater effect on the rate of onset of blocking drugs than on the rate of recovery. Times to 80% onset and recovery (T80) were measured in the preparations described in Figure 5. Priming significantly decreased the T80 for onset. However, the smaller increase in T80 for recovery was not significant.

twitch (339 ± 49 sec) and 90% steady-state TOF fade (314 ± 47 sec) were not significantly different (paired t test). Thus, a difference in the time constants for block-of-twitch and TOF fade could not be discerned.

Discussion

The results of our present studies using an *in vitro* mouse phrenic nerve diaphragm preparation indicate that the block/fade relationship can differ between onset of and

FIGURE 5 Steady-state measurements of the block/fade relationship do not differ between increasing (onset) and decreasing (recovery) concentrations of dTC. The least square fit line for onset is dashed ($m = 1.1 \pm 0.091$, $X\text{-int} = -28 \pm 5.6$, $r = 0.90$). The line for recovery is solid ($m = 1.2 \pm 0.10$, $X\text{-int} = -19 \pm 4.7$, $r = 0.88$). The lines do not differ significantly in slope or X-intercept.

recovery from blocking drugs. They are consistent with the findings of clinical studies on TOF fade,²⁻⁴ as well as an earlier one on tetanic fade.⁸ The results of our present study also indicate that differences between the onset of and the recovery from blocking drugs are not due to neuromuscular fatigue. In addition, these differences are not due to other time-related factors because there was no change in the onset or in the recovery of block/fade relationships over time.

These results also suggest strongly that the time course of drug distribution affects the amount of TOF fade that occurs at any particular block-of-twitch level. This conclusion is supported by the observations that factors which affect the rate of neuromuscular block (i.e., dose and priming), also affect the relationship between block-of-twitch and TOF fade. In addition, the differences in the block/fade relationship between the onset of and the recovery from blocking drugs were eliminated when the block-of-twitch and TOF fade were measured at steady-state levels. These differences were eliminated despite progressive, though transient, changes in blocking drug concentration.

The results of our studies with priming are consistent with the clinical results of Naguib *et al.*⁹ These authors additionally reported that some priming conditions which decreased the onset time did not significantly affect the block/fade relationship. Thus, the extent to which the altered time course affects the block/fade relationship may be limited. However, a clinical implication of the results of our studies is that alterations in blocking drug time course (i.e., distribution or elimination) may some-

FIGURE 6 The block/fade relationship does not differ between dTC (circles, dashed line: $m = 1.2 \pm 0.15$, $X\text{-int} = -24 \pm 7.8$, $r = 0.86$), pancuronium (squares, solid line: $m = 0.94 \pm 0.13$, $X\text{-int} = -31 \pm 10$, $r = 0.93$) and vecuronium (triangles, dotted line: $m = 0.94 \pm 0.17$, $X\text{-int} = -39 \pm 14$, $r = 0.81$) when measurements are made at steady-state.

times alter the amount of TOF fade for a given degree of block-of-twitch. Thus, TOF fade criteria for tracheal intubation, reversal of block-of-twitch or extubation might change.

The fade TOF had been observed clinically to be more pronounced throughout the recovery than at the onset of block. This was not the case *in vitro*. Unlike the phrenic nerve preparations that we used *in vitro*, recovery from block is much slower than the onset of block under clinical conditions. Indeed, clinical recovery (but not onset) occurs at pseudo-equilibrium. We suspect that the greater fade during recovery than onset observed under clinical conditions parallels the greater fade during steady-state than non-steady-state observed *in vitro* (compare Figures 1 and 5).

It has been observed that the block/fade relationships of various blocking drugs can differ.^{5,6} However, like similar findings with tetanic fade these observations were not made at steady-state levels of block-of-twitch. We did not observe drug-dependent differences in the block/fade relationship at steady-state *in vitro*. It is plausible that differences among blocking drugs in the block/fade relationships that were observed clinically are due to pharmacokinetic differences rather than pharmacodynamic differences between these same drugs. This is to suggest that they may be due to differences in the rates of distribution and elimination rather than to differences in potencies at their site(s) of action. We speculate that the differences in block/fade relationships among blocking drugs may not be detectable in the clinical setting if measured during steady-state drug infusions which would

compensate for pharmacokinetic differences between the drugs.

Power and Jones¹² suggest that the differences in the block/fade relationship that can be observed between the onset and the recovery of blocking drugs are due to the different binding rates of prejunctional (fade) and postjunctional (twitch) acetylcholine receptors (hereafter referred to as the dual receptor hypothesis). Neither the functional nor binding characteristics of the prejunctional acetylcholine receptor are known. For these reasons, we cannot disprove the dual receptor hypothesis.

We suspect that it would be difficult to explain our data using Power and Jones's suggestion. Our suspicions are based on the research of Graham *et al.*² who used a two-site model of drug action to explain the block-of-twitch and TOF fade results with onset and recovery in their patients. They calculated the mean half-life of the drug-postjunctional receptor complex to be three minutes, and the mean half-life of the drug-prejunctional receptor complex was calculated to be six minutes. Graham *et al.* note that this is substantially larger than might be expected from other studies.² Indeed it seems unlikely that the differences in the drug-receptor binding and dissociation which occur on a millisecond time scale for dTC for the postjunctional acetylcholine receptor¹³ could explain the differences between the onset and recovery from neuromuscular block which occur over minutes. In addition, it is not clear that the dual receptor model would explain our studies with dose and priming and our observation that the block/fade relationships of onset and recovery are not parallel.

A second reason for being unable to explain our data using the dual receptor hypothesis is that if prejunctional and postjunctional acetylcholine receptors have different affinities, the magnitude of the differences would likely differ among drugs. However, we did not find any evidence of this when the block/fade relationship for dTC, vecuronium and pancuronium were determined at steady-state.

A third and conclusive reason for being unable to explain our data using the dual receptor hypothesis is that in our specifically designed experiment (and from an informal analysis of our other data) we could not discern a difference in the time constants for block-of-twitch and TOF fade. Thus, in the *in vitro* mouse phrenic nerve-diaphragm preparation we cannot confirm the essence of the dual receptor hypothesis (the difference between the half-lives of block-of-twitch and fade). However, we can reproduce the phenomenon that the dual receptor hypothesis is meant to explain (the difference between onset and recovery block/fade relationships). It maybe that *in vivo* and *in vitro* systems show similar alterations in the block/fade relationships but for different mechanistic

reasons. However, we suspect that apparent dual receptor kinetics^{2,12} is an artifact of the greater pharmacokinetic complexity of the clinical situation. Additional human research is needed to resolve this issue.

We speculate that, as an alternative to the dual receptor hypothesis, neuromuscular preparations (whole tissues) are made up of several pharmacokinetic compartments. These compartments also differ in their block/fade relationships. It is well known that the muscles are composed of different types of motor units and that activity-dependent pharmacologic differences between motor unit types have been detected.¹⁴ Differences in block/fade relationships of whole tissues could be explained by differences in the contribution over time of each compartment to total block-of-twitch and total TOF.¹⁵ Conceptually, this explanation of the block/fade relationship differs from the dual receptor hypothesis.^{2,9,12} It explains multiple sites of drug action as differences between neuromuscular functions (e.g., in regard to drug perfusion and sensitivity to TOF fade) rather than as differences within the neuromuscular functions (i.e., block-of-twitch vs TOF fade receptors). A similar pharmacokinetic explanation of the data of Graham *et al.*² relying on differences among compartments of muscle rather than differences between receptors has recently been put forward by Bartkowski and Epstein.¹⁶

We have demonstrated that the block/fade relationship for blocking drugs is not fixed and that it can be altered by conditions that change the time course of the action of blocking drugs (onset, recovery, dose, priming). The recognition that TOF fade is related to the pharmacokinetics of blocking drugs even under *in vitro* conditions, demonstrates the variability of TOF fade as an index of neuromuscular block; and represents a complication in the mechanistic interpretation of TOF fade.

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