Depression of Synaptic Efficacy in High- and Low-Output *Drosophila* Neuromuscular Junctions by the Molting Hormone (20-HE)

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Ruffner, Marvin E., Stuart I. Cromarty, and Robin L. Cooper. Depression of synaptic efficacy in high- and low-output *Drosophila* neuromuscular junctions by the molting hormone (20-HE). *J. Neurophysiol.* 81: 788–794, 1999. The molt-related steroid hormone, 20-hydroxyecdysone (20-HE), was applied to muscles 6 and 7 of third instar larval of *Drosophila melanogaster* neuromuscular junction preparations to examine if rapid, nongenomic responses could be observed as was shown recently to occur in crustacean neuromuscular junctions. At a dose of 10 μM, the excitatory junction potentials were reduced in amplitude within minutes. To elucidate the site of action of the hormone, focal-macropatch recordings of synaptic currents were obtained over the neuromuscular junctions. The results showed that the high-output (Is) and the low-output (Ib) motor nerve terminals, which innervate muscles 6 and 7, released fewer synaptic vesicles for each stimulation while exposed to 20-HE. Because the size and shape of synaptic currents from spontaneous releases did not change, the effects of the 20-HE are presynaptic. The rapid effects of this hormone may account in part for the quiescent behavior associated with molts among insects and crustaceans.

INTRODUCTION

Hormones have essential roles in developmental changes in the life of the fruit fly *Drosophila melanogaster*, from the instar stages to the pupa and to the adult forms. Hormones associated with developmental changes during metamorphosis are known as ecdysteroids, one of which is 20-hydroxyecdysone (20-HE), currently regarded as the active form of ecdysone (Riddford 1985; Steel and Davey 1985). This particular group of hormones is important in causing the behavioral and physical changes that occur during the development stages of each molt (Truman 1996). In *D. melanogaster*, the highest levels of ecdysteroid are measured during the third instar larva to prepupal formation and in the pupa stage preceding the adult stage (White et al. 1997). It has been shown that motor neurons in the hawkmoth, *Manduca sexta*, undergo various responses such as apoptosis, regression, and regrowth during various stages of development where levels of ecdysteroids are also high (Truman and Reiss 1995). Thus it has been observed that steroids show both physiological and anatomic effects on neurons (Jacobs and Weeks 1990; Levine and Weeks 1996; Thummel 1996).

It has been established that the actions of ecdysteroids described to date within these insects indicate a steroid-based genomic effect (Levine and Weeks 1996; Segraves 1994; Thummel 1996). Currently there is a vast amount of knowledge showing the genomic action of various steroids and hormones on gene expression in insects. Besides the generalized steroidal effects, such as those reported for estradiol, aldosterone, vitamin D3, and cortisol, within mammalian systems, there is substantial documentation of nongenomic effects by direct binding to membrane-bound receptors that result in rapid action of cellular processes. These nongenomic cellular processes include activation of the inositol triphosphate (IP_3), guanosine 3′,5′-cyclic monophosphate (cGMP) and tris(hydroxymethyl)aminomethane signaling pathways and increased release of internal calcium (Thummel 1996; Wehling 1995). The nongenomic mechanics of steroid actions are not well understood. However, from recent studies it has been shown that quick changes occur in synaptic transmission in response to 20-HE exposure (Cooper and Ruffner 1998; Cromarty and Kass-Simon 1996, 1998).

The following study investigates the neuromodulatory effect of 20-HE on the quantal release of synaptic transmission. This study is an attempt to understand the mechanistic action of 20-HE on *D. melanogaster* motor neurons at the neuromuscular junctions of high- and low-output terminals during the third instar larva stage. We concentrated on the nongenomic effects of 20-HE in synaptic transmission by measuring the excitatory junction potentials (EJPs) and the excitatory junction currents (EJCs). EJP amplitudes are seen to depress in the presence of 20-HE due to a reduction in EJCs, thus demonstrating that 20-HE behaves as a neuromodulator. To explain the alteration in the EJPs, investigation of the synaptic efficacy at the motor nerve terminals was performed by recording spontaneous and evoked currents by quantal analysis. From this analysis, we have addressed pre- and postsynaptic effects induced by 20-HE. The results help to explain the behavior of the animals at the time of molt when the molt hormones are at their peak in that they exhibit a reduction in overall movements.

METHODS

Chemicals

The 20-HE and physiological salts were obtained from Sigma. The 20-HE solution was made that day of experimentation at a concentration of 10 μM in saline. The entire bathing solution during the experiment was exchanged rapidly (<1 min) two times with the

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20-HE containing saline. After the 20-HE application, the preparations were rinsed with saline and a saline containing 5 mM Ca^{2+} to determine if transmission increased.

Electrophysiology

The wild-type Canton S fly strain was raised at 21°C on cornmeal-agar-dextrose-yeast medium. Only third instar larvae were used for these studies.

The larval dissections were preformed as described previously (Stewart 1996; Stewart et al. 1994). In brief, the preparations were slit along the mid-dorsal longitudinal axis and pinned flat. The preparation dish consisted of a glass slide with magnetic tape adhered to one side. A hole in the center of the magnetic strip allowed the preparation to be viewed with transmitted light. Dissecting pins were bent and glued to paper clips. Paper clips are maneuvered easily on the magnetic tape to hold the fillet preparation in place. This type of recording dish has been described previously for pinning ganglia isolated from the leech ventral nerve cord (Muller et al. 1981).

The recording arrangement and solutions were the same as previously described (Neckameyer and Cooper 1998; Stewart et al. 1994). In brief, the physiological saline contained (in mM) 1.0 $CaCl_2 0.2H_2O$, 70 NaCl, 5 KCl, 10 $NaHCO_3$, 5 trehalose, 115 sucrose, 5 *N*,*N*-bis(2-hydoxyethyl)-2-aminoethanesulfonic acid (BES). All experiments were performed at room temperature (19–22°C). Intracellular recordings were made with the use of microelectrodes filled with 3 M KCl having a resistance of 30–60 mΩ. Responses were recorded with a 1X LU head stage and an Axoclamp 2A amplifier. Intracellular recordings were made in muscles 6 and 7. Focal, macropatch recordings were made with a 10-μm diam fire-polished glass electrode placed directly over nerve terminals that were viewed under a ×40 water immersion lens (Nikon, NA 0.55). By grossly misaligning the light condenser on the Nikon Optiphot microscope, an image of the nerve terminals as viewed under Nomarski optics was visible (suggestions by Dr. Len Kaczmarek in a seminar, Yale). To record synaptic currents, a 0.1X LU head stage was used. Electrical signals were recorded to VHS tape (Vetter, 400) as well as on-line to a PowerMac 9500 via a MacLab/4 s interface (ADInstruments). All events were measured and calibrated with the MacLab Scope software version 3.5.4. Averages of 1,000 traces of evoked currents were made to obtain an overall average as presented for the Ib and Is terminals. Recordings presented of the EJPs consisted of an average of 10–20 events. The average in each of the five preparations was used to calculate the mean ± SE. All recordings were made at 0.5-Hz simulation frequency and taken from muscle 6.

Focal macropatch recordings

On determining the terminal region to record, a photograph was taken for later documentation.

In each trace, a trigger artifact was visualized that was used as a reference point to measure the time to evoked responses. Evoked and spontaneous events were analyzed to determine mean quantal content, *m*, as previously described in detail (Cooper et al. 1995b). Mean quantal content determined by two approaches was implemented for all the synaptic current recordings to compare the various approaches used while exposed to 20-HE. Peak measures gave values close to those obtained by charge measures as noted earlier when only exposed to saline (Cooper et al. 1995b). Measurements of the maximum peak of each evoked event, including failures, provided an average evoked peak. This value then was divided by the mean peak amplitude of spontaneous events to provide the mean quantal content determined by the peak amplitude (m_{pk}) approach (Del Castillo and Katz 1954). It should be noted that the time of peak evoked events were varied, thus the point in time in which the measurements were made was allowed to shift to obtain the true peak in each evoked response. The area under the trace or charge (pA × ms) of the evoked events and failures similarly was divided by the mean charge of the spontaneous to provide the mean quantal content (m_{ch}) by the charge approach. Histograms of the evoked events were made for each trace within a recorded period and for any spontaneous events throughout the recording to determine if shifts in peak and charge distributions occurred on the addition of 20-HE and the high-Ca^{2+}-containing solution.

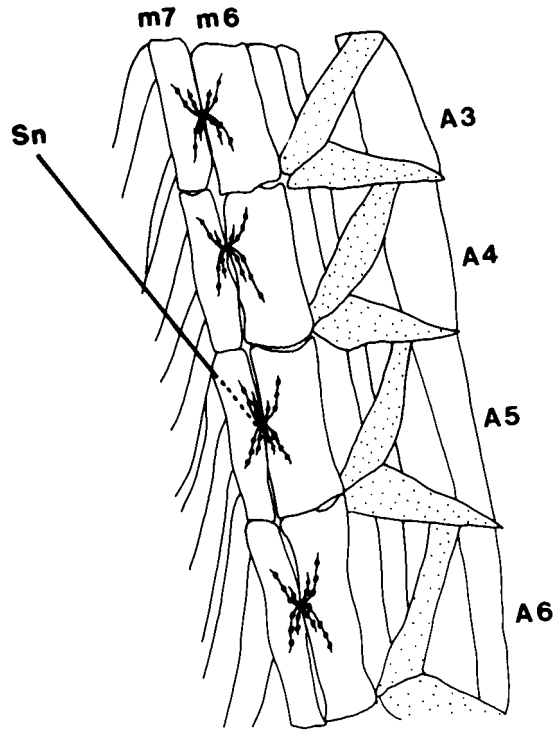

FIG. 1. Schematic diagram of the 3rd instar *Drosophila* larva preparation. Ventral abdominal muscles, m6 and m7 muscles, are innervated by 2 excitatory axons contained in one segmental nerve (Sn). Segmental nerve is only shown for segment A5. Nerve terminals, Is and Ib, are represented schematically on the m6 and m7 muscles.

RESULTS

Innervation

The ventral longitudinal abdominal muscles, m6 and m7, can be observed readily in a preparation when a longitudinal middorsal incision is made and the preparation is spread out followed by removing the internal organs (Fig. 1). Muscles m6 and m7 are innervated by both type Ib (big varicosities) and Is (small varicosities) motor neurons (Atwood et al. 1993). The Ib neuron gives rise to a smaller EJPs than the Is motor neuron on these muscles.

Past physiological observations (Jan and Jan 1976) from these muscles have shown different types of EJPs, but recent studies that explored a more suitable physiological bathing medium have shown that the shape of the EJPs and the length in viability of the preparation is highly dependent on the bathing medium being used (Stewart et al. 1994). The EJPs that arise from type Ib and Is can be distinguished readily based on the size of their response within a preparation by intracellular recording in the muscle fibers. Figure 2 shows a typical EJP response of an Is and an Ib neuron. If both terminals are recruited, then a summed EJP is recorded. The selective exci-

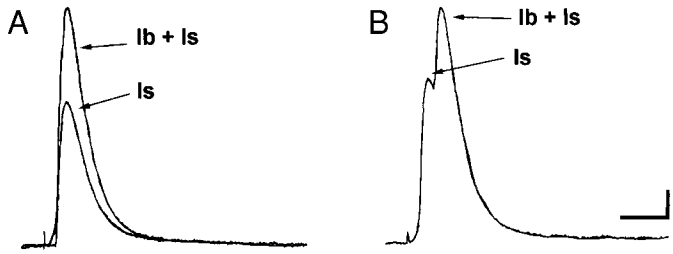

FIG. 2. Recording of excitatory junctional potentials (EJPs). Segmental nerve 4 was stimulated while the EJPs were recorded with an intracellular electrode in the muscle 6. *A*: isolated responses from either stimulation of just the Is or in combination of Is and Ib motor neurons are shown. *B*: when altering the stimulus duration or intensity, occasionally Ib can activated with a slight delay and the 2 responses can be observed in a single trace. Scale bars: 10 mV, 20 ms for *A* and *B*.

tation of either the Is or Ib axon is done by altering the stimulus polarity and/or intensity given to the segmented nerve root.

Effects of 20-HE on EJPs

Exchanging the bathing medium to one containing 10 μM 20-HE resulted in a rapid reduction in the combined Is and Ib EJP amplitudes (Fig. 3*A*). The reduction normally occurred within the 1st min after the exchange of the bathing medium. The response continued to decline as long as the 20-HE was present. On three rapid exchanges of bathing medium back to the initial medium, the EJPs still continued to decline (Fig. 3*B*). The EJP amplitudes, for the most part, then could be revived by the addition of a bathing medium five times higher in Ca^{2+} (1–5 mM) as shown in Fig. 3. When the preparation is exposed to only a high-calcium-containing saline without prior exposure to 20-HE, the EJP amplitude increases above the basal levels. This indicates that 20-HE still causes a depression in synaptic release even with exposure to high calcium (Fig. 3, *A* and *C*). Because there was variability in the EJP amplitudes among preparations, an average of the initial EJP amplitudes, over the first 1 min in each of the five preparations that were exposed to 20-HE, was set to 100% for normalization. The EJP amplitude values shown were a result of an average over the preceding 1 min during a 0.5-Hz nerve stimulation frequency. Figure 4 depicts the variability in the rate of reduction in the EJP amplitude during the exposure to 20-HE. In all the preparations, there was a continuous decrease in the EJP amplitudes over time during the exposure to 20-HE and an enhancement on a higher calcium containing medium in the absence of 20-HE.

The effects of 20-HE were not selective to the Is or Ib terminals because the EJPs decreased for both terminals. As expected from studies in which high calcium alone was added (Kurdyak et al. 1994), both terminals increased the evoked synaptic output on exposure to a higher intracellular calcium concentration.

Effect of 20-HE on EJCs

The EJC recordings selected over varicosities of either Is or Ib terminals with a focal macropatch electrode revealed that 20-HE reduced the total amount of synaptic charge (Fig. 5). Focal recordings made directly over visualized varicosities allowed one to determine the quantal content for the location and to assess the effects on both pre- and postsynaptic sites.

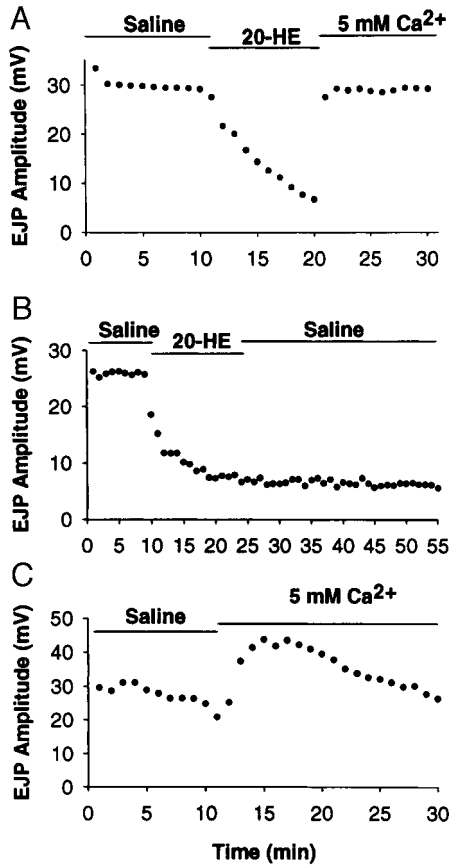

FIG. 3. Changes in the summed Is and Ib EJP amplitude elicited by 20-hydroxyecdysone (20-HE) over time. *A*: 20-HE decreases the EJP amplitude over a short period of time, while after wash out of 20-HE and addition of 5 mM Ca^{2+}, the EJP amplitude is restored partially. Ability of the neuron to increase transmitter output after a strong reduction indicates that the preparation is still viable. *B*: although the effects of 20-HE are not readily reversed by washing the preparation with saline. *C*: effect of high-calcium-containing saline without prior exposure to 20-HE results in the EJP amplitude increasing above the control levels. This indicates that the partial reversal in the effect of 20-HE with exposure to high calcium, shown in *A*, is still under an influence of the prior 20-HE exposure.

The sites along the Is and Ib terminals exhibited a range of synaptic efficacies as measured in other arthropod (crayfish) motor nerve terminals (Cooper et al. 1995a). The data shown were from representative sites of the Is and Ib terminals when clear visual distinctions could be made of the two terminal endings.

The measure of evoked ''charge'' instead of evoked peak ''current'' amplitudes is a more reliable approach in determining quantal content when events do not appear in synchrony. Release that occurs with a latency jitter results in a broader and smaller evoked current that would underestimate mean quantal content. These differences in estimating mean quantal content have been dealt with in an earlier study of this preparation (Cooper et al. 1995b).

The focal macropatch electrodes used in this study had 10- to 12-μm ID, which results in at least the corresponding length of terminal being recorded. Because there is substantial variation of evoked release among varicosities along a given terminal as well as between the Is and Ib terminals, an average was taken of the evoked change over the period of recordings during the control saline bath exposure. This average response

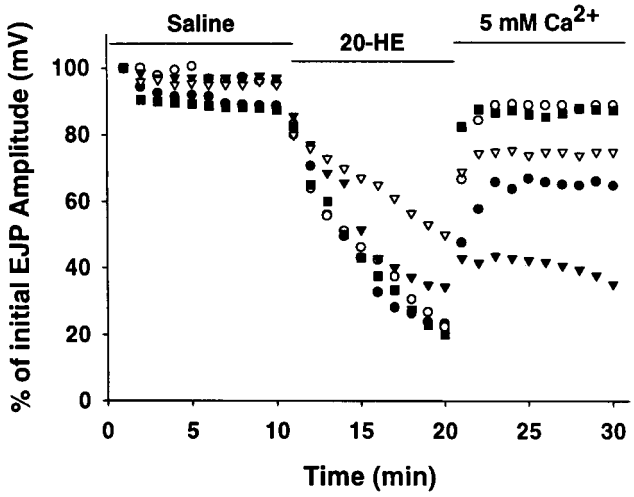

FIG. 4. Normalized EJP amplitudes of several preparations from Is motor neurons. Graphic representation showing the depression in EJP amplitudes during a 20-min time period in the presence of 20-HE in 5 preparations. Amplitude values are normalized to the initial EJP in each preparation. After wash out of 20-HE, 5 mM Ca^{2+} increased synaptic release to varying degrees in each preparation.

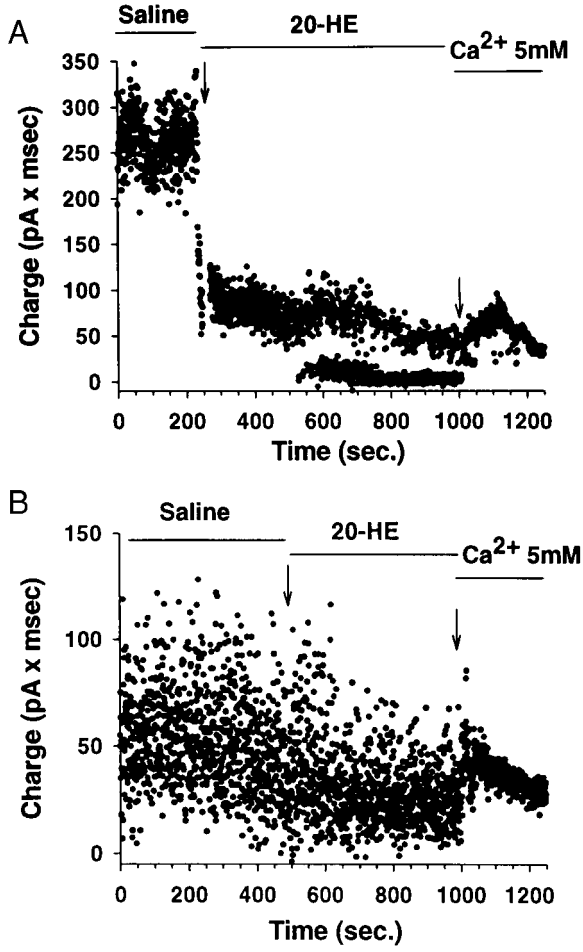

FIG. 5. Effect of 20-HE on synaptic currents. Current influx into the muscle fiber measured by a focal macropatch electrode in the high-output (Is, *A*) and low-output (Ib, *B*) nerve terminals is seen to decrease over time in the presence of 20-HE. Addition of Ca^{2+} (5 mM) increases vesicular release at the nerve terminals causing an increase in the evoked excitatory junctional current (EJC).

during the initial phase of the control period was set to 100% for normalization among preparations. After normalization the trend was seen readily that showed that the higher output (Is) terminals decreased more dramatically than the Ib, low-output terminals (Fig. 6, Table 1). Both terminals displayed a transient increase in the evoked response when a higher concentration calcium medium was exchanged. Due to the fact that in the presence of a higher calcium solution the muscle produces larger contractions, the seal resistance of the macropatch electrode and its location over the terminal was hard to maintain. Thus the transient increase usually was followed by an artifactual reduction in the charge measure. The intracellular EJPs maintained responses in higher calcium concentrations, supporting the fact that the focal-patch condition could not be maintained with the large muscle contractions.

To obtain estimates of the mean quantal content, the current area or charge was calculated (pA × ms) under the curve for both evoked and spontaneous measurements (Cooper et al. 1995b). Then by dividing the averaged evoked response by the average of the spontaneous events, the mean quantal content was estimated. As mentioned above, this method is preferred when latency jitter in release occurs (Borst and Sakmann 1996; Cooper et al. 1995b).

The mEJCs indicate the unitary nature of the evoked events. There was no discernible difference in the distribution of the area of the mEJCs between the saline bath and the 20-HE-treated preparations, indicating that the postsynaptic receptors were not being altered by the presence of 20-HE. Figure 7 represents the general findings in that there were more failures in evoked, EJCs in the presence of 20-HE, although the spontaneous or miniature excitatory postsynaptic currents (mEJC) still occurred with relatively the same size and shape. The increase in the number of failures in the presence of 20-HE is apparent in the superimposed histograms. The distribution of spontaneous events is not shifted as a result of 20-HE application (Fig. 7, *right*).

Estimates of mean quantal content (m_{charge}) of patches of the Is and Ib terminals generally showed that the Is had higher output varicosities than the Ib terminals. It has been reported (Stewart 1996) that there is variation along the length of the terminal that probably accounts for the variations that we also observed (Table 1). It was not our purpose to quantitate the synaptic efficacy along the lengths of terminals, so we did not pursue this issue because it previously has been dealt with physiologically and structurally (Atwood and Cooper 1995, 1996a; Atwood et al. 1993). We did resolve the fact that 20-HE reduced synaptic efficacy in both Is and Ib terminals and that 20-HE is working through a presynaptic component. As similarly shown in tonic crayfish neuromuscular junctions (Cooper and Ruffner 1998), 20-HE did not result in any measurable effect on the spontaneous events recorded from Ib or Is terminals innervating m6 and m7 muscles.

Because the synaptic responses continued to diminish after the application of 20-HE and washing, there was concern that the nerve terminal could not recover or enhance release, thus the bathing medium was changed to saline containing 5 mM calcium, which is well known to cause an increase in synaptic transmission. The increases with calcium resulted in a reversal of the decline induced by 20-HE. The rapid rise in the response showed that the ions were able to reach the presynaptic calcium channels within one minute.

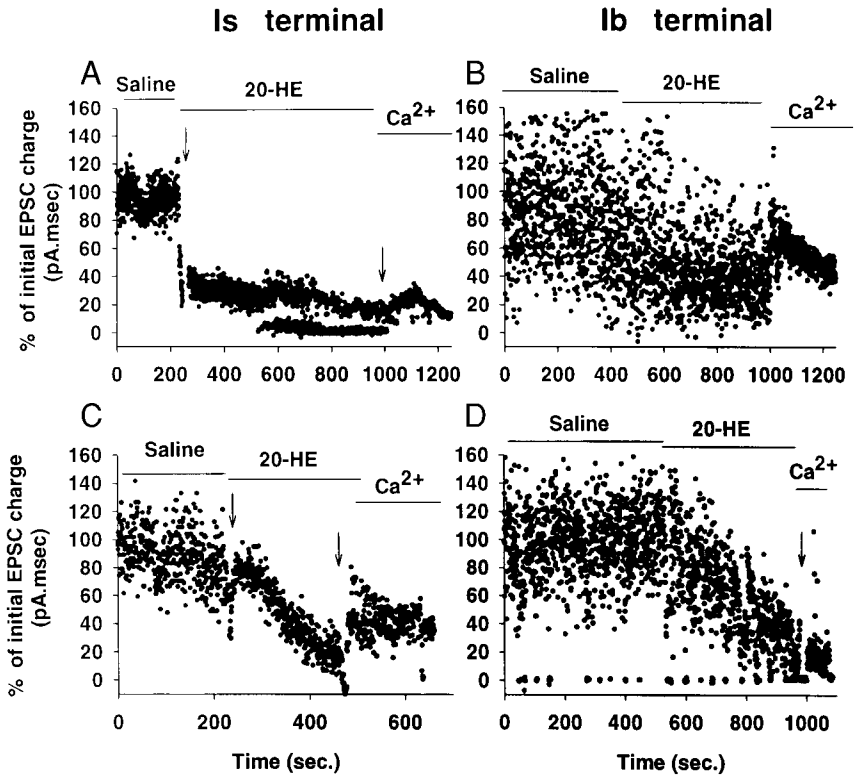

FIG. 6. Normalized EJC amplitudes of several preparations from Is and Ib neuromuscular junctions. All the preparations show a substantial decrease from a small patched region of the nerve terminal. Thus the decrease of transmission for the entire length of the terminal will be large and is likely to account for the decrease in the EJP amplitudes. The values shown are normalized to 100% by taking an average of the 1st 5 initial EJCs in each preparation and setting the averaged value to 100%. *A* and *C* are from Is terminals, and *B* and *D* are from Ib terminals. Each graph is from a separate preparation.

DISCUSSION

The larval *Drosophila* neuromuscular preparations of the body wall muscles 6 and 7 serve a distinct advantage to compare the effects of neurmodulators because high- (Is) and low-output (Ib) nerve terminals innervate the same muscle fiber. The synaptic structural differences, which in part account for the physiologically observed differences, have been investigated (Atwood and Cooper 1995; Atwood et al. 1993; Kurdyak et al. 1994; Stewart et al. 1994). Many features that account for the high- and low-output differences in *Drosophila* are also evident among crustacean motor nerve terminals (Atwood and Cooper 1996b; Bradacs et al. 1997; Cooper et al. 1995a, 1996a–c).

In this study, we have presented evidence that indicates that the probability of vesicular release at high- (Is) and low-output (Ib) motor nerve terminals in the wandering third instar larval stage of *D. melanogaster* is reduced in the presence of the steroid molting hormone, 20-HE. The overall effect in a reduction of vesicular fusion is that the EJPs are smaller due to the fact that there is a smaller influx of current into the muscle fiber. Hence, evoke EJCs and mEJCs were recorded to support the inference that 20-HE produces a reduction in quantal release from the excitatory motor neuron.

TABLE 1. *Mean quantal content determined by charge measures (m_{charge})*

Terminal	Experiment	Saline	20-HE	Percent Difference
Ib	Preparation 1	9.65	7.00	↓ 27.46
	Preparation 2	3.59	1.31	↓ 63.42
	Preparation 3	3.64	1.67	↓ 54.12
	Preparation 4	6.08	3.77	↓ 38
Mean				45.75
Is	Preparation 5	6.211	3.86	↓ 37.85
	Preparation 6	9.03	2.44	↓ 72.94
	Preparation 7	17.64	3.21	↓ 81.79
Mean				64.19

The mean quantal content determined by measuring evoked charge (m_{charge}) in the saline groups as well as in the 20-hydroxyecdysone (20-HE) groups. The percent difference was calculated by the following method: {[(saline) − (20-HE)]/(saline)} × 100%. There is a trend for a greater decrease in the m_{charge} for the Is terminals than that of the Ib terminals in the presence of 20-HE.

The rapid effects 20-HE has on these motor nerve terminals suggests that it is acting through a nonconventional, nongenomic mechanism. The EJCs at both high- and low-output neuromuscular junctions showed a rapid reduction in size on the application of 20-HE. The physiological changes measured in this study correlate well with the fact that there are many developmental changes occurring during this stage of development in *Drosophila*. During the transition between the third instar larva and pupa formation, physiological changes occur in preparation for the development of the pupal stage. Because rearrangement of muscles and nerves occur during this period, it is not surprising that the neurons are sensitive to the neuromodulatory effects of 20-HE and that muscular contractions would be suppressed to avoid damaging the forming pupa.

The quantal analysis of the spontaneous currents indicates that 20-HE does not affect the postsynaptic receptors in these glutamatergic junctions because no notable change in the amplitude or charge could be measured. These findings suggest that 20-HE's site of action is at the presynaptic neuron. There maybe an ecdysone receptor located on the presynaptic neuron that binds 20-HE with certain affinity, and this binding in turn has an effect on (one or more) of the processes of the synaptic vesicle formation, docking, or release. This speculation re-

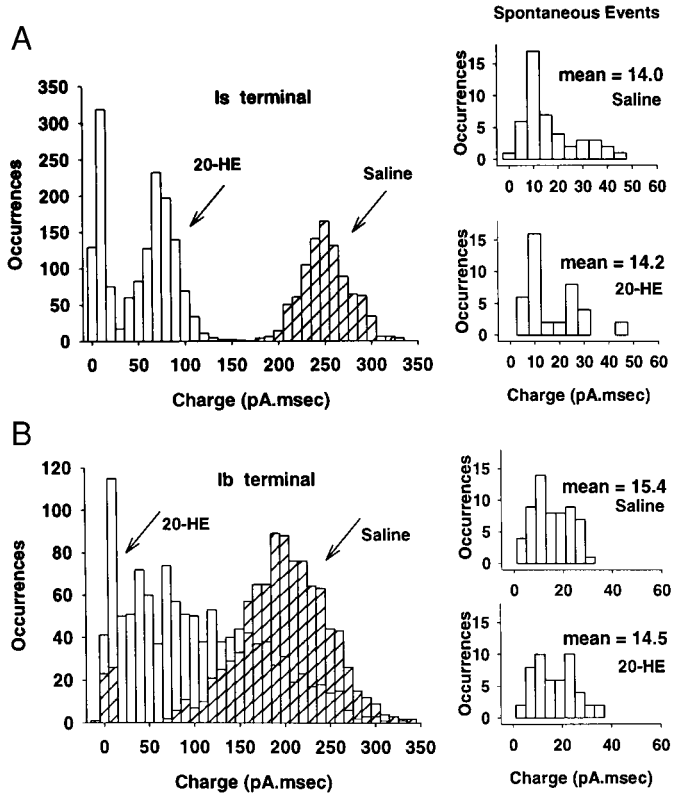

FIG. 7. Changes in the quantal content induced by 20-HE. Peak-evoked and spontaneous EJCs are plotted in histograms to show overall shifts in the evoked response (large graphs) with relatively no change in the amplitudes of spontaneous events (*insets* for saline and 20-HE). Responses were recorded during a 0.5-Hz stimulation frequency for 1,000 trials in saline and for the 1st 1,000 trials in the presence of 20-HE (10 μM). Note the size of the spontaneous events did not shift while the preparation was exposed to 20-HE. This experimental procedure was followed for both the Is terminals (*A*) and the Ib terminals (*B*). Results indicates a presynaptic site of action for 20-HE.

mains to be tested and attempts are being made to localize ecdysone receptors on the nerve terminals.

The rapid nongenomic effects observed conforms well to current studies reported in lobsters (Cromarty 1995; Cromarty and Kass-Simon 1996, 1998) and supports the emerging concept of short-term steroid modulation on biological activity (Schumacher 1990; Wehling 1995).

We have not yet elucidated the cellular sites of 20-HE's action, but it is possible that it might be functioning by affecting the presynaptic mGlu receptors of the motor nerve terminals, which in crustaceans is known to act as a negative feedback although the effects of these receptors are readily reversible (Miwa et al. 1990). Recent evidence has shown 20-HE to have a rapid nongenomic effect on crayfish motor nerve terminals by decreasing the number of vesicles that are released during evoked stimulation in the opener muscle of the walking legs (Cooper and Ruffner 1998), whereas exposure to 20-HE of the opener muscle of the claw in lobsters has shown an enhancement of the postsynaptic potentials (Cromarty and Kass-Simon 1996, 1998). In addition, the tonic superficial, abdominal flexor muscle of lobsters showed a decrease in the EJP amplitude in the presence of 20-HE (Cromarty and Kass-Simon 1998). The differential effects among neuromuscular junctions warrants further investigations into the mechanism of action. Work is currently underway with the fly mutant ''ecdysonless'' to help in elucidating the mechanisms.

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