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THE RISING PHASE OF THE MINIATURE ENDPLATE CURRENT AT THE FROG NEUROMUSCULAR JUNCTION

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(1) The rising phase of minature endplate currents was recorded at the frog's neuromuscular junction using both the two electrode voltage clamp and a single external electrode, or Strickholm, voltage clamp. (2) The $Q(10)$ of the miniature endplate current rising phase was 2.3 in a variety of solutions selected to alter presynaptic behavior. (3) Increasing the solution's viscosity by an amount sufficient to slow the diffusion coefficient of acetylcholine by a third has no effect on the duration of the rising or the decay phase. This solution does seem to further slow the miniature endplate current decay phase, but not the rising phase, after inhibition of the acetylcholinesterase. (4) As the membrane potential is made more positive, the miniature endplate current rising phase is prolonged, with an *e*-fold slowing per 170 mV change. (5) It is concluded that neither presynaptic nor subsynaptic events determine the rising phase of miniature endplate currents at the frog neuromuscular junction. Rather, the limiting step occurs within the membrane and is most likely a change in the binding constant of the receptor for the acetylcholine molecule.

Introduction

At the frog neuromuscular junction, transmitter is released randomly in small packets [1] producing a brief current (the miniature endplate current) at the postsynaptic membrane. This current flows through channels opened by acetylcholine. The closing of these channels is limited by a step which occurs within the membrane, as shown by the existence of a voltage-dependent rate of miniature endplate current decay [2,3,4]. Anderson and Stevens [5] analyzed acetylcholine noise spectra and showed that the rate of miniature endplate current decay (α) was identical to the rate at which acetylcholine channels closed at the frog neuromuscular junction. The observed Q_{10} of 2.3 is consistent with a membrane, but not a diffusion, limited process.

The events determining the early time course of the miniature endplate current are not the same as those of the nerve-evoked endplate current. The rising phase of the miniature endplate current lasts 1/4 as long as the rising phase of the nerve-evoked endplate current [6,7]. The endplate current results from an asynchronous release of quanta extending over a period of 2 ms, while in the miniature endplate current the release is quantal and presumably instantaneous. Thus the early nerve-evoked endplate current time course is determined by presynaptic events.

The rising phase of the spontaneous miniature endplate current may result from the time required for acetylcholine to diffuse across the subsynaptic cleft, or it may be a membrane-determined event as with the decay phase. The first possibility is unlikely since the calculations of Eccles and Jaeger [8] suggested

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Abbreviations: α, closing rate of acetylcholine channels; γ, single channel conductance; V_r, reversal potential.

that the diffusion of acetylcholine was complete early in the miniature endplate current rising phase, and so simple diffusion alone does not explain miniature endplate current rising phase. Gage and McBurney [9] confirmed this discrepancy but found a Q_{10} of 1.2 for the miniature endplate current rise time, as would be expected for diffusion. There is conflicting evidence concerning the second possibility: the miniature endplate current rise time may be longer [2] or shorter [9,10] at increasingly more positive membrane potentials.

The purpose of this paper is to determine which step limits the opening of the acetylcholine channel. The voltage and temperature dependence of the rising phase and of the rate at which acetylcholine channels open was examined in miniature endplate currents recorded by extracellular electrodes. The information gained in this study was used to explain the non-exponential shape of the miniature endplate current decay after inhibition of the acetylcholinesterase, with normal and with slowed diffusion. Preliminary reports have appeared elsewhere [11,12].

Materials and Methods

The endplates used in these experiments were from the sartorius of the frog *Rana pipiens*. This muscle was stretched to 120% of its resting length to increase the miniature endplate current frequency [13,14]. The muscle was placed, perimysium side down, in a 2-ml lucite chamber, which was cooled by a Peltier device controlled by a feedback circuit (±0.2°C). A dissecting microscope, fitted with polaroid filters, was adequate for finding the smallest myelinated motoneurons. Since the endplates were unmyelinated, they could be detected only by probing the muscle fiber with the voltage electrode (3–5 MΩ) until external miniature endplate currents or rapidly rising (<2 ms) internally recorded miniature endplate potentials were seen. Low resistance (0.5–2 MΩ) electrodes for passing current were manufactured by grinding ordinary micropipettes against a brass piece embedded with diamond dust (0–1 μm particles). Electrodes were filled with 3 M KCl for internal recording or the appropriate Ringer's solution for external recording.

The normal Ringer's solution was 2.5 mM KCl, 115 mM NaCl, 1.8 mM $CaCl_2$ and 3 mM phosphate buffer. The pH was adjusted to 7.2. The recordings were regularly made shortly after changing to a 6.5 mM KCl Ringer's solution to increase miniature endplate current frequency. The high viscosity Ringer's solution was a 10% solution of 500 000 molecular weight dextran (Sigma, St. Louis, MO) dissolved in the 6.5 mM KCl Ringer's solution. In this solution, the viscosity was increased 20-fold over the standard Ringer's solution [12] as measured by a Cannon-Fenske No. 100 viscometer. The diffusion coefficient of acetylcholine (D_{ACh}) was slowed 38%, from $(8.8 \pm 0.3) \cdot 10^{-6}$ to $(5.6 \pm 0.2) \cdot 10^{-6}$ cm^2 · s ($n = 4$) [15] as measured by diffusion of radioactive acetylcholine from a capillary tube [16]. This value compares well with that of Krnjevic and Mitchell [17], who measured D_{ACh} in agar to be $(9.8 \pm 0.4) \cdot 10^{-6}$ cm^2 · s at 20°C. Dreyer and Peper [18] reported $D_{ACh} = (11.9 \pm 0.4) \cdot 10^{-6}$ cm^2 · s, and Peper et al. [19] reported $D_{ACh} = (10.4 \pm 0.4) \cdot 10^{-6}$ cm^2 · s using similar techniques, both values being obtained at 23°C. The low ionic strength Ringer's solution had 4/5 of the NaCl replaced isosmotically with sucrose. High magnesium Ringer's solution contained 6.5 mM KCl, 109 mM NaCl, 3 mM phosphate buffer, 0.08 mM CaCl, and 5.5 mM $MgCl_2$. Neostigmine was used at about 0.01 mM ($(2–5) \cdot 10^{-6}$ μg/ml). To reach this concentration, 0.3 to 1 ml of a 1/10 dilution of 1/1 000 neostigmine hydrochloride (Roche, New Jersey) was added directly to the bath and mixed or allowed to diffuse to equilibrium for 30 min. This concentration has been thought to completely inhibit junctional acetylcholinesterase (see, for instance Ref. 3). However, neostigmine inhibits little more than three quarters of the activity of junctional acetylcholinesterase, even at supra-maximal doses [20].

Two methods of recording miniature endplate currents were used, a two-electrode voltage clamp and a Strickholm (patch) clamp. Both methods used a preamplifier with capacity neutralization whose bandwidth was 30 kHz for the two-electrode clamp and 100 kHz for the patch clamp. In both cases, miniature endplate currents were captured by a device which incorporated the features of the digital recording system previously described [21] but also included a transient capturing circuit so that the initial baseline, the miniature endplate current rising phase, and a portion of the decay phase was stored for analysis. The time resolution of this circuit was

5 μs. Since the signal was greater than half of the range of the 8-bit A-D converter, the resolution of the amplitude was 0.2 to 0.5%. A detailed description is available [15].

The two-electrode clamp had the standard configuration [2,3,15,22]. The bandwidth of this clamp was 10 kHz as measured using a model circuit, and 5–10 kHz using a clamped muscle fiber [15]. This is approximately the bandwidth of an exponentially rising function which has a rise time of 70 μs [23]. Thus, this method did not accurately record the miniature endplate current rise time, but was used to compare data reported in this paper with that from previous reports. All studies of the miniature endplate current decay phase used this clamp exclusively.

In contrast, extracellular electrodes accurately record the early time course of miniature endplate current [24,25]. As shown by Strickholm [26,27], extracellular electrodes can be modified such that a patch of membrane can be polarized away from the resting potential. The time constant of such a system is less than 10 μs and the signal to noise ratio is better than six. Thus, these records are an accurate measure of the miniature endplate currents rising phase.

All values are given to two significant digits as mean ± S.E. and P values were obtained from the Student's t-test.

Results

Miniature endplate currents recorded by the Strickholm voltage clamp method grew to their peak value within 200 to 900 μs depending on temperature and membrane potential. They then decayed as a single exponential, just as when recorded by the standard two-electrode voltage clamp [6,14]. However, in a variety of solutions, the rising phase was briefer when recorded by the Strickholm clamp than when recorded by the two-electrode clamp (Table I). Just as with the two-electrode clamp, the Strickholm electrode also recorded occasional miniature endplate currents which required 1–2 ms to reach their peak amplitude, decayed more slowly than usual, and yet were no larger than the more common type. These slow miniature endplate currents were not included in the data of this paper. A third type of miniature endplate current, shown in Fig. 2, appeared to be the result of two, nearly simultaneous, individual miniature endplate currents. While the statistics of random processes [22] predict that only one miniature endplate current in 10^5 or less would be followed by a second miniature endplate current within 0.5 ms, (at the observed miniature endplate current frequency of 2 s^{-1}), actually eight could be discerned in a series of 250. These eight were also excluded from the analysis in this paper.

TABLE I

RISE TIMES OF MINIATURE ENDPLATE CURRENT

The 20–80% rise times of miniature endplate currents were recorded internally and externally. Neostigmine prolonged the rise time, but increasing the solution's viscosity had no significant effect. All experiments were conducted at 10 ± 1°C, and the number of endplates examined is given in parenthesis.

	Recorded rise time (μs)	
	Externally	Internally
Control	250 ± 30 (13)	500 ± 60 (8)
High viscosity (η)	280 ± 80 (15)	–
Neostigmine	470 ± 60 (4)	640 ± 60 (7)
Neostigmine-high η	520 ± 60 (10)	710 ± 110 (7)

Miniature endplate current rising phase

Fig. 1 shows a digitized record of a single miniature endplate current displayed first at a slow then at a fast sweep speed, allowing measurement of both the decay and the rising phase of the same event. Since the very early part of the miniature endplate current was obscured by the baseline noise, the precise origin and initial kinetic properties are uncertain. The duration of the miniature endplate current rising phase was approximated by the time required to grow from 20% to 80% of the peak amplitude (the rise time, t_r). In this example the rise time was 240 μs. The mean rise time for all experiments at 10 ± 0.5°C was 250 ± 30 (13 endplates).

The miniature endplate current rise time was doubled by an 8°C increase bath temperature. As illustrated by Fig. 2, the mean rise time at 9.5 ± 0.5°C was significantly different from that at 21 ± 1°C (250 ± 30 vs. 140 ± 10 μs; n = 13,20; $P < 0.001$). The temperature dependence is seen as clearly in individ-

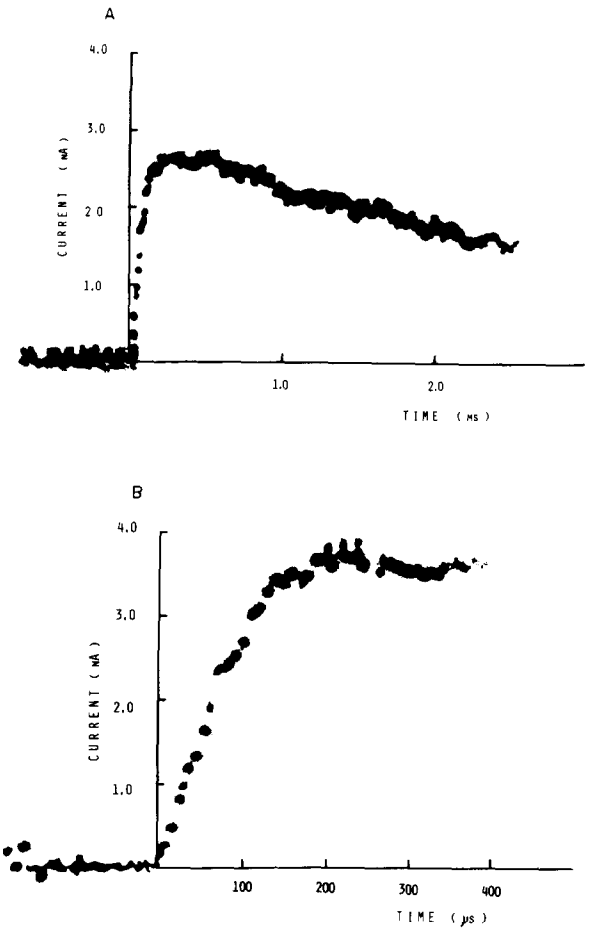

Fig. 1. The rising phase of a miniature endplate current. A sample miniature endplate current recorded using the Strickholm clamp is displayed at two sweep speeds. The digitized record was retouched for clarity. The sweep speed of the upper figure was 2.5-times that in the lower. The rise time (240 μs) was close to the average for this experiment (280 ± 30 μs, n = 23). The resting potential was −70 mv and bath temperature was 9.5°C. The amplitude of the current was calculated from the recorded electrode and seal resistance as well as the recorded voltage record.

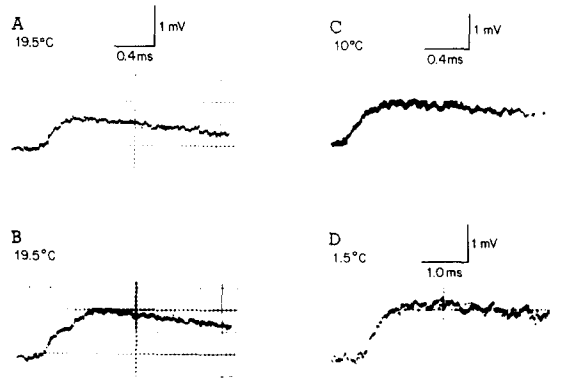

Fig. 2. Temperature dependence of the rising phase. These records were obtained using the Strickholm clamp in the low ionic strength Ringer's solution. Record of A is a typical miniature endplate current, showing the usual abrupt rise (t_r = 130 μs). Record B is representative of those miniature endplate currents with prolonged rising phases which have inflections. Cooling the preparation to 10°C slowed the average rise time 41%. Record C shown here had a rise time within one standard deviation of the mean (t_r = 170 μs). Cooling the preparation further to 1.5°C slowed the rise time two-fold. The example shown had a rise time within a standard deviation of the mean (t_r = 510 μs).

ual experiments; the most complete example, plotted as open circles in this figure, had a Q_{10} very similar to the mean (2.1 vs. 2.3 for the mean, see Table II).

At no temperature did modifying solutions in a way designed to effect presynaptic or subsynaptic events have an effect on the rising phase as long as the cholinesterase was intact. For instance, the rise time was unaffected by raising the magnesium concentration or by decreasing the ionic strength of the bathing solution, as can be seen in Fig. 3. Slowing the diffusion coefficient 38% by raising the solution's viscosity [14] had no significant effect on the rise time (280 ± 80 μs vs. 250 ± 30 μs for control, all at 9.5 ± 0.5°C).

The duration of the miniature endplate current growth phase was as voltage dependent as was the miniature endplate current decay rate. Fig. 4 shows that the miniature endplate current rise time was longer at +37 mV, 190 μs, than at −80 mV, 130 μs. Also apparent is the more rounded peak at +37 mV, compared to −80 mV, which again demonstrates that the processes which cause the growth of the miniature endplate current are prolonged at more positive membrane potentials. Table III shows that the mean rise time was prolonged by 55%. For comparison, the rate of decay was increased by 64%. Another fiber in the same muscle was examined first at the resting potential, next at a hyperpolarized potential and finally at the resting potential again. As there was no significant difference between the first and last group, the values were combined and are shown in Table III. Although the recordings took place over a

TABLE II

TEMPERATURE DEPENDENCE OF MINIATURE ENDPLATE CURRENT PARAMETERS

Amplitudes are tabulated as recorded; insufficient information is available to convert the values to currents. Membrane potentials ranged from −72 to −85 mV. All values differ, $P < 0.005$.

Parameter	Parameters at			Q_{10}
	19°C	10°C	1.5°C	
t_r (μs)	130 ± 10	180 ± 10	570 ± 30	2.3
α (ms^{-1})	0.58 ± 0.02	0.28 ± 0.01	0.11 ± 0.06	2.5
Amplitude (mV)	−3.1 ± 0.1	−2.6 ± 0.1	−1.8 ± 0.1	1.4
N	32	30	36	

small voltage range, the parameters have the same magnitude of voltage dependence as in the previous fiber. Finally, the voltage dependence of the miniature endplate current rising phase was examined in a third fiber from another muscle by alternatively recording a series of miniature endplate currents at the resting potential and then at +25 mV, for a total of five runs (Table IV). Note that the bath temperature is 10°C colder than the previous two examples. The rising phase was again slowed with the rise time increased by about one-third for a 95 mV depolarization. The results of this repeated sampling demonstrated the reproducibility of these measurements.

Action of neostigmine

The cleft acetylcholinesterase was pharmacologically blocked in order to prolong the lifetime of the neurotransmitter. Neostigmine, at the supramaximal concentration of 10^{-6} M for 30 min blocked 80% of the cholinesterase activity [20] and modified the behavior of both the rising and falling miniature endplate current phases. The rise time was slowed 2-fold, to 470 ± 60 μs, $n = 4$. If in addition to containing

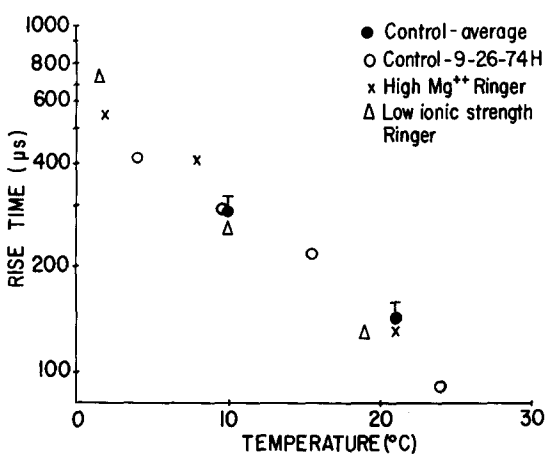

Fig. 3. Temperature dependence of miniature endplate current rise time. The average control rise times at 10°C and 20°C in Fig. 2 are plotted in a logarithmic scale against bath temperature. Also included are the average values of the rise time in the low ionic strength (illustrated in Fig. 3) and the high magnesium, low calcium Ringer's solution. For comparison, average values from a single experiment are also included. The slope of the line of best fit through these points gives a Q_{10} 2.3.

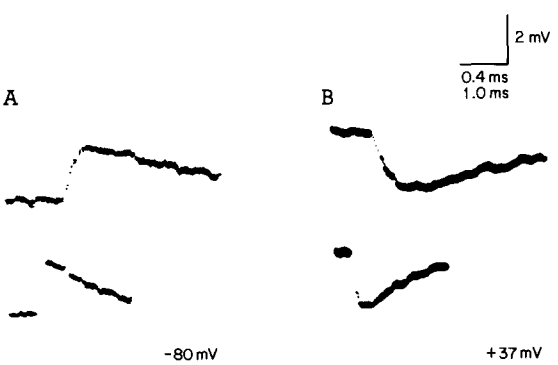

Fig. 4. Voltage dependence of the miniature endplate current rising phase. Two individual records of representative miniature endplate currents recorded with the Strickholm clamp at −80 and +37 mV are displayed. The rising phase of each record is shown twice, with the upper record at the faster sweep speed. Record A was obtained at the resting potential (−80 mV) and had a rise time of 130 μs. The rising phase was visibly slower at a holding potential of +37 mV, with a rise time of 190 μs.

TABLE III

VOLTAGE DEPENDENCE OF MINIATURE ENDPLATE CURRENT

Experiments of 5-7-75. The first and second row of each parameter are from two endplates on the same muscle. The temporal sequence of experiments is left to right. Bath temperature was 21°C.

Parameter		Parameter at holding potential		
		−104 mV	−80 mV	+37 mV
$\alpha(ms^{-1})$	1	–	0.60 ± 0.01	0.93 ± 0.04
	2	0.47 ± 0.09	0.54 ± 0.04	–
$t_r(\mu s)$	1	–	140 ± 20	230 ± 40
	2	120 ± 10	140 ± 10	–
N	1	–	36	32
	2	15	26	–

neostigmine the solution was made very viscous, slowing the diffusion of acetylcholine by one-third, the rise time was prolonged, but not to a significant extent (520 ± 60 μs, $n = 10$, $P < 0.2$). Note that the high viscosity solution alone had no effect on miniature endplate current rise times (Table I).

In agreement with previous studies [3], neostigmine slowed the half-time of decay by a factor of two to three, but did not significantly change its voltage dependence (Table V). However, closer examination showed that after treatment by neostigmine, few miniature endplate currents could be described as a single exponential; instead the currents decayed far more slowly than control miniature endplate currents, but progressively faster and faster than expected for a single exponential (Fig. 5A). While similar in nature to those shown by Katz and Miledi [28] for externally recorded miniature endplate currents in neostigmine, no records had as extreme a plateau as theirs. If all miniature endplate currents

TABLE IV

VOLTAGE DEPENDENCE OF MINIATURE ENDPLATE CURRENT

Experiments of 4-29-75. Runs at −70 mV were alternated with those at +25 mV. Bath temperature was 21°C. The mean value of each parameter is different at these two potentials ($P < 0.1$).

Parameter	Parameter at potential E	
	−70 mV	+25 mV
$\alpha(ms^{-1})$	0.28 ± 0.01	0.47 ± 0.02
	0.25 ± 0.01	0.58 ± 0.03
	0.27 ± 0.01	–
Mean	0.27 ± 0.01	0.52 ± 0.02
$t_r(\mu s)$	260 ± 10	280 ± 10
	240 ± 10	360 ± 10
	260 ± 10	–
Mean	250 ± 10	320 ± 40
Amplitude (mV)	−3.9 ± 0.1	3.1 ± 0.1
	−2.6 ± 0.1	1.9 ± 0.1
	−3.0 ± 0.01	–
Mean	−3.1 ± 0.01	2.5 ± 0.1

TABLE V

MEAN VOLTAGE DEPENDENCE OF APPARENT RATES OF MINIATURE ENDPLATE CURRENT DECAY

The least-squares regression of the apparent rate of miniature endplate current decay (α) on voltage (E) is given, assuming that $\alpha = B \exp(AE)$: B and A are constants. The apparent rate of decay was obtained by measuring the apparent half time of the miniature endplate current decay in each of the solutions listed. The temperature was 16.5 ± 1°C for all values except the high viscosity solution, where the temperature was 9.5 ± 0.03°C. If the Q_{10} is 2.7 [3], the value of B would become 690 s^{-1} at 17°C for high viscosity solution.

	A (V^{-1})	B (s^{-1})	Temp. (°C)	N
Control	7.3 ± 0.5	890 ± 30	17	5
High viscosity	5.7 ± 1.0	360 ± 30	9.5	4
Neostigmine	5.9 ± 1.0	260 ± 40	17	6
Neostigmine-high η	6.8 ± 1.1	170 ± 20	17	6

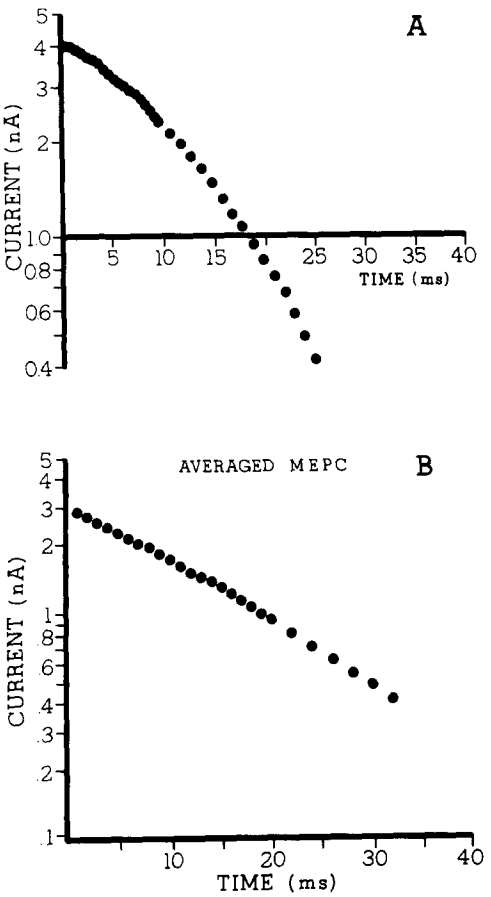

Fig. 5. Curvature of miniature endplate current decay phase after neostigmine treatment. The miniature endplate current whose decay phase is plotted semilogarithmically in A was recorded after 30 min exposure to neostigmine. As do most such plots, this semilogarithmic record curves downward. The average of ten miniature endplate currents recorded at 100 mV in this fiber is shown in B and appears to decay as a single exponential. Temperature was 16.5°C.

recorded during a single run were averaged (Fig. 5B) the results do decay as single exponentials, just as seen for nerve-evoked endplate potentials [3]. Thus, neostigmine-treated endplates differ from the control, non-drug treated, endplate in so far as individual miniature endplate current records suggest quite a different kinetic scheme than the averaged record.

Increasing the solution's viscosity slowed the half-time of the neostigmine treated miniature endplate current decay by a third (Table V). By contrast, without neostigmine treatment the solution's viscosity had no effect on the rate of miniature endplate current decay (1.3 ± 0.4 ms for control, 1.4 ± 0.5 ms for high viscosity, $n = 5$ and 4, respectively, all at 9.5°C and −100 mV). Two neostigmine experiments were examined in detail, one with the control solution and one with the high viscosity solution. The whole of the slowing in the high viscosity solution occurred within the first half of the miniature endplate current (Table VI). That is, only the initial rate of decay was slowed; the final rate of decay of neostigmine treated miniature endplate currents in control and high viscosity neostigmine solutions was the same. The rate constants associated with all portions of the miniature endplate current remain voltage dependent. Table VII shows that both the early and the late decay rates became faster as the membrane potential became

TABLE VI

VISCOSITY EFFECT ON NEOSTIGMINE MINIATURE ENDPLATE CURRENT APPARENT DECAY RATES

The rate constants of miniature endplate current decay were obtained either from the apparent half-times of decay of the original records (average) or from the early (initial) or late (final) rates of miniature endplate current as measured by eye from semilogarithmic plot of these records. The experiment with control viscosity included miniature endplate currents recorded at 15 different holding potentials; for consecutive holding potentials were chosen to obtain initial and final apparent rate constants. The values for the control solution at +50 mV (*) were extrapolated from results obtained at +30 mV. Temperature was 16.5°C. The experiment with a high viscosity solution included two different holding potentials. Temperature was 9.5°C.

Solution viscosity	Normal	High
Holding potential (mV)	−100	−90
N	10	17
$\alpha(s^{-1})$ initial	150 ± 20	90 ± 10
final	200 ± 15	180 ± 20
Rise time (μs)	[a]	620 ± 70
Holding potential (mV)	+50	+50
N	*	7
$\alpha(s^{-1})$ initial	490	240 ± 20
final	530	480 ± 110
Rise time (μs)	[a]	980 ± 150

[a] Not available.

more positive. The rising phase could be measured in the neostigmine-high viscosity experiment shown in Table VI; just as in the drug-free case, the rising phase became slower as the membrane potential was made more positive.

Discussion

The sizable temperature dependence of the miniature endplate current rising phase, as well as its indifference to the solution's viscosity, argues strongly that the kinetics of this phase are not governed by free diffusion of acetylcholine alone. The voltage dependence of the duration of the rising phase further suggests that the rate limiting step occurs within the post-synaptic membrane.

Temperature dependence

Two observations exclude free diffusion as the explanation for the miniature endplate current rise time. The Q_{10} of the miniature endplate current rising phase is 1.9 times that of free diffusion (Robinson and Stokes, 1959). Furthermore, increasing the solution's viscosity by a third does not significantly slow the rise time until the acetylcholinesterase is inhibited, and then only by a small amount. The diffusion of acetylcholine is certainly slowed within the subsynaptic cleft since the miniature endplate current decay was prolonged by an average of 50% after the solution's viscosity was increased, but only when the acetylcholinesterase was inhibited. Alternatively, this could have resulted from slowed diffusion of the transmitter only along the surface of the muscle, not in the cleft. However, were this the case, the rise time would not be prolonged. Furthermore, since the transmitter might be released near the surface or deep within the cleft there would be considerable variation in rise time and early decay phase from one miniature endplate current to the next. Neither result was actually seen. Furthermore, it is unlikely that a privileged volume immediately around the acetylcholine receptor could exist which excludes macromolecules, since molecules as large as immunoglobulins are known to attack these receptors in the disease myasthenia gravis [29]. Finally, free diffusion of the transmitter is complete in a few tens of microseconds, not the hundreds of microseconds which was the observed duration of the rising phase. That is, the quanta of acetylcholine released at the frog twitch fiber could be completely bound by the morphologically defined sites contained within 0.3 μm^2, requiring less than 15 μs, if diffusion were the same as in the bulk solution [30]. Thus, unless the diffusion coefficient of acetylcholine within the cleft were three orders of magnitude slower than in bulk solution, or the diffusion distances were greater than the shortest route [31], the rising phase kinetics must be governed by steps other than the cleft lifetime of acetylcholine.

The principal reason for including the neostigmine experiments in this paper was to demonstrate that the diffusion of acetylcholine through the cleft can be slowed by adding a high molecular weight dextran to the solution (Table V). However, additional lessons can be learned. First, the rising phase is slowed by inhibiting the cleft acetylcholinesterase. Thus, when active, this enzyme effectively limits the spread of transmitter from the point of release. Second, an averaged record gave very different results from an individual miniature endplate current record. This behavior was the converse of the two-exponential decay seen after local anesthetics [2] which was the result of the summed opening blockade and then closing of many identical channels [32]. In contrast to the fast then slow decay of miniature endplate currents after addition of local anesthetics, the miniature endplate currents decayed slowly at first and then faster toward the end after neostigmine treatment. Each record differed from the next, but when averaged, this characteristic shape was no longer apparent and the result appeared to be a single exponential (Fig. 5). Therefore, in cases such as neostigmine, it is the individual miniature endplate current records that must be examined, not the average.

Three possible reasons exist for the transformed shape of the miniature endplate current decay phase after neostigmine treatment. First, the lifetime of the bound agonist may be prolonged [33, 28; 34 for d-tubocurare]. Thus, the late portion of the miniature endplate current decay phase which was sensitive only to the membrane potential, and not the solution's viscosity may be due to increased affinity of the receptor for the agonist. The earlier portion of the miniature endplate current would reflect the buffered diffusion of acetylcholine from the cleft,

which would be modified not only by increasing the viscosity to slow free diffusion but also by increasing the membrane potential to prolong the lifetime of the bound complex. A second general explanation would be that the holding current iontophoreses the positively charged acetylcholine away from the receptors at the more positive membrane potentials. While the sign of the current is as expected for the observed decay kinetics, such a mechanism would explain neither the shape of the decay phase nor the dependence of only a part of the decay phase on the solution's viscosity. Furthermore, there was no correlation between the amplitude of the current (and presumably the amount of acetylcholine available to carry current) and the rate of decay [15]. Finally, the channel itself may close at a slower rate in the presence of neostigmine. Unfortunately, methods such as noise analysis have given only ambiguous answers [35], but this would readily explain the slow final decay of the neostigmine-treated miniature endplate current.

Voltage dependence

When the membrane's potential was made more positive, the miniature endplate current rising phase was as prolonged as the decay phase was shortened. This is unlikely to be caused by the current required to maintain the membrane holding potential, since this current would tend to sweep the cation acetylcholine away from the subsynaptic cleft and so shorten, not prolong, the rising phase. Thus, the effect must be due to the membrane potential and the rate limiting step for the growth of the miniature endplate current must lie within the membrane itself. Since the open conformation of the receptor is less stable at more positive membrane potentials, a non-conducting intermediate state must be more stable and so have a more prolonged life to explain the slower rising phase. Such behavior would explain why more channels are open at positive potentials than negative at the miniature endplate current peak, as opposed to the steady state [2,36]. The increased affinity constant would predict a slower loss of acetylcholine from the cleft, due to 'buffering' by the receptors. However, the lack of effect of raising the solution's viscosity and the estimate that virtually all released transmitter is bound by the receptors [37] argue that buffered diffusion does not account for the prolonged rising phase.

The voltage dependence of the miniature endplate current rising phase has been examined in other species. In the toad, the rising phase has either no voltage dependence or the same as reported here. However, unlike the frog, ordinary, non drug-treated miniature endplate currents of the toad occasionally have a two-exponential decay phase, and so in their case, the rising phase may be governed by different mechanisms [38]. In snake slow muscle fibers, Dionne and Parsons [39,40] convincingly demonstrated a voltage dependent step other than the well known voltage dependent closing of the channel [5], namely the unbinding of the agonist. This behavior was readily apparent at this synapse because the rates involved are slower than at the amphibian or snake fast fibers, allowing use of the standard two-electrode clamp as well as noise analysis and voltage jumps. Indeed, the only model to fit their data consistently was that proposed first by del Castillo and Katz in 1957 [41]:

$$A + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AR \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} AR^*$$

where A is the agonist, R the receptor, AR the closed or non-conducting agonist receptor complex and AR^* the conducting moiety. The rate constants (k, k_{-1}, α and β) are as shown. In the snake slow fibers, the kinetics of miniature endplate current, acetylcholine noise and current relaxations following voltage steps are explained only if the rate constants k_{-1} and α become faster as the membrane potential becomes more positive (β changes little with voltage and the behaviour of k_1 is unknown). Such a study in frog twitch muscles is precluded by the limited voltage frequency response of the two-electrode voltage clamp.

Finally, step changes in the membrane potential of rat myotubes lead to relaxations in acetylcholine-induced current consistent with this report of a voltage-dependent miniature endplate current rising phase [42].

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References

1 Katz, B. (1966) Nerve, Muscle and Synapse, McGraw-Hill, New York
2 Gage, P. and Armstrong, C. (1968) Nature 218, 363–365
3 Magleby, K. and Stevens, C. (1972) J. Physiol. (Lond.) 223, 151–171
4 Gage, P. and McBurney, R. (1972) J. Physiol. (Lond.) 226, 79–94
5 Anderson, C. and Stevens, C. (1973) J. Physiol. (Lond.) 235, 655–691
6 Gage, P. and McBurney, R. (1972) J. Physiol. (Lond.) 226, 79–94
7 Takeuchi, A. and Takeuchi, N. (1959) J. Neurophysiol. 22, 396–411
8 Eccles, J. and Jaeger, J. (1958) Proc. R. Soc. B 148, 38–56
9 Gage, P. and McBurney, R. (1975) J. Physiol. (Lond.) 244, 385–407
10 Hamill, O. (1968) Ph.D. Thesis, University of New South Wales
11 Dwyer, T. (1976) Fed. Proc. 35, 697
12 Dwyer, T. (1977) Fed. Proc. 36, 485
13 Hutter, O. and Trautwein, W. (1956) J. Physiol. (Lond.) 133, 610–625
14 Ypey, D. (1975) Pflugers Arch. 355, 291–306
15 Dwyer, T. (1977) Ph.D. Thesis, University of Rochester
16 Robinson, R. and Stokes, R. (1959) Electrolyte Solutions, Butterworth, London
17 Krnjevic, K. and Mitchell, J. (1962) J. Physiol. (Lond.) 153, 562–572
18 Dreyer, F. and Peper, K. (1975) Nature 253, 641–643
19 Peper, K., Dreyer, F. and Muller, K. (1970) Cold Spring Harbor Symp. Quant. Biol. 40, 187–192
20 Kordas, M. (1975) J. Neuropharm. 14, 791–800
21 Bezanilla, F. and Armstrong, C. (1977) Am. J. Physiol. 232, C211-C215
22 Kordas, M. (1969) J. Physiol. (Lond.) 204, 493–502
23 Aseltine, J. (1958) Transfer Method in Linear Systems Analysis, McGraw-Hill, New York
24 Fatt, P. and Katz, B. (1952) J. Physiol. (Lond.) 117, 109–128
25 Del Castillo, J. and Katz, B. (1956) J. Physiol. (Lond.) 132, 630–649
26 Strickholm, A. (1961) J. Gen. Physiol. 44, 1073–1088
27 Strickholm, A. (1962) J. Cell. Comp. Physiol. 60, 149–167
28 Katz, B. and Miledi, R. (1973) J. Physiol. (Lond.), 231, 549–574
29 Drachmann, R. (1978) New Eng. J. Med. 298, 136–142 and 186–193
30 Mathews-Bellinger, J. and Salpepter, M. (1978) J. Physiol. (Lond.) 279, 179–213
31 Gage, P. and McBurney, R. (1975) J. Physiol. (Lond.) 244, 385–408
32 Neher, E. and Steinbach, J.H. (1978) J. Physiol. (Lond.) 277, 153–176
33 Eccles, J., Katz, B. and Kuffler, S. (1942) J. Neurophysiol. 5, 211–230
34 Armstrong, D. and Lester, H. (1979) J. Physiol. (Lond.) 294, 365–386
35 Steinback, J. and Stevens, C. (1976) in Frog Neurobiology (Llinas, R. and Precht, W., eds.), pp. 33–92, Springer-Verlag, Berlin
36 Dionne, V. and Stevens, C. (1975) J. Physiol. (Lond.) 251, 215–270
37 Adams, P. and Sackmann, B. (1978) Biophys. J. 21, 53a
38 Gage, P., McBurney, R. and Van Helden, D. (1975) J. Physiol. (Lond.) 274, 279–298
39 Dionne, V. and Parson, R. (1981) J. Physiol. (Lond.) 310, 145–158
40 Dionne, V. (1981) J. Physiol. (Lond.) 310, 159–190
41 Del Castillo, J. and Katz, B. (1957) Proc. Roy. Soc. B 146, 362–368
42 Horn, R. and Broderick, M. (1980) J. Gen. Physiol. 75, 297–321