

Inhibition of Mobilization of Acetylcholine:

The Weak Link in Neuromuscular Transmission During Partial Neuromuscular Block with d-Tubocurarine

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The authors have demonstrated earlier, by direct measurement of acetylcholine (ACh), that d-tubocurarine (d-Tc) and other nondepolarizing muscle relaxants decrease the release of ACh from the indirectly stimulated mouse hemidiaphragm preparation. It was the purpose of the present study to determine whether the decrease of the stimulated release of ACh and the increase in the intensity of the partial neuromuscular block observed at higher stimulation rates is caused by the inhibition of mobilization of ACh from reserve depots to release sites or by inhibition of the release process itself. To attain our objective, the authors have investigated the influence of the progressive increase of the rate of stimulation from 0.1 to 1, 2, 3, and 5 Hz on the force of contraction on the *in vitro* phrenic nerve—hemidiaphragm and *in vivo* sciatic nerve—tibialis anterior preparation in the absence of drugs and after about 20% neuromuscular block produced by d-Tc or by Mg^{2+} . The latter is known to inhibit the Ca^{2+} dependent release of ACh. In the absence of drugs increasing the stimulation rate increased the force of contraction, *in vivo* and *in vitro*, during both indirect and direct stimulation. In the phrenic nerve-hemidiaphragm preparation the increase was significant at 1, 2, 3, and 5 Hz ($P < 0.001$) with both types of stimulation. In the sciatic nerve-tibialis anterior preparation the force of contraction was only higher at 3 and 5 Hz ($P < 0.01$). The similar magnitude of the increase of the force of contraction during direct and indirect stimulation indicates that it is caused by facilitation of the contraction of the muscle fibers. When about 20% steady state neuromuscular block was produced by d-Tc at 0.1 Hz, increasing the stimulation rate to 1, 2, 3, and 5 Hz significantly ($P < 0.001$) increased the intensity of the neuromuscular block both *in vitro* and *in vivo*. In contrast, when similar neuromuscular block was produced by Mg^{2+} , increasing the stimulation rate, increased, rather than decreased the force of contraction ($P < 0.001$) both *in vitro* and *in vivo*. The dissimilar effect of increasing stimulation rates on the intensity of the partial neuromuscular block, namely increase by d-Tc and antagonism by Mg^{2+} , indicates that d-Tc inhibits mobilization of ACh. Inhibition of mobilization of ACh contributes significantly to the neuromuscular effect of d-Tc at higher stimulation rates. (Key words: Acetylcholine, mobilization of; release of. Ions, magnesium: acetylcholine release. Neuromuscular relaxant, d-tubocurarine: acetylcholine mobilization.)

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ACETYLCHOLINE (ACh), the physiological transmitter of neuromuscular transmission, is stored in synaptic vesicles containing a quantum[¶] (several thousand molecules) of ACh.¹ A part of these vesicles are assumed to be in close proximity to the prejunctional membrane of the motor nerve terminal, and are readily available for release by the nerve impulse.² As the readily available store of ACh is used up it is replenished from a larger reserve store located at some distance from the prejunctional membrane.³ It has been suggested that the mobilization of ACh stored in synaptic vesicles is facilitated by a positive-feedback mechanism through nicotinic receptors located on the motor nerve terminal and that nondepolarizing neuromuscular blocking agents inhibit this mechanism.⁴ The profound tetanic fade, caused by concentrations or doses of nondepolarizing neuromuscular-blocking agents which only cause moderate neuromuscular block at slow (*e.g.*, 0.1 Hz) stimulation, rates has been attributed to the inhibition of this positive-feedback mechanism.⁴ It has been subsequently demonstrated, by direct measurement of ACh, that nondepolarizing neuromuscular blocking agents inhibit the evoked release of ACh.⁵

The integrity of neuromuscular transmission is dependent on the interaction of ACh, released by the nerve impulse, with sufficient number of cholinergic receptors of the postjunctional membrane. There is a high safety margin of neuromuscular transmission.⁶ Five to ten times as much ACh is released by the nerve impulse than that necessary for the depolarization of the postjunctional membrane, and the development of the endplate- and action-potential, when all the cholinergic receptors are free to interact with ACh. Furthermore, if the release of ACh is not inhibited, as long as at least 30% of the postjunctional cholinergic receptors are available for interaction with ACh, neuromuscular transmission will be unimpeded at slow stimulation rates.⁶ It has been suggested that, at higher stimulation rates, the nicotinic feedback mechanism of ACh mobilization further increases the safety margin of neuromuscular transmission.⁴

It may be assumed that when partial, steady state, neuromuscular block is produced either *in vitro* or *in vivo*, the proportion of presynaptic and postsynaptic receptors oc-

[¶] Since only quantal (vesicular) ACh plays a role in neuromuscular transmission, in this paper, the term ACh will always refer to quantal ACh.

cupied by (interacting with) nondepolarizing neuromuscular blocking agents is constant. Therefore, under these conditions, the inhibition of the rate of mobilization of ACh from reserve to release sites should also be constant. In other words, the amount of ACh mobilized per unit of time from reserve to release sites should be the same as long as the time available between stimuli remains unchanged. However, when by increasing the rate of stimulation, the time interval between stimuli is decreased, the amount of ACh mobilized between subsequent stimuli should decrease in proportion to the increase of the stimulation rate. This in turn should reduce the amount of ACh available for immediate release. Because of the decreased quantity of ACh released the statistical probability of interaction between the unchanged number of free postsynaptic cholinergic receptors and ACh should diminish and the intensity of the neuromuscular block should increase. If d-Tc indeed inhibits mobilization of ACh then the effect of higher stimulation rates should be accentuated in the presence of partial d-Tc-induced neuromuscular block.

It is also conceivable, however, that d-Tc, instead of inhibiting the mobilization, similar to Mg^{2+} , inhibits the Ca^{2+} -dependent process of ACh release.^{7,8} To test the validity of the hypothesis that d-Tc inhibits mobilization of ACh^{3,4}, the influence of increasing the stimulation rate from 0.1 to 1, 2, 3, and 5 Hz on the partial neuromuscular block was investigated on *in vitro* and *in vivo* rat nerve-muscle preparations. The dissimilar effect of increasing stimulation rates, namely facilitation of the d-Tc block and antagonism of the Mg^{2+} block, to be demonstrated, indicates that d-Tc indeed inhibits mobilization of ACh.

Methods

The experiments were carried out on the *in vitro* phrenic nerve-hemidiaphragm preparation and the *in vivo* sciatic nerve-tibialis anterior preparations of male, Sprague-Dawley rats of 350 to 450 g body weight. The experiments were approved by the Institutional Animal Care and Use Committee.

In the *in vitro* experiments, rats were lightly anesthetized with ether and decapitated. The hemidiaphragms, with the phrenic nerve attached, were suspended in modified Krebs' solution,⁹ containing the same concentration of Ca^{2+} (1.1 mM) and Mg^{2+} (0.9 mM) as rat or human plasma. The bath was aerated with 95% O_2 -5% CO_2 , and its temperature was kept constant at 37° C. Under these conditions, the pH was 7.38-7.42. A diagram of the experimental preparation has been published.¹⁰

In the *in vivo* experiments, rats were anesthetized with 0.5 mg·kg⁻¹ urethane and 35 mg·kg⁻¹ pentobarbital injected intraperitoneally. Bipolar platinum electrodes were placed on the sciatic nerve in the gluteal region and

the nerve was crushed by a strong ligature proximal to the electrodes.

In *in vitro* experiments, the tendon of the hemidiaphragm and *in vivo* the distal tendon of the tibialis anterior were attached to FT03 transducers. The preparations were stimulated (Grass Model® S88 stimulator) indirectly, through the corresponding nerve, or directly through two needle electrodes, inserted about 1 cm apart into the muscle, with supramaximal square wave impulses. To eliminate the indirect component of direct stimulation,¹¹ neuromuscular transmission was completely inhibited *in vitro* by the addition of 5 × ED95 d-Tc to the organ bath and *in vivo* by the continuous iv infusion of 0.3 mg·kg⁻¹·min⁻¹ vecuronium before start of direct stimulation. The stimulus duration was 0.2 ms with indirect and 2 ms with direct stimulation. The optimal resting tension of the muscles, 10-12 g in *in vitro* and 25-30 g *in vivo*, was determined for each preparation before the start of the experiments. The stimulation frequency was increased sequentially from 0.1 to 1, 2, 3, and 5 Hz. Stimulation at each frequency was continued until the force of contraction of the muscle, quantitated by the transducer, and continuously recorded (Grass Model® 7B polygraph) became stable. A 3-min rest period was allowed between stabilization of the force of contraction and the start of stimulation at the next higher rate. Higher than 5 Hz stimulation rates were not used, because stimulation frequencies above 10 Hz cause a rapid decay of the force of contraction even in the absence of drugs.

In various experiments, the effect of increasing stimulation rates on the force of contraction was determined during indirect or direct stimulation in the absence of drugs or in preparations, in which about 20% neuromuscular block was produced at 0.1 Hz by 0.2 to 0.3 μg d-Tc or 3.0 to 3.5 mM Mg^{2+} . All *in vitro* and *in vivo* data summarized in tables 1 and 2 represent the means ± SEM of 8 experiments.

The statistical significance of the changes caused by increasing stimulation rates on neuromuscular transmission or force of contraction was tested with ANOVA followed by Tuckey's test.¹² $P < 0.05$ was considered significant.

Results

During indirect stimulation, in the absence of d-Tc, increasing sequentially the stimulation rate from 0.1 to 1, 2, 3, and 5 Hz caused a progressive increase of the force of contraction in both the *in vitro* phrenic nerve-hemidiaphragm and the *in vivo* sciatic-tibialis anterior preparation of rats. In the phrenic nerve-hemidiaphragm preparation the force of contraction became progressively greater ($P < 0.001$) at 1, 2, 3, and 5 Hz than at 0.1 Hz. In the sciatic nerve-tibialis anterior preparation the in-

TABLE 1. The Effect of Increasing Stimulation Rates on the Force of Contraction of the Indirectly or Directly Stimulated *In Vitro* and *In Vivo* Nerve-Muscle Preparations

Preparation	Stimulation	Force of Contraction at Equilibrium Expressed as Percent of Control at 0.1 Hz at the Stimulation Rates (Hz) Indicated				
		0.1	1	2	3	5
<i>In vitro</i> * Phrenic nerve—hemidiaphragm	Indirect†	100	108.8 ± 0.8†	113.7 ± 0.7	114.7 ± 0.9	112.8 ± 1.3
	Direct†‡	100	110.4 ± 0.7	118.6 ± 1.2	119.2 ± 0.8	118.2 ± 1.6
<i>In vivo</i> * Sciatic nerve—tibialis anterior	Indirect§	100	100.8 ± 0.8	103.1 ± 0.8	105.5 ± 1.1	106.9 ± 1.5
	Direct†§	100	102.5 ± 1.3	105.3 ± 1.1	109.4 ± 1.6	114.8 ± 1.9

* All values are means ± SEM of eight experiments.

† NM transmission completely inhibited *in vitro* with 5 μM d-Tc and *in vivo* with the continuous infusion of 0.3 mg · kg⁻¹ · min vecuronium before start of stimulation.

‡ Force of contraction higher at 1, 2, 3, or 5 Hz than at 0.1 Hz ($P < 0.001$).

§ Force of contraction high at 3 and 5 Hz ($P < 0.01$) than at 0.1 Hz.

crease of the force of contraction was significantly greater only at 3 and 5 Hz ($P < 0.01$) than at 0.1 Hz (see table 1). When neuromuscular transmission was completely inhibited with d-Tc before start of direct stimulation, increasing the stimulation rate from 0.1 Hz increased the force of contraction in the phrenic nerve-hemidiaphragm preparation at 1, 2, 3, and 5 Hz ($P < 0.001$) and in the sciatic nerve-tibialis anterior preparation at 3 and 5 Hz ($P < 0.01$) (see table 1).

When during indirect stimulation at 0.1 Hz, 20% neuromuscular block was produced with d-Tc, increasing the stimulation rate from 0.1 to 1, 2, 3, and 5 Hz caused a progressive decrease ($P < 0.001$) of neuromuscular transmission in both the phrenic nerve-hemidiaphragm and the sciatic nerve-tibialis anterior preparations (see table 2). In contrast, in the presence of about 20% Mg²⁺ induced neuromuscular block increasing the stimulation rate from 0.1 to 1, 2, 3, and 5 instead of decreasing, increased the force of contraction ($P < 0.001$) (see table 2 and fig. 1).

The intensity of the partial neuromuscular block caused by Mg²⁺ at 0.1 Hz was significantly increased by the addition of a low concentration of d-Tc to the organ bath

(see fig. 2). Subsequent increase of the stimulation rate from 0.1 to 1, 2, 3, and 5 Hz caused a progressive increase of the neuromuscular block. The rate of increase of the intensity of the neuromuscular block was similar to that caused by d-Tc without the preliminary addition of Mg²⁺.

Discussion

The findings presented indicate that in the absence of drugs, when the positive feedback mechanism of ACh mobilization is not inhibited and the postsynaptic cholinergic receptors are free to interact with ACh, increasing the rate of indirect stimulation from 0.1 to 1, 2, 3, and 5 Hz caused a progressive increase in the force of contraction of both the *in vitro* phrenic nerve-hemidiaphragm and the *in vivo* sciatic nerve-tibialis anterior preparation of rats. Similar observations were made earlier in human subjects by Slómic *et al.*¹³ The increase in the force of contraction caused by increasing stimulation rates during indirect stimulation and direct stimulation, when the influence of neuromuscular transmission was eliminated, were similar. Therefore, it may be assumed that the site of the facilitating effect of increasing stimulation rates is

TABLE 2. The Effect of Increasing Stimulation Rates on the about 20% d-Tubocurarine (d-Tc) or Magnesium (Mg²⁺) Induced Neuromuscular Block at 0.1 Hz of *In Vitro* and *In Vivo* Preparations

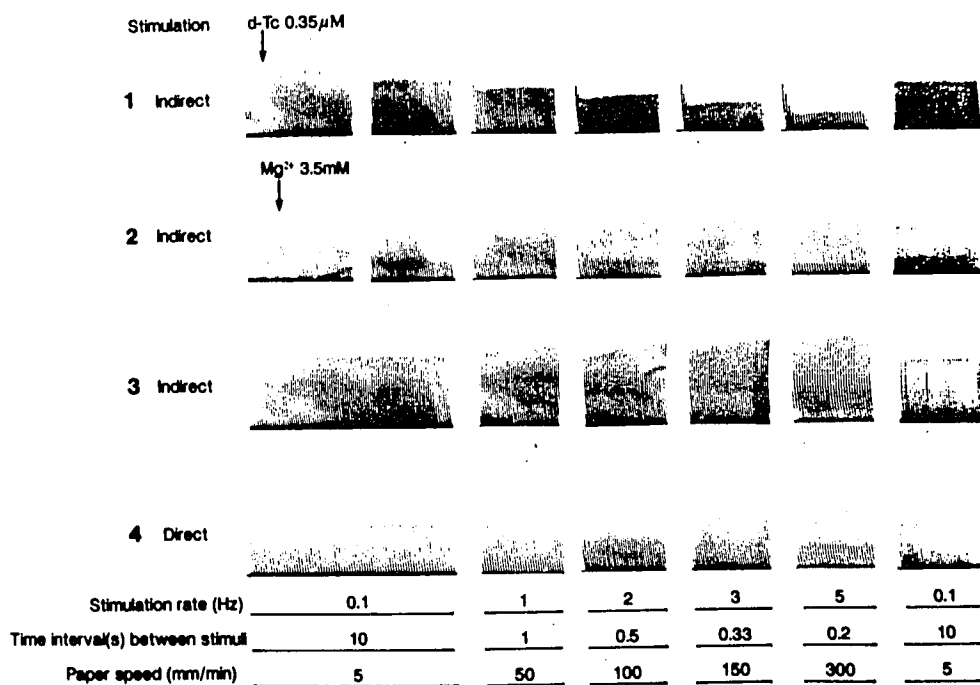
Preparation	Compound	Neuromuscular Block at Equilibrium at the Stimulation Rates (Hz) Indicated				
		0.1	1	2	3	5
<i>In vitro</i> * Phrenic nerve—hemidiaphragm	d-Tc†	20.3 ± 0.5	43.3 ± 2.0	76.0 ± 5.2	89.1 ± 3.9	95.6 ± 1.4
	Mg ²⁺ ‡	20.1 ± 0.6	12.2 ± 1.1	7.3 ± 0.2	4.0 ± 0.3	5.6 ± 0.4
<i>In vivo</i> * Sciatic nerve—tibialis anterior	d-Tc†	29.7 ± 1.5	43.8 ± 1.0	74.9 ± 0.7	84.0 ± 0.7	90.8 ± 0.6

* All values are mean ± SEM of eight experiments.

† Neuromuscular block is greater ($P < 0.001$) at all higher stimulation rates than at 0.1 Hz.

‡ Neuromuscular block is less ($P < 0.001$) at all higher stimulation rates than at 0.1 Hz.

FIG. 1. The influence of increasing stimulation rates on the force of contraction under various experimental conditions. Rat phrenic nerve-hemidiaphragm preparation. Note that during indirect stimulation, if partial neuromuscular block is produced by d-Tc, a compound that inhibits mobilization of ACh, increasing the stimulation rate decreases the force of contraction (first tracing). In contrast, when partial neuromuscular block is induced by Mg^{2+} , an agent that inhibits release of ACh, increasing the stimulation rate increases the force of contraction (second tracing). In the absence of drugs, increasing the stimulation rate increases the force of contraction both during indirect stimulation (third tracing) and direct stimulation of the completely curarized preparation (fourth tracing). Resuming stimulation at 0.1 Hz returned force of contraction to its original value.



not the neuromuscular junction but the muscle fiber. The mechanism of this increase could not be determined in this study.

In the presence of about 20% steady state d-Tc block, when less than 30% of the postsynaptic cholinergic receptors are available for interaction with ACh,⁶ and when presumably the rate of mobilization of ACh from reserve depots to release sites is also diminished,⁴ increasing the stimulation rate from 0.1 to 1, 2, 3, and 5 Hz significantly increased the neuromuscular block. Under steady state conditions, increasing the rate of stimulation is not likely to cause any change in the proportion of the pre- or post-

synaptic cholinergic receptors occupied by d-Tc. Therefore, presumably, inhibition of the mobilization of ACh by d-Tc, combined with the shorter time available for mobilization of ACh and the resultant decrease of ACh necessary for the transmission of the nerve impulse, will increase the intensity of the neuromuscular block.

It is conceivable that during partial d-Tc block, when the majority of the postsynaptic cholinergic receptors are occupied by d-Tc, the shorter time available between stimuli for the mobilization of ACh could alone explain the increase of the intensity of neuromuscular block observed at higher stimulation rates. This hypothesis, how-

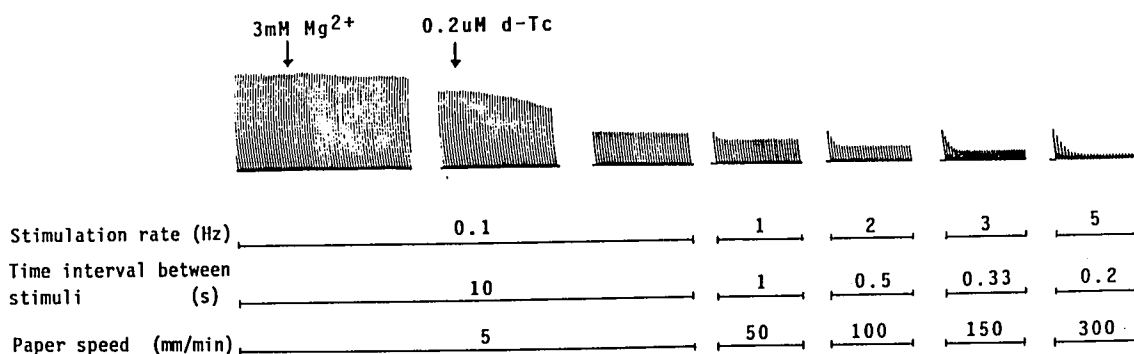


FIG. 2. At 0.1 Hz, about 20% neuromuscular block caused by Mg^{2+} was increased to about 70% by the addition of an otherwise ineffective concentration of d-Tc. Increasing the stimulation rate caused a progressive increase in the intensity of the neuromuscular block. The observations presented in this figure indicate that when the stimulated release of ACh is partially inhibited by Mg^{2+} , inhibition of its mobilization by d-Tc will increase the intensity of the neuromuscular block at low, 0.1 Hz, and even more at higher stimulation rates.

ever, would only be acceptable if under physiological conditions ACh would be mobilized from reserve to release sites at a constant rate, which is independent of the stimulation frequency. In other words, the quantity of ACh mobilized per unit of time would be the same during stimulation at 0.1 or 50 Hz. Under physiological conditions at 50 Hz, neuromuscular transmission in mammals remains constant for at least 5 s (250 stimuli).¹⁴ At 50 Hz, the time interval between stimuli is 0.02 s. During this 0.02 s, the quantity of ACh mobilized and available for release is sufficient to maintain neuromuscular transmission. At 0.1 Hz, the time available for mobilization of ACh between stimuli is 10 s, 500 times longer than at 50 Hz. This means that if the rate of mobilization of ACh would be constant and independent from the stimulation rate, ACh release at 0.1 Hz would have a 500-fold safety margin. The other possibility would be that the stores of ACh immediately available for release would be sufficient, without any significant replenishment from reserve stores, to maintain neuromuscular transmission elicited by 250 impulses over 5 s. It is unlikely that neuromuscular transmission would operate with a highly uneconomical, 500-fold safety margin, or that the readily releasable stores would contain enough ACh for the transmission of 250 impulses. These considerations support the hypothesis that the rate of ACh mobilization is greater at high than at low stimulation rates.^{3,4} This assumption is further corroborated by observations made of the influence of stimulation frequency on the intensity of the neuromuscular block of the nerve muscle preparation partially blocked by d-Tc. If there was a 3 min rest period between stimulating the preparation at progressively higher rates, neuromuscular transmission elicited by the first stimulus, as indicated by the measurement of force of contraction of the muscle, was always the same (see fig. 1). This indicates that during this rest period enough ACh was mobilized to maintain neuromuscular transmission elicited by the first impulse. However, at 1 Hz or higher stimulation rates, the force of contraction elicited by the second and subsequent stimuli decreased significantly in proportion to the increased stimulation frequency. As already discussed, in the absence of drugs, enough ACh was mobilized in 0.02 s to maintain neuromuscular transmission elicited by 250 consecutive stimuli. Consequently, it may be assumed that the progressive decay of neuromuscular transmission caused by increasing the stimulation rate from 0.1 to 1, 2, 3, and 5 Hz during 20% d-Tc block must have been caused by the partial inhibition of the increased rate of ACh mobilization elicited, under physiological conditions, by higher stimulation frequencies. However, before accepting the validity of this assumption, other possible causes that could decrease the readily releasable stores of ACh must be eliminated.

Theoretically, the availability of ACh essential for neu-

romuscular transmission, in addition to the inhibition of its rate of mobilization from reserve to release sites, may be caused by diminution of the size of the reserve stores by: 1) inhibition of cholineacetyltransferase (EC 2.3.1.6) the enzyme responsible for the synthesis of ACh;¹⁵ 2) inhibition of the re-uptake of choline, essential for the synthesis of ACh;¹⁶ and 3) the packaging of ACh into synaptic vesicles.^{17,18} These three possibilities may be excluded since the neuromuscular block caused by these mechanisms, at the stimulation rates used in this study, develops very slowly.¹⁵⁻¹⁸ In contrast, in the presence of d-Tc, the maximal effect of increasing the stimulation rate developed within seconds (see fig. 1). The possibility that increasing stimulation rates inhibited the release of ACh can be eliminated by comparing their influence on the partial d-Tc or Mg²⁺ block.

It is generally accepted that Mg²⁺ competes with Ca²⁺ at the ACh release sites of the motor nerve terminal.^{7,8} Increasing the extracellular [Mg²⁺] inhibits the evoked release of ACh.¹⁹ In addition, Mg²⁺ also has a postsynaptic inhibitory effect on neuromuscular transmission and inhibits depolarization of the postjunctional membrane by exogenous ACh or carbachol.⁷ In addition, higher than 10 μ M extracellular Mg²⁺ also compete with Ca²⁺ necessary for the formation of the contractile actomyosin complex and inhibits the force of contraction of the completely curarized, directly stimulated muscle.** It is of interest that while the presynaptic effect of Mg²⁺ and Ca²⁺ and their effect on the development of the contractile force of the muscle are mutually antagonistic, they both inhibit depolarization of the postjunctional membrane by ACh.¹⁹ In agreement with this, in the rat hemidiaphragm preparation in the absence of drugs, increasing the extracellular [Ca²⁺] from the physiological 1.1 mM to 2.0 mM, instead of increasing, slightly inhibits neuromuscular transmission.** It is conceivable that Mg²⁺ could inhibit synthesis of ACh, re-uptake of choline, or packaging of the synaptic vesicles with ACh. These possibilities could be excluded, because of the relatively fast onset of the neuromuscular effect of Mg²⁺. Furthermore, if any of the above mechanisms would be inhibited, the rate of replenishment of the pool of ACh readily available for release would also be inhibited, and decreasing the time available for replacement of the ACh released by the nerve impulse, by increasing stimulation rates, should increase the intensity of the partial Mg²⁺ block. In fact, the opposite is true: increasing stimulation rate, instead of decreasing, increased the force of contraction of the indirectly stimulated *in vitro* hemidiaphragm preparation (see table 2). By process of elimination it may be concluded that increased extracellular [Mg²⁺] inhibits neuromuscular transmission primarily by inhibiting the rate of release of ACh. Mo-

** Foldes FF, unpublished data.

bilization of ACh is not inhibited and therefore the supply of readily releasable ACh may be even higher than in the absence of high extracellular $[Mg^{2+}]$. Because of this, the release of ACh at the reduced rate permitted by $[Mg^{2+}]$, that cause partial neuromuscular block, can be maintained even at high stimulation rates. Whatever other inhibitory effect of the extracellular $[Mg^{2+}]$ may have on neuromuscular transmission appears to be more than compensated for by the facilitation of the contractile force of the muscle caused by higher stimulation rates. In agreement with this, during partial Mg^{2+} block increasing stimulation rates, instead of decreasing, increase the force of contraction of the muscle (see table 2, fig. 1).

In clinical practice, the influence of increasing the stimulation rate from 0.1 to 2.0 Hz $(T4/T1)^{20}$ is used for the evaluation of the state of neuromuscular transmission. The use of the T4/T1 ratio represents a special application of the general principle described in this study. The findings presented indicate that the absence of any decrease, or a moderate increase of the force of contraction during stimulation with 1 or 2 s trains of 5 Hz stimuli, would be a more convincing sign of the reestablishment of neuromuscular transmission than the observation of the T4/T1 ratio.

In conclusion, the data presented support the assumption that d-Tc and other nondepolarizing muscle relaxants²¹ inhibit mobilization of ACh from reserve depots to release sites. Inhibition of mobilization of ACh contributes significantly to the neuromuscular blocking effect of nondepolarizing relaxants at higher stimulation rates. In view of the fact that in mammals (including humans), all voluntary muscle movements are initiated by short trains of tetani,²² the inhibitory effect of d-Tc and other nondepolarizing muscle relaxants on mobilization of ACh may have considerable clinical significance. Our observations also confirm earlier reports^{7,8,19} that the primary neuromuscular effect of Mg^{2+} is the inhibition of the rate of the evoked release of ACh.

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