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THE EFFECT OF γ-AMINOBUTYRIC ACID ON THE INPUT CONDUCTANCE AND MEMBRANE POTENTIAL OF ASCARIS MUSCLE

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1 Twin intracellular recordings were made from the bag region of *Ascaris* muscle in order to make conductance measurements. The preparation was bathed in a cool (22°C) Ringer solution to abolish the large spontaneous depolarizing potentials and to improve stability for recording.

2 The resting membrane potential was -31 ± 1 mV, mean ± s.e. mean ($n = 17$). The current–voltage plots were linear in the hyperpolarizing direction but showed evidence of delayed rectification during the application of depolarizing currents. The input conductance of the bag was measured from the slope of these plots during the application of hyperpolarizing current. The resting conductance of the bags was 2.4 ± 0.2 μS, mean ± s.e. mean ($n = 12$).

3 When the preparation was perfused with γ-aminobutyric acid (GABA) in concentrations greater than 3 μM, a dose-dependent increase in conductance associated with a hyperpolarizing potential was recorded. The log dose-response relationship obtained from 6 preparations was sigmoidal and had an ED_{50} of !3 μM.

4 When Cl^- in the Ringer was replaced by SO_4^{2-}, the GABA-induced conductance changes decreased and were associated with depolarizing potentials.

5 Voltage responses were recorded in the bag region during the iontophoretic application of GABA to different regions of the muscle cell. The largest responses were recorded when GABA was applied to the bag region. Smaller responses were recorded when GABA was applied to the arms and syncytial regions. The responses of the bags were dose-dependent and were antagonized by bath-applied picrotoxin.

6 An extrapolation method using 10^{-5} M GABA suggested that the GABA reversal potential was about 30 mV more negative than the resting membrane potential. This was confirmed by means of a two microelectrode voltage clamp technique. The reversal potential was thus estimated as -61.2 ± 2.2 mV mean ± s.e. mean ($n = 27$).

Introduction

Ascaris suum is a worm-like intestinal parasite of the pig. The muscle cells of *Ascaris* are composed of three parts called, the spindle, the bag and the arm (Figure 1a). The spindle is the elongated fibrillar part which is the contractile region. It lies under the hypodermis and is arranged longitudinally. The nucleus of the cell is located in the bag region of the muscle. The bag is balloon shaped, about 200 μm in diameter and fills the body cavity. The arm, a thin process, reaches transversely from the bag to one of the longitudinally running nerve cords. The arms come together to form a syncytium over the nerve cord and are electrically coupled in this region (De Bell, Del Castillo & Sanchez, 1963). Spontaneous depolarizing potentials of 2 to 10 mV amplitude superimposed on a resting potential of −30 mV can be recorded in the bag region (Jarman, 1959). Acetylcholine depolarizes these cells (Del Castillo, De Mello & Morales, 1963), while piperazine and γ-aminobutyric acid (GABA) hyperpolarize them (Del Castillo, De Mello & Morales 1964a, b). The inhibitory receptors responsible for the hyperpolarization were believed to be located exclusively at the syncytium because hyperpolarizing responses to bath-applied piperazine were reported to be largest in bags located near the nerve cord. It was also reported that iontophoretic application of piperazine only produced responses when applied near the region of the syncytium (Del Castillo *et al.*, 1964a). However, Brading & Caldwell (1971) sectioned the arms of muscle cells and still recorded GABA-induced

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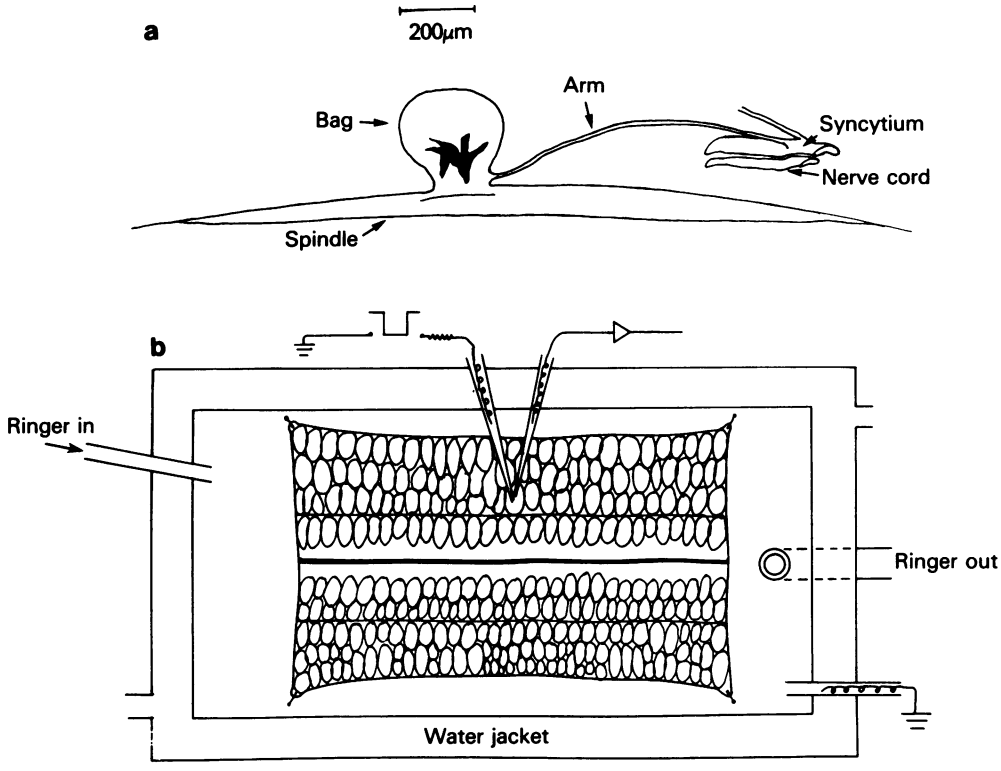

Figure 1 (a) Diagram of the *Ascaris* muscle cell illustrating its anatomical features. (b) Experimental chamber and preparation. A section of *Ascaris* is pinned, cuticle side down, onto Sylgard in a chamber containing 1.5 ml Ringer solution and perfused at a rate of 6 ml/min. The chamber is fitted with a water jacket to maintain the preparation at 22°C. Two potassium acetate filled micropipettes are illustrated, inserted into the bag region of the muscle cell. One pipette was used for current injection, the other was used for recording voltage responses. During the iontophoresis of γ-aminobutyric acid to different parts of the muscle cell, one pipette was used for iontophoresis and one was placed in the bag for recording voltage responses. During the voltage clamp-experiments a third GABA filled pipette was placed over the bag, between the voltage and current electrodes.

hyperpolarizations in the bags. They suggested that the bags may also possess GABA receptors. These studies have been reviewed by Gerschenfeld (1973). The present paper describes the effects of bath-applied GABA on the conductance of single muscle cells and the results of GABA iontophoretic experiments which show that there are inhibitory receptors sensitive to GABA located on the bag region of the muscle cells. A preliminary account of some of these observations has been presented to the British Pharmacological Society (Martin 1980).

Methods

Specimens of *Ascaris suum* were obtained from the local slaughter house and transported to the laboratory in warm Locke solution. The worms were then placed in fresh Locke solution which was replaced daily and maintained at 37°C in a water bath. Experience with different media suggested that this solution was most satisfactory for maintaining the *Ascaris* in a healthy condition. The *Ascaris* survived for over 1 week under these conditions but were used for experiments within 4 days.

In most experiments a cylindrical section of the worm, about 2 cm long was obtained from 6 cm behind the head. These sections were cut along the lateral line and the intestine removed. The preparation was then pinned, cuticle side down, onto Sylgard in a chamber containing 1.5 ml of Ringer solution (Figure 1b). The chamber was surrounded by a water jacket which maintained the preparation at about 22°C. A caudal 2 cm section was used during the iontophoretic experiments because the anatomy of the muscle in this region more readily permitted the application of GABA to different regions of the muscle cell.

The preparation was perfused at a constant rate of 6 ml/min with Ringer solution. This rate was temporarily increased during the initial application of GABA and during washing. The Ringer contained (mM): NaCl 135, KCl 3.0, $CaCl_2$ 3.0, $MgCl_2$ 15.7 and glucose 3, buffered to pH 7.6 with 5mM Tris-maleate. Although this solution contains higher Cl^- than *Ascaris* haemolymph (Brading & Caldwell, 1964), it has proved satisfactory for electrical recording. In experiments on the effects of the removal of Cl^-, Cl^- salts were replaced by SO_4^{2-} salts and the osmolarity maintained by the addition of sucrose.

Micropipettes were placed under the visual control with a dissecting microscope and micromanipulators. For conductance measurements, two potassium acetate (2 M) filled micropipettes with resistances of 15 to 25 MΩ were used, one for recording voltage and the other for current injection.

They were connected via Ag/AgCl wires to high input impedance preamplifiers. Similar micropipettes were filled with 2 M GABA, pH 2.5, for the iontophoresis experiments. GABA was then applied as a cation by a micro-iontophoresis programmer, Model 160 (W-P instruments Inc.). Retaining currents of about 10 nA were used. A 1GΩ resistor was used as a constant current source for the current micropipette, while the current flowing was measured from the voltage applied to the 1GΩ resistor. The bath electrode was an Agar-KCl bridge. In order to estimate the GABA reversal potentials directly, a two microelectrode voltage clamp technique was used with the iontophoretic application of GABA to the bag. The micropipettes were made and filled as described previously except that the current micropipettes had resistances of 4 to 6 MΩ. The gain of the control amplifier was usually set at 1000. The current injected into the cell was measured from the voltage drop across a 500 KΩ resistor connected in series with the current electrode. No correction was made for the resistance (Rs) in series with the membrane capacity. Rs is partly due to the experimental bath and agar bridge. It was estimated to be approximately 10 KΩ, so that when a transmembrane current (Im) of 200 nA was passed a potential error of 2 mV (Im·Rs) in the voltage recording would have been produced. A maximum of 200 nA was passed as the holding current when clamping the bag at −80 mV (it was uaually much less at −80 mV). The efficiency of the clamp was tested by placing a second voltage measuring electrode in the bag on the opposite side to the first. The difference in potential recorded by these two pipettes was less than 5% in preparations undamaged by the electrode penetration. Voltage clamp studies were only carried out on bags with resting input conductances less than 4 μS.

The drugs used were GABA (Sigma) and picrotoxin (Sigma). GABA was applied to the bath via the Ringer during experiments on conductance changes. Picrotoxin was applied in a similar manner during the iontophoresis experiments.

Responses were monitored on a cathode ray oscilloscope and on a 2 channel pen recorder.

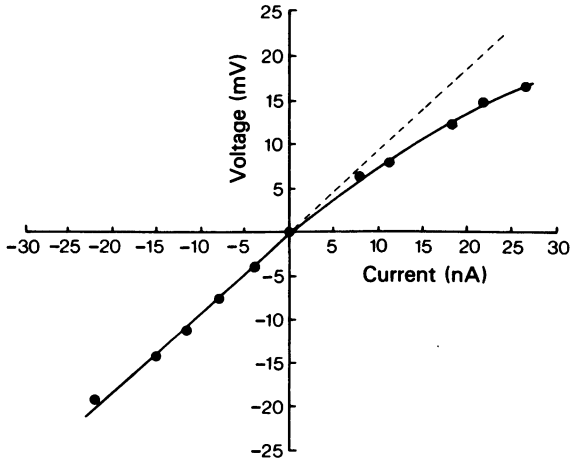

Figure 2 Current-voltage relationship observed in the bag region. Ordinate scale: voltage responses; abscissa scale; transmembrane current. The relationship is linear during the application of hyperpolarizing current but shows evidence of delayed rectification during the application of depolarizing current.

Results

The experiments described here were conducted on muscle cells of 50 preparations. At temperatures of 37°C, large spontaneous depolarizing potentials associated with pacemaker activity at the syncytium were recorded in the bag region. The large depolarizing potentials were therefore abolished by cooling the preparation to 22°C. This procedure had the added advantage of improving the stability of the muscle preparation. Under these conditions the membrane potential was stable and measured -31 ± 1 mV, mean ± s.e. mean ($n = 17$).

To determine current-voltage relationships, two micropipettes were placed in the bag, one for current injection and one for recording voltage responses (Figure 2). These plots showed evidence of delayed rectification when depolarizing currents were injected but there was a linear current-voltage relationship when hyperpolarizing currents were injected. The time constant of the bag was about 6 ms. The input conductance was therefore measured from the slope of the current-voltage plots obtained during the injection of hyperpolarizing current pulses of 250 ms duration. The resting input conductance of the bags measured 2.4 ± 0.2 μS, mean ± s.e. mean ($n = 12$).

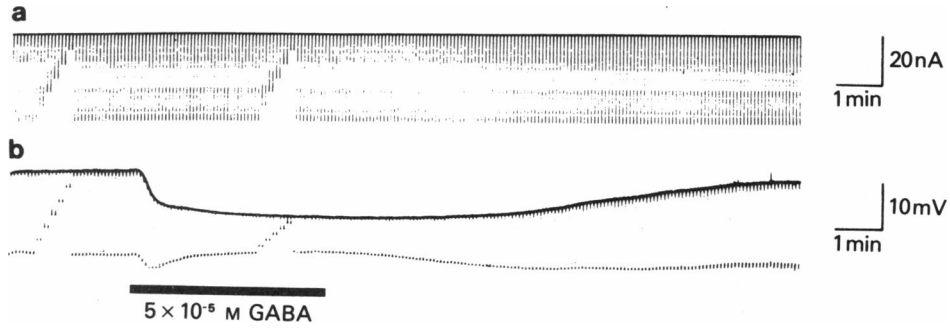

Figure 3 Effect of γ-aminobutyric acid (GABA) on input conductance. The top trace (a) represents hyperpolarizing current pulses of 250 ms (downward deflections) duration; (b) represents the hyperpolarizing voltage responses (downward deflections). The application of 5×10^{-5} M GABA increased the resting input conductance from 2.1 μS to 4.9 μS.

γ-Aminobutyric acid-induced conductance changes

Bath-applied GABA in concentrations greater than 3 μM caused a reversible increase in conductance, which was associated with a hyperpolarizing potential (Figure 3). Various concentrations of GABA were applied separately in ascending concentrations to 6 cells from 6 preparations and ΔG measured in bags located at different distances from the nerve cord. The dose-dependent nature of the response is illustrated by the sigmoidal log dose-response relationship shown in Figure 4. The concentration of GABA producing the half maximal effect (the ED_{50}) was 13 μM. No consistent signs of desensitization were observed even during the application of high doses of GABA.

Ionic mechanism

Cl^- is known to be involved in the hyperpolarizing response to piperazine and GABA (Del Castillo *et al.*, 1964a; Brading & Caldwell, 1971). The effect of low Cl^- on the GABA conductances was tested in 5 experiments. The resting input conductances of the bags decreased slightly in the low Cl^- Ringer, indicating a small Cl^- permeability in the absence of GABA. The GABA-induced conductance changes decreased when the preparations were perfused with low Cl^- Ringer in all experiments and were associated with depolarizing potentials. These GABA-induced conductance changes continued to decline in the low Cl^- Ringer over a period of 20 to 30 min. For example, in one experiment 2×10^{-5}M GABA produced a change in input conductance of 3.2 μS in normal Ringer but after perfusing the preparation with low Cl^- Ringer solution for 8 min, 2×10^{-5} M GABA produced a smaller conductance change of 2.8 μS. After 17 min the GABA-induced conductance change fell to 0.8 μS. The GABA conductances returned to near control levels after perfusing the preparations with the normal

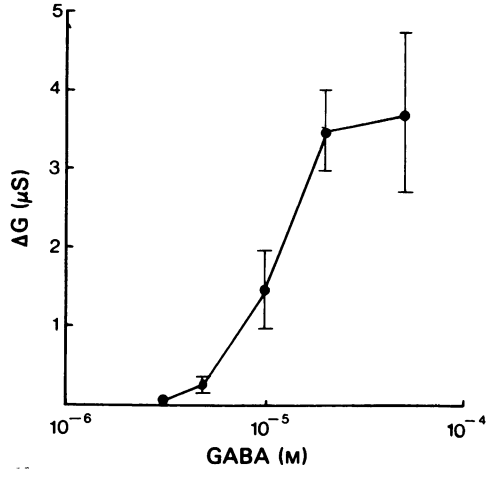

Figure 4 The log dose-response relationship for the change in input conductance of the bags produced by different molar concentrations of γ-aminobutyric acid (GABA). This relationship was obtained from 6 preparations and each point represents the mean of 5 or 6 observations; vertical lines show s.e. means. The ED_{50} was 13 μM.

Ringer. In the crayfish, Takeuchi & Takeuchi (1967) attributed the progressive reduction of GABA conductances in low Cl^- Ringer to the gradual removal of intracellular Cl^-.

γ-Aminobutyric acid iontophoresis

The bath-applied GABA experiments showed that large conductance changes could be observed in bags located well away from the nerve cord. It seemed unlikely that these conductance changes could be explained by inhibitory receptors exclusively located at the syncytium as suggested by Del Castillo *et al.* (1964a). The resistance of the arm should have limited

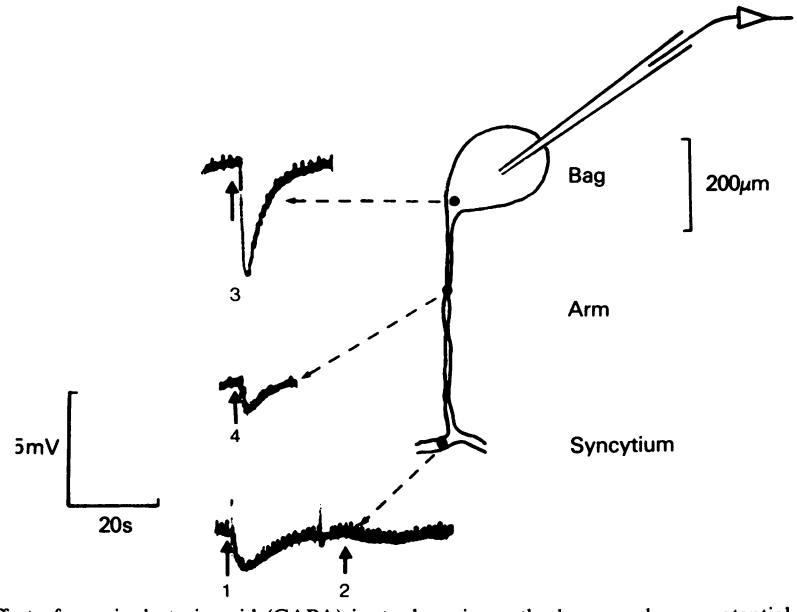

Figure 5 Effect of γ-aminobutyric acid (GABA) iontophoresis on the bag membrane potential when GABA is applied to different regions of the muscle cell. This preparation was made from a caudal section of *Ascaris*. A standard current pulse of 0.4 μC (at the vertical arrow) was used to deliver GABA to the different regions marked (●). Even though this preparation was at 22°C, small depolarizing potentials were still observed. The effect of GABA release over the syncytium was tested first (1). The GABA pipette was raised 50 μm but then only caused a small response (2). Application of GABA to the bag produced the largest response (3). No response to GABA iontophoresis was observed when the GABA pipette was placed 100 μm from the edge of the bag, except for small responses observed over the arm (4).

the size of the conductance changes observed in bags located at a distance from the nerve cord. GABA was therefore applied iontophoretically to the bags to detect the presence of GABA receptors on the bag. When GABA was released from a micropipette placed over the bags of muscle cells, a dose-dependent hyperpolarization was invariably recorded even when the bags were located 2 mm from the nerve cords.

Figure 5 illustrates the results of a typical experiment in which GABA was applied to different regions of the muscle cell of a caudal section of *Ascaris*. In this particular experiment a standard current pulse of 0.4 μC was used to deliver the GABA to the different regions of the muscle cell. The response at the syncytium was tested first (Figure 5 (1)). When the GABA pipette was raised 50 μm only a small response was observed (Figure 5 (2)). The response at the bag was tested next (Figure 5 (3)). Typically it was larger than at the syncytium. No response was observed when the GABA pipette was removed 100 μm from the bag except for small responses over the arm (Figure 5 (4)).

Picrotoxin antagonism

Picrotoxin was used in order to confirm that the above responses were due to the action of GABA and not due to the direct action of the iontophoretic current. The effects of bath-applied picrotoxin were tested on 4 preparations. The results of one of these experiments is illustrated in Figure 6. In all these preparations picrotoxin antagonized the actions of GABA when applied in concentrations of 50 to 500 μM. The antagonism of picrotoxin was dose-dependent but was not easily washed off. The reduction of the slope of the dose-response curves indicated that picrotoxin acted as a non-competitive antagonist.

Reversal potential

Extrapolation of current voltage plots like those shown in Figure 7 using bath-applied 10^{-5}M GABA suggested that the reversal potential was about 30 mV more negative than the resting membrane potential. However, this method of estimating the reversal potential assumes that conductance values are independent of the membrane potential. The reversal potential was therefore estimated directly. GABA was applied iontophoretically to the bag and the potential of the bag altered by means of a two microelectrode voltage clamp system. The results of a typical experiment are shown in Figure 8a and b. It can be seen that the peak of the outward GABA-induced current

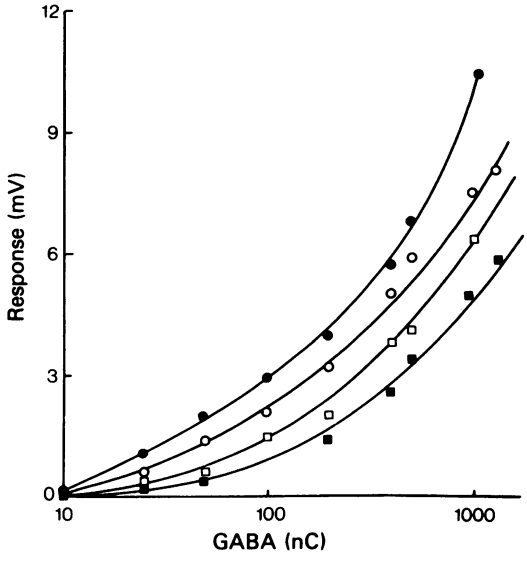

Figure 6 Antagonism of γ-aminobutyric acid (GABA) by picrotoxin. Peak hyperpolarizations recorded in the bag (ordinate scale) are plotted against the iontophoretic current (log scale abscissa). The GABA pipette was placed over the bag. The control responses (●) were observed initially. Picrotoxin 5×10^{-5} M (○) was then applied in the bath and the responses re-determined. Picrotoxin 5×10^{-4} M (■) was then applied and the responses determined. Finally the preparation was washed for 20 min and the responses again determined (□). Picrotoxin antagonized the GABA responses in a dose-dependent manner. The antagonism was only partially reversed by 20 min washing.

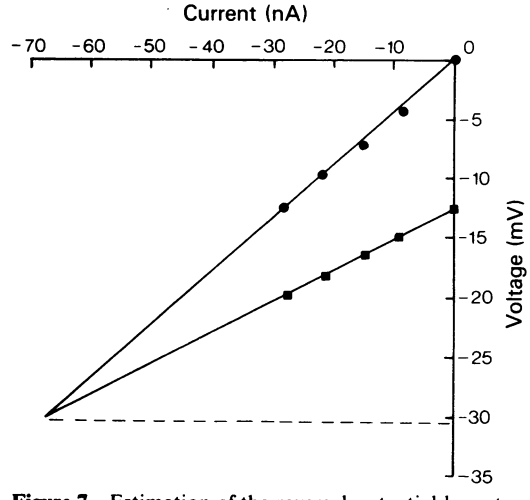

Figure 7 Estimation of the reversal potential by extrapolation. The current-voltage relationships were determined without γ-aminobutyric acid (GABA, ●) and then in the presence of 10^{-5} M GABA (■). The resting membrane potential (−30 mV) was plotted as zero on the ordinate scale with negative potentials indicating hyperpolarization. The hyperpolarizing current is plotted on the abscissa scale. GABA 10^{-5} M hyperpolarized the bag by 13 mV and increased the resting conductance from 2.3 μS to 4.2 μS. The intersection of the two current voltage slopes gives an estimate of 31 mV for the reversal potential.

at −35 mV declines in amplitude as the membrane potential is increased and is reversed in direction near −55 mV. The estimated value of the GABA reversal potential from 27 such experiments was −61.2 mV ± 2.2 mV mean ± s.e. mean. The speed of the response is also interesting since there was several seconds between the iontophoretic pulse and the peak of the GABA current (Figure 8a). This delay occurred even though the GABA pipette was placed directly over the bags and was measured in 27 experiments as 3.6 ± 0.3 s (mean ± s.e. mean).

Discussion

Conductance changes

The experiments described in this paper show that GABA produces a dose-dependent and reversible increase in input conductances associated with a hyperpolarizing potential. The involvement of Cl^- was confirmed by the effect of low Cl^- Ringer on the GABA-induced conductance changes and membrane potential. Brading & Caldwell (1971) have suggested that the main action of GABA may be to inhibit an electrogenic transport system. However, this suggestion was made following an analysis of the membrane potential using KCl filled micropipettes for recording. The inhibition of an electrogenic transport system by GABA does not explain the marked effects of low Cl^- Ringer on the conductance changes, nor the fact that the value (−61.2 ± 2.2 mV) of the reversal potential estimated in the present experiments was close to the value predicted by a Nernst Cl^- electrode (−64.8 mV) with an intracellular Cl^- concentration of 13.7 mM (Brading & Caldwell 1971).

Iontophoresis of γ-aminobutyric acid

The iontophoresis of GABA on the bag region confirmed the presence of inhibitory receptors on the bag. The results contrast with the experiments of Del Castillo *et al.* (1964a) which suggested that there were no inhibitory receptors on the bag. However, they applied piperazine not GABA iontophoretically. Piperazine is less potent (about 100 times) than GABA (Del Castillo *et al.*, 1964b) and may act more slowly (Constanti & Nistri, 1976).

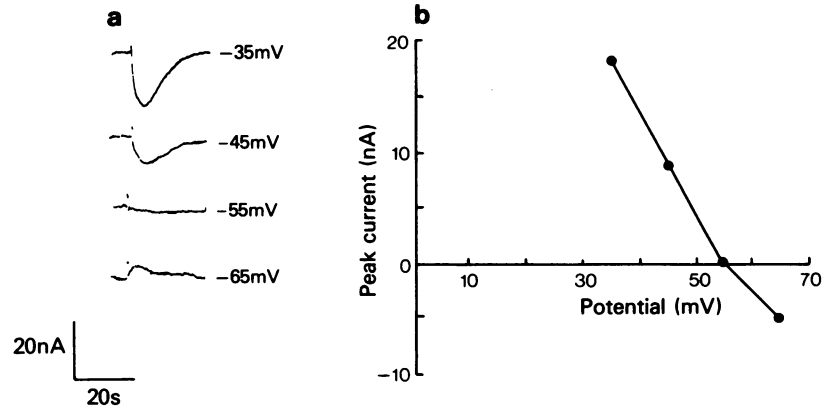

Figure 8 Estimation of the reversal potential by the two microelectrode voltage clamp technique, (a) γ-Aminobutyric acid (GABA) was applied directly over the bag using an iontophoretic current of 1 μA for 500 ms (during the artefact) and the GABA-induced current was observed at different holding potentials. A downward deflection indicates an outward GABA-induced current. Note that the peak of the GABA current occurred several seconds after the application of GABA and that the reversal potential is near −55 mV. (b) Graph of the peak GABA current (positive values indicate an outward current on the ordinate scale) against the membrane potential (positive values on the abscissa scale indicate increased hyperpolarization).

The physiological significance of the presence of GABA receptors on the bag is obscure since it is a region devoid of synapses. However this observation sets no precedent since there are other examples in invertebrates where somatic membranes possess receptors without synapses (Tauc & Gerschenfeld, 1962; Kerkut, Pitman & Walker, 1969). The GABA responses produced by iontophoresis at the syncytium were usually smaller than those produced at the bag. This observation may be explained in part by shielding of inhibitory receptors at the synctium as suggested by Del Castillo *et al.* (1964) as well as the decrement between the syncytium and bag associated with electronic spread.

The slow response following γ-aminobutyric acid iontophoresis

The peak of the GABA-induced voltage or current responses occurred several seconds after the iontophoretic GABA pulse, even though the GABA pipette was placed directly over the bag. The membrane of the bag region of *Ascaris* muscle is covered by several other thin membranes (Ellory, 1967). One explanation for the delay of several seconds between the GABA pulse and the peak of the response might be due to diffusion across this layer. However, without further analysis it is not possible to exclude other factors such as uptake , slow dissociation of GABA from receptors or rate limiting intermediate steps between receptor activation and membrane conductance increases. (Diamond & Roper, 1973).

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