Co-release and interaction of two inhibitory co-transmitters in rat sacral dorsal commissural neurons

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Conventional whole-cell recording configuration was used to characterize the interaction between two inhibitory neurotansmitters, GABA and glycine, in synaptic bouton preparation obtained from rat sacral dorsal commissural nucleus (SDCN). The co-release of GABA and glycine as well as the interaction between their corresponding receptors was demonstrated. Furthermore, taking advantage of pure glycinergic terminal preparation, the possible interaction between GABA and glycine at synaptic level was studied. The results revealed a novel cross-modulation between the two inhibitory cotransmitters systems. This interaction may contribute to sensory processing such as nociception in the mammalian deep dorsal horn under physiological and/or pathological conditions. *NeuroReport* 13:977–981 © 2002 Lippincott Williams & Wilkins.

Key words: Co-release; GABA; Glycine; Sacral dorsal commissural nucleus

INTRODUCTION

It is well accepted that individual neurons in the CNS release a single fast transmitter. However, recent studies have demonstrated that the two main fast inhibitory transmitters GABA and glycine are co-released from the same presynaptic terminals even from the same presynaptic vesicles [1–4]. In addition, it is also becoming evident that neurotransmitter receptors can interact with each other [5–9], exerting powerful modulation upon receptor activation adapting transmitter signaling to various functional situations.

The sacral dorsal commissural nucleus (SDCN), which represents the area just dorsal to the central canal in the lower lumbar and sacral spinal cord, receives primary inputs form pelvic organs and is involved in nociceptive transmission [10]. Therefore, the regulation of excitability of SDCN neurons has a major impact on the pelvic sensory processing especially nociception [11,12]. Our previous observations indicated both GABA and glycine neurotansmissions are present in the rat SDCN [11,12]. Here we use whole-cell patch-clamp recordings to examine the co-release and possible interaction of these two neurotransmitters in mechanically dissociated SDCN neurons retaining functional synaptic terminals [11,13].

MATERIALS AND METHODS

Synaptic bouton preparation: Neurons from the sacral dorsal commissural nucleus (SDCN) were mechanically dissociated as described previously [11,13]. In brief, 2-week-old Wistar rats were killed by decapitation and a segment of lumbosacral (L5–S4) spinal cord was dissected out. Then, 400 μm transverse slices were obtained at 400 μm with a vibratome tissue slicer (VT1000S, Leica instruments Ltd, Wetzlar, Germany). A vibration-isolation system [14,15] was then used to mechanically dissociate the SDCN neurons. Briefly, a fire-polished glass pipette mounted on a vibrator touched lightly and vibrated horizontally at about 5–10 Hz on the surface of the SDCN of the spinal slice under the control of a pulse generator. The vibration-dissociation lasted for about 3 min and the slices were then removed from the dish. Within 20 min of dissociation, isolated neurons had attached to the bottom of the culture dish and were ready for electrophysiological experiments.

Solutions and drugs: The ionic composition of the incubation solution was (mM): NaCl, 124; $NaHCO_3$, 24; KCl, 5; KH_2PO_4, 1.2; $CaCl_2$, 2.4; $MgSO_4$, 1.3; glucose, 10; aerated with 95% O_2/5% CO_2 to a final pH of 7.4. The standard external solution contained (mM): NaCl, 150; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2; glucose, 10; HEPES, 10; pH 7.3 adjusted with Tris-base. The osmolarity of all bath solutions was adjusted to 325–330 mOsm/l with sucrose (3300, Norwood, Massachusetts, USA). The patch pipette solution for whole-cell patch recording was (mM): KCl, 120; NaCl, 30; $MgCl_2$, 1; $CaCl_2$, 0.5; EGTA, 5; Mg-ATP, 2; HEPES, 10. The internal solutions were adjusted to a pH of 7.2 with Tris-base.

When the current–voltage (I-V) relationship for miniature inhibitory postsynaptic currents (mIPSCs) was examined, 0.3 μM tetrodotoxin (TTX) and 0.2 mM $CdCl_2$ were added to the standard external solution. Drugs were applied using a rapid application technique termed the Y-tube method throughout the experiments [12]. This system allows a complete exchange of external solution surrounding a neuron within 20 ms.

Electrophysiological recordings and data analysis: Whole-cell patch-clamp recording was carried out at room temperature (22–25°C) using a patch-clamp amplifier (200B, Axon Instruments, Foster City, CA, USA), sampled and analyzed using a Digidata 1320A interface and a computer with the Clampex and Clampfit software (Version 8.0.1, Axon Instruments). The resistance between the recording electrode filled with pipette solution and the reference electrode was 4–6 MΩ. In most experiments, 80–90% series resistance was compensated. Synaptic events were counted using Mini Analysis Program (version 4.3.3, Synaptosoft Inc.). Cumulative data were compared using the Kolmogorov–Smirnov test (K-S test) for significant difference. Difference in mean amplitude and frequency were tested by Student's paired two-tailed *t*-test. All statistical results are given as mean ± s.e.m. The membrane potential was held at −50 mV throughout the experiment.

RESULTS

Functional interaction between $GABA_A$ receptor ($GABA_AR$) and glycine receptor (GlyR) in the sacral dorsal commissural nucleus (SDCN) neurons: Our previous studies have demonstrated that both $GABA_AR$ and GlyR are present in the acutely dissociated SDCN neurons [11,12]. In addition, state-dependent cross-inhibition between $GABA_AR$ and GlyR has been observed in rat hippocampus CA1 neurons recently [9]. This stimulated us to study whether the two receptors interact in SDCN neurons.

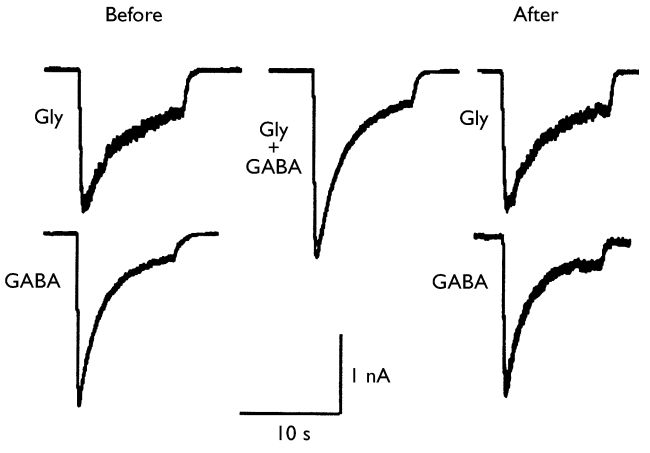

Fig. 1. Interaction between GABA and glycine at the receptor level in rat SDCN neurons. 1 mM GABA- and 1 mM glycine-induced currents had partial occlusion. Responses caused by individual application of GABA and glycine after co-application were similar to the individual responses obtained before co-application. Similar results were obtained from other seven neurons (see text).

To examine the possible interaction of $GABA_AR$ and GlyR, GABA and glycine were exogenously applied to the mechanically dissociated SDCN neurons. As the idea that $GABA_AR$ and GlyR are pharmacologically distinct ligand-gated ion channels appears well supported, it could be expected that these receptor-mediated responses are additive [8,9]. Thus, we compared the total currents predicted by a simple summation of the two individual currents with the observed currents evoked by coapplication of GABA and glycine. GABA- and glycine-induced currents showed partial occlusion (Fig. 1). Co-application of saturating concentrations of GABA (1 mM) and glycine (1 mM) activated a current response that was only 51.4 ± 3.1% ($n = 8$, $p < 0.001$) of the linear sum of the individual currents induced by 1 mM GABA and 1 mM glycine (Fig. 1).

Co-release of GABA and glycine in SDCN neurons: As the co-release of GABA and glycine has recently been demon-

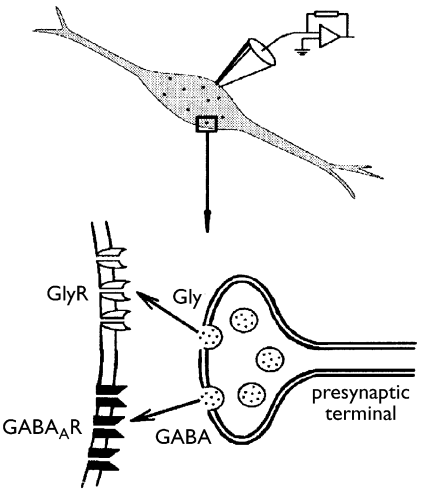

Fig. 2. Co-transmission of GABA and glycine to the SDCN neurons. Schematic of recording setup. Recordings were made from mechanically dissociated SDCN neurons retaining functional synaptic terminals.

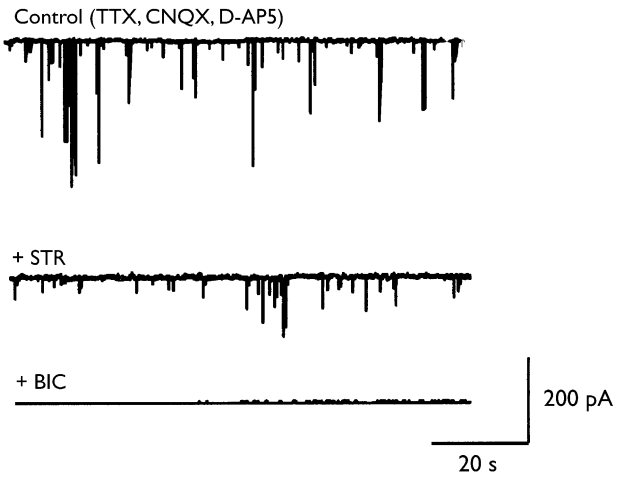

Fig. 3. Bicuculline (BIC, 1 μM) completely blocked the strychnine (STR, 1 μM)-resistant mIPSCs.

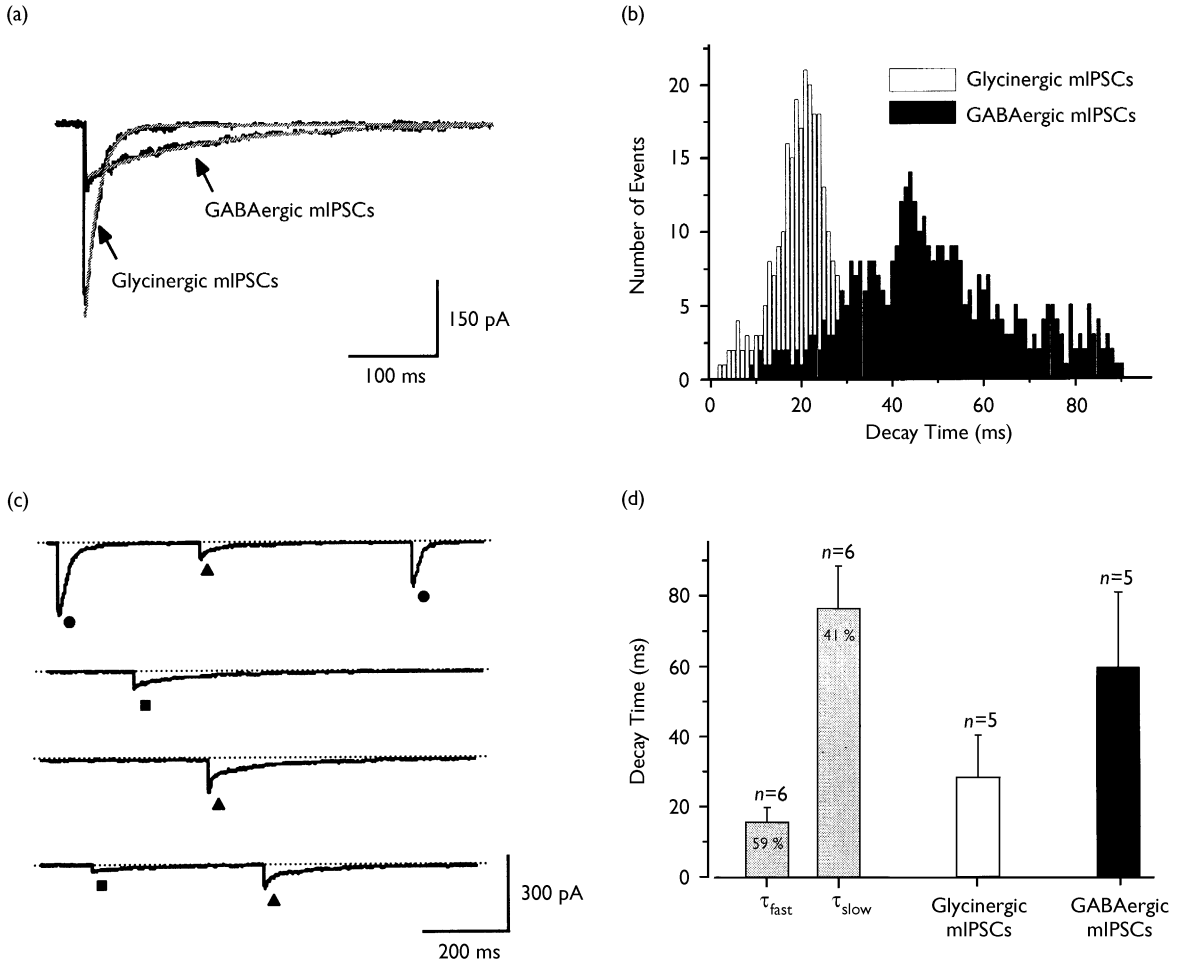

Fig. 4. Cotransmission of GABA and glycine to the SDCN neurons. (**a**) Typical GABAergic and glycinergic mIPSCs recorded in the presence of 30 μM sodium pentobarbital. Note the difference between their decay time. (**b**) GABAergic and glycinergic mIPSCs can be distinguished by the decay time distribution. (**c**) Three types of mIPSCs can be distinguished in the presence of pentobarbital; the slow GABAergic mIPSCs (■), the fast glycinergic mIPSCs (●) and the dual-component mIPSCs (▲). (**d**) Average decay times of the three types of mIPSCs are shown. Dual-component mIPSCs account for bi-exponential fit and the mean of τ_{fast} and τ_{slow} is comparable to the decay time of glycinergic and GABAergic mIPSCs respectively.

strated in the ventral horn [1] and superficial dorsal horn [4] of the rat spinal cord, it is interesting to know whether this is also the case in the deep dorsal horn (i.e. SDCN). To address this issue, a novel preparation of mechanically isolated SDCN neurons retaining functional synaptic terminals, so-called synaptic bouton preparation, was used (Fig. 2).

In the presence of TTX (0.3 μM), CNQX (3 μM) and D-AP5 (10 μM), miniature inhibitory postsynaptic currents (mIPSCs) were recorded in these neurons. In a small population of the neurons (seven of 25), application of 1 μM strychnine (STR), a selective GlyR antagonist, completely blocked the mIPSCs. This suggested that the pure glycinergic terminals were preserved in some neurons. In the rest of the neurons (18 of 25), the STR-resistant mIPSCs were abolished completely by 1 μM $GABA_AR$ antagonist bicuculline (BIC; Fig. 3), suggesting that functional GABAergic and glycinergic transmissions both existed in these neurons and they could be dissected pharmacologically.

To investigate the co-release of GABA and glycine, the neurons receiving both GABAergic and glycinergic inputs were selected and sodium pentobarbital (PB, 30 μM) was used to enhance the differences between the two types of mIPSCs [2]. In the presence of pentobarbital (PB), the decay time constant of the GABAergic mIPSCs differed significantly from that of glycinergic mIPSCs with means of 59.6 ms and 28.2 ms ($n = 5$, $p < 0.001$), respectively (Fig. 3). The two separate distributions of mIPSCs in the presence of PB made it easy to distinguish glycinergic events from slower decaying GABAergic events (Fig. 3). Interestingly, some individual mIPSCs (18.2 ± 7.1% of the total number of mIPSCs, $n = 6$) showed dual components which could be fitted appropriately with bi-exponential function (Fig. 3). The average decay time constants of slow ($\tau_{slow} = 76.3 \pm 12.1$ ms) and fast ($\tau_{fast} = 15.6 \pm 4.2$ ms) components of the dual-component mIPSCs were comparable to those of the pharmacologically isolated glycinergic and GABAergic events, respectively (Fig. 3). Because miniature synaptic currents are thought to reflect the release of transmitters from single vesicles, the above observations suggested that the dual-component events were possibly resulted from the co-release of GABA and glycine [1,2].

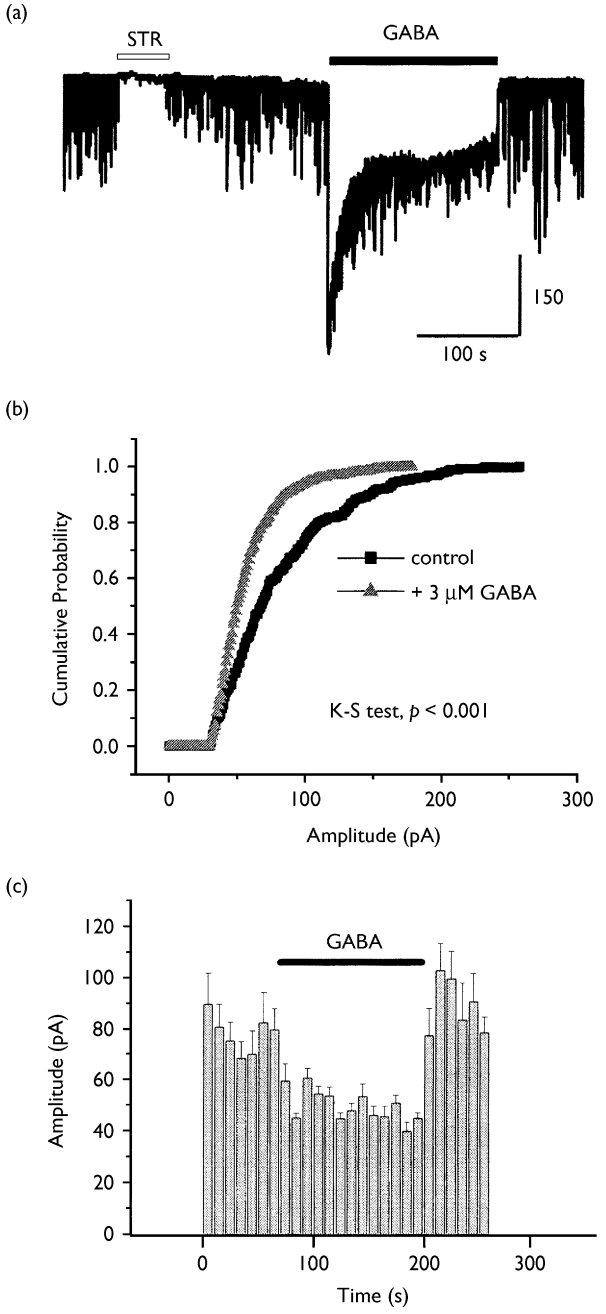

Fig. 5. GABA-modulated glycinergic mIPSCs. (**a**) Pure glycinergic mIPSCs could be completely abolished by 1 μM STR (left). Exogenously applied 3 μM GABA induced an inward current in the same neuron (right). (**b**) Cumulative probability plots of mIPSCs amplitude show a significant difference between events in control and during application of GABA. (**c**) Pooled results for the mean mIPSCs amplitude as a function of time show that 3 μM GABA decreased the amplitude of glycinergic events.

The effect of exogenously applied GABA on glycinergic mIPSCs: We then hypothesized that co-released GABA and glycine would interact at the synaptic level. If this is the case, GABA should affect glycinergic synaptic transmission and vice versa. To test this possibility, GABA was locally applied to the SDCN neurons receiving pure glycinergic input (Fig. 4) [11]. The inward currents were elicited by application of 3 μM GABA in all the tested neurons ($n = 9$) which could be selectively blocked by 1 μM BIC. On average, 3 μM GABA decreased the amplitude of glycinergic mIPSCs by $22.5 \pm 19.3\%$ (Fig. 4b,c) in eight of nine neurons tested (n=8, $p < 0.01$). Meanwhile, there existed changes in the frequency of the mIPSCs. GABA 3 μM depressed the frequency of the glycinergic mIPSCs in five of nine neurons while it was increased in the rest of the neurons (data not shown). The results suggested that GABA had both postsynaptic and presynaptic modulation of glycinergic synaptic transmission (Fig. 5).

DISCUSSION

The present experiments indicated that the currents carried by $GABA_AR$ and GlyR in SDCN neurons were occluded when they were activated simultaneously. The result together with previous reports [9,16,17] suggests that these two anionic ionotropic receptors interact functionally. This stimulated us to explore the co-release of GABA and glycine in the mechanically dissociated SDCN neurons retaining functional synaptic terminals. The present data showed that both GABAergic and glycinergic synaptic inputs converged onto the same SDCN neurons. Furthermore, some dual-component mIPSCs were possibly due to the co-release of glycine and GABA from the same presynaptic vesicles. These results strengthen the conclusions of the previous morphological [18] and electrophysiological studies [1–4] in the ventral and superficial dorsal horn of the spinal cord, in which co-released GABA and glycine act as co-transmitters.

To study the interaction between GABA and glycine *in situ*, a model of pure glycinergic presynaptic terminal preparation was used to exclude the interference of other transmitter systems. GABA was applied exogenously to study its effect on glycinergic mIPSCs, thus mimicking the co-release and possible interaction between GABA and glycine [7]. The results showed that GABA has partial occlusion of the amplitude of the glycinergic mIPSCs. This observation was expected as we had demonstrated that $GABA_AR$ and GlyR cross-inhibited each other on the SDCN neurons (Fig. 1). The changes in the frequency of the glycinergic mIPSCs during the application of the GABA might due to the presynaptic $GABA_A$ or $GABA_B$ receptors. The present experiment extends the interaction of GABA and glycine from the receptor level to the synaptic level.

Our study supports the idea that GABA- and glycine-mediated co-transmission might be a general principle of inhibition, especially in the mammalian spinal cord. Due to the difference between the decay time of GABAergic and glycinergic mIPSCs, the co-release of GABA and glycine would finely tune their inhibitory effect *in vivo* [1]. The cross-inhibition between GABA and glycine receptors could brake excessive inhibition, reflecting the activity balance and plasticity of the neurons when receiving fast inhibitory co-transmissions [9]. Thus, the interaction between these two inhibitory transmitter systems may contribute critically to the sensory processing such as nociception in the mammalian spinal dorsal horn.

CONCLUSION

We have obtained evidence of interaction between GABA and glycine either at receptor or synaptic level in rat SDCN neuron. The two transmitters may be co-released from the same presynaptic terminal, and their corresponding receptors, $GABA_AR$ and GlyR cross-inhibit each other. The interaction may reflect the activity balance and plasticity of the neurons when receiving both GABAergic and glycinergic transmitters, which would finely tune their inhibitory effect on the postsynaptic neurons.

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