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DO MOTOR-NERVE TERMINALS HAVE γ-AMINOBUTYRIC ACID RECEPTORS?

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1 γ-Aminobutyric acid (GABA, 0.1 to 1 mM) had no significant effect on the amplitude, rise time, half decay time or frequency of miniature endplate potentials (m.e.p.ps) at the frog or mouse neuromuscular junctions *in vitro*.

2 Addition of GABA (1 mM) to preparations previously treated with 11 mM K^+-Ringer did not cause any further increase in m.e.p.p. frequency. GABA also failed to increase the m.e.p.p. frequency in a low Cl^--Ringer.

3 GABA (0.1 to 1 mM) did not reduce the high m.e.p.p. frequency induced by veratrine (20 to 40 mg/l).

4 GABA (0.5 to 1 mM) did not affect the amplitude of the extracellularly-recorded nerve terminal spike, whereas 15 mM [K^+] reduced the spike.

5 The quantal content (*m*) of the evoked endplate potential was not significantly altered by GABA; 9 mM [K^+] significantly increased *m*.

6 When external d.c. potential differences were recorded in a three-chambered bath, GABA (0.1 to 1 mM) produced a very small depolarization if applied to the phrenic nerve trunk, but not if applied to the pre-terminal axon/motor nerve terminal region. Carbachol (0.3 to 1 mM) evoked a small depolarization when applied to the nerve terminal chamber.

7 These results fail to provide evidence for the existence of GABA receptors on motor nerve terminals.

Introduction

In the vertebrate peripheral nervous system, receptors for the central inhibitory transmitter 4-aminobutanoic acid (GABA) show a surprisingly wide distribution. For example, they have been detected on sympathetic (De Groat, 1970; Bowery & Brown, 1974; Adams & Brown, 1975) and sensory (De Groat, 1972; Feltz & Rasminsky, 1974; Deschenes, Feltz & Lamour, 1976; Gallagher, Higashi & Nishi, 1978) neurones, and on unmyelinated axons in vagal and sympathetic nerve trunks (Brown & Marsh, 1978). On these tissues, GABA produces a membrane depolarization probably mediated by an increase in Cl^- permeability (see Nishi, Minota & Karczmar, 1974; Adams & Brown, 1975; Deschenes *et al.*, 1976; Gallagher *et al.*, 1978).

The observations on peripheral axons raised the question whether comparable receptors might exist on unmyelinated motor nerve terminals. Effects compatible with the presence of nerve terminal GABA receptors have been detected in the autonomic nervous system: GABA reduces the release of acetylcholine from the preganglionic fibres in the rat superior cervical ganglion (Brown & Higgins, 1979) and depresses the quantal content of the excitatory postsynaptic potential (e.p.s.p.) in the bullfrog sympathetic ganglion (Koketsu, Shoji & Yamamoto, 1974; Kato & Kuba, 1980).

Although neuromuscular transmission is usually regarded as being insensitive to GABA (see Florey & McLennan, 1955), previous tests at this site have been rather restricted in scope. Further, there is one brief report (Hofmann, Feigen & Genther, 1962), that GABA reduces m.e.p.p. frequency and amplitude in rat intercostal muscle.

In the present study, the effect of GABA has been examined on prejunctional function in the frog isolated sartorius and mouse hemidiaphragm preparations by means of conventional electrophysiological methods.

Methods

Experimental procedures

Mouse muscle Phrenic nerve-hemidiaphragm muscles were dissected from small (20 to 40 g) homebred mice

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(originally of Tuck No. 1 strain). Solutions were superfused over the preparation at ≃30 ml/min (bath volume 2 to 3 ml, temperature 21 to 23°C). Mammalian Ringer contained, (mM): NaCl 125, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 24, $NaH_2PO_4.2H_2O$ 1 and glucose 11, bubbled continually with 95% O_2 and 5% CO_2, pH 7.3 to 7.4. High K^+-Ringer was made by substituting NaCl for KCl in equimolar amounts; low Cl^--Ringer was made by substituting sodium propionate for NaCl.

Frog muscle Sciatic nerve-sartorius muscles were obtained from *Rana temporaria*. Frog Ringer contained, (mM): NaCl 111, KCl 1.9, $CaCl_2$1.8, $NaHCO_3$ 2.4 and $NaH_2PO_4.2H_2O$ 0.85. Hyperosmotic glycerol Ringer for frog or mouse was made by incorporating 400 mM glycerol into one litre of normal Ringer; High K^+-Ringer was made by substituting NaCl for KCl in equimolar amounts. Only superficial endplates were used.

Recording Conventional techniques were employed for intracellular recording (Fatt & Katz, 1951). Membrane potential was monitored using an 8 to 15 MΩ glass microelectrode filled with 1.5 M tripotassium citrate (tip potential < -5 mV). Current microelectrodes were filled with 0.6 M potassium sulphate (resistance <5 MΩ). Recordings were displayed on a Tektronix D13 oscilloscope which was also used as a preamplifier to drive a Racal Thermionic Store 4D tape recorder (Ampex 786 tape; FM channel: 7.5 ips; bandwidth 0 to 2.5 kHz). A low pass variable filter (typically used at 30 kHz) was employed to improve the signal-to-noise ratio without attenuating m.e.p.p. amplitude significantly. The membrane potential was also recorded on a Bryans 28000 potentiometric recorder. For extracellular focal recording, a coarse 2 to 5 MΩ glass microelectrode filled with 2 M NaCl in a 2% w/v Agar gel was employed.

Glycerol treatment Muscles were bathed in glycerol Ringer for approximately 30 to 40 min followed by washing in normal Ringer. (Howell & Jenden, 1967; Howell, 1969; Eisenberg, Howell & Vaughan, 1971).

Endplate criteria Before an endplate was accepted for recording and analysis certain criteria had to be satisfied: (i) m.e.p.p. rise time <1 ms and amplitude >0.1 mV; (ii) m.e.p.p. to noise ratio $\geqslant 2$; (iii) stable membrane potential (not varying by >5 mV during entire recording at an endplate, 0.5 to 1.5 h); (iv) endplate potential (e.p.p.) rise time <2 ms; (v) in glycerol-treated preparations, e.p.ps were resolved from muscle action potentials by double stimulating the nerve (Miyamoto, 1975).

Determination of quantal content The quantal content (m) of the evoked e.p.p. was determined by the direct method, i.e. the mean e.p.p. amplitude divided by the mean m.e.p.p. amplitude obtained at the same endplate. The method was thought to be the most accurate for the experimental procedure employed (Hubbard, Llinás & Quastel, 1969; Ginsborg & Jenkinson, 1976). If a small change in membrane potential (<5 mV) occurred during the recording of a series of e.p.p.s, then their amplitudes were corrected back to the original membrane potential by multiplying by a correction factor (Katz & Thesleff, 1957a). All e.p.ps were corrected for non-linear summation by using Martin's correction (Martin, 1955). The application of Martin's correction to e.p.ps with amplitudes >20 mV and >12 mV in glycerol- and magnesium-treated preparations respectively may produce substantial overcorrection from their true amplitudes (Miyamoto, 1978). For this reason, e.p.ps with amplitudes <12 mV and <20 mV in magnesium- and glycerol-treated junctions were preferred for analysis. Standard correction formulae were used (Hubbard *et al.*, 1969; Ginsborg & Jenkinson, 1976).

For the calculation of m, 200 e.p.ps and 200 m.e.p.ps were recorded for control measurements and repeated in the presence of the drug. Trains of e.p.ps were evoked at 1 Hz, but the first 20 e.p.ps were not used for analysis because of transient changes in release causing initial e.p.ps to have higher amplitudes than when a steady state for release had been attained (Hubbard *et al.*, 1969; no qualitative difference in the first twenty e.p.ps for control and GABA-treated preparations was observed). If any obvious drift in e.p.p. amplitude occurred, the data were rejected. If a small drift was suspected, then e.p.ps were subdivided into groups of ten and the mean amplitude and variance was calculated for each group; this usually revealed any underlying trends in the data.

Statistical analysis of results

For the analysis of m, a paired t test was applied as control and drug measurements were made at the same endplate. Values of m were subjected to a test for normality by using the rankit method for small samples (Bliss, 1967). The method involves ranking the values of m in order of their values and transforming each observation into a rankit by use of tables. Plotting the rankits against the respective observations should produce a straight line for normally distributed data. Although the samples of m are quite small (5 values in each group) the observation that all the groups containing five values of m all show approximate linearity on the rankit vs. observation graph was thought to indicate that the results may be normally distributed.

The rankit plot seems to justify the use of a parametric test in this case, which also allowed some

measure of sensitivity of the system to detect significant changes in m (i.e., $P < 0.05$). By calculating the mean values of m for magnesium- and glycerol-treated preparations, an estimation of the percentage decrease in m which was necessary for significance at the 5% level, could be obtained. By arbitrarily decreasing the values of m, and comparing these values to the control values of m by the paired t-test, indicated whether or not the difference was significant.

Drugs

The drugs used and their respective sources were as follows: GABA (BDH); carbachol (Sigma Chemical Co.); veratrine (Sigma); muscimol (Flurochem Ltd.).

Results

General membrane properties

GABA, in concentrations of 100 μM to 1 mM, had no statistically significant effect on the muscle membrane potential, input resistance, capacitance or membrane time constant in these preparations.

Effects on spontaneous transmitter release

(i) *GABA in normal and high K^+ Ringer* Concentrations of GABA ranging from 100 μM to 1 mM had no significant effect on the frequency of m.e.p.ps recorded in frog and mouse endplates. In 18 fibres in the mouse, control m.e.p.p. frequency was 1.2 ± 0.14 Hz., and in the presence of GABA (1 mM) it was 1.4 ± 0.16 Hz. In 15 fibres in the frog, control frequency was 2.5 ± 0.31 Hz., and in the presence of GABA it was 2.6 ± 0.29 Hz. Also GABA did not cause any detectable change in the amplitude, rise time or decay time of m.e.p.p.s.

If GABA depolarized the motor nerve terminals, the m.e.p.p. frequency would be expected to increase. However, if the depolarization was very small, then the expected increase in m.e.p.p. frequency may also have been very small and undetectable by the present recording method. In an attempt to improve the sensitivity of the system, the sample size of m.e.p.p.s during a set time interval was increased by raising the external potassium concentration in the Ringer to 11 mM. This allowed m.e.p.p. sampling at a steeper portion of the m.e.p.p. frequency-terminal depolarization curve such that a small extra depolarization would then produce a proportionately larger increase in m.e.p.p. frequency (Liley, 1956; Miyamoto & Volle, 1974). M.e.p.p. frequency in the diaphragm increased approximately five times in an 11 mm K^+-Ringer (from 1.19 ± 0.1 Hz. to 5.92 ± 0.08 Hz., in 20 fibres, Figure 1a). The addition of GABA (1 mM) to this

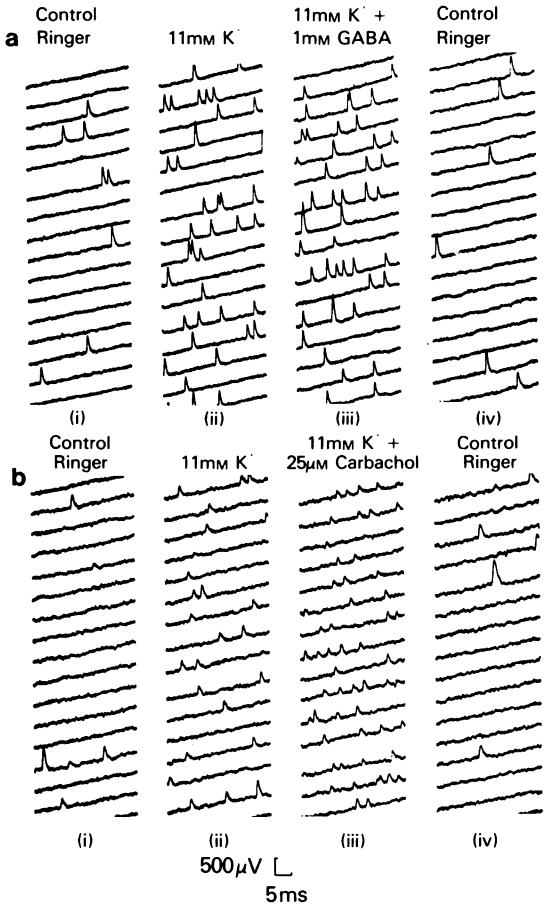

Figure 1 Effect of high K^+-Ringer solution, GABA and carbachol on m.e.p.p. frequency in the mouse hemidiaphragm. Records (a) and (b) were taken from different endplates: (a) shows the effect of 11 mM K^+ and 11 mM K^+ + 1 mM GABA on m.e.p.p. frequency; (b) shows the effect of 25 μM carbachol + 11 mM K^+-Ringer. Note the increased frequency and reduced m.e.p.p. amplitude in the latter record. The GABA and carbachol raster records were taken after 3 min exposure to a given test solution.

solution did not alter the m.e.p.p. frequency (6.08 ± 14 Hz.) significantly, whereas carbachol (25 μM) produced the expected further increase (12.14 ± 0.18 Hz.) (Figure 1b; cf. Miyamoto & Volle, 1974). Smaller doses of GABA (100 to 500 μM) were also tested in case the larger dose was inducing a rapid desensitization of the nerve terminal receptors (Ginsborg, 1971) as previously observed for cholinomimetic drugs (Hubbard, Schmidt & Yokota, 1965); however, smaller doses of GABA also had no effect on m.e.p.p. frequency in high K^+ Ringer.

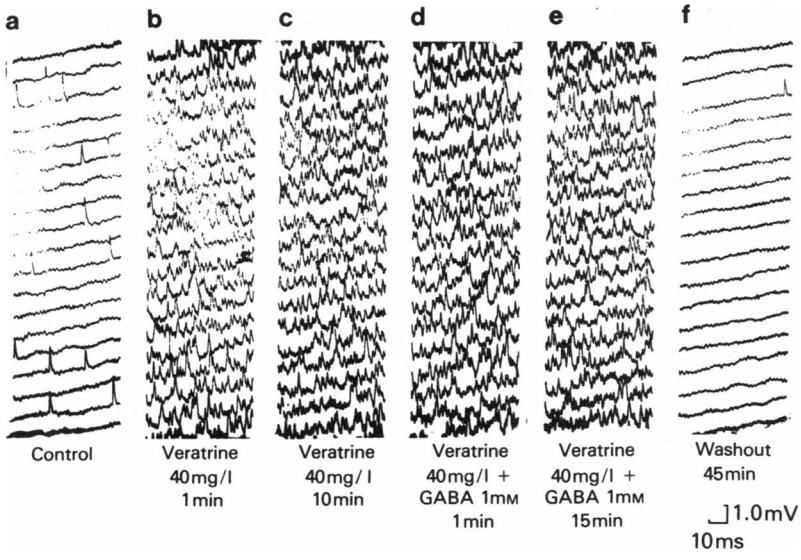

Figure 2 Effect of veratrine and GABA on m.e.p.ps recorded from mouse hemidiaphragm: (a) to (f) are raster records of m.e.p.ps recorded in different solutions. The times shown beneath records (b) to (f) indicate times when the records were made following drug application.

(ii) *GABA and veratrine* The experiment of Hofmann *et al.* (1962) on rat intercostal muscle showing a decrease in m.e.p.p. frequency with GABA in the presence of veratrine (a mixture of the alkaloids cevadilline, sabadine, cevadine, and veratridine) was repeated on the mouse hemidiaphragm using similar concentrations of drugs. The application of veratrine (20 to 40 mg/l) produced a vast increase in m.e.p.p. frequency (> 50 Hz Figure 2); however, the addition of 1 mM GABA with veratrine caused no noticeable reduction in m.e.p.p. frequency (still > 50 Hz) back to control levels even after 10 min contact. After prolonged washing with control Ringer, the m.e.p.p. frequency decreased well below the control level ($\ll 1$ Hz), and m.e.p.p. amplitude began to merge into the baseline noise (< 100 μV). Superfusing GABA over the preparation, following veratrine treatment, produced no further reduction in frequency and amplitude relative to a control Ringer wash. Thus GABA appeared to have no detectable action of its own in this instance. Moreover, prior treatment of the preparation with 1 mM GABA did not prevent the vast increase in m.e.p.p. frequency produced by 20 mg/l veratrine or 50 μM veratridine. These results therefore contrast with those described by Hofmann *et al.* (1962).

The impaled cells began to depolarize irreversibly and muscle fasciculations were common 1.5 h after superfusing veratrine over the preparation (resting potentials were typically reduced to −15 to −30 mV).

(iii) *GABA in low Cl^- Ringer* If the presumed terminal depolarizing response to GABA is mediated by Cl^- ions, as is thought to be the case in peripheral nerves (Brown & Marsh, 1978), and the terminal E_{Cl} is close to the resting potential, any depolarization would probably be small. However, replacing 60% of external Cl^- with an impermeable anion (propionate) should cause a transient shift in E_{Cl} in the depolarizing direction: if GABA were applied at this point, it should produce a larger depolarization until Cl^- shifts cause the E_{Cl} to return to near the resting potential (Adams & Brown, 1975). However, although m.e.p.p. frequency, in five endplates, increased in a low Cl^--proprionate medium, addition of GABA (1 mM) in this medium did not cause a further increase in m.e.p.p. frequency.

Effect on nerve terminal spike

Mouse hemidiaphragm Focal extracellular nerve terminal spikes were recorded in preparations where excitation-contraction coupling had been blocked with 8 to 12 mM magnesium ions. An agent which depolarizes nerve terminals may be expected to reduce the amplitude of the nerve action potential in the terminal region, as has been shown for externally applied depolarizing currents on rat phrenic nerve terminals (Hubbard & Willis, 1968), so monitoring the extracellular current at the nerve terminal should provide a measure of presynaptic polarization.

GABA in concentrations ranging from 0.5 to 1 mM had no significant effect on the size of the nerve ter-

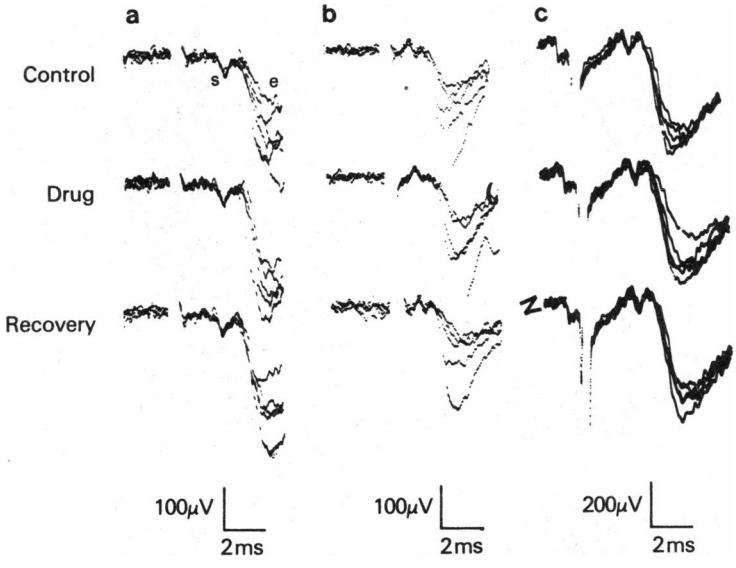

Figure 3 Effect of GABA on the focal extracellularly recorded nerve terminal spike at mouse hemidiaphragm endplates. The traces showing nerve terminal spikes (s) and endplate current (e) were recorded at 1 Hz stimulus frequency and all records are five superimposed sweeps at different sweep speeds in the presence of (a) 9 mM K^+-Ringer, (b) 15 mM K^+-Ringer and (c) 700 μM GABA respectively. The above drugs were applied to different preparations.

minal spike recorded from four endplates, whereas a significant reduction ($P < 0.05$) was found in 1 of 4 endplates exposed to 9 mM K^+-Ringer and in all 3 endplates exposed to 15 mM K^+-Ringer (Figure 3). A similar effect of a high external K^+ concentration was reported at the squid giant synapse (Erulkar & Weight, 1977).

Effect on quantal content (m)

On the magnesium treated frog sartorius, no significant ($P > 0.05$) difference was found, on five endplates, between the mean value of *m* before (14.9 ± 2.3) and after (15.0 ± 2.3) 1 mM GABA. However, application of 9 mM K^+-Ringer caused a significant ($P < 0.05$) increase in *m* from 18.1 ± 2.9 to 32.1 ± 4.6 in 2 endplates. Glycerol-treated frog or mouse muscle also showed no significant change in the value of *m* upon addition of GABA; in five endplates in the frog, the control *m* was 102.8 ± 11.4, and in the presence of GABA 102.5 ± 10.3, and in five endplates in the mouse the control *m* was 56.5 ± 8.6 and after 1 mM GABA it was 56.9 ± 9.5.

Extracellular depolarizations

Three experiments were carried out in a three-chambered bath, to determine whether gross extracellular potential changes could be recorded when GABA was applied to various superfusion chambers.

Mouse hemidiaphragm Initially, the potential difference between chambers 2 and 3 (Figure 4) was monitored while GABA (0.1 to 1 mM), was perfused through chamber 2. A very weak depolarization was observed manifest as a very small shift in the recorded d.c. level; this confirms earlier reports on the rat isolated phrenic nerve (Brown & Marsh, 1978).

To test whether GABA would depolarize the terminal region of the phrenic nerve, the potential difference between chambers 1 and 3 was monitored while GABA (0.1 to 1 mM) was perfused through chamber 1.

Although some part of the pre-terminal axon must have been present in chamber 1, no depolarization of the nerve terminal/axon region was observed in the presence of GABA. Muscimol (100 μM), a potent GABA-mimetic agonist, also failed to produce a depolarization when applied to chamber 1. However, perfusing carbachol (0.3 to 1 mM) through chamber 1 produced a small depolarization.

Discussion

By analogy with previous experiments on peripheral neurones and axons (see Introduction), these experiments were initiated on the assumption that GABA receptors, if present on motor nerve terminals, would induce a membrane depolarization, probably by increasing Cl^- permeability. Such a terminal depolarization should be revealed as an increase in m.e.p.p.

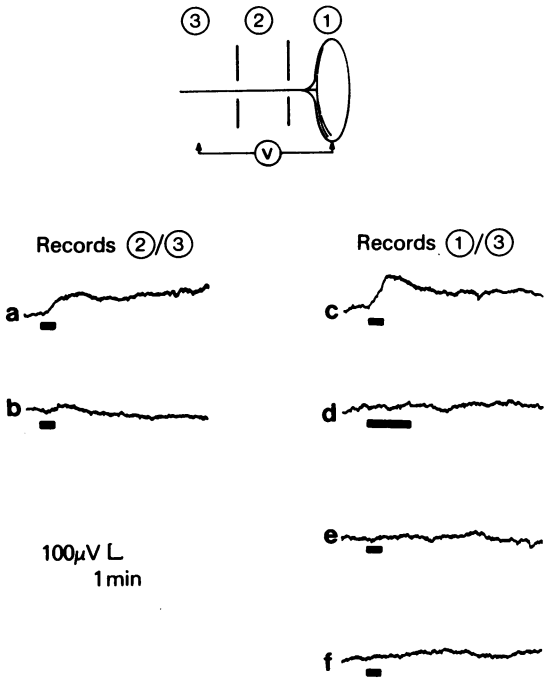

Figure 4 Chamber bath for recording external changes in potential difference of the mouse phrenic/hemidiaphragm preparation. Chamber (1) contains the hemidiaphragm, preterminal axon, and terminal axon. Chambers (2) and (3) contain the phrenic nerve trunk. Partitions between chambers were sealed with thin Perspex strips and Dow Corning silicone grease. Mouse Ringer (bubbled with O_2/CO_2) was perfused through the chamber at 25°C via a Watson-Marlow pump (flow rate 10 ml/min). The d.c. level was recorded via two Ag/AgCl wires embedded in 2% agar/mouse Ringer between chambers (2) and (3) or (1) and (3). Records (a) and (b) were obtained by recording across chambers (2) and (3) while the drugs perfused through chamber (2). (a): Carbachol 0.6 mM; (b): GABA 1 mM (note small depolarization). Records (c) to (f): obtained by recording across chambers (1) and (3) while the drugs perfused through chamber (3). (c): Carbachol 0.8 mM; (d): GABA 100 μM; (e): GABA 1 mM; (f): muscimol 100 μM (note absence of depolarization in records d to f). Bars indicate drug application times. The records were obtained on the same diaphragm.

frequency and/or a reduction in m, but in practice, neither change could be detected.

The test for changes in m is not particularly sensitive, because of the normal variation in the values of m observed. Quantal content would have to be reduced by approximately 20% to obtain significance at the 5% level. Further the relationship between terminal depolarization and m may be more complex than assumed, since elevation of K^+ increases m (Takeuchi & Takeuchi, 1961; Edwards & Ikeda, 1962; Branisteanu, Miyamoto & Volle, 1976), whereas acetylcholine decreases m (Hubbard *et al.*, 1965). However, in the bullfrog sympathetic ganglion, GABA (0.1 to 1 mM) decreased m by approximately 42%, a decrease which should have been resolvable by the present techniques (Kato, Kuba & Koketsu, 1978). Thus it may be concluded that the effects of GABA on evoked transmitter release at the neuromuscular junction, if any at all, were rather small.

The failure of GABA to increase spontaneous m.e.p.p. frequency is less equivocal for the following reasons: (i) large increases in frequency were detectable at small elevations of $[K^+]_o$. (ii) Tests in the presence of elevated $[K^+]_o$, which enabled the effect of carbachol on m.e.p.p. frequency to be resolved (Figure 1; Miyamoto & Volle, 1974), still revealed no effect of GABA (Figure 1). This would suggest that GABA does not depolarize nerve terminals or that the GABA depolarization-m.e.p.p. frequency curve is more complex than initially assumed. In the bullfrog sympathetic ganglion, GABA did not affect the frequency of m.e.p.s.ps (Kato *et al.*, 1978; Kato & Kuba, 1980). In accordance with the results for m.e.p.p. frequency and GABA, no change in the extracellularly recorded nerve terminal spike could be detected, nor could a depolarization of the terminal/preterminal region of the phrenic nerve be recorded under conditions where nerve trunks showed a small depolarization (cf. Brown & Marsh, 1978).

The inability to record any effect with GABA may result from factors other than the absence of receptors *per se*. (i) There may have been rapid receptor 'desensitization' (Katz & Thesleff, 1975b). This is unlikely since the perfusion rates were such as to reveal a clear depolarization of multifibre nerve preparations. Also no transient effects on m.e.p.p. frequency or e.p.p. amplitude were observed in the first seconds of drug application. (ii) The resting Cl^- permeability might be sufficiently high that $E_{Cl} \simeq E_m$ or, if $E_{Cl} \neq E_m$ at rest, a very rapid redistribution might occur across the membrane when P_{Cl} is changed. Both seem unlikely as GABA still had no effect when E_{Cl} was shifted in a depolarizing direction by reducing $[Cl^-]_o$, whereas the same procedure produced a sustained increase in m.e.p.p. frequency. (iii) The ability of GABA to gain 'access' to the receptors may have been impeded by rapid glial uptake (see, for example, Bowery, Brown, White & Yamini, 1979). This also seems unlikely in view of the previous tests on nerve trunks (where access would be slower) and the equal ineffectiveness of muscimol on the terminal.

The most reasonable conclusion, therefore, is that the depolarizing GABA receptors previously noted in other peripheral unmyelinated nerves are indeed absent from motor nerve terminals. This is rather curious because the motor neurone soma is sensitive to

GABA (Curtis, Phillis & Watkins, 1959). However, previous experiments on mixed nerve trunks indicated that myelinated axons were indeed relatively insensitive to GABA (Brown & Marsh, 1978). It was suggested that this might reflect either an absence of receptors in myelinated axons or the ineffectiveness of nodal receptors resulting from the high safety factor of saltatory conduction. The present experiments suggest the former to be more likely and that the motor nerve terminal may represent an exception to the otherwise ubiquitous distribution of GABA receptors on exposed nerve membranes.

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