Interactions Between GABA and Glycine at Inhibitory Amino Acid Receptors on Rat Olfactory Bulb Neurons

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Trombley, Paul Q., Brook J. Hill, and Michelle S. Horning. Interactions between GABA and glycine at inhibitory amino acid receptors on rat olfactory bulb neurons. J. Neurophysiol. 82: 3417–3422, 1999. Whole cell voltage-clamp electrophysiology was used to examine interactions between GABA and glycine at inhibitory amino acid receptors on rat olfactory bulb neurons in primary culture. Membrane currents evoked by GABA and glycine were selectively inhibited by low concentrations of bicuculline and strychnine, respectively, suggesting that they activate pharmacologically distinct receptors. However, GABA- and glycine-mediated currents showed cross-inhibition when the two amino acids were applied sequentially. Application of one amino acid inhibited the response to immediate subsequent application of the other. In the majority of neurons, GABA inhibited subsequent glycine-evoked currents and glycine inhibited subsequent GABA-evoked currents. In a small proportion of neurons, however, GABA inhibited glycine-evoked currents but glycine had little effect on GABA-evoked currents. The reverse was true in other neurons, suggesting that alterations in chloride gradients alone did not account for the cross-inhibition. Furthermore, no cross-inhibition was observed between GABA- and glycine-evoked currents in some neurons. The amplitude of the current evoked by the coapplication of saturating concentrations of GABA and glycine in these neurons was nearly the sum of the currents evoked by GABA and glycine alone. In contrast, the currents were not additive in neurons demonstrating cross-inhibition. These results suggest that olfactory bulb neurons heterogeneously express a population of inhibitory amino acid receptors that can bind either GABA or glycine. Interactions between GABA and glycine at inhibitory amino acid receptors may provide a mechanism to modulate inhibitory synaptic transmission.

INTRODUCTION

GABA and glycine are the dominant inhibitory neurotransmitters in the vertebrate CNS and mediate the fast synaptic components of inhibitory transmission. The traditional view is that glycineric neurotransmission is restricted to the spinal cord and brain stem and GABA dominates inhibitory transmission in higher brain regions such as the cortex (for reviews see MacDonald and Olsen 1994; Nicoll et al. 1990; Stepheson and Dolphin 1989). However, experimental evidence suggests less clear distinctions. For example, in many brain regions glycineric and GABAergic terminals and/or receptors are colocalized (Ottersen et al. 1988; Todd and Sullivan 1990; Triller et al. 1987; Yazulla and Yang 1988). Furthermore, gephyrin, a protein believed to be selectively associated with glycine receptors and involved in the stabilization of glycine receptors at the postsynaptic membrane (Kirsch et al. 1993), recently has been reported to also be associated with GABA<sub>Δ</sub> receptor subunits in the rat olfactory bulb (Giustetto et al. 1998). This suggests that GABA and glycine receptors may share some common structural components. Other studies have demonstrated the corelease of GABA and glycine (Jonas et al. 1998) and even nonselective binding of GABA and glycine to inhibitory amino acid receptors (e.g., Baev et al. 1992; Lewis and Faber 1993).

The majority of the electrophysiological evidence supports the notion that GABA is the primary inhibitory transmitter in the vertebrate olfactory bulb. This notion is based primarily on the results of pharmacological analyses of inhibitory synaptic potentials (IPSPs). However, Jahr and Nicoll (1982) have reported that not all of the early component of the IPSP can be blocked by the GABA receptor antagonist, bicuculline. This result is significant in light of our recent results which demonstrate that glycine receptors can be blocked by picrotoxin, but are bicuculline resistant (Trombley and Shepherd 1994). This may suggest that some of the bicuculline-resistant component of the IPSPs observed by Jahr and Nicoll (1982) was mediated by the synaptic release of glycine and activation of postsynaptic glycine receptors.

Evidence suggests that glycine may contribute to inhibitory transmission in the olfactory bulb. Some investigators have reported that glycine immunoreactivity is concentrated in the external plexiform layer (EPL), the neuropil surrounding mitral cell bodies, and in periglomerular cells (Pourcho et al. 1992; Schell et al. 1997; van den Pol and Gorcs 1988). These investigators also observed strong glycine-receptor immunoreactivity throughout the EPL and mitral cell layer, with much weaker immunoreactivity in the glomerular layer and granule cell layer. Glycine-receptor immunoreactivity was most evident surrounding presumptive mitral cells. These results are supported by in situ hybridization studies that have demonstrated the expression of glycine receptor subunit mRNAs in the olfactory bulb (Malosio et al. 1991).

Glycine-receptor and GABA<sub>Δ</sub>-receptor ion channels have nearly identical ionic selectivity and single-channel subconductance substates despite different dwell times in these substates (Bormann et al. 1987). Structural studies, however, demonstrate that although they share nearly identical amino acid sequences, GABA<sub>Δ</sub> and glycine receptors have no subunits in common and are derived from different genes (Betz 1990). However, there have been several reports suggesting interactions between GABA and glycine and inhibitory amino acid receptors (Baev et al. 1992; Barker and McBurney 1979; Lewis and Faber 1993). Further support for potential interactions between GABA and glycine comes from the recent demonstra-

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tion in rat spinal cord slices that GABA and glycine can be coreleased from the same synapses and even from individual synaptic vesicles (Jonas et al. 1998).

In the present study, we use whole cell patch-clamp electrophysiology to provide evidence supporting the notion that olfactory bulb neurons may express populations of inhibitory amino acid receptors that can bind both GABA and glycine.

METHODS

Neuronal cultures

The care and use of animals used for these experiments followed USDA guidelines and protocols approved by our institutional Animal Care and Use Committee. The methods used for preparation of primary cultures of olfactory bulb neurons are reported in detail elsewhere (Trombley and Blakemore 1999). The olfactory bulbs were dissected from rat pups ranging in age from E18 to P2. The meninges were removed and the bulbs cut into small pieces and incubated at 37°C for 1 h in a low-calcium buffer containing 200 units of papain. The bulb tissue was dissociated into a single-cell suspension by gentle trituration and plated in 35 mm culture dishes onto a confluent monolayer of previously prepared olfactory bulb astrocytes. The cell density was ~300,000 cells/dish. The neuronal growth media contained 10% serum extender (Serum Extender, Collaborative Research). Overgrowth of back-serum (Gibco, USA), 6 gm/l glucose, and a nutrient supplement density was monolayer of previously prepared olfactory bulb astrocytes. The cell trituration and plated in 35 mm culture dishes onto a confluent monolayer of previously prepared olfactory bulb astrocytes. The cell density was ~300,000 cells/dish. The neuronal growth media consisted of 92.5% minimal essential medium (MEM), 7.5% fetal bovine serum (Gibco, USA), 6 gm/l glucose, and a nutrient supplement (Serum Extender, Collaborative Research). Overgrowth of background cells was prevented by adding cytosine-β-D-arabinofuranoside (10^{-5} M) one day after plating neurons.

Electrophysiology

Whole cell voltage-clamp recordings were made at room temperature from olfactory bulb neurons after 2–14 days in culture. The 35-mm culture dish was used as the recording chamber and was perfused at 0.5–2.0 ml/min with a bath solution containing (in mM) 162.5 NaCl, 2.5 KCl, 2 CaCl₂, 10 HEPES, 10 glucose, and 1 MgCl₂. The pH was adjusted to 7.3 with NaOH. The final osmolarity was 325 mosM. Patch electrodes pulled from borosilicate glass to a final electrode resistance of 4–6 MΩ. These electrodes were filled with a solution containing (in mM) 145 CsCl, 1 MgCl₂, 10 HEPES, 4 Mg-ATP, 0.5 Mg-GTP, and 1.1 EGTA (pH 7.2, osmolarity 310). Drugs were diluted in the bath solution and applied using a gravity-fed flow-pipe perfusion system assembled from an array of 600-μm OD, square glass barrels. The flow pipes were positioned near the neuron using an electronic manipulator (Warner Instruments) and flow was controlled with pinch clamps. The speed of solution changes allowed peak drug responses to occur within 100–200 ms. For experiments examining cross-inhibition, current amplitudes for the first and second agonists in a series were measured at their peaks. The first agonist in a series contributes little to the peak amplitude of the second because the second agonist displaces the first. Neurons were always perfused using a barrel containing bath solution except during application of drugs. In the text and figures “control” solution is the bath solution. Applied agonists and antagonists were GABA, glycine, bicuculline, and strychnine (Sigma).

Experimental procedure

To examine membrane currents evoked by GABA and glycine, whole cell recordings were made from mitral/tufted cell bodies with an Axoclamp 2B amplifier (Axon Instruments) using either the discontinuous (switch frequency of 10–15 kHz) or continuous single-electrode, voltage-clamp mode. Membrane currents were filtered at 1–3 kHz, digitized at 5–10 kHz, and analyzed using AxoGraph and AxoData software (Axon Instruments).

RESULTS

Selective antagonists were used to determine whether GABA and glycine activated pharmacologically distinct inhibitory amino acid receptors. All neurons examined responded to both GABA and glycine. Under voltage-clamp at −60 mV, flow-pipe application of a saturating concentration of glycine (1 mM) evoked an inward current that desensitized during the period of application. Saturating concentrations were used to ensure that all glycine-sensitive receptors were activated. Co-application of 1 μM strychnine blocked most of the current evoked by glycine [94 ± 4% reduction (SD), n = 12, Fig. 1A]. In contrast, bicuculline, up to 30 μM (the highest concentration examined), had no affect on currents evoked by 1 mM glycine (n = 7, Fig. 1C).

Flow-pipe application of a saturating concentration of GABA (1 mM) also evoked a desensitizing inward current. In contrast to the glycine-evoked current, the current evoked by GABA was usually unaffected by 1 μM strychnine (2 ± 5% reduction, n = 9, Fig. 1B). However, the GABA<sub>A</sub> receptor antagonist, bicuculline (30 μM), inhibited the GABA-evoked current (72 ± 16% reduction, n = 8, Fig. 1D). The effects of simultaneous application of strychnine or bicuculline on peak GABA- or glycine-evoked currents were similar to their effects on the sustained components shown in Fig. 1.

Sequential application of saturating concentrations of GABA and glycine were used to examine potential cross-sensitivity among inhibitory amino acid receptors for these agonists. In most neurons (~90%, n = 27), preapplication of GABA dramatically reduced the amplitude of the current evoked by the subsequent application of glycine (Fig. 2A, 79 ± 12% reduction, n = 27). However, in a small percentage (~15%, n = 4) of the neurons in which GABA reduced the response to glycine, preapplication of glycine had little effect on the amplitude of the response to GABA (Fig. 2A). In other neurons, the opposite effect was observed. Preapplication of
Glycine inhibited the amplitude of the response to GABA (Fig. 2B, 75 ± 18% reduction, n = 32) whereas preapplication of GABA had little effect on the subsequent response to glycine in a small percentage of these neurons (≈10%, n = 3, Fig. 2B).

To examine further whether GABA and glycine acted on a shared population of receptors, we compared the results from cross-inhibition with the sum of the responses to individual applications of GABA and glycine. Figure 3A shows the data from a neuron in which preapplied glycine reduced the current evoked by subsequent application of GABA. In this neuron, preapplied GABA had relatively little effect on the current evoked by subsequently applied glycine. The data shown in

**FIG. 2.** Cross-inhibition between glycine and GABA receptors. A: in some OB neurons GABA cross-inhibited the glycine-evoked current (top trace) whereas in the same neuron glycine had little effect on the GABA-evoked current (bottom trace). The 1st part of each current trace reflects the control amplitude for either GABA or glycine. B: in other OB neurons, GABA had little effect on the glycine-evoked current (top trace) whereas in the same neuron glycine cross-inhibited the GABA-evoked current (bottom trace).

**FIG. 3.** Currents evoked by glycine and GABA are not additive when glycine and GABA receptors cross-inhibited. A: in this neuron, glycine cross-inhibited the GABA-evoked current (top trace) but GABA did not substantially cross-inhibit the glycine-evoked current (bottom trace). Ba: current evoked by a saturating concentration of glycine. Bb: current evoked by a saturating concentration of GABA. Bc: current evoked by the combination of saturating concentrations of glycine and GABA. Amplitude of the current evoked by glycine alone is marked a; by GABA alone, b; and by the combination of glycine and GABA, c. A drawing of the amplitude expected if the currents were additive is marked a + b.
Fig. 3B illustrates the lack of summation of the currents in response to the coapplication of GABA and glycine. When both agonists were applied simultaneously at saturating concentrations, the current evoked was less than the sum of the currents evoked by saturating concentrations of GABA and glycine alone (Fig. 3B).

Fig. 5. Direction of current flow did not influence cross-inhibition. Reversal potential for chloride was near 0 mV. A: cross-inhibition of the glycine-evoked current, by the preapplication of GABA, was similar at −30 mV (outward Cl− flux) and at +30 mV (inward Cl− flux). Bottom trace: control response to glycine. B: similarly, the cross-inhibition of the GABA-evoked current, by the preapplication of glycine, was similar at −30 mV and at +30 mV. Bottom trace: control response to GABA.
Cross-inhibition did not occur in about 10% of the neurons examined. That is, preapplication of one amino acid did not reduce the amplitude of the current evoked by subsequent application of the other (Fig. 4A, n = 5). In these neurons, the current amplitudes evoked by saturating concentrations of GABA and glycine were additive. The amplitude of the current evoked by simultaneous application of 1 mM glycine and 1 mM GABA was approximately equal to the sum of the amplitudes of the currents evoked by glycine and GABA alone (Fig. 4B).

We next examined the effects of sequential application of GABA and glycine at different holding potentials to determine if the cross-inhibition was affected by the direction of chloride movement. The intracellular and extracellular solutions contained similar chloride concentrations to produce a chloride reversal potential near 0 mV. At −30 mV, GABA and glycine evoked inward currents (outward chloride flux) and at +30 mV they evoked outward currents (inward chloride flux). The degree of cross-inhibition of glycine-mediated currents by GABA (Fig. 5A), or GABA-mediated currents by glycine (Fig. 5B), was similar at both positive and negative holding potentials.

Discussion

In the majority of neurons examined (~90%), application of one inhibitory amino acid (either GABA or glycine) reduced the amplitude of the currents evoked by immediate subsequent application of the other (cross-inhibition). Therefore the results from the present study suggest the possibility that some inhibitory amino acid receptors expressed by olfactory bulb neurons may bind either GABA or glycine.

Although this is the first report of cross-inhibition between GABA and glycine in the olfactory system, it is consistent with several reports by other investigators concerning other brain regions. Barker and McBurney (1979) reported that sustained iontophoretic application of either GABA or glycine in mouse spinal cord neurons depressed the response to subsequent application of the other. The response depression was not associated with a change in the driving force. More recently, Lewis and Faber (1993) reported cross-inhibitory effects between GABA and glycine in rat medullary neurons. Baev et al. (1992) have reported that inhibitory amino acid receptors from lamprey spinal neurons can be gated by either GABA or glycine, show complete cross-inhibition, and are blocked by picrotoxin, strychnine, and bicuculline. They have proposed that inhibitory amino acid receptors in these neurons are composed of a single type of receptor ion channel complex that is sensitive to both GABA and glycine. However, interactions between GABA and glycine at inhibitory amino acid receptors have not been universally reported. Nelson et al. (1977) reported that GABA and glycine do not cross-inhibit each other in mouse spinal neurons and Jonas et al. (1998) reported that coreleased GABA and glycine did not affect GABA-evoked currents. In these neurons, the opposite effect was observed; GABA inhibited glycine-evoked currents but not the reverse.

In some neurons, there was no cross-inhibition in either direction. GABA did not affect glycine-evoked currents and glycine did not affect GABA-evoked currents. In these neurons, the current amplitude evoked by coapplication of saturating concentrations of GABA and glycine was nearly the sum of the amplitudes of the currents evoked by glycine and GABA alone. This is the expected result if GABA and glycine activate distinct populations of receptors.

In neurons in which one amino acid inhibited currents evoked by the other, the current amplitude evoked by coapplication of saturating concentrations of GABA and glycine was less than the sum of the amplitudes of the currents evoked by glycine and GABA alone. This is the expected result if GABA and glycine share some proportion of receptors.

The results from experiments which demonstrate cross-inhibition in only one direction (e.g., GABA inhibits glycine evoked currents but not the reverse) suggest that in some neurons a proportion of glycine-activated receptors (gated by glycine and evoking a membrane current) can bind GABA, but that GABA-activated receptors in these same neurons are unaffected by glycine. Because glycine does not significantly inhibit the current evoked by GABA in these neurons, the proportion of glycine-activated receptors which is affected (inhibited) by GABA must not be gated by GABA. Otherwise this proportion of the GABA-activated current should be sensitive to cross-inhibition by glycine (these receptors should generate a current to GABA and also be affected by glycine). In these instances, GABA may bind to the receptor, and although not evoke a current, prevent glycine from activating the receptor. Alternatively, GABA may bind to the receptor and cause the receptor to switch to a desensitized state.
without spending a significant amount of time in an open state. A similar argument holds for those neurons in which some proportion of GABA-activated receptors can be inhibited by glycine, but that glycine-activated receptors are unaffected by GABA.

It has been proposed that the whole cell recording configuration may not be adequate to “clamp” the intracellular chloride concentration (e.g., Grassi 1992; Huguenard and Alger 1986). Large membrane currents evoked by GABA or glycine could alter the intracellular chloride concentration and, consequently, the driving force. This could “inhibit” the current amplitude to subsequent receptor activation until the intracellular concentration recovered. In our experiments, the lack of effect on cross-inhibition by altering membrane potential suggests that the direction of current flow through the channel was not an important factor. However, this result does not rule out the possibility that the cross-inhibitory effect is due to inadequate “clamping” of the intracellular chloride concentration (intracellular depletion with inward currents, intracellular chloride build-up with outward currents). Furthermore, the heterogeneity in our results could be explained by inadequate concentration clamp in some neurons and adequate concentration clamp in others. Perhaps an adequate concentration clamp occurs when most of the receptor-mediated currents are evoked by somatic receptors and an inadequate clamp occurs in neurons when most of the receptors are dendritic—somewhat analogous to the “space clamp error” of voltage observed with voltage-clamp recording. However, these possibilities do not adequately explain all of the results either, since there was cross-inhibition in only one direction in some neurons (e.g., GABA inhibited glycine, but glycine had no effect on GABA). An inadequate concentration clamp should not affect one population of receptors without affecting the other. The most parsimonious explanation is that some receptors can bind both GABA and glycine.

Our results demonstrate substantial cross-inhibition between currents activated by GABA and glycine that cannot be fully explained by alterations in the chloride gradient. These results suggest the heterogeneous expression of a population of inhibitory amino acid receptors that can bind either GABA or glycine. The recent report by Jonas et al. (1998), demonstrating corelease of GABA and glycine from single synaptic vesicles, suggests a mechanism in which both agonists would be in the synaptic cleft at the same time, permitting one amino acid to alter the receptor response to the other. Under such circumstances, cross-inhibition could provide a mechanism to limit the amplitude of inhibitory synaptic transmission during high-frequency discharge. This may contribute to odor information processing by the olfactory bulb by limiting reciprocal inhibition of the most active circuits, while maintaining surround inhibition.

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