Cotransmission of GABA and Glycine to Brain Stem Motoneurons

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O’Brien, Jennifer A. and Albert J. Berger. Cotransmission of GABA and glycine to brain stem motoneurons. J. Neurophysiol. 82: 1638–1641, 1999. Using whole cell patch-clamp recording in a rat brain stem slice preparation, we found that γ-aminobutyric acid (GABA) and glycine act as cotransmitters to hypoglossal motoneurons (HMs). Focal application of GABA and glycine onto a single HM revealed that GABA A and glycine receptors are present on the same neuron. To demonstrate that HMs receive both GABAergic and glycineric synaptic inputs, we simultaneously recorded GABA A- and glycine-receptor-mediated spontaneous miniature inhibitory postsynaptic currents (mIPSCs) in single HMs. GABAergic and glycineric mIPSCs were differentiated based on their kinetics and modulation by pentobarbital. Specifically, GABA A-receptor–mediated events decayed more slowly than glycine-receptor–mediated events. GABAergic response decay kinetics were prolonged by pentobarbital, whereas glycineric response decay kinetics remained unchanged. The distinct kinetics of the glycine- and GABA A-receptor-mediated synaptic events allowed us to record dual component mIPSCs, mIPSCs that are mediated by both receptor types. These data suggest that GABA and glycine are colocalized in the same presynaptic vesicle and are coreleased from presynaptic terminals opposed to motoneurons.

INTRODUCTION

γ-Aminobutyric acid (GABA) and glycine are the two main inhibitory neurotransmitters in the CNS. Each neurotransmitter activates a different family of ionotropic receptors that are permeable to chloride ions. GABA A receptors are blocked preferentially by biccuculline and are modulated by pentobarbital (Macdonald and Olsen 1994), whereas glycine receptors are preferentially blocked by strychnine and are insensitive to modulation by pentobarbital (Rajendra et al. 1997).

In the spinal cord and brain stem, inhibitory synaptic transmission can be mediated by GABA A and/or glycine. Recently, Jonas et al. (1998) demonstrated in spinal cord that GABA and glycine are coreleased from the same presynaptic terminal, resulting in coactivation of the corresponding receptors on target spinal cord neurons. It is not known if this phenomenon is restricted to spinal cord or whether other CNS regions, such as brain-stem motoneurons, receive dual-transmitter (GABA and glycine) inhibitory synaptic inputs. Here we demonstrate that both GABA and glycine act as cotransmitters to visualized hypoglossal motoneurons (HMs) by using whole cell patch-clamp techniques to record miniature inhibitory postsynaptic currents (mIPSCs), which presumably are due to release of single presynaptic vesicles (Katz 1969).

METHODS

Sprague-Dawley rats (1- to 5-d old) were anesthetized by injection (intramuscular) of a ketamine-xylazine mixture (200 and 14 mg/kg, respectively). After decapitation, the brain stem was removed and transverse brain stem slices (250–300 μm) were prepared. During slicing, incubation (1 h at 37°C), and recording, the slices were perfused by a Ringer solution containing (in mM) 119 NaCl, 26.2 NaHCO 3 , 1 Na 2 HPO 4 , 2.5 KCl, 11 glucose, 2.5 CaCl 2 , and 1.4 MgSO 4 . Using near-infrared DIC optics, HMs were identified based on their characteristic location and morphology (Umemiya and Berger 1994).

Whole cell patch-clamp recordings were performed at room temperature. Patch electrodes (resistance 1–5 MΩ) were filled with (in mM) 145 CsCl, 10 HEPES, 10 ethylene glycol-bis(β-aminoethyl ether)-N,N′,N′,N′-tetraacetic acid (EGTA), 2 MgCl 2 , 2 ATP-Mg, and 0.2 GTP-tris (pH 7.2). HMs were voltage-clamped —50 to —65 mV. Access resistance was always <15 MΩ. Data were filtered at 2 kHz and digitized at 5 kHz using pCLAMP software (Axon Instruments). GABA (200 μM) and glycine (200 μM) were applied (10—50 ms) onto the same HM using a dual channel picospritzer (General Valve) and a double barrel glass pipette. Five current traces were averaged and peak current amplitude measured using pCLAMP software (Axon Instruments).

Spontaneous mIPSCs were analyzed by software developed in our laboratory. Decay kinetics were measured as the time for the mIPSC to decay to 37% of its peak amplitude. All results are presented as means ± SD unless otherwise stated. The Kolmogorov-Smirnov statistical test (KS-test) was used to assess differences in mIPSC data. An unpaired t-test was used to assess differences in mean values from different conditions. Drugs used included: biccuculline methiodide (Sigma), strychnine hydrochloride (Sigma), tetrodotoxin (TTX, Alomone Labs), 6,7-dinitroquinoxaline (DNQX, RBI), N-(—)-2-amino-5 phosphopentoic acid (AP5, RBI), and sodium pentobarbital (Abbott labs).

RESULTS

To determine whether GABA A and glycine receptors were present on the same HM, GABA and glycine were applied focally via a dual-barreled pipette onto a single voltage-clamped HM (V h = —50 mV, Fig. 1A, top). Experiments were performed in the presence of TTX (0.5–1 μM) and DNQX (10–20 μM) to block Na + -dependent action potentials and non-N-methyl-D-aspartate (NMDA) glutamate receptors, respectively. Under these conditions, both GABA A and glycine receptor-mediated responses were present in five of five HMs studied. Addition of 5 μM biccuculline abolished almost all of the GABA A-receptor–mediated response (Fig. 1A, middle, n = 4). Addition of strychnine (1 μM) abolished almost all of the remaining glycine-receptor–mediated response (Fig. 1A, bottom, n = 4). These data show that both types of receptors are present on the same motoneuron.

Because it has been reported that biccuculline also can block...
glycine-receptor–mediated responses and strychnine can block GABA A-receptor–mediated responses (Jonas et al. 1998), we next performed dose-response experiments. Bath application of 5 μM bicuculline blocked 96.7 ± 1.1% of the GABA A-receptor–mediated response (n = 4, Fig. 1B), whereas it blocked only 5.7 ± 7.5% of the glycine-receptor–mediated response (n = 4, Fig. 1C). A greater dose of bicuculline (10 μM) also blocked 27.5 ± 4.5% of the glycine-receptor–mediated response (n = 4, Fig. 1C). Bath application of 500 nM strychnine blocked 96.6 ± 4.0% of the glycine-receptor–mediated response (n = 4, Fig. 1C), while inhibiting the GABA A-receptor–mediated response by only 11.3 ± 10.5% (n = 4, Fig. 1B). A greater dose of strychnine (10 μM) also abolished 96.4 ± 4.0% of the GABA A-receptor–mediated responses (n = 4, Fig. 1B).

To determine if both GABAergic and glycineergic synaptic currents are present in the same HM, we recorded spontaneous mIPSCs in the presence of TTX (0.5–1 μM), DNQX (20 μM) and AP5 (25 μM) with HMs voltage-clamped at −65 mV. Under these conditions, two populations of mIPSCs could be distinguished based on their sensitivity to antagonists and decay kinetics (Table 1). Spontaneous mIPSCs recorded in the absence of strychnine and bicuculline exhibited fast and slow decay kinetics (n = 6, Fig. 2, A1 and B1). Addition of the glycine receptor antagonist strychnine (500 nM) abolished the majority of responses with fast decay kinetics (Fig. 2A2, n = 3). The remaining mIPSCs were blocked by 5 μM bicuculline, confirming that these events are mediated by GABA A receptors (data not shown, n = 3). In a separate experiment, when bicuculline was added first, the mIPSCs with slower decaying kinetics were selectively abolished (Fig. 2B2, n = 3). The remaining glycineergic responses were blocked by 500 nM strychnine (data not shown, n = 3).

We computed the cumulative probability distribution of decay times for events recorded in control conditions (Fig. 2, A3 and B3, ▲) versus events recorded in the presence of either strychnine or bicuculline. When strychnine was added, the cumulative probability of events shifted to the right (Fig. 2A3, ○, n = 3). When bicuculline was added, the cumulative probability of events shifted to the left (Fig. 2B3, ■, n = 3). Comparing these distributions shows that the observed distribution in the absence of blockers lies between those of the glycineergic and GABAergic events. This indicates that in the control condition both types of events contribute to the overall population of control mIPSCs.

To investigate whether GABA and glycine are contained within and released from the same presynaptic vesicle, it was

**FIG. 1.** GABA A- and glycine-receptor–mediated responses recorded in a single hypoglossal motoneuron (HM). A: sequential GABA and glycine focal applications onto the cell soma (top). Addition of the GABA A receptor antagonist bicuculline (Bic) to the extracellular solution abolished the GABAergic response (middle). Addition of the glycine receptor antagonist strychnine (Stry) abolished the remaining glycineergic response (bottom). All traces are the average of 5 sequential GABA and glycine applications. B: inhibition of the peak GABAergic response by bicuculline and strychnine. Plotted are the mean percent inhibition (±SD) of the control GABAergic response after bath application of biculline (5 μM, ■, n = 4) and 2 concentrations of strychnine (500 nM and 10 μM, □, n = 4). Note that the higher dose of strychnine almost completely inhibits the GABAergic response. C: inhibition of the peak glycineergic response by strychnine and bicuculline. Plotted are the mean percent inhibition (±SD) of the glycine response after bath application of strychnine (500 nM, □, n = 4) and 2 concentrations of bicuculline (5 and 10 μM, ■, n = 4). Higher dose of bicuculline abolishes 27.8 ± 4.5% of the glycineergic response.

**TABLE 1.** Comparison of mIPSC amplitude and decay kinetics with and without pentobarbital present in the extracellular solution

<table>
<thead>
<tr>
<th>Type of mIPSCs</th>
<th>Events Recorded in the Absence of Pentobarbital</th>
<th>Events Recorded in the Presence of Pentobarbital</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amplitude, pA</td>
<td>Rise time T_{10-90}, ms</td>
</tr>
<tr>
<td>Control</td>
<td>−45.1 ± 11.6</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>GABAergic</td>
<td>−33.4 ± 4.9</td>
<td>3.0 ± 0.9</td>
</tr>
<tr>
<td>Glycinergic</td>
<td>−33.0 ± 6.9</td>
<td>1.8 ± 0.7</td>
</tr>
</tbody>
</table>

Values are means ± SD. Decay kinetics were measured as the time for miniature postsynaptic current (mIPSC) to decay to 37% of its peak amplitude. * GABAergic mIPSC decay times were significantly prolonged by pentobarbital (P ≤ 0.05, t-test).
necessary to distinguish GABAergic and glycinergic mIPSCs in the same cell based on differences in their decay kinetics (Fig. 3B1). To increase the difference between the GABAergic and glycinergic kinetics, we recorded mIPSCs in the presence of pentobarbital (25–50 μM). Pentobarbital prolonged the decay kinetics of GABAergic events without affecting the kinetics of glycine-receptor–mediated events (Table 1). The distributions of decay times for glycinergic and GABAergic mIPSCs recorded in the presence of pentobarbital are shown in Fig. 3B2. The prolonged GABAergic events recorded in isolation have a significantly different distribution from faster decaying glycinergic events, thus making it possible to distinguish GABAergic from glycinergic events.

If GABA and glycine are coreleased from the same presyn-
aptic vesicle, we would predict that mIPSCs should have both a fast decaying glycinergic component and a slow decaying GABAergic component. When recorded in the presence of pentobarbital, mIPSCs had three different types of decay kinetics. There were fast decaying glycinergic events (Fig. 3A1, ■) and slow decaying GABAergic events (Fig. 3A1, ○). There were also dual component mIPSCs having both fast and slow decay components (Fig. 3A1, ▲). These dual component mIPSCs are due to release of both GABA and glycine from the same vesicle because presumably mIPSCs are due to release of a single presynaptic vesicle.

We plotted the distribution of decay kinetics from events recorded in control conditions and this resulted in a skewed frequency distribution (Fig. 3A2) rather than two separate distributions indicative of isolated glycinergic and GABAergic events (Fig. 3B2). This result strongly suggests that dual component mIPSCs, involving the corelease of GABA and glycine from a single vesicle, occur under these conditions.

**DISCUSSION**

Our data suggest that GABA and glycine act as cotransmitters to HMs. We also have demonstrated that these can be coreleased from the same presynaptic vesicle. These data are consistent with and extend a previous study demonstrating dual component mIPSCs in spinal cord neurons (Jonas et al. 1998).

Biochemical and anatomic experiments suggest that GABA and glycine can be colocalized in the same neuron and synaptic vesicle. It is known that GABA and glycine are transported by the same vesicular transporter (Burger et al. 1991). Colocalization of GABA and glycine in single neurons has been shown using immunohistochemistry in many areas including spinal cord and trigeminal nucleus. (Dumba et al. 1998; Taal and Holstege 1994; Todd and Sullivan 1990). Although colocalization of GABA and glycine in neurons projecting to HMs has not been demonstrated, there is evidence that brain stem neurons, projecting to HMs, can contain either GABA or glycine. Furthermore these neurons are located in the same region of the brain stem (Li et al. 1997).

Why are GABA and glycine co-released? It is possible that corelease of GABA and glycine may be important for development because these experiments were performed using neonatal rats. During this time in development, the chloride gradient in neonatal HMs, causes glycine receptor (Singer et al. 1998) and probably also GABA_A receptor activation to depolarize HMs. A combination of slow GABAergic responses and fast glycinergic responses may lead to a depolarization great enough to activate voltage-gated calcium channels. Also there is evidence that calcium influx due to GABA_A-receptor activation is important for neuronal development (Cherubini et al. 1991; Obrietan and van den Pol 1995). There may be a developmental change in proportions of synaptically activated GABA_A and glycine receptor as has been seen in other systems (Gao and Ziskind-Conhaim 1995; Kotak et al. 1998). If the developmental switch is due to a change in postsynaptic receptor type, corelease of both neurotransmitters would ensure that release of vesicular contents causes a postsynaptic effect. An important issue, and as yet unknown, is whether GABA and glycine are coreleased throughout development or if this phenomena is specific only to HMs of neonatal animals.

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