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L-Glutamate activates excitatory and inhibitory channels in *Drosophila* larval muscle

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Muscle fibers from *Drosophila* larvae show an L-glutamate-sensitive membrane potential. Bath-applied L-glutamate depolarizes the muscle in the range from 0.5 to 20 μM. Greater concentrations of the agonist repolarize the fibers. The repolarizing effect disappears if chloride is replaced by sulfate in the external medium. Intracellular recordings show the occurrence of depolarizing and hyperpolarizing spontaneous miniature postsynaptic potentials (smpp). Patch-clamp studies indicate the presence of two types of receptor channels: (i) an anion-selective channel activated by both L-glutamate and GABA. In outside out-patches, bathed in symmetrical 140 mM Cl^- and 200 μM GABA, the channel displays conductance substates of 40, 80 and 110 pS. In the presence of 200 μM L-glutamate only the 40 and 80 pS substates are observed; (ii) a cation-selective channel activated only by L-glutamate that has a conductance of 104 pS in cell-attached patches (128 mM Na^+ outside). The presence of these two types of receptor channels in *Drosophila* muscle may explain the effect of bath-applied L-glutamate on membrane potential and the presence of inhibitory and excitatory smpp.

L-Glutamate; Membrane potential; Ion channel; Conductance substate

1. INTRODUCTION

In *Drosophila* L-glutamate is the transmitter at the neuromuscular junction [1,2]. The excitatory effects of L-Glu in *Drosophila* larval muscle synapses were first demonstrated by Jan and Jan [1]. The presence of inhibitory synapses in this type of muscle has not been explored, but GABA can induce a hyperpolarizing response in *Drosophila* neuromuscular junctions in culture [2]. The current carrier responsible for this hyperpolarizing response was not identified. Arthropod muscles possess both excitatory and inhibitory synapses [3,4] and, more recently, excitatory and inhibitory receptor channels activated by L-Glu have been described [5–7]. However, single-channel currents activated by L-Glu have not been recorded in *Drosophila* muscle. Using intracellular recording techniques, we obtained evidence for the occurrence of inhibitory synapses and for the presence of inhibitory receptors activated by L-Glu in *Drosophila*. Furthermore, using the patch-clamp technique we demonstrate the existence of two types of receptor channels in this preparation. One type is anion-selective and activated by L-Glu and GABA whereas the other is a cation-selective channel, activated only by L-Glu.

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2. MATERIALS AND METHODS

Experiments were carried out in muscle fibers from early-stage wild-type *Drosophila* larvae at room temperature (21°C). Only the anterior segments of ventrolateral longitudinal muscles were used. Larvae were pinned down to a layer of sylgard covering the bottom of a petri dish and dissected along the midline under 5 ml of standard buffer containing 128 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 4 mM $MgCl_2$, 36 mM sucrose, 5 mM Hepes, pH 7 [1]. The chloride-free buffer was prepared following Hodgkin and Horowicz [8]. The solutions containing different concentrations of L-Glu were prepared each day by diluting a freshly made 1 M L-Glu stock solution.

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The microelectrodes used in intracellular studies were pulled in a vertical puller (Kopf, model 700 C), filled with 3 M KCl and had resistances of 20–30 MΩ. Muscle membrane potential was measured relative to ground provided by a silver/silver chloride electrode immersed in the bath. In the experiments in which Cl^- was replaced by sulfate, the ground electrode was connected to the bath through a 3 M KCl agar bridge.

Patch pipettes, having resistances of about 5 MΩ fire-polished, treated with Silgard, and filled with appropriate solution, were sealed to muscle fibers after treating the cells with collagenase type IV (Sigma). The patch-clamp circuit was home-built. Single-channel current records, filtered at 1 kHz, were stored in magnetic tape, digitized, displayed on paper and analyzed by hand to build the current-voltage curves and conductance histograms. Kinetic analysis of single-channel data was carried out by computer. The experimental records were low-pass filtered at 1 kHz and sampled at 10 kHz. Channel open dwell times were analyzed as described by Sigworth and Sine [9]. Open dwell time distributions were displayed in the form of logarithmically binned histograms to reveal multiexponential components more readily and fitted to probability density functions using a maximal likelyhood algorithm.

3. RESULTS

3.1. *Effects of bath-applied L-Glu*

In preliminary patch-clamp experiments we failed to detect channel activity induced by L-Glu in cell-attached patches using pipettes filled with buffers containing up to 50 μM L-Glu. One possible explanation for the failure to record channel activity induced by L-Glu is receptor desensitization [1]. Thus, it was of interest to determine the range of concentrations in which the agonist is effective in activating excitatory receptors present in larval muscle. A simple way of doing this is to measure the effects of bath-applied L-Glu on membrane potential using intracellular microelectrodes, inasmuch as activation of excitatory receptors must lead to cell depolarization. The result of such study is summarized in fig.1A. Here, ΔE_m represents the difference in membrane potential measured in the presence of L-Glu minus the resting potential measured in the absence of the agonist as a function of [L-Glu]. The results show that ΔE_m is a skewed bell-shape function of agonist concentration. It shows a maximum at about 20 μM L-Glu. Higher concentrations of L-Glu lead to a gradual decrease in ΔE_m values which indicate a repolarizing effect of the agonist. At above 500 μM L-Glu, membrane potential is only a few millivolts more positive than the control resting potential, measured in the absence of L-Glu. This last result coincides with that obtained by Jan and Jan [1] who reported that equilibrating larval muscle in a solution containing 1 mM L-Glu did not significantly change the resting potential. Note, however, that the results shown in fig.1A indicate that the effects of L-Glu on membrane potential depend on the concentration of agonist. The results summarized in fig.1A are obtained regardless of whether the membrane potential measurements are made first in the presence of high [L-Glu] and then measured at lower concentrations of the agonist or vice versa. All L-Glu ef-

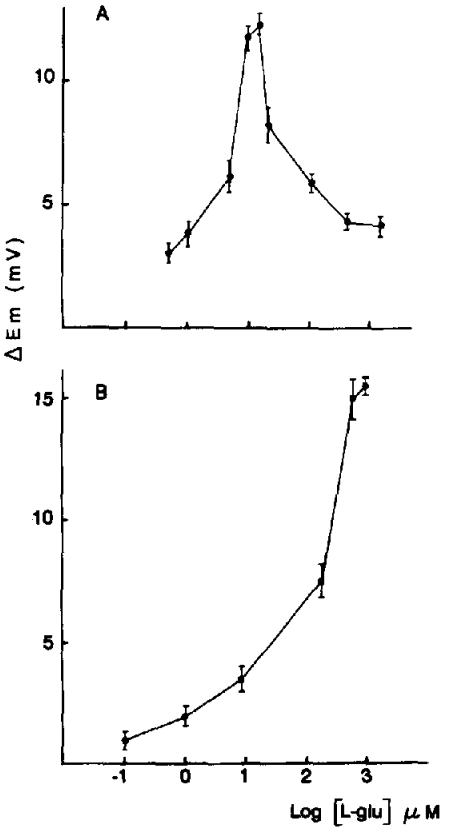

Fig.1. Difference in membrane potential (ΔE_m) as a function of bath-applied L-Glu. (A) ΔE_m vs [L-Glu] in standard buffer. After dissection muscle fibers were equilibrated for 5 min in standard buffer. The anterior segments of longitudinal ventrolateral muscles were then impaled to determine the control resting potential. The experimental chamber was then flushed with several volumes of buffer containing the appropriate concentration of L-Glu. Membrane potential was measured 1 or 2 min after addition of agonist. The control resting potential was also measured at the end of the experiment by perfusing the chamber with several volumes of agonist-free buffer. (B) ΔE_m vs [L-Glu] in chloride-free buffer. In this case chloride was totally replaced by sulfate.

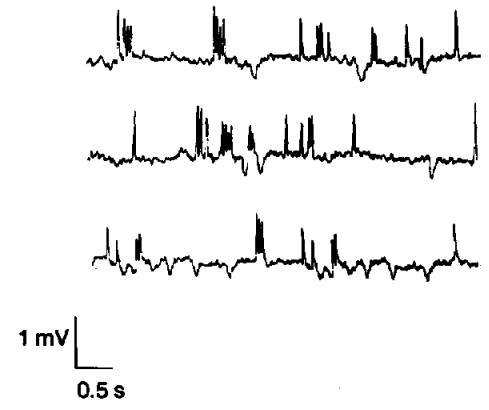

Fig.2. Spontaneous miniature postsynaptic potential. Records were obtained under the same ionic conditions as those described for fig.1A.

fects on membrane potential are reversible. However, as documented in fig.1B, the shape of the ΔE_m vs [L-Glu] curve is dramatically changed if chloride is replaced by sulfate from the external buffer. In this case L-Glu has only a depolarizing effect.

3.2. *Spontaneous miniature postsynaptic potentials*

Spontaneous miniature postsynaptic potentials (smpp) can be recorded from longitudinal ventrolateral muscle using intracellular electrodes. Such records usually reveal the occurrence of two types of smpp, depolarizing and hyperpolarizing, as shown in fig.2. Depolarizing smpp are always of greater amplitude and shorter time course than the hyperpolarizing smpp. Because of their small amplitude, hyperpolarizing potentials can be easily obscured by the background noise. However, under good recording conditions they are well discerned. Average decay times for depolarizing events are 16 ± 1 ms (mean ± SE) and for hyperpolarizing events 75 ± 8 ms (mean ± SE). Both depolarizing and hyperpolarizing smpp are abolished when the muscle is exposed to L-Glu concentrations above 200 μM.

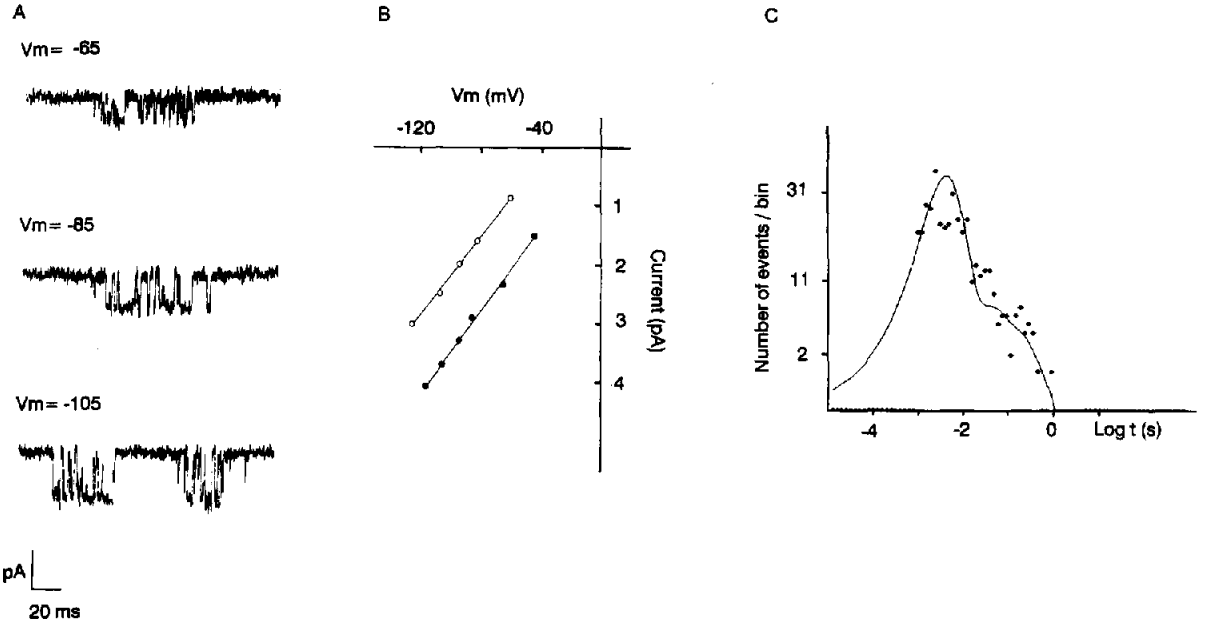

Fig.3. Anion-selective channels activated by L-Glu in cell-attached patches. (A) L-Glu-activated single-channel currents. Patch pipettes contained 128 mM Na^+, 2 mM K^+, 0.1 mM Ca^{2+}, 4 mM Mg^{2+}, 130 mM acetate, 10 mM Cl^-, 200 μM L-Glu, 5 mM Hepes, pH 7.0. (B) Current-voltage curves of anion-selective channels activated by L-Glu obtained at two different Cl^- concentrations in the patch pipette. Pipettes were filled with either 130 mM acetate, 10 mM Cl^- buffer (full circles) or 90 mM acetate, 50 mM Cl^- (open circles). (C) Histogram and fitted probability density functions of open dwell times. Single-channel current records were obtained under the same conditions as in (A) at an applied voltage of −65 mV. The ordinate is displayed as a square root scale. Data were best fitted with three exponential distributions with time constants of 4, 22, and 150 ms and fraction of events of 70, 19, and 11%, respectively. Histogram obtained from 364 events. Cut-off time was set at 1 ms.

3.3. *L-Glutamate and GABA activate anion-selective channels*

To explore the possibility that the effects of bath-applied L-glutamate may originate from activation of excitatory and inhibitory receptors, we directly measured single-channel currents induced by L-Glu. Single-channel currents were recorded in cell-attached and outside-out patches. At above 50 μM, anion-selective single-channel currents were readily detected in cell-attached patches. The channel openings are grouped in bursts. Fig.3A shows single-channel records at a higher time and current resolution. Under these conditions it is apparent that the channel displays two conductance states. However, conductance substates can also be occasionally detected. Current-voltage relationships for the open channel at two different chloride concentrations inside the patch pipette are shown in fig.3B. The full circles represent currents obtained when the chloride concentration in the pipette was 10 mM. Under this condition, the straight line fitted to the experimental points extrapolates to a reversal potential near zero. Raising the concentration of chloride to 50 mM inside the pipette shifts the current-voltage curve by nearly 40 mV (open circles) indicating that the channel is perfectly anion-selective. The slope conductance for both chloride concentrations is about 38 pS. The distribution of open times is well fitted by the sum of three exponential components with time constants of 4, 22, and 150 ms (fig.3C). The fast component accounts for 70% of all transitions, the slower components accounting for 19 and 11%, respectively. Thus, at 200 μM glutamate the channel has access to at least three kinetically distinct open states. Measurements of single-channel currents activated by L-Glu beyond -40 mV are difficult because depolarization activates other conductance pathways. Channel activity appears to decay with time, perhaps due to receptor desensitization. This decay occurs a few minutes after the recording conditions have been established. The apparent time constant for this process is about 2 min. We also observed activation by GABA of anion-selective channel in cell-attached patches. GABA-activated anion channels have a slope conductance of 23 pS (not shown).

Activation by L-Glu and GABA of anion-selective channels can also be demonstrated in outside-out patches. Fig.4A shows records before (control) and after perfusion with a solution containing 200 μM GABA. It is clear from these records that single-channel currents do not display a unique open state conductance value under these experimental conditions. This is documented in the conductance histogram shown in fig.4B derived

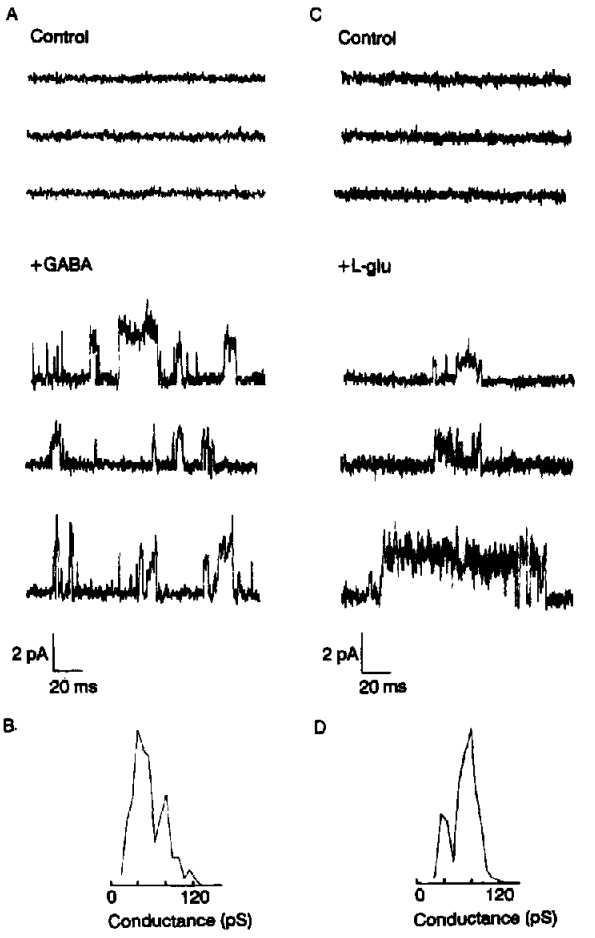

Fig.4. Anion-selective channels activated by GABA and L-Glu in outside-out patches. Patch pipettes had resistances of 1–2 MΩ and the solution on both sides of the patch was standard buffer. (A) Single-channel currents were measured in the absence of GABA for several minutes (control). The chamber was then perfused with 5 vols of standard saline containing 200 μM GABA. The applied voltage in both the control and experimental record was -30 mV. (B) Conductance histogram of single-channel currents activated by GABA in outside-out patches. (C) Current record obtained in an outside-out patch previous to the addition of L-Glu (control) and after perfusion with 200 μM L-Glu. Both sides of the patch were exposed to standard buffer. Applied voltage was 30 mV. (D) Conductance histogram of L-Glu-activated single-channel currents in outside-out patches.

from data obtained in symmetrical solutions containing 140 mM chloride. The conductance histogram obtained in the presence of GABA shows two well defined peaks at 40 and 80 pS and a minor peak at 110 pS. Similar results can be obtained with L-Glu (fig.4C,D). The conductance histograms shown in fig.4D indicate the occurrence of only two major peaks at 40 and 80 pS. Note that the peak at 80 pS is more prominent in the presence of L-Glu while the 40 pS peak is more probable in the case of GABA. In the presence of chloride gradients channels activated by GABA and L-Glu in outside-out patches behave as predicted by the Nernst equation for a chloride electrode.

3.4. *Excitatory receptor channels activated by L-Glu*

We also observed the activation of high-conductance cationic channels by L-Glu. These channels were not detected in the presence of GABA. The frequency of appearance of this type of channels is very low and they were observed in only five out of four hundred patches. This can be due to a low channel density or desensitization. Treatment with concanavalin A (conA), which abolishes desensitization of excitatory channels activated by L-Glu in other preparations [7,10,11], did not improve the chances of detecting this type of channel in *Drosophila*. Fig.5A shows the activation by L-Glu of channels with a slope conductance of 104 pS (fig.5B). Channel activity appears in bursts as shown in the upper panel of fig.5A. The distribution of open dwell times shows a fast and a slow component. The fast component, which predominates, has a time constant of 1.5 ms, while that of the slow component is 16 ms. This result indicates that at 50 μM L-Glu, the cationic L-Glu-activated channel has at least two open states. Kerry et al. [12] have recently reported that the number of distinct open states the channel has access to is [L-Glu]-dependent. At 10 μM L-Glu two accessible open states are found whereas at 100 μM L-Glu the channel has access to three kinetically distinct open states [12]. The reversal potential estimated from extrapolation of the current-voltage relationship shown in fig.5B, is close to 0 mV and indicates that excitatory channels show a poor selectivity between Na^+ and K^+.

4. DISCUSSION

Here, we provide evidence supporting the idea that L-Glu activates two types of receptor channels in *Drosophila* larval muscle. The excitatory role of this neurotransmitter has been previously documented by Jan and Jan [1]. However, L-Glu-activated channels have not been previously described in *Drosophila* larval muscle. Our studies indicate that, similar to other arthropods, L-Glu-activated excitatory channels in *Drosophila* possess a large unitary conductance [5–7]. In addition, we have demonstrated for the first time the presence of anion-selective receptor channels activated by

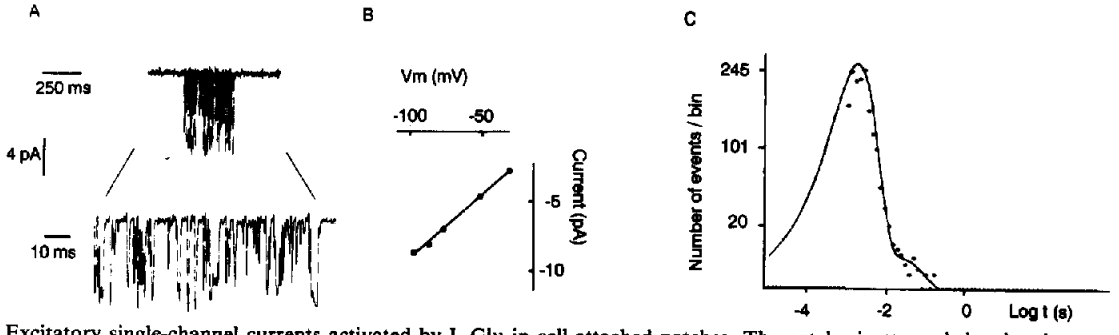

Fig.5. Excitatory single-channel currents activated by L-Glu in cell-attached patches. The patch pipette and the chamber contained standard buffer. Voltage was −105 mV. (A) Bursts of channel activity obtained with a pipette containing 50 μM L-Glu. Upper trace: The record is shown at low time resolution. Lower trace: a segment of the upper trace is shown at higher time resolution. (B) Current-voltage relationship of excitatory single-channel current activated by 50 μM L-Glu. Slope conductance is 104 pS. (C) Histogram and fitted probability density functions of open dwell times. Single-channel records were obtained under the same conditions as in (A) at an applied voltage of −70 mV. The ordinate is displayed as square root scale. Data were best fitted with two exponential distributions with time constants of 1.5 and 16 ms and fraction of events of 98 and 2%, respectively. Histogram obtained from 1647 events. Cut-off time was set at 1 ms.

L-Glu and GABA. Channel activation was demonstrated in both cell-attached, and outside-out patches. In cell-attached patches anion channels activated by L-Glu and GABA display mostly only two conductance states. On the other hand, in outside-out patches, under conditions in which both sides of the patch are exposed to standard buffer, the presence of subconductance states is apparent. The frequency of occurrence of conductance substates seems to depend on the type of agonist as found for the inhibitory channels activated by L-Glu and GABA in crayfish muscle [13]. A correlation between open conductance substate and type of agonist has also been reported in excitatory L-Glu receptors in vertebrates [14,15]. In addition, in cell-attached patches, channels activated by GABA have longer open times than those activated by L-Glu. The effects of agonist concentration on channel activation and the pharmacological profile of inhibitory receptor channels present in *Drosophila* larval muscle are currently under study.

The presence of two types of receptor channels activated by L-Glu in *Drosophila* larval muscle may explain the effects of bath-applied agonist on membrane potential. Thus, in the presence of chloride in the external buffer, L-Glu up to concentrations $\leq 20\,\mu M$, gradually depolarizes the muscle membrane, but higher agonist concentrations induce a repolarizing effect. On the basis of the present results we suggest that activation by L-Glu of excitatory receptor channels induces the membrane depolarization seen in fig.1A,B. However, higher concentrations of L-Glu also activate inhibitory receptor channels which are able to repolarize the muscle membrane to levels not very different from the resting potential measured in the absence of the agonist. This is supported by the fact that when chloride is replaced by sulfate from the external solution, only the depolarizing effect of L-Glu is observed. On the other hand, bath-applied GABA had only a small hyperpolarizing or no effect on membrane potential when chloride is present in the external solution.

Records of smpp revealed the occurrence of depolarizing and hyperpolarizing events. The occurrence of hyperpolarizing smpp strongly suggests the presence of inhibitory synapses in *Drosophila* larval muscle. However, we have not directly unveiled the actual nature of the chemical transmitter. GABA-sensitive inhibitory synapses and anion channels activated by GABA and L-Glu have been reported in other arthropods [13]. Furthermore, it has been shown that GABA induces hyperpolarizing responses in *Drosophila* neuromuscular junction in culture [2].

Further studies of L-Glu receptors in *Drosophila* muscle, at the single-channel level, should lead to their complete pharmacological characterization. Definition of such a pharmacological profile is a required condition to attempt the identification of L-Glu receptor neurological mutants in this attractive experimental system.

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