GABA\textsubscript{B} Receptors Are the First Target of Released GABA at Lamina I Inhibitory Synapses in the Adult Rat Spinal Cord

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Ch\`{e}ry, Nade\`ge and Yves De Koninck. GABA\textsubscript{B} receptors are the first target of released GABA at lamina I inhibitory synapses in the adult rat spinal cord. J Neurophysiol 84: 1006–1011, 2000. We have previously provided functional evidence that glycine and GABA are contained in the same synaptic vesicles and coreleased at the same synapses in lamina I of the rat spinal dorsal horn. However, whereas both glycine receptors (GlyRs) and GABA\textsubscript{A} receptors (GABA\textsubscript{A}Rs) are expressed on the postsynaptic target, under certain conditions inhibitory events appeared to be mediated by GlyRs only. We therefore wanted to test whether GABA\textsubscript{B} receptors could be activated in conditions where GABA released was insufficient to activate GABA\textsubscript{A}Rs. Focal stimulation in the vicinity of visually identified lamina I neurons elicited monosynaptic IPSCs in the presence of ionotropic glutamate receptor antagonists. Pairs of stimuli were given at different interstimulus intervals (ISI), ranging from 25 ms to 1 s to study the depression of the second of evoked IPSCs (paired pulse depression; PPD). Maximal PPD of IPSCs was 60 ± 14\% (SE) of the conditioning pulse amplitude, at ISI between 150 and 200 ms. PPD was observed with IPSCs evoked at stimulus intensities where they had no GABA\textsubscript{A}R component. PPD of small evoked IPSCs was not affected by the GABA\textsubscript{A}R antagonist bicuculline but significantly attenuated by 10^{-3}–10^{-2} \text{M} M3G52432, a specific GABA\textsubscript{B} receptor antagonist. These data indicate that, under conditions where GABA released is insufficient to affect postsynaptic GABA\textsubscript{A}Rs at lamina I inhibitory synapses, significant activation of presynaptic GABA\textsubscript{B} receptors can occur.

INTRODUCTION

We have recently shown that GABA and glycine are coreleased from the same synaptic vesicles at inhibitory synapses on lamina I neurons of the spinal cord. However, we have also shown that miniature (action potential independent) or small evoked IPSCs (mIPSCs) involve activation of glycine receptors (GlyRs) only, even though the postsynaptic neurons expressed both GlyRs and GABA\textsubscript{A} receptors (GABA\textsubscript{A}Rs). Activation of postsynaptic GABA\textsubscript{B} receptors could be detected only by enhancing the affinity of GABA\textsubscript{A}Rs with a benzodiazepine or following stimuli of sufficient intensity to allow synchronous activation of a sufficient number of terminals, presumably because under such conditions GABA could spill over from synapses to reach extrasynaptic GABA\textsubscript{A}Rs (Ch\`{e}ry and De Koninck 1999a). We therefore wanted to test whether presynaptic GABA\textsubscript{B} autoreceptors could be activated at stimulus intensities where GlyR-mediated IPSCs are elicited, but GABA\textsubscript{A}R activation is not detectable. This is because GABA\textsubscript{B} receptors (GABA\textsubscript{B}Rs) may display a greater affinity for GABA than do GABA\textsubscript{A}Rs (Isaacson et al. 1993; Yoon and Rothman 1991). In many regions of the CNS GABA\textsubscript{B}Rs are often found localized on axonal endings (Bowery 1993), indicating that they might play a role in the modulation of neurotransmitter release. A classical test of GABA\textsubscript{B}Rs activation on synaptic release of GABA in the brain is the study of paired-pulse depression (PPD) of inhibitory synaptic events where one analyzes the response to a test stimulus following a conditioning stimulus at different interstimulus intervals (ISIs) (Davies et al. 1990; Otis et al. 1993). Thus we studied PPD of evoked IPSCs by using focal electrical stimuli conditions whereby the phasic release of GABA is insufficient to activate GABA\textsubscript{A}Rs on spinal lamina I neurons. Our findings indicate that, under those circumstances, the amount of GABA released from synaptic terminals first serves to activate GABA\textsubscript{B} receptors. Preliminary accounts of this study have been reported in abstract form (De Koninck and Ch\`{e}ry 1999).

METHODS

Slicing procedure

Adult male Sprague–Dawley rats (weighing 150–250g) were anesthetized with pentobarbital sodium (30 mg/kg), and spinal cord slices were obtained as described previously (Ch\`{e}ry and De Koninck 1999a; Ch\`{e}ry et al. 2000). Briefly, rats were perfused with ice-cold sucrose artificial cerebrospinal fluid (ACSF; in which 126 mM NaCl was replaced with 252 mM sucrose; see below for a description of normal ACSF) and rapidly decapitated. The spinal cord was removed by hydraulic extrusion, and the cervical and lumbar segments (2 cm long) were isolated and glued, lateral side down, on a brass platform with cyanoacrylate cement, in a chamber filled with oxygenated ice-cold sucrose ACSF. Parasagittal 400-\( \mu \)m-thick slices were cut, incubated in sucrose-ACSF at room temperature (23–28°C) for 30 min, and transferred to normal ACSF for at least one hour prior to electrophysiological recordings. Next, the slices were transferred to a recording chamber under a Zeiss Axioscope equipped with infrared differential interference contrast (IR-DIC) and water immersion-objectives for visualization of neurons in thick live tissue. The slices were perfused at \( \sim 2 \text{ml/min} \) with oxygenated ACSF containing (in mM) 126 NaCl, 2.5 KCl, 2 CaCl\(_2\), 2 MgCl\(_2\), 10 glucose, 26 NaHCO\(_3\), 1.25 NaH\(_2\)PO\(_4\) (pH 7.35; 300–310 mOsm), and the glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, 10 \( \mu \)M; Tocris Cook-
**Drug application**

Bicuculline methiodide (10–20 μM; RBI), strychnine hydrochloride (100 nM = 0.5 μM; RBI) and CGP52432 (([3-[[3,4-dichlorophenyl]methyl]amino]propyl[(diethoxymethyl) phosphinic acid; 10–30 μM; Ciba-Geigy) were used to block GABA<sub>A</sub>Rs, GlyRs, and GABA<sub>B</sub>Rs, respectively. The glutamate receptor antagonists CNQX and d-AP5 were used to isolate monosynaptic IPSCs. The action potential blocker tetrodotoxin (TTX, 1 μM; RBI) was used to record miniature IPSCs and the benzodiazepine flunitrazepam (1 μM; Sigma) was used to potentiate GABA<sub>A</sub>Rs and thus unmask the GABA<sub>A</sub>R components of mIPSCs.

**Whole cell recordings and data analysis**

For whole cell voltage-clamp recordings of IPSCs, patch pipettes were pulled from borosilicate glass capillaries (with an inner filament, WPI) using a two-stage vertical puller (Narishige PP-83). To record mIPSCs and evoked IPSCs, the pipettes were filled with an intracellular solution composed of (in mM) 110 CsCl, 10 HEPES, 2 MgCl<sub>2</sub>, 2 mM ATP (Sigma), 0.4 mM GTP (Sigma), 11 mM BAPTA (Sigma), 1 mM CaCl<sub>2</sub> and 0.5% Lucifer yellow (Sigma). The pH was adjusted to 7.2 with CsOH and the osmolarity ranged from 260 to 280 mOsm (pipette resistance 3 MΩ). For the paired pulse experiments, 110 mM CsCl was replaced with 110 mM Cs-gluconate and 5 mM CsCl, and the membrane was held at 0 mV to avoid the confounding effect of action potential generation. Recordings were obtained by lowering the patch electrode onto the surface of visually identified neurons in lamina I. While monitoring current responses to 5 mV pulses, a brief suction was applied to form >GΩ seals. An Axopatch 200B amplifier (Axon Instruments) with >80% series resistance compensation was used for the recording. The access resistance was monitored throughout each experiment. Only recordings with access resistance between 7–20 MΩ were considered acceptable for analysis of evoked IPSCs and only recordings with stable access throughout the entire administration of antagonists were used for further analysis. Monosynaptic IPSCs were evoked by focal electrical stimulation using a patch micropipette. Square-wave constant paired-pulses (200–300 μs duration) were applied at a frequency of ~0.3 Hz, at different interstimulus intervals, ranging from 25 ms to 1 s. The electrode was placed within 20–50 μm of cell body of lamina I neurons. For analysis of the data, traces were low-pass filtered at 10 kHz and stored on a videotape, using a digital data recorder (VR-10B, Instrutech). Offline, the recordings were low-pass filtered at 2–3 kHz and sampled at 10–20 kHz on an Intel Pentium-based computer and analyzed using software designed by Y. De Koninck (Chéry and De Koninck 1999a; De Koninck and Mody 1994).

**Statistical analysis**

Student t-tests were used to analyze the differences between the kinetic and amplitude parameters of the IPSCs. The critical value for statistical significance was set at \( P < 0.05 \). All the data are expressed as mean ± SE, unless otherwise indicated.

**RESULTS**

Whole cell patch-clamp recordings from identified lamina I neurons were performed using a previously described parasag-
for 200 m, namely GABAB receptors. GABA B autoreceptors are found may still be sufficient to activate another receptor subtype, A, completely blocked by strychnine (Fig. 2). Glycine may be subthreshold to activation of GABA A Rs, it critically associated with activation of presynaptic GABAB Rs (Da - 1993). Failure to detect a GABA A R-mediated component to mIPSCs in lamina I neurons was due to a subthreshold activation of GABA A Rs, Chéry and De Koninck 1999a). We confirmed that at these synapses both GlyRs and GABA A Rs can be activated during individual mIPSCs by adding flunitrazepam to enhance the sensitivity of GABA A Rs. Figure 1 illustrates that, while under normal conditions, all mIPSCs are antagonized by 100 nM strychnine, in the presence of flunitrazepam, an additional slowly rising and slowly decaying GABA A R-mediated component appeared in the large majority of events. Following the application of flunitrazepam, >85% of the mIPSCs had a dual kinetic with a very prolonged second component (rise time, 4.1 ± 0.9 ms; decay, 52.8 ± 8.9 ms). Given that mIPSCs represent the activation of postsynaptic receptors by single vesicles of transmitter (Edwards et al. 1990), these results indicate corelease of GABA and glycine from the same synaptic vesicles and thus from the same terminals. This evidence is consistent with previous reports indicating that GABA and glycine are taken up by the same vesicular transporter (Burger et al. 1991; Chaudhry et al. 1998; Dumoulin et al. 1999) and with evidence at the motoneuron synapse that stimulation of single inhibitory interneurons produces mixed GlyRs and GABA A R-mediated IPSCs (Jonas et al. 1998).

While under certain conditions, the GABA coreleased with glycine may be subthreshold to activation of GABA A Rs, it may still be sufficient to activate another receptor subtype, namely GABA B receptors. GABA B autoreceptors are found predominantly in laminae I–II of the dorsal horn (Bowery 1993) and may have a greater affinity for the inhibitory transmitter than GABA A Rs. To test this hypothesis, we sought to detect activation of GABA B receptors under conditions where GABA A Rs are not activated (i.e., conditions in which inhibitory currents are mediated by GlyRs only). Figure 2 illustrates that IPSCs evoked by focal stimuli at low intensity (<100 µA for 200 µs) in the vicinity of identified lamina I neurons were completely blocked by strychnine (Fig. 2A; n = 12). In the presence of strychnine, GABA A R-mediated evoked IPSCS were only obtained on increasing the stimulus intensity (Fig. 2B).

Using such stimuli that resulted in pure GlyR-mediated IPSCS, we studied the paired-pulse depression (PPD) of IPSCS evoked in lamina I neurons. Paired-pulse depression is typically associated with activation of presynaptic GABA A Rs (Davies et al. 1990). A conditioning current and a test current were applied focally at different interstimulus intervals (ISIs; see Fig. 3). The ISIs ranged from 25 ms to 1 s. When the ISI was shorter than the decay of the conditioning IPSCS, an overlap in time of the conditioning and test currents was observed. Thus a digital subtraction was used to obtain accurate values for the peak of the test IPSCS, as previously described (Otis et al. 1993). Figure 3 illustrates PPD of evoked IPSCs in a lamina I neuron. The maximal depression of the test IPSCS (60 ± 14% of the amplitude of the conditioning IPSC; P < 0.01) was observed at 150–200 ms ISIs (n = 6).

Up to 20 µM bicuculline failed to affect the amplitude of the conditioning pulse (Fig. 4B; nor the PPD ratio), indicating that IPSCS evoked by minimal stimuli do not involve activation of postsynaptic GABA B Rs. PPD of small evoked IPSCs was reversed following bath application of 10–30 µM CGP52432 (Fig. 4C), a specific GABA B receptors antagonist. This suggests that GABA B autoreceptors appear to be the first target of GABA released at inhibitory synapses in lamina I neurons. The evoked IPSCs were completely abolished by strychnine (Fig. 4B), confirming that they are selectively mediated by GlyRs.

**DISCUSSION**

Our findings indicate that, while GABA and glycine appear to be released from the same synaptic terminals in lamina I, the amount of GABA released on activation of a few synaptic terminals may be subliminal to the activation of postsynaptic GABA A receptors, yet the released GABA may be sufficient to significantly activate presynaptic GABA B receptors.

The predominant localization of GABA B receptors in superficial laminae of the spinal cord (Malcangio et al. 1993) and their preferential occurrence on synaptic terminals in many CNS regions (Bowery 1993) indicate that they may have an important role in the modulation of GABA release in the dorsal horn.

Given the evidence of both GABA and glycine are contained in the same terminals and most likely in the same synaptic vesicles (this study and Burger et al. 1991; Chaudhry et al. 1998; Chéry and De Koninck 1999a; Dumoulin et al. 1999;
Jonas et al. 1998) and that small evoked IPSCs were mediated exclusively by glycine provided an ideal setting to test whether activation of GABA_B receptors occurs in conditions where GABA_A receptor activation may not be detectable, because the release event could be measured independent of a GABA_A component using the GlyR-mediated event. In this study we were able to show GABA_B-mediated PPD of GlyR IPSCs. Thus our results provide evidence that GABA_B autoreceptors are present on glycnergic interneurons that also contain GABA (Todd and Spike 1993), where they modulate the release of both inhibitory transmitters from interneurons terminals. This evidence is consistent with the recent demonstration of presynaptic inhibition of both GABA and glycine release at spinal interneuron-motoneuron synapses by the GABA_B agonist baclofen (Jonas et al. 1998).

The released GABA is likely originating from the same terminal as the released glycine because results from immunocytochemical studies indicate that virtually all of the glycnergic neurons and terminals in the superficial dorsal horn also contain GABA (Mitchell et al. 1993; Todd and Sullivan 1990) and evidence indicate that in these terminals, glycine and GABA are packaged in the same synaptic vesicles (Burger et al. 1991; Chêry and De Koninck 1999a; Dumoulin et al. 1999; Jonas et al. 1998). It remains however that some GABAergic neurons do not contain glycine. Thus it is possible that some of the GABA released may originate from separate terminals from those releasing the glycine. In such case, our results
have observed a recruitment of GABAA Rs to synaptic junctions of the pharmacology of GABAergic synapses. This is of particular interest in light of evidence that such synaptic arrangements may be an important means to control excitability in this area and thus the relay of nociceptive input to the brain. Interestingly, the results of binding studies in dorsal horn slices and spinal cord synaptosomes indicate that GABA A receptors on GABAergic terminals may be distinct from the heteroreceptors present on glutamate-releasing terminals (Bonanno et al. 1998; Teoh et al. 1996). Increasing the release of GABA may be particularly useful to enhance inhibition in lamina I, given our recent evidence that GABA A Rs are only activated under conditions that promote spillover of GABA from synapses in lamina I (Chéry and De Koninck 1999a). Thus it may be possible to selectively antagonize GABA B autoreceptors to favor inhibitory transmission mediated by both GABA and glycine in lamina I without significantly affecting GABA B heteroreceptors (present on glutamate-containing synaptic terminals). Selective targeting of GABA B autoreceptors may prove particularly useful for the treatment of chronic pain states (Henry 1982).

The issue of whether distinct interneurons are responsible for GABA A- and GABA B-mediated inhibition has been discussed for quite some time and several lines of evidence indicate that these two classes of receptors may be differentially activated at some synapses (Benardo 1994; Newberry and Nicoll 1984; Nurse and Lacaille 1997; Otis and Mody 1992; Segal 1990; Solis and Nicoll 1992; Sugita et al. 1992). In cases of mixed GABA A and GABA B responses, recruitment of few inhibitory interneurons appear to often preferentially activate GABA A Rs, while activation of GABA B autoreceptors often requires recruitment of a larger number of afferents as it likely promotes GABA spillover from synapses (Dutar and Nicoll 1988; Isaacson et al. 1993; Nurse and Lacaille 1997; Otis and Mody 1992; Ouardouz and Lacaille 1997). The converse scenario appears to apply to lamina I inhibitory synapses and could be attributed to the fact that in this case GABA A Rs, like GABA B Rs, are likely located at a distance from the release site (i.e., a preferential extrasynaptic distribution of GABA A Rs (Chéry and De Koninck 1999a)). Differential subsynaptic distribution of GABA A Rs may thus be an important determinant of the pharmacology of GABAergic synapses. This is of particular interest in light of evidence that such synaptic arrangement can be altered under certain conditions. For example, we have observed a recruitment of GABA A Rs to synaptic junctions at lamina I synapses following peripheral nerve injury (Chéry and De Koninck 1999b).

Thus GABAergic inhibition appears to be modulated in a selective manner in lamina I, whereby GABA B autoreceptors may be the first target of GABA released in this spinal area. Such regulation of GABA release may have important physiological implications, notably under conditions that favor hyperexcitability in the dorsal horn.

In summary, these data indicate that under conditions where GABA release is insufficient to significantly affect postsynaptic GABA A Rs on lamina I neurons, it may rather serve to activate GABA A receptors to regulate the release of both glycine and GABA.

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