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A STUDY OF THE INHIBITORY ACTION OF γ-AMINOBUTYRIC ACID ON NEUROMUSCULAR TRANSMISSION IN THE CRAYFISH

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SUMMARY

1. The effect of γ-aminobutyric acid (GABA) on the neuromuscular junction of the crayfish (*Cambarus clarkii*) was investigated. The drug was applied iontophoretically to a junction and the potential changes were recorded intracellularly as well as extracellularly with micro-electrodes.

2. The amplitude of the potential produced by iontophoretically applied L-glutamate was decreased by simultaneous application of GABA to the same junctional area. When the membrane potential was clamped at the resting level the clamping current for the L-glutamate injection was not changed by GABA application, indicating that there is no appreciable competition between L-glutamate and GABA for the glutamate-sensitive receptor.

3. The excitatory nerve was stimulated repetitively and the excitatory junctional potentials (e.j.p.s) were recorded extracellularly with a micro-electrode placed on the surface of the muscle. The average size of the extracellular e.j.p.s was reduced by the application of GABA.

4. The number of quanta released per impulse calculated from the extracellular e.j.p.s was decreased during the application of GABA, while the quantum size remained unchanged.

5. The size of the excitatory presynaptic nerve spike was decreased by the application of GABA. However, no appreciable change was observed in the inhibitory nerve spike.

6. These results indicate that in addition to its post-synaptic action GABA acts on the excitatory presynaptic nerve terminal and reduces the release of the transmitter.

INTRODUCTION

It has been reported that two kinds of mechanism are concerned in the synaptic inhibition of crustacean neuromuscular transmission: (i) a post-synaptic conductance increase (Fatt & Katz, 1953; Boistel & Fatt, 1958;

Grundfest, Reuben & Rickles, 1959; Kuffler, 1960; Dudel & Kuffler, 1961*b*); and (ii) presynaptic inhibition which decreases the release of the excitatory transmitter (Dudel & Kuffler, 1961*b*).

γ-Aminobutyric acid (GABA) occurs in high concentration in the inhibitory axon of the lobster (Kravitz, Kuffler & Potter, 1963), and it mimics the inhibitory transmitter in increasing the post-synaptic membrane conductance in a manner similar to the inhibitory transmitter (Boistel & Fatt, 1958; Grundfest *et al.* 1959; Kuffler, 1960; Dudel & Kuffler, 1961*b*; Grundfest & Reuben, 1961; Takeuchi & Takeuchi, 1964*b*, 1965). The similarity between the effects of GABA and of neural inhibition has been extended to their actions on the presynaptic nerve terminal (Dudel & Kuffler, 1961*b*; Dudel, 1962).

On the other hand it has been observed that L-glutamate mimics the excitatory transmitter at the neuromuscular junction in the crayfish (Takeuchi & Takeuchi, 1964*a*). The present experiments were designed to investigate the inhibitory action of GABA on the glutamate potential and on the presynaptic axons by means of iontophoretic application of drugs to a single junctional area.

While the manuscript of this paper was being prepared, Dudel's work appeared (1965*b*, *c*). Some of the present results are in agreement with those of Dudel's which were obtained by the perfusion technique of drug application.

METHODS

The abductor muscle of the dactylopodite in the first or second walking leg of the crayfish (*Cambarus clarkii*) was used. Experimental procedures were similar to those in earlier studies (Takeuchi & Takeuchi, 1964*a*, 1965). When the potential changes of the presynaptic nerve were recorded with an extracellular micro-electrode, the potential was usually small and care was taken to avoid contamination by potentials from other parts of the nerve fibre than that under investigation. For this purpose another micro-electrode was used as an indifferent electrode; it was placed just above the tip of the recording electrode, and the potential was recorded differentially.

In analysing the size distribution of the extracellular e.j.p.s, the excitatory nerve was stimulated repetitively and the e.j.p.s were recorded on moving film at the speeds of 5–10 cm/sec.

Micropipettes for extracellular recording were filled with 3 M-NaCl or 3 M sodium methylsulphate. Intracellular electrodes were usually filled with 3 M-KCl but sometimes potassium propionate was used.

RESULTS

The effects of GABA on the glutamate potential

Previous reports showed that L-glutamate and GABA exert their actions at the junctional areas and that they mimic the action of the excitatory and the inhibitory transmitters respectively (Takeuchi & Takeuchi, 1964*a*, 1965). When GABA was applied iontophoretically to the area where the glutamate-sensitive receptor was localized, GABA decreased the amplitude

of the glutamate potential. Since the glutamate- and the GABA-sensitive receptors are located in the same area, this action of GABA might be due to the increase in the conductance of the post-synaptic membrane. However, there is also a possibility that GABA competes with L-glutamate for its receptor in a curare-like manner as was first proposed by Fatt & Katz (1953) (see also Curtis & Watkins, 1960).

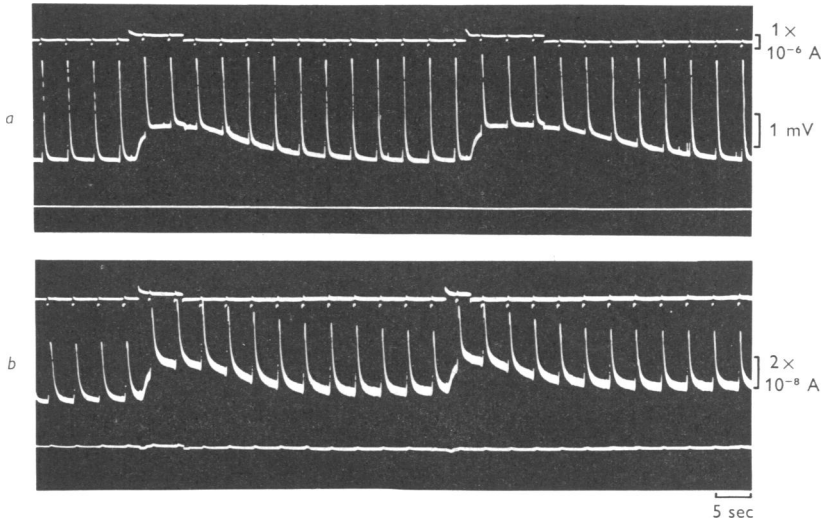

Fig. 1. Effect of GABA on the glutamate-induced potential. Glutamate and GABA were applied electrophoretically through separate pipettes to a junctional area. Upper traces are monitored injection currents, upward deflexion, GABA injection; downward deflexion, L-glutamate injection. *a*, intracellularly recorded glutamate- (brief depolarization) and GABA-potentials (slow depolarization). *b*, the clamping current for the L-glutamate injection (brief deflexions) and for the GABA injection (slow deflexions) when the membrane potential was clamped at the resting level. Lower trace: the clamped membrane potential.

An example is shown in Fig. 1*a*. Brief pulses of L-glutamate were applied to a junctional area and they produced brief glutamate potentials (middle trace). GABA was applied to the same area from a separate micropipette. Injection currents are monitored on the upper beam where downward deflexions represent the glutamate injection and upward deflexions the GABA injection. On the application of a steady dose of GABA, the membrane was slowly depolarized. The brief glutamate potentials were superposed on the GABA potential and their amplitude was decreased to about 70 % of the control during the action of GABA.

If the decrease in the amplitude of the glutamate potential is due to the increase in the post-synaptic membrane conductance by the action of GABA, it would be expected that, if the membrane potential was clamped

at the resting level, the clamping current for the glutamate injection should not change during the application of GABA (cf. Takeuchi & Takeuchi, 1959). Figure 1*b* gives an example of voltage clamping which was obtained from the same junction as used in Fig. 1*a*. The middle trace shows the clamping currents and the bottom trace the clamped membrane potential. Brief inward current was produced by the injection of L-glutamate and a slow inward current by GABA injection. During the GABA action, the glutamate-induced currents were superimposed on the GABA-induced current and the former showed little or no change in their amplitude.

A similar result was also obtained when L-glutamate was applied to a junction and both extracellular and intracellular glutamate potentials were recorded simultaneously from a single junctional area. On the application of GABA to the same junctional area, the amplitude of the intracellular glutamate potential was decreased by about 30%, while little if any, change was observed in the amplitude of the extracellular glutamate potential.

The above results show that the decrease in the amplitude of the glutamate potential during the action of GABA is due to the increase in the conductance of the post-synaptic membrane and there is no appreciable competition between L-glutamate and GABA for the glutamate-sensitive receptor.

Effect of GABA on extracellular e.j.p.s

The excitatory nerve was stimulated repetitively and the excitatory junctional potentials (e.j.p.s) were recorded extracellularly with the recording micro-electrode placed on the surface of the muscle. An example of simultaneously recorded extracellular and intracellular potential changes is presented in Fig. 2. The excitatory nerve was stimulated at 20/sec and about fifteen traces were superimposed. The middle trace shows the extracellular e.j.p.s preceded by the extracellular presynaptic nerve spike and the bottom trace the simultaneous intracellular e.j.p.s. A GABA filled micropipette was brought near to the tip of the extracellular recording electrode and varying doses of GABA were applied. Figure 2*b* and *c* were obtained during the steady application of GABA. The upper traces show the monitored injection current. In *b*, two extracellular e.j.p.s are observed while in the control (*a* and *d*) many more extracellular e.j.p.s of large size are observed. In *c*, where a larger dose of GABA was applied, no extracellular e.j.p. was recorded. In these cases no appreciable change is observed in the intracellular e.j.p.s. This may be due to the fact that GABA was applied to a small area and it produced little decrease of the post-synaptic membrane resistance, while the intracellular e.j.p.s were recorded from many junctions distributed over the muscle surface. By

analogy with the presynaptic inhibition produced by the inhibitory nerve stimulation, this result suggests that the site of action of GABA is presynaptic with less transmitter released per impulse (Dudel & Kuffler, 1961*b*).

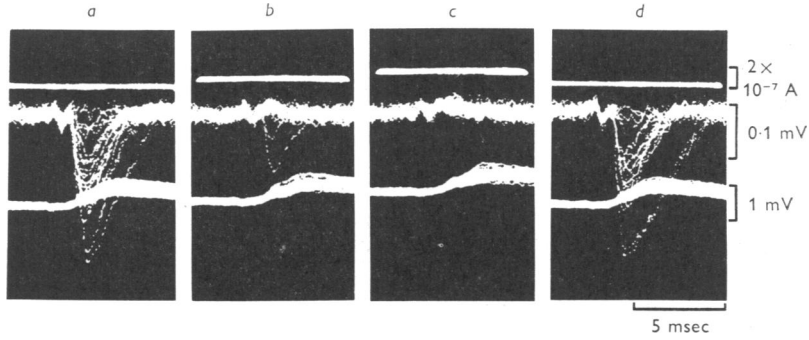

Fig. 2. Effect of GABA on the extracellular e.j.p.s. Upper traces, monitored injection current. Middle traces, extracellular e.j.p.s. recorded at a single junctional area. Lower traces, intracellular e.j.p.s. *a*, before, *b*, and *c* during and *d* after the steady electrophoretic application of GABA to the junction. The excitatory nerve was stimulated at 20/sec and about fifteen traces are superimposed.

Another example is given in Fig. 3. The upper traces are monitored injection currents of GABA applications, the middle traces extracellular e.j.p.s and the bottom traces the intracellular e.j.p.s produced at a stimulation rate of 20/sec. Doses of GABA were increased from *a* to *c*. A GABA potential was produced in the intracellular record of Fig. 3*b* and *c* and at the same time the extracellular e.j.p.s disappeared during the GABA action. At smaller doses, even when no appreciable GABA potential was observed, the extracellular e.j.p.s had already disappeared (Fig. 3*a*).

When an extracellular electrode was located in the neighbourhood of a junctional area, in some cases an upward (positive) deflexion was observed following a presynaptic nerve spike. The upward deflexion had a similar time course to that of the extracellular e.j.p.s and it coincided with the rising phase of the intracellular e.j.p.s. The upward deflexion may be due to the return current of e.j.p.s through the muscle membrane around active spots located in the neighbourhood of the recording electrode. In some cases the downward extracellular e.j.p.s were superimposed on the small upward deflexions. When GABA was topically applied to the junction where the tip of the recording electrode was placed, the downward deflexion disappeared and in some cases small upward deflexions remained. In this case transmission of this junction may be blocked, leaving the neighbouring junctions less affected. Thus the return current from the neighbouring junctions may remain and produce a small upward deflexion.

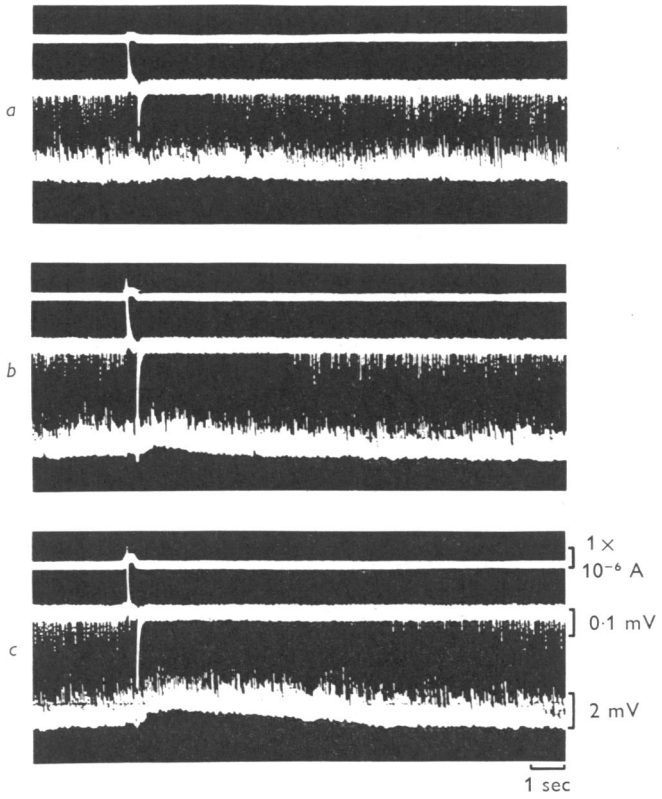

Fig. 3. Effect of GABA on the extracellular e.j.p.s. Upper traces, monitored injection current. Middle traces, extracellular e.j.p.s. Lower traces, intracellular e.j.p.s. The excitatory nerve was stimulated at 20/sec and the dose of GABA was increased from *a* to *c*.

Statistical treatment

It has been shown that stimulation of the inhibitory nerve reduces the number of released quanta of the excitatory transmitter while their size remains unchanged, i.e. the inhibition acts at a presynaptic site on the excitatory nerve terminals (Dudel & Kuffler, 1961*b*). If GABA also has an action on the presynaptic nerve terminal as well as on the post-synaptic membrane, it would be expected that GABA would reduce the number of quanta released from the excitatory nerve terminal. In order to test this idea a statistical analysis used by del Castillo & Katz (1954), Boyd & Martin (1956), Liley (1956) and Dudel & Kuffler (1961*a*) was performed.

Extracellular e.j.p.s were recorded and GABA was applied electrophoretically from a micropipette placed near the tip of the extracellular

recording electrode. Figure 4 gives the size distribution of extracellular e.j.p.s from a single junctional area at a stimulation rate of 9/sec. The upper graph is a control and the lower graph is obtained during the steady

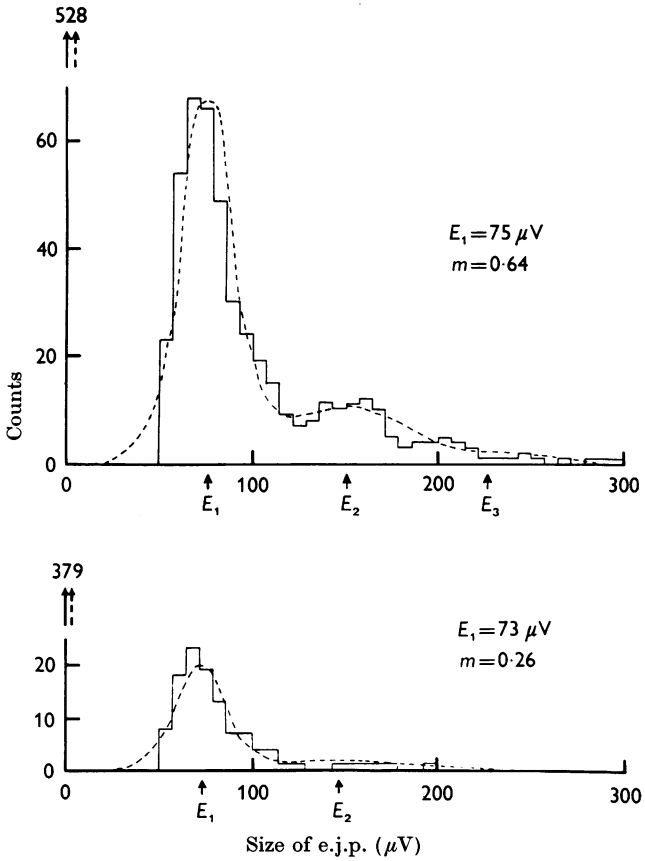

Fig. 4. Histogram of size distribution of extracellularly recorded e.j.p.s from a single junctional area at stimulation rate of 9/sec. Upper graph shows control and lower graph during the application of GABA. m = quantum content; E_1 = unit quantum size. Interrupted lines are theoretical distributions for each m and E_1 with standard deviation of 14 μV. Number of stimuli is 998 for control and 490 for GABA application.

application of GABA. Quantum content m was calculated assuming a Poisson distribution, from the equation

$$n_0 = n\mathrm{e}^{-m},$$

where n is the number of stimuli and n_0 is the number of failures of the response. The size of the average unit potential E_1 was obtained from the relation

$$E_1 = \frac{\bar{E}}{m},$$

where $\bar{E}$ is the average size of the extracellular e.j.p. The quantum content m was 0·64 in the control and it was decreased to 0·26 by the application of GABA, while the unit potential E_1 was 75 and 73 μV, respectively. The interrupted line was drawn assuming a Gaussian distribution of the e.j.p. size and a standard deviation of 14 μV. Table 1 gives quantum content with and without GABA application. The reduction of m depends on the dose of GABA and at larger doses transmission could be completely blocked.

TABLE 1. Effect of GABA on quantum content of e.j.p.s. GABA was applied locally by electrophoretic injection (+GABA). n = number of stimuli, m = average quantum content of extracellular e.j.p. determined from $m = \log_e n/n_0$, n_0 = number of transmission failures, $\bar{E}$ = average size of extracellular e.j.p., E_1 = size of quantum determined from $E_1 = \bar{E}/m$

Expt. no.		Stim. rate	n	m	$\bar{E}$ (μV)	E_1 (μV)
1	Control	9/sec	482	0·65	49	74
	+GABA	9/sec	268	0·28	21	76
2	Control	9/sec	256	0·60	51	82
	+GABA	9/sec	222	0·23	18	78
3	Control	9/sec	260	0·65	51	78
	+GABA	9/sec	268	0·34	24	71
4	Control	9/sec	340	0·53	38	71
	+GABA	9/sec	280	0·19	13	67
5	Control	10/sec	201	0·35	25	65
	+GABA	10/sec	293	0·17	13	78
6	Control	10/sec	183	0·41	34	82
	+GABA	10/sec	289	0·16	13	81
7	Control	10/sec	292	0·58	48	82
	+GABA	10/sec	385	0·064	5	81

The above result shows that GABA reduces the number of released quanta, while producing no change in their size.

Effect of GABA on the presynaptic nerve spike

A decrease in the amplitude of the presynaptic nerve spike by inhibitory nerve stimulation has been observed at the crayfish neuromuscular junction (Dudel, 1963). If GABA acts on the presynaptic nerve terminal and reduces the release of the excitatory transmitter as the inhibitory transmitter does, it would be expected that the application of GABA would reduce the amplitude of the presynaptic nerve spike.

The excitatory nerve was stimulated repetitively and both extracellular and intracellular e.j.p.s were recorded simultaneously. Presynaptic nerve spikes of various sizes and time courses were recorded preceding the extracellular e.j.p.s. The presynaptic nerve spikes were usually diphasic (positive–negative) but sometimes positive monophasic spikes were recorded. When

the electrode was placed at the point where largest extracellular e.j.p.s were recorded, the presynaptic spike was relatively small and slow and the positive phase was prominent. At a critical recording position where large extracellular e.j.p.s were recorded, sometimes the frequency of the spontaneous miniature discharge increased, suggesting an injury of the nerve terminal.

A GABA-filled micropipette was brought near the tip of the extracellular recording electrode and various doses were applied. Presynaptic spikes recorded at various positions with and without GABA are presented in Fig. 5. Figure 5*A* was obtained before, *B* during and *C* after the steady application of GABA. In *b*, presynaptic spikes are small and have a prominent positive phase. The extracellular e.j.p.s are relatively large, suggesting that the electrode was placed in proximity to the nerve terminal. On the application of GABA the amplitude of presynaptic spikes, especially the negative phase, was reduced and the e.j.p.s disappeared. At larger doses of GABA the presynaptic spike was further reduced. Figure 5*c* was obtained from the nerve branch proximal to the junctional area. In contrast to *b* the presynaptic spike is much larger and has a sharp negative phase, and no extracellular e.j.p.s were observed. Application of GABA near the recording electrode now produced no appreciable change in the presynaptic spike; at larger doses, the presynaptic spike was even increased by about 10%. Such an increase at nerve branches proximal to the terminal has also been seen by Dudel (1963), when the inhibitory nerve was stimulated. In this case, one may assume that the large dose of GABA applied near the nerve branch diffused to the nerve terminal and increased the conductance there, resulting in greater current density at a point proximal to the terminal. As expected the post-synaptic membrane was depolarized during GABA application. In *a* is shown another example which may be recorded near the junction. The presynaptic spikes are relatively large and have a dominant negative phase and small extracellular e.j.p.s are observed. However, GABA still reduced the presynaptic spike size. The changes in the amplitude of the presynaptic spikes obtained at various recording positions are summarized in Table 2.

The above results indicate that GABA-sensitive receptors are located on the presynaptic terminals of the excitatory axons. It was then investigated whether or not the inhibitory nerve terminal is also sensitive to GABA. Since it is known that the inhibitory and the excitatory junctions are found in close proximity to each other (Takeuchi & Takeuchi, 1965), the excitatory and the inhibitory axons were stimulated repetitively and responses were recorded at a single junctional area. In Fig. 6 responses produced by the excitatory and the inhibitory nerve stimulations are presented in *a* and *b*, respectively. In *a*, the presynaptic spike and both

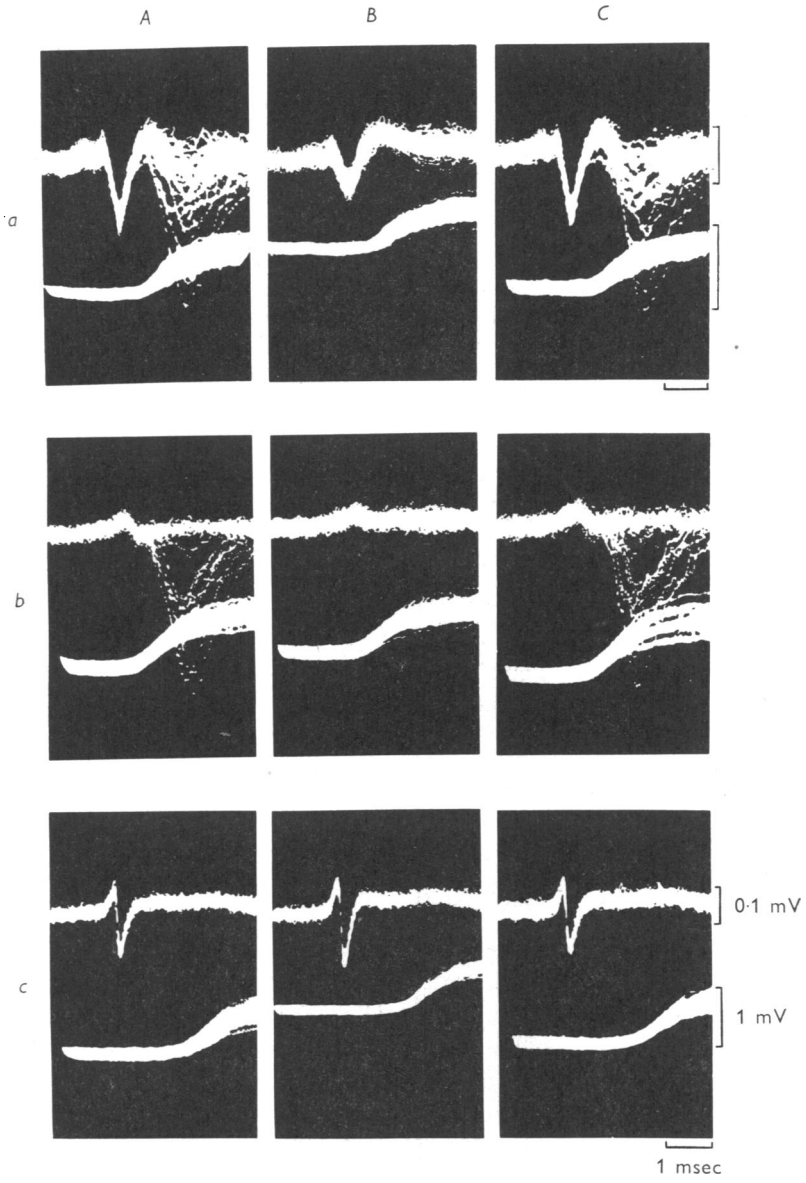

Fig. 5. Effect of localized application of GABA on the presynaptic nerve spikes. *A*, before, *B*, during and *C* after the steady electrophoretic application of GABA. Upper traces, extracellularly recorded potential changes. Lower traces, intracellular e.j.p.s. The excitatory nerve was stimulated at 15/sec (*a*) and at 20/sec (*b* and *c*). *c* is obtained from a point proximal to *b* and dose of GABA applied in *c* is 3·3 times that in *b*. Calibrations are same for *b* and *c*. Calibrations: intracellular record, 1 mV; extracellular record, 0·1 mV; time, 1 msec.

extracellular and intracellular e.j.p.s are observed. However, in *b* the extracellular and the intracellular i.j.p.s are small and only the presynaptic spike is visible. When stimulation of the inhibitory nerve preceded stimulation of the excitatory nerve, the number of extracellular e.j.p.s was reduced, confirming that this junction was supplied by the inhibitory nerve terminal. GABA was applied electrophoretically to this junction. Figure 6*A* was obtained before, *B* during and *C* after the application of GABA. During the steady application of the same dose of GABA, the

TABLE 2. Effect of localized application of GABA on the presynaptic spike size. Recording positions are estimated from the size of extracellular e.j.p.s, the size and the configuration of the presynaptic spikes. Effect of GABA on the inhibitory presynaptic spikes is compared with that on the excitatory presynaptic spikes recorded at the same junctional area. Presynaptic spike sizes are mean value of 8 to 40 measurements, which are made from the superimposed traces of about 20 sweeps

				Presynaptic spike size		
Expt. no.	Nerve stimulation	Recording position	Relative dose of GABA	Control mean ±S.D. (μV)	+GABA mean ±S.D. (μV)	Ratio of change (%)
1	Excit. n. 20/sec	Junction	0·4	33±2·6	19±6·3	58
2	Excit. n. 20/sec	Junction	1	28±0·5	10±7·3	35
3	Excit. n. 15/sec	Junction	3	33±2·6	11±8·7	33
4	Excit. n. 20/sec	Near junction	1	46±3·3	28±5·4	60
5	Excit. n. 15/sec	Near junction	1	44±3·4	24±2·1	55
6	Excit. n. 15/sec	Near junction	1	57±5·5	36±4·6	63
7	Excit. n. 15/sec	Near junction	1·5	70±2·0	38±4·8	54
8	Excit. n. 15/sec	Near junction	2	67±2·6	28±1·8	42
9	Excit. n. 15/sec	Proximal	4	80±4·0	84±3·7	105
10	Excit. n. 20/sec	Proximal	3	162±4·6	185±8·2	114
11	Excit. n. 20/sec	Proximal	3	147±4·3	166±7·5	112
12	Excit. n. 20/sec	Nerve trunk	3	486±8·5	495±14·4	102
13	Excit. n. 20/sec	Near junction	1	60±2·8	42±5·3	70
	Inhib. n. 20/sec	Near junction	1	69±3·9	71±3·5	103
14	Excit. n. 20/sec	Near junction	1	123±7·3	78±6·0	63
	Inhib. n. 20/sec	Near junction	1	126±4·7	128±4·4	101

excitatory presynaptic spike was decreased in amplitude and the extracellular e.j.p.s disappeared, whereas no change was observed in the inhibitory presynaptic spike (Fig. 6*b*). The effects of GABA application on the amplitudes of the excitatory and the inhibitory presynaptic spikes obtained at a single junctional area are compared in Table 2. This result indicates that the GABA-sensitive receptor is localized on the excitatory presynaptic terminal, but not on the inhibitory terminal.

DISCUSSION

The present investigation shows that there is no appreciable competition between L-glutamate and GABA for the glutamate-sensitive receptor and the decrease in the amplitude of glutamate potential during the GABA application is due to the post-synaptic conductance increase. This result

also provides further evidence that the glutamate-sensitive receptor is different from the GABA-sensitive receptor (Takeuchi & Takeuchi, 1965). There is a good analogy between L-glutamate and GABA action and neural excitation and inhibition. It is therefore an attractive idea to assume also that the neurally released transmitters do not interact competitively at the post-synaptic sites. Accordingly the inhibitory transmitter would have two main sites of actions, namely on receptors of motor nerve terminals and on post-synaptic inhibitory receptor sites.

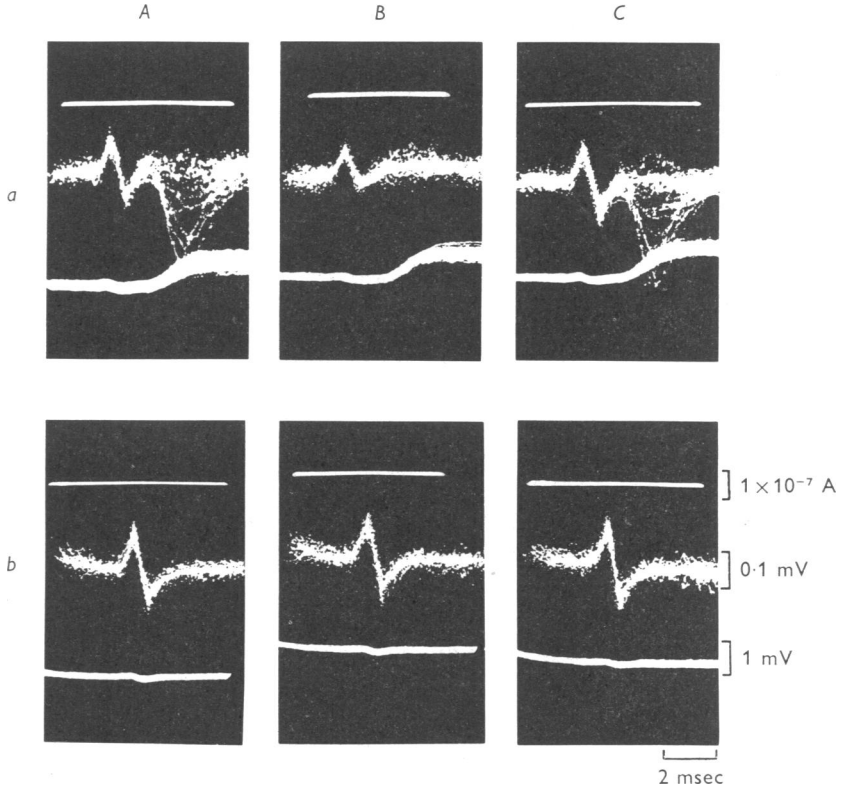

Fig. 6. The effect of localized application of GABA on the excitatory (*a*) and the inhibitory presynaptic spikes (*b*) produced at the stimulation rate of 20/sec. *A*, before; *B*, during and *C* after the steady application of GABA. Upper trace: monitored injection current. Middle trace: extracellular record. Lower trace: intracellular record.

A presynaptic inhibitory action of GABA was previously thought to occur because it closely resembled neural inhibition (Dudel & Kuffler, 1961*b*). Present evidence supporting this conclusion is: (i) the average size of extracellular e.j.p.s was reduced by GABA injection, (ii) the

number of quanta was decreased by GABA injection without change in their size, and (iii) the size of excitatory presynaptic spike was decreased by GABA injection. Essentially the same conclusion was obtained by Dudel (1965*b*, *c*). The method of application of drugs, however, was different in his experiment, where drugs were applied in the bathing fluid. The amplitude of the presynaptic spike may be one of the factors which control the amount of transmitter released from the nerve terminal. Therefore a change in the size of extracellularly recorded presynaptic spikes may be a measure of the action of drugs on the nerve terminal. In this connexion it seems interesting that GABA decreased the size of excitatory presynaptic spikes but had no effect on the inhibitory spikes. This result suggests that the chemoreceptor sites are localized on the excitatory nerve terminal but not on the inhibitory terminal.

The extracellularly recorded presynaptic spikes showed various configurations depending on the recording position (Fig. 5, see also Dudel, 1963, 1965*a*). Similar changes in the configuration of the presynaptic spike were also observed at the giant synapse of *Loligo* and the frog neuromuscular junction (Takeuchi & Takeuchi, 1962; Katz & Miledi, 1965). This change in the configuration may be due either to the blind end of the nerve terminal, as in the case of the frog neuromuscular junction, or to the fact that a full-sized action potential does not invade the nerve terminal. On the application of GABA, the amplitude of the presynaptic spike was reduced depending on the dose applied, even when it had a prominent negative phase. It might be considered that this potential was recorded at a point some distance proximal to the terminal and that at this point the potential was not solely electrotonic but some active response occurred. This effect may arise from local changes either in the amplitude or in the time course of the membrane action potential by GABA action. A simple explanation may be that since GABA is supposed to have little influence on the membrane potential (Dudel, 1965*c*; Takeuchi & Takeuchi, 1966), the reduction of the spike size will occur by the increase in the membane conductance; the increased conductance near the terminal will decrease the 'stimulatory action' of the proximal action potential. If the response were graded near the terminal, one could explain the reduction of the presynaptic spike size.

The time course of GABA action on the presynaptic receptor was similar to that on the post-synaptic one (Fig. 3), suggesting that both receptors are equally accessible from outside. The nature of the presynaptic receptors seems similar to that of receptors in the post-synaptic membrane in that they showed no appreciable desensitization to prolonged injection of GABA. Further similarity between the presynaptic and the post-synaptic receptors is the permeability change produced by GABA.

Some results suggest that chloride permeability of the presynaptic terminal is increased by the action of GABA as it is in the post-synaptic membrane (Takeuchi & Takeuchi, 1966). However, a different action of β-guanidino-propionic acid on the presynaptic nerve terminal and on the post-synaptic membrane has been reported (Kuffler, 1960; Dudel, 1965*b*).

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