Independence of and Interactions between GABA-, Glutamate-, and Acetylcholine-Activated Cl Conductances in *Aplysia* Neurons

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In certain *Aplysia* neurons, glutamate, GABA, and acetylcholine (ACh) all elicit desensitizing Cl-dependent responses. This fact and the finding that the glutamate and GABA responses "cross-desensitize" led to the suggestion (Swann and Carpenter, 1975; King and Carpenter, 1987) that the responses to these transmitters were mediated by the same receptor–channel complex.

This hypothesis is incompatible with the demonstration given here that the GABA- and glutamate-gated channels are clearly distinct; the GABA channel, but not the glutamate channel, shows outward rectification (Matsumoto, 1982; King and Carpenter, 1987, 1989) and is selectively blocked by intracellular sulfate. Exploiting these distinctive characteristics and the independent expression of the receptors in some cells, we have been able to reevaluate the so-called cross-desensitization by analyzing the ability of GABA, glutamate, and other agonists to interact with each of the receptor molecules.

The cross-desensitization was found to be exclusively attributable to the ability of GABA to interact with the glutamate receptor (Oyama et al., 1990). The GABA receptor is unaffected by glutamate. Nevertheless, in cells expressing both receptors, glutamate can reduce the GABA response by auto-desensitizing the part of the response that is mediated by the glutamate receptor. No interactions were observed between ACh-induced responses and either of the responses elicited by the amino acids.

The invertebrate glutamate-gated Cl channels that have been cloned resemble the vertebrate glycine receptor (Vassilatis et al., 1997). Our pharmacological evaluation of the molluscan glutamate receptor points in the same direction.

Key words: GABA; glutamate; acetylcholine; cross-desensitization; chloride channel; Aplysia*; mollusk; neuron; pharmacological characteristics; sulfate*

A number of studies on vertebrate neurons have described interactions between inhibitory Cl responses elicited by GABA, glycine, and β-alanine, e.g., between responses to GABA and glycine (Barker and McBurney, 1979; Hamill et al., 1983; Baev et al., 1992; Lewis and Faber, 1993; Trombley et al., 1999), between responses to β-alanine and either GABA or glycine (Choquet and Korn, 1988), and between responses to glycine and β-alanine (Krishtal et al., 1988). The demonstration that recombinant homomeric glycine receptors (formed from subunits cloned from zebrafish) can be activated by GABA (Fucile et al., 1999) might well explain certain of the interactions observed in vertebrate neurons between GABA- and glycine-induced responses.

In invertebrates, many different neurotransmitters, including glutamate but not glycine, gate Cl channels. In cells in which different transmitters activate Cl conductances, interactions observed between the responses have led to the suggestion that different transmitters share the same receptor molecule. For example, in crayfish muscle, a single Cl channel is activated by similar concentrations of glutamate, GABA, and acetylcholine (ACh) (Franke et al., 1986; Zufall et al., 1988). In certain identified *Aplysia* neurons, these same three transmitters often increase Cl conductance in the same cell, but studies evaluating interactions between these responses have yielded conflicting conclusions. King and Carpenter (1987) observed bi-directional "cross-desensitization" between the GABA and glutamate responses and suggested that, as in crayfish muscle, these two amino acids share the same receptor molecule. However, Ikemoto and Akaike (1988) failed to observe any interactions, and Oyama et al. (1990) observed that, whereas the glutamate response was diminished in the presence of GABA, only a

distinctive slowly desensitizing, high-threshold element of the GABA response was diminished by glutamate. They proposed that this element of the GABA response was attributable to GABA activation of the glutamate receptor. In contrast, similar studies on other invertebrate ganglionic neurons revealed no interactions between GABA and glutamate Cl-dependent responses (lobster, Cleland and Selverston, 1998; crab, Duan and Cooke, 2000).

In the present study, we have reexamined the interactions between the glutamate-, GABA-, and ACh-activated responses in *Aplysia* neurons. By exploiting our finding that the GABA-gated channel has distinguishing characteristics and the fact that, in certain cells, only one of the relevant receptors is expressed, we have shown that all three of these transmitters open Cl channels by binding to distinct and independent molecular entities. Nevertheless, interactions were shown to exist between the GABA and glutamate responses. These seemingly bi-directional interactions can be entirely explained by the ability of GABA to interact with the glutamate receptor (Oyama et al., 1990).

MATERIALS AND METHODS

Experimental preparation. The experiments described in this paper were performed on cells from either the buccal, pleural, or cerebral ganglia of *Aplysia*.

The ganglia were prepared as described previously (Kehoe, 1985). For the study of the coexpressed GABA, glutamate, and ACh receptors mediating Cl-dependent responses, two series of cells were used: the medial cells of the pleural ganglia (Kehoe, 1972b) and selected cells from the buccal ganglia (including B3 and B6) (Gardner and Kandel, 1977). For examining glutamate-induced Cl-dependent responses in cells having no GABA receptor, we used small unpigmented cells from the pleural ganglia (Ascher et al., 1978), whereas, for the evaluation of GABA-induced Cl-dependent responses in cells having no glutamate-induced Cl response, we chose small, unpigmented cells located on the base of the midline of the dorsal surface of the cerebral ganglion.

Electrodes, voltage clamp, recording procedures, and treatment of data. For experiments illustrated in Figures 2*A–C*, 3*B*, 4*B*, 6*C*, and 10*A*, whole-cell patch-clamp methods (Hamill et al., 1981) were used (for details, see Kehoe, 1994). Pipettes of 200–500 kΩ ensured a good exchange, and data were retained only from cells for which the access resistance remained constant throughout the experiment. In all other experiments, recordings were made in conventional microelectrode voltage clamp using hand-pulled microelectrodes made with a de Fonbrune microforge and filled

with 0.5 M K$_2$SO$_4$. Although cells with intact axons were used in these experiments, and obviously the voltage in much of the axon was not controlled by the somatically placed electrodes, clamp error cannot explain the changes in response amplitude studied in these experiments. No evidence of a failure to control somatic voltage was seen in the voltage traces, and the control of the somatic voltage was evident from the unchanging amplitude of one of the two agonist-induced responses in experiments in which the other agonist response was markedly reduced. Activation of axonal membrane was minimized by the somatic application of the agonist and by its rapid removal by the flow from the control tube, as well as by a continual superfusion of the entire ganglion. For an additional description of the characteristics of the two-electrode voltage clamp, see Kehoe (1985).

Continuous recordings were made on a Servogor 340 paper recorder and on a digital audio tape recorder. Records of currents elicited by agonist applications were digitized and sampled on-line on a Dell (Round Rock, TX) computer, via a Cambridge Electronic Design (Cambridge, UK) 1401 interface, using the Whole Cell Eectrophysiology program from Strathclyde Electrophysiological Software.

External and internal solutions. The artificial seawater (ASW) bathing the ganglion contained (in mM): 480 NaCl, 10 KCl, 10 CaCl$_2$, 50 MgCl$_2$, and 10 Na-HEPES, pH 7.8. The internal solution used in the whole-cell clamp experiments illustrated here contained (in mM): 411 K$_2$SO$_4$, 8.3 Na$_2$SO$_4$, 1 CaCl$_2$, 2 MgCl$_2$, and 1.5 EGTA, buffered with 20 K-HEPES to pH 7.4. In a few nonillustrated experiments, a KCl-based solution was used to show that the relevant factor causing the diminution of the GABA response in whole-cell perfusion experiments came from the presence of sulfate ions in the internal solution. The KCl-based internal solution contained (in mM): 496 KCl, 10 NaCl, 2 MgCl$_2$, 1 CaCl$_2$, 1.5 EGTA, and 20 K-HEPES, pH 7.4.

Fast application system. The fast application system used here has been described previously in detail (Johnson and Ascher, 1987; Kehoe, 1994). Three glass tubes held together with heat-shrink tubing were moved laterally so that the appropriate (ASW or agonist-containing) tube was directly in front of the cell being studied. The central tube, called the "control tube," contained ASW that continually bathed the cell under study except when the tubes were moved laterally to permit a 2 sec application of one of the agonists. Solenoid-driven valves were placed in the line of the tubing feeding each of the agonist-containing glass tubes, permitting the flow of agonist only when the relevant tube was directly facing the cell. Although the concentration jump occurring at the tip of the tubes is very rapid, the relevant measurement is of course the speed at which the solutions flowing from the tube envelop the cell. An estimation of this exchange was made by switching from normal ASW in the control tube (containing 10 mM K) to high-K seawater (containing 154 mM K). The time to reach 67% of the maximal current induced by the high-K seawater was found to be on the order of 70–80 msec.

The duration of agonist application in all experiments was 2 sec, and the interval between applications was 1 min. Because in most experiments there was an alternation between two different agonists (e.g., GABA and glutamate), for a given agonist, a 2 min interval separated two successive applications. With the agonist concentrations used here, this regimen yielded control responses of a constant amplitude. The antagonists were applied only through the control tube (see exception in Fig. 9A). This implies that, during the 2 sec application of the agonist, a partial wash of the preapplied antagonist occurs. The application of desensitizing agonists through the control tube resulted in a similar partial wash during the 2 sec agonist application. This led, at some concentrations, to particularities in the recorded currents that are discussed in the relevant text describing the figures.

Drugs. GABA, L-glutamic acid (monosodium salt), ACh, β-alanine, tubocurarine chloride (TC), strychnine hydrochloride, and pentobarbital were all obtained from Sigma (St. Louis, MO); methyllycaconitine citrate (MLA) was obtained from Research Biochemicals (Natick, MA); and *cis*-4-aminocrotonic acid (CACA), *trans*-4-aminocrotonic acid (TACA), hypotaurine, and bicuculline methochloride were obtained from Tocris Cookson (Bristol, UK).

Experimental n values, statistical analyses, and experimental controls. Each conclusion that has been illustrated in this paper was drawn from a minimum of three experiments (with the exception of that illustrated in Fig. 2B, for which only two experiments were performed). Statistical analyses using either the ANOVA test (Figs. 1C, 8) or the Student's *t* test (other figures) quantified the effects of the various manipulations on the responses to the different agonists.

RESULTS

Comparison of glutamate- and GABA-gated Cl responses

Glutamate, GABA, and ACh each elicits a rapidly activated inhibitory response in a number of neurons in *Aplysia* central ganglia. In a given experiment and at a given time, the reversal potential of this response is the same for all three of the transmitters and has been shown, for the response to ACh, to be equal to the Cl equilibrium potential (Kehoe, 1972a). Such cells offer an easily controlled comparison of these receptor-operated channels and provide a good preparation for the evaluation of any possible interactions between them. In this paper, only the glutamate and GABA responses will be described in detail, but some data will be presented concerning the ACh-induced Cl-dependent responses (Kehoe and McIntosh, 1998) that coexist with the glutamate and GABA Cl responses in the medial cells of the pleural ganglia, as well as in selected cells of the buccal ganglia (e.g., B3 and B6) (Gardner and Kandel, 1977).

A given molluscan neuron often expresses more than one receptor type for a given transmitter. That is the case for glutamate in the medial cells studied here, because, in addition to gating a Cl channel in those cells, glutamate also activates a nonspecific cationic channel, thereby eliciting a two-component response (King and Carpenter, 1987). Because the cationic response desensitizes very rapidly, it does not interfere with the peak of the glutamate-activated increase in Cl conductance and has consequently not been pharmacologically eliminated for these experiments.

Differential voltage dependence of the glutamate- and GABA-gated Cl conductances

Examples of the responses to glutamate and GABA in the two different cell types are shown in Figure 1. In the medial cells (Fig. 1A), it can be seen that the Cl current elicited by glutamate (outward at −26 mV, inward at −66 mV, with the reversal potential in this cell at −46 mV) is preceded by a very rapidly desensitizing cationic inward current. The only response elicited by GABA in the same cells is a Cl-dependent one, hence no change in current is seen at E$_{Cl}$ (−46 mV). Figure 1B illustrates records from one of the selected buccal cells in which both glutamate and GABA activate only Cl-dependent responses. (Given the rapidity of the desensitization of the glutamate cationic response, such a response might be missed in these large cells, which are often a few hundred micrometers in diameter. For cells of this size, the fast application tubes must be pulled back further from the cell surface, and the speed of the concentration jump at the level of the membrane is thereby reduced.)

An examination of the outward- and inward-going responses to the two amino acids reveals that, when the holding potential is fixed on either side of (but equidistant from) E$_{Cl}$, the inward and outward currents elicited by glutamate are of equal amplitude, whereas the inward current elicited by GABA is always smaller than the outward current. This was shown to be true in both cell types. In Figure 1, A and B, the response recorded at a holding potential more negative than E$_{Cl}$ has been multiplied by −1 and is represented by a *dashed line* to permit an easy comparison with the amplitude of the outward current. The *vertical dashed line* crossing the recordings indicates the end of the agonist application and the return to ASW flowing from the control tube.

A statistical evaluation of the differential voltage dependence of the GABA- and glutamate-induced conductances is presented in Figure 1C, and the results obtained from an ANOVA of these data are presented in the legend to Figure 1. The outward rectification of the GABA-induced conductance has been described previously (Matsumoto, 1982; King and Carpenter, 1987, 1989).

Differential effect of intracellular sulfate ions on the glutamate and GABA responses

The differential voltage dependence of the Cl conductances activated by glutamate and GABA suggests that the two transmitters gate different types of Cl channels.

This impression was reinforced by the differential effect of intracellular sulfate ions observed here on the responses to GABA and glutamate. The experiments illustrated in Figure 2A–C were performed in whole-cell patch-clamp mode, so the solution in the pipette was constantly exchanging with the cytoplasm, hence altering the internal Cl concentration and thereby provoking changes in E$_{Cl}$. Consequently, in Figure 2, A and B, periodic adjustments throughout the experiments were made to ensure that the holding potential remained at a constant distance from E$_{Cl}$. Under these
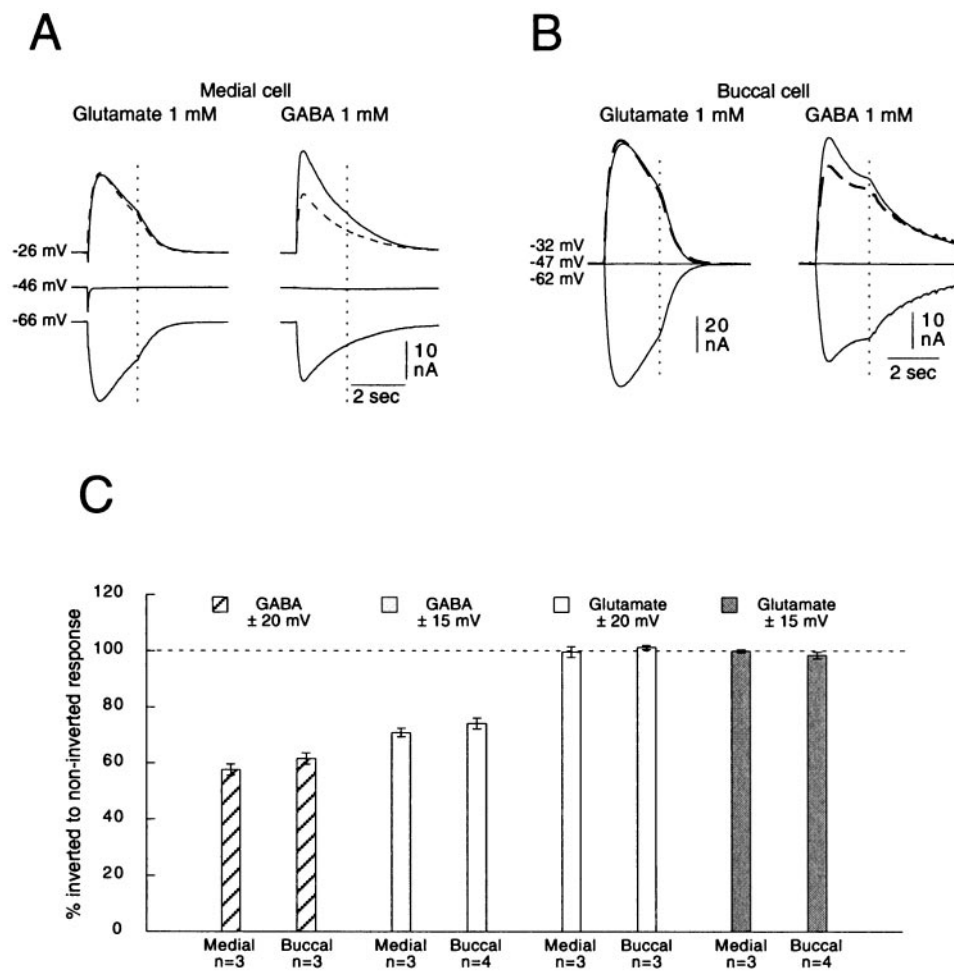
Figure 1. Differential voltage dependence of glutamate- and GABA-activated Cl conductances measured in two-electrode voltage clamp in medial and buccal cells. *A*, Glutamate and GABA responses in a medial cell. Glutamate elicited a two-component response consisting of a rapidly desensitizing cationic element (seen here as an inward current at all holding potentials) and an inhibitory, Cl-dependent element that is manifested by an outward current at -26 mV, an inward current at -66 mV, and 0 net current at E_{Cl} (-46 mV). The inward and outward Cl currents, measured at holding potentials 20 mV on either side of E_{Cl}, were of equal amplitude. The *dashed line* represents the inward current multiplied by -1 for easier comparison with the outward current measured at -26 mV. GABA elicited a pure Cl-dependent response in the medial cells, so 0 net current is recorded at E_{Cl}. Again, the *dashed line* represents the inward GABA-induced current multiplied by -1. It can be seen that the GABA response measured at a holding potential 20 mV more negative than E_{Cl} is much smaller than that measured 20 mV less negative than E_{Cl}. The *vertical dashed line* indicates the end of the 2 sec agonist application and the return to the flow of ASW over the cell. *B*, Glutamate and GABA responses in a buccal neuron. Note that no cationic response to glutamate can be detected in this cell. Here the Cl currents were measured at holding potentials 15 mV on either side of E_{Cl}. As was the case for the medial cell, the glutamate-activated conductance in the buccal cell showed no voltage dependence, whereas that induced by GABA showed outward rectification. *C*, It can be seen that the mean percent of inverted to noninverted responses is clearly different for the GABA and glutamate responses and that, for the GABA response, there is an evident difference in the same measurement as a function of voltage. This impression was verified by an ANOVA that reveals that the probability under the null hypothesis of the observed main effect of agonists is $p \leq 0.0001$ and that of the observed main effect of voltage is $p \leq 0.0006$. In addition, the interaction between voltage and agonist is highly significant ($p \leq 0.0001$). In contrast, the differences observed between cell types do not permit the rejection of the null hypothesis ($p = 0.2025$).

conditions, the amplitude of the glutamate response remained constant (mean of six experiments was 99.1% of control; $\sigma = 2.13\%$), whereas that to GABA decreased progressively to an average of 37.7% of the control value ($\sigma = 8.33\%$) after ~20 min whole-cell perfusion ($n = 6$; $p \leq 0.0001$). Figure 2*B* shows that the blocking effect of sulfate on the GABA response is completely independent of both membrane voltage and the direction of current flow. In this experiment made at ± 15 mV on either side of E_{Cl}, the inward/outward current ratio was maintained at 68%, despite a sulfate-induced reduction in the GABA response amplitude to 36% of the initial value. In the second such experiment (data not shown) performed at ± 10 mV on either side of E_{Cl}, the inward/outward current ratio was maintained at 78% throughout the sulfate-induced diminution of the absolute response amplitude, which reached 57% of the initial amplitude after 20 min whole-cell recording.

Figure 2*C* illustrates another protocol for studying the differential effect of internal sulfate ions on the GABA and glutamate responses. In this experiment, the holding potential was held constant while E_{Cl} became more negative because of the whole-cell perfusion. Because of the resulting increase in driving force for Cl ions, the response to glutamate increased, whereas, despite the change in driving force, the response to GABA decreased. This simplified protocol, which of course leads to the same conclusion, was used in experiments to be described later evaluating differential effects of intracellular sulfate on responses elicited by various agonists.

It is known that GABA responses in some mammalian neurons show rundown when intracellular ATP is washed out by whole-cell

perfusion (Shirasaki et al., 1992). This however was not the explanation for the observation made here, because the results were unchanged by the inclusion of ATP and GTP in the K_2SO_4-based internal solution. Furthermore, when KCl-based, ATP-free solutions were used (three experiments), GABA and glutamate responses changed proportionally as intracellular perfusion progressed (data not shown). Finally, the same differential blockade of the GABA response by sulfate ions was seen (Fig. 2*D*) when 0.5 M K_2SO_4 was simply injected by pressure into a cell in which recordings were made with conventional two-electrode voltage clamp, thereby avoiding any possibility of washout associated with whole-cell perfusion (see figure legend for a statistical résumé of the relevant experiments).

Pharmacological distinctions between the glutamate and GABA responses

One of the factors that has held back the identification of glutamatergic and GABAergic synapses in molluscan nervous systems is the paucity of pharmacological tools that have been found to be selective for the different Cl-dependent responses activated by these different transmitters. In an effort to distinguish further between the glutamate- and GABA-induced Cl responses and to provide compounds that could eventually assist in identifying synapses using these neurotransmitters, a number of potential agonists and antagonists were evaluated.

β-alanine

β-alanine (1 mM) was found to elicit in both the medial and buccal cells a Cl-dependent response that reflects the activation of the

Figure 2. Selective blockade by sulfate ions of the GABA-induced Cl-dependent response. *A,* Whole-cell recording with K_2SO_4-based internal solution in a buccal cell. The membrane potential was maintained at 15 mV less negative than E_{Cl}. Whole-cell perfusion with a sulfate-containing internal solution caused a selective diminution of the GABA-induced Cl-dependent response. From six experiments in which the effect of sulfate was measured 20 min after control responses, the glutamate response was maintained at 99.1% of control ($\sigma = 2.13\%$), whereas that to GABA decreased to 37.7% control ($\sigma = 8.33\%$; $p \leq 0.00002$). *B,* A similar experiment on a medial cell response to GABA illustrated at ±15 mV on either side of E_{Cl}. It can be seen that the reduction of the GABA response induced by a 20 min whole-cell recording with an intracellular sulfate-based solution is independent of membrane voltage and of the direction of current flow, with inward current being 68% of the outward current before and after reduction of the response by sulfate to 36% of the control value (see Results for additional data). *C,* Whole-cell recording with K_2SO_4-based internal solution of a medial cell response to 1 mM glutamate and 1 mM GABA. Holding potential was maintained at −30 mV, and E_{Cl} shifted to more negative values as the internal solution entered the cell. The increased driving force on Cl ions caused an increase in the glutamate response, but the blocking effect of sulfate ions counteracted and dominated the facilitory effect of an increased driving force on the GABA response. *D,* Two-electrode voltage-clamp recording of glutamate and GABA responses from a medial cell held at −30

A

Block of GABA response by intracellular sulphate (cell held at 15 mV less negative than E_{Cl})

Glutamate 1 mM

10 nA

5 sec

GABA 1 mM

0   5   10   15   20

Duration of whole cell recording (min)

B

Block of GABA response by intracellular sulphate occurs on both sides of E_{Cl} (±15 mV).

—— Recorded currents
—— Inverted inward currents
- - 68 % of outward current at 0 and 20 min

5 nA

2 sec

C

Glutamate 1 mM   GABA 1 mM

—— Control
- - - 10 min in whole cell with K_2SO_4

−30 mV

10 nA

2 sec

D

Glutamate 1 mM   GABA 1 mM

—— Control
- - after K_2SO_4 injections

5 nA

2 sec

HP: 20 mV less negative than E_{Cl}

mV. From a third microelectrode introduced into the cell, three brief pressure injections of 0.5 M K_2SO_4 were made. These three injections caused the selective and progressive decline in the amplitude of the GABA-induced Cl-dependent response. From three such experiments, the average GABA response measured at 13–15 min after the first injection was 26% of the control amplitude ($\sigma = 5.57\%$), whereas the average glutamate response was 102.6% of control ($\sigma = 4.16\%$; $p \leq 0.00004$).
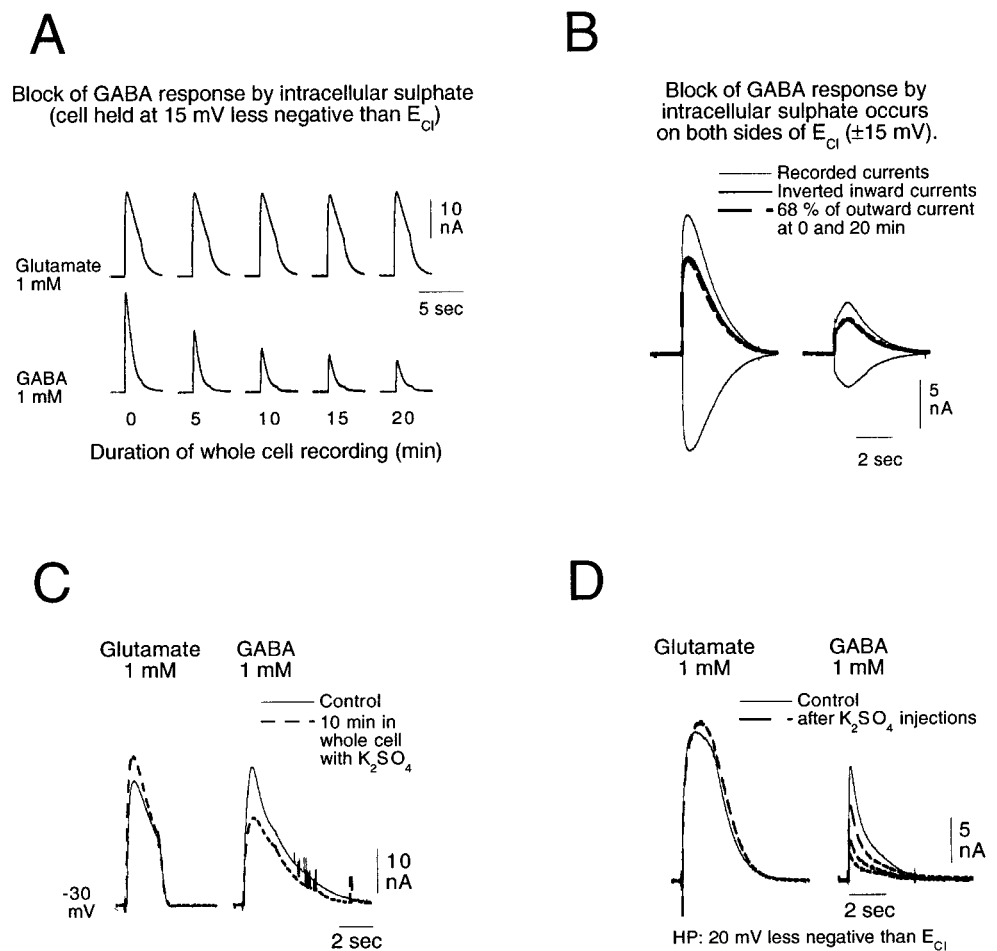
glutamate rather than of the GABA receptor. The response to β-alanine was, on average, 45% of the peak amplitude of the response to 1 mM glutamate in the same cells ($n = 5$; $\sigma = 5.59\%$). Like the glutamate-activated conductance, that activated by β-alanine showed no voltage dependence (Fig. 3*A*). Furthermore, the response to β-alanine, like the glutamate response, was not blocked by intracellular sulfate. In a cell held at −30 mV (Fig. 3*B*), the increase in outward-going currents in response to both glutamate and β-alanine as whole-cell perfusion proceeds reflects the perfusion-induced change in E_{Cl}. The two responses show the same percent increase. The mean difference in increases for the two responses in such experiments was, for $n = 3$, 1% ($\sigma = 0.8\%$). Another manifestation of the fact that β-alanine activates the glutamate receptor preferentially can be seen in Figure 3*C* in which 50 μM β-alanine is shown to selectively desensitize–block the glutamate Cl-dependent response (for $n = 5$, mean glutamate response in β-alanine was 8% of control with $\sigma = 3.67\%$; mean GABA response in β-alanine was 81.8% of control with $\sigma = 8.93\%$; $p \leq 0.0002$). This selectivity of β-alanine is not maintained at higher concentrations (see below) (Ikemoto et al., 1988a).

β-alanine is primarily known for its preferential action on vertebrate glycine receptors (Wu et al., 1993; Jonas et al., 1998). No glycine-induced Cl-dependent responses have as yet been reported in invertebrate neurons, and even at 10 mM, glycine failed to increase Cl conductance in any of the cell types used in this study (three experiments for each cell type; data not shown).

In addition to activating a Cl conductance, glutamate has been shown to act on two other pharmacologically and ionically distinct receptors: one mediating a cationic excitatory response (see the

rapid inward current in the medial cells) (Kehoe, 1978; King and Carpenter, 1987), and the other, a K-dependent inhibitory response (Kehoe, 1978, 1994; Katz and Levitan, 1993). β-alanine neither activated nor blocked the glutamate receptors mediating these two responses (results from three experiments studying each of the two other glutamate receptors).

TACA

The GABA analog TACA activates a conductance that, like that activated by GABA, is voltage-dependent (Fig. 4*A*) and is blocked by intracellular sulfate ions (Fig. 4*B*). Furthermore, TACA (100 μM) preferentially desensitizes–blocks the GABA response (Fig. 4*C*) (Fig. 4*A–C,* see legend for statistical analyses of the findings illustrated).

Hypotaurine

Hypotaurine, an endogenous amino acid shown to have inhibitory effects in cerebellar slices (Okamoto and Sakai, 1981), had no agonist effect on any of the cells tested. However, it did preferentially block the Cl-dependent response to glutamate, having only a very slight effect on the Cl-dependent response to GABA (Fig. 5*A,* see legend for statistical evaluation of relevant experiments). Hypotaurine had no blocking effect on either the cationic or the K-dependent glutamate response (three experiments on each response type; data not shown).

CACA

CACA, an agonist of $GABA_C$ vertebrate receptors (Johnston, 1996), induced only a weak current in the cells studied here, but,
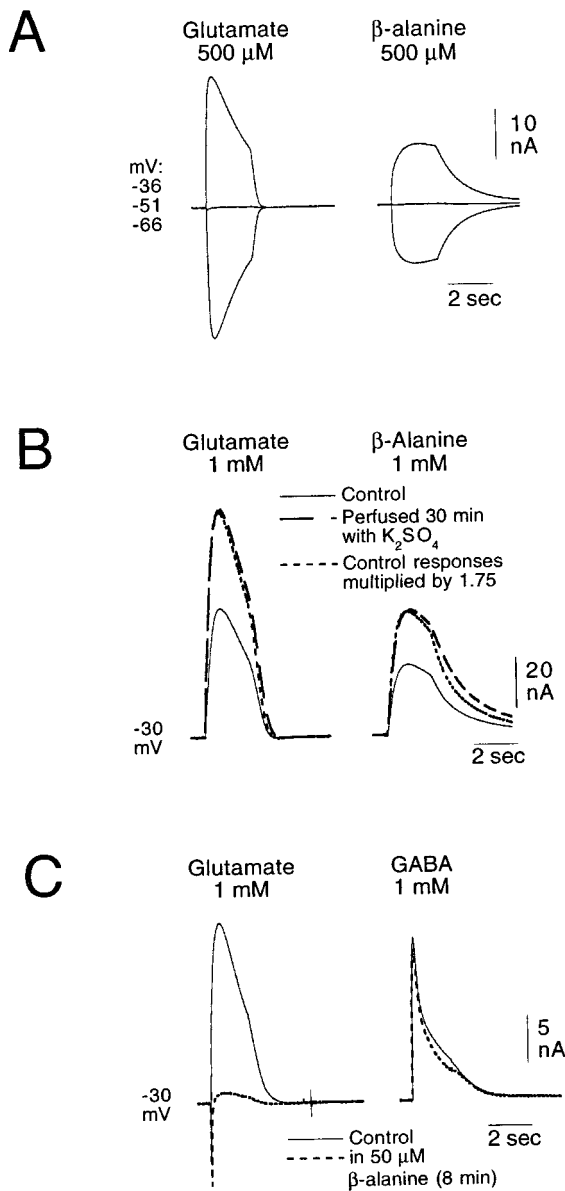
Figure 3. β-alanine selectively activates the glutamate receptor in the buccal and medial cells. *A,* Responses of a buccal cell to glutamate and β-alanine were measured at 15 mV either side of E_{Cl} using a two-electrode voltage clamp. Note that the β-alanine-induced inward and outward currents measured at 15 mV on either side of E_{Cl}, like those to glutamate, are of equal amplitude (mean inward to outward current for glutamate was 99.3% with $\sigma = 1.15\%$; that for β-alanine was 100% with $\sigma = 2.52\%$; $p = 0.56$). *B,* Whole-cell recording with a K_2SO_4-based internal solution of responses to glutamate and β-alanine in a buccal cell held at −30 mV. Note that the glutamate- and β-alanine-induced responses increased proportionately as E_{Cl} changed over time with whole-cell perfusion using the sulfate-based solution (mean difference between changes in the glutamate and β-alanine responses in sulfate was 1%; $\sigma = 0.8\%$). *C,* Two-electrode voltage-clamp recordings of glutamate and GABA responses from a medial cell held at −30 mV. β-alanine (50 μM) applied through the control tube induced no change in holding current but selectively desensitized–blocked the response to glutamate (for $n = 5$, mean glutamate response in β-alanine was 8% of control with $\sigma = 3.67\%$; mean GABA response in β-alanine was 81.8% of control with $\sigma = 8.93\%$; $p \le 0.0002$).

like TACA, preferentially desensitized–blocked the GABA Cl-dependent response, having only a very weak blocking effect on the glutamate response (Fig. 5*B*, see legend for statistical analysis of three experiments of this type).

A number of other compounds (TC, strychnine, bicuculline, and pentobarbital) were tested at 100 μM on the glutamate- and GABA-induced Cl-dependent responses (data not shown). TC
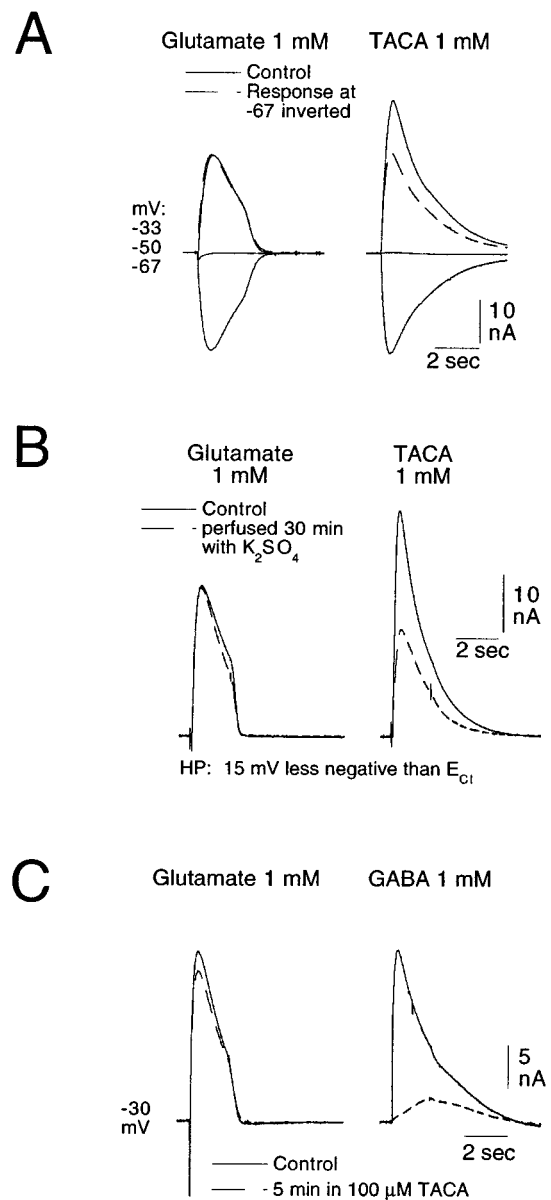
Figure 4. TACA selectively activates the GABA receptor in the buccal and medial cells. *A,* Responses of a buccal cell to glutamate and TACA measured with a two-electrode voltage clamp at 17 mV on either side of E_{Cl}. Note that the amplitude of the TACA-induced currents measured at 17 mV on either side of E_{Cl} are not of equal amplitude, with the TACA-induced inward current being smaller than the TACA-induced outward current. The inward and outward currents elicited by glutamate at the same holding potentials are of equal amplitude (mean inward to outward current for glutamate was 102% with $\sigma = 2.39\%$; that for TACA was 59.2% with $\sigma = 6.06\%$; $n = 5$; $p \le 0.0001$). *B,* Whole-cell recording with a K_2SO_4-based internal solution of responses to glutamate and TACA of a buccal cell held at 15 mV less negative than E_{Cl}. Note that there is a selective diminution in the TACA-induced response. Pooling data from experiments using the protocol illustrated in *B* with experiments in which the holding potential was held constant, it was found that sulfate caused a mean relative diminution in the TACA response to 37.5% ($\sigma = 9.76\%$; $n = 3$). *C,* Two-electrode voltage-clamp recordings of glutamate and GABA responses from a medial cell held at −30 mV. TACA (100 μM) applied through the control tube induced no change in holding current but selectively desensitized–blocked the response to GABA. A quantitative evaluation of this reduction was made by measuring the response in TACA at the time corresponding to the peak control response, because the TACA block was rapidly reduced during the 2 sec application of agonist solutions, which contained no TACA. Such an analysis of five experiments yielded a mean reduction in the glutamate response to 78% of the control ($\sigma = 10.7\%$) and a mean reduction in the GABA response to 7.3% of the control ($\sigma = 7.9\%$), with $p \le 0.0002$.
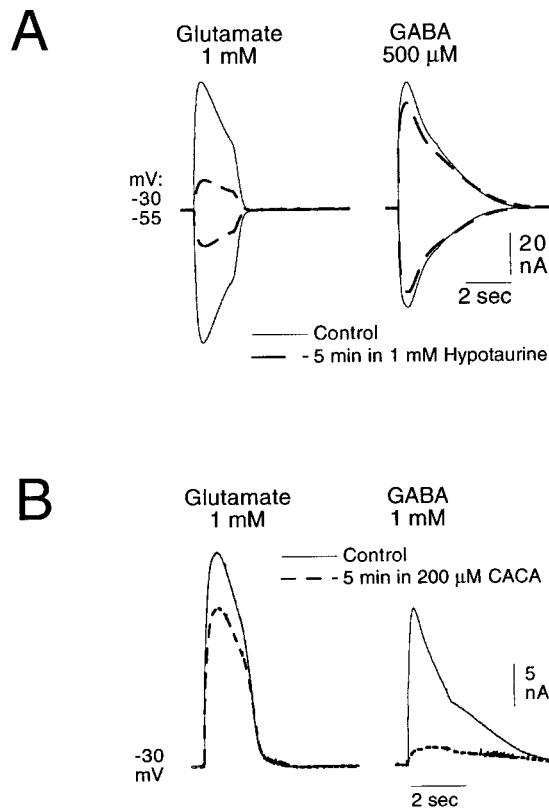
Figure 5. Preferential block of the glutamate Cl-dependent response by hypotaurine and preferential block–desensitization of the GABA Cl-dependent response by CACA. *A*, Effect of 1 mM hypotaurine on equal amplitude responses of a medial cell to 1 mM glutamate and 500 μM GABA measured in two-electrode voltage clamp at 17 mV on either side of E_{Cl}. Note that hypotaurine, which induced no change in holding potential, preferentially blocked the glutamate-induced response. The glutamate response was reduced to 20.2% ($\sigma = 6.33\%$) of the control, whereas that to GABA was only reduced to 87.7% ($\sigma = 12.6\%$) of the control ($n = 3$; $p \leq 0.001$). *B*, Effect of CACA on glutamate and GABA responses of a buccal cell measured in two-electrode voltage clamp at −30 mV. CACA (200 μM) applied through the control tube induced no change in holding current but preferentially desensitized–blocked the response to GABA. The GABA response was reduced to 13.7% ($\sigma = 4.04\%$) of the control, whereas that to glutamate was only reduced to 82.3% ($\sigma = 8.02\%$) of control ($n = 3$; $p \leq 0.0002$).

and strychnine were without effect on GABA responses but caused a small reduction in the glutamate response. The glutamate response in 100 μM strychnine was reduced to an average of 86% of the control value ($n = 3$; $\sigma = 2\%$). Bicuculline caused a slight diminution in both the glutamate and GABA responses, whereas pentobarbital reduced the GABA response (Ikemoto et al., 1988a) to ~20–30% of the control amplitude (three experiments) but had practically no effect on the glutamate response. Even with 1 and 10 μM concentrations, the effect of pentobarbital was to reduce rather than potentiate the GABA response.

Differential expression of GABA and glutamate receptors in different cells

Cells in which the glutamate but not the GABA receptor is expressed reveal that GABA can activate the glutamate receptor

In a group of small identifiable cells in the right pleural ganglion (Ascher et al., 1978) in which glutamate activates a marked Cl-dependent response, GABA was shown to elicit a very weak response (Fig. 6*A*) that does not share the characteristics of the GABA responses seen in the medial or buccal cells. The GABA-activated Cl conductance in the small pleural cells was shown to be independent of voltage (Fig. 6*B*, response to 10 mM GABA) and unaffected by intracellular sulfate ions (Fig. 6*C*). Furthermore, like the response to glutamate in all of the cells studied, it was markedly
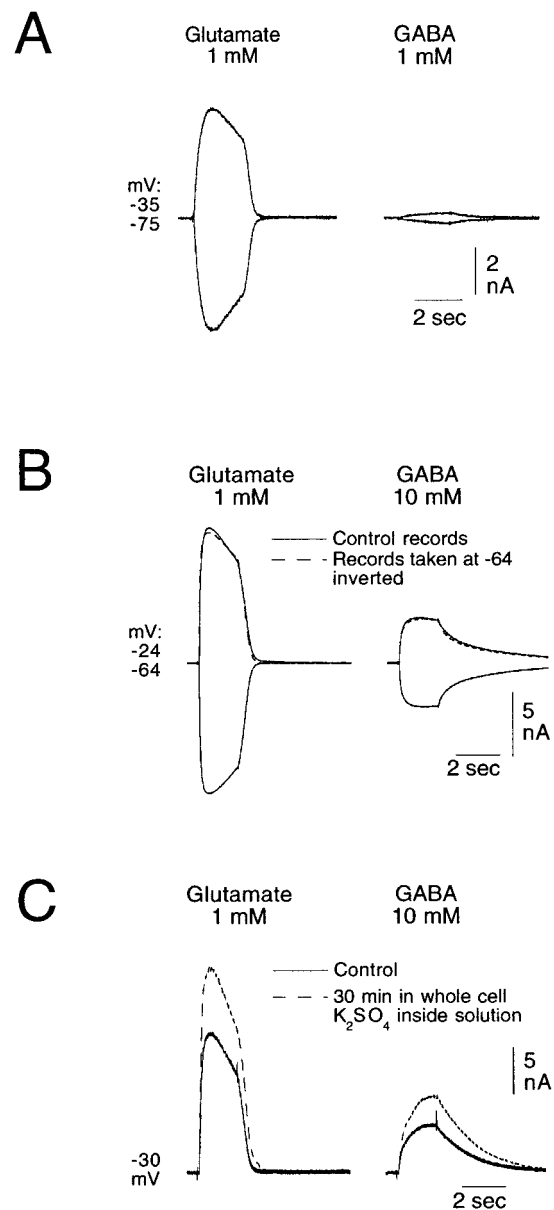
Figure 6. Recordings of glutamate and GABA responses in cells from an identifiable group situated near the pleural-pedal connective in the right pleural ganglion (Ascher et al., 1978). *A*, Responses to 1 mM glutamate and 1 mM GABA recorded in two-electrode voltage clamp 20 mV on either side of E_{Cl}. The response to 1 mM GABA in these cells is only 3.83% of that to glutamate ($n = 3$; $\sigma = 0.289\%$). The glutamate-activated conductance in these cells, as in the buccal and medial cells, showed no voltage dependence. *B*, Using a 10 mM concentration of GABA, the response became large enough to compare it with the response to 1 mM glutamate recorded, again, in a small pleural cell. It can be seen that, in these cells, both glutamate and GABA elicited Cl conductances that are independent of voltage over the range tested. The mean percent of inward to outward current ($n = 3$) for the glutamate response was 100, with $\sigma = 1.89\%$; that for GABA was 99.7, with $\sigma = 0.57\%$ ($p = 0.684$). *C*, Recordings, made with whole-cell voltage clamp using a K_2SO_4-based internal solution, of the responses to 1 mM glutamate and 10 mM GABA in a small pleural cell held at −30 mV. The GABA-induced response in these cells behaved like the glutamate-induced response, i.e., both increased as intracellular perfusion with the K_2SO_4-based solution progresses and E_{Cl} becomes more negative. The effects of internal sulfate on the response to 1 mM glutamate and on the response to 10 mM GABA in these cells did not differ (mean glutamate response change was 172% with $\sigma = 24.1\%$; mean GABA response change was 172% with $\sigma = 21.7\%$; $n = 3$; $p = 0.973$).

reduced by 50 μM β-alanine (Fig. 7*A*), weakly blocked by 100 μM TACA (Fig. 7*B*), markedly reduced by 1 mM hypotaurine (Fig. 7*C*), and only slightly affected by 200 μM CACA (Fig. 7*D*). In all of these respects, the current elicited by GABA in these cells resembles the
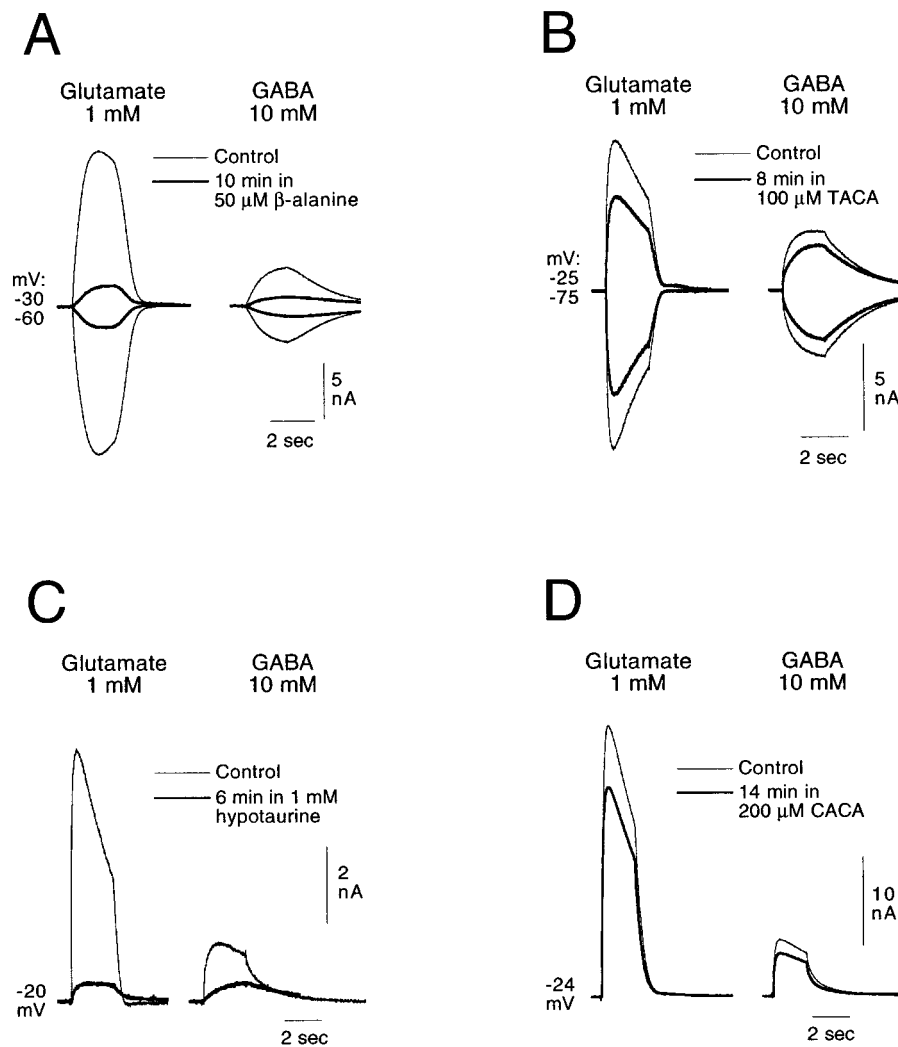
Figure 7. The effect of pharmacological compounds shown to act on either the glutamate- or GABA-induced Cl-dependent responses confirm that the response to GABA in the small pleural cells (Ascher et al., 1978) is mediated by the glutamate receptor. All recordings were made in two-electrode voltage clamp with cells held at fixed potentials (see figure for values). *A*, The response to both 1 mM glutamate and 10 mM GABA were markedly reduced by 50 μM β-alanine applied through the control tube (response to glutamate reduced to 12.5% of control with $\sigma = 0.86$%; that to GABA was reduced to 16.7% of control with $\sigma = 4.04$%; $n = 3$; $p = 0.156$). *B*, Both the response to 1 mM glutamate and that to 10 mM GABA were only weakly reduced by 100 μM TACA applied through the control tube (response to glutamate reduced to 69.7% of control with $\sigma = 6.51$%; that to GABA reduced to 71.3% of control with $\sigma = 5.69$%; $n = 3$; $p = 0.755$). *C*, Hypotaurine applied through the control tube blocked both the response to 1 mM glutamate and that to 10 mM GABA (response to glutamate reduced to 19.2% of control with $\sigma = 11.3$%; that to GABA reduced to 21.7% of control with $\sigma = 2.89$%; $n = 3$, $p = 0.729$). *D*, CACA (200 μM) applied through the control tube only weakly blocked the response to both 1 mM glutamate and 10 mM GABA (response to glutamate reduced to 82% of control with $\sigma = 3.46$%; that to GABA reduced to 79.7% of control with $\sigma = 1.53$%; $n = 3$; $p = 0.346$).

response to glutamate seen in the medial and buccal cells (see figure legend for a statistical evaluation of these findings). This suggests that it is mediated by the glutamate-sensitive Cl channel and that the GABA-gated Cl receptor–channel complex is not expressed in these cells.

As was seen in Figure 6*A*, GABA activation of the glutamate receptor yields a response that is much smaller than that elicited by the same concentration of glutamate (for $n = 3$, the average response to 1 mM GABA is 3.83% that of the response to 1 mM glutamate). Nevertheless, there appears to be little difference in the concentrations of the two amino acids needed to elicit a threshold response via the glutamate receptor. When testing 20 and 50 μM glutamate and 20 and 100 μM GABA on the pleural cells, no response could be elicited with 20 μM of either agonist. A response was elicited by 50 μM glutamate in all five cells (average of 1268 pA), whereas 100 μM GABA elicited a response (average of 142 pA) in four of the five cells. The fifth cell responded to 200 μM, the next higher concentration tested.

GABA activation of the glutamate receptor explains the observation of cross-desensitization

Cross-desensitization between transmitter-activated Cl channels has been described frequently in both vertebrate and invertebrate preparations (invertebrate, Franke et al., 1986; Zufall et al., 1988; King and Carpenter, 1987, 1989; vertebrate, Barker and McBurney, 1979; Choquet and Korn, 1988; Krishtal et al., 1988; Baev et al., 1992; Lewis and Faber, 1993; Trombley et al., 1999). In view of the finding that GABA can activate the glutamate receptor mediating the Cl-dependent response, it is not surprising to see that

GABA has a desensitizing–blocking effect on the response to glutamate. Even at low concentrations (e.g., 1 and 10 μM, which induce no visible current in cells having only the glutamate receptor), GABA significantly reduces the glutamate response in all cells expressing the glutamate receptor (pleural, medial, and buccal cells) (Fig. 8, recordings from a pleural cell). After application of a given GABA concentration, the glutamate response was allowed to stabilize, and all measurements describing the diminution of the glutamate response by GABA refer to such "stabilized" responses. The diminution in the glutamate response increased with increasing concentrations of GABA (Fig. 8). No statistical differences were seen in the percent diminution in these stabilized responses for a given concentration of GABA, whether or not that concentration had been preceded by another, weaker concentration or applied singly (*t* tests comparing such experiments yielded $p = 0.385$ for 10 μM GABA and $p = 0.305$ for 100 μM GABA). Consequently, for describing the diminution in the glutamate response as a function of GABA concentration, all data for a given concentration have been lumped together. The response to 1 mM glutamate in 1 μM GABA was reduced to 85.1% of the control amplitude ($\sigma = 7.16$%; $n = 7$), in 10 μM GABA to 70.2% ($\sigma = 11.8$%; $n = 7$), and in 100 μM GABA to 34.9% ($\sigma = 4.22$%; $n = 8$).

It should be mentioned at this point that changes in E_{Cl} cannot account for the GABA-induced diminutions in the amplitude of the glutamate response: (1) E_{Cl} was continually monitored, and evaluations of the glutamate response were always made at a holding potential of a constant distance from E_{Cl}; (2) application of GABA had the same desensitizing effect on the glutamate response at E_{Cl} and at less negative potentials; (3) GABA had a marked

Effect of 1, 10, and 100 μM GABA on response to 1 mM Glutamate
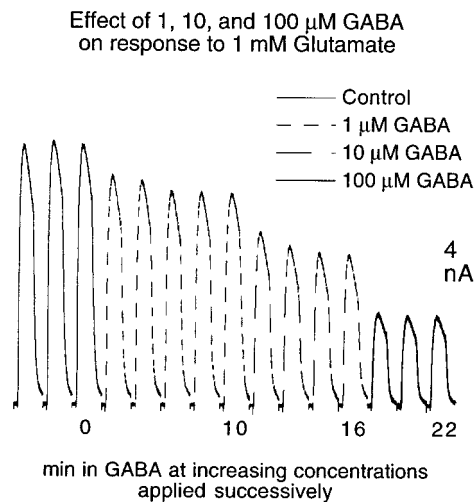
Figure 8. Effect of GABA on the response of a pleural cell to 1 mM glutamate recorded in two-electrode voltage clamp. Increasing concentrations of GABA (1, 10, and 100 μM) applied successively through the control tube caused an increasing diminution in the response of a pleural cell to 1 mM glutamate. Notice that no GABA-induced change in holding potential can be detected. The cell was held at 20 mV less negative than E_{Cl}. See Results for a detailed résumé of related experiments. The response to 1 mM glutamate in 1 μM GABA was reduced to 85.1% of the control amplitude ($\sigma = 7.16\%$; $n = 7$), in 10 μM GABA to 70.2% ($\sigma = 11.8\%$; $n = 7$), and in 100 μM GABA to 34.9% ($\sigma = 4.22\%$; $n = 8$).

desensitizing effect at concentrations that elicited no current in the cell in which the glutamate responses were being evaluated; and (4) in many neurons (e.g., the medial cells), whereas a Cl current gated by the GABA receptor blocked the glutamate response, a similar Cl current gated by the glutamate receptor failed to affect the GABA response.

Likewise, it should be pointed out that an action of GABA on presynaptic neurons can be rejected as a possible source for the cross-desensitization effect. A number of similar experiments (data not shown) performed here on isolated cells in culture confirmed completely the findings from cells in the intact ganglion. Furthermore, the experiments of King and Carpenter (1987) and Oyama et al. (1990), both of which showed similar GABA-induced diminution in glutamate responses, were performed on isolated cells.

Although GABA clearly desensitizes–blocks the glutamate-induced Cl-dependent response, it has no effect on either the glutamate-induced cationic or the glutamate-induced K-dependent response (three experiments made for each response type; data not shown).

Cells in which the GABA but not the glutamate receptor is expressed reveal that glutamate cannot activate the GABA receptor

There are identifiable cells in the cerebral ganglion in which GABA, but not glutamate, elicits a Cl-dependent response. The response to glutamate in these cells is a pure cationic one and that to GABA is a pure Cl-dependent one, as illustrated in Figure 9A, *first column*. Although, as has been shown above, GABA can effectively activate (Figs. 6, 7) and block (Fig. 8) the glutamate Cl-dependent response, the opposite is not true. In Figure 9A, glutamate added to the control tube completely desensitized the glutamate excitatory response in the cerebral cells but had no effect on the GABA Cl-dependent response (Oyama et al., 1990). Even at a 10 mM concentration, glutamate failed to elicit an increase in Cl conductance in these cells and failed to diminish the GABA-induced Cl-dependent response (mean response to 1 mM GABA in the presence of 10 mM glutamate for four experiments was 100% of control; $\sigma = 2.36\%$).

The failure of glutamate to block the Cl-dependent response to GABA can also be seen in the medial cells in which both glutamate and GABA receptors mediating Cl-dependent responses are ex-

pressed (Fig. 9B) (mean response to 1 mM GABA in the presence of 1 mM glutamate for four experiments was 102% of control; $\sigma = 2.46\%$). See the figure legend of Figure 9, A and B, for a more detailed description.

It was mentioned above when discussing the agonist activity of β-alanine on the glutamate receptor that β-alanine could, at higher concentrations, activate the GABA receptor. The cerebral cells used for Figure 9A permitted the confirmation of this activation, because β-alanine in these cells elicited a weak voltage-dependent response that was unaffected by 1 mM glutamate and markedly blocked by 100 μM TACA, all of these findings consistent with the hypothesis that β-alanine in these cells was activating, albeit weakly, the GABA receptor. Because the response to 1 mM β-alanine in these cells was very weak (~3% of that to 1 mM GABA), the analyses of this response were made using 10 mM β-alanine. The amplitude of the response in the cerebral cells to 10 mM β-alanine was ~21% of that to 1 mM GABA ($n = 3$; $\sigma = 4.77\%$). The mean percent inward to outward current ($n = 3$) for β-alanine in these cells was indistinguishable from that for GABA (51.3 and 52.3%, with $\sigma = 2.52\%$ and 1.15%, for β-alanine and GABA, respectively, measured at 20 mV ± E_{Cl}). Comparing these results with the β-alanine response in cells having only the glutamate receptor (Fig. 3) reveals the preferential, but incompletely selective, activation by β-alanine of the glutamate receptor.

In cells in which the glutamate receptor is highly expressed, the response to 1 mM GABA often contains a detectable component mediated by the glutamate receptor

In light of the finding that GABA can activate the glutamate receptor (Figs. 6, 7), it is interesting to turn our attention to the differences in kinetics of the response to 1 mM GABA in the medial versus buccal neurons. The expression of the glutamate-gated Cl channel is much greater in the buccal than in the medial cells, with the response to 1 mM glutamate being typically approximately twice that to 1 mM GABA in the buccal cells, and vice versa in the medial cells.
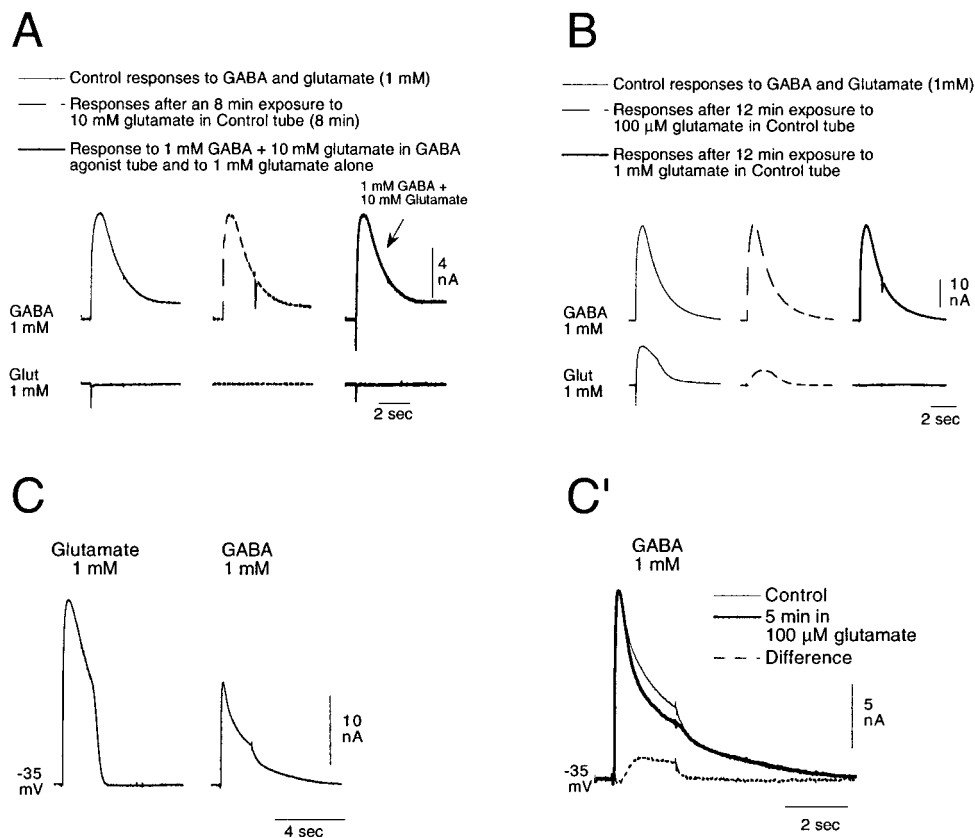
In the medial cells, the decay of the response to 1 mM GABA is almost always smooth, with no change in rate of decay manifested at the end of the 2 sec GABA pulse (Figs. 1A, 9B). At this concentration, the decay appears to depend exclusively on the "off rate" of the desensitized GABA receptor. That is not the case for the response to glutamate, which shows a sharp change in decay rate when the control flow replaces the agonist flow.

In the buccal cells, on the other hand, the response to 1 mM GABA often shows an "atypical" decay (Figs. 1B, 9C). As can be seen in Figure 9C', the "hump" sometimes seen in the GABA response of the buccal cells disappears with a "desensitizing" application of 100 μM glutamate in the control tube, leaving a decay typical of the GABA response mediated by the voltage-dependent, sulfate-sensitive GABA receptor. The transformation in the GABA response in the presence of 100 μM glutamate was evaluated by approximating the integrals of the GABA responses in the presence and absence of 100 μM glutamate and subtracting these integrals. The mean reduction in the control GABA response thus obtained was 10.3% ($n = 6$; $\sigma = 3.94\%$). This 10% diminution by 100 μM glutamate of the GABA response in some buccal cells should be compared with the total failure of even 10 mM glutamate to affect the GABA response in the cerebral cells, which are lacking the glutamate receptor. The element of the response desensitized by 100 μM glutamate (Fig. 9C', *Difference trace*) can hence be attributed to a current resulting from the activation by GABA of the glutamate receptor.

Comparison of the amino acid receptors with the two ACh receptors mediating Cl-dependent responses

It was shown recently by Kehoe and McIntosh (1998) that two pharmacologically distinct receptors mediate the ACh-induced increase in Cl conductance. One receptor mediates a rapidly desensitizing response, and the other mediates a sustained response. These Cl-dependent cholinergic responses are seen, alongside the

A

—— Control responses to GABA and glutamate (1 mM)

— - Responses after an 8 min exposure to
10 mM glutamate in Control tube (8 min)

—— Response to 1 mM GABA + 10 mM glutamate in GABA
agonist tube and to 1 mM glutamate alone

B

—— Control responses to GABA and Glutamate (1mM)

— - Responses after 12 min exposure to
100 μM glutamate in Control tube

—— Responses after 12 min exposure to
1 mM glutamate in Control tube

C

C'

Figure 9. The Cl-dependent response to GABA measured in two-electrode voltage clamp in a cerebral cell (*A*), a medial cell (*B*), and a buccal cell (*C, C'*) in the absence or presence of glutamate. *A,* In the cerebral cell control records, GABA elicits a pure Cl-dependent response (*top trace*), whereas glutamate elicits a pure cationic current (*bottom trace*). Glutamate (10 mM) flowing through the control tube (*middle column*) desensitized the glutamate cationic current but failed to affect the GABA response. The inward-going current seen on the downswing of the GABA response (*middle GABA record*) reveals a recovery from desensitization of the glutamate cationic response, elicited here by the glutamate flowing from the control tube at the end of the 2 sec application of the glutamate-free, 1 mM GABA solution. In the *third column*, it can be seen that, even when 10 mM glutamate was added to the 1 mM GABA solution, there was no blocking effect of glutamate on the GABA response. Note that the response to the glutamate–GABA agonist application is biphasic, consisting of a cationic response elicited by 10 mM glutamate and a Cl-dependent response elicited by 1 mM GABA. *B,* Responses to GABA (*top trace*) and glutamate (*bottom trace*) recorded from a medial cell in the absence and presence of 100 μM or 1 mM glutamate flowing through the control tube. Note that both the cationic and Cl-dependent response to glutamate were desensitized by the glutamate in the control tube, whereas no desensitizing or blocking effect of the glutamate can be detected on the response to GABA. The presence of 1 mM glutamate in the control tube can be detected on the downswing of the GABA response (*final record* in *B*) because the return of the control tube containing 1 mM glutamate elicited a weak cationic current after the 2 sec application of glutamate-free GABA-containing solution. *C,* Responses of a buccal cell to glutamate and GABA. *C',* Effect of the application of 100 μM glutamate in the control tube on the response of the buccal cell to GABA. Note that the atypical decay of the GABA response, i.e., a change in rate of desensitization at the end of the 2 sec application, disappeared in the presence of 100 μM glutamate, presumably because glutamate desensitized the element of the GABA response that was mediated by the glutamate receptor (*Difference*).

glutamate- and GABA-induced Cl responses, in the medial cells and in selected buccal cells but are not always associated with them in other cells. For example, on the small pleural cells that express no GABA receptor but do express the glutamate receptor mediating a Cl-dependent response, ACh elicits a pure cationic response (Ascher et al., 1978).

The ACh-activated conductance, like that activated by glutamate, fails to show a voltage dependence over the range of holding potentials studied here (Kehoe and McIntosh, 1998), and neither of the two Cl-dependent responses elicited by ACh was affected by intracellular sulfate ions (Fig. 10*A*). Whereas the response to ACh after perfusion with a sulfate-based pipette solution remained essentially unchanged (98.8% of control; $\sigma = 3.4\%$), that to GABA was markedly reduced (35.7% of control; $\sigma = 19\%$; $n = 3$; $p = 0.005$).

The pharmacology of the Cl-dependent ACh responses has been described in detail by Kehoe and McIntosh (1998) in which the effects of 100 μM TC and strychnine can be seen. MLA, like α-bungarotoxin (Kehoe and McIntosh, 1998), blocked both of the cholinergic Cl-dependent responses but had no effect on either the glutamate- or GABA-induced responses (Fig. 10*B*, records taken from a buccal cell). The ACh response in MLA, measured at the time corresponding to the peak of the control ACh response, was only 1% of the control ($n = 3$; $\sigma = 0.5\%$); the responses to glutamate and GABA in the presence of MLA were 103% ($\sigma = 0.58\%$) and 102% ($\sigma = 3.4\%$) of control, respectively, for the two sets of responses. Neither glutamate nor GABA desensitized–blocked the ACh responses (Fig. 10*C,C'*), and ACh, in turn, had no desensitizing–blocking effect on the responses to the two amino acids (Fig. 10*D,D'*) (Ikemoto and Akaike, 1988). These latter experiments, however, had to be performed in cells that did not

respond to ACh with a strong nondesensitizing Cl current (Kehoe and McIntosh, 1998). In a cell in which such persistent outward ACh-induced currents exist, the application of either glutamate or GABA solutions (which did not contain ACh) would cause a change in current because of the washing off of ACh, giving a false estimation of either the GABA- or glutamate-activated current. Statistical analyses of the results illustrated in Figure 10 are presented in the figure legend.

DISCUSSION

The experiments described here have shown that the receptor–channel complexes mediating the Cl-dependent responses to glutamate, GABA, and ACh in *Aplysia* neurons are independent entities. Nevertheless, cross-desensitization does occur between the GABA and glutamate responses and was shown to result exclusively from the ability of GABA to interact with the glutamate receptor.

These findings are compatible with the results obtained by King and Carpenter (1987) but not with their conclusion that the glutamate and GABA responses gate the same receptor–channel complex. On the other hand, our results are incompatible with those of Ikemoto and Akaike (1988), who saw no interactions between the GABA and glutamate responses. These same authors participated in a later study (Oyama et al., 1990) in which interactions were seen, and GABA was shown to elicit both a low-threshold and a high-threshold response. In the 1990 study (Oyama et al.), the authors proposed that the high-threshold GABA response they observed was attributable to the activation, by GABA, of the glutamate receptor, a hypothesis confirmed by the results presented here.

A

Progressive and selective block of GABA response recorded with K$_2$SO$_4$ inside solution

B

ACh 100 µM   Glutamate 250 µM   GABA 500 µM

Figure 10. Comparison of the amino acid Cl-dependent responses with the cholinergic Cl-dependent response. *A*, Recordings from a buccal cell in whole-cell voltage clamp with K$_2$SO$_4$-based internal solution of responses to 500 µM GABA and 500 µM ACh. These records were taken at a holding potential 20 mV less negative than E$_{Cl}$ and show that the whole-cell perfusion with a sulfate-containing internal solution selectively diminished the GABA response. *B*, MLA blocked the ACh-induced Cl-dependent responses at 2 µM but had no effect, even at 10 µM, on the responses to either glutamate or GABA. These responses were recorded from buccal cells in two-electrode voltage clamp. *C, C'*, Neither 1 mM glutamate (*C*) nor 1 mM GABA (*C'*) added to the control tube affected the response to 200 µM ACh recorded in two-electrode voltage clamp from a buccal cell. The peak ACh response in the presence of 1 mM glutamate was 99.7% of the control (σ = 3.9%; *n* = 5), and the peak ACh response in the presence of 1 mM GABA was 97.9% of the control (σ = 3.47%; *n* = 5). *D, D'*, ACh (200 µM) had no effect on either the glutamate (*D*) or GABA (*D'*) response recorded from a buccal cell in two-electrode voltage clamp. The mean peak responses to both glutamate and GABA in the presence of 200 µM ACh were 99.7% of control (σ = 2.08 and 1.53% for glutamate and GABA, respectively; *n* = 3).

C, C'

D, D'

Glutamate 1 mM   GABA 1 mM
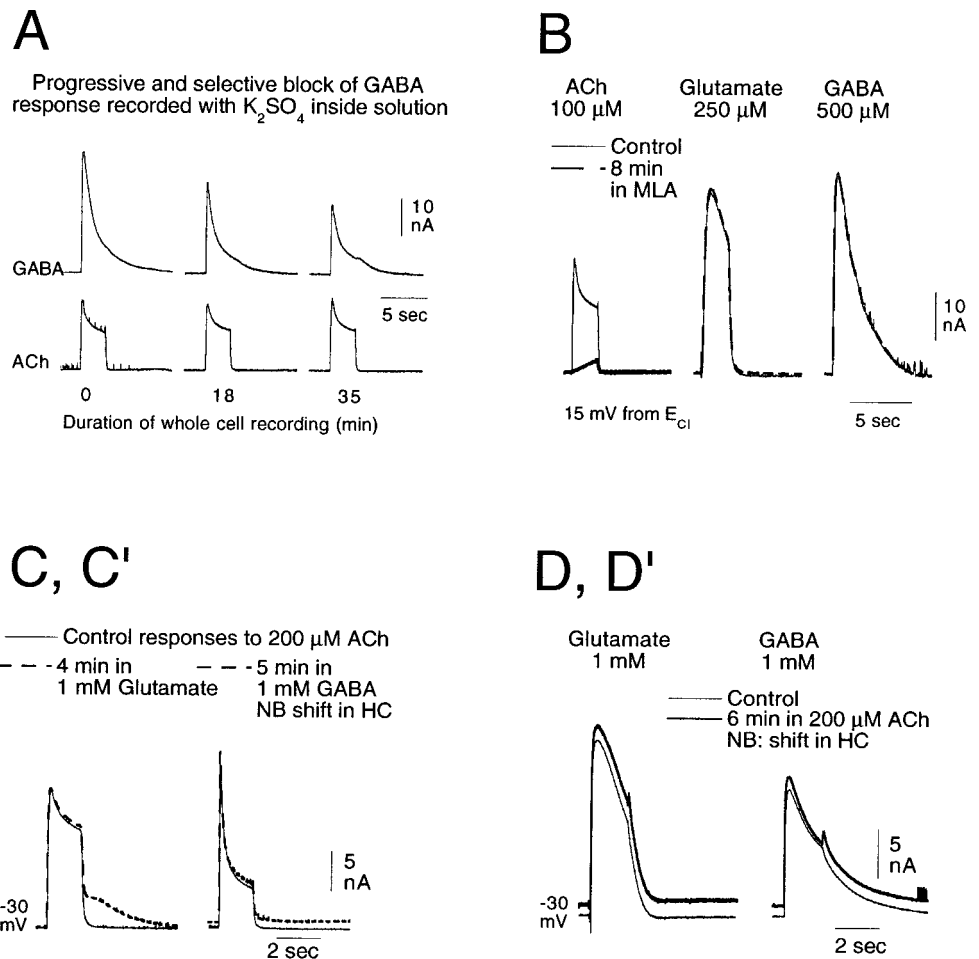
Glutamate and GABA activation of the glutamate receptor

In our study, the concentrations of GABA and glutamate required for threshold activation of the glutamate receptor were found to be remarkably similar. That is, to consistently obtain a discernible response to glutamate, a concentration of 50 µM was needed; GABA was shown to activate the same receptor at only twice that concentration. In contrast, the average currents elicited by 1 and 10 mM GABA in cells having only the glutamate receptor (Fig. 6*A,B*) were only ~4 and 36%, respectively, of the response of the same cell to 1 mM glutamate.

The low concentrations of GABA that are able to affect the sensitivity of the glutamate receptor (Fig. 8) suggest that, if both GABA and glutamate receptors are coexpressed on the same subsynaptic membrane, liberation of GABA on that membrane would be able to cross-desensitize the glutamate receptor. The protocol used here did not permit an evaluation of the kinetics of the GABA-induced desensitization. However, from the work of Oyama et al. (1990), it is clear that a 10 sec application of 300 µM GABA is sufficient to cause a very marked diminution in the response to the same concentration of glutamate.

Although the data strongly suggest that direct activation by GABA of the glutamate receptor mediates the cross-desensitization process, the molecular mechanism by which GABA and β-alanine desensitize the glutamate receptor remains to be established. The fact that the response to glutamate shows auto-desensitization suggests that there exists a "desensitized state" of the glutamate receptor, and GABA and β-alanine may act by favoring the transition toward this state. However, the strength of the cross-desensitization appears to be correlated with neither the strength of activation by the "inhibiting" compound nor the rate of desensitization it induces in the "inhibited" receptor. The inhibi-

tion induced by GABA and β-alanine may be, to some degree, a partial agonist effect. That is, GABA and β-alanine may compete with glutamate for binding to the same site and inhibit the glutamate response because they induce a much lower probability of opening of the channels.

Relationship between the invertebrate glutamate receptor and the vertebrate glycine receptor

The invertebrate glutamate receptors gating Cl channels that have thus far been cloned show greater resemblance to the vertebrate glycine receptor than to any other receptor (Cully et al., 1994; Vassilatis et al., 1997). Furthermore, no one has reported evidence for a glycine-gated Cl channel in invertebrate neurons. Conversely, the only glutamate-gated Cl channels thus far seen in vertebrates appear to be associated with glutamate transporters (for review, see Fairman and Amara, 1999). It thus appears reasonable to relate the invertebrate glutamate receptors to the vertebrate glycine receptors. Despite the failure of the glutamate receptor studied here to be blocked by 100 µM strychnine, its sensitivity to β-alanine reinforces this parallel (Wu et al., 1993; Jonas et al., 1998).

A recent study evaluating the differential activation by glycine and GABA of a recombinant homomeric glycine receptor revealed a 200- to 300-fold difference in the EC$_{50}$ values of the activation curves for the two amino acids (Fucile et al., 1999). In contrast, in a study measuring the activity of GABA and glycine on a recombinant homomeric human GABA ρ receptor, the difference in sensitivity to the two amino acids was much greater, on the order of 10,000-fold (Calvo and Miledi, 1995).

In previously published experiments on isolated *Aplysia* neurons, concentration–response curves were obtained for the glutamate response (Ikemoto et al., 1988b) and for the high-threshold GABA response hypothesized to be mediated by the glutamate receptor

(Oyama et al., 1990) and yielded EC_{50} values of 130 and ~6 mM, respectively. The difference in EC_{50} values is higher (~46-fold) than that between the threshold values referred to above (twofold), probably because of a smaller Hill coefficient of the GABA response. Yet the difference is surprisingly small compared with the failure of even very high concentrations of glutamate (10 mM) to activate the GABA receptor, for which the EC_{50} for activation by GABA was estimated to be 64 μM (Ikemoto et al. (1988a).

These studies together suggest a high selectivity of both the vertebrate and invertebrate GABA receptor, whereas both the vertebrate glycine receptor and the invertebrate glutamate receptor show some sensitivity to GABA.

However, many molecular variations of GABA and glycine receptors exist in vertebrate neurons, and this diversity may explain the wide range of findings concerning cross-desensitization between these ligand-gated Cl channels. For example, whereas Choquet and Korn (1988) found no interaction between GABA and glycine responses in cultured chick spinal neurons, Baev et al. (1992), studying lamprey spinal cord neurons, found that there was total cross-desensitization between the responses to the same amino acids. They concluded that only one receptor–channel complex, responding to both amino acids, was expressed on these neurons. Recently, Trombley et al. (1999) described all possible cross-desensitization profiles between GABA and glycine responses in rat olfactory bulb neurons in primary culture, with the profile depending on the neuron studied. Their findings suggest that more than one type of receptor for each amino acid is expressed in these cells.

A similar diversity must exist in invertebrate neurons because, whereas the glutamate receptor of molluscs is desensitized by even low concentrations of GABA, no interaction between the two amino acid responses is seen in some invertebrate ganglionic neurons (lobster, Cleland and Selverston, 1998; crab, Duan and Cooke, 2000).

The ACh-activated receptor–channel complex

Although we have no data that permit us to discriminate between the properties of the ACh-gated channel(s) (Kehoe and McIntosh, 1998) and those of the glutamate-gated channel, the fact that the ACh and glutamate receptor channel complexes are not consistently coexpressed permits us to conclude that, even if the channels per se are identical, the receptor–channel complexes for the different transmitters are distinct molecular entities.

$GABA_A$ or $GABA_C$?

The object of the pharmacological evaluation of the GABA receptor in this study was primarily to differentiate it from the receptor underlying the glutamate response. However the finding that TACA is a more effective agonist than CACA suggests that the molluscan GABA receptor resembles the $GABA_C$ rather than the $GABA_A$ receptor (Johnston, 1996), a conclusion likewise drawn from previous studies showing that the molluscan receptor is not blocked by bicuculline and is not potentiated by either diazepam (Ikemoto et al., 1988a; Kim and Takeuchi, 1990) or pentobarbital (Ikemoto et al., 1988a) (but see Kim and Takeuchi, 1990). The molluscan GABA receptor desensitizes rapidly, a characteristic associated with the $GABA_A$ receptor. However, rapidly desensitizing $GABA_C$ receptors have been described recently in vertebrates (Han et al., 1997; Boué-Grabot et al., 2000).

Sulfate blockade of the GABA channel

The observation made here that intracellular sulfate ions selectively block the GABA response was exploited in these experiments as a "tool" for differentiating the GABA receptor–channel complex from the glutamate and ACh receptor–channel complexes, and the means by which this blockade occurs have not as yet been examined. However, the block is clearly independent of voltage over the range tested (Fig. 2*B*). Furthermore, a differential permeability of the GABA receptor channel to sulfate ions cannot explain its differential sensitivity to internal sulfate. It was shown

previously (Kehoe, 1972a) that sulfate cannot pass through the channels opened by ACh, and it was shown here that partial substitution of intracellular Cl by sulfate alters the inversion potential of all three agonist-induced responses to the same extent.

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