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At Least Three Sequential Steps are Involved in the Tetanus Toxin-Induced Block of Neuromuscular Transmission

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Summary. Tetanus toxin causes a block of the neuromuscular transmission. The kinetic aspects of the block were studied in vitro on the mouse phrenic nerve-hemidiaphragm exposed to toxin (1 μg/ml).

1. The toxin action on the nerve ending involves three sequential steps: binding, "translocation" and paralysis.

2. Diffusion and binding of tetanus toxin molecules to the presynaptic membrane is complete in about 60 min. The binding step is irreversible, independent of transmitter release and of the temperature. Tetanus antitoxin, however, inactivates the bound toxin molecules.

3. After a second step which is probably due to a "translocation" of the toxin molecules into or through the presynaptic membrane the antitoxin molecules are now ineffective to prevent the toxin-induced inhibition of transmitter release. This so called "translocation" step requires transmitter release and therefore depends strongly on the frequency of nerve stimulation.

4. The paralytic step does not depend on the transmitter release. It, however, depends strongly on temperature with a break in the Arrhenius-plot around 33° C which suggests the involvement of a phase transition rather than of an enzymatic activity of the toxin.

Key words: Tetanus toxin – Neuromuscular junction – Binding – Translocation – Paralysis

Introduction

In mammals tetanus toxin produces a spastic paralysis which is attributed to its action on the central nervous system. But it acts as well at the motor endplates inducing a block of neuromuscular transmission. This could be verified by clinical observations and was supported by local application of the toxin to skeletal muscles of whole animals (for references see Habermann et al. 1980).

Recently the paralysis induced by tetanus toxin could also be demonstrated on the isolated phrenic nerve-hemidiaphragm preparation of the mouse (Habermann et al. 1980). This system allows to study directly the processes leading to the development of paralysis and to compare them with the action of botulinum toxin (Simpson 1973, 1980; Habermann et al. 1980). For both toxins the time course of muscle paralysis depended strongly on temperature, on transmitter release and on the time at which antitoxins were added to the poisoned preparations. From these experiments it was concluded that so far no differences exist between the action of both toxins except that the tetanus toxin concentration necessary to block the transmitter release was about 500 times higher than for botulinum A toxin (Habermann et al. 1980). No paralysis could be produced with comparable tetanus toxin concentrations in the isolated rat diaphragm preparation although the toxin has been shown to paralyse this muscle when applied by local injection in vivo (Kryzhanovsky 1973). This indicated that the time course of the effect is largely influenced by diffusion of the toxin molecules to the motor endplates.

In order to describe these and further aspects of tetanus toxin paralysis by a plausible theory, we started with the assumption that three sequential steps similar to those postulated for the botulinum A toxin by Simpson (1980) are involved in the action of toxin at the mouse diaphragm. The aim of the present investigation was to show the involvement of different steps and to characterize them. A preliminary report of these studies has been published (Schmitt et al. 1981).

Methods

The left mouse phrenic nerve-diaphragm was mounted in an organ bath filled with 10 ml Krebs-Ringer solution (in mmol/l: NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11) bubbled with 95% O_2 and 5% CO_2. The pH was 7.4. The time required for changing the bath temperature from 18° C to 37° C was about 2 min.

The N. phrenicus was stimulated via two ring platinum electrodes by supramaximal stimuli of 3–6 V amplitude and of 0.1 ms pulse duration.

The isometric muscle contractions were recorded via a force transducer on a pen recorder with a cut-off frequency of 150 Hz. The resting tension was always set to about 15 mN. In control experiments the contraction amplitude of the muscle stimulated with 0.1 or 1 Hz was constant for at least 6 h. Stimulation frequencies beyond 1.5 Hz led to a progressive decline of the contraction amplitude. The diaphragms were always taken from mice of 30 g weight to reduce variations in the time course of poisoning due to diffusion of toxin molecules to the motor endplates. Under different experimental conditions such as low or high temperature, nerve stimulation etc. the different sequential steps involved in toxin induced paralysis could be separated and the single steps could be studied without distortion from the others.

Tetanus toxin was kindly provided by Dr. Bizzini (Institut Pasteur, Paris, France). Its LD_{50} (mice, s.c. in the neck

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region) was about 3 ng/kg. Tetanus antitoxin (horse, Fermo-Antiserum, 5,000 U/ml) was from the Behringwerke, Marburg, FRG. In all experiments a final tetanus toxin concentration of 1 µg/ml was used. If tetanus antitoxin was applied the concentration was 25 U/ml which represented at least a tenfold excess over toxin (Habermann et al. 1980).

Results

Figure 1 shows an original recording of the muscle contractions of the diaphragm poisoned with tetanus toxin. After a latency of about 60 min the amplitude decreases and after about 120 min it is disappeared. At this point the application of 4-aminopyridine (500 µmol/l) did not restore muscle contractions.

1. Diffusion of Toxin Molecules to the Motor Endplates

The weight of mice and so of the diaphragms strongly influenced the time course of poisoning. The paralytic time was longer in thicker preparations, most probably because the diffusion of the toxin macromolecules is impeded by connective tissue and muscle fiber layers. Therefore the diffusion to its target had to be separated from the action of tetanus toxin on the nerve ending.

To find out the diffusion time the diaphragms were incubated in tetanus toxin at 18° C. At this temperature the poisoned muscles showed no signs of paralysis up to 6 h (Habermann et al. 1980 and this paper). At varying time intervals the toxin was washed out and the muscle warmed up to 37° C. The time required from rewarming to 50 % reduction of the contraction was defined as the paralytic time t_p. After 60 min incubation a nearly constant, minimum t_p-value was reached suggesting that the diffusion process was complete (Fig. 2). Increasing the nerve stimulation frequency from 0.1 Hz to 1.5 Hz shortened the paralytic time (see below) without changing the curve. Therefore to avoid the distortion of the paralytic time by the slow diffusion process all muscles were preincubated with toxin for 60 min at 18° C before starting the other experimental procedures.

2. Binding of Tetanus Toxin to Presynaptic Membrane

Bound Toxin Molecules Cannot be Washed Out. In all experiments the muscle was examined at 37° C and 0.1 Hz nerve stimulation to see if it has the normal contraction amplitudes. It was then cooled to 18° C, the nerve stimulation was stopped and tetanus toxin applied. In the first experiment (Fig. 3a) the muscle was rewarmed after 60 min to 37° C and the nerve stimulated with 0.1 Hz. Tetanus toxin was present in the bath solution throughout. Within about 70 min the contraction faded to 50 %.

In the second experiment (Fig. 3b) the muscle was incubated again in toxin for 60 min. But now the tissue was extensively washed with toxin free solution for another hour at 18° C without nerve stimulation. Then the muscle was rewarmed to 37° C and the nerve stimulated. The obtained t_p-value was statistically not different from that without washing. Any significant release of bound toxin from the nerve terminal membrane should have delayed the paralysis.

Inactivation of Bound Toxin Molecules by Antitoxin. The experiment shown in Fig. 3b was repeated except that the

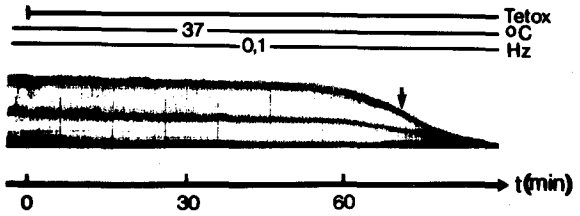

Fig. 1. Time course of the development of muscle paralysis induced by tetanus toxin (*Tetox*). The mouse diaphragm was poisoned with 1 µg/ml toxin at zero time and stimulated continuously with 0.1 Hz at 37° C. The *arrow* indicates the paralytic time t_p where the contraction amplitude decreased to 50 % of its initial value

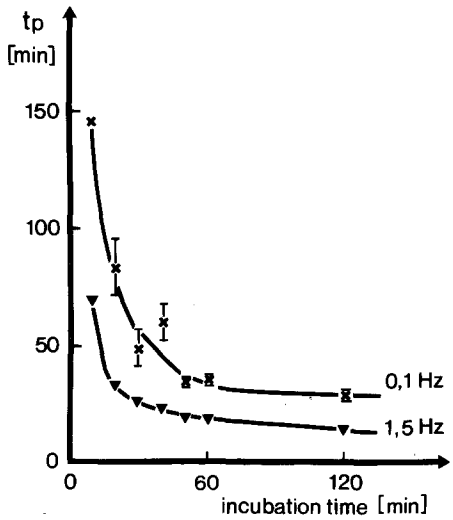

Fig. 2. Dependence of the paralytic time t_p on the tetanus toxin preincubation time at 18° C of the nerve-muscle preparation. Details of the experimental procedure are given in the text. The diaphragm was continuously stimulated with 0.1 Hz (crosses, each point is the mean value ± S.E.) or 1.5 Hz (triangles, single data points)

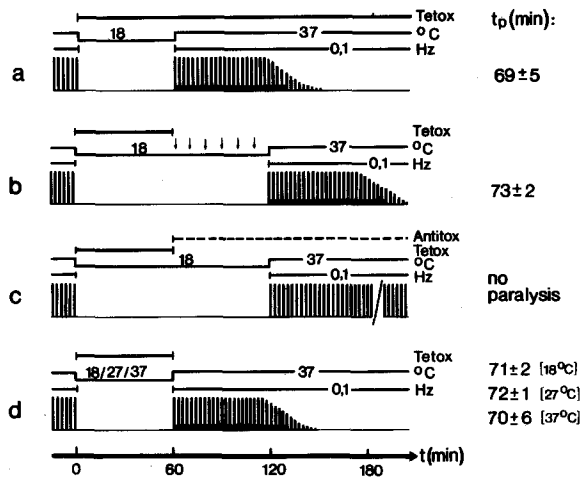

Fig. 3. Characterization of the binding step. The different experimental protocols are presented in *a* to *d* schematically. In each protocol the upper line indicates the presence of toxin (*Tetox*) or antitoxin (*Antitox*) (*dashed line*) in the bath solution, the middle one the degree of bath temperature, and the lower one the nerve stimulation with the respective frequencies. The thick line at the bottom of the contraction amplitudes demonstrates how the paralytic times t_p are defined (see Fig. 1). The numbers on the right are the mean t_p-values ± S.E. of three preparations. In *b* the downward *arrows* indicate the repeated washing of the preparation with toxin free solution

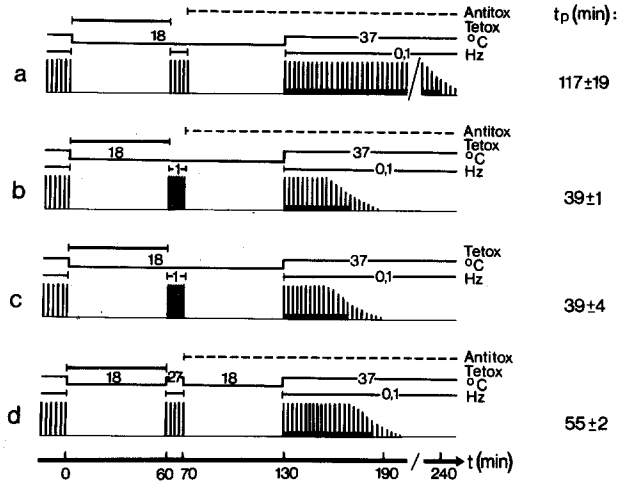

Fig. 4. Characterization of the "translocation" step of tetanus toxin. Schematic drawing of the protocol, together with the mean t_p-values ($\bar{x} \pm$ S.E., $n = 3$). The paralytic time t_p (thick line at the bottom of the contraction amplitudes) is the time between the rewarming of the muscle preparation to 37° C (130 min taken as zero time) and the reduction of the contraction amplitude to 50% of its initial value. For further explanations see Fig. 3

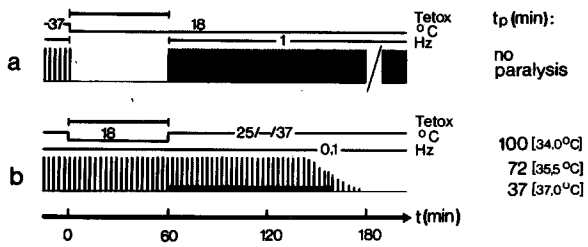

Fig. 5a and b. Temperature dependence of the paralytic step. Schematic drawing of the experimental protocol. (**a**) No signs of paralysis at 18° C. (**b**) At higher temperatures a muscle paralysis could be observed. Examples of paralytic times at different temperatures (in parentheses) are given on the right (*single data points*). For details see text

tetanus antitoxin was applied after 1 h toxin incubation. The antitoxin completely prevented the occurrence of paralysis (Fig. 3c).

The Toxin Binding Does not Depend on Temperature. When the toxin incubation was performed at three different temperatures without nerve stimulation no differences in the t_p-values were obtained (Fig. 3d).

These experiments clearly show that 1 h incubation at low temperature and without nerve stimulation is sufficient to cause not only a complete diffusion but also a complete binding of toxin molecules to the presynaptic membrane.

3. *'Translocation' of Toxin Molecules Within the Membrane*

'Translocation' Depends Strongly on Transmitter Release. The experiment in Fig. 3c clearly demonstrates that under special conditions the paralytic effect of tetanus toxin can be totally abolished by antitoxin (see also Habermann et al. 1980). However, the antitoxin is ineffective if the nerve is stimulated for 10 min with 0.1 Hz before the application of antitoxin (Fig. 4a). This indicates that a process linked with the transmitter release is crucial for the development of paralysis. We called this step 'translocation' following the nomenclature of Simpson (1980) without knowing whether or not there is a real transport of toxin molecules into or through the presynaptic membrane.

The delayed onset of paralysis shown in Fig. 4 as compared to the values of Fig. 3 suggests that during the short and low frequency nerve stimulation not all the bound toxin molecules were involved in the translocation process. So a part could still be reached and inactivated by antitoxin. To test this hypothesis the experiment in Fig. 4 a was repeated with the stimulation frequency raised to 1 Hz (Fig. 4b). The paralytic time was dramatically shortened to about 40 min demonstrating once more the importance of transmitter release in the development of toxin induced paralysis.

After Termination of the 'Translocation' Step Antitoxin has no Effect on Muscle Paralysis. If the experiment in Fig. 4b is repeated without antitoxin application no change in t_p-value was found (Fig. 4c). Obviously the condition of 1 Hz for 10 min is sufficient to translocate all toxin molecules necessary to produce the paralysis in the shortest time. So if the 'translocation' step is completed antitoxin has no further influence on the development of paralysis.

Temperature Dependence. To find out the influence of temperature on the 'translocation' process the experiment in Fig. 4a was repeated with the only difference that the short stimulation period was done at 27° C instead of 18° C (Fig. 4d). The paralytic time was reduced by a factor of 2. This finding allows two interpretations. Either the 'translocation' step is directly temperature dependent, or the shortening of t_p is due to an increased transmitter release induced by higher temperature. Electrophysiological experiments with intracellular recordings show an increase of the quantal content of endplate potentials by 40% (Glavinovic 1979) to 80% (own observations) upon increasing the temperature from 18° C to 27° C. Choosing a frequency of 0.2 Hz in the 18° C-period in Fig. 4a yielded t_p-values of about 75 min. Therefore, the shortening of t_p at 27° C can be explained partly by an increased transmitter release.

4. *The Paralytic Step*

Development of Paralysis Depends Strongly on Temperature. After 10 min nerve stimulation with 1 Hz all the paralysis inducing toxin molecules are translocated (Fig. 4c) and inaccessible for antitoxin (Fig. 4b). At this stage warming the muscle to 37° C led to paralysis in a short time. However, if the temperature was kept at 18° C throughout the experiment no signs of paralysis were observed during 6 h even if the nerve was stimulated with 1 Hz (Fig. 5a).

To elucidate the temperature dependence of the paralytic step the muscle was kept at 18° C and the nerve stimulated for 1 h to allow completion of the diffusion, binding and 'translocation' processes. Then the temperature was raised to different values some of them indicated in Fig. 5b. The result of this experiment is expressed in terms of an Arrhenius-plot (Fig. 6). In a temperature range between 25° C and about 32° C t_p decreases exponentially with the inverse of the absolute temperature. At about 33° C the slope of the relationship changes abruptly. While in the range below 33° C a Q_{10}-value of about 4 can be calculated, the relationship

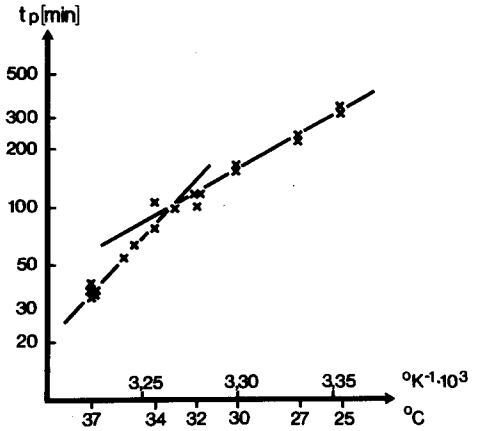

Fig. 6. Dependence of the paralytic time t_p on temperature presented by an Arrhenius-plot. The t_p-values are plotted semilogarithmically against the reciprocal of the absolute temperature, K^{-1} (Kelvin). Temperatures in °C are given below the abscissa. Three data points are taken from Fig. 5b. The solid lines are drawn by eye. The transition temperature is about 33° C. In the range of 25 to 33° C the slope of the line is equivalent to a Q_{10}-value of 4

Table 1. The main properties of the three sequential steps involved in the blockade of neuromuscular transmission due to tetanus toxin

	1. Binding	2. 'Translocation'	3. Paralysis
Inhibition by antitoxin	yes	no	no
Transmitter release indispensible	no	yes	no
Temperature dependence	no	weak	extreme
Neuromuscular transmission	normal	normal	blocked

beyond the transition temperature shows an extreme temperature dependence (see discussion).

To find out how long exposure to high temperature was required to induce the paralytic step the temperature was raised to 37° C for 10 min and then lowered again to 18° C. No paralysis was observed. Only after 15 min at 37° C the paralytic process was triggered. But then it could not be inhibited or delayed by lowering the temperature to 18° C again.

Transmitter Release Does not Influence the Paralytic Process. Higher or lower nerve stimulation frequencies in the rewarming period of the experiments shown in Fig. 4b, c and 5b produced only slight changes of about 10% in the paralytic time. So no significant influence on the paralytic step could be attributed to the transmitter release.

The main properties of each step are summarized in Table 1.

Discussion

Our results allow the distinction of three different steps for the tetanus toxin induced blockade of neuromuscular transmission. The steps have their specific properties as summarized in Table 1.

Simpson (1980) proposed a three step mechanism of the botulinum A toxin induced neuromuscular block using the isolated rat phrenic nerve-hemidiaphragm preparation. There is, however, no report of such steps in the development of the tetanus toxin induced muscle paralysis.

Our study on the binding step could basically be compared to the results obtained with botulinum A toxin by Simpson (1980). A similar binding step of tetanus and botulinum A toxin action has been demonstrated for the inhibition of transmitter uptake (Habermann et al. 1981) and transmitter release (Bigalke et al. 1981) in brain particles.

The 'translocation' and the paralytic steps show different characteristics in our system from those proposed for botulinum A toxin. For the latter there has been only weak evidence that the nerve evoked transmitter release tends to accelerate the translocation process (Simpson 1980). However, our experiments strongly indicate that the translocation step has an absolute dependence on transmitter release whether spontaneous or nerve evoked. Also a nerve activity-dependent paralytic step for botulinum A toxin has been suggested (Simpson 1980). Such a conclusion could not be drawn from our present study. It may be possible that in this respect tetanus and botulinum A toxin have different action.

The word 'translocation step' may favour the concept that the toxin molecules move through the membrane and therefore are not accessible to antitoxin. It is known that tetanus toxin molecules have to be incorporated at the motor endplates to reach the central nervous system by axonal flow. But we do not know whether the same toxin molecules are also responsible for the block of transmitter release. Alternatively the toxin molecules might bind to sites near the active zones of the nerve terminal. The transmitter release should allow the incorporation of the toxin macromolecule with only its toxic moiety into the membrane, while one or more antigenic sites remain directed to the synaptic cleft and thus accessible to the antitoxin which no longer inactivates the toxic part of the macromolecule.

In our study the temperature dependence of the paralytic process shows a break in the Arrhenius-plot around 33° C with a steep slope to higher temperature values. Therefore any direct or indirect enzymatic effect of the toxin seems quite improbable. More likely the break may result from a phase transition of some unknown nerve ending structure involved in the paralytic process of tetanus toxin. To trigger this last step the temperature must be high for a minimum of time (about 15 min at 37° C). As soon as this process has started, the development of paralysis cannot be stopped by reducing the temperature to 18° C.

In the present study we have shown the methodology which allows a distinct separation of three sequential steps involved in the action of tetanus toxin on the neuromuscular junction. Our results have also shown some differences in the 'translocation' and paralytic steps for tetanus toxin when compared to those with botulinum toxin (Simpson 1980). This finding is important because so far it seems that tetanus and botulinum A toxin are very similar in their action on motor endplates and brain synaptosomes. But it is doubtful if both toxins share their sites of action. Results obtained with electrophysiological methods strongly suggest different effects of both toxins on the coupling between nerve depolarization and transmitter release (Dreyer and Schmitt 1981).

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