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A COMPARATIVE STUDY OF THE ACTION OF γ-AMINOBUTYRIC ACID AND PIPERAZINE ON THE LOBSTER MUSCLE FIBRE AND THE FROG SPINAL CORD

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1 The effects of γ-aminobutyric acid (GABA) and piperazine were compared on two *in vitro* preparations, the lobster muscle fibre and the frog spinal cord.

2 Both GABA and piperazine increased the membrane conductance of single lobster muscle fibres without changing the membrane potential; sigmoidal log dose-conductance curves for these agents were obtained and a similar model expressed the receptor interaction of both substances.

3 The actions of GABA and piperazine on lobster muscle were antagonized by picrotoxin and were Cl^--dependent.

4 In the frog spinal cord GABA depolarized the dorsal roots presumably by mimicking the activity of the transmitter depolarizing the primary afferents; sigmoidal log dose-response curves for GABA were obtained.

5 On the dorsal roots piperazine produced either depolarizations or biphasic responses; these were mainly indirect effects as was shown by experiments in the presence of tetrodotoxin (TTX).

6 The effects of GABA on the dorsal root (in TTX-treated cords) were antagonized by picrotoxin whereas those of piperazine were more resistant to this alkaloid. The GABA-induced responses appeared to be largely Na^+-dependent while both Na^+ and Cl^- seemed to mediate the effects of piperazine.

7 It is proposed that piperazine has GABA-agonist activity on lobster muscle but little GABA-like activity on the frog spinal cord.

Introduction

γ-Aminobutyric acid (GABA) is considered to be an inhibitory transmitter in several areas of the vertebrate central nervous system (Curtis & Johnston, 1974; Krnjević, 1974) and at many invertebrate synapses (Gerschenfeld, 1973). However, the characteristics of GABA-evoked responses differ according to the animal species or tissue being investigated (Curtis, Duggan, Felix, Johnston & McLennan, 1971; Bowery & Brown, 1974; Nistri, Constanti & Quilliam, 1974; Shank, Pong, Freeman & Graham, 1974; Straughan, 1974). At the lobster neuromuscular junction and in the frog spinal cord, GABA has an inhibitory action (Barker & Nicholl, 1973; Gerschenfeld, 1973), but its effects on the membrane potential and its ionic dependence differ in the two species.

The present study was prompted by the reports that piperazine, an anthelmintic agent, mimics the activity of the natural inhibitory transmitter (possibly GABA) at the *Ascaris* neuromuscular junction (del Castillo, de Mello & Moralles, 1964) and has a GABA-like agonist effect on crayfish muscle (Iravani, 1965a,b). When applied microiontophoretically, piperazine can depress the activity of rat cerebral neurones (Shinozaki & Konishi, 1970) although little attention has been paid to its action on the vertebrate central nervous system, even when isolated cases of neurotoxicity in humans have been described (Parsons, 1971). In order to examine further the possibility that the anthelmintic and central neuronal depressant actions of piperazine involve an interaction with GABA receptors, we carried out a quantitative comparison of the effects of GABA and piperazine on two *in vitro* preparations, the lobster muscle fibre and the frog spinal cord.

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Methods

The lobster muscle fibre preparation

Lobsters (*Homarus vulgaris*) were obtained from a local supplier and kept in aerated artificial sea water at

5°C until used. The opener muscle of the first or second walking leg was exposed and superfused at room temperature with crustacean Ringer solution of the following composition (mM): NaCl 522, KCl 12, $CaCl_2$ 21, $MgCl_2.6H_2O$ 5, Tris maleate 10; pH was adjusted to 7.6 with 0.1N NaOH.

The membrane potential at the centre and tendon end of a single superficial fibre in the central portion of the muscle was monitored continuously with two glass microelectrodes filled with 1.5 M tripotassium citrate connected to unity gain voltage followers (F.E.T.—operational amplifiers).

The membrane potential was recorded differentially with respect to the potential of the bathing fluid, monitored by a Ag/AgCl-Agar bath reference electrode. A third microelectrode filled with 0.6 M K_2SO_4 was inserted within 50 μm of the central voltage electrode and used to pass rectangular hyperpolarizing current pulses of constant amplitude (800 ms; 0.25 Hz) through the membrane. The current injection circuit was completed through a second bath electrode connected in series with a 40 MΩ resistor. This same electrode also kept the bath fluid at zero (earth) potential. Electrotonic potentials (e.t.ps) at the centre (V_0) and at the end (V_L) of the fibre were displayed on a Tektronix 502A oscilloscope and recorded on a Devices MX4 chart recorder. The output of the current injection circuit was also recorded on a separate channel.

Calculation of the membrane conductance

The membrane conductance of single muscle fibres was calculated by the method of Takeuchi & Takeuchi (1967) using the short cable equations (Weidmann, 1952; Vaughan, 1974). The general analysis has been fully described elsewhere (Takeuchi & Takeuchi, 1967; Feltz, 1971; Earl & Large, 1974). In the present method, the three microelectrodes were kept within the fibre throughout the experiment without causing any obvious damage to the cell. This allowed an average value of the 'resting' length/space constant ratio (L/λ) to be calculated from some 20 to 30 individual measurements made throughout the day using the relation

$$L/\lambda = \cosh^{-1}(V_0/V_L) \qquad (1)$$

V_0/V_L was the ratio of hyperpolarizing e.t.ps at the middle (V_0) and end (V_L) of the fibre. This average value of L/λ was then used in the calculation of the resting membrane conductance at any instant from

$$g_m L = (I_0/2V_0)(L/\lambda)\coth(L/\lambda) \qquad (2)$$

where g_m was the conductance per unit length (mho.cm^{-1}) and I_0/V_0 the input conductance (mho). The current/voltage relation of the lobster fibre was sufficiently linear in the hyperpolarizing direction to allow the input conductance to be estimated directly from the ratio of current to e.t.p. amplitude at the centre of the fibre without introducing serious error (imposed hyperpolarizations were always $\leqslant$ 15 mV).

The modified length/space constant ratio (L/λ^*) in the presence of GABA or piperazine was calculated by an iterative procedure (Earl & Large, 1974) using the average L/λ and the ratio of e.t.ps at the centre of the fibre in the presence (V_0^*) and absence (V_0) of drug. L/λ^* and V_0^* were then substituted into eqn (2) to give the modified conductance $g_m^* L$ and the actual change in membrane conductance was obtained by subtracting the resting conductance value calculated immediately before the addition of the drug

$$\Delta g_m L = g_m^* L - g_m L \qquad (3)$$

The iterative method for calculating L/λ^* avoided the use of V_L^* which, during large conductance changes, often became too small to measure with accuracy.

The frog spinal cord preparation

Frogs (*Rana temporaria*) were kept in a tank with circulating water at 5°C until used. The spinal cords were removed following dorsal laminectomy, hemisected and fixed on the bottom of a 0.5 ml bath as previously described (Nistri, 1975). The frog Ringer solution had the following composition (mM): NaCl 109, KCl 4 mM, $CaCl_2$ 1.5, $NaHCO_3$ 1.27, glucose 4; and was gassed with 95% O_2 and 5% CO_2 (pH 7.2). Stimulations and recordings were made through the VIIIth or IXth pair of lumbar roots placed in paraffin-filled side chambers. The whole preparation was maintained at 13 to 14°C throughout the experiment. Rectangular pulses (1 Hz; 0.1 ms; supramaximal voltage) were delivered through platinum electrodes.

Two types of potential were recorded: (1) the ventral root potential (VRP) evoked by orthodromic stimulation of the adjacent dorsal root, and (2) the dorsal root potential (DRP) evoked by antidromic stimulation of the adjacent ventral root. These potentials were recorded differentially with Ag/AgCl-Agar electrodes, displayed on a storage oscilloscope and a 502A Tektronix oscilloscope and photographed with a Polaroid camera. The potentials and the root d.c. polarization level were recorded on a two channel pen recorder.

Drugs

All the drugs were adjusted to the pH of the Ringer solutions and applied to the two preparations via the bathing fluid. The drugs used were obtained from the following sources; GABA from B.D.H., piperazine citrate, tetrodotoxin, picrotoxin and sodium glutamate from Sigma; dibenamine hydrochloride was kindly donated by Smith, Kline & French. The doses of piperazine quoted in the text on a molar basis refer to the citrate salt. Dibenamine hydrochloride was diluted

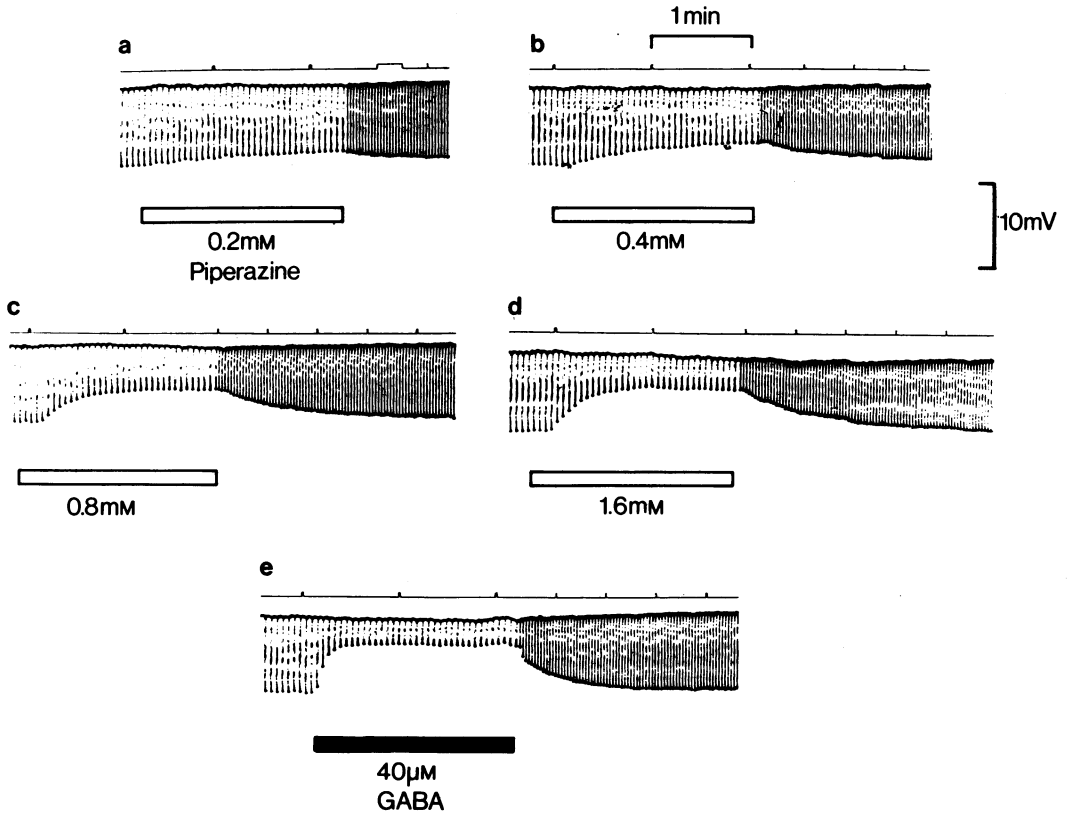

Figure 1 The effect of piperazine (0.2 mM to 1.6 mM, open bars) on the hyperpolarizing electrotonic potentials (downward deflections) recorded at the centre of a single lobster muscle fibre (resting potential = −76 mV) in response to intracellular current pulses applied via a second central microelectrode (800 ms; 1.45×10^{-7}A). (a to d) reduction in amplitude of the hyperpolarizing potentials (indicating increases in membrane conductance) during application of successively increasing concentrations of piperazine (with intermediate washing). In (e), the muscle was bathed with γ-aminobutyric acid (GABA) solution (40 μM, filled bar) over a similar period. Note that the onset/decline rates for the action of piperazine were much slower than those for GABA. Chart speed was halved during decline of responses.

immediately before use from freshly made stock solutions (10% w/v) in 90% ethanol. In this case GABA solutions contained the same small amount of 90% ethanol and produced responses no different from those obtained with control doses.

Results

Effects of GABA or piperazine on lobster muscle fibres

The bath application of GABA (5 μM to 640 μM) produced a reversible and dose-related increase in the membrane conductance with little or no change in the resting potential. Such an effect, already described in our laboratory (Constanti & Quilliam, 1974; Nistri & Constanti, 1975), had a typically fast onset and did not fade despite the continued presence of GABA (see below). A typical example of GABA action on the e.t.p. recorded from a single fibre is shown at the bottom of Figure 1. Figure 1 also shows the effects of piperazine on the same fibre. As with GABA, this compound (0.1 to 3.2 mM) produced dose-related increases in membrane conductance unaccompanied by a change in membrane potential, and with no visible desensitization. Piperazine however, was much less potent than GABA on a molar basis. Moreover, both the onset and the decline of the effects of piperazine were slower than those of GABA, even when similar conductance changes were obtained in each case. Piperazine responses usually required about 2 min to attain equilibrium and 5–10 min for.

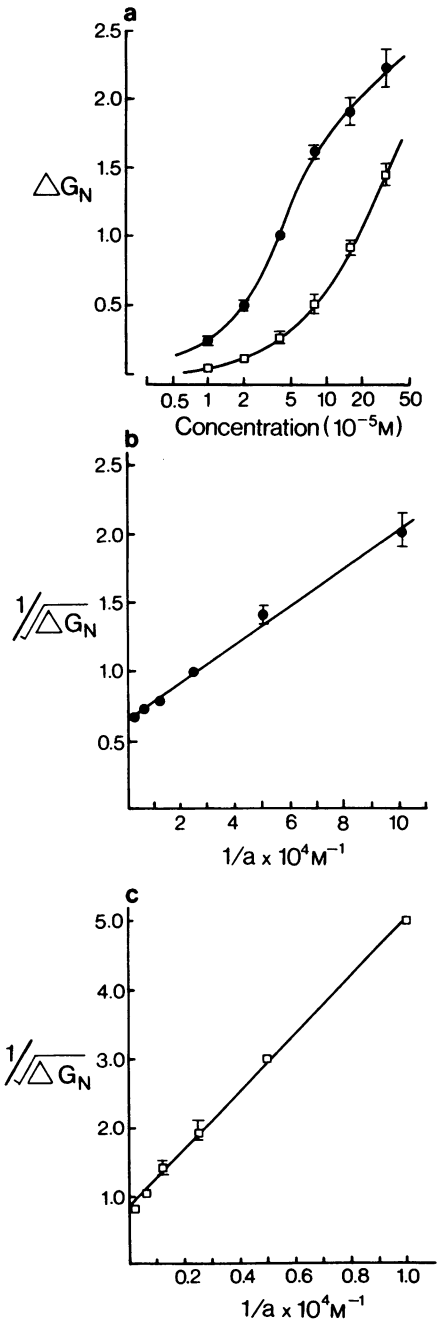

Figure 2 (a) Normalized log dose-conductance curves for γ-aminobutyric acid (GABA) (●) and piperazine (□). Points represent mean of 5 experiments. Vertical lines show s.e. mean. Errors not shown were within the size of the points. Ordinate scale represents normalized increase in membrane conductance (ΔG_N); abscissa scale gives concentration of drug added to the superfusing solution. ΔG_N was calculated with respect to the 40 μM (4×10^{-5}M) GABA response (see text). Note that the piperazine curve has been displaced one log unit to the left along the abscissa scale in order to facilitate comparison. (b and c); Double reciprocal transformations ($1/\sqrt{\Delta G_N}$ vs $1/a$) of the normalized GABA and piperazine conductance curves (data from Figure 2a). The plots were drawn on the basis of the two independent binding-site models. ΔG_N represents normalized conductance increase and 'a' is the drug concentration (10 μM; 10^{-5}M). Error intervals indicated are the transformed standard errors. (●) GABA plot: concentration range represented, 10 μM (10^{-5}M) to 320 μM (3.2×10^{-4}M). Weighted regression line had slope $= +0.143$: intercept $= 0.639$: $r = 0.998$: Estimated value of $K_{GABA} = 22.4$ μM (2.24×10^{-5}M). (□) Piperazine plot: concentration range 0.1 mM (10^{-4}M) to 3.2 mM (3.2×10^{-3}M) (note difference in ordinate and abscissa scales). Weighted regression line had slope $= +4.238$; intercept $= 0.822$; $r = +0.999$. Estimated value $K_{Pip} = 516$ μM (5.16×10^{-4}M).

recovery, whereas equilibrium GABA responses were normally attained within 30 s of changing from control to test solution and required 4 to 5 min for recovery of the resting conductance.

Owing to the very small size of the e.t.p. during large conductance changes, the maximal conductance could not be measured with accuracy. A normalization procedure was therefore adopted whereby all GABA- and piperazine-induced conductance changes measured on any single fibre were expressed as fractions of the conductance change produced by 40 μM GABA in that fibre. The normalized conductance increase expressed as ΔG_N was then used to construct normalized log dose-conductance curves. This procedure also allowed the pooling of conductance data from different muscle fibres, of varying diameter, length and resting conductance.

Figure 2a shows the normalized log dose-conductance curves for GABA and piperazine, the latter curve having been displaced one log unit to the left along the abscissa scale to facilitate comparison. Each point represents the mean (with standard error) of 5 experiments in which GABA and piperazine curves were constructed on a single fibre. In the range studied, the piperazine curve appeared to be parallel with the GABA curve suggesting a similar mechanism of action at receptor level. When the dose-conductance curves for both compounds were plotted on linear coordinates (not shown), the curves showed an initial sigmoidicity, suggesting that more than one molecule of each drug was involved in the receptor interaction; in addition the Lineweaver–Burk double-reciprocal plots of these data showed a marked non-linearity.

Model for GABA or piperazine receptor interaction in the lobster muscle

Takeuchi & Takeuchi (1967; 1969) proposed that at least two molecules of GABA were required for the activation of the unit conductance change on crayfish muscle and they used a high-cooperativity receptor model to describe their data. In the present case, a better overall fit to the GABA data was provided by a simple two independent binding-site model (for a discussion on different receptor models see Werman, 1969) although this does not imply that the independent model is more physically likely than any other. This model assumed the presence of two equivalent agonist binding sites at the receptor, either of which could initially interact with a GABA molecule. The binding of the first molecule was considered not to influence the binding of the second, and when both sites had been activated, the unit conductance channel would be opened. The properties of the model can be represented by the equation:

$$\Delta g/\Delta g_{max} = [a/(a+K)]^2 \qquad (4)$$

where $\Delta g/\Delta g_{max}$ = fractional equilibrium conductance changes (measured as $\Delta g_m L$), a is concentration and K is the apparent agonist/receptor dissociation constant (= 1/affinity constant). A convenient check for conformity with this model was provided by the double-reciprocal transformation of eqn (4). However, since all responses were normalized with respect to 40 μM GABA, it was also necessary to normalize eqn (4) thus:

$$\Delta G_N = [a/(a+K)]^2/[4/(4+K)]^2$$

Taking the square root of the reciprocal gives:

$$1/\sqrt{\Delta G_N} = [\alpha + \alpha K \cdot (1/a)] \qquad (5)$$

where $\alpha = 4/(4+K)$. A plot of $1/\sqrt{\Delta G_N}$ vs $1/a$ should thus be linear with slope (αK) and intercept α. K can then be estimated from the ratio slope/intercept.

Figure 2b and c shows such double-reciprocal transformations of normalized piperazine and GABA dose-conductance curves presented in Figure 2a. In each case, the regression lines were estimated using a weighted linear regression analysis so that equal emphasis could be provided for all the points along the line. Each point was assigned a weight = $1/\text{var}(\Delta G_N)$. Both plots were linear in the concentration ranges studied in good agreement with the proposed model, but the piperazine (Pip) double-reciprocal plot had the steeper slope. The values of K_{GABA} and K_{Pip} estimated from the slope/intercept ratio were 22.4 μM (2.24×10^{-5} M) and 516 μM (5.16×10^{-4} M) respectively, indicating the relatively lower affinity of piperazine for the GABA receptor. If both GABA and piperazine could produce the same maximal conductance change, then the ordinate intercepts should be identical. However, the intercept value of the piperazine plot (0.82) was slightly higher than that from the GABA plot (0.64), possibly indicating that piperazine produces a smaller maximum than GABA.

Effects of low Cl^- solution on GABA and piperazine-induced responses

The absence of membrane potential changes during the action of GABA or piperazine suggested a similarity in the reversal potentials. In crustacean muscle, GABA-evoked conductance changes involve an increase in Cl^- permeability (Boistel & Fatt, 1958; Takeuchi & Takeuchi, 1967). In order to determine whether the conductance increase produced by piperazine was also Cl^- mediated, some experiments were carried out in which the external Cl^- (586 mM) of the normal solution was reduced to 325 mM; the impermeant isethionate was used as a substitute. Approximately equieffective doses of piperazine and GABA having no effect on membrane potential in normal solution (Figure 3a and b), when applied soon after changing to low Cl^- produced almost identical membrane depolarizations (Figure 3c and d). These depolarizations were reversed to hyperpolarizations (about 2 mV) on returning to a normal solution (Figure 3e and f). These changes in membrane potential were in a direction consistent with the imposed change in the Cl^- equilibrium potential, suggesting that both GABA and piperazine were producing similar changes in Cl^- permeability.

Combination experiments on the lobster muscle

Iravani (1965a,b) reported that piperazine was a partial agonist at the crayfish GABA receptor. At low GABA concentrations, piperazine increased the GABA effect while at high GABA concentrations the effect was decreased. This phenomenon was not observed in the present case. The result of a typical GABA-piperazine combination experiment is shown in Figure 4a. The GABA curve was first measured, then it was repeated with various concentrations of GABA combined with a fixed concentration of piperazine. The combination of GABA with piperazine produced approximately additive effects with no evidence of mutual inhibition at high GABA doses. Similar results were obtained with higher fixed piperazine concentrations.

Effects of picrotoxin on the GABA and piperazine-induced responses

Picrotoxin, a potent GABA antagonist at the crustacean neuromuscular junction (Takeuchi & Takeuchi, 1969; Constanti & Quilliam, 1974; Earl & Large, 1974; Nistri *et al.*, 1974; Shank *et al.*, 1974), was also tested as an inhibitor of piperazine-induced responses. Figure 4b shows the piperazine dose-

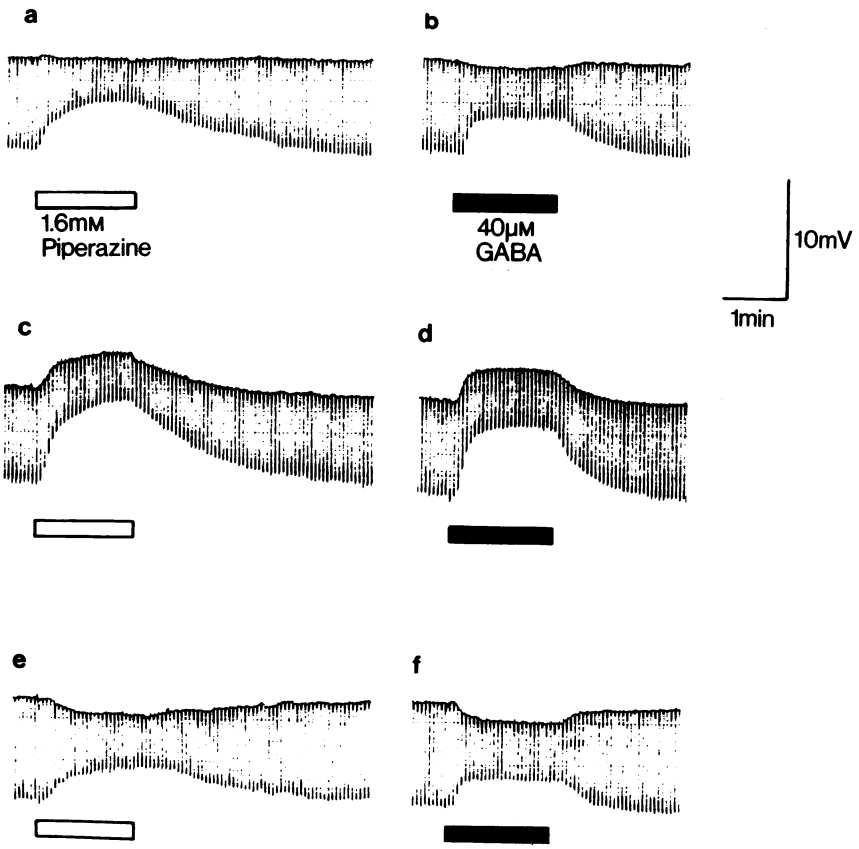

Figure 3 Hyperpolarizing electrotonic potentials (downward deflections) recorded at the centre of a single lobster fibre in response to central intracellular current pulses. (800 ms; 1.25×10^{-7}A). Records show effect of changing from normal external solution (586 mM Cl^-) to a low chloride (325 mM) solution chloride replaced with isethionate) on the membrane potential and conductance changes produced by γ-aminobutyric acid (GABA, 40 μM-filled bars) and piperazine (1.6 mM-open bars). (a,b) Control matched responses to piperazine and GABA respectively, measured in normal solution; (c,d) 5 and 10 min respectively after changing to low chloride solution, both piperazine and GABA now produced membrane depolarizations; (e,f) 5 and 10 min respectively after returning to normal bath solution, both piperazine and GABA now hyperpolarized the membrane.

conductance curve in normal solution and in the presence of picrotoxin (0.5 μM).

This concentration has been previously shown (Constanti & Quilliam, 1974) to displace the GABA curve in a non-parallel fashion with a reduction of the apparent maximal conductance change of about 65%. Figure 4b shows that the piperazine curve was also displaced to the right, although in the range studied there was no indication of a maximum.

According to Furchgott (1966) the alkylating agent dibenamine will bind irreversibly to a wide variety of membrane receptors. It was therefore of interest to test the effect of GABA in the presence of dibenamine (0.1 mM). However, despite the high concentration used, no consistent antagonism could be demonstrated.

Effects of GABA or piperazine on the frog spinal cord.

Bath application of GABA (0.1 mM) or piperazine (0.1 mM) produced a reversible depression of the VRP and DRP (Figure 5), but the onset and the decline in rates of action of piperazine were slower than those of GABA. The GABA-induced decrease in VRP was associated with either a depolarization or a hyperpolarization of the ventral root while piperazine often depolarized the same root. The decrease in DRP amplitude produced by GABA was always

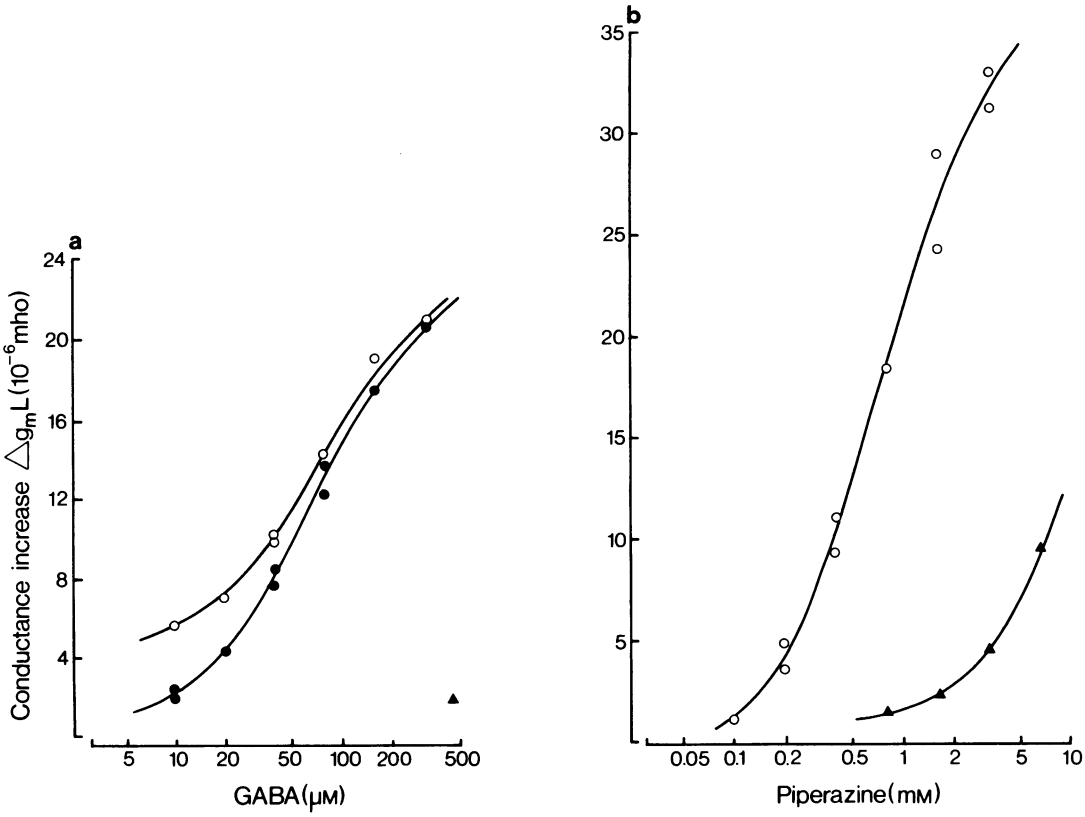

Figure 4 (a) Interaction between γ-aminobutyric acid (GABA) and piperazine. (●), Control GABA log dose-conductance curve measured in normal solution. Ordinate scale represents the increase in membrane conductance ($\Delta g_m L$) where g_m is the membrane conductance per unit length, and L is the half-length of the muscle fibre. Abscissa scale gives the concentration of applied GABA. (○), GABA 'combination' curve obtained by combining various concentrations of GABA with a fixed concentration (0.4 mM) of piperazine. (▲), Conductance change produced by 0.4 mM piperazine. All measurements were made on the same fibre. (b) Effect of picrotoxin on the piperazine log dose-conductance curve. (○), Piperazine-evoked increase in membrane conductance ($\Delta g_m L$) measured in normal solution; (▲), in 0.5 μM picrotoxin. Measurements were made on a different fibre from that of Figure 4a.

accompanied by a dorsal root depolarization while piperazine produced either a biphasic response consisting of an initial small hyperpolarization followed by a depolarization, or a pure depolarizing response. Since the action of GABA on the dorsal root was very consistent and is considered to be implicated in the depression of the primary afferents (Barker & Nicholl, 1973), the subsequent experiments dealt with the dorsal root responses to GABA. In order to avoid indirect synaptic effects from interneuronal activity the cord was treated with tetrodotoxin (TTX; 1 μg/ml) and the action of GABA was observed as a change in the d.c. polarization level of the dorsal root. Such responses therefore represented electronic spreading of the depolarization along the root (Barker & Nicoll, 1973). In the TTX-treated cord the GABA-evoked depolarizations were dose-related and had a very fast onset. However, in the continued presence of GABA, these responses declined rapidly suggesting desensitization (see inset of Figure 6a). The GABA log dose-response curve was sigmoidal (Figure 6a) but the apparent maximum was influenced by tissue desensitization. GABA dose-response curves in cords not treated with TTX had a very similar shape to those in the presence of this toxin, suggesting that any indirect effect of GABA on the dorsal root was minimal. The dorsal root responses to GABA were closely reproducible even after many hours provided that 8–10 min elapsed between each administration of this substance.

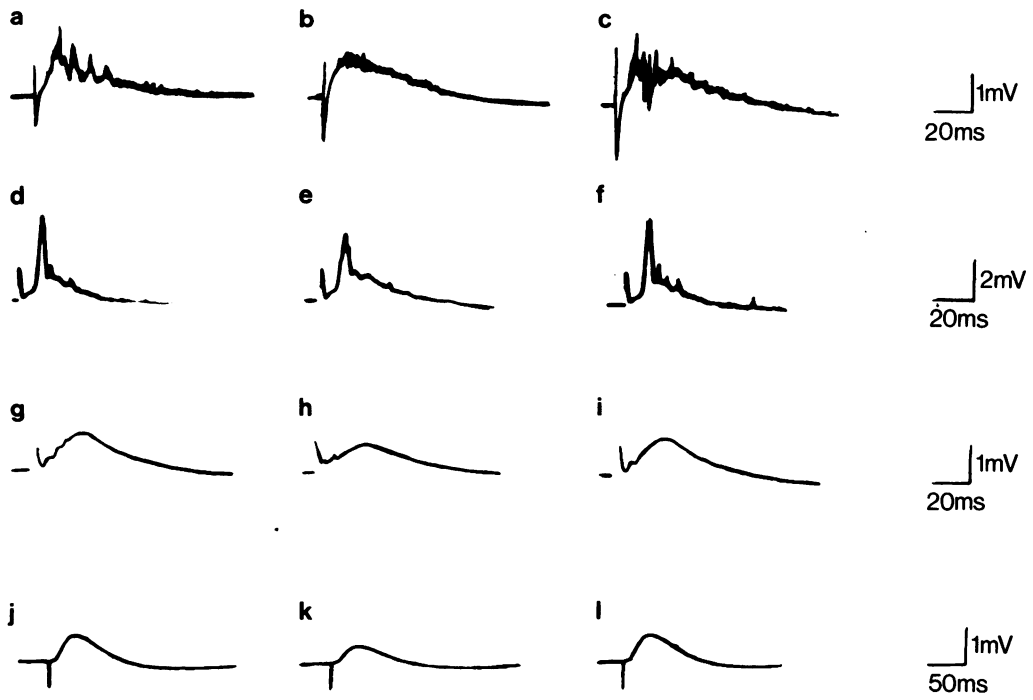

Figure 5 Effects of γ-aminobutyric acid (GABA) or piperazine on the frog ventral root potential (VRP) and dorsal root potential (DRP). (a and d) Control VRPs; (b and e) VRPs 10 min after GABA (0.1 mM) or piperazine (0.1 mM) respectively; (c and f) recovery (20 min later); (g and j) control DRPs; (h and k) DRPs after 10 min in the presence of GABA (0.1 mM) or piperazine (0.1 mM) respectively; (i and l) recovery (20 min later).

The dorsal root responses to piperazine were more variable than those to GABA. As previously mentioned, biphasic effects (hyperpolarizations followed by depolarizations) were often observed (see Figure 8). A decline of the response to piperazine during the continued application of the drug was not easily found (see inset in Figure 7). The cord was usually less sensitive to piperazine than to GABA on a molar basis and a considerable variability in the cord sensitivity to piperazine was encountered.

When piperazine log dose-response curves were compared in the presence or in the absence of TTX, a marked depression of the curve in the presence of TTX was noted (Figure 7). In order to ensure reproducibility of the dorsal root responses, the doses of piperazine had to be administered every 12–15 minutes.

In an attempt to analyze the nature of the GABA and piperazine interaction with the receptor sites in TTX-treated cords, the dose-response curves previously obtained were transformed into log-log plots. This type of analysis is useful when the tissue responses are recorded as depolarizations rather than as conductance changes (Werman, 1969). In the range studied, the GABA or piperazine log-log plots were linear with a limiting slope of 0.55 and 0.82 respectively. This suggested that one GABA or piperazine molecule was interacting with a single receptor site.

Effects of picrotoxin or ionic substitutions on the cord responses

The effect of picrotoxin (10 μM) on the GABA or piperazine action was tested in TTX-treated spinal cords. Although picrotoxin is a well-known GABA antagonist on the frog dorsal root (Barker & Nicoll, 1973), conventional log dose-response curves of this phenomenon have not been presented to show the mechanism of inhibition. Figure 6b shows that in the presence of picrotoxin the GABA dose-response curve was strongly depressed and shifted to the right whereas the piperazine curve was less affected (see Figure 7). The action of picrotoxin was slowly reversible over a period of 90 min or more.

High concentrations of dibenamine (up to 0.5 mM for 30–45 min) were used in an attempt to block the effects of GABA or piperazine. However, no clear

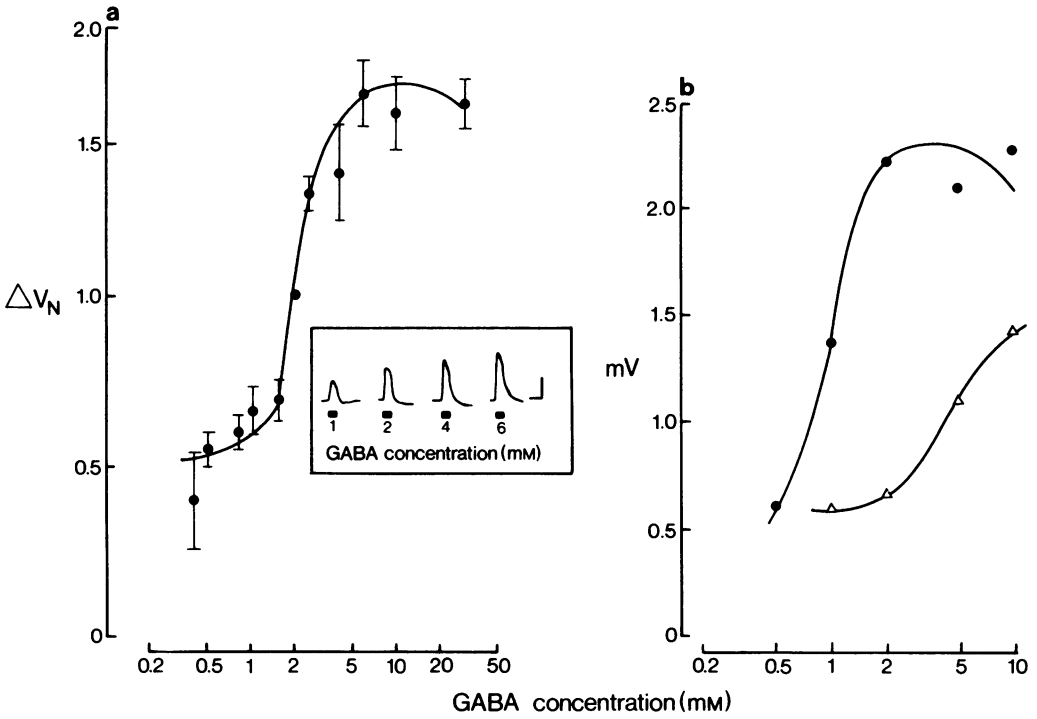

Figure 6 (a) Normalized log dose-response curve for γ-aminobutyric acid (GABA) obtained from the dorsal root of a tetrodotoxin (TTX)-treated cord. Each point (●) is the mean of 9 experiments. Vertical lines show s.e. mean. Abscissa scale: GABA concentration; ordinate scale: normalized depolarizations (ΔV_N was calculated by dividing all GABA depolarizations of any single cord by the response to 2 mM GABA in that cord). Inset: examples of GABA-induced depolarization on the dorsal root. In this and the following figures depolarizations are indicated by upward deflections of the pen. (Calibration; 1 mV; 1 min). (b) Log dose-response curves for GABA.(●) and GABA in the presence of picrotoxin (△) obtained from a dorsal root of a TTX-treated cord. Picrotoxin was applied at a concentration of 10 μM for 20 minutes. Note the depression and shift of the GABA curve.

antagonism of the actions of these substances was found.

The ionic dependence of the GABA or piperazine effects on TTX-treated cords was also tested (Figure 8). Ninety per cent substitution of the Na^+ content of the bathing medium with Li^+, abolished the effects of GABA after 60 min whereas the biphasic effect of piperazine was converted into a simple hyperpolarization. No potentiation of the actions of these two substances was observed, in contrast with the report of Nishi, Minota & Karczmar (1974). The 90% substitution of external Cl^- with the impermeant isethionate ion did not alter the depolarization produced by GABA although the after-hyperpolarization was enhanced. However the biphasic response to piperazine was converted into a depolarization.

In contrast, the equimolar substitution of external Ca^{2+} with Mg^{2+} did not greatly alter the effect of GABA or piperazine. In low Na^+ medium the depolarization of the dorsal root produced by glutamate, a potent excitatory amino acid, was also abolished (not shown).

Combination experiments in the frog cord (tetrodotoxin-treated)

Following the procedure described for the lobster preparation, GABA-piperazine combination experiments were carried out to test for possible mutual hindrance. The combination of GABA with a fixed dose of piperazine (2.5 mM) produced approximately additive effects over most of the GABA concentration range. However, in view of the strong tissue desensitization to high doses of GABA, it was not possible to reach any definite conclusion about mutual interaction on this preparation.

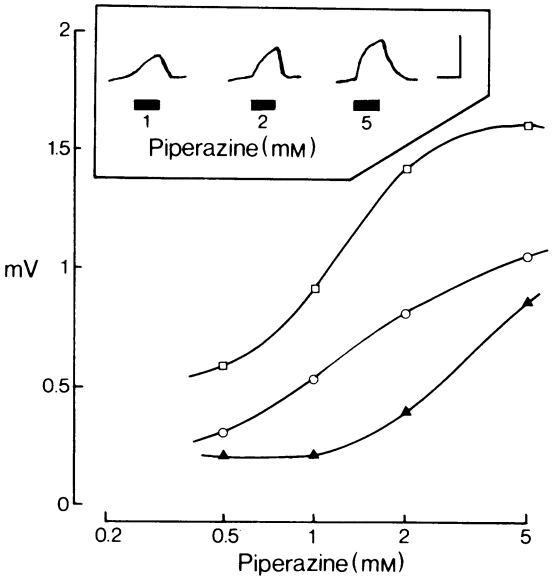

Figure 7 Log dose-response curves for piperazine obtained from a dorsal root of the frog spinal cord. Abscissa scale: piperazine concentrations; ordinate scale: depolarizations (mV). (□) Indicates the curve in the absence of tetrodotoxin (TTX); (○) in the presence of 1 μg/ml TTX; (▲) in the presence of TTX and picrotoxin (10 μM for 20 minutes). Inset: examples of depolarizations produced by piperazine on a dorsal root of a TTX-treated cord. (Calibration: 1 mV; 1 min).

Discussion

Similar actions of GABA and piperazine on the lobster muscle

On the lobster muscle fibre the conductance responses to the application of GABA or piperazine had several points in common. First, both compounds produced a reversible and dose-related increase in membrane conductance without changing the membrane potential. Second, the log dose-conductance curves for the two drugs were well fitted by an independent binding model, according to which two agonist molecules were supposed to bind to a single receptor site without mutual interaction. Thirdly, an increase in Cl^- permeability appeared to mediate the effects of GABA and piperazine and fourthly, the actions of both drugs could be antagonized by picrotoxin. Piperazine thus behaved like a GABA agonist with lower affinity for the receptor than GABA itself ($K_{Pip} = 516\ \mu M$; $K_{GABA} = 22.4\ \mu M$), despite the absence of any structural similarity between the piperazine and GABA molecules. The above results are in general agreement with those of del Castillo *et al.* (1964) who suggested that the paralyzing effect of piperazine on *Ascaris* muscle was mimicking the action of the natural inhibitory transmitter (possibly GABA) although piperazine was 100 times less potent than GABA on this preparation.

According to the mass action law, when two drugs of similar efficacy (Stephenson, 1956) compete for a common receptor site, the combined effect of the two drugs would be a competitive synergism (Ariëns & Simonis, 1964). If one of the drugs were acting as a partial agonist, then mutual hindrance would occur (see also Constanti & Quilliam, 1974). The results of the present combination experiments would therefore suggest that GABA and piperazine were acting as agonists of similar efficacy although of different affinity. The only major difference between the effects of GABA and piperazine was the slow onset and offset rate of action of the latter. This might be due to a slow diffusion rate of this compound into and out of the region of the receptors. Alternatively, piperazine may be unable to share with GABA the tissue uptake process that may be important in terminating the action of this amino acid (Curtis & Johnston, 1974). Another possibility could be that piperazine dissociates from the receptors at a slower rate than GABA. All these hypotheses, which can also be proposed to explain the slow onset and offset of the responses observed in the frog spinal cord, need to be confirmed experimentally.

Differences between the actions of GABA and piperazine on the frog cord

In the frog spinal cord GABA and piperazine depressed both VRP and DRP. To clarify the mechanism of this effect, changes in dorsal root d.c. levels were also examined. Despite the presence of TTX the dorsal root responses to GABA always consisted of a depolarization with characteristic fading which suggested rapid receptor desensitization. The log dose-response curve for GABA was steep with a maximum difficult to establish with certainty. The ventral root responses were more variable consisting of hyperpolarizations or depolarizations according to the preparations. On the dorsal (and sometimes on the ventral) roots piperazine often produced biphasic responses (hyperpolarizations followed by depolarizations) with little fading. These depolarizing components of the piperazine effect gave log dose-response curves less steep than those for GABA, and the effect of piperazine was found to be largely mediated by interneurones since the addition of TTX strongly reduced it. Picrotoxin antagonized the effect of GABA in an apparently non-competitive fashion but was less effective in reducing the action of piperazine. The slopes of the log-log plots for GABA and piperazine (0.55 and 0.82 respectively) may be interpreted as one molecule of GABA or piperazine interacting with the spinal receptors.

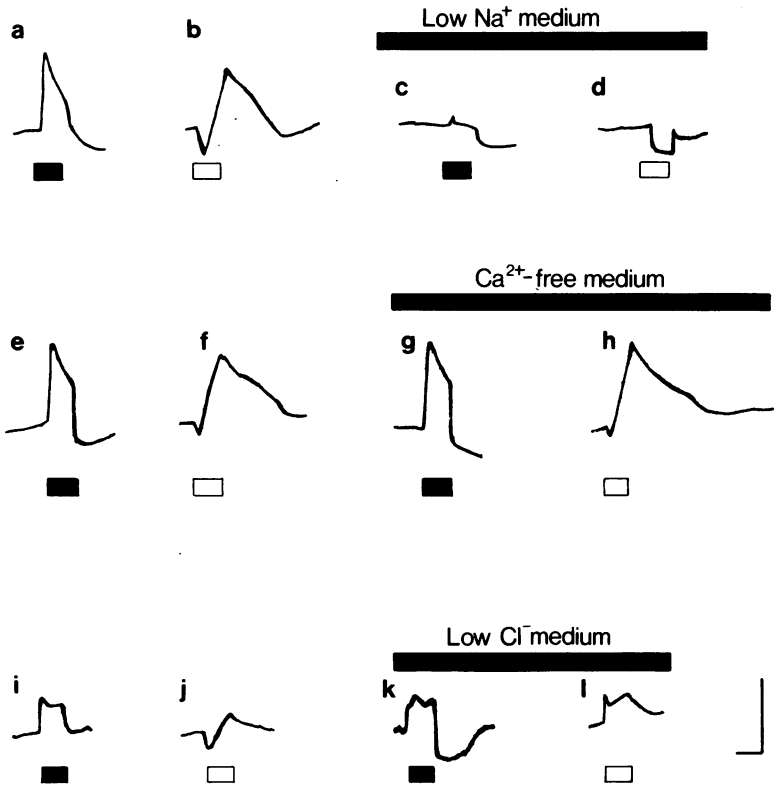

Figure 8 Effects of ionic replacement on γ-aminobutyric acid (GABA) (filled bars) or piperazine (open bars) evoked dorsal root responses of tetrodotoxin (TTX)-treated cords. (a and b) control responses to GABA (2.5 mM) and piperazine (2 mM); (c and d) responses to the same doses after 60 min in low Na^+ medium (90% substitution made with equimolar Li^+); (e and f) recovery 60 min later; (g and h) responses to the same doses after 30 min in Ca^{2+}-free medium (substitution made with equimolar Mg^{2+}); (i and j) control responses to GABA (3 mM) and piperazine (2 mM); (k and l) responses to the same doses after 30 min in low Cl^- medium (90% substitution made with equimolar isethionate). Calibration bars for all responses: 1 mV; 1 min. Note the biphasic effect of piperazine in (b) and (j).

In the case of GABA at least, the low value of this slope might depend on the rapid desensitization and thus not express the actual number of binding molecules. The Na^+ dependence of the action of GABA on the dorsal root has already been described (Barker & Nicoll, 1973) and found also in our study. Nishi *et al.* (1974) were unable to find a Na^+ dependence and suggested that Cl^- was the ion involved. In the present report a blockade of the effect of GABA was seen in a low Na^+ but not in a low Cl^- medium. Therefore, although extracellular recordings cannot provide conclusive evidence about the ionic species involved in a depolarization, the effect of GABA on the dorsal roots appeared to be at least partly dependent on external Na^+. In the case of piperazine, Cl^- appeared to mediate the hyperpolarizing component and Na^+ the depolarizing one.

Different characteristics of GABA responses on lobster muscle and in the frog spinal cord

It is important to emphasize the difference between the effects of GABA on the lobster muscle and the frog cord. The differences in membrane potential response and ionic dependency in the two tissues suggest a difference in the nature of the ionophore involved in the GABA-evoked permeability change. The results obtained with piperazine indicate that it is behaving like a GABA agonist on the lobster muscle but has only weak GABA-mimetic activity on the frog spinal cord, where it is likely to act mostly via interneuronal pathways. A possible extrasynaptic action of piperazine on both lobster muscle and frog dorsal root terminals cannot, of course, be excluded. Since piperazine only mimics the action of GABA on lobster

muscle, it is tempting to suggest that the GABA receptor binding site(s) in the lobster and in the frog are different. However, this interpretation should only be considered as tentative. If a potent competitive GABA antagonist existed, then a comparison of pA_x values (Schild, 1947) using GABA and piperazine as agonists would be of great value in clarifying this matter. Unfortunately, no sufficiently potent competitive antagonist of GABA on these preparations is yet available and the classical irreversible antagonist of monoamine receptors and cholinoceptors, dibenamine (Furchgott, 1966) proved to be ineffective in suppressing the responses to GABA in both preparations.

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