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Actions of γ-aminobutyric acid on neurones of guinea-pig myenteric plexus

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1 The effects of γ-aminobutyric acid (GABA) applied by ionophoresis, pressure ejection and superfusion to myenteric neurones of the guinea-pig ileum were investigated by intracellular recording techniques.

2 Ionophoretic or pressure application of GABA (10 pC–30 nC) caused membrane depolarizations of AH neurones but not S neurones. This depolarization was associated with a conductance increase. It reversed polarity at a membrane potential of −18 mV when intracellular electrodes contained KCl, and −39 mV when electrodes contained K acetate, citrate or sulphate.

3 The ionophoretic depolarization was antagonized by bicuculline (1–30 μM) in an apparently competitive manner.

4 During prolonged or repeated ionophoretic application of GABA, both the depolarization and conductance increase desensitized.

5 Superfusion of GABA (1–100 μM) caused a membrane depolarization in AH neurones, associated with an increase in membrane conductance. The increase in conductance was always smaller than that evoked by ionophoresis of GABA.

6 Bicuculline only partially depressed the depolarization induced by superfusion of GABA, particularly slowing its rising phase. ß-*p*-Chlorophenyl GABA (baclofen) (10 μM) caused a depolarization similar to that observed with GABA in the presence of bicuculline.

7 The depolarization induced by baclofen and GABA (in presence of bicuculline) superfusion did not decline during prolonged applications; superfusion of GABA but not baclofen reversibly reduced or eliminated the effects of GABA ionophoresis.

8 It is concluded that GABA has two effects on the membrane of myenteric neurones. The first is a bicuculline-sensitive, rapidly desensitizing chloride activation: the second is a bicuculline-insensitive, non-desensitizing depolarization.

Introduction

γ-Aminobutyric acid (GABA) and its synthesizing enzyme, glutamic acid decarboxylase (GAD), both occur in nerves of the myenteric plexus (Jessen *et al.*, 1979). These neurones accumulate GABA by an uptake system having properties similar to that in brain (Krantis & Kerr, 1981), and can release GABA when electrically stimulated (Taniyama *et al.*, 1982). These findings suggest the possibility that GABA may play a physiological role within the enteric nervous system. We tested this by examining the mechanisms of action of exogenous GABA on the membranes of myenteric neurones, with the aim of comparing these actions with those of transmitters released within the myenteric plexus.

Methods

Intracellular recordings were made from neurones lying within ganglia of the myenteric plexus of the guinea-pig ileum. The techniques for isolation of the ganglia, superfusion with physiological salt solutions, and intracellular recordings have been fully described (Nishi & North, 1973). The superfusing solution was pumped at 1–2 ml min^{-1} and heated so that its temperature over the tissue was 37°C. Its composition was (mM): NaCl 117, KCl 4.7, NaH_2PO4 1.2, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, glucose 11, gassed with O_2 95% and CO_2 5%. Unless otherwise stated, recording electrodes were filled with potassium chloride (2 M), and cells were impaled under direct vision as they lay in a shallow bath (volume

1–2 ml) on the stage of a microscope (Zeiss Nomarski, total magnification ×500). GABA was applied by ionophoresis from micropipettes containing 100 mM–1 M solutions (pH 4.5); the tips of these pipettes were placed within 5 μm of the soma of the impaled cell. Glycine (100 mM–1 M, pH 4.5) and acetylcholine chloride (ACh) (500 mM) were also used for ionophoresis. In some experiments, GABA was applied by application of a brief (10–50 ms) pressure pulse to a micropipette containing GABA (100 μM–10 mM, in physiological saline, pH 7.4), positioned 10–20 μm from the impaled cell. Other drugs were applied by changing the superfusing solution to one which differed only in its content of drug(s). Figures in this paper indicate the period during which a tap was turned, allowing the changed superfusing solution to reach the tissue. There was a delay of 45–60 s between turning this tap and the first arrival at the tissue of the changed solution. The ratio of flow rate to bath volume ensured complete exchange within 1 min. Substances used were GABA (Sigma), bicuculline methiodide (Pierce), (±)-β-*p*-chlorophenyl-GABA (baclofen) (Dr H. Proudfit), muscimol (Sigma), picrotoxin (Sigma), nipecotic acid (Sigma), ß-alanine (Sigma), ACh chloride (Sigma) tetrodotoxin (Sigma) and glycine (Sigma).

Results

Ionophoretic applications of GABA

GABA depolarized myenteric neurones when applied by ionophoresis (Figure 1). Typical ionophoretic currents were 10–100 nA for 1–300 ms (10 pC–30 nC); essentially similar results were obtained with pressure application. This effect of GABA could not be mimicked by glycine even when applied with charges of 200 nC. The most striking feature of this action of GABA was its selectivity between the main cell types of the myenteric plexus. S neurones, defined by the existence of a nicotinic fast e.p.s.p. (Nishi & North, 1973; Hirst *et al.*, 1974), were only rarely depolarized by GABA (5 of 52 cells) despite application of up to 200 nC. AH neurones have no fast e.p.s.p. but have a prolonged calcium-dependent afterhyperpolarization (Nishi & North, 1973; Hirst *et al.*, 1974). These cells were almost always depolarized by GABA (81 of 89 neurones). The absolute sensitivity to GABA varied among cells; on a given neurone the amplitude of the depolarization was dependent on the ionophoretic charge ejected. This action of GABA was presumably a direct action on the impaled cell, since it was unchanged by superfusion with tetrodotoxin (TTX, 300 nM–1 μM) or by calcium-free, 10 mM magnesium solutions. In about 10% of neurones, the GABA depolarization was followed by a hyperpolarization lasting for up to 20 s.

Conductance changes The GABA depolarization was accompanied by a decrease in the cell input resistance measured from the amplitude of hyperpolarizing electrotonic potentials. Larger depolarizations had larger conductance increases, and with increased amounts of GABA the conductance change continued to increase although the amplitude of the depolarization became maximal. In 13 cells in which the fall in input resistance was 50.0 ± 4.0% (mean ± s.e.mean), the mean GABA depolarization was 24 mV from a resting level of −60 mV.

If the GABA depolarization results from opening of ion channels the change in conductance of the cell should be related to the amplitude of the depolarization (△V) by $\triangle V = (1 - R'/R)(E_{rev,R} - E_m)$ where R′ is the input resistance during GABA application, R is the control input resistance, $E_{rev,R}$ the membrane potential at which no driving force exists and E_m is the membrane potential at which the GABA response is evoked (Ginsborg, 1967; Morita *et al.*, 1982). We found no correlation between △V and (1−R′/R) ($r = 0.125$, $v = 38$, $P = 0.5$), presumably because hyperpolarizing electrotonic potentials arise from currents carried not only by chloride ions, and because an increase in potassium conductance is likely to occur because of the depolarization caused by GABA (Nishi & North, 1973). Therefore, we repeated these experiments in 20 cells during recording with electrodes filled with CsCl (1 M), after impalement for at least 30 min to allow caesium to enter the cell (effects on spike duration had reached steady state). For CsCl filled electrodes △V and (1−R′/R) were linearly related ($r = 0.600$, $P = < 0.005$). The slope of the regression line and the mean resting potential of −63 ± 1.7 mV gave an estimate for $E_{rev,R}$ of −10 mV.

Reversal potential The amplitude of the GABA depolarization changed linearly with the membrane potential at which it was evoked. The GABA depolarization had a reversal potential of −18 ± 3.6 mV ($n = 12$) (Figure 1). When the recordings were made with electrodes containing potassium acetate the reversal potential was −39 ± 1.2 mV ($n = 6$) (Figure 1). Similar results were obtained with K citrate or K sulphate in the recording electrodes. With caesium chloride filled electrodes the GABA response reversal potential was not different from that observed with potassium chloride filled electrodes.

Changing the extracellular chloride concentration from 125 to 10 mM (isethionate substitution) increased the amplitude of the GABA response to 150% of its control value and increased the response duration.

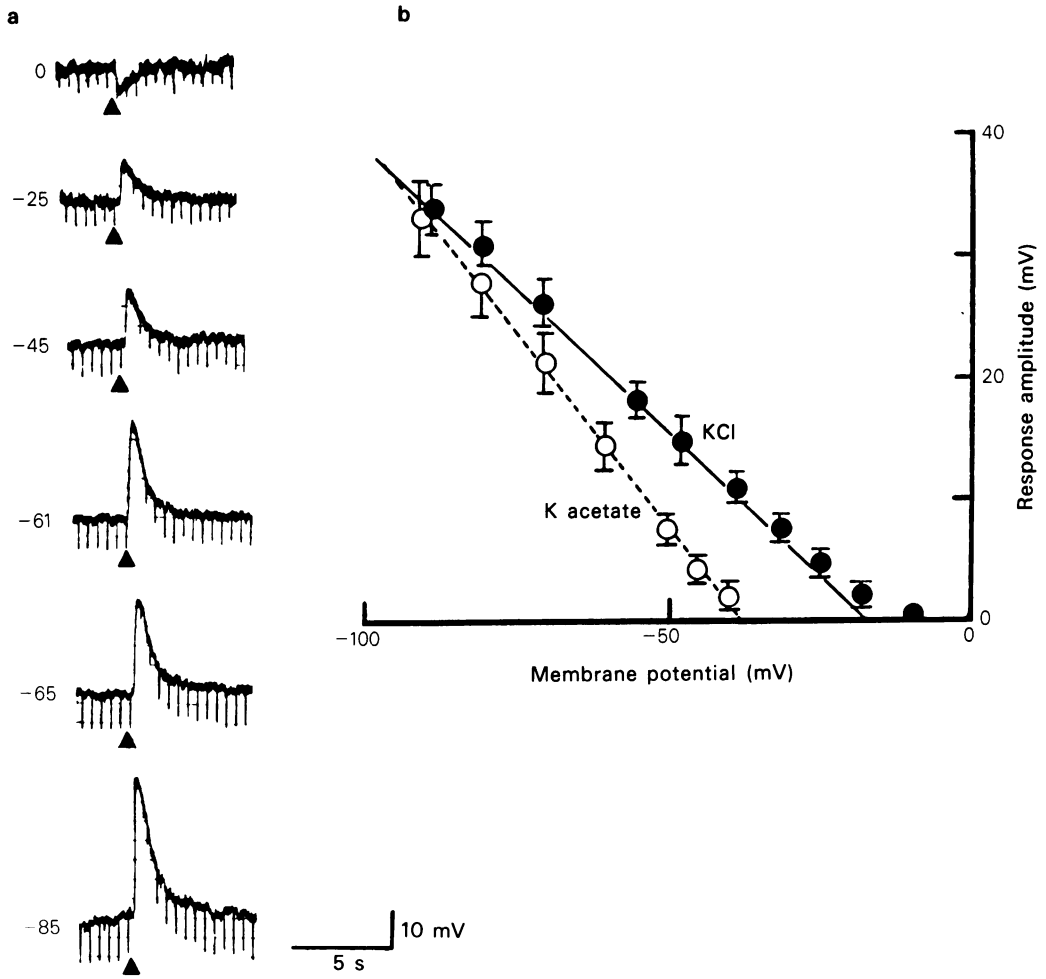

Figure 1 The depolarization induced by ionophoresis of GABA in AH neurones. (a) Changing the membrane potential by passing steady current through the recording electrode changed the amplitude of the depolarization. Membrane potentials indicated in mV; downward deflections in this and other figures are electrotonic potentials resulting from repeated passage of a fixed current pulse. (b) Relation between membrane potential and the amplitude of the GABA depolarization determined in 12 cells penetrated with KCl electrodes and in 6 cells penetrated with K acetate electrodes. Vertical bars are s.e.means.

Antagonists Bicuculline and picrotoxin reversibly depressed or abolished the depolarizations evoked by ionophoresis of GABA (Figure 2). The effective concentrations were 100 nM–10 μM. Equal depolarizations evoked by ionophoresis of glycine and ACh were not affected by bicuculline. Higher concentrations of bicuculline (30 μM) depolarized the membrane and increased the input resistance.

Uptake blockers Nipecotic acid (1–10 μM) and ß-alanine (10–30 μM) had no marked effects on the responses to GABA. This does not necessarily imply that uptake does not contribute to the time course of the response, since the concentrations may be too low to block uptake (Schon & Kelly, 1974; Krogsgaard-Larsen & Johnston, 1975). However, higher concentrations (100 μM–1 mM) themselves caused membrane depolarizations of 5–8 mV. These were generally associated with a depression in the peak amplitude of the GABA response, without any change in its time course.

Low sodium containing solutions inhibit neuronal GABA uptake (Iversen & Neal, 1968). Experiments performed to test the hypothesis that GABA uptake was occurring with ionophoretic application were equivocal. We found that a reduction in sodium ion

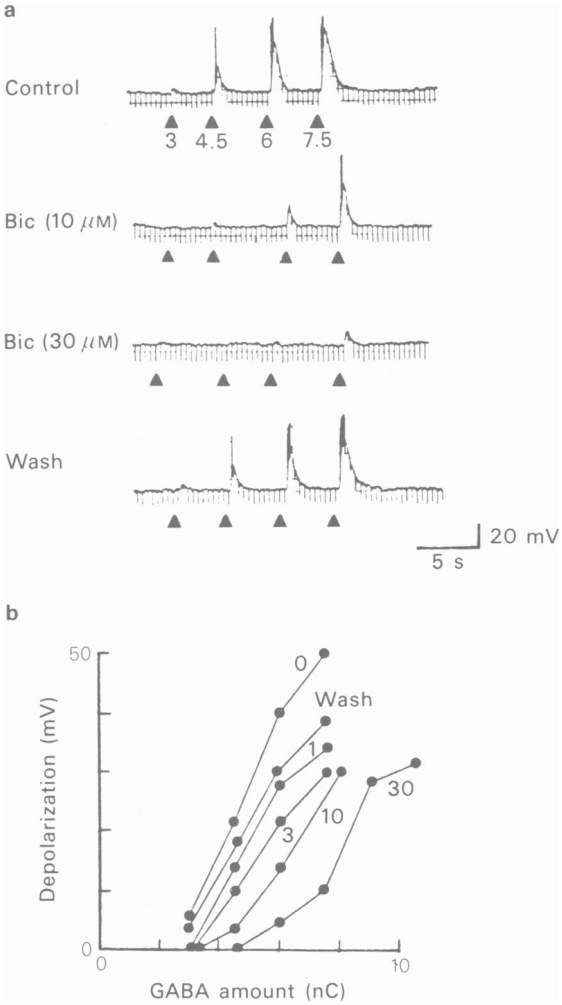

Figure 2 The depolarizing effect of GABA is antagonized by bicuculline. (a) Responses to GABA applied by ionophoresis (the amount of charge ejected is indicated in nC below the triangles) in control and in the presence of 10 and 30 μM bicuculline (Bic). (b) Dose-response curves to GABA in another neurone before and during superfusion with bicuculline in the concentrations indicated (μM).

concentration (choline substitution) from 143 to 26 mM usually depressed the amplitude of the GABA response during the first 10–20 min of perfusion. More prolonged perfusion (20–30 min) with sodium-free solutions caused an increase in the duration of the response to GABA.

Desensitization Long applications (several seconds) of GABA, by applying direct current or a succession of pulses to the ionophoresis pipette, evoked responses which rapidly declined from their initial amplitude, and then reached a steady state depressed level for as long as the GABA was applied (up to 30s). The conductance change associated with the GABA depolarization showed desensitization with a time course similar to that of the potential change. Following such a prolonged application of GABA, the response to brief applications slowly recovered its control level with a half time of approximately 1 min.

Superfusion of GABA

Superfusion with GABA (1–100 μM) caused a membrane depolarization in 58 of 66 AH cells and in 5 of 52 S cells. The depolarizations were frequently excitatory in that they reached threshold for action potential generation. The depolarization began within 10–20 s of the GABA reaching the tissue and achieved its peak amplitude in 20–30 s. The typical depolarization by superfusion of a high concentration of GABA (30–100 μM) had a rapid initial rate of rise to its peak amplitude, and then declined slightly to a steady state which was maintained throughout the period of superfusion (Figures 3 and 4). The membrane potential returned to its control level within 2–10 min of washing with GABA-free solution. This action of GABA was unaltered by TTX (1 μM), implying that it probably did not result from action potential generation and release of transmitter from surrounding cells. The sensitivity to GABA varied from cell to cell, but in a given neurone the response varied with the concentration applied. Increasing concentrations of GABA increased the peak amplitude but particularly the rate of rise of the depolarization (Figure 3).

Conductance change The GABA depolarization was accompanied by a decrease in input resistance. The mean fall in resistance caused by 100 μM GABA was to 28.0 ± 0.7% (mean ± s.e.mean, $n = 10$): the depolarization caused by this concentration was 22.5 ± 0.73 mV from the resting level of 62.5 ± 2.0 mV. This conductance increase still occurred when the depolarization was annulled by passing small hyperpolarizing currents through the recording electrode (Figures 3 and 4). The conductance changes were always smaller than those evoked by ionophoresis of GABA.

Ionic mechanism The response was unaffected by substitution of choline chloride for sodium chloride (final concentration 25 mM). This indicates that the GABA depolarization is probably not due to the entry of sodium ions associated with GABA uptake (Constanti *et al.*, 1980). The role of potassium ions was assessed by recording with CsCl filled electrodes; GABA depolarizations were apparently the same

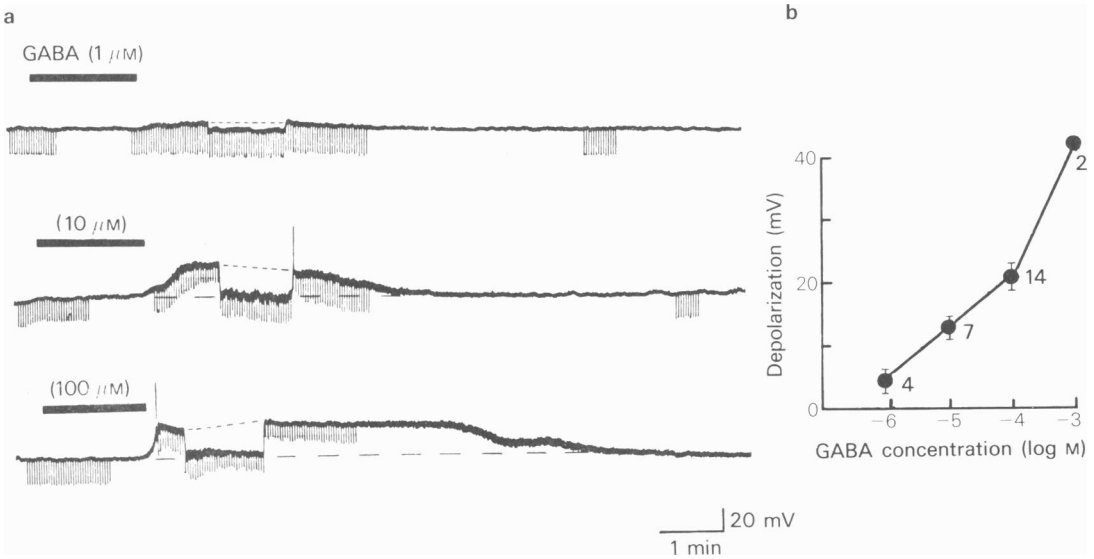

Figure 3 Dose-dependent depolarization induced by superfusion of GABA. (a) Responses to increasing concentrations of GABA in a single AH neurone. The downward deflections in the voltage recording lasting about 1 min result from passage of steady current to restore the membrane potential to its resting level. (b) Dose-response curves to increasing concentrations of GABA applied by superfusion. Bars indicate s.e.mean for number of GABA applications indicated. The 27 applications of GABA were made during recordings from 15 neurones, having resting potentials ranging from − 58 mV to − 65 mV.

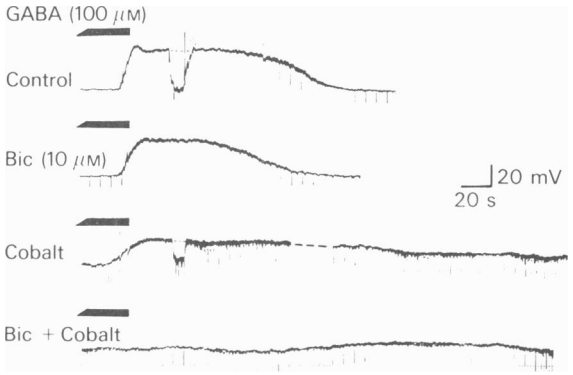

Figure 4 The depolarization induced by superfusion of GABA is antagonized by bicuculline (Bic) and cobalt. GABA was superfused for 1.5 min in each recording. The solid bars indicate only the last part of the superfusion period; there was a delay of about 1 min between the start of the superfusion period and the arrival of GABA at the tissue (see Methods). Bicuculline (10 μM) depressed the rate of rise of the GABA response. Cobalt (2 mM) depolarized the membrane 12 mV (resting level was − 62 mV), increased the membrane resistance and prolonged the duration of the response. Cobalt and bicuculline together almost abolished the response to GABA. Downward deflections are electrotonic potentials caused by passing a constant current pulse across the cell membrane. The longer downward deflections in two of the traces result from passing steady current to restore the membrane potential to its resting level. There is a break in the third trace of 2.6 min.

whether Cs or K was used in the recording electrode. The amplitude of the response to GABA perfusion was reduced by 15–20% by calcium-free/high (20 mM) magnesium or by addition of cobalt (2 mM) solutions. However, cobalt itself always depolarized the neurones and increased the input resistance. We noticed that GABA depolarizations were prolonged when GABA was applied by superfusion in calcium-free or cobalt containing solutions.

Antagonists Concentrations of bicuculline (1–10 μM) which markedly reduced the responses to ionophoresis of GABA did not greatly depress the final steady-state amplitude of the depolarization caused by superfusion of GABA. However, increasing concentrations of bicuculline (10–30 μM) progressively reduced the rate of onset of the response and depressed the initial peak response (Figure 4). Depolarizations resulting from superfusion of ACh were not affected by bicuculline. In the presence of bicuculline, the GABA depolarization was almost abolished by the addition of cobalt (2 mM) (Figure 4).

Desensitization When the superfusion with GABA was continued for 5 min the depolarization reached its peak level and after a small initial decline the potential remained at the same depolarized level throughout the period of application. The membrane potential returned to its original level within 5–8 min after changing to a solution free of GABA. Applica-

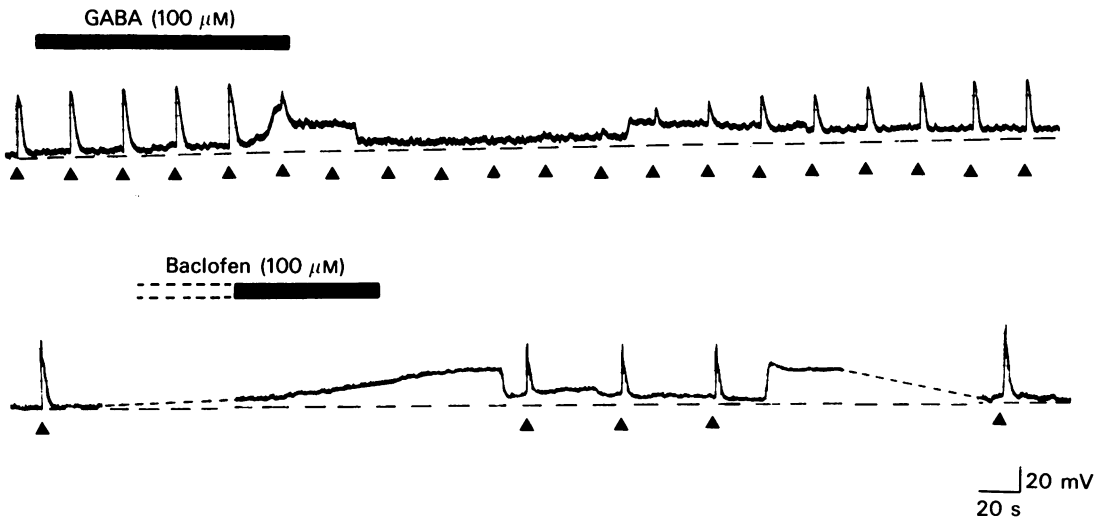

Figure 5 Comparison of the effects of GABA and baclofen on the response to ionophoretic application of GABA. (a) The response to GABA ionophoresis (3 nC, ▲) was completely blocked during the depolarization induced by superfusion of GABA (100 μM). This cell was hyperpolarized to −72 mV so as to increase the amplitude of the responses to GABA. (b) In another neurone, the response to GABA ionophoresis (3 nC, ▲ was depressed by only 30% during an equivalent depolarization induced by superfusion of baclofen (100 μM). Notice that the time course of the baclofen depolarization is quite different from that evoked by GABA; the initial rapid depolarization is absent (see also Figure 4). The longer downward deflections in the two traces result from passing steady current to restore the membrane potential to its control level. There are two breaks in the bottom trace of 2 and 3.3 min.

tion of 100 μM GABA at the time of steady state depolarization by 30 μM GABA caused an additional depolarization.

In contrast to these findings, responses to ionophoresis of GABA were completely blocked by superfusion with GABA (Figure 5). The degree of blockade of the ionophoretic response was proportional to the concentration of GABA perfused. The percentage reductions (± s.e.means) in the amplitude of the ionophoretic responses were: 1 mM GABA, 100% ($n=3$); 100 μM GABA, 75 ± 3% ($n=6$); 10 μM GABA, 57 ± 7% ($n=6$); 1 μM GABA, 36 ± 9% ($n=4$). In many cells, when 1 μM GABA was used, the only detectable effect of the GABA superfusion was the block of the ionophoretic response. Thus, the depolarization induced by GABA superfusion showed little tendency to desensitize, but superfusion of GABA readily and reversibly abolished the response to GABA ionophoresis.

GABA analogues and other amino acids

Muscimol mimicked the effects of GABA, in a similar concentration range, and muscimol blocked the responses to ionophoresis of GABA. ß-*p*-Chlorophenyl-GABA (baclofen) also mimicked the GABA depolarizaton, although higher concentrations were required and the response rose more slowly (Figure 5). Baclofen depolarizations were unaffected by bicuculline, but were reversibly depressed by cobalt (2 mM). During baclofen depolarizations, the responses to GABA ionophoresis were depressed, but never by more than 30%. This contrasted strongly with the effect of GABA superfusion (above); superfusion with sufficient GABA to cause a depolarization equivalent to that caused by baclofen always completely blocked responses to GABA ionophoresis. Glycine and ß-alanine mimicked the depolarizing effect of GABA only when superfused at 100 times higher concentrations: they did not affect responses to GABA ionophoresis.

Discussion

There are two major findings of the present investigation. The first is that GABA opens chloride channels in the membrane of AH type myenteric neurones, but has relatively little effect on S cells. The activation of chloride conductance is sensitive to bicuculline and rapidly densensitizing. In these respects it resembles the responses to GABA described in many other neurones (Krnjević & Schwartz, 1967; Davidoff, 1972; Adams & Brown, 1975; Gallagher *et al.*, 1978; Désarmenien *et al.*, 1980). The selectivity of GABA action on AH neurones as distinct from S

neurones is interesting. Although the functional role of AH cells is incompletely understood, there is some evidence that they include both afferent neurones in the peristaltic reflex arc as well as neurones which release acetylcholine (North, 1982; Szerb, 1982). The blockade by bicuculline and the apparent desensitization by superfusion with GABA or muscimol, but not baclofen, suggest the involvement of a $GABA_A$ receptor (Bowery *et al.*, 1980; Hill & Bowery, 1981). In this respect, the response of AH neurones is essentially identical to that of afferent cells in the dorsal root ganglion (Gallagher *et al.*, 1978).

The second finding is that GABA depolarizes myenteric neurones even in the presence of bicuculline, and baclofen has a similar action. The finding that baclofen only weakly depresses the responses to GABA ionophoresis, whereas GABA superfusion completely eliminates them, speaks to an interaction with a distinct receptor, such as that termed $GABA_B$ by Hill & Bowery (1981). The ionic basis of this depolarization is unknown. One possibility is a non-somatic action of GABA, leading to the observation that the depolarization is associated with a relatively small conductance increase (Barker & Ransom, 1978; Alger & Nicoll, 1979; Andersen *et al.*, 1980). Another possibility is a release by GABA of other depolarizing agents. The reduction of the response by cobalt might suggest this; however, such an action must involve TTX-resistant transmitter release and furthermore, GABA and baclofen both 'depress' the evoked release of two depolarizing transmitters in the myenteric plexus (Cherubini & North, 1984). A third possibility is that GABA and baclofen act on $GABA_B$ receptors to block a constant inward calcium current. Such an inward calcium current contributes to the resting potassium conductance in myenteric neurones (Grafe *et al.*, 1980).

When GABA is applied to the intact myenteric plexus within the guinea-pig ileum it causes a release of acetylcholine (ACh) (Kleinrok & Kilbinger, 1983). This effect is also blocked by bicuculline (Kaplita *et al.*, 1982; Ong & Kerr, 1983; Giotti *et al.*, 1983; Kleinrok & Kilbinger, 1983). This indicates that some AH neurones release ACh, a suggestion which has been made previously (Szerb, 1982; North, 1982). We do not know whether such an action of GABA is important for the normal operation of the enteric nervous system. Krantis & Kerr (1981) did find that bicuculline alone reduced the rate at which guinea-pig isolated colon expelled faecal pellets. However, bicuculline-sensitive synaptic potentials which are due to an increase in chloride conductance have not been reported in either type of myenteric neurone. This might be due to the problems involved in the selective activation of GABA containing neurones. In conclusion, the present findings are compatible with, but provide no direct evidence for, a neurotransmitter role for GABA in the myenteric plexus.

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