Relative Contribution by GABA or Glycine to Cl\(^-\)-Mediated Synaptic Transmission on Rat Hypoglossal Motoneurons In Vitro

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The relative contribution by GABA and glycine to synaptic transmission of motoneurons was investigated using an hypoglossus nucleus slice preparation from neonatal rats. Spontaneous, miniature, or electrically evoked postsynaptic currents (sPSCs, mPSCs, ePSCs, respectively) mediated by glycine or GABA were recorded under whole cell voltage clamp after blocking excitatory glutamatergic transmission with the broad-spectrum glutamatergic mGluR agonist (trans-50\(\mu\)M) trans-1-aminocyclopentane-1,3-dicarboxylic acid (6M), which in control solution increased the frequency of both glycine and GABA-mediated sPSCs. In fact, the broad-spectrum mGluR agonist, which in control solution increased the frequency of both GABAergic and glycine-mediated sPSCs, enhanced the frequency of glycine-mediated mPSCs only. These results indicate that, unlike spontaneous GABAergic transmission, glycine-mediated neurotransmission was essentially independent of network activity. There was a consistent difference in the kinetics of GABAergic and glycine-mediated events as GABAergic events had significantly slower rise and decay times than glycine-mediated ones. Such a difference was always present whenever sPSCs, mPSCs, or ePSCs were measured. Finally, GABAergic and glycine-mediated mPSCs were differentially modulated by activation of glutamate metabotropic receptors (mGluRs), which are abundant in the hypoglossus nucleus. In fact, the broad-spectrum mGluR agonist, which in control solution increased the frequency of both GABAergic and glycine-mediated sPSCs, enhanced the frequency of glycine-mediated mPSCs only. These results indicate that on brain stem motoneurons, Cl\(^-\)-mediated synaptic transmission is mainly due to glycine rather than GABA and that GABAergic and glycine-mediated events differ in terms of kinetics and pharmacological sensitivity to mGluR activation or TTX.

INTRODUCTION

The hypoglossus nucleus is a brain stem structure in which more than 90% of local cells are motoneurons (Viana et al. 1990). Hypoglossal motoneurons are cholinergic elements (Davidoff and Schulze 1988; Lewis et al. 1971) that innervate tongue muscles and are thus important for functions such as swallowing, respiration, sucking, and vocalization (Lowe 1980). Disorders of hypoglossal motoneurons appear to be implicated in syndromes like sleep apnea in man (Gauda et al. 1987; Wiegand et al. 1991). Studies of neonatal rat hypoglossal motoneurons have demonstrated that these cells receive synaptic inputs of excitatory or inhibitory nature (Aldes et al. 1988; Berger and Isaacscon 1999; Li et al. 1997; O’Brien et al. 1997; Rekling 1992; Yang et al. 1995). The major inhibitory neurotransmitters on motoneurons are thought to be GABA or glycine, which operate via activation of distinct postsynaptic receptors gating Cl\(^-\) channels (for a recent review, see Rekling et al. 2000). GABA receptors mainly belong to the GABA\(_A\) class and are reversibly blocked by bicuculline while glycine receptors are antagonized by strychnine (Barnard et al. 1993; Kushe et al. 1995; Nistri 1983; Rajendra et al. 1997). The co-existence of GABA and glycine in this nucleus raises the question of their relative contribution to synaptic microphysiology of motoneurons. Electrophysiological studies carried out with the spinal cord slices (Jonas et al. 1998) have recently suggested that GABA and glycine may be released by the same presynaptic fiber, a notion that would classify these two substances as co-transmitters. On dorsal horn interneurons, GABA is co-released with ATP (Jo and Schlichter 1999), indicating that the same presynaptic fiber can control postsynaptic activity via more than one transmitter.

A recent report (O’Brien and Berger 1999) has suggested that also on hypoglossal motoneurons Cl\(^-\)-mediated, miniature postsynaptic currents (mPSCs) are generated by co-release of GABA and glycine from the same presynaptic fiber. Nevertheless it is still unclear how many inhibitory synapses can operate in this fashion and if co-release takes place also in the case of network-mediated, spontaneous events (sPSCs) and of responses evoked by electrical stimulation of afferent inputs (ePSCs). Furthermore it is not yet known if the GABA and glycine co-release process might be subjected to modulation via receptors located on presynaptic fibers. In particular, as metabotropic glutamate receptors (mGluRs) largely influence synaptic transmission presynaptically (Cochilla and Alford 1998; Del Negro and Chandler 1998; Gereau and Con 1995; Manzoni and Bockaert 1995; Nakanishi 1994; Netzband et al. 1997; Salt and Eaton 1995; Sayer et al. 1992; Schoppa and Westbrook 1997; Schrader and Tasker 1997; Shigemoto et al. 1998; Del Negro and Chandler 1998; Gereau and Conn 1995; Jo and Schlichter 1999), indicating that the same presynaptic fiber can control postsynaptic activity via more than one transmitter.
The present study of neonatal rat hypoglossal motoneurons is aimed at addressing to what extent glycine or GABA contributed to Cl\(^-\)-mediated synaptic events, their basic properties and sensitivity to tetrodotoxin (TTX) or to the broad spectrum metabotropic receptor agonist \((\pm)-1\)-aminocyclopentane-trans-1,3-dicarboxylic acid (t-ACPD). The present observations suggest that, at least in the case of neonatal motoneurons, the vast majority of Cl\(^-\)-mediated events were not apparently due to co-release of GABA and glycine. These two neurotransmitters seemed to generate kinetically distinct postsynaptic signals, perhaps caused by differential location of their receptors.

**METHODS**

**Slice preparation**

Experiments were carried out on brain stem slices obtained from 0- to 5-day-old Wistar rats terminally anesthetized with 0.2 ml urethan (10% ip). The entire procedure (including animal handling and care) is in accordance with the Animal Welfare Act and was approved by the Local Authority Veterinary Service. Thin brain stem slices were prepared following the procedure described by Viana et al. (1994). In brief, the brain stem was isolated and placed in modified ice-cold Krebs solution (see following text) to dissect out the lower medulla, which was then pinned to an agar block placed inside a Vitrabrate chamber (filled with ice-cold Krebs solution gassed with 95% O\(_2\)-5% CO\(_2\)). Two-hundred-micrometer-thick slices were cut and transferred to an incubation chamber for 1 h at 32°C containing continuously oxygenated Krebs medium. The incubation temperature was later lowered to ambient level and the slices maintained under this condition for at least 1 h before use.

**Recording and electrical stimulation**

For electrophysiological experiments, brain stem slices placed in a small recording chamber were viewed with an infrared video-camera to identify single hypoglossal motoneurons within the XII nucleus. Unless otherwise stated, all cell recordings were obtained with whole cell patch-clamp electrodes (3–5 M\(\Omega\) DC resistance) while cells were clamped at \(-70\) mV holding potential (\(V_h\)). Seal resistance was usually more than 2 G\(\Omega\). After seal rupture, series resistance (5–25 M\(\Omega\)) was routinely monitored and compensated (usually by 30%, range 20–60%). Voltage-pulse generation and data acquisition were performed with a PC using pClamp 6.1 software. Currents elicited by voltage steps were filtered at 3–10 kHz and sampled at 5–10 kHz.

For extracellular stimulation (0.2 Hz; 0.2 ms; variable intensity) of GABAergic or glycinerigic cells, a single bipolar tungsten electrode was placed in the lateral reticular formation. After stabilization of the synaptic response, stimulus intensity was adjusted to obtain 25–50% failures for 100 stimuli. Evoked synaptic currents were then stored in a PC as individual files and averaged with pCLAMP software (version 6.1) after discarding failed events.

**Solutions and drugs**

For slice preparation and subsequent incubation, the solution (in mM) was 130 NaCl, 3 KCl, 26 NaHCO\(_3\), 1.5 Na\(_2\)HPO\(_4\), 1 CaCl\(_2\), 5 MgCl\(_2\), and 10 glucose (290–310 mOsM). For electrophysiological recordings, the extracellular control solution (in mM) was 140 NaCl, 3 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, and 10 glucose (pH 7.4; 290–310 mOsM). Unless otherwise stated, 2 mM kynurenic acid was routinely added to block glutamaterigic ionotropic currents. The patch pipette solution (in mM) was 120 CsCl, 9 NaCl, 2 MgCl\(_2\), 1 CaCl\(_2\), 10 HEPES, 10 EGTA, and 2 Mg-ATP at pH 7.2 (270–290 mOsM). Drugs were applied via the extracellular solution (superfused at 2–5 ml/min) for a minimum of 5–10 min to reach equilibrium conditions. Pilot experiments were performed to assess the antagonism selectivity of bicuculline or strychnine under the present conditions. On cells bathed with a solution containing 1 \(\mu M\) tetrodotoxin (TTX) and 2 mM kynurenic acid, bicuculline (10 \(\mu M\)) did not significantly (\(P > 0.05; n = 10\) cells) reduce the amplitude of peak currents evoked by application of 100 \(\mu M\) glycine. Likewise, strychnine (0.4 \(\mu M\)) did not affect significantly the amplitude of the peak current evoked by 100 \(\mu M\) GABA (\(P > 0.05; n = 7\) cells). These results demonstrate that at the concentrations used in the present study bicuculline or strychnine retained their antagonism selectivity against GABA\(_A\) or glycine receptors, respectively.

The following drugs were used: kynurenic acid (Sigma), bicuculline methiodide (Sigma), strychnine hydrochloride (Sigma), GABA (Sigma), glycine (Sigma), TTX (Affiniti, UK), t-ACPD (Tocris, UK).

**Data analysis**

Cell input resistance (\(R_{\text{in}}\)) was calculated by measuring the current response to 10- or 20-nV hyperpolarizing pulses (from \(-70\) mV \(V_h\)), or from the slope of the linear part of the \(I-V\) relation obtained by applying a slowly rising voltage signal (ramp test). Detection of single postsynaptic currents was done with AxoGraph 3.5 (Axon Instruments, Foster City, CA) software that uses the method of minimizing the sum of squared errors between data and a template function approximating the width and time course of a typical synaptic event as described by Clements and Bekkers (1997). Rise time of captured single events was measured between 10 and 90% of peak amplitude, while for average events it was measured between 20 and 80% of peak amplitude to minimize errors due to any single event misalignment. Exponential fitting of captured event decay was carried out with the Chebyshev algorithm provided by AxoGraph software. The decay of the vast majority of pharmacologically identified glycinergic or GABAergic mPSCs was best fitted by a monoexponential function. For a small minority of mPSCs (up to 20% of all events) the time course of decay was multiphasic and, as a first approximation, was fitted by a single time constant, representing the weighted average of the individual components. Sigma Plot (Jandel Scientific, San Rafael, CA) and Clampfit (Axon Instruments) softwares were used for linear regression analysis of experimental data. Paired or unpaired t-test was used to assess differences in mean values; \(P < 0.05\) was considered as the acceptable level of statistical significance.

**Immunohistochemistry**

Immunohistochemical detection of neuronal choline acetyltransferase was carried out as described by Ballerini et al. (1999) using anti-choline acetyltransferase polyclonal antibodies (Chemicon International, Temecula, CA). Immunocytochemical detection of \(\alpha_1\), \(\alpha_2\), \(\beta_2\), and \(\beta_3\) subunits of the GABA\(_A\) receptor was performed by using polyclonal antiseraa (Santa Cruz Biotechnology, Santa Cruz, CA) against a peptide mapping the amino terminus of the \(\alpha_1\) or \(\alpha_2\) subunits and a monoclonal antibody against the \(\beta_2/3\) subunit of the same receptor (Mize and Butler 1997). For this purpose, the whole brain stem was removed and fixed in paraformaldehyde (4% in phosphate buffer solution; PBS) for 24 h at 4°C, then in sucrose (30% in PBS) for cryoprotection for the same period of time. Transverse sections (20-\(\mu M\) thin) were cut with a sliding microtome at the level of the lower medulla from frozen blocks of tissue and placed in wells containing the fixative until further use. After washing with Triton X-100 (0.2% in PBS), incubating for 30 min in H\(_2\)O\(_2\) (3% in PBS), and further washing, free-floating sections were incubated overnight at 4°C in the solution containing primary antibodies (diluted 1:100 in PBS containing 0.2% Triton X-100 and 10% fetal calf serum; FCS). After washing with PBS, slices were incubated for 1 h in the solution containing the secondary antibody (diluted 1:100 or 1:50 in 10% FCS in PBS). After further washing, sections were then incubated at room temperature for 1 h with the ABC kit for alkaline phosphatase, washed, and developed for 20–30 min in buffer containing (for 10
Results

The database of the present study comprises 77 motoneurons with $64 \pm 3$ pF somatic capacitance and $290 \pm 20$ MΩ input resistance ($R_{in}$). The vast majority of cells in this area were positively stained for choline acetyltransferase (see Fig. 1A), and were thus identified as motoneurons (cf. Viana et al. 1990). While the presence of glycine receptors on neonatal hypoglossal motoneurons has previously been reported (Singer and Berger 1999; Singer et al. 1998), the localization of $\text{GABA}_A$ receptors on motoneurons of the same age is uncertain. For this reason, we investigated the immunocytochemical presence of $\alpha_1$, $\alpha_2$, and $\beta_2$–3 subunits of $\text{GABA}_A$ receptors. The $\alpha_1$ subunit (Fig. 1B) or the $\beta_2$–3 subunits (not shown) were not detected. Conversely, Fig. 1, C and D, shows, at low and high power, motoneuron somata extensively labeled by the $\alpha_2$ subunit antibody.

Characteristics of GABAergic and glycineric sPSCs on hypoglossal motoneurons

In kynurenic acid solution spontaneous synaptic activity routinely consisted of inward currents occurring on average at $1.5 \pm 0.3$ Hz ($n = 19$ cells; see example in Fig. 2A). Under our recording conditions (symmetrical $\text{Cl}^-$ concentration across the cell membrane and $-70$ mV $V_h$), glycineric as well as GABAergic events were inwardly directed. To separate them pharmacologically, we used 10 $\mu$M bicuculline or 0.4 $\mu$M strychnine, respectively, as shown in Fig. 2A, middle and bottom; note that strychnine was applied after bicuculline had been washed out and synaptic events had returned to control frequency and amplitude (data not shown). On a random sample of 11 cells exposed to bicuculline, spontaneous currents occurred at an average frequency of $1.3 \pm 0.4$ Hz, a value (90 ± 20%) not significantly different from control. Further addition of strychnine to the same cells in the presence of bicuculline completely abolished any residual spontaneous activity (data not shown), indicating that under the present conditions, spontaneous currents were mediated by activation of glycine and $\text{GABA}_A$ receptors.

After addition of strychnine to the control solution, spontaneous currents occurred at a frequency of $0.9 \pm 0.3$ Hz ($n = 13$ cells); this corresponded to a decrease by 69 ± 8% in control event frequency. Any residual activity was completely abolished by subsequent addition of bicuculline (data not shown). The action of strychnine could not be fully reversed even after more than 30 min washout. Figure 2B (left) exemplifies the mean time course of glycineric or GABAergic sPSCs (obtained by averaging all events recorded from 2 cells in the presence of bicuculline or strychnine). After scaling, the GABAergic response had slower decay (and rise time) than the glycineric one (Fig. 2B, right). Table 1 confirms that GABAergic sPSCs had average kinetics significantly slower than glycineric ones.

Characteristics of GABAergic or glycineric mPSCs

Figure 3A shows sample traces (obtained at $-70$ mV $V_h$ in control solution; see top 2 records), which comprised spontaneously occurring inward currents (mPSCs) in the continuous presence of TTX. These events appeared at random (at a $2.2 \pm 0.5$-Hz frequency) with relatively fast onset (2.7 ± 0.1 ms) and monoeponential decay (14.3 ± 0.7; $n = 22$ cells). Figure 3A (middle) shows that bicuculline reduced (by 17%) the frequency of all mPSCs and did not abolish those with multiphasic decay (see *) in accordance with a recent report (Singer et al. 1998). Subsequent addition of strychnine (plus bicuculline; Fig. 3A, bottom) completely suppressed any synaptic activity, indicating that in kynurenic acid solution mPSCs were due to
activation of GABA and glycine receptors. Pharmacologically isolated glycineric mPSCs occurred at 1.6 ± 0.5 Hz (n = 19 cells) and made up 70 ± 10% (n = 16 cells; P < 0.05) of the total events. Figure 3B shows that there was no correlation between glycineric mPSC amplitude and rise time, making it unlikely that they were merely shaped by electrotonic filtering (Soltesz et al. 1995; Ulrich and Lüsch 1993). Likewise we found no correlation between mPSC amplitude and decay time (r = 0.11, slope = 0.09 ± 0.03 ms/pA, P < 0.005). Histograms of the amplitude of glycineric mPSCs displayed a skewed distribution as shown in Fig. 3C (see also Singer and Berger 1999). In accordance with the stochastic nature of the underlying releasing process (Fatt and Katz 1952), glycineric mPSCs occurred at random as indicated by the histogram of the inter-event intervals (Fig. 3D, in which their distribution is well fitted by a single exponential). As shown in Fig. 3E, glycineric mPSCs reversed at −3 ± 5 mV, a value close to the one predicted for Cl⁻-mediated currents.

Likewise, GABAergic mPSCs were pharmacologically isolated by adding 0.4 μM strychnine to kynurenic acid and 1 μM TTX containing solution (Fig. 4A). Subsequent application of bicuculline blocked any residual activity. Note that on four cells 0.4 μM strychnine fully abolished all synaptic events even if exogenous application of GABA (100 μM) still elicited inward currents as large as in control solution (98 ± 5%; data not shown). Whenever present, pharmacologically isolated GABAergic mPSCs occurred at 0.5 ± 0.2 Hz (n = 13 cells) and represented 22 ± 8% (n = 7 cells; P < 0.05) of all mPSCs. The large majority of GABAergic mPSCs decayed monoexponentially as only up to 15% of them had complex kinetics of decay. The amplitude of GABAergic mPSCs was not correlated to their rise time (Fig. 4B) or decay (r = −0.34, slope = −0.18 ± 0.03 ms/pA, P < 0.0001) and was distributed in a skewed fashion (Fig. 4C). The inter-event distribution could be

![FIG. 2. Spontaneous synaptic currents in kynurenic acid solution comprise glycine- or GABA-mediated events. A, top: inward currents recorded at −70 Vₒ in the presence of kynurenic acid (2 mM). Middle: in the presence of bicuculline, a number of spontaneous events remain. Bottom: after bicuculline washout and application of strychnine, spontaneous events are also detected. B, left: average synaptic current due to activation of glycine or GABA_A receptors (561 or 102 events, respectively). Right: after normalizing the amplitude of glycine- or GABA-mediated currents, the different time course of these responses becomes apparent. The fitting function overlaps almost completely the average records. All data are from the same cell.](http://jn.physiology.org/)

![FIG. 3. Properties of glycineric miniature postsynaptic currents (mPSCs). A, top: 2 consecutive traces of mPSCs in control solution, * events with complex decay. Middle: after adding bicuculline, glycineric mPSCs can be recorded, some of which with complex decay (see *). Bottom: further application of strychnine leads to full suppression of synaptic activity. B: plots of glycineric event amplitude against corresponding rise time. The slope value is −0.081 ± 0.002 ms/pA while the correlation coefficient r is −0.85. The probability that the correlation coefficient is significantly different from 1 is <0.0001. C: amplitude distribution histograms for glycineric mPSCs. Most events are grouped between −10 and −30 pA. D: histograms of distribution of inter-event intervals for responses shown in B and C. A single exponential function (time constant = 180 ± 10 ms) can adequately fit the interval distribution. E: current-voltage relation for glycineric mPSCs. The calculated reversal potential is −3 ± 5 mV.](http://jn.physiology.org/)
accordance with the calculated reversal potential for Cl
shows that GABAergic mPSCs reversed at 
2
1
constant
test). Figure 5
contrast the properties of glycinergic and GABAergic synaptic events. A: average mPSCs recorded from the same cell in the presence of strychnine (GABAergic events; \( n = 37 \)) or in the presence of bicuculline (glycinergic events; \( n = 526 \)) in kynurenic acid and TTX solution. Note slower time course of GABAergic mPSCs (fitting functions are virtually superimposed on average records). B: histograms show, for the same cell, the distribution of decay values for all mPSCs in control solution, glycinergic mPSCs, and GABAergic mPSCs. Note that to maximize the capture of GABAergic events (which occurred at low frequency) records, bottom, were analyzed for 10 min instead of the customary 5 min used for the top and middle. C: histograms depicting the sensitivity of glycinergic or GABAergic synaptic responses to TTX. Application of TTX brings about a large fall in the frequency of GABA-mediated events and a smaller, albeit significant, reduction in their mean amplitude (\( n = 6 \)). Glycine-mediated events are essentially unchanged by TTX (\( n = 4 \)).

session. When we first isolated pharmacologically GABA-
or glycine-mediated PSCs and then applied TTX, a major difference between GABA- and glycine-mediated transmission became apparent. As indicated by the histograms of Fig. 5C, pharmacologically isolated glycine-mediated events did not change in frequency or amplitude after adding TTX. This finding indicates that spontaneous glycine-mediated neurotransmission was essentially independent of network activity. Conversely, GABAergic events were slightly, yet significantly, reduced in amplitude and dramatically decreased (by more than two-thirds) in frequency, outlining the requirement for strong network activity to express spontaneous GABAergic events.

fitted with a monoexponential function to indicate the stochastic occurrence of such events (Fig. 4D). Figure 4E shows that GABAergic mPSCs reversed at 
2
1
mV in accordance with the calculated reversal potential for Cl
. Figure 5A shows the average kinetics of glycinergic and GABAergic mPSCs, which differed significantly in rise time, amplitude, and decay (see Table 2 for full description of data). Figure 5B compares histograms for the mPSC decay in control solution and after pharmacological application of bicuculline or strychnine to dissect mPSCs into glycinergic or GABAergic ones. The control histogram and the one for the glycinergic mPSCs appear similar because glycinergic responses represent the large majority of events. However, the cumulative plots for control, glycinergic or GABAergic event decay were significantly different (\( P < 0.0001 \); Kolmogorov-Smirnov test; not shown).

The experiments reported so far relied on protocols in which TTX was applied from the beginning of the recording

| TABLE 2. Mean characteristics of mPSCs |
|-----------------|----------|----------|-----------|----------|
|                | Rise, ms | Amplitude, pA | Decay, ms | Frequency, Hz |
| Glycinergic     | 2.9 ± 0.2 | -23 ± 2 | 12.8 ± 0.8 | 1.6 ± 0.5 |
| GABAergic       | 4.4 ± 0.6** | -12 ± 1* | 25 ± 3** | 0.5 ± 0.2* |

Values are means ± SE. \( n \), number of motoneurons. ** Significant statistical difference between glycinergic and GABAergic miniature PSC (mPSC) average values with \( P < 0.001 \). * \( P < 0.05 \) (t-test).
Characteristics of GABAergic or glycinergic ePSCs

ePSCs were evoked by electrical stimuli applied to the lateral reticular formation (ipsilateral to the patched motoneuron) (see Borke et al. 1983; Travers and Norgren 1983; Umemiya and Berger 1995). Since ePSCs appeared with a relatively short, constant latency after applying weak pulses, it seems probable that these were mainly monosynaptic events. Further support for this notion was obtained in experiments like the one shown in Fig. 6, A and B, in which step-wise increments in ePSC amplitude were observed whenever the stimulus strength was increased from one range of intensity to the next. Within each stimulus range the synaptic response remained constant, indicating that there was no gradual recruitment of additional fibers. The constant amplitude of synaptic currents for each range of stimulus intensity is plotted in Fig. 6B. Using the same pharmacological antagonists employed for testing spontaneous events, we investigated, in isolation, glycinergic (Fig. 7A) or GABAergic evoked currents (Fig. 7B). For both types of ePSC, the calculated reversal potential was 10 ± 3 mV (Fig. 7, C and D). ePSCs had average rise and decay times (Fig. 7E) similar to those of sPSCs and mPSCs. These data thus confirmed the comparatively slow kinetics of all GABAergic synaptic currents.

Although GABA- or glycine-mediated currents were kinetically distinct, it seemed interesting to find out whether, on the same cell, they might be evoked by the same electrical stimulus and what relative contribution each component might make to the composite Cl–-mediated current. The example of Fig. 7F shows a cell in which average bicuculline- or strychnine-sensitive (after bicuculline washout) evoked responses were recorded in kynurenic acid solution. Co-application of these antagonists fully blocked any evoked response. Digital summation of these two components gave a waveform that could be almost completely superimposed to the control event in input resistance (30+6%) and in synaptic activity, despite

of electrically evoked transmission could be found in less than 20% of recorded motoneurons.

Modulation of sPSCs and mPSCs by mGluR activity

The broad-spectrum mGluR agonist t-ACPD was tested on 18 cells on which it evoked a slowly rising, persistent inward current (on average = 20 ± 1 pA) together with a large increase in input resistance (30 ± 10%) and in synaptic activity, despite the presence of kynurenic acid. This observation indicates that the enhancing action of t-ACPD on synaptic activity was mainly targeted to Cl–-mediated events. To check whether glycineric or GABAergic events were differentially affected by the activation of mGlurRs, bicuculline or strychnine was added to separate sPSC.
Figure 8A shows typical traces of glycinergic sPSCs (in bicuculline solution) before and after addition of t-ACPD, while Fig. 8B depicts similar data with GABAergic sPSCs. In both cases, there was a large increase in sPSC frequency. These results are quantified (Fig. 8C) in terms of changes in normalized frequency or amplitude for glycinergic (■) and GABAergic (□) events before and after t-ACPD.

We next studied the effect of t-ACPD on mPSC pharmacologically separated as above. As shown by the representative traces in Fig. 9A, the frequency of glycinergic mPSCs was enhanced in a reversible fashion. On the other hand, the frequency and amplitude of GABAergic mPSCs remained insensitive to t-ACPD (Fig. 9B). The histograms of Fig. 9C provide statistical analysis of average data, confirming that the process of GABA release from pharmacologically isolated GABAergic terminals in the presence of TTX was unaffected by t-ACPD while the comparable process of glycine release was t-ACPD sensitive.

**DISCUSSION**

The principal finding of the present study is that glycinergic synaptic events differ from GABAergic ones in terms of kinetics, frequency, and sensitivity to TTX or t-ACPD. These data do not provide evidence for substantial co-release of these transmitters at this early postnatal stage and are interpreted in terms of kinetically distinct synaptic roles for glycine and GABA.

![Image](http://jn.physiology.org/)

**FIG. 8.** Frequency up-regulation by (±)-1-aminocyclopentane-trans-1,3-dicarboxylic acid (t-ACPD, 50 μM) of spontaneous postsynaptic currents (sPSCs) always recorded in kynurenic acid solution. A: effect of t-ACPD on glycinergic currents (left, control responses) in the presence of bicuculline. B: effect of t-ACPD on GABAergic currents in the presence of strychnine. C: histograms of fractional changes in sPSC amplitude or frequency in the presence of t-ACPD. Note significant increase in frequency but not in amplitude (glycine: n = 3, GABA: n = 5; * P < 0.05).

**FIG. 9.** Differential modulation of GABAergic or glycinergic mPSCs by t-ACPD in the presence of kynurenic acid (kyn. ac.) solution. A: glycinergic miniature events in control (left), t-ACPD (middle), or washout of t-ACPD. Note large and reversible increase in frequency. B: GABAergic miniature events in control (left), t-ACPD (middle), or washout of t-ACPD. Note lack of effect of t-ACPD. C: histograms of fractional changes in amplitude or frequency of mPSCs mediated by glycine (n = 4) or GABA (n = 10). Note significant increase in frequency (but not in amplitude) of glycinergic events only (* P < 0.05).

**Electrophysiological characteristics of spontaneous synaptic events**

In the present investigation, synaptic currents were always studied during pharmacological block of glutamatergic ionotropic receptors and were found to reverse near 0 mV, in accordance with the value predicted by the Nernst equation for Cl−-mediated responses. Pharmacological separation of glycine-mediated events from those mediated by GABA_A receptors allowed us to ascertain the relative contribution of each transmitter to the observed activity. Parallel experiments were carried out to establish that strychnine or bicuculline retained their receptor selectivity at the concentrations used in the present study. Because 0.4 μM strychnine did not affect the inward current evoked by 100 μM GABA and 10 μM bicuculline was ineffective against 100 μM glycine, we concluded that each antagonist retained the expected receptor specificity of action. It should also be noted that co-application of bicuculline and strychnine fully suppressed any synaptic activity, indicating that the synaptic currents measured under the present conditions were only mediated by glycine or GABA_A receptor activation.

In general, glycinergic events were significantly faster than GABAergic events and occurred at higher frequency. The effect of TTX on glycinergic or GABAergic events was dramatically different. In fact, the frequency of glycinergic events remained substantially unchanged (and their amplitude was not significantly depressed), whereas GABAergic events were reduced in amplitude and, especially, in frequency. This observation indicated that asynchronous release of GABA largely...
depended on network activity while the release of glycine did not.

The difference in kinetics between glycinergic and GABAergic events was also found when mPSCs were analyzed. In fact, GABAergic events were significantly slower, smaller and occurred at lower frequency than their glycinergic counterparts. Despite these differences in kinetic behavior, in control solution it was not possible to identify, within the cumulative histogram of event decay, separate components due to either glycinergic or GABAergic mPSCs because in control solution the histogram was largely shaped by glycinergic events.

In some cases 0.4 μM strychnine completely suppressed any Cl⁻-mediated synaptic events even if exogenously applied GABA was still able to elicit robust inward currents. The contribution of any tonic (action potential-independent) release of GABA to the overall synaptic activity of resting hypoglossal motoneurons must therefore have been quite small even though GABA_A receptors were functional on these cells and demonstrably present at somatic level. In fact, our immunocytochemical data indicated the presence of the GABA_A receptor α2 subunit on the cell body of neonatal motoneurons. This subunit is a major constituent of developing GABA_A receptors, while the α1 subunit, which could not be found in the present experiments, is indeed typical of adult GABA_A receptors (Fritschy and Mohler 1995; Fritschy et al. 1994; Mohler et al. 1996).

It is interesting that a small fraction of mPSCs displayed complex decay kinetics that might have suggested simultaneous activation of GABA_A and glycine receptors by synaptic co-release of both transmitters (Jonas et al. 1998; O’Brien and Berger 1999). Nevertheless subsequent application of either bicuculline or strychnine failed to abolish this subpopulation of mPSCs: this result suggests that a high order of mPSC decay is not per se indicative of co-release mechanism. Complex decay might reflect spatial segregation of synaptic receptors or heterogeneous receptors for the same transmitters (Lewis and Faber 1996a,b).

**Differences between GABAergic and glycinergic inputs**

The large reduction in GABAergic events brought about by acute application of TTX suggests that, even in the absence of glutamate-mediated synaptic transmission, GABAergic interneurons were spontaneously active because they either received a nonglutamatergic drive or fired spikes due to the operation of their intrinsic conductances. When glutamatergic transmission was blocked, glycine releasing neurons were not spontaneously active. Full elucidation of the mechanisms underlying such a different behavior is not currently available. However, one likely explanation is that glycinergic interneurons might have had their somata severed during slice preparation. In this case, glycine release would have merely reflected spontaneous quantal discharge of this neurotransmitter, a process undoubtedly endowed with either a high probability of release or a high-density of active synaptic sites (Singer and Berger 1999) in view of the large, frequent events detected in the present study. Vice versa, a substantial number of functional GABAergic neurons might have been preserved in the slice preparation: their collective network behavior would have been thus responsible for their strong sensitivity to TTX action. However, immunocytochemical studies show that GABAergic and glycinergic premotoneurons are distributed in several areas of the brain stem and are often not spatially segregated, although their relative preponderance in each projection pathway is unclear (Li et al. 1997). Another possibility would be that glycinergic boutons had a much higher probability of release than GABAergic ones, either because glycinergic terminals possess a more efficient release machinery or because locally released GABA inhibits its own release (Lim et al. 2000).

**Electrophysiological characteristics of stimulus-evoked synaptic currents**

Minimal stimulation of afferent inputs is supposed to activate one or very few presynaptic inputs to the recorded cell (Raastad 1995). In the present experiments, this stimulation protocol was applied to the fibers originating from the lateral reticular formation (Umemiya and Berger 1995). When examining GABA or glycine mediated responses separately, their overall properties like rise and decay times were similar to those of spontaneous PSCs mediated by glycine or GABA, respectively.

On eight cells, recording stability was sufficiently long to allow studying the probability of failures first in control solution, then in the presence of bicuculline, and, finally, in the presence of strychnine (after bicuculline washout). On two cells only an approximately equivalent number of failures was found under all three experimental conditions, an observation that would make compatible co-release of glycine and GABA in this limited number (less than 20%) of cases. However, other data (differential TTX sensitivity, persistence of complex decay mPSCs during antagonist application) did not support the co-release hypothesis. Further clarification of this issue might be provided by pair recording from a single presynaptic fiber and its postsynaptic cell, a hardly achievable aim with a slice preparation. To explore the co-release question with alternative strategies, we tested the effect of up-regulating Cl⁻-dependent synaptic transmission with t-ACPD, which is known to modulate glycine- or GABA-mediated synaptic transmission on other neurons (Bond and Lodge 1995; Chu and Habits 1998; Miles and Ponder 1993).

**Differential modulation of glycine- or GABA-mediated transmission by t-ACPD**

In analogy with other studies (Dong et al. 1996; Schoppa and Westbrook 1997), t-ACPD induced an inward current, associated with a large increase in the frequency of spontaneous PSCs. The action of t-ACPD is complex and exerted at pre- and postsynaptic level via distinct receptor subclasses (Nakanishi 1994). It seems likely that on hypoglossal motoneurons the effects of this substance on spontaneous events were chiefly generated at presynaptic level since the main change was in frequency rather than amplitude. In the case of mPSCs, t-ACPD increased the frequency of glycinergic events only, presumably via a presynaptic site of action. There is no current evidence for selective up-regulation of glycine receptors by metabotropic receptor activation at postsynaptic level. The differential action by t-ACPD on glycinergic versus GABAergic mPSCs suggests that the action of this substance was not a mere epiphenomenon of a rise in cell input resistance that enabled detection of electrotonically remote events.

The present observations also help to clarify the question of...
co-release of GABA and glycine from the same presynaptic cell. The fact that t-ACPD enhanced glycine release without affecting GABA release in TTX solution suggests that co-release did not take place. Co-release might have taken place in limited instances when some inputs were electrically stimulated although another possibility is that even “minimal stimuli” might have activated a larger number of fibers than anticipated. In the latter case, summation and/or occlusion of inputs would have been likely. These data thus suggest that caution is necessary when considering the possibility of co-release on the basis of minimal stimulation experiments in which unequivocal control over presynaptic inputs is lacking.

_Can differences in kinetics between glycinergic and GABAergic events be due to spatial segregation of their receptors?_

The time course of GABAergic sPSCs, mPSCs, and ePSCs was always slower than the one of glycine-mediated responses. The slow decay of GABA receptors was (see also Banks and Pearce 2000; Banks et al. 1998; Rossi and Hamman 1998) was unlikely due to electrotonic filtering of remotely generated responses as there was no apparent correlation between amplitude and rise time (or decay) of GABAergic events (Soltész et al. 1995; Ulrich and Lüscher 1993). Although the synaptic location of these receptors on hypoglossal motoneurons has not yet been proven with ultrastructural studies, GABA receptors containing the α2 subunit (essential to confer them functional properties) (Fritschy et al. 1997) were readily found on the soma of these cells. The same subunit has also been detected on motoneuron soma in the adult rat (Fritschy and Mohler 1995). Likewise, glycine receptors are found on the cell body of neonatal (Singer et al. 1998) and adult (Racca et al. 1998) hypoglossal motoneurons. These results collectively suggest that GABA and glycine receptors have dendritic as well as somatic location. In light of these considerations, it seems that the slow kinetics of GABAergic events perhaps require a different interpretation.

It seems unlikely that some intrinsic properties of GABA receptors were responsible for the slow kinetics as α2-subunit-containing receptors generate fast onset responses (Lavoie et al. 1997; McCellan and Twyman 1999). Another possibility is that GABA receptors spread out beyond the subsynaptic area and were thus activated by transmitter spillover (Barbour and Haussier 1997; Brickley et al. 1996; Faber and Korn 1988; Isaacson et al. 1993; Kullmann and Asztely 1996; Rossi and Hamman 1998). The slow rise time of GABA-mediated events might then represent the time integral required to activate a sparse population of receptors (Clements 1996; Jones et al. 1998; Kruk et al. 1997; Maconochie et al. 1994; Uteshev and Pennefather 1996, 1997). In conjunction with this hypothesis, the small amplitude of GABA events may indicate a low density of subsynaptic GABA receptors.

This proposal is in accordance with the mechanisms of GABAergic transmission operation in the dorsal horn of the spinal cord (Chery and de Konick 1999) or in the hippocampus (Banks and Pearce 2000; Banks et al. 1998), where most GABA receptors are extrasynaptic and are activated mainly by GABA spillover. The _en passant_ varicosities made by GABAergic fibers on hypoglossal motoneurons (Takasu and Hashimoto 1988) might represent the structures involved in this form of synaptic transmission in the hypoglossal nucleus.

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