EXTRACELLULAR EXCITATORY JUNCTIONAL CURRENT AT THE CRAYFISH NEUROMUSCULAR JUNCTION

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The nature of the falling phase of the synaptic current was studied in more detail at the crayfish neuromuscular junction and fundamental data relating to focally recorded excitatory junctional potentials (e.j.p.s) was complemented. The decay of extracellular e.j.p.s at the crayfish neuromuscular junction was almost exponential. The decay time constant of extracellular e.j.p.s varied considerably from one e.j.p. to another, the mean of the decay time constant being about 1 ms at the normal resting potential at a bath temperature of 22°C. There was no detectable correlation between the decay time constant and the amplitude of extracellular e.j.p.s which were induced by a single stimulation of the excitatory axon. Prolonged application of glutamate hardly affected the decay time constant of extracellular e.j.p.s. The significance of the study on the crayfish neuromuscular system was discussed.

Keywords — excitatory junctional current; glutamate; desensitization; decay time constant; crayfish neuromuscular system

INTRODUCTION

The neuromuscular system of the crayfish resembles very closely the mammalian central synapses, and it provides us with insight into chemical transmission processes at many synapses. The detailed mechanisms of synaptic transmission at the crayfish neuromuscular junction are well documented. After being released from presynaptic structures, the transmitter undergoes reversible combination with junctional neuro-receptors to form the receptor-transmitter complex. Consequently, a sequence of events is started, which ultimately increases the conductance of junctional membrane of the muscle fibre. The time course and relative amplitude of this conductance increase can be measured by recording the end-plate current. The time course of the end-plate current seems to be related to several factors, e.g., the quantum content of the end-plate current, the degree of synchronization of the released quanta, the spread of transmitter along the receptive surface, the dissociation of the receptor-transmitter complex, and diffusion of the released transmitter from the synaptic cleft. The mechanism of enzymatic destruction is well documented only at cholinergic synapses, where cholinesterases play an important role in elimination of the released transmitter. At the crayfish neuromuscular junction, however, there is no evidence for the enzymatic destruction of the released transmitter, and diffusion from the synaptic cleft would be the most important mechanism for elimination of the released transmitter.

It has been recently shown that various effects of some drugs can be explained in terms of a reversible blocking of open end-plate channels. Some open channel blockers markedly affect the falling phase of the end-plate current, so the end-plate current sometimes provides crucial clues for analysis of the mode of action of the drug. Therefore, it is necessary to have a good knowledge of the nature of end-plate currents. The falling phase of the miniature end-plate current at the cholinergic end-plate is approximately an exponential function of time.
neuromuscular junction of crustacean and locust where glutamate is the putative excitatory transmitter, the junctional current decayed quasi-exponentially. 9,15,36 These suggest that the falling phase of the end-plate current is apparently determined by a first-order chemical reaction. The synaptic current can be derived from extracellular recordings of the synaptic potential. In the present study the nature of the falling phase of the synaptic current was studied in more detail and fundamental data relating to focally recorded excitatory junctional potentials (e.j.p.s) was complemented at the crayfish neuromuscular junction.

METHODS

The methods used were similar to those reported previously. 37 The crayfish opener muscle of the dactyl in the first walking leg was used in all experiments. The neuromuscular preparation consisting of the muscle and the excitatory nerve fibre was prepared. A nerve bundle containing excitatory axons to the muscle was exposed and stimulated with a suction electrode. Potential changes were recorded either intracellularly from the muscle with a 3 M KCl filled microelectrode or extracellularly from the neuromuscular junction with a 2 M NaCl filled microelectrode. The recording system consisted of conventional d.c. and a.c. amplifiers and an oscilloscope, in most cases with condenser coupling (time constant: 0.1 s) to monitor focally recorded extracellular junctional potential. Individual muscle fibres of the crayfish opener muscle are innervated by dual excitatory and inhibitory axons whose endings converge onto many regions of the muscle membrane. Extracellular e.j.p.s were differentially recorded from a single junctional site. Differential recording greatly reduced non-specific field potentials generated by the inevitable activation of other synapses on the fibre. In some experiments, the membrane potential of muscle fibres was clamped with an intracellular microelectrode to test the effect of the membrane potential on extracellular e.j.p.s at various membrane potential. For checking the exponential decay of extracellular e.j.p.s, they were plotted semilogarithmically on a Hewlett-Packard desk computer. Since it was possible to fit results to a straight line by the method of least-squares, the decay time constant of extracellular e.j.p.s was determined from the slope of the straight line. Regressions were calculated between 80 % and 20 % of the peak response.

RESULTS

Exponential Decay — The excitatory axon was stimulated at a frequency of 12.5/s and the e.j.p.s were focally recorded with an extracellular microelectrode placed on the surface of the crayfish opener muscle. Various types of extracellular e.j.p.s were recorded from a single junctional site, as shown in Fig. 1. The amplitude of extracellular e.j.p.s was much varied. One should obtain a binomial or Poisson distribution in the amplitude histogram of nerve-evoked synaptic potentials at the crayfish neuromuscular junction. 2,38,39 The extracellular e.j.p.s rise in about 0.4 ms to its peak value and then decay quasi-exponentially to base line after a brief slow non-exponential phase. The mean value of the rise time was 0.37 ± 0.02 (S.E.) ms at the resting membrane potential (88.1 ± 0.4 mV) in 5 preparations and the number of observations in each preparation ranged from 50 to 90. Fig. 2 shows the rise time distribution of extracellular e.j.p.s and the relationship between their rise time and the peak amplitude. There was no correlation between them. Fig. 3 shows the semilogarithmic plots of the focally recorded e.j.p.s. Both A and B in Fig. 3 were obtained from the same junctional spot. In A, the plots were seen to fall close to a straight line, but as shown in B, the decay of e.j.p.s was not always completely exponential. The degree of the data to fit the straight line was compared by simply calculating the correlation coefficient (r) between the time and natural logarithm of the potential in its time. The values of the correlation coefficient in A was 0.999, and 0.982 in B. Squares of the correlation coefficient (r²) should indicate the relative extent of linear regressions of semilogarithmic plots. Therefore, the values of r² were calculated in some preparations. In Fig. 3C, a
histogram of $r^2$ was shown. These figures suggest that the falling phase of the crayfish excitatory junctional current can be regarded as being almost exponential. The decay time constant of extracellular e.j.p.s can be determined from the slope of semilogarithmic plots of the falling phase. For the experiment shown in Fig. 4, the distribution of the decay time constant was examined. The decay time constant varied considerably from one extracellular e.j.p. to another. The number of observations in each preparation was more than 50. The mean of the decay time constant was 1.06 ±0.05 (mean ± S.E., ms, $n=6$) at the resting membrane potential (88.3 ±0.4 mV, $n=6$).

**Relationship between e.j.p. Amplitude and Decay Time Constant**—At the frog neuromuscular junction large end-plate currents decay more slowly in the presence of anticholinesterases than small end-plate currents. Computer simulation also showed that the time course and the amplitude of the end-plate current depended on the quantity of the released transmitter under the assumption that the released transmitter was not hydrolyzed by an enzyme. Magleby and Terrar demonstrated at the frog neuromuscular junction that the second of two successive end-plate currents decayed more slowly than the first in the presence of anticholinesterases. These can be explained by the hypothesis that repeated binding of ACh to receptors can contribute to the prolongation of end-plate currents which occurs when acetylcholinesterase is blocked by an inhibitor. So the relationship between the amplitude and the decay time constant of extracellular e.j.p.s was examined at the crayfish neuromuscular junction. E.j.p.s at the individual junction are quantal and the amplitude of extracellularly recorded e.j.p.s corresponds approximately to the amount of the released transmitter. When the excitatory axon was stimulated at intervals of 50

**FIG. 1. Families of Various Types of Extracellularly Recorded e.j.p.s**
The excitatory axon was stimulated at a frequency of 12.5/s. Since the quantum content was relatively small and consequently nerve impulses often failed to induce extracellular e.j.p.s. Sometimes, repetitive e.j.p.s were observed.
ms, there was no detectable correlation between the decay time constant and the amplitude of extracellular e.j.p.s (Fig. 5).

Effect of Membrane Potential on Quantal Content — As shown in Fig. 1, repetitive junctional potentials sometimes appeared even when the excitatory axon was once stimulated with a relatively long interval at the normal membrane potential. Hyperpolarization increased the incidence of repetitive appearance of e.j.p.s at a single

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

**FIG. 2.** *A*: The Rise Time Distribution of Extracellular e.j.p.s. Extracellular e.j.p.s were induced by the stimulation of the axon at a frequency of 10/s. Number of observations was 84, the mean value of the rise time being $0.35 \pm 0.03$ (S.E.) ms.

*B*: The Relationship between the Rise Time and the Peak Amplitude of Extracellular e.j.p.s (Correlation Coefficient = 0.277)

The data were obtained from the same preparation as that in *A*.

**FIG. 3.** *A, B*: Semilogarithmic Plots of the Falling Phase of Extracellularly Recorded e.j.p.s at a Bath Temperature of $22^\circ C$

The membrane potential was $-90$ mV. Abscissa, time (ms). Ordinate, e.j.p. amplitude (mV). Both data in *A* and *B* were obtained from the same junctional spot. *C*: Histogram of squares of correlation coefficient ($r^2$). Ordinate, percent of e.j.p.s counted ($n = 58$). Abscissa, size of $r^2$. 
junction (Fig. 6). Onodera and Takeuchi\(^9\) suggested that a large hyperpolarizing current from an intracellular clamp electrode might have some effect on the nerve terminal and change the release process of the transmitter. One would expect that a quantal analysis gives a solution to the problem. A quantal analysis of extracellular e.j.p.s at various membrane potentials, which were controlled by the voltage clamp techniques, showed that hyperpolarization increased the quantal content of extracellular e.j.p.s in 4 of 6 preparations. When the membrane potential varied from \(-90\) mV to \(-120\) mV, an increase in the quantal content was \(5-10\) \% of the control quantal content at the resting membrane potential. Table 1 shows a typical case of the increased quantal content. An increase in unit size results in the enlargement of the peak amplitude of extracellular e.j.p.s due to the increase in the driving force. An increase in the probability of the quantal release may justify repetitive appearance of e.j.p.s. Relationship between the membrane potential and the decay time constant of extracellular e.j.p.s was also examined (Table I). This is in good agreement with early data.\(^9,12,42\)

**Decay in the Presence of Agonist** — Prolonged application of glutamate to the crayfish neuromuscular junction induces desensitization of the glutamate receptor, and as a result, the

**FIG. 4. Histogram of the Decay Time Constant of Extracellular e.j.p.s at a Bath Temperature of 22°C**

The membrane potential was \(-88.7\) mV. Excitatory axon was stimulated at a frequency of 20/s. Ordinate, percent of e.j.p.s counted. Abscissa, size of the decay time constant (ms). The mean value was \(1.13 \pm 0.02\) (mean \(\pm\) S.E. ms, \(n = 88\)).

**FIG. 5. Relationship between the Peak Amplitude and the Decay Time Constant of Extracellular e.j.p.s**

E.j.p.s were induced by stimulation of the excitatory axon at a frequency of 20/s. Correlation coefficient was 0.112 (\(n = 87\)).

**FIG. 6. Recordings of Repetitive Junctional Currents**

Membrane potential: \(-128\) mV, stimulation intervals 90 ms.
amplitudes of both e.j.p.s and glutamate responses are decreased. This is one of the important bases of the argument that glutamate is an excitatory transmitter at this site. Desensitization is not restricted to glutamate receptors, but glutamate particularly induces marked desensitization of the receptor. So the effect of neuroreceptor desensitization caused by glutamate on the decay time constant of extracellular e.j.p.s was examined. Extracellular e.j.p.s were induced by double pulses at an interval of 100 ms (pulse interval: 3.3 ms). Desensitization of the neuroreceptor was induced by bath application of glutamate in a concentration of $10^{-4}$M. The perfusion of glutamate led to rapid development of depolarization of the muscle membrane which then slowly subsided despite the continued presence of glutamate, and then the depolarization declined to a steady plateau level (about 5 mV). At this stage, the mean amplitude of extracellular e.j.p.s recorded from a junctional spot was reduced to about 1/2, however, the decay time constant of the extracellular e.j.p. was hardly affected (Fig. 7). In higher concentrations of glutamate, desensitization of the receptor markedly developed, and as a result, the extracellular e.j.p.s were reduced to a noise level and it was impossible to record them with reasonable accuracy.

**DISCUSSION**

The study of the crayfish neuromuscular junction will serve in some respects as a useful introduction to the problem of central nervous system.¹¹ The crayfish neuromuscular junction provides a good model for studying the mechanism of action of drugs on synaptic transmission in mammalian central nervous system. Since no specific and selective glutamate antagonists has been found at the crayfish neuromuscular junction, studies of action of drugs affecting glutamate responses are useful for the pharmacological identification of the excitatory transmitter at this site, and experiments comparing the effect of drugs on e.j.p.s with those on the glutamate-induced depolarization may provide crucial evidence for the identification of a transmitter. Identification of the crayfish excitatory transmitter promotes the establishment of mammalian central transmitter. Although glutamate is a putative excitatory transmitter at this site, there is a lack of evidence for pharmacological identification. The prior success in establishing GABA as an inhibitory transmitter, first at the crayfish neuromuscular junction, then at mammalian central synapses, gave us confidence in the approach as well as clues to the necessary method of research. For these reasons it is of great value to study the detailed mechanism of the crayfish synaptic transmission. However, there have been only a few experimental works relating to the effect of drugs on the binding of the transmitter with the receptor at this neuromuscular junction.

**TABLE I. Effects of the Membrane Potential on the Quantal Content, Unit Size and the Decay Time Constant of Extracellular e.j.p.s**

<table>
<thead>
<tr>
<th>Membrane potential (mV)</th>
<th>Number of observations</th>
<th>Quantal content</th>
<th>Unit size (µV)</th>
<th>Decay time constant (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-70</td>
<td>195</td>
<td>0.72</td>
<td>119.1</td>
<td>0.91</td>
</tr>
<tr>
<td>-90</td>
<td>92</td>
<td>0.76</td>
<td>124.4</td>
<td>0.88</td>
</tr>
<tr>
<td>-125</td>
<td>74</td>
<td>0.84</td>
<td>143.8</td>
<td>0.78</td>
</tr>
<tr>
<td>-160</td>
<td>100</td>
<td>1.11</td>
<td>168.8</td>
<td>0.68</td>
</tr>
</tbody>
</table>

*The membrane potential of muscle fibres was clamped with an intracellular microelectrode which was inserted near the junctional spot. Resting membrane potential was - 90 mV. Excitatory axon was stimulated in a frequency of 15/s and the excitatory junctional potential was extracellularly recorded.*
Crayfish Neuromuscular Junctional Current

In the present examination, there was no relationship between the amplitude and the rise time of extracellular e.j.p.s, and their decay time constant was not related to their peak amplitudes. In the crayfish the innervation of the fibre differs from that of the fast muscle in vertebrates in many respects, but the nature of the nerve-evoked junctional current in the crayfish opener muscle seems to resemble that of cholinergic miniature end-plate currents than the nerve-evoked end-plate current. Nerve-evoked e.j.p.s at the crayfish neuromuscular junction result from synchronous releasing of the transmitter, and the rise rate (the ratio of the peak amplitude of extracellular e.j.p.s to their time-to-peak) should provide crucial clues for analysis of the mode of action of the drug. In previous reports, the rise rate of the synaptic current was measured in order to examine whether a drug was an open channel blocker or not. If a different mechanism from the open channel block, say, the binding of the drug to the free reactive receptor or the closed channel, is operating to reduce the effectiveness of the excitatory transmitter in opening channel, the block should be evident throughout the rising phase.

The process involved in inducing the synaptic current can be in general summarized in the following kinetic scheme:

\[ A + R \stackrel{k_1}{\longrightarrow} AR \stackrel{\alpha}{\longrightarrow} AR^* \stackrel{\beta}{\longrightarrow} AR' \]

where \( A \) is an agonist molecule, \( R \) the free receptor in its initial reactive form, \( AR \) the agonist-receptor complex associated with a closed ionic channel, \( AR^* \) the open conformation of this complex, and \( AR' \) is the desensitized form of the complex. \( k_1, k_{-1}, \alpha, \beta, k_4 \) and \( k_c \) are the rate constant. In the ordinary neuromuscular transmission the desensitization mechanism does not seem to play an appreciable role, and the desensitized form \( AR' \) should be negligible.

The value of the backward rate constant \( \beta \) is related to the decay time constant of the synaptic current. It seems unlikely that the variation of the decay time constant from one e.j.p. to another is due to the change of the value of \( \beta \) according to time and circumstance, because the value of the rate constant should be invariable except when the temperature changes. The variation of the decay time constant seems to be related to diffusion of the transmitter from the synaptic cleft.

FIG. 7. Histogram of the Decay Time Constant of Extracellular e.j.p.s in the Presence of Glutamate (10^{-4} M) at a Bath Temperature of 22\(^\circ\)C

The membrane potential of the muscle fibre was -86.0 mV. A: in the absence of glutamate. B: glutamate 10^{-4} M. Ordinate, percent of e.j.p.s counted. Abscissa, size of the decay time constant (ms). The mean value in A was 0.96 ± 0.03 (S.E.), \( n = 72 \) and 0.97 ± 0.02 (S.E.), \( n = 73 \) in B.
During prolonged application of glutamate, desensitization process of the glutamate receptor goes on in spite of the fact that the value of the desensitization rate constant $k_d$ is relatively small\(^{45}\), and the concentration of $AR^*$ is gradually increased. This results in the decrease in the concentration of the free reactive receptor because the value of $k_r$ is smaller than that of $k_d$\(^{46}\). The decrease in the concentration of the free receptor results in the reduction of the response to the newly released transmitter. So, in the equilibrium condition, prolonged application of the agonist should not affect the decay time constant of the synaptic current. If the reverse process of the desensitizing step could not be ignored, there is a possibility that the decay time constant of extracellular e.j.p.s may be affected by prolonged application of glutamate. In the case of the cholinergic end-plate, the decay of ACh currents which were induced by iontophoretic application became rapid in the continued presence of bath-applied ACh with anticholinesterases\(^{40}\). This is due to inhibition of delayed diffusion of iontophoretically applied agonist\(^{21}\). The present study demonstrated that the decay of the synaptic current at the crayfish neuromuscular junction did not change in the continued presence of the agonist.

Nerve endings converge onto many regions of the opener muscle and the quantal content of extracellular e.j.p.s is relatively small at the crayfish neuromuscular junction, and consequently nerve impulses often fail to induce e.j.p.s at each junctional spot. Miniature e.j.p.s was hardly observed in the crayfish neuromuscular preparation. The number of quanta released per impulse was four or five at the most, and the largest amplitude of extracellular e.j.p.s is less than 1 mV at the stimulation interval of more than 5 ms. When the synaptic delay was measured from the time between the peak of the presynaptic nerve spike and the start of the e.j.p.s, it ranged between 0.4 and 2.9 ms at $23^\circ$C\(^{47}\). In the present study repetitive appearance of e.j.p.s was also observed. Therefore, computer-averaged e.j.p.s are irrelevant for analysing the time course of extracellular e.j.p.s at the crayfish neuromuscular junction. The present study demonstrated that there was no detectable difference in the decay time constant of extracellular e.j.p.s within the limits of several quanta, but there might be no denying a possibility that large extracellular e.j.p.s decay apparently faster due to desensitization of the neuroreceptor than they did fundamentally. However, in the light of the desensitization rate constant of the crayfish glutamate receptor,\(^{45}\) it seems unlikely that desensitization of the neuroreceptor is induced by the excitatory transmitter itself even if it is released at a minimum interval by train pulses.

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**REFERENCES**

9. K.Onodera and A.Takeuchi: Ionic mechanism of the


27) H.Shinozaki and M.Ishida: Excitatory junctional responses and glutamate responses at the crayfish neuromuscular junction in the presence of chlorisondamine, *Brain Res.*, “in press”.


38) G.D.Bimter and J.Harrison: A reconsideration of the Poisson hypothesis for the transmitter release at the crayfish neuromuscular junction, *J. Physiol.* (London),


