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Glutamate and *N*-methyl-D-aspartate affect release from crayfish axon terminals in a voltage-dependent manner

(feedback inhibition/presynaptic autoreceptors/synaptic modulation/quantal release)

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ABSTRACT **In the crayfish neuromuscular junction, the excitatory transmitter is glutamate. The present study shows that at concentrations as low as 5×10^{-7} M, glutamate affects the depolarization-evoked release of neurotransmitter. Furthermore, the effect of glutamate on release is voltage-dependent and depends on the level of the depolarizing pulse. Nerve terminals were exposed to 5×10^{-7} M tetrodotoxin and then depolarized to different levels by a macropatch electrode. Depending on the amplitude of the depolarizing pulse, glutamate (5×10^{-7} to 1×10^{-5} M) had a dual effect on release. At small depolarizing pulses, glutamate reduced release, whereas at large depolarizing pulses, it enhanced it. Glutamate at 10^{-6} M had no significant effect on action-potential-induced release. At 10^{-4} M glutamate, the action-potential-induced release was always inhibited. *N*-Methyl-D-aspartate was found to mimic one of the effects of glutamate: *N*-methyl-D-aspartate (10^{-7} to 10^{-5} M) reduced release at small depolarizing pulses but had no effect with larger depolarizations. 2-Amino-5-phosphonovaleric acid blocked the effect of *N*-methyl-D-aspartate.**

Notable among the mechanisms that modulate release of neurotransmitter is the activation of presynaptic autoreceptors that usually produce feedback inhibition on release. For example, the activation of the metabotrophic glutamate receptor reduces the release of glutamate in rat neostriatal slices (ref. 1 and see also ref. 2). To date, however, the mechanism underlying the presynaptic effect of glutamate has not been analyzed, nor has the full extent of its possible physiological role been elucidated.

A glutamatergic system that might contribute to the understanding of presynaptic autoreceptors is the neuromuscular system of the crayfish. In the crayfish, both the opener muscle system and the deep extensor abdominal muscle system have been extensively studied, and in both systems quantal release can be measured directly (3, 4). The neuromuscular system of the crayfish is of further interest because it shows many types of presynaptic plasticity (5). Moreover, there is evidence that crustacean axon terminals possess glutamate receptors. Glutamate depolarizes crayfish axon terminals (6) and hyperpolarizes lobster axon terminals (7). Moreover, Miwa *et al.* (8) found that glutamate (10^{-5} to 10^{-4} M), but not *N*-methyl-D-aspartate (NMDA), depressed release in lobster neuromuscular synapses.

Until recently, it was thought that invertebrate nervous systems do not possess NMDA receptors (9). However, Pfieffer-Linn and Glantz (10) have now demonstrated the presence of NMDA receptors in the crayfish optic lobe, and Dale and Kandel (11) have found that glutamate is the neurotransmitter in *Aplysia* sensory neurons. In that system, the action of glutamate on the postsynaptic receptors depends on the postsynaptic cell membrane potential, a phenomenon reminiscent of the voltage dependence of NMDA receptors.

In these earlier reports, release was induced by action potentials. Therefore, it was not possible to determine whether the presynaptic response to glutamate autoreceptors depends on the level of the depolarizing pulse.

In the present study, we tested whether presynaptic effect of glutamate and NMDA on release in crayfish axon terminals could be revealed by depolarizing the axon terminals to different levels [in the presence of tetrodotoxin (TTX)]. We found that glutamate (5×10^{-7} to 1×10^{-5} M) had a dual effect on release: it reduced release at small depolarizing pulses and enhanced release at larger depolarizing pulses. We found that NMDA (10^{-7} to 10^{-5} M) reduced release at small depolarizing pulses but had no effect on release at larger pulses. Thus, NMDA mimicked only one of the effects produced by glutamate. In this report, we concentrate only on the inhibitory effects of glutamate and NMDA on transmitter release.

METHODS

The deep extensor abdominal muscle and the opener muscle of the crayfish *Procambarus clarkii* were used. Crayfish were imported from Louisiana and kept in tanks with running fresh water. The animals were fed fish fillets twice a week. Technical details for isolation of the abdominal and opener muscles and mounting of the nerve muscle preparations are given by Arechiga *et al.* (12) and Hochner *et al.* (13). For action-potential-evoked release, the excitor axon was stimulated with a small suction electrode placed on the axon. Single quantum events were recorded with a macropatch electrode (12, 13). For direct depolarizations of the axon terminal, the preparation was treated with 5×10^{-7} M TTX, and negative current pulses of variable amplitudes were given through the same macropatch electrode (12). The level of depolarization depends on the amplitude of the negative current pulse and on the seal resistance of the macropatch electrode (≈150 kΩ). Thus, a current pulse of −0.1 μA depolarizes the terminal by ≈15 mV (14). Although the exact level of depolarization is not known, larger negative current pulses produce larger depolarizations.

It should be emphasized that depolarization is achieved by shifting the extracellular potential to more negative values. In essence, the level of depolarization does not depend on the membrane resistance of the nerve terminal, which is much larger than the seal resistance. Thus, even if glutamate (or NMDA) decreases the membrane resistance of the terminal, this should not affect the actual level of depolarization achieved by a given pulse amplitude. A detailed discussion of the conditions that determine the extent of depolarization may be found in Katz and Miledi (15) and Dudel (14).

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Abbreviations: NMDA, *N*-methyl-D-aspartate; TTX, tetrodotoxin; APV, 2-amino-5-phosphonovaleric acid.

Current traces were digitized at 50 kHz (Neurocorder, Neurodata Instruments) and stored on video tapes. In parallel, the traces were digitized at 50 kHz and fed into a Compaq 386/33 computer for on-line analysis.

Determination of Quantal Content. In the crayfish neuromuscular system, a single quantum can be easily recorded (see Fig. 1*A* and ref. 13). Presynaptic effects may, therefore, be detected by directly counting the number of quanta released. The quantal content is evaluated as follows: a given number of pulses is applied, and the total number of released quanta is counted and divided by the number of pulses. Since the number of released quanta is counted, this method is not affected by postsynaptic factors or by the size of the quantum, providing that a single quantum can be resolved. At higher glutamate concentrations, the postsynaptic receptors partially desensitize, but Fig. 1*A* shows that the single quantum could be easily discerned from the noise level at glutamate concentrations as high as 10^{-4} M, where the quantum size is significantly smaller than in the control.

Counting quanta is valid even at high concentrations of NMDA. This is because the quantum size was not altered in the presence of NMDA at any concentration. This is to be expected given that postsynaptic glutamate receptors in the crayfish neuromuscular junction are of the non-NMDA type (16).

Experiments were carried out at 12 ± 0.5°C. At this temperature, evoked release ends within 5 or 6 ms. Hence, the quantal content was obtained by counting the number of quanta released during a period of 10 ms after each pulse. For each pulse amplitude, 256–512 pulses were administered. For this number of pulses, the quantal content at repeated runs was found to vary by 10–15%. Therefore, when the quantal content was <0.1, 1024–2048 pulses were administered. The rate of stimulation was 5 Hz.

Determining Presynaptic Membrane Potential and Conductance. To monitor the resting potential or membrane conductance, one or two microelectrodes (respectively) were inserted into a secondary branch of the excitor axon of the opener muscle. The insertion point was near a junction with a tertiary nerve terminal. The electrical distance from release sites must have been short, as a small depolarization of 10–12 mV was sufficient to produce release of transmitter at a nearby release site (release was recorded with the macropatch electrode). For these experiments, we used an upright Nikon microscope (Optiphot 2) with a long working distance (1.6 mm) objective of ×40. The resistance of the electrodes was 18 MΩ and the distance between them was 12–15 μm. For gentle insertion of the two electrodes, we used two Burleigh inchworm systems. The two electrodes were connected to an Axoclamp amplifier. One electrode served to pass a constant current pulse, and the second monitored the voltage drop. Usually, the constant current pulse had an amplitude of 7 nA and a duration of 30 ms.

The small narrow chamber containing the nerve–muscle preparation had a volume of 0.8 ml. The total volume including the fluid in the perfusion tubes was 2 ml. The fluid was circulated using a Gilson peristaltic pump (rate flow 2 ml/min). Drugs were added to the pump reservoir; it took ≈2 min for the final concentration of the drug to be reached in the chamber. This technique limits the resolution to the steady-state effects of the drug. For this purpose, the quantal content was determined continuously before and after addition of the drug, until the maximal effect was obtained. The quantal content was established every minute (256 pulses at 5 Hz) or every 2 min (512 pulses at 5 Hz). In experiments where TTX was added, the electrode fluid also contained TTX. The van Harreveld solution contained 220 mM NaCl, 5.4 mM KCl, 13.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, and 10 mM Tris maleate (pH 7.4). NMDA and 2-amino-5-phosphonovaleric acid (APV) were purchased from Research Biochemicals (Natick, MA).

RESULTS

Effect of Glutamate on Release Produced by Graded Depolarization. We measured the quantal content produced by depolarizing pulses of different amplitudes (Fig. 1*B*). Quantal content increased with the amplitude of the negative current pulse until a peak was reached, after which release declined as the amplitude of the depolarizing pulse began to approach the equilibrium potential for Ca^{2+} (17). After establishing the control curve, glutamate (10^{-6} M) was added and the quantal content for a single level of depolarization was measured. Only when the effect was maximal and stable was the quantal content for different pulse amplitudes determined (Fig. 1*B*). It is readily apparent that glutamate exhibited a dual effect on release. At smaller current pulses (−0.2 to −0.4 μA), glutamate reduced release by 100–50%, respectively. This reduction is consistently larger than the fluctuations encountered on repeated determinations of quantal content (10–15%). With larger current pulses, glutamate enhanced release. Thus the control and the glutamate curves crossed each other.

Fig. 1*C* shows that there was variability in the response of a given release site to glutamate as a function of the depolarizing pulse intensity. This variability exists because it is impossible to know the absolute level of depolarization produced by the depolarizing current (due to changes in the patch electrode location and its pressure on the preparation). Because of this, it is incorrect to average results from different preparations and they are, therefore, presented individually (Fig. 1*C*). Despite these technical difficulties, it is clear that when release is presented as the percentage of control (in the absence of glutamate) for each preparation, all

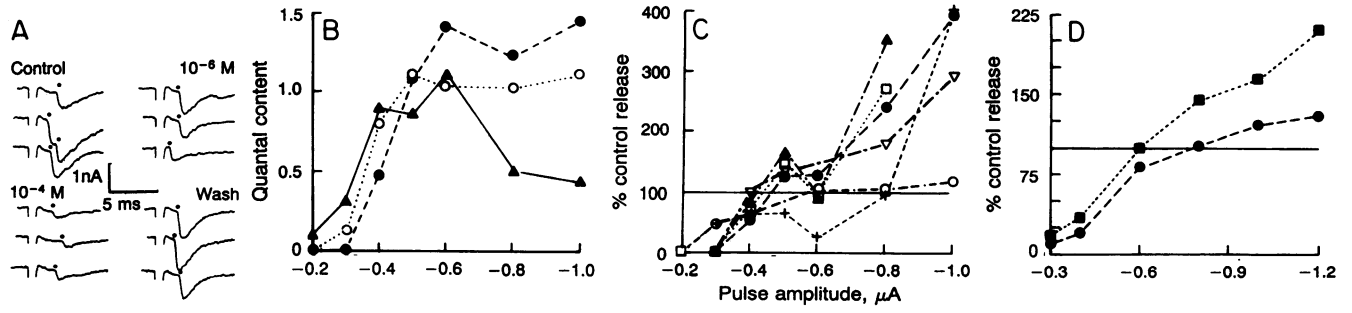

FIG. 1. Effects of glutamate on release. (*A*) Records showing single quantum events obtained in control (*Upper Left*) and after wash (*Lower Right*) conditions and in the presence of glutamate at 10^{-6} M (*Upper Right*) and 10^{-4} M (*Lower Left*). (*B*) Quantal content at different pulse amplitudes. ▲, Control; ●, with 10^{-6} M glutamate; ○, after a 20-min wash. (*C*) Results of six experiments with 10^{-6} M glutamate, presented as a percentage of control release at each pulse amplitude. (*D*) Effects of glutamate at two concentrations (■, 5×10^{-7} M; ●, 1×10^{-6} M). The data are presented as a percentage of control release (no glutamate) at each pulse amplitude.

experiments show the same trend: release was inhibited by small depolarizing currents and enhanced at large currents. The same trend was observed in 13 additional experiments (data not shown for the sake of clarity).

The effect of glutamate varied with concentration. The lowest glutamate concentration that affected release was 10^{-7} M (three experiments). In most experiments (22 experiments), however, 10^{-7} M glutamate had no effect on release, and 5×10^{-7} M was required. Altogether, we varied the glutamate concentration from 10^{-7} to 10^{-5} M. In most experiments, for a given site, only one concentration of glutamate was used. This is because only partial recovery was seen even after a 20–30 min wash with normal solution (Fig. 1*B*). In three experiments, however, we were able to test for effects of two concentrations of glutamate (Fig. 1*D*; 5×10^{-7} M and 1×10^{-6} M) at the same site. At the higher concentration, the reduction of release was more pronounced, and the point at which the glutamate curve crossed the control curve shifted to the right.

Is There a Correlation Between Quantal Content and the Degree to Which Glutamate Reduces Release? As the pulse amplitude increases (as in the experiments shown in Fig. 1), two factors, depolarization and quantal content, vary concomitantly: membrane depolarization increases and, for moderate depolarizations, Ca^{2+} influx increases, resulting in a higher quantal content.

Relief of the glutamate-mediated block, seen at large pulse amplitudes, might depend on either of the two factors. It could be "depolarization-dependent," that is, the block could be relieved to become less effective at high depolarizations. Or, it could be "quantal-content-dependent," that is, the block could become less effective as quantal content increases.

Since it is impossible to determine the absolute level of depolarization (see above), we could not examine the first possibility. Therefore, we looked for a correlation between the quantal content obtained at a narrow range of small pulse amplitudes and the degree of block produced by glutamate. Had the degree of block been determined by quantal content, one would expect it to decline as the quantal content increased. Such a correlation was not observed over a large range in quantal contents (0.13–1.55). In contrast, in all experiments, the degree of block was reduced at the larger pulse amplitudes irrespective of quantal content (see Fig. 1*C*).

We cannot rule out the possibility that the lack of correlation between quantal content and block is due to some uncontrolled factor. Nevertheless, our results suggest that as the pulse amplitude increases, it is the increase in membrane depolarization that is responsible for relief of the glutamate block.

Effects of NMDA on Release. The finding that the presynaptic action of glutamate is voltage-dependent led us to investigate the effects of NMDA on release at different pulse amplitudes. Fig. 2*A* shows one example. Control release was measured in normal van Harreveld solution at different pulse amplitudes. Then, 10^{-5} M NMDA was added, and 10 min later the data with NMDA were obtained. NMDA reduced release in response to small depolarizing pulses but had no effect on release at the larger pulses where the control curve and NMDA curve merge. The same behavior is seen in Fig. 2*C*, where results of nine experiments are shown. In Fig. 2*C*, the data are presented as a percentage of control release (in the absence of NMDA) at each pulse amplitude.

As with glutamate, no correlation was found between quantal content and the degree of block of release by NMDA. This suggests that the NMDA-mediated block also depends on depolarization. This conclusion is supported by the observation that at the large pulse amplitudes, where the control quantal content decreased (see Figs. 1*B* and 2*A*), the degree of block produced by both glutamate (Fig. 1*B*) and NMDA (Fig. 2*B*) did not increase. In fact, NMDA was not effective at these levels of depolarization and glutamate even enhanced release.

Like the effect of glutamate, the effect of NMDA was concentration-dependent. For example, in an experiment where the effect of NMDA on release was measured at different concentrations (with full recovery between runs),

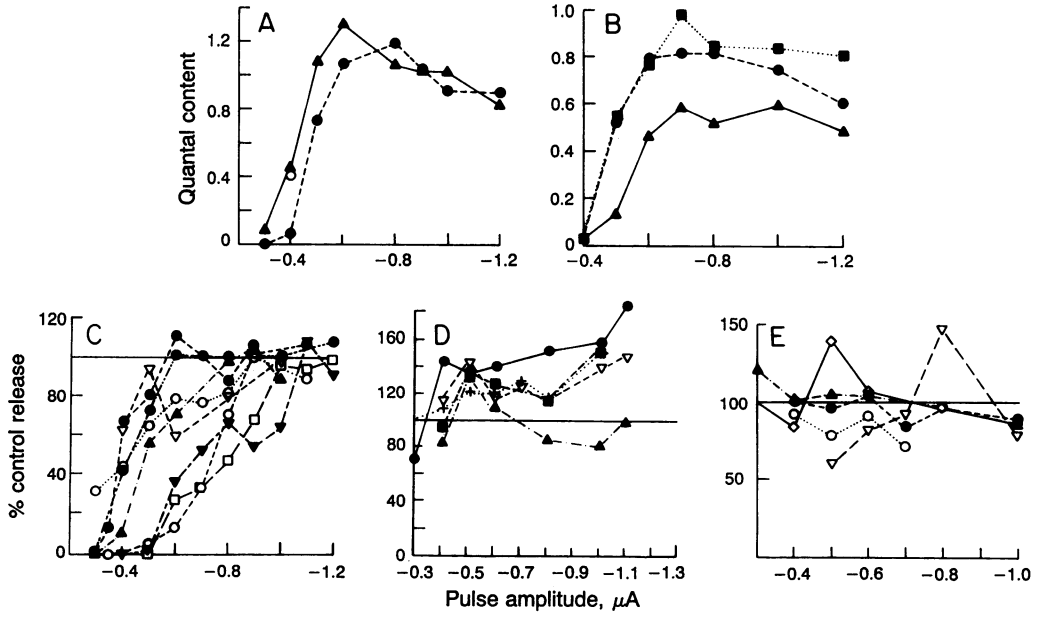

FIG. 2. Effects of NMDA on release. (*A*) Control release (▲) and release 10 min after application of 10^{-5} M NMDA (●). After a 20-min wash, recovery is shown for release at −0.4 μA (○). (*B*) Effect of APV. ▲, Control; ■, 10^{-4} M APV. Ten minutes after the addition of APV, 10^{-5} M NMDA was added and effects were measured 10 min later (●). (*C*) Results of nine experiments with 10^{-5} M NMDA presented as a percentage of control (in the absence of NMDA) at each pulse amplitude. (*D*) Results of five experiments where 10^{-4} M APV was added to control. (*E*) Results of the same five experiments as in *D*, but after the addition of 10^{-5} M NMDA. Here the "control" curve is that of release in the presence of APV.

for a pulse amplitude of −0.2 μA, 10^{-7} M NMDA reduced the quantal content by 25%, 10^{-6} M NMDA reduced it by 80%, 10^{-5} M NMDA reduced it by 90%, and 10^{-4} M NMDA produced a complete block. NMDA did not increase release at any concentration and at any pulse amplitude (up to −1.4 μA). Thus NMDA mimicked only one of the effects of glutamate.

APV Blocks the Effect of NMDA on Release. Since APV blocks the NMDA receptor in vertebrates (18), we tested the effect of APV on the NMDA-mediated block of release. First, a control curve was obtained (Fig. 2*B*). The preparation was then incubated with 10^{-4} M APV for 10 min, after which release was again measured at all pulse amplitudes (Fig. 2*B*). The increase in release observed in the presence of APV (see also Fig. 2*D*) could be due to APV's counteracting the inhibition (at resting membrane potential) caused by endogenous low concentrations of glutamate that are present tonically in the synaptic cleft. In view of the finding that APV increased release at all pulse amplitudes, however, it is possible that APV in addition to counteracting inhibition also enhanced release directly.

In the presence of 10^{-4} M APV, 10^{-5} M NMDA did not reduce release (Fig. 2 *B* and *E*). In this aspect, the putative crayfish presynaptic receptors resemble the postsynaptic NMDA receptors of vertebrates.

Dependence of the NMDA Effect on Mg^{2+} Concentration. In vertebrates, the NMDA receptor is blocked by Mg^{2+} and this block is relieved by depolarization (19). We therefore tested for effects of various extracellular Mg^{2+} concentrations on the presynaptic action of NMDA. The normal Mg^{2+} concentration in the van Harreveld solution is 2.5 mM. We lowered the Mg^{2+} concentration to "zero" (no added Mg^{2+}) and increased the Mg^{2+} concentration to 20 mM. Because Mg^{2+} itself affects release, experiments were carried out in different preparations. First, the control curve at the desired Mg^{2+} concentration was established and then NMDA was added. The results of three experiments in "zero" Mg^{2+} (Fig. 3*A*) and four experiments in 20 mM Mg^{2+} (Fig. 3*B*) reveal that NMDA reduced release in a voltage-dependent manner and to a similar extent at the three Mg^{2+} concentrations (zero and 20 mM in Fig. 3 and 2.5 mM in Fig. 2*C*). This similarity, in the degree of block, once again supports the conclusion that the NMDA-mediated block is independent of quantal content (the quantal content at the high Mg^{2+} concentrations was lower) but depends on depolarization. Comparison of Fig. 3*A* to Figs. 3*B* and 2*C* suggests that the inhibition of release shows a steeper voltage dependence at low Mg^{2+} concentrations. It seems, however, that in the crayfish, unlike in vertebrates, the presynaptic receptor is not significantly affected by the concentration of extracellular Mg^{2+}.

Effects of Glutamate on Action-Potential-Evoked Release. The amplitude of the action potential in crayfish axon terminals near the release sites is not known, but it can be inferred from intracellular recording in secondary branches of the excitatory axon where it was measured to be 90–110 mV (13). This level of depolarization may be compared to a direct depolarization produced by a pulse of −0.6 to −0.7 μA. Fig. 1*C* shows that at these pulse amplitudes, 10^{-6} M glutamate either had no effect or slightly increased release. It was therefore of interest to test for effects of glutamate on the release induced by an action potential. For these experiments the excitatory axon was stimulated (in the absence of TTX) and single quantum events were recorded with the macropatch electrode. The results differed somewhat in different experiments. In four experiments, 10^{-6} M glutamate had no effect on action-potential-induced release; in two experiments, a slight reduction was observed; and in four experiments, 10^{-6} M glutamate slightly increased release. We therefore conclude that at low concentrations, glutamate has no effect on action-potential-evoked release. This result is consistent with the lack of effect of low glutamate concentrations on release produced by moderate depolarizations (Fig. 1*C*). Note, however, that higher concentrations of glutamate reduced release at the same depolarization at which lower concentrations of glutamate were ineffective or even increased release (Fig. 1*D*). We therefore tested for effects of higher concentrations of glutamate on action-potential-evoked release. Fig. 4 shows an example where the effects of two concentrations of glutamate (10^{-6} M and 10^{-4} M) were tested on the same site. It can be seen that 10^{-6} M glutamate had no effect on release, whereas 10^{-4} M glutamate reduced release by ≈80%, with recovery after wash. The lack of effect of low concentrations of glutamate on action-potential-evoked release and inhibition of release at high glutamate concentrations is consistent with the voltage-dependent effect of glutamate observed under direct depolarization (Fig. 1). Similar results were obtained in six additional experiments.

Effect of Glutamate and NMDA on Membrane Potential and Conductance of the Excitatory Axon. Glutamate has been found to cause a slight depolarization of crayfish axon terminals (6). On the other hand, glutamate hyperpolarized the excitatory nerve terminals of the lobster by increasing the potassium conductance (7). The hyperpolarization was small, in the range of a few millivolts, and the increase in conductance was ≈20%. In a later study, Miwa *et al.* (8) reported that NMDA had no effect.

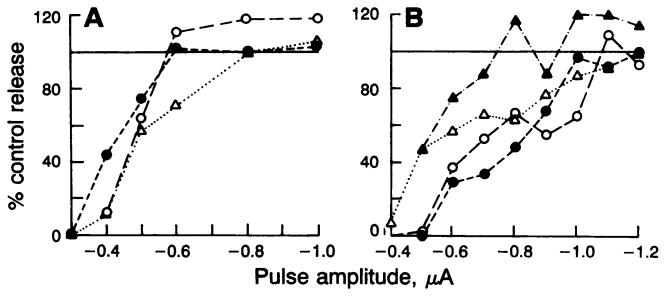

FIG. 3. Dependence of the 10^{-5} M NMDA effect on Mg^{2+} concentration. (*A*) Three experiments (○, ●, and △) with "zero Mg^{2+}" (no Mg^{2+} added). (*B*) Four experiments (▲, △, ○, and ●) with 20 mM Mg^{2+}. The data are presented as a percentage of release (without NMDA) at each pulse amplitude.

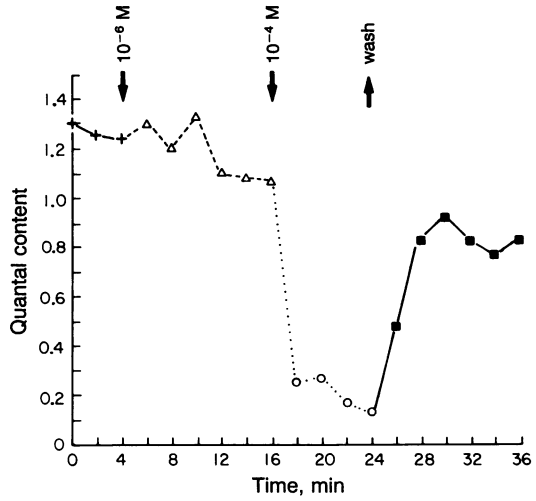

FIG. 4. Effect of 10^{-6} and 10^{-4} M glutamate on action-potential-evoked release in the opener muscle. +, Control; △, 10^{-6} M glutamate; ○, 10^{-4} M glutamate, ■, wash.

Since we found that both glutamate and NMDA reduced release, it was of interest to check for effects of these two compounds on membrane potential and conductance. The axon was treated with TTX and one (for membrane potential measurements) or two (for conductance measurements) microelectrodes were inserted into the presynaptic secondary branch. In different preparations, the resting potential of TTX-treated axons (12°C) varied between 68 and 72 mV (-70.1 ± 0.5 mV; mean ± SEM; $n = 10$). Glutamate was tested at 1×10^{-6}, 1×10^{-5}, 1×10^{-4}, and 3×10^{-4} M and was found to have no effect on the resting potential; the maximal fluctuations in resting potential were ≈1 mV. At the end of each experiment, the electrode was withdrawn, and the zero potential was found to be the same (<1 mV change). Thus, in our experiments, glutamate at a concentration as high as 3×10^{-4} M had no effect on the resting membrane potential. Similarly, NMDA in concentrations as high as 10^{-3} M had no effect on the resting potential.

Glutamate at concentrations of 10^{-6} to 10^{-5} M had no measurable effect on the membrane conductance. When 10^{-4} M glutamate was tested, the resistance fell by 15–20% (eight experiments), and there was full recovery within 20 s of wash. Glutamate at 3×10^{-4} M reduced resistance by 30–33% (five experiments). Complete and rapid recovery was recorded after wash.

Application of NMDA, in contrast, did not change membrane resistance even at 10^{-3} M (six experiments). To make sure that the preparation remained viable, glutamate (10^{-4} M) was superfused (after washout of NMDA), and an increase in membrane conductance was again recorded.

DISCUSSION

The most significant result shown here is that the effect of glutamate on release of neurotransmitter is voltage-dependent: glutamate inhibits release at small depolarizations and enhances release at large depolarizations. In the present work, however, we focused on the inhibitory effect of glutamate.

The mechanism of this voltage-dependent effect of glutamate is at present unknown. Like the muscarinic receptor (20), the putative glutamate receptor may bind glutamate with either high or low affinity, depending on the membrane potential. Another possible mechanism could involve inhibition by glutamate at moderate depolarizations, but not at high depolarizations of N-type Ca^{2+} channels, similar to what has been observed in bullfrog sympathetic neurons (21). Because of the lack of correlation between quantal content and the degree of inhibition, this possibility seems unlikely to acount for the full extent of the voltage-depedent inhibition.

Does the voltage-dependent effect of glutamate have physiological implications? Experimental results described here suggest that it does and that the voltage-dependent effect might bear on the basic mechanism of evoked release. The finding that release was blocked at small levels of depolarizations (at the range of resting potential) by a glutamate concentration as low as 10^{-7} M is consistent with the following hypothesis. At the resting membrane potential, the endogenous concentrations of glutamate normally present at the synaptic cleft might keep the "release machinery" in a tonic blocked state. Then, when the action potential reaches the nerve terminal, the block is relieved, and with the concurrent influx of Ca^{2+}, evoked release commences. Evoked release then stops with membrane repolarization due to restoration of the block. The increase in release in the presence of APV, particularly at low depolarizations, further supports this hypothesis.

The normal concentration of glutamate at the synaptic cleft cannot be higher than 10^{-7} M, as 10^{-7} M glutamate was sufficient to inhibit release at small depolarizations. This means that the endogenous blocking action of glutamate occurs at low concentrations. This is to be expected if, at the resting potential, glutamate does indeed keep the release machinery in a tonic blocked state.

Several authors looked for the effect of NMDA on release in crayfish and found no effect (8). The finding that NMDA reduces release only at low depolarizations may explain their results, given that they studied the effects of NMDA on action-potential-evoked release.

In conclusion, we have obtained suggestive evidence for the presence of glutamate and NMDA receptors on the excitatory nerve terminals in crayfish. However, our results do not exclude the possibility of indirect effects of glutamate and NMDA. If indeed NMDA receptors do exist in these presynaptic terminals, they differ from the vertebrate NMDA receptors especially since their activation did not produce an increase in membrane conductance.

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