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# BICUCULLINE/BACLOFEN-INSENSITIVE GABA RESPONSE IN CRUSTACEAN NEURONES IN CULTURE

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## Summary

Neurones were dissociated from thoracic ganglia of embryonic and adult lobsters and kept in primary culture. When γ-aminobutyric acid (GABA) was applied by pressure ejection, depolarizing or hyperpolarizing responses were produced, depending on the membrane potential. They were accompanied by an increase in membrane conductance. When they were present, action potential firing was inhibited.

The pharmacological profile and ionic mechanism of GABA-evoked current were investigated under voltage-clamp with the whole-cell patch-clamp technique. The reversal potential of GABA-evoked current depended on the intracellular and extracellular $Cl^-$ concentration but not on extracellular $Na^+$ and $K^+$. Blockade of $Ca^{2+}$ channels by $Mn^{2+}$ was also without effect. The GABA-evoked current was mimicked by application of the $GABA_A$ agonists muscimol and isoguvacine with an order of potency muscimol>GABA>isoguvacine. *cis*-4-aminocrotonic acid (CACA), a folded and conformationally restricted GABA analogue, supposed to be diagnostic for the vertebrate $GABA_C$ receptor, also induced a bicuculline-resistant chloride current, although with a potency about 10 times lower than that of GABA. The GABA-evoked current was largely blocked by picrotoxin, but was insensitive to the $GABA_A$ antagonists bicuculline, bicuculline methiodide and SR 95531 at concentrations of up to 100 $\mu mol\,l^{-1}$. Diazepam and phenobarbital did not exert modulatory effects. The $GABA_B$ antagonist phaclophen did not affect the GABA-induced current, while the $GABA_B$ agonists baclophen and 3-aminopropylphosphonic acid (3-APA) never evoked any response.

Our results suggest that lobster thoracic neurones in culture express a chloride-conducting GABA-receptor channel which conforms to neither the $GABA_A$ nor the $GABA_B$ types of vertebrates but shows a pharmacology close to that of the novel $GABA_C$ receptor described in the vertebrate retina.

## Introduction

The first experiments demonstrating the inhibitory action of γ-aminobutyric acid (GABA) were performed on invertebrate neurones. Florey (1954) showed that a substance extracted from mammalian brain (Factor I), now identified as GABA (Bazemore *et al.* 1956), had an

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inhibitory effect on the stretch receptor neurone in the crayfish *Astacus astacus*. GABA was also demonstrated to be active on the isolated cardiac ganglion of the horseshoe crab *Limulus polyphemus* (Burgen and Kuffler, 1957). It is now generally accepted that GABA functions as an inhibitory transmitter in the peripheral and central nervous system of invertebrates (Robinson and Olsen, 1988). In vertebrates, GABA is thought to be the predominant inhibitory transmitter in the central nervous system, where two well-characterized classes of GABA receptors can be distinguished. $GABA_A$ receptors are ligand-gated $Cl^-$ channels that are antagonized by bicuculline and picrotoxin and are allosterically modulated by barbiturates and benzodiazepines (reviewed by Johnston, 1986). In contrast, $GABA_B$ receptors regulate $K^+$ and $Ca^{2+}$ channels through GTP-binding proteins and intracellular messenger pathways (reviewed by Bowery *et al.* 1991). It is becoming clear that, in invertebrates, GABA receptors present similarities in pharmacological profile with the mammalian ones without fitting precisely into the classification developed for mammalian brain. Invertebrates have specific binding sites similar to the GABA/muscimol sites of mammalian $GABA_A$ receptors, but most of them differ from those of vertebrates by a markedly reduced sensitivity to bicuculline and by a distinctly different benzodiazepine pharmacology and a weaker affinity binding to picrotoxin (Lunt, 1991).

Recent observations indicate that the vertebrate retina contains a new type of GABA receptor (Feigenspan *et al.* 1993; Qian and Dowling, 1993), with unusual pharmacological properties, as proposed earlier for $GABA_C$ receptors (Johnston *et al.* 1975; Drew *et al.* 1984). These receptors are activated by an analogue of GABA in a folded conformation, *cis*-4-aminocrotonic acid (CACA), but are insensitive to the $GABA_A$ antagonist bicuculline and to the $GABA_B$ agonist baclofen. Moreover, the GABA response in these receptors is not modulated by barbiturates and benzodiazepines (Feigenspan *et al.* 1993; Qian and Dowling, 1993). Here we report that GABA receptors in lobster thoracic neurones in culture have a pharmacological profile that resembles that of the $GABA_C$ receptor in the vertebrate retina. Part of this work has been published in the form of abstracts (Jackel *et al.* 1993).

## Materials and methods

### *Animals*

Egg-bearing female lobsters (*Homarus gammarus*) were obtained from commercial suppliers (Aiguillon-Marée, Arcachon) and maintained in laboratory tanks with circulating sea water at a temperature of 13 °C and a 12 h:12 h day:night cycle. Electrophysiological studies were performed on isolated neurones from adult lobsters of both sexes and from embryos at 60–100 % of development. Lobster embryos were staged by measuring the size of the pigmented area of the eye with an ocular micrometer (Perkins, 1972).

### *Cell culture*

Embryos were removed from the egg and pinned to the bottom of a Sylgard-lined dish. Muscles surrounding the nerve cord were removed and the five thoracic ganglia dissected. Thoracic ganglia from adult lobster were desheathed prior to enzyme treatment. All following steps were identical for the two preparations.

The method we used to culture neurones from marine crustaceans was adapted from the method originally developed for crayfish (Krenz *et al.* 1990). Briefly, thoracic ganglia were incubated for 1–2 h in either collagenase/dispase (Boehringer Mannheim) or subtilisin (Sigma), both 2 mg $ml^{-1}$ (collagenase/dispase in $Ca^{2+}$/$Mg^{2+}$-free medium). The enzyme was removed by rinsing in sterile medium containing 10 % foetal calf serum and gentamycin (50 $\mu$g $ml^{-1}$, Sigma) and streptomycin (100 $\mu$g $ml^{-1}$, Sigma). The following steps were carried out under sterile conditions in a laminar flow hood and with sterile-filtered solutions. Ganglia were washed again by transferring them through three volumes of medium. In the final culture dish, the cells were removed from the ganglia by gentle suction through fire-polished micropipettes. Culture dishes were coated with concanavalin A (500 $\mu$g $ml^{-1}$, Sigma) in order to facilitate cell adhesion. The medium contained equal parts of Leibovitz's L15 medium and double-strength saline. The final composition of the salts in the culture medium was (in mmol $l^{-1}$): 490.55 NaCl, 15.42 KCl, 14.3 $CaCl_2$, 10.41 $MgSO_4$, 0.49 $MgCl_2$, 0.67 $Na_2HPO_4$, 0.22 $KH_2PO_4$, 3.91 $Na_2SO_4$, 10.0 Hepes. The pH was adjusted to 7.45 with NaOH and osmolality was 1042 mosmol $l^{-1}$.

After 24 h, the culture medium was replaced with medium containing 10 % foetal calf serum. The cultures were stored for 3–5 days before experimentation. Under these conditions many neurones survived and grew processes (Fig. 1).

### *Solutions*

The solutions used for electrophysiological experiments were made up as follows. Standard external solution contained (in mmol $l^{-1}$): 521 NaCl, 12.74 KCl, 13.67 $CaCl_2$,

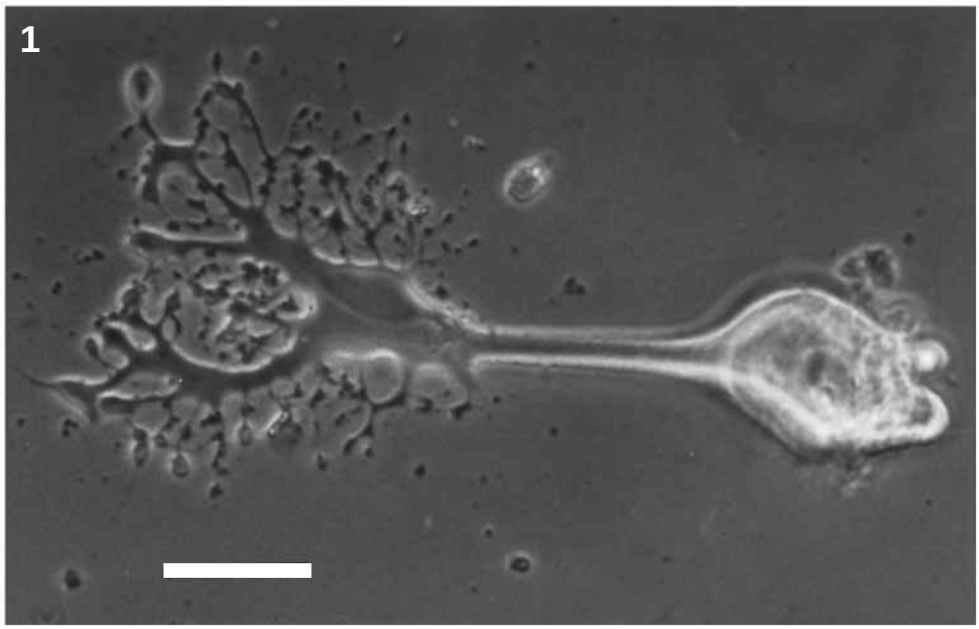


Fig. 1. Isolated neurone from thoracic ganglion of an adult lobster after 3 days in culture. Scale bar, 50 $\mu$m.

10 $MgSO_4$, 3.91 $Na_2SO_4$, 5 Hepes (pH adjusted to 7.45 with HCl). Standard internal solution contained (in mmol $l^{-1}$): 48.37 NaCl, 20.63 KCl, 139.68 $K_2SO_4$, 0.5 $CaCl_2$, 5 $MgCl_2$, 5 sodium ATP, 5 maleic acid, 11 Tris base, 5 EGTA, 550 D-mannitol (pH adjusted to 7.45 with NaOH). In some experiments this medium was supplemented with 0.2 mmol $l^{-1}$ sodium GTP.

The intracellular solution for high $Cl^-$ contained (in mmol $l^{-1}$): 48.37 NaCl, 300 KCl, 0.5 $CaCl_2$, 5 $MgCl_2$, 5 NaATP, 5 maleic acid, 11 Tris base, 5 EGTA, 430 D-mannitol (pH adjusted to 7.45 with KOH). For low $Cl^-$, the composition of the intracellular solution was (in mmol $l^{-1}$): 8.37 NaCl, 40 sodium isethionate, 20.63 KCl, 139.68 $K_2SO_4$, 0.5 $CaCl_2$, 5 $MgCl_2$, 5 sodium ATP, 5 maleic acid, 11 Tris base, 5 EGTA, 550 D-mannitol (pH adjusted to 7.45 with KOH).

### *Electrical recordings*

Neurones were investigated under current- and voltage-clamp using the patch-clamp technique in the whole-cell configuration (Hamill *et al.* 1981). Patch pipettes were pulled from borosilicate glass capillaries with filament (GC150TF-10, Clark Electromedical Instruments) on a vertical pipette puller (L/M-3P-A, List Medical). Fire-polished pipettes were filled with standard intracellular solution (described above) and had resistances of 5–8 MΩ. High-resistance seals formed easily when gentle suction was applied after touching the cell membrane. Rupture of the patch for whole-cell recording was obtained either by applying a brief pulse of suction or a brief pulse of current.

Experiments were performed with a patch-clamp amplifier (Axopatch 1D, Axon Instruments). Cells were clamped at a holding potential of −50 mV, unless otherwise stated. Series resistance values were obtained for each experiment from the patch-clamp amplifier settings after compensation and varied between 6 and 20 MΩ. Stimulations and data acquisition were performed with the pClamp software package (Axon Instruments, USA, version 5.5.1) running on a 20 MHz 386 microcomputer (Packard-Bell, 320SX) equipped with a Labmaster DMA acquisition system (TL-1 interface, Axon Instruments). Data were stored on the computer hard disk and processed later with data analysis programs of the pClamp program package. Curves were fitted using the Sigmaplot software (Jandel Scientific, version 5.0).

In order to determine liquid junction potentials in the presence of different external solutions, the pipette tip (containing the internal standard solution) was first placed into a bath filled with standard external solution and the junction potential between the pipette and the bath was compensated to zero. The external solution was then replaced by modified extracellular solutions and the deviation for each solution was measured. All present data were corrected for junction potentials, which varied between 1 and 4 mV. Results are presented as individual values or means ± standard error (S.E.M.), unless otherwise stated in the text. All experiments were performed in standard extracellular solution (unless otherwise noted) at a temperature of about 17 °C.

### *Drugs*

Drugs used in the present experiments were γ-aminobutyric acid (GABA), muscimol, 3-aminopropylphosphonic acid (3-APA), bicuculline and bicuculline methiodide, all

purchased from Sigma. Phaclofen, (−)-baclofen-(b-p-chlorophenyl)GABA (baclofen), 2-(3-carboxypropyl)-3-amino-6-(4-methoxy)-phenyl pyridazinium bromide (SR 95531) and isoguvacine–HCl were purchased from RBI. *cis*-4-aminocrotonic acid (CACA) was purchased from Tocris Neuramin, Bristol, UK. Diazepam and phenobarbital were a gift from the Cooperation Pharmaceutique Française. All drugs were either dissolved in the external solution or, when insoluble in saline, first dissolved in methanol at a concentration of 10 mmol $l^{-1}$ and further diluted in saline to their final concentration. Drugs were stored at −20 °C and thawed just before use, except for bicuculline which was prepared fresh.

### *Application of drugs*

Application of drugs was performed by pressure ejection (Picospritzer II, General Valve Corp., USA) from glass pipettes pulled on the vertical pipette puller. In order to minimize desensitization of the GABA receptor during repetitive application, drugs were applied at 90 s intervals. Comparisons between agonists were undertaken with equivalent pipettes the first of which contained GABA and the second muscimol, isoguvacine or CACA. Both pipettes were positioned at a distance of 40–70 $\mu$m from the cell body. Drugs were applied at a concentration of 1 mmol $l^{-1}$ with brief pulses of pressure (68.94 kPa) of increasing duration. Several experiments with Fast Green added to the pipette solution showed that the ejected bolus of drug did mix with the stream of saline before hitting the cell. For studies of the effect of antagonists and modulators on the GABA-induced current we used both bath application and pressure ejection (see Table 1). For pressure ejection we used the same pipette configuration as described above. The antagonists were applied for 30 s, followed immediately by a brief pulse of GABA (50 ms).

## Results

### *GABA-induced responses*

Under current-clamp conditions, application of GABA suppressed the firing of action potentials (Fig. 2A). In this experiment, the cell fired overshooting action potentials at a membrane potential of −33 mV. Each action potential (AP) is followed by an after-hyperpolarization potential (AHP) of 12 mV. Application of GABA at a concentration of 1 mmol $l^{-1}$ for 50 ms (Fig. 2A, arrow) slightly hyperpolarized the cell and transiently inhibited firing.

The response to GABA was then analyzed under voltage-clamp conditions. Fig. 2B shows the response of a cell held at a potential of −60 mV, a potential at which GABA evoked a large transient depolarization in unclamped neurones. Applying GABA at a concentration of 1 mmol $l^{-1}$ evoked an inward current of 250 pA. Fig. 2C shows the same cell as in Fig. 2B but, in addition to the holding potential of −60 mV, hyperpolarizing pulses of −20 mV were applied repetitively in order to monitor variations in membrane conductance. Pressure ejection of GABA increased the amplitude of the current, indicating that the GABA response is associated with an increase in membrane conductance.

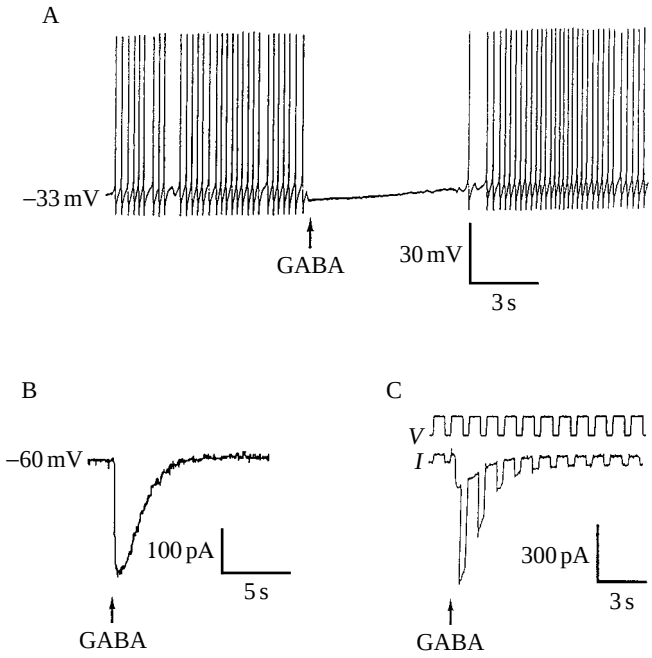


Fig. 2. GABA-evoked responses. (A) Current-clamp recording from an isolated neurone which fired action potentials after 3 days in culture. Application of GABA (arrow, 1 mmol $l^{-1}$ in the pipette) for 50 ms slightly hyperpolarized the cell and inhibited the generation of action potentials. (B) Under voltage-clamp, GABA induced an inward current in a cell held at a membrane potential of −60 mV ($E_{Cl}$=−49 mV). (C) From a holding potential of −60 mV, repetitive voltage pulses of −20 mV revealed an increase in membrane conductance after GABA application.

### *Ionic mechanism of the GABA response*

#### *Equilibrium potential*

In order to determine the reversal potential for the GABA-induced current, GABA was applied in 50 ms pulses at various holding potentials. Fig. 3A shows the original recordings from one such experiment. In Fig. 3B the peak currents are plotted against the holding potentials. The solid line is the linear regression through the data points. The *I–V* relationship is linear within the range of voltages applied. In this cell, the GABA response reversed at a membrane potential of −46 mV. The same type of experiment was repeated in 23 other cells from embryos and in five cells from adults. Mean reversal potential values were −42.7±4.9 mV for embryonic cells and −49.6±1.8 mV for adult ones (no significant difference; Student's *t*-test, $P$>0.05). Both values are close to the equilibrium potential for $Cl^-$ calculated from the Nernst equation ($E_{Cl}$=−49 mV). The observations that GABA produced membrane responses that inverted at a potential close to the equilibrium potential for $Cl^-$ and that it induced an increase in membrane conductance suggest that opening of $Cl^-$ channels underlies the GABA-induced response. To test this hypothesis, we modified the intracellular and extracellular concentrations of $Cl^-$.

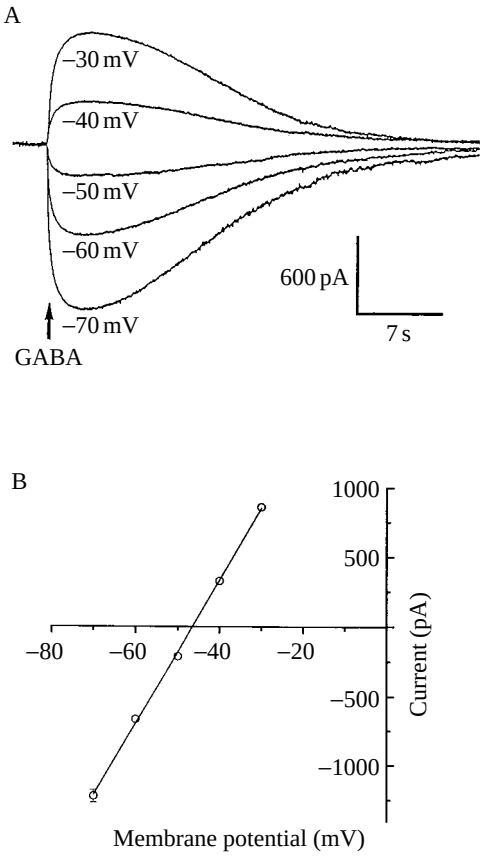


Fig. 3. Reversal potential of the GABA-induced current. (A) Responses to GABA (1 mmol $l^{-1}$ in the pipette) when applied for 50 ms at the indicated holding potentials. (B) Plot of the peak current against different holding potentials. Each point is the mean ± S.E.M. of two GABA applications; error bars are omitted when smaller than symbols. Regression through data points yields a reversal potential of −46 mV, which coincides well with the imposed $Cl^-$ equilibrium (−49 mV).

*Effects of altering intracellular chloride concentration*

Both low and high intracellular chloride solutions were used. In the first case, the normal $Cl^-$ concentration was decreased by 50 % by replacement with sodium isethionate to obtain a final $Cl^-$ concentration of 40 mmol $l^{-1}$ (see Materials and methods). This shifted the equilibrium potential for $Cl^-$ to −66 mV according to the Nernst equation. For the high-chloride solution, the intracellular concentration of $Cl^-$ was modified by increasing $[Cl^-]$ to 359 mmol $l^{-1}$ with KCl (see Materials and methods) thus shifting the equilibrium potential for $Cl^-$ to −11 mV.

The recordings in Fig. 4Ai show GABA-evoked currents at different voltages when the imposed equilibrium potential for $Cl^-$ was −66 mV, −49 mV and −11 mV. The interpolated reversal potentials are indicated by the arrows. Fig. 4Aii illustrates the current–voltage relationships obtained from the above data. The *I–V* relationships

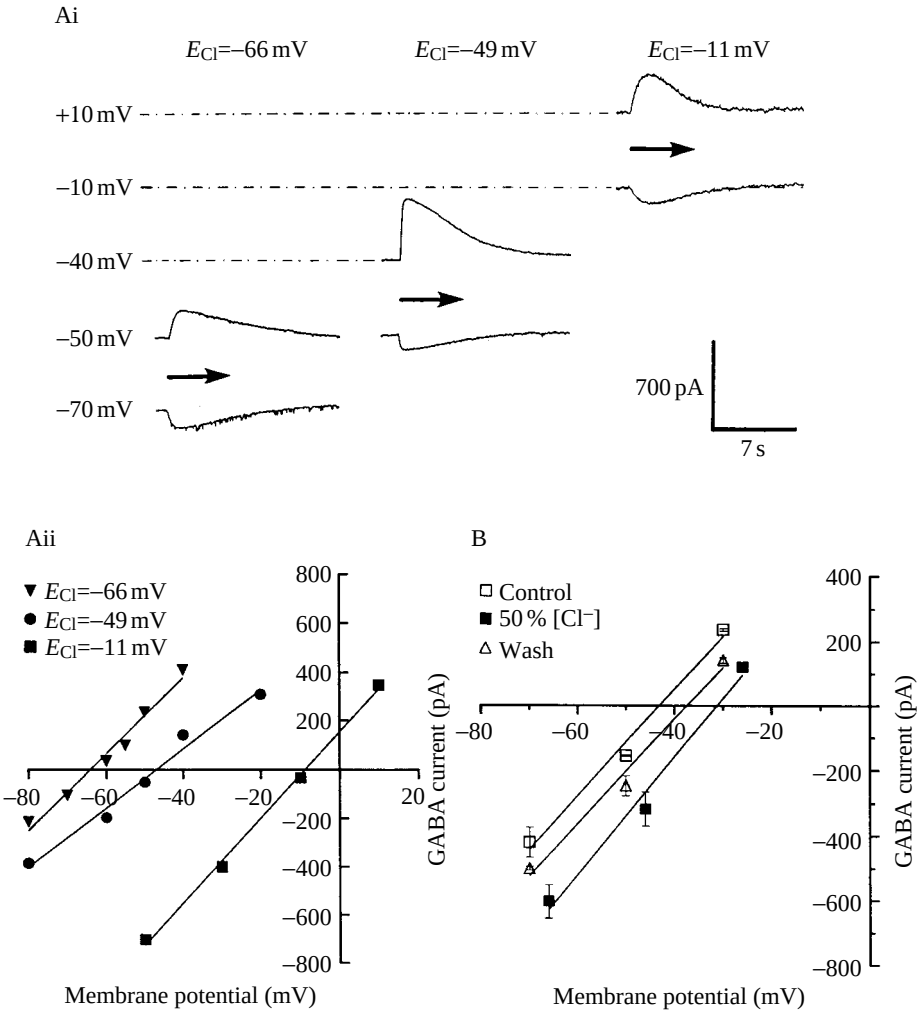


Fig. 4. Dependence of the GABA-induced current on chloride. (A) Dependence on intracellular chloride concentration. (Ai) GABA-evoked currents at different voltages when the imposed equilibrium potential for $Cl^-$ was −66 mV (left-hand panel), −49 mV (middle panel) and −11 mV (right-hand panel). Arrows indicate reversal potentials for the GABA responses. (Aii) Current–voltage relationships obtained from experiments in Ai. The solid lines are linear regressions through the data points. In normal saline (circles) the response reversed at −48 mV, in high $[Cl^-]_i$ (squares) at −11 mV, and in low $[Cl^-]_i$ (triangles) at −63 mV. These values are close to those calculated from the Nernst equation. (B) Dependence on extracellular chloride concentration. *I–V* curve for control conditions ($[Cl^-]_o$=566 mmol $l^{-1}$, open squares), for decreased $[Cl^-]_o$ (283 mmol $l^{-1}$, filled squares) and on return to normal solution (open triangles). NaCl was replaced by sodium methanesulphonic acid. Data points are the mean values ± S.E.M. of two GABA applications. The reversal potential of the GABA-induced current was −42.6 mV in control saline and was shifted to −31.1 mV in 50 % chloride saline.

remained linear over the range of membrane potentials tested and were only shifted along the voltage axes in parallel with the modified intracellular $Cl^-$ concentrations. In these experiments, the GABA current reversed at −48 mV under control conditions (circles). The reversal potential was −11 mV for high intracellular [$Cl^-$] (squares) and −63 mV for low intracellular [$Cl^-$] (triangles). Mean values for embryonic cells determined from five experiments of this kind gave reversal potentials of −59±1.33 mV for low-chloride and −6.1±2.2 mV for high-chloride solutions. These values coincide well with the $Cl^-$ equilibrium potentials calculated from the Nernst equation. This result confirms the involvement of $Cl^-$ in the GABA response.

*Effects of altering extracellular chloride concentration*

In order to test whether the equilibrium potential of the GABA response in embryonic cells was also affected by changing the extracellular concentration of $Cl^-$, alterations were made with the appropriate substitutions of NaCl by sodium methanesulphonic acid in the superfusion solution. Fig. 4B demonstrates the effect of substitution of 50 % of the $Cl^-$. The lines drawn through data are linear regressions for each condition. Data points are mean values ± S.E.M. of two GABA applications. Again, the *I–V* relationships are linear and are shifted along the voltage axes with modified extracellular $Cl^-$ concentration. Under control conditions, the GABA-evoked current reversed at −42.6 mV in this cell ($E_{Cl}$=−49 mV). In the modified external solution, the reversal potential for the GABA-induced current in the experiment shown in Fig. 4B was shifted to −31.1 mV ($\Delta V$=−11.5 mV). In most experiments, however, the change in reversal potential was only 9.76±0.47 mV (*N*=4). This value represents only half the change in reversal potential predicted by the Nernst equation (−18 mV).

*Effects of altering extracellular cation concentrations*

Since ionotropic cation-selective GABA receptors have been described in crustaceans (see Discussion), we wished to assess whether other ions might also be involved in the GABA response studied here. Therefore, we measured the response of embryonic cells to GABA when the extracellular concentrations of $Na^+$, $K^+$ and $Ca^{2+}$ were modified.

In 50 % of the cells tested (*N*=10), the reversal potential of the GABA current was not displaced when the extracellular $K^+$ concentration was either increased 2.5-fold (K×2.5) or decreased to half of the standard concentration (K/2). An example of one such response is shown in Fig. 5A. When the peak values were plotted against the different membrane potentials, a linear relationship was observed. The reversal potential of the GABA-induced current in this cell was −41 mV for all three conditions. Mean values of five experiments where cells responded in the same way to GABA applications were −40.86±0.29 mV for controls, −40.6±0.29 mV for increased [$K^+$] (29.84 mmol $l^{-1}$) and −42.05±0.55 mV for decreased [$K^+$] (6.37 mmol $l^{-1}$). In the other 50 % of the cells, we observed a slight change of the reversal potential of the GABA-evoked current (data not shown). In these cells, the GABA current reversed at a membrane potential of −46.2±2.2 mV under control conditions. In the presence of saline containing twice the normal $K^+$ concentration, the reversal potential was found to be −40.3±1.6 mV.

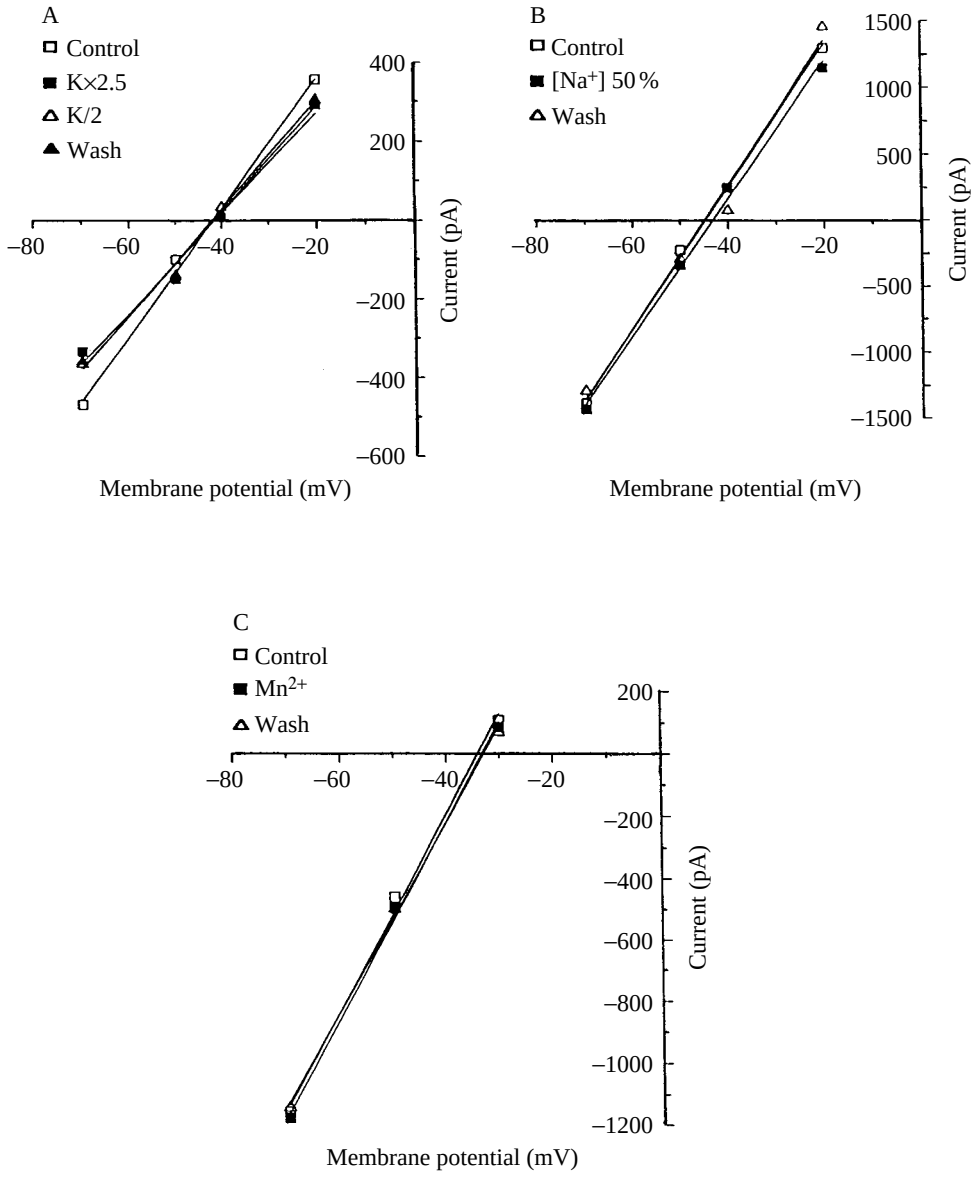


Fig. 5. Insensitivity of the GABA response to alterations of extracellular cation concentrations. (A) *I–V* curve for GABA-induced current in control saline (open squares), in K×2.5 (filled squares), in K/2 (open triangles) and after washout (filled triangles). For all conditions, the reversal potential was found to be −41 mV. (B) Current–voltage plot for GABA-evoked current under control conditions (open squares), in saline containing 50 % $Na^+$ (filled squares) and after washout (open triangles). $Na^+$ was replaced by glucamine. Substitution of $Na^+$ did not affect the GABA-induced current. (C) *I–V* curve for GABA-induced current in control conditions (open squares), in solution containing 5 mmol $l^{-1}$ $Mn^{2+}$ (filled squares) and on return to normal solution (open triangles). In this cell, the GABA current reversed at a potential of −37 mV and was unaffected by saline containing $Mn^{2+}$. A, B and C are from different cells; solid lines were obtained from linear regressions through data points.

Superfusion of the cells with K/2 shifted the reversal potential of the GABA current to −52.4±3.4 mV. These values represent changes of only one-third to one-quarter of those predicted by the Nernst equation for $K^+$, namely 5.9 mV instead of 23 mV for K×2.5 and 6.2 mV instead of 18 mV for K/2. In these experiments the GABA current always reversed at a membrane potential closer to the chloride equilibrium potential (−49 mV) than to the $K^+$ equilibrium potential ($E_{Kcontrol}$=−79 mV, $E_{K\times2.5}$=−56 mV, $E_{K/2}$=−97 mV). These findings further suggest that the GABA current is not carried by $K^+$.

Fig. 5B shows the results when the extracellular $Na^+$ concentration was modified. GABA was applied at various membrane potentials and the peak current was measured under control conditions and when 50 % of the $Na^+$ was replaced by glucamine. Solid lines were obtained from linear regressions through the data points. In all tested cells (*N*=5) the equilibrium potential of the GABA response was not affected by $Na^+$ replacement. Furthermore, the slopes of the solid lines did not change, showing that the amplitude of the response for all membrane potentials was not modified. These results led us to conclude that $Na^+$ is not involved in the GABA-induced current.

In order to determine whether the GABA response was partially dependent upon $Ca^{2+}$, the potent calcium-channel blockers $Mn^{2+}$ and $Cd^{2+}$ were added to the external saline at a concentration of 5 mmol $l^{-1}$. Fig. 5C illustrates one experiment where the extracellular solution contained $Mn^{2+}$. In this cell, the reversal potential of the GABA-induced current was at −37 mV (open squares) and was unaffected by the presence of $Mn^{2+}$ (filled squares). Furthermore, the amplitude of the GABA current was the same for controls and for saline containing $Mn^{2+}$. $Cd^{2+}$ was also without effect on the GABA current (*N*=5, data not shown). From the above results, it can be concluded that the current induced by GABA is not mediated by $Na^+$ or $Ca^{2+}$, but that $Cl^-$ is the main carrier of the GABA-induced current. We cannot, however, exclude some involvement of $K^+$ in the GABA response but, if this is so, its influence is small.

Further evidence that the GABA current is mediated by chloride ions was obtained from experiments with picrotoxin (PTX). PTX is thought to block the $Cl^-$ ionophore either directly or following binding to a closely located site in the vertebrate $GABA_A$ receptor (Barker *et al.* 1983). Fig. 6A shows the effect of PTX on a single cell clamped at a potential of −60 mV. As can be seen, PTX reduced the amplitude of the GABA-induced current by about 60 %. This effect was only partially reversible, even following prolonged superfusion with control saline. Fig. 6B illustrates the current–voltage relationship for the same cell in control saline and in saline containing PTX. The reduction of the GABA current by PTX was proportional at all membrane potentials, so that a linear relationship was maintained. That the blockade is independent of voltage is further illustrated in Fig. 6C, which summarizes the results from five experiments performed on embryonic cells. Normalized currents remaining after application of PTX are represented as a percentage of the control value, recorded at three different membrane potentials. The percentage of blockade is almost identical at the three potential values. Mean values of PTX block were 73.8±7.3 % for embryonic (*N*=5) and 64.5±2.3 % for adult cells (*N*=3). This difference is not statistically significant (Student's *t*-test, $P>0.05$). Higher doses of PTX did not increase the percentage of blockade. 50 $\mu$mol $l^{-1}$ PTX

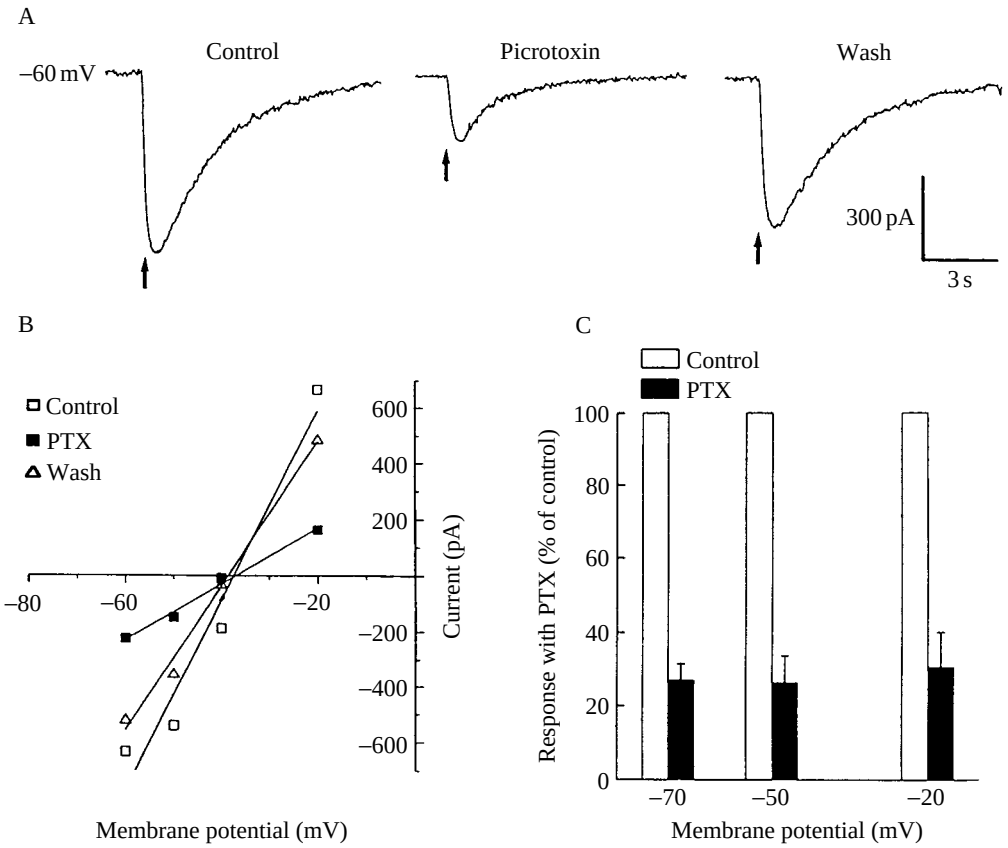


Fig. 6. Blockade of the GABA-induced current by picrotoxin (PTX). (A) Whole-cell recordings of current evoked by 50 ms application of GABA (arrow, 1 mmol $l^{-1}$ in the pipette) under control conditions (left), in the presence of 10 $\mu$mol $l^{-1}$ picrotoxin (middle) and after washout (right), at a holding potential of −60 mV ($E_{Cl}$=−49 mV). In the presence of PTX, the GABA current is reduced by about 60 %. (B) Current–voltage plot for the same cell in control saline (open squares) and in saline containing 10 $\mu$mol $l^{-1}$ PTX (filled squares). The PTX effect is largely reversible (open triangles). (C) GABA-induced current (open bars) compared with the remaining current after PTX treatment (filled bars) at three different membrane potentials. Currents are normalized. Each bar represents mean values + S.E.M. of five cells.

produced 59.3±3.6 % of blockade in three adult cells. The difference between the effects of 10 and 50 $\mu$mol $l^{-1}$ PTX is not statistically significant (Student's *t*-test, $P$>0.05).

### *The pharmacological profile of the thoracic GABA receptor*

#### *Effects of $GABA_A$ agonists and antagonists*

Having established that GABA induces a membrane current carried mainly by chloride ions in *Homarus gammarus* thoracic neurones in culture, we attempted to determine the pharmacological properties of the GABA receptor activating the chloride conductance. The results are summarized in Table 1.

Table 1. *Actions of GABA agonists and antagonists on lobster neurones in culture*

| Compound | Concentration | Relative current (%) | Percentage block | Number of cells |
|---|---|---|---|---|
| Specific $GABA_A$ agonists | | | | |
| GABA | 1 mmol $l^{-1}$* | 100 | | 10 |
| Muscimol | 1 mmol $l^{-1}$* | 120±8.9 | | 5 |
| Isoguvacine | 1 mmol $l^{-1}$* | 85±5.5 | | 5 |
| Specfic $GABA_B$ agonists | | | | |
| 3-APA | 1 mmol $l^{-1}$* | NE | | 5 |
| Baclofen | 1 mmol $l^{-1}$* | NE | | 10 |
| Putatively specific $GABA_C$ agonists | | | | |
| CACA | 1 mmol $l^{-1}$* | 68±16 | | 3 |
| Specific $GABA_A$ antagonists | | | | |
| Bicuculline | 100 $\mu$mol $l^{-1}$*† | | NE | 9 |
| Bicuculline methiodide | 100 $\mu$mol $l^{-1}$*† | | NE | 5 |
| SR 95531 | 100 $\mu$mol $l^{-1}$*† | | NE | 5 |
| Picrotoxin (on GABA) | 50 $\mu$mol $l^{-1}$*† | | 73.8±7.3 | 5 |
| Picrotoxin (on CACA) | 50 $\mu$mol $l^{-1}$*† | | 68.6±9.3 | 4 |
| Specific $GABA_B$ antagonists | | | | |
| Phaclofen | 500 $\mu$mol $l^{-1}$* | | NE | 5 |
| Modulators | | | | |
| Diazepam | 10 $\mu$mol $l^{-1}$*† | NE | | 7 |
| Phenobarbital | 100 $\mu$mol $l^{-1}$* | NE | | 7 |

Concentrations in the second column represent concentrations of compounds in the pipette when applied by pressure ejection (*) and/or as final concentrations in the bath when applied by perfusion (†).

Currents in the third column are percentages relative to GABA-evoked currents in paired applications.

Values are means ± S.E.M.

NE, no effect; 3-APA, 3-aminopropylphosphonic acid; SR95531, 2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl) pyridazinium bromide.

As a first step, we tested several specific $GABA_A$ receptor agonists on embryonic and adult neurones. Muscimol and isoguvacine are known to be agonists at the $GABA_A$ receptor in vertebrates (Barker and Mathers, 1981). When applied by pressure ejection onto cells clamped at a holding potential of −60 mV, muscimol and isoguvacine induced inward currents. Fig. 7A shows the response of one embryonic cell to GABA and isoguvacine and Fig. 7B shows the response to GABA and muscimol in another cell. Note that the amplitudes of the currents were different, although the agonists were applied with identical ejection parameters. The muscimol-induced current was larger than the GABA-evoked current. Isoguvacine evoked the smallest current and the duration of the response was also shorter (see below). *I–V* curves for muscimol and isoguvacine demonstrated that the current inverted at the same potential as the GABA-induced current (data not shown).

In order to determine whether the GABA- and muscimol-evoked responses are mediated by the same receptor, both substances were applied to the same voltage-

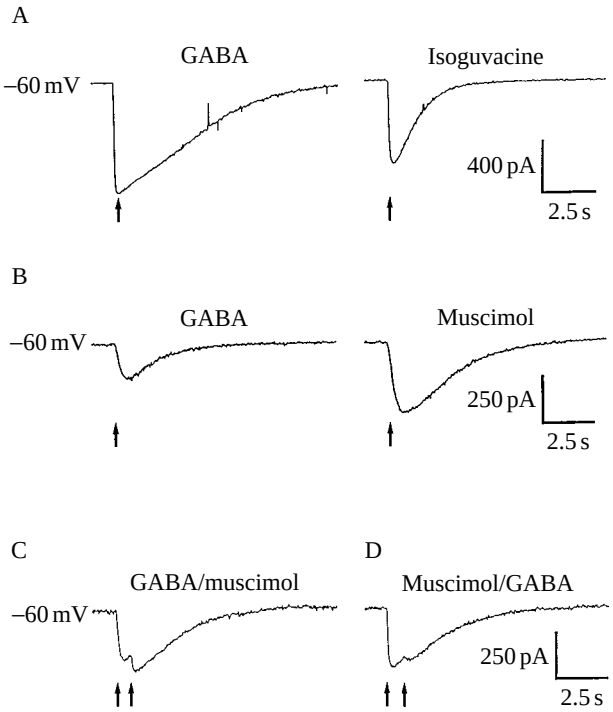


Fig. 7. Sensitivity of the receptor to the $GABA_A$ agonists muscimol and isoguvacine. Inward currents were induced by application of either 1 mmol $l^{-1}$ isoguvacine (A) or 1 mmol $l^{-1}$ muscimol (B) for 50 ms (arrow) at a holding potential of −60 mV ($E_{Cl}$=−49 mV). (C,D) When muscimol and GABA were applied successively, the evoked responses were not additive, indicating activation of the same receptor population.

clamped neurones with a short interval of 1 s. Fig. 7C shows the response of one embryonic cell when first GABA and then muscimol were applied. Fig. 7D shows the same cell when muscimol was applied first. In both cases, the amplitudes of the responses were not additive, indicating that both muscimol and GABA bound to and activated a common population of receptors.

We then studied the dose–response relationships for GABA and for some of its agonists. Fig. 8A illustrates an example of dose–response relationships for GABA and muscimol in one embryonic cell, and Fig. 8B that for GABA and isoguvacine in another neurone. The peak currents are plotted against increasing pulse duration. The solid lines are best fits to each population of data points and were calculated from the following equation:

$$I = I_{max} \times t^n/(t_{50}{}^n + t^n),$$

where $I$ is the GABA-, muscimol- or isoguvacine-activated current, $I_{max}$ is the maximal recorded current when the response saturates, $t$ is the duration of the pulse, $t_{50}$ is the pulse duration inducing 50 % of $I_{max}$, and $n$ the Hill coefficient. The amplitude of GABA-induced currents as well as responses to the agonists increased in a concentration-dependent manner. However, we did observe differences in the maximal response for

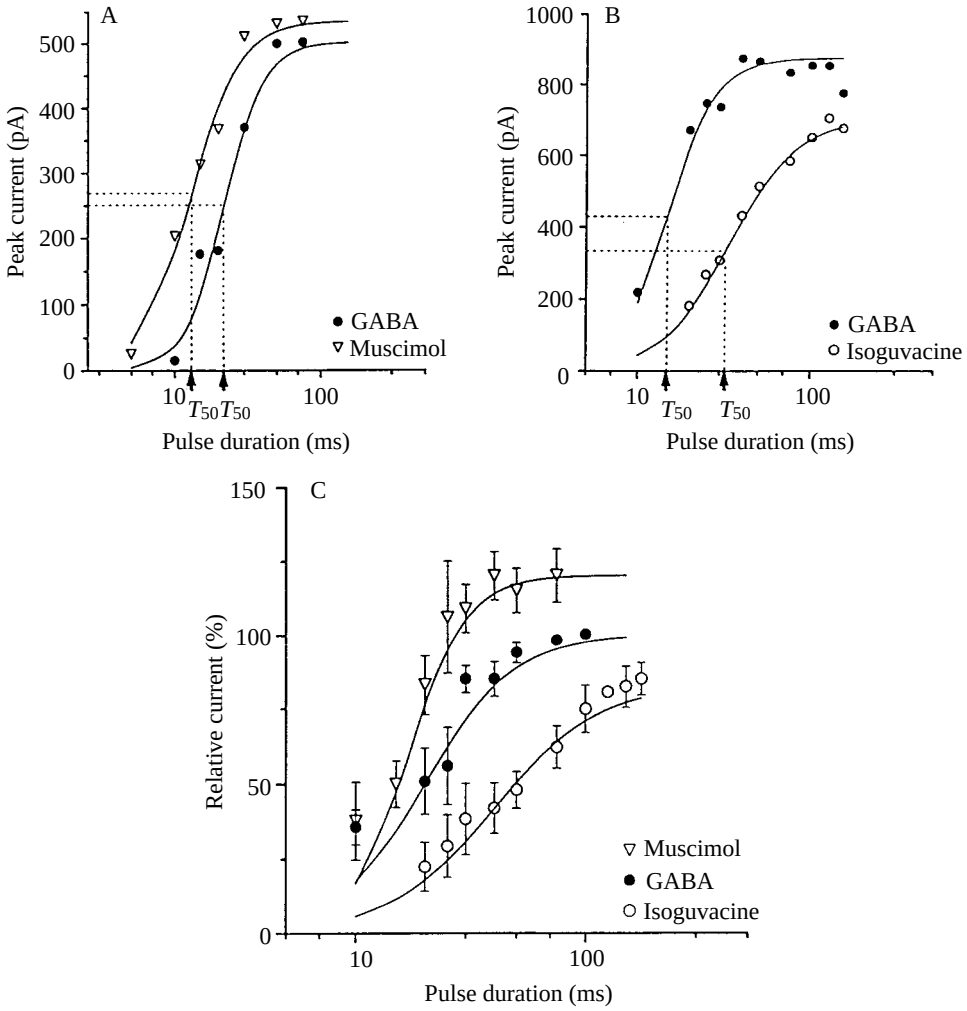


Fig. 8. Dose–response relationships for GABA-, muscimol- and isoguvacine-induced currents. (A) Dose–response curves for GABA (filled circles) and muscimol (open triangles) in the same neurone. The measured amplitude of the peak current is plotted against the duration of the ejection pulse, taken as a measure of the agonist concentration (1 mmol $l^{-1}$ in the pipette, see text). The continuous curves are Michaelis functions fitted to the data points, with maximum responses of 551 pA and 500 pA for muscimol and GABA, respectively. The $T_{50}$ values indicating the half-maximal response were 13 ms for muscimol and 22 ms for GABA. (B) Dose–response relationship from another cell for GABA (filled circles) and isoguvacine (open circles). The maximum response was 852 pA for GABA and 675 pA for isoguvacine, and the $T_{50}$ values were 32 ms for isoguvacine and 15 ms for GABA. (C) Comparison of the dose–response relationships for GABA and its agonists. The peak currents of each cell were expressed as a percentage of the maximal response to GABA and were averaged. The points are mean values ± S.E.M. with $N$=5 for muscimol, $N$=5 for isoguvacine and $N$=10 for GABA (of which five are controls for muscimol and five are controls for isoguvacine). The potency gradient was muscimol>GABA>isoguvacine.

GABA and for its agonists. This indicates that the 'intrinsic activity' for the thoracic GABA receptor of muscimol is probably greater than that of GABA, while that of isoguvacine is smaller (GABA=100 %, muscimol=120±8.97 %, isoguvacine=84.8±5.5 %). From these results, we deduced a rank order of potency of muscimol>GABA>isoguvacine. Although we did not measure complete dose–response curves for all the three substances in adult neurones, in experiments in which we tested the three agonists in parallel they revealed the same order of potency.

The graphs in Fig. 8A,B also indicate that in these two cells GABA, muscimol and isoguvacine are characterized by different $T_{50}$ values (the concentration of an agonist necessary to produce 50 % of the maximal response). Although the $T_{50}$ values are not actual concentrations, they give an indication of the affinity for the receptor (see Discussion). In the first cell, the $T_{50}$ value for muscimol-induced current ($T_{50}$=13 ms) is smaller than that for the GABA current ($T_{50}$=22 ms), indicating a higher affinity of muscimol for the receptor. The highest $T_{50}$ value was found for isoguvacine ($T_{50}$=32 ms), suggesting that its affinity for the receptor is the smallest in the cells tested. Dose–response relationships for all agonists measured in embryonic cells are summarized in Fig. 8C. The peak currents of each cell were expressed as a percentage of the maximal response to GABA and averaged. Mean values for each agonist ($N$=10 for GABA, $N$=5 for isoguvacine, $N$=5 for muscimol) are plotted as a function of pulse duration. The mean $T_{50}$ values determined from the averaged dose–response curves were 17 ms for muscimol, 20 ms for GABA and 40 ms for isoguvacine.

Although $GABA_A$ agonists activated GABA receptors in lobster thoracic neurones, the latter were completely insensitive to $GABA_A$ antagonists. Bicuculline is a specific competitive antagonist at the vertebrate $GABA_A$ receptor. We tested bicuculline as well as its water-soluble analogue bicuculline methiodide (Fig. 9A) for effects on the GABA-evoked currents at concentrations of up to 100 $\mu$mol $l^{-1}$. In 10 experiments on embryonic neurones and in six on adult neurones, the GABA-induced current was completely unaffected by these two compounds (Fig. 9A). The synthetic $GABA_A$ receptor antagonist SR 95531 was also without effect up to concentrations of 100 $\mu$mol $l^{-1}$ (Fig. 9B, $N$=5).

*Effects of modulators*

The vertebrate $GABA_A$ receptor is associated with binding sites for benzodiazepines and barbiturates, compounds which enhance the action of GABA. We tested two drugs, phenobarbital and diazepam, for modulatory effects on the GABA response in embryonic and adult neurones. Both substances were without effect: the GABA response in the presence of the modulators was not significantly different from that in control saline in embryonic ($N$=10) and adult ($N$=4) cells. Together with the insensitivity to $GABA_A$ antagonists, these findings indicate that the GABA receptors of thoracic neurones in culture differ from vertebrate $GABA_A$ receptors.

*Effects of $GABA_B$ agonists and antagonists*

We next tested whether $GABA_B$ receptors might be involved. We examined the effects of the two specific $GABA_B$ agonists, namely baclofen (Hill and Bowery, 1981) and 3-APA (both tested in embryonic and adult cells, $N$=13). In all experiments, neither agent

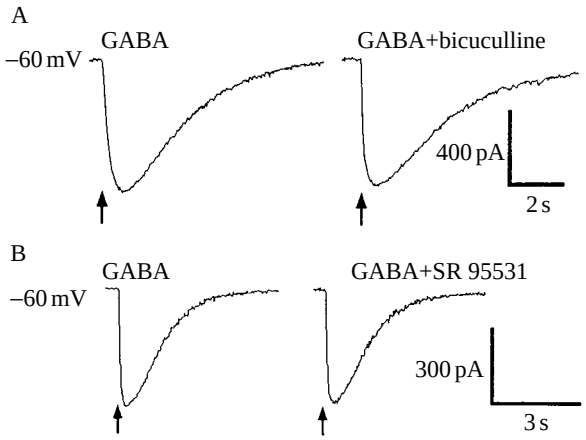


Fig. 9. Resistance of GABA-evoked current to $GABA_A$ antagonists. (A) Whole-cell recordings of current evoked by 50 ms application of GABA (arrow, 1 mmol $l^{-1}$ in the pipette) under control conditions (left-hand panel) and in the presence of 100 $\mu$mol $l^{-1}$ bicuculline (right-hand panel), at a holding potential of −60 mV ($E_{Cl}$=−49 mV). (B) Comparison of GABA-evoked current in another cell, under control conditions (left-hand panel) and in the presence of the synthetic antagonist SR 95531 (right-hand panel). The GABA response is completely unaffected by the two antagonists.

induced a response up to a concentration of 1 mmol $l^{-1}$ (tested over a holding potential range of −70 to −10 mV). Phaclofen, a highly selective antagonist of the vertebrate $GABA_B$ receptor (Kerr *et al.* 1987; Dutar and Nicoll, 1988) failed to inhibit the GABA-induced current up to a concentration of 1 mmol $l^{-1}$ ($N$=5). We thus conclude that $GABA_B$ receptors are either not expressed in lobster thoracic neurones in culture, or, if they are present, do not affect any conductance under our experimental conditions.

*Effect of* cis*-4-aminocrotonic acid (CACA)*

Insensitivity to bicuculline and baclofen, as demonstrated in our cells, is a characteristic of a third class of GABA receptors recently described in vertebrates, designated $GABA_C$ (Drew *et al.* 1984; Johnston, 1986). This type of receptor is thought to be specifically activated by folded GABA analogues such as *cis*-4-aminocrotonic acid (CACA). Thus, we examined the response of our cells to CACA.

In standard saline, CACA, like GABA, induced inward currents when applied at holding potentials more negative than −50 mV (Fig. 10A). Fig. 10B shows the reversal potentials of the CACA-induced current determined with different solutions in the patch pipette, i.e. standard solution ($E_{Cl}$=−49 mV), low-chloride ($E_{Cl}$=−11 mV) and high-chloride ($E_{Cl}$=−66 mV) internal solutions. The data points (filled circles, means ± S.E.M., $N$=3) correlate well with theoretical values for the chloride equilibrium potential calculated from the Nernst equation (dotted line). This indicates that the CACA-induced current, like the GABA response (see Fig. 4), is carried mainly by $Cl^-$. Moreover, Fig. 10C indicates that CACA and GABA act on the same population of receptors. When

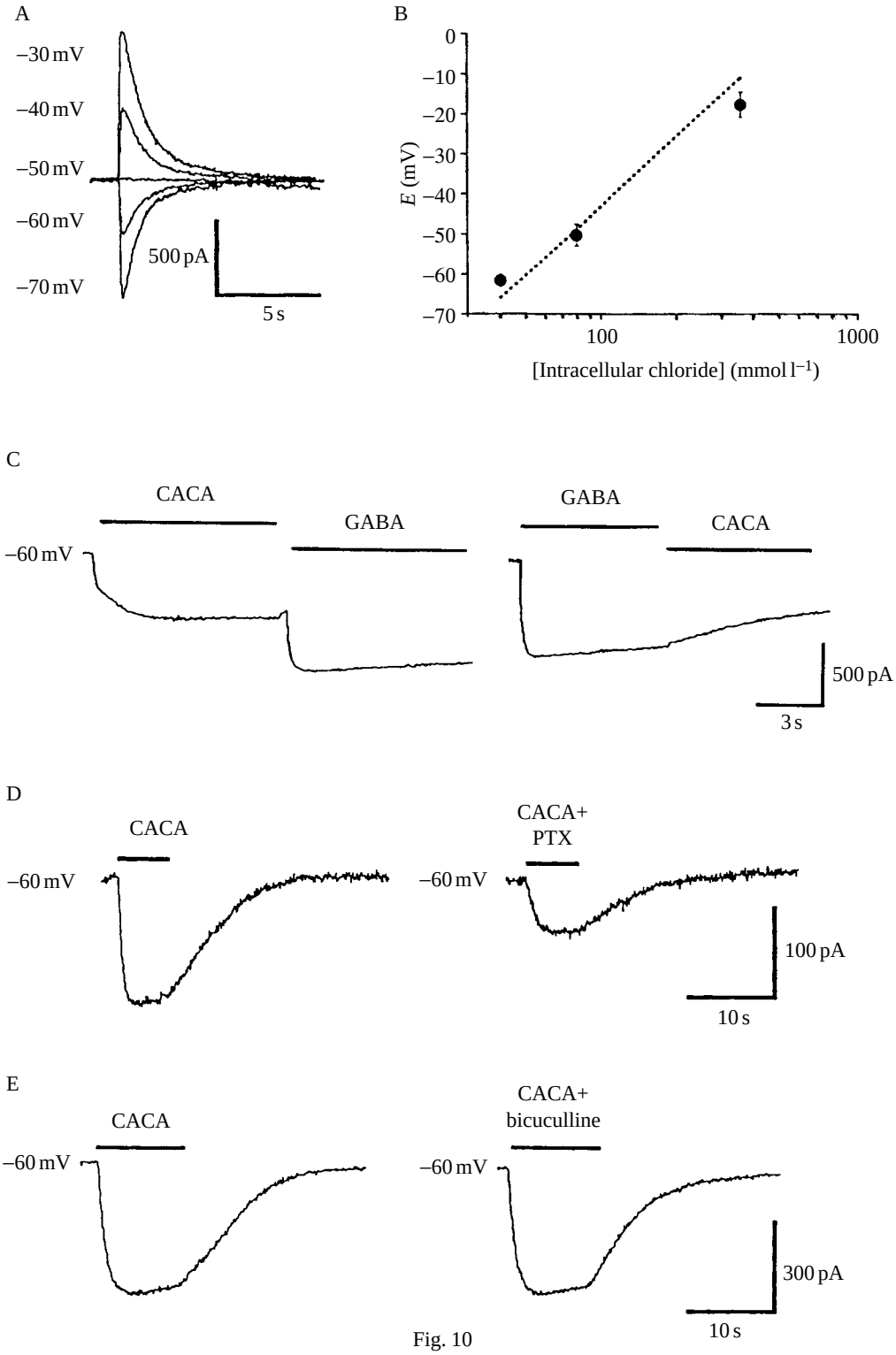
A
−30 mV
−40 mV
−50 mV
−60 mV
−70 mV
500 pA
5 s
B
0
−10
−20
−30
−40
−50
−60
−70
E (mV)
100
1000
[Intracellular chloride] (mmol l−1)
C
CACA
GABA
GABA
CACA
−60 mV
500 pA
3 s
D
CACA
CACA+
PTX
−60 mV
−60 mV
100 pA
10 s
E
CACA
CACA+
bicuculline
−60 mV
−60 mV
300 pA
10 s

Fig. 10

CACA and GABA were applied successively, the responses of the two agonists were not additive, the summated response being of the same amplitude as the response for GABA alone.

Similarities between CACA and GABA responses are further illustrated in Fig. 10D,E, which shows that, like the GABA response, the CACA-induced current is substantially decreased in the presence of PTX (50 $\mu$mol l$^{-1}$ in Fig. 10D), but completely unaffected by bicuculline (100 $\mu$mol l$^{-1}$ in Fig. 10E). In three tested cells, PTX reduced the CACA-induced current by 68.6±9.3 % (mean ± S.E.M.), while bicuculline was without effect on the CACA response (*N*=4). Finally, desensitization of the response was slow during prolonged application of CACA as well as GABA. In the experiment documented in Fig. 11A, desensitization over 30 s was only 18.6 % for the CACA-induced current and 26 % for the GABA-induced current. In four cells evaluated in the same way, the mean values for desensitization were 20.25±6.5 % for GABA and 12.2±4.4 % (mean ± S.D.) for CACA. Furthermore, in three cells in which we investigated the rate of desensitization to GABA and CACA, we did not detect any significant difference between the two ligands (not shown).

Despite their similarities, however, the CACA and GABA responses showed quantitative differences. First, during prolonged applications as shown in Fig. 11A, the CACA-evoked current took 2–3 times as long (2.75±0.52 s, *N*=7) as the GABA current (0.96±0.25 s, *N*=7) to reach its peak. Second, as shown by Fig. 11A and by the dose–response curves for one adult cell in Fig. 11B, the maximal amplitude for the CACA-evoked current (758 pA) was smaller than that for GABA (847 pA). In three tested thoracic neurones, the mean value of the CACA response was 68±16 % of the GABA response. Finally, Fig. 11B shows that the $t_{50}$ value was much smaller for GABA (68 ms) than for CACA (445 ms), indicating a lower affinity for the receptor for CACA than for GABA. Thus, the rank order of potency is extended to muscimol>GABA>isoguvacine>CACA.

## Discussion

Although many invertebrate GABA receptors appear to be related to vertebrate

Fig. 10. Responses to the $GABA_C$ agonist *cis*-4-aminocrotonic acid (CACA). (A) Whole-cell recordings of currents in response to 50 ms application of CACA (1 mmol l$^{-1}$ in the pipette) at various holding potentials. (B) Reversal potentials of CACA responses determined as in A with various intracellular chloride concentrations are plotted against intracellular chloride concentration. Data points are mean values ± S.E.M. of three cells. The dotted line represents theoretical values calculated from the Nernst equation. (C) Successive applications of CACA and GABA evoke a summated inward current (left-hand panel) of the same amplitude as the current evoked by GABA alone (right-hand panel, GABA applied first), again indicating activation of the same receptor population. Horizontal bars show the duration of agonist applications. (D) Response to 1 mmol l$^{-1}$ CACA from a single cell (left-hand panel) is decreased in the presence of 50 $\mu$mol l$^{-1}$ PTX (right-hand panel). (E) CACA-induced current in another cell (left-hand panel) is totally insensitive to the $GABA_A$ antagonist bicuculline (100 $\mu$mol l$^{-1}$, right-hand panel). C, D and E are at a holding potential −60 mV; $E_{Cl}$=−49 mV.

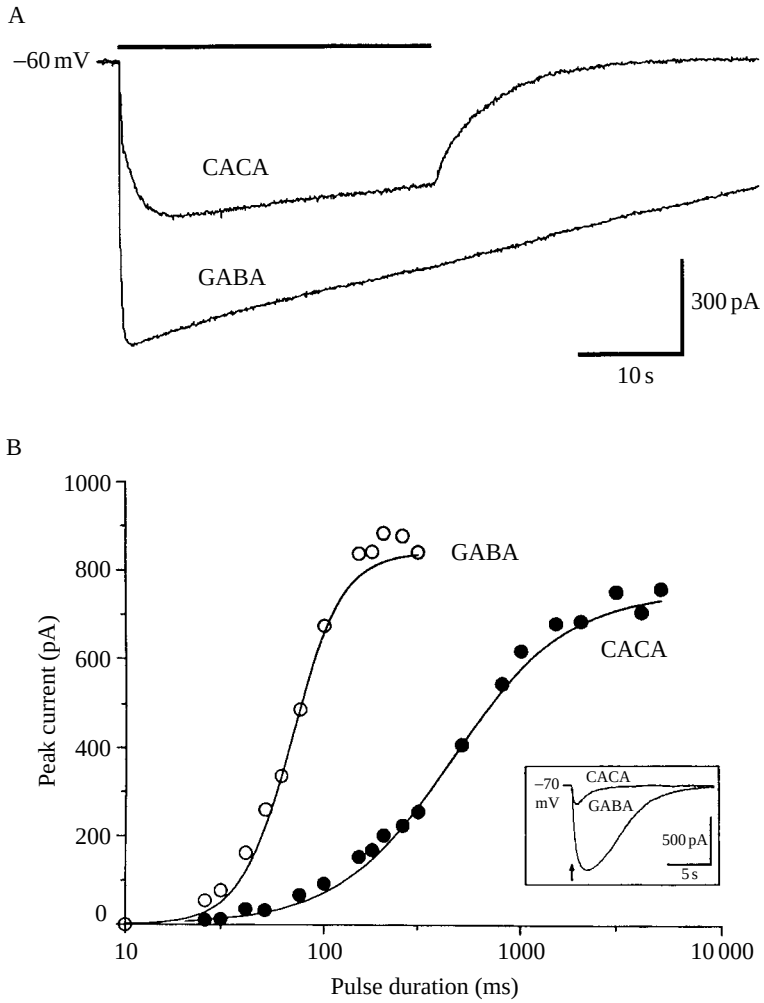


Fig. 11. (A) Moderate desensitization of currents evoked during prolonged application of GABA and CACA. Inward currents evoked in the same cell by application of GABA and CACA for 30 s at a holding potential of −60 mV ($E_{Cl}$=−49 mV). See text for comments. (B) Dose–response relationships for CACA- and GABA-induced currents. Responses of the same cell to increasing doses of GABA and CACA. The same experimental conditions and representation as in Fig. 8. Both currents increased in a concentration-dependent manner. The $T_{50}$ values indicating the half-maximal response were 68 ms for GABA and 445 ms for CACA. Inset: a comparison of inward currents induced by 200 ms application of GABA and CACA at a holding potential of −70 mV ($E_{Cl}$=−49 mV).

$GABA_A$ receptors in that they consist of a chloride channel and respond to the $GABA_A$ agonist muscimol, other characteristics of their pharmacological profile do not fit into the conventional $GABA_A$ and $GABA_B$ receptor classification. In the present study, we have shown that the pharmacological characteristics of GABA receptors on thoracic neurones from adult and embryonic lobster in culture are similar, instead, to those of a novel type of

receptor described in vertebrates as $GABA_C$ (Johnston, 1986). So far, membrane current responses from individual neurones that present this novel type of pharmacology have been reported only in vertebrate retinal cells (Feigenspan *et al.* 1993; Qian and Dowling, 1993). Our work presents the first example of such responses in an invertebrate. We found that all neurones tested responded to GABA, and that the receptors investigated were remarkably homogeneous with respect to their ionic mechanism and pharmacological profile. Moreover, we did not detect any significant difference between the GABA receptors in neurones cultured from embryonic and from adult lobsters.

### *Ionic mechanisms of the GABA-evoked current*

We have shown that GABA evoked, in lobster thoracic neurones in culture, an inward current accompanied by an increase in membrane conductance. The GABA-evoked current reversed close to the equilibrium potential for $Cl^-$. When the intracellular concentration of $Cl^-$ was modified by changing the pipette solution, the reversal potential of the GABA-induced response coincided with the equilibrium potential of $Cl^-$ calculated from the Nernst equation. When the external chloride concentration was decreased, the change in reversal potential of the GABA-induced current was less than predicted by the Nernst equation. However, the difference between calculated and measured values may be due to changes in the internal $Cl^-$ concentration resulting from a substantial resting $Cl^-$ conductance. This has been reported for neurones of the stomatogastric nervous system (Marder and Paupardin-Tritsch, 1978), in which the resting $Cl^-$ conductance can be 2.7 times the resting $K^+$ conductance (Golowasch, 1990). Assuming a high $Cl^-$ conductance, the $E_{Cl}$ in cultured thoracic neurones would tend to equilibrate to the resting membrane potential or, in our experimental conditions, to the holding potential when the external chloride concentration is changed, resulting in a smaller than expected shift in reversal potential of the GABA response. Alternatively, active extrusion of chloride, which has been demonstrated for the crayfish stretch receptor (Aickin *et al.* 1982) and for a variety of neurones in the vertebrate central nervous system (Thompson *et al.* 1988), could also compensate partially for any strong transmembrane $Cl^-$ gradient in our preparation.

In crustacean nervous systems, the reversal potential for $Cl^-$-mediated GABA responses may vary depending on the type of neurone. For instance, this potential is $-70$ mV in the abdominal stretch receptor cell of the crayfish (Kuffler and Eyzaguirre, 1955), about $-50$ mV in neurones of the stomatogastric ganglion (Marder and Paupardin-Tritsch, 1978) and $-35$ mV in crayfish primary afferent terminals of a leg proprioceptor (El Manira and Clarac, 1991; El Manira, 1992). In the latter case, as well as in crayfish primary afferent terminals of tail mechanoreceptors (Kennedy *et al.* 1980), the reversal potential for the $Cl^-$-mediated GABA response is clearly more positive than the resting potential, and GABA provokes a depolarization of the terminal. Under our whole-cell recording conditions, both the reversal potential of the GABA response and the resting membrane potential depend on ion concentrations in the pipette, so the type of GABA response of an intact thoracic neurone cannot be determined. A GABA-induced depolarization, however, has been reported *in vivo* for leg motoneurones in crayfish thoracic ganglia (El Manira, 1992).

In invertebrate preparations, GABA can activate other conductances as well. In crustacean neuromuscular junction, it gates both a $Cl^-$ and a $K^+$ conductance (Fuchs and Getting, 1980), as it does in the stomatogastric nervous system (Marder and Paupardin-Trisch, 1978). In molluscan neurones, GABA receptors may control conductances for sodium, potassium and chloride (Yarowsky and Carpenter, 1978). In our experiments, changing the extracellular concentration of $Na^+$, as well as blocking $Ca^{2+}$ channels, had no effect on the reversal potential of the GABA response. Changing the extracellular concentration of $K^+$ was also without effect in 50 % of the tested cells. From these results, we conclude that, as in the vertebrate $GABA_A$ and $GABA_C$ receptors and in many invertebrate GABA receptors, the GABA-induced current described here is mediated by an increase in $Cl^-$ conductance. In some of the thoracic cells, however, changing the extracellular concentration of $K^+$ induced slight shifts in the reversal potential of the GABA current. One possible explanation for this would be the existence of a $K^+/Cl^-$ cotransporter, as reported, for instance, for CA3 pyramidal cells in organotypic hippocampal slice cultures, where it causes a hyperpolarizing shift in $E_{GABA}$ when the external concentration of $K^+$ is decreased (Thompson and Gähwiler, 1989). An alternative possibility is a partial involvement of $K^+$ itself in the GABA response, assuming some heterogeneity in the GABA receptor type in thoracic neurones. However, preliminary results obtained with single-channel recordings did not indicate any such diversity in the ionic bases of the GABA response analyzed here (C. Jackel, W.-D. Krenz and F. Nagy, in preparation).

*Complex pharmacology of invertebrate GABA receptors*

GABA receptors in the vertebrate nervous system are generally classified into two main families: $GABA_A$ and $GABA_B$ receptors. The former constitute a $Cl^-$ channel thought to be associated directly with binding sites for GABA and with modulatory sites for benzodiazepines, barbiturates and steroids (for a review, see Grayson *et al.* 1991; Ticku, 1991). The $GABA_B$ receptors, in contrast, regulate various conductances ($K^+$ and $Ca^{2+}$) *via* intracellular second-messenger systems (Bowery *et al.* 1991).

Pharmacological profiles of invertebrate GABA receptors do not fit well into this vertebrate scheme. Sensitivity for the vertebrate $GABA_A$ agonists muscimol and isoguvacine has been demonstrated, with varying potency orders, in a variety of invertebrate preparations, such as insect somata (Robinson and Olson, 1988; Rauh *et al.* 1990; Sattelle, 1990), molluscan neurones (for a review, see Nistri and Constanti, 1979), *Limulus polyphemus* heart (Benson, 1989), crustacean primary afferents (El Manira and Clarac, 1991) and crustacean muscle (for a review, see Nistri and Constanti, 1979). However, insensitivity to bicuculline, the blocking effect of which is a characteristic property of the vertebrate $GABA_A$ receptor, is common in invertebrate GABA-gated chloride channels. It has been reported, for example, for insect neuronal somata (Lees *et al.* 1987; Neumann *et al.* 1987; Benson, 1988; Satelle, 1990), for central nervous system neurones (Walker and Roberts, 1982) and for heart muscle (Benson, 1989) of *Limulus polyphemus* and in *Ascaris suum* muscle (Holden-Dye *et al.* 1988). Inhibition of GABA responses by bicuculline was shown in lobster (Shank *et al.* 1974; Constanti, 1978) and in crayfish muscle (Takeuchi and Onodera, 1972). Finally, picrotoxin (PTX),

which is a potent inhibitor of GABA-mediated inhibition in vertebrates (for a review, see Simmonds, 1983), thought to act by blocking the GABA-dependent $Cl^-$ channel (Takeuchi and Takeuchi, 1969), has variable effects depending on the invertebrate preparation under study (for a review, see Lunt, 1991). In crustaceans, for instance, blockage of the GABA response by PTX has been reported in lobster and crayfish muscle (Takeuchi and Takeuchi, 1969; Shank *et al.* 1974; Constanti, 1978) and in crayfish primary afferents (El Manira and Clarac, 1991). In the stomatogastric ganglion of the crab, however, PTX has no effect on GABA-induced currents and has been shown, instead, to block glutamate responses (Marder and Paupardin-Tritsch, 1978). Also, PTX is not active on the *Limulus polyphemus* heart GABA-receptor (Benson, 1989).

In the present study, the current induced by activation of the GABA receptors described was also blocked by up to 70% by PTX. Moreover, the lobster thoracic neurones investigated here were sensitive to the $GABA_A$ agonists muscimol and isoguvacine. The dose–response relationships presented for the different agonists were based on the duration of the ejection pulse. Because the ejected volume is linearly related to the pulse duration (McCaman *et al.* 1977; Sakai *et al.* 1979), it was possible to demonstrate that the GABA- and agonist-induced currents were dose-dependent and that the Michaelis–Menten equation, with $t_{50}$ values replacing $C_{50}$ values, could be fitted to the data points to yield the familiar sigmoid dose–response curve. In this way, the relative potencies of the different ligands could be compared, although the absolute $C_{50}$ values were not available. In our preparation, the rank order of agonist potency was muscimol>GABA>isoguvacine. This is in accordance with most cases in both vertebrates (e.g. Nakagawa *et al.* 1991) and invertebrates (e.g. Benson, 1989), although in some preparations, such as in *Ascaris suum* muscle cells, GABA has the highest potency when applied to the receptor (Holden-Dye *et al.* 1988).

### *$GABA_C$-like pharmacology of lobster thoracic neurones*

Besides its sensitivity to the chloride ionophore blocker PTX and to a number of $GABA_A$ receptor agonists, the GABA receptor described here shows a pharmacological profile resembling that of a novel type of receptor preferentially expressed in vertebrate retina. This receptor was characterized in oocyte expression studies (Cutting *et al.* 1991; Polenzani *et al.* 1991; Shimada *et al.* 1992; Woodward *et al.* 1992) and was recently studied *in situ* (Feigenspan *et al.* 1993; Qian and Dowling, 1993). The retinal receptor shows unusual pharmacological properties as proposed earlier for $GABA_C$ receptors (Drew *et al.* 1984; Johnston, 1986) and shares these with the lobster receptor described in the present paper.

First, the current induced by GABA, muscimol and isoguvacine was entirely unaffected by application of the $GABA_A$ antagonists bicuculline, bicuculline methiodide and the synthetic SR 95531. Second, receptors in the thoracic neurones were totally unresponsive to $GABA_B$ agonists such as baclofen and 3-APA, and the $GABA_B$ antagonist phaclofen had no effect on the GABA-evoked current. Note that, for these experiments, we supplemented the intracellular solution with sodium GTP since the action of baclofen is mediated *via* interactions with GTP-binding proteins (Bowery *et al.* 1991). Third, The GABA-evoked chloride currents in the thoracic neurones are not

modulated by benzodiazepines and barbiturates. Fourth, receptors of the lobster thoracic neurones are sensitive to CACA, a folded GABA analogue with restricted conformation, which is thought to activate specifically the $GABA_C$ receptor (Johnston *et al.* 1975). CACA produced membrane responses that inverted at the same potential as GABA-evoked currents and were reduced to the same extent by PTX, suggesting that CACA, like GABA, increases a $Cl^-$ conductance. CACA yields a dose–response curve with a $T_{50}$ value about ten times longer and a lower maximal response than those of GABA, indicating a lower affinity of CACA for the receptor. This is consistent not only with properties of the GABA responses recorded in retinal neurones, where GABA was reported to be about ten times as potent as CACA (Feigenspan *et al.* 1993; Qian and Dowling, 1993) but also with those of the GABA responses recorded extracellularly from neurones of the frog optic tectum (Sivilotti and Nistri, 1989). Fifth, both the GABA- and the CACA-induced responses described in the present paper show only moderate desensitization, a feature also encountered in the vertebrate retina.

Thoracic ganglia are made up of different types of interneurones and motoneurones, and the dissociated thoracic neurones in our preparation are necessarily a heterogeneous population. Their GABA receptors, however, appeared remarkably similar with respect to their ionic mechanism and pharmacological profile in all neurones tested, suggesting strongly the existence of only one type of GABA receptor in lobster thoracic neurones. This hypothesis is reinforced by preliminary results obtained with single-channel recordings indicating that bicuculline-resistant GABA and CACA responses are mediated by the same $Cl^-$ channel (C. Jackel, W.-D. Krenz and F. Nagy, in preparation). This is in strong contrast with the vertebrate retina, where both bicuculline-resistant and bicuculline-sensitive receptors are present in different neurones (Qian and Dowling, 1993) or in the same neurones associated with two populations of channels with different unitary conductances (Feigenspan *et al.* 1993). This raises the question of how widespread the bicuculline-resistant $GABA_C$-like receptors are in crustaceans. To our knowledge, sensitivity to CACA has not been investigated so far in any other crustacean preparation. CACA has been tested only in a few invertebrate excitable cells, such as the *Limulus polyphemus* heart (Benson, 1989) and *Ascaris suum* muscle (Holden-Dye *et al.* 1988), where it was shown to be inactive.

At present, the functional significance of a GABA receptor with the characteristics described in this study is a matter of speculation. Weak and slow desensitization may enable sustained inhibition on continued release of GABA. The fact that we did not detect any significant difference between the GABA responses in neurones from embryonic and adult lobsters could also indicate the involvement of an embryonic type of receptor. This receptor might be expressed in adult neurones after isolation in culture, in a way similar to the appearance of the embryonic nicotinic acetylcholine receptor in adult vertebrate skeletal muscle following denervation (Mishina *et al.* 1986; Witzemann *et al.* 1991).

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