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955 — THE RATE-LIMITING STEPS OF THE RISING PHASE OF MINIATURE END PLATE CURRENTS IN THE MOUSE DIAPHRAGM *

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SUMMARY

To gain insight about the processes controlling the opening rate of postsynaptic channels following the release of a quantum of acetylcholine, the onset rate of miniature end plate currents (MEPCs) was evaluated at different temperatures and in a high viscosity medium.

The onset rate of MEPCs, measured as the rise time from 10% to 50% of the peak amplitude, is remarkably sensitive to temperature in the range from 9 °C to about 19 °C, while it is almost constant at higher temperatures (from 20 to 39 °C).

The viscosity of the medium affects the onset rate of MEPCs significantly: when 10% Dextran is added to the bath solution, the rise time is slowed down by about 20%.

The present results suggest that at high temperatures (above about 19 °C) the rate of the rising phase of MEPCs is limited only by the rate of acetylcholine diffusion; at low temperatures (below about 19 °C), the binding of neurotransmitter and/or the subsequent protein conformational change leading to opening of the acetylcholine channel become so slow as to reduce greatly the onset rate of MEPCs.

The relative contribution of these three processes to the overall kinetics of the rising phase of MEPCs is not investigated here.

INTRODUCTION

The release of a quantum of acetylcholine (Ach) at the neuromuscular junction induces a transient current inflow called miniature end plate current (MEPC). While consistent information about the falling phase of MEPCs has been collected in recent years, the nature of the processes determining the time course of the rising phase is still not well established.

Possible rate-limiting steps of the MEPCs rising phase are:

(i) Ach diffusion through the synaptic cleft;
(ii) binding of Ach to postsynaptic receptors;
(iii) conformational change of channel proteins leading to channel opening.

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The aim of the present work was to try to clarify, at least in part, which steps actually limit the rising rate of MEPCs in the mouse phrenic-diaphragm preparation. To this end, we measured the onset rate of MEPCs at different temperatures and at medium viscosity. The diffusion rate of Ach is scarcely modified by temperature but it is sensitive to medium viscosity; on the contrary, the rate of binding and of protein conformational change is believed to be very sensitive to temperature and is probably insensitive to medium viscosity. Therefore, according to the effects of temperature and viscosity on the onset rate of MEPCs, we will be able to infer whether the rate of Ach diffusion and/or the rate of binding and conformational change are capable of affecting the time course of the MEPC rising phase.

Data in the literature are controversial. Gage and McBurney [1] reported that the rising phase of MEPCs is relatively insensitive to temperature while Dwyer [2] and Head [3] proposed a pure exponential dependence of the rising phase of MEPCs upon temperature. Dwyer [2] suggests that the process of Ach diffusion through the synaptic cleft does not limit the onset rate of MEPCs but Land *et al.* [4,5] reach the opposite conclusion. Finally, the binding process is considered by some authors to be very fast both with respect to diffusion and to conformational change [6,7] while, according to others [4], it seems to have an actual weight in limiting the MEPC rising phase.

MATERIALS AND METHODS

The experiments were performed using mouse phrenic-diaphragm preparations placed in a 30 cm^3 recording chamber filled with Krebs solution bubbled with 95% O_2 and 5% CO_2 which was renewed several times during the experiment.

MEPCs were recorded by means of extracellular electrodes with resistances of 0.5–1 MΩ and tips covered with Sylgard resin. The frequency response of the leading off apparatus was tested by injecting a sinusoidal current of constant amplitude and increasing frequency through the recording electrode still placed against the muscle and recording the MEPCs. The resulting voltage deflection maintained almost constant values (the voltage decrease was less than 5%) up to at least 7 kHz, a frequency response adequate to resolve the time course of the MEPC rising phase. In fact, its highest frequency content, evaluated by frequency analysis, was always less than 7 kHz.

A number of MEPCs were recorded at various temperatures, digitized with a sampling rate of 100 kHz and stored on disk.

The analysis was performed off-line; the signals were accurately examined on the computer display and the first point followed only by points of increasing amplitude was taken as the starting point of the MEPC rising phase. This criterion was usually unambiguous owing to the good signal-to-noise ratio of the recorded signals. Only at low temperatures (below about 13° C), when MEPCs display smaller amplitudes, was the starting point sometimes not clearly identifiable. However, the long rise

times exhibited by these MEPCs make the possible errors negligible. The averaging of ten rising phases, synchronized at their starting points, was performed thereafter.

As an estimate of the onset rate of MEPCs, we used the time taken to rise from 10% to 50% of the peak value.

The temperature, measured by a small thermistor placed close to the muscle, was changed by simply adding cold or hot saline to the bath and waiting for about 30 s to avoid temperature gradients. The bath had a thermal inertia sufficient to maintain its temperature to at least within $\pm 0.5°C$ during the recording time.

The viscosity of the Krebs solution was increased by adding 10% Dextran, *m.w.* 500 000; this is known to decrease the Ach diffusion rate in water by about a third [2].

RESULTS AND DISCUSSION

Two MEPCs, recorded at 21 and 37°C respectively, are shown in Fig. 1. The rising phases, at these two temperatures, are over in about 250 μs; the falling phases are much slower and exhibit the usual exponential decay very sensitive to temperature.

An example of the effect of temperature on the MEPC onset rate is shown in Fig. 2, where the averaging rising phases of two MEPCs, recorded at 6.8 and 16.1°C respectively, are compared.

The rise times recorded at different temperatures, in the entire range from 7°C to 39°C, are plotted in Fig. 3. Simple visual inspection of the plot suggests that from 39°C to about 19°C the rise times maintained almost constant values, while from 19°C to 7°C there was a remarkable dependence upon temperature. To establish more quantitatively that the effect of temperature on the rise times tends to disappear in a higher temperature range, where the rise times attain almost constant values, we fitted the experimental data with an exponential function and an exponential function plus a constant term. This second function, shown in Fig. 3,

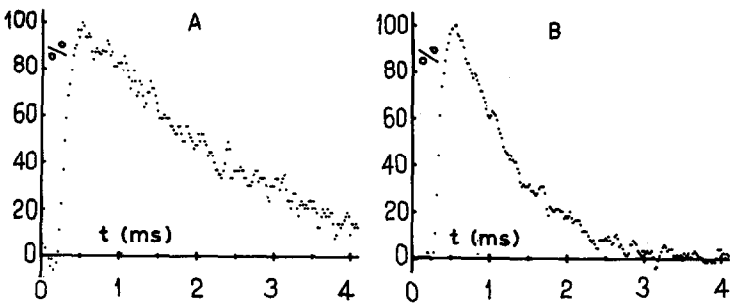

Fig. 1. MEPCs recorded at 21°C (left, A) and 37°C (right, B). In this example, to avoid excessive crowding of points during the falling phase, the time interval between subsequent points is 20 μs. MEPCs have been plotted as a percentage of the maximum amplitudes.

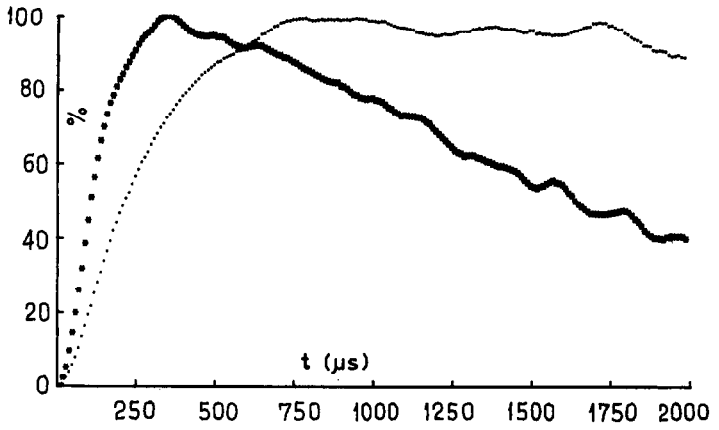

Fig. 2. Rising phase of two MEPCs recorded at 6.8° C (•) and 16.1° C (*), respectively. Each signal is the average from ten MEPCs.

gives a very nice fit of the data and a standard error from squared residuals much less than a simple exponential. Fitting analysis therefore confirms that the rise times tend exponentially to a constant value (about 70 μs) with increasing temperature.

This kind of relationship between temperature and the onset rate of MEPCs suggests that the kinetics of channel opening is limited by at least two processes, one sensitive to temperature and one independent of temperature. The temperature-sensitive process, as we mentioned in the Introduction, can be reasonably identified with the two combined processes of neurotransmitter binding and conformational

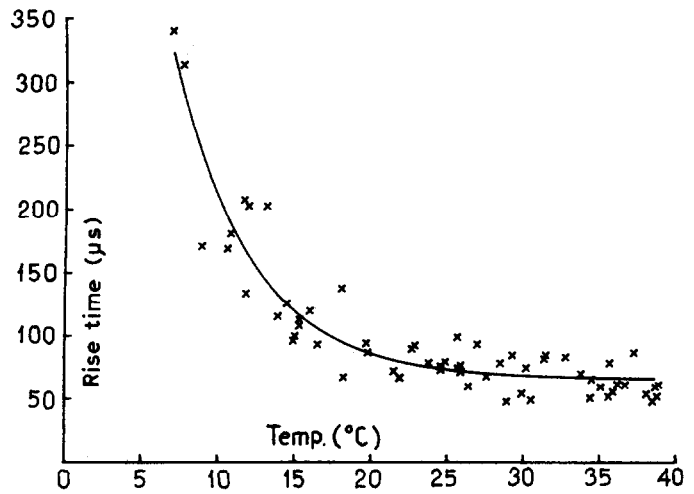

Fig. 3. Effect of temperature on the rise time of MEPCs.

TABLE 1

Effect of 10% Dextran on the rise times of MEPCs recorded at 21° C. Comparison with the control was performed by using the *t*-test

	Rise time (μs, means $\pm$ s.d.)	Obser-vations	End plates
Control	76.1 ± 8.0	13	6
Dextran	92.4 ± 11.3 $p < 0.001$	13	5

change of channel proteins, while the temperature-independent process can be identified with the process of Ach diffusion to the postsynaptic receptors. At low temperatures, the first process is slow and affects the onset rate of MEPCs significantly; by increasing the temperature, it becomes so fast as to be negligible when compared to the Ach diffusion rate and therefore the kinetics of channel opening become independent of temperature.

This view is supported by data obtained by varying the Ach diffusion rate using media of different viscosities. Table 1 shows that the rise time of MEPCs, which turned out to be 76 ± 8 μs in plain Krebs solution at 19° C, becomes significantly slower (92.4 ± 11.3 μs) in high viscosity Krebs solution. Thus, the process of Ach diffusion through the synaptic cleft seems to have an actual weight in limiting the time course of the MEPC rising phase.

It seems to be worthwhile to compare the present data with previous information reported in the literature. Referring to the effect of temperature on the onset rate of MEPCs, direct examination of Dwyer's data (see Table II of Ref. 2) reveals that 3.16 is the fractional increase of the rising times on varying the temperature from 10° C to 1.5° C and only 1.38 from 19° C to 10° C. This supports our data, although the author concludes that a simple exponential relation exists between the rise times and temperature.

On the contrary, Head's data on the rat diaphragm [3] do not indicate that the rise times tend to attain a constant value with increasing temperature.

Concerning the process of Ach diffusion, it results from the kinetic model elaborated by Land *et al.* [4] that 60% (from 20% to 80%) of a quantum of Ach diffuses to the postsynaptic membrane in about 50 μs. This time is similar to the onset time we measured at higher temperatures, where we propose that diffusion is the only rate-limiting step.

Even Dwyer's [2] results about the effect of Dextran on the rising phase of MEPC might not be incompatible with ours. The mean onset rate of MEPCs measured by Dwyer in high viscosity solution in fact proved to be longer than that of controls (about 10%) both with Ach-esterase intact and with Ach-esterase inhibited. This difference might not have been statistically significant due to the high scatter of data.

From the above discussion it is therefore possible to suggest that, in the mouse diaphragm, at high temperatures the opening rate of postsynaptic channels is

limited only by the rate of Ach diffusion while at low temperatures it is further slowed down by the process of neurotransmitter binding and conformational change. More experimental and theoretical work is required to give stronger support to this conclusion and to quantify the relative importance of the above-mentioned processes in controlling the opening rate of postsynaptic channels.

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