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Functional roles of presynaptic $GABA_A$ receptors on glycinergic nerve terminals in the rat spinal cord

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$GABA_A$ receptor-mediated presynaptic depolarization is believed to induce presynaptic inhibition of excitatory synaptic transmission. We report here the functional roles of presynaptic $GABA_A$ receptors in glycinergic transmission of the rat spinal cord. In mechanically dissociated rat sacral dorsal commissural nucleus (SDCN) neurons attached with native glycinergic and GABAergic nerve terminals, glycinergic spontaneous inhibitory postsynaptic currents (sIPSCs) were isolated from a mixture of both glycinergic and GABAergic sIPSCs by perfusing the SDCN nerve cell body with ATP-free internal solution. Under such experimental conditions, exogenously applied muscimol (0.5 μM) depolarized glycinergic presynaptic nerve terminals and significantly increased glycinergic sIPSC frequency to 542.7 ± 47.3 % of the control without affecting the mean current amplitude. The facilitatory effect of muscimol on sIPSC frequency was completely blocked by bicuculline (10 μM) or SR95531 (10 μM), selective $GABA_A$ receptor antagonists. This muscimol-induced presynaptic depolarization was due to a higher intraterminal Cl^- concentration, which is maintained by a bumetanide-sensitive Na–K–Cl cotransporter. On the contrary, when electrically evoked, this muscimol-induced presynaptic depolarization was found to decrease the action potential-dependent glycine release evoked by focal stimulation of a single terminal. The results suggest that $GABA_A$ receptor-mediated presynaptic depolarization has two functional roles: (1) presynaptic inhibition of action potential-driven glycinergic transmission, and (2) presynaptic facilitation of spontaneous glycinergic transmission.

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GABA is a primary inhibitory neurotransmitter throughout the mammalian CNS. $GABA_A$ receptor-mediated inhibitory actions are generally due to an increase in the Cl^- conductance leading to direct postsynaptic hyperpolarization. In sensory afferent terminals, the activation of presynaptic $GABA_A$ receptors also induces presynaptic inhibition to reduce the electrically stimulated neurotransmitter release resulting from inactivation of Na^+ channels and/or shunt of the presynaptic membrane by depolarizing the presynaptic terminals (Segev, 1990; Stuart & Redman, 1992; Graham & Redman, 1994; Cattaert & El Manira, 1999). Such a $GABA_A$ receptor-mediated presynaptic depolarization is based on a higher intracellular Cl^- concentration ($[Cl^-]_i$) which results from a Na–K–Cl cotransporter (NKCC) (for review, Alvarez-Leefmans, 1990; Kaila, 1994; Russell, 2000). Electron and confocal microscope studies have also provided some evidence for the existence of GABAergic axo-axonic synapses or GABA immunoreactivity on the excitatory nerve terminals or *en passant* boutons (Atwood *et al.* 1984; Lamotte d'Incamps *et al.* 1998). These observations support the idea that $GABA_A$ receptors on the excitatory nerve axon or terminal represent morphological substrates for presynaptic inhibition.

Most spinal neurons receive both GABAergic and glycinergic inputs (Todd & Sullivan, 1990; Schneider & Fyffe, 1992; Bohlhalter *et al.* 1994). In addition, there is much convincing evidence that GABAergic boutons make axo-axonic synapses with other presynaptic nerve terminals in the spinal cord (Todd *et al.* 1995; Maxwell *et al.* 1997). These results suggest the possibility that GABA, through $GABA_A$ receptors, can modulate the inhibitory GABAergic or glycinergic neurotransmissions at presynaptic sites. To test this hypothesis, we utilized mechanically isolated sacral dorsal commissural nucleus (SDCN) neurons retaining both glycinergic and GABAergic nerve terminals. In such SDCN neurons, we isolated the glycinergic sIPSCs from a mixture of both glycinergic and GABAergic sIPSCs without using bicuculline, a selective $GABA_A$ receptor antagonist. This procedure allowed us to selectively focus on the functional action of presynaptic $GABA_A$ receptors on glycinergic transmission. In addition, we also attempted to determine whether $GABA_A$ receptor-mediated presynaptic depolarization could induce presynaptic inhibition for glycinergic transmission elicited by the selective focal electrical stimulation of a single glycinergic bouton.

METHODS

All experiments conformed to the guiding principles for the care and use of animals approved by the Council of the Physiological Society of Japan and all efforts were made to minimize both the number of animals used and their suffering.

Preparation

Wistar rats (10–12 days old) were decapitated under pentobarbital anaesthesia (50 mg kg^{-1}, I.P.). A segment of the lumbosacral (L6–S2) spinal cord was dissected and transversely sliced at a thickness of 350 μm using a microslicer (VT1000S; Leica, Nussloch, Germany). Slices containing the sacral dorsal commissural nucleus (SDCN) were kept in a control incubation medium (see below) saturated with 95 % O_2 and 5 % CO_2 at room temperature (21–24 °C) for at least 1 h before mechanical dissociation. For dissociation, slices were transferred into a 35 mm culture dish (Primaria 3801; Becton Dickinson, Rutherford, NJ, USA) containing the standard external solution (see below). The region of the SDCN was identified under a binocular microscope (× 20, SMZ-1; Nikon, Tokyo, Japan). The details of mechanical dissociation have previously been described (Rhee *et al.* 1999). Briefly, mechanical dissociation was accomplished using a custom-built vibration device and a fire-polished glass pipette oscillating at about 40–60 Hz (0.1–0.2 mm). The tip of the fire-polished glass pipette was lightly placed on the surface of the SDCN region using a micromanipulator. The tip of the glass pipette was then vibrated horizontally for about 2 min. The slices were next removed, and mechanically dissociated neurons (5–10 neurons per dish) were allowed to settle and adhere to the bottom of the dish for 15 min. Such neurons undergoing dissociation retained a short portion of their proximal dendrites (Fig. 1*A*).

Electrical measurements

All electrical measurements were performed using the conventional whole-cell patch recording mode at a holding potential of −30 to −40 mV using a patch clamp amplifier (CEZ-2300; Nihon Kohden, Tokyo, Japan), except when examining *I–V* relationships. Patch pipettes were made from borosilicate capillary glass (1.5 mm o.d., 0.9 mm i.d.; G-1.5; Narishige, Tokyo, Japan) in two stages on a vertical pipette puller (PB-7; Narishige). The resistance of the recording pipettes filled with internal solution was 5–6 MΩ. Electrode capacitance and liquid junction potential were compensated, but the series resistance was not. Neurons were viewed under phase contrast on an inverted microscope (×400, Diaphot; Nikon). Current and voltage were continuously monitored on an oscilloscope (VC-60 23; Hitachi) and a pen recorder (RECTI-HORIT-8K; Sanei, Tokyo, Japan) and recorded on a digital-audio tape recorder (RD-120TE; TEAC). The membrane currents were filtered at 1 kHz (E-3201A Decade Filter; NF Electronic Instruments, Tokyo, Japan), digitized at 4 kHz, and stored on a computer equipped with pCLAMP 8.0 (Axon Instruments). When recording, 10 mV hyperpolarizing step pulses (30 ms in duration) were periodically delivered to monitor the access resistance. All experiments were performed at room temperature (21–24 °C).

Identification and focal stimulation of a single bouton

Identification and focal stimulation of single boutons were performed as described in our previous report (Akaike *et al.* 2002). Briefly, FM 1–43 (10 μM) in 45 mM high K^+ solution, in which 40 mM NaCl in a standard external solution was replaced with equimolar KCl, was perfused across a dissociated single neuron for 30 s, and then washed out with a standard external solution. The fluorescent spots indicating the existence of putative presynaptic nerve terminals were visualized with an inverted microscope equipped with barrier filters (excitation of 390–490 nm, long-pass filter of 590 nm). Fluorescence imaging was recorded by a CCD camera (CoolSNAP, Photometrics, AZ, USA) and the fluorescence intensity was measured by Lumina Vision software (Mitani Corporation, Chiba, Japan) (Fig. 1*A*).

The stimulation pipette was made by the same method as described for the recording pipette. Its inner diameter was 0.5–0.6 μm. Short negative pulses (5–10 μA, 100 μs in duration) at 0.2 Hz through the glass pipette filled with a standard external solution were applied using a stimulus isolation unit (SS-202J; Nihon Koden). The stimulation pipette was placed close to the surface of a single bouton identified with FM 1–43. The evoked current was elicited in an 'all-or-none' fashion when the stimulation pipette was positioned just above a bouton, and was highly sensitive to 0.3 μM tetrodotoxin (TTX) (Akaike *et al.* (2002).

Data analysis

Spontaneous inhibitory postsynaptic currents (sIPSCs) were detected and analysed in pre-set epochs before, during and after each experimental condition, using the MiniAnalysis Program (Synaptosoft, NJ, USA). Briefly, spontaneous events were automatically screened using an amplitude threshold of 5 pA and were then visually accepted or rejected based upon the rise (0.5–1.5 ms) and decay times (8.0–20.0 ms). In complex waveforms where the event starts to rise before the previous event goes back to the baseline, the baseline for the second event was estimated by extrapolating the decay of the first peak at the location of the double peak. Then the peak amplitude of the second event was determined from this calculated baseline but not from the onset point of the event (Fig. 1*C*). The average values of sIPSC frequency and amplitude during the control period (10–15 min) were calculated, and the frequency and amplitude of all the events during agonist application (1–2 min) were normalized to these values. The effect of the agonist was quantified as a percentage increase in sIPSC frequency compared to the control value. The amplitude of evoked IPSCs (eIPSCs) was analysed using pCLAMP 8.0. Numerical values were reported as means ± standard error of the mean (S.E.M.) using values normalized to the control levels. Possible differences in the amplitude and frequency distribution were tested by Student's paired two-tailed *t* test using their absolute values but not normalized ones. Values of $P < 0.05$ were considered to be significant. On the other hand, the inter-event intervals and amplitudes of a large number of sIPSCs obtained from the same neuron were examined by constructing cumulative probability distributions and compared using the Kolmogorov-Smirnov (K-S) test with StatView software (SAS Institute, Inc., Cary, NC, USA).

Solutions

The incubation medium consisted of (mM): 124 NaCl, 5 KCl, 1.2 KH_2PO_4, 24 $NaHCO_3$, 2.4 $CaCl_2$, 1.3 $MgSO_4$ and 10 glucose saturated with 95 % O_2 and 5 % CO_2. The pH was about 7.45. The standard external solution consisted of (mM): 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 Hepes. The Ca^{2+}-free external solution consisted of (mM): 150 NaCl, 5 KCl, 5 $MgCl_2$, 2 EGTA, 10 glucose and 10 Hepes. The Na^+-free external solution consisted of (mM): 150 *N*-methyl-D-glucamine-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 Hepes. These external solutions were adjusted to a pH of 7.4 with Tris-base. For recording sIPSCs, external solutions contained 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 20 μM DL-2-amino-5-phosphonovaleric acid

(AP5) to block glutamatergic currents. The internal (patch-pipette) solution for the whole-cell patch recording consisted of (mM) 145 caesium methanesulfonate, 5 TEA-Cl, 5 CsCl, 2 EGTA and 10 Hepes, and was adjusted to a pH of 7.2 with Tris-base. For the depression of the postsynaptic GABA$_A$ response, most experiments were performed with the above internal solution, which did not contain ATP (Shirasaki *et al.* 1992; Harata *et al.* 1997; Jang *et al.* 2001).

Drugs

The drugs used in the present study included TTX, bicuculline, strychnine, CNQX, AP5, EGTA, muscimol, diazepam, bumetanide, *cis*-aminocrotonic acid (CACA), baclofen and 2-[3-carboxypropyl-3-amino-6-[4-methoxyphenyl]pyridazinium bromide (SR95531) from Sigma (St Louis, MO, USA), and FM 1–43 (Molecular probes, OR, USA). CNQX, bicuculline and bumetanide were dissolved in dimethyl sulfoxide at 10 mM as a stock solution. All solutions containing drugs were applied by the 'Y-tube system' for rapid solution exchange (Akaike & Harata, 1994).

RESULTS

Mechanically dissociated SDCN neurons preserve the functional presynaptic nerve terminals

After brief mechanical dissociation of the SDCN region, we found that neurons were bipolar (Fig. 1*Aa*) or triangular in shape (10–15 μm in somatic diameter). To identify the functional presynaptic nerve terminals, we applied FM 1–43 to dissociated SDCN neurons (Fig. 1*Ab*). Note that several fluorescent spots regarded as the putative presynaptic nerve terminals were located on the soma and proximal dendrites of a neuron. When 0.5 μM muscimol was applied to a neuron, one of the fluorescent spots (arrow in Fig. 1*Ac*) was quickly destained but the other (arrowhead in Fig. 1*Ac*) was not (Fig. 1*B*). However, these two fluorescent spots were further destained by subsequent addition of 20 mM K^+ solution (Fig. 1*Ad* and *B*). The results suggest that mechanically dissociated SDCN neurons preserve the functional presynaptic nerve terminals which are sensitive to the stimulation of muscimol or high K^+ solution.

Separation of glycinergic sIPSCs from a mixture of both glycinergic and GABAergic sIPSCs

To investigate whether exogenously applied GABA influences the glycinergic transmission, glycinergic sIPSCs were isolated from GABAergic ones by perfusing the SDCN nerve cell body with ATP-free internal solution, which rapidly induces a selective rundown of the GABA$_A$

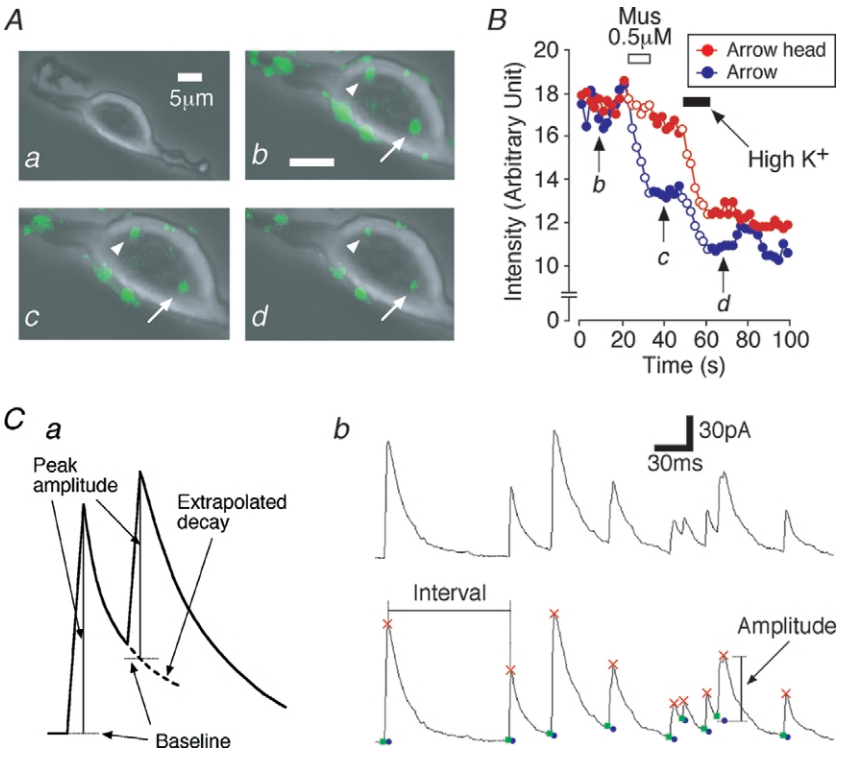

Figure 1. Mechanically dissociated SDCN neurons and the method for detection of sIPSCs

A, representative photographs of a mechanically dissociated SDCN neuron before loading FM 1–43 (*a*), after loading FM 1–43 (*b*), after application of 0.5 μM muscimol (*c*) and after application of 20 mM K^+ solution (*d*). In *b*, *c* and *d*, the fluorescence signals were superimposed on *a*. *B*, typical time courses of the fluorescence intensities of two presynaptic nerve terminals during application of muscimol or high K^+ solution. Red and blue points were analysed from arrowhead and arrow in *A*, respectively. Open circles represent periods during the drug application. *C*, schematic illustration of the sIPSC detection method (*a*), and a typical trace of sIPSCs at expanded time scale (upper) and its detection result (lower) (*b*). Red: peak of events; green: onset; blue: calculated baseline.

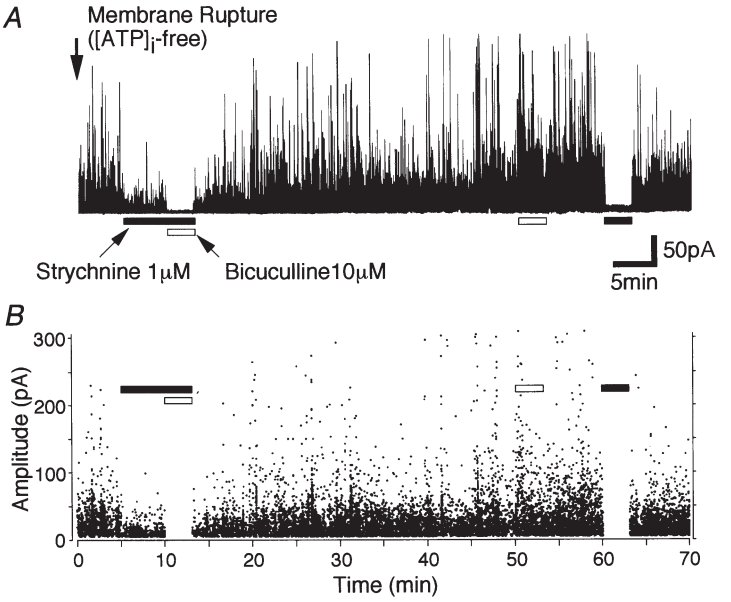

Figure 2. Separation of glycinergic sIPSCs

A, typical trace of sIPSCs obtained from a single SDCN neuron at a holding potential, V_H, of −30 mV. The pipette solution did not contain ATP. *B*, all points scatter plots of sIPSCs obtained from *A*. Each point represents a single IPSC. Note that the cumulative application of strychnine (1 μM) and bicuculline (10 μM) eliminated sIPSCs 10 min after membrane rupture, whereas 50 min after membrane rupture, bicuculline itself did not affect sIPSCs but strychnine completely blocked all sIPSCs.

response (Shirasaki *et al.* 1992; Harata *et al.* 1997; Jang *et al.* 2001). Note that under such experimental conditions the presynaptic nerve terminals maintain the native intra-terminal environment. In SDCN neurons well perfused with ATP-free pipette solution, the spontaneous outward currents were recorded at a holding potential of −30 mV in the presence of both 10 μM CNQX and 20 μM AP5. By about 20 min after membrane rupture, these sIPSCs were a

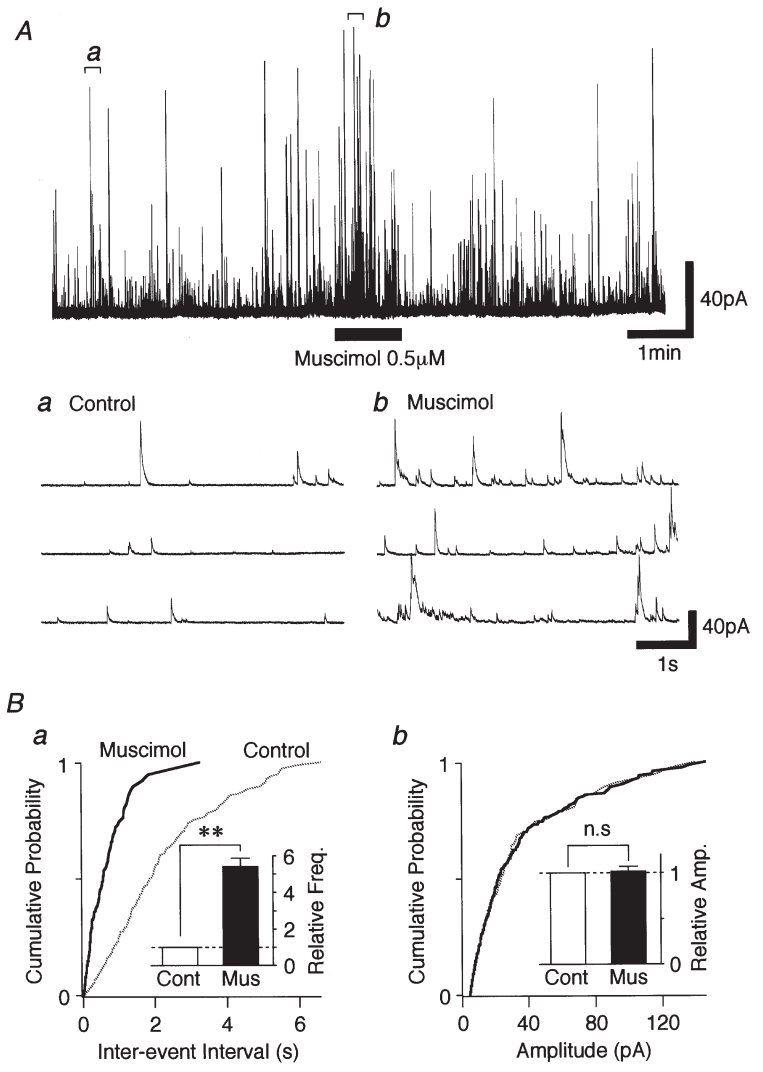

Figure 3. Muscimol acts presynaptically on the glycinergic nerve terminals

A, typical trace of glycinergic sIPSCs observed before, during and after application of 0.5 μM muscimol. Insets (*a* and *b*) represent the regions indicated in the upper trace with an expanded time scale. *B*, cumulative probability plots for the inter-event interval (*a*; $P < 0.01$, K-S test) and amplitude (*b*; $P = 0.46$, K-S test) of glycinergic sIPSCs shown in *A;* 603 events for the control and 328 events for muscimol were plotted. Insets, each bar represents the mean ± S.E.M. ($n = 12$), normalized to the control. Cont: control; Mus: muscimol; ** $P < 0.01$; n.s: not significant.

mixture of both GABAergic and glycinergic ones, since the cumulative application of 1 μM strychnine and 10 μM bicuculline substantially eliminated all sIPSCs (Fig. 2*A* and *B*). However, sIPSCs at about 50 min after membrane rupture were completely eliminated by adding only strychnine (Fig. 2*A* and *B*). These results clearly indicate that most of the sIPSCs are glycinergic about 50 min after membrane rupture, and that the ATP-free pipette solution does not affect glycinergic sIPSCs. Under such experimental conditions, the reversal potential for sIPSCs estimated from the current–voltage (*I–V*) relationship was about −64.8 mV ($n = 4$, data not shown), which was consistent with the theoretical Cl^- equilibrium potential (E_{Cl}) of −69.9 mV calculated by the Nernst equation using 161 mM $[Cl^-]_o$ and 10 mM $[Cl^-]_i$.

On the other hand, we also tested the rundown of the GABA$_A$ receptor-mediated postsynaptic currents. The postsynaptic responses induced by the exogenous application of 0.5 μM muscimol, a GABA$_A$ receptor agonist, were gradually attenuated and substantially occluded within 50 min of the membrane rupture (data not shown). In all subsequent experiments, muscimol and related drugs were applied at least 50 min after the membrane rupture in order to completely abolish the contamination of both GABAergic sIPSCs and postsynaptic GABA currents.

GABAergic modulation of glycinergic sIPSCs

In most of the neurons tested (107 of 131 neurons), 0.5 μM muscimol greatly increased glycinergic sIPSC frequency to 542.7 ± 47.3 % of the control ($n = 10$, $P < 0.01$) without affecting the mean current amplitude (101.9 ± 5.5 % of the control, $P = 0.901$) (Fig. 3*A* and *B*). Figure 3*Ba* and *b* shows cumulative probability distributions of the inter-event interval and amplitude of glycinergic sIPSCs, respectively. Muscimol significantly shifted the distribution of sIPSC frequency to the left but did not affect the amplitude, thus suggesting that muscimol acts presynaptically to increase the probability of spontaneous glycine release.

To confirm the involvement of presynaptic GABA$_A$ receptors in an increase of glycinergic sIPSC frequency, we tested the effect of diazepam, a GABA$_A$ receptor modulator, on glycinergic sIPSCs. As predicted, 1 μM diazepam increased the facilitatory action of muscimol on glycinergic sIPSCs (Fig. 4*Aa* and *b*, and *B*). In the presence of diazepam, 0.5 μM muscimol further increased sIPSC frequency to 718.3 ± 107.8 % of the diazepam condition

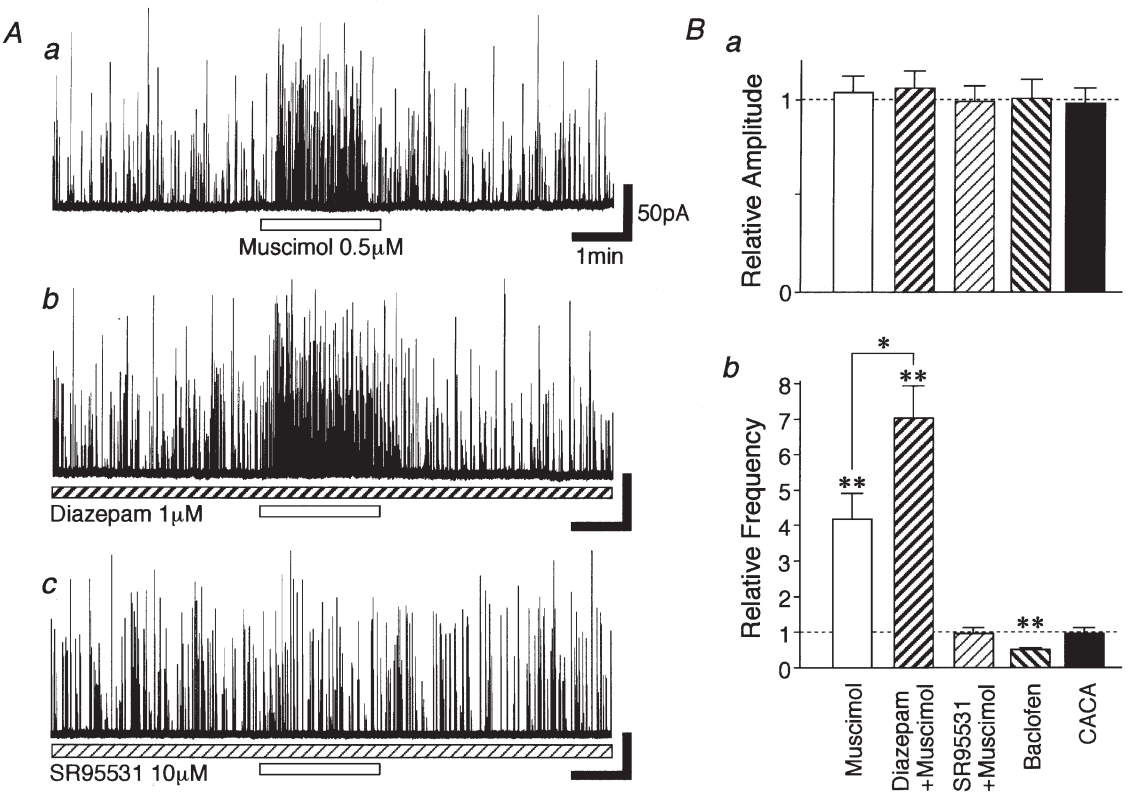

Figure 4. GABA$_A$ receptors are responsible for glycinergic sIPSC facilitation

A, typical traces of glycinergic sIPSCs observed before, during and after application of 0.5 μM muscimol in the absence of (*a*) and in the presence of (*b*) 1 μM diazepam, and in the presence of 10 μM SR95531 (*c*). All traces were recorded from the same neuron. *B*, each bar for amplitude (*a*) and frequency (*b*) was normalized to the control. Muscimol ($n = 5$), diazepam + muscimol ($n = 5$), SR95531 + muscimol ($n = 5$), 30 μM baclofen ($n = 9$), 10 μM CACA ($n = 6$). * $P < 0.05$, ** $P < 0.01$.

($n = 5$, $P < 0.01$) without affecting the mean current amplitude (Fig. 4*B*). Muscimol action on glycinergic sIPSC frequency was completely blocked in the presence of 10 μM bicuculline (data not shown) or 10 μM SR95531 ($n = 5$), specific $GABA_A$ receptor antagonists (Fig. 4*Ac* and *B*). Baclofen (30 μM), a selective $GABA_B$ receptor agonist, reduced the sIPSC frequency to 51.3 ± 3.8 % of the control ($n = 9$, $P < 0.01$) without affecting the mean current amplitude (Fig. 4*B*). However, CACA (10 μM, $n = 6$), a selective $GABA_C$ receptor agonist, had no effect (Fig. 4*B*). In postnatal 30-day-old SDCN neurons, on the other hand, we also found that 0.5 μM muscimol increased glycinergic sIPSC frequency to 441.3 ± 75.4 % of the control ($n = 4$, $P < 0.05$). These results clearly indicate that an increase in sIPSC frequency results from $GABA_A$ receptor activation of the glycinergic presynaptic nerve terminals.

Muscimol depolarizes glycinergic presynaptic nerve terminals

We examined which mechanisms are involved in the $GABA_A$ receptor-mediated sIPSC frequency facilitation. Firstly, the effect of Cd^{2+}, a general voltage-dependent Ca^{2+} channel (VDCC) blocker, on muscimol-induced facilitation of sIPSC frequency was tested. The application of 100 μM Cd^{2+} itself reduced the frequency of sIPSCs to 59.3 ± 8.7 % of the control ($n = 5$, $P < 0.01$) without affecting the mean current amplitude (90.9 ± 5.9 %, $n = 5$, $P = 0.13$) (Fig. 5*B*). In the presence of Cd^{2+}, muscimol action on sIPSC frequency (Fig. 5*Aa*) was completely occluded to 112.6 ± 11.7 % of the Cd^{2+} condition ($n = 5$, $P = 0.24$, Fig. 5*Ab* and *B*). Since Cd^{2+} itself is known to block $GABA_A$ receptors (Kumamoto & Murata, 1995; Fisher & Macdonald, 1998), the effect of Ca^{2+}-free external solution on $GABA_A$ receptor-mediated sIPSC frequency facilitation was also tested. In a Ca^{2+}-free external solution, sIPSC frequency was greatly reduced to 53.5 ± 6.1 % ($n = 6$, $P < 0.01$) of the control without affecting the mean current amplitude (90.4 ± 7.2 %, $n = 6$, $P = 0.22$) (Fig. 5*C*). The facilitatory effect of muscimol on sIPSC frequency was also completely occluded to 106.7 ± 5.8 % of the Ca^{2+}-free condition ($n = 6$, $P = 0.45$, Fig. 5*Ac* and *C*). These results suggest that muscimol-induced sIPSC frequency facilitation is mediated by Ca^{2+} influx from the extracellular space via VDCC activation.

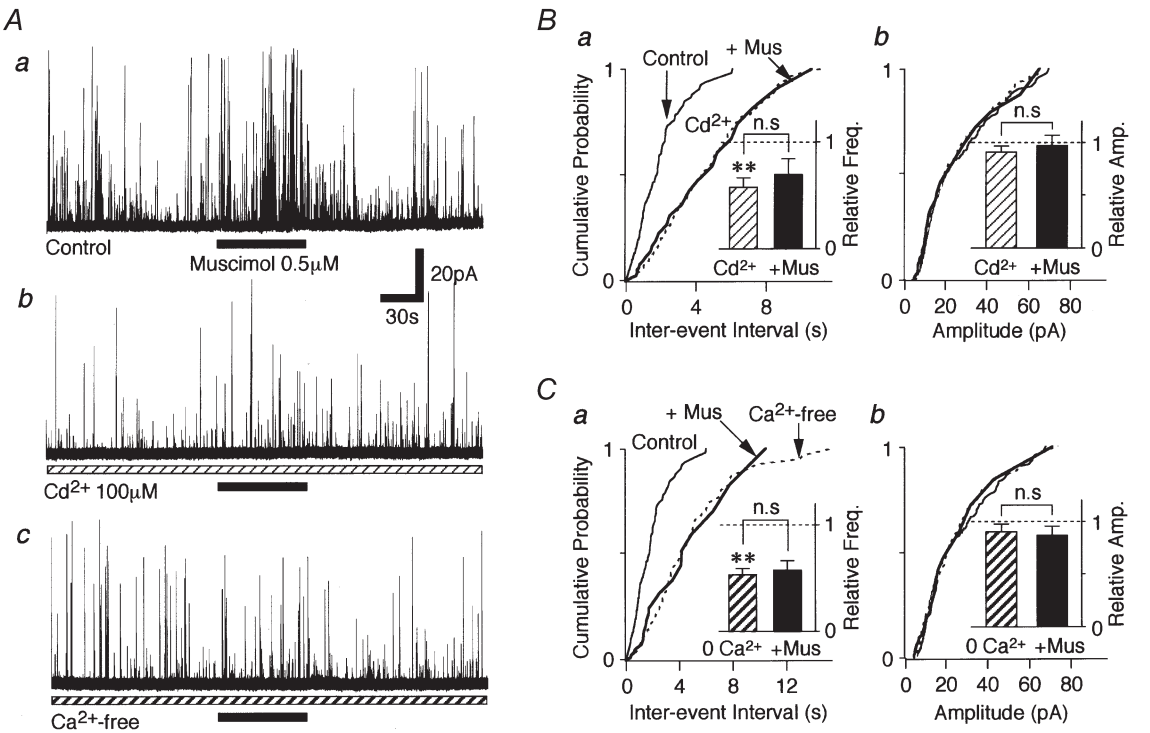

Figure 5. Muscimol-induced sIPSC facilitation is mediated by Ca^{2+} influxes through VDCCs

A, typical traces of glycinergic sIPSCs observed before, during and after adding 0.5 μM muscimol in a standard solution (*a*), in the presence of 100 μM Cd^{2+} (*b*) and in a Ca^{2+}-free external solution (*c*). All traces were obtained from the same neuron. *B*, cumulative probability plots for inter-event interval (*a*; $P = 0.68$) and amplitude (*b*; $P = 0.17$) of sIPSCs shown in *Aa* and *b*; 402 events for control, 335 events for Cd^{2+} and 28 events for muscimol were plotted. Insets, all columns represent the mean of 5 neurons and are normalized to the respective control. *C*, cumulative probability plots for the inter-event interval (*a*; $P = 0.15$) and amplitude (*b*; $P = 0.33$) of sIPSCs shown in *Aa* and *c*; 402 events for the control, 225 events for Ca^{2+}-free and 26 events for muscimol were plotted. Insets, each bar is the mean of 6 neurons and normalized to the control. 0 Ca^{2+}: Ca^{2+}-free; ** $P < 0.01$; n.s: not significant.

We next tested whether muscimol-induced sIPSC frequency facilitation results from the direct VDCC opening or occurs via the activation of TTX-sensitive Na^+ channels. TTX (0.3 μM) also significantly decreased the glycinergic sIPSC frequency to 56.9 ± 7.7 % of the control ($P < 0.01$, $n = 8$) without affecting the mean current amplitude (91.5 ± 6.0 % of the control, $P = 0.25$). This suggests that TTX-sensitive Na^+ channels contribute to the generation of glycinergic sIPSCs. In the presence of TTX however, muscimol could still facilitate glycinergic sIPSC frequency to 272.7 ± 20.4 % of the TTX condition ($n = 8$, $P < 0.01$, Fig. 6*A* and *B*), but this facilitation ratio was significantly lower than the control ratio ($P < 0.05$, ANOVA test). These results suggest that muscimol-induced depolarization activates TTX-sensitive Na^+ channels and, in part, directly activates VDCCs to facilitate glycine release.

The involvement of NKCC in GABA$_A$ receptor-mediated presynaptic depolarization

Since muscimol-induced facilitation of sIPSC frequency seems to be mediated by presynaptic depolarization, the activation of presynaptic GABA$_A$ receptors may depolarize the glycinergic nerve terminals. This suggests the possibility that the $[Cl^-]_i$ of presynaptic nerve terminals might be maintained at higher levels than predicted for a passive distribution. In immature CNS neurons or peripheral sensory neurons, the NKCC has been reported to play a pivotal role in the generation of higher $[Cl^-]_i$ and GABA-induced depolarization (Alvarez-Leefmans, 1990; Plotkin *et al.* 1997; Clayton *et al.* 1998; Russell, 2000). We thus investigated the effect of bumetanide, a selective NKCC blocker (Haas, 1989; Xu *et al.* 1994), on the muscimol-induced facilitation of sIPSC frequency.

Muscimol action on sIPSC frequency was gradually attenuated in the presence of 10 μM bumetanide (Fig. 7*A* and *B*), in which the facilitation ratios were reduced from 445.7 ± 89.1 % for the first application to 227.4 ± 68.1 and 179.4 ± 46.4 % for the second and third applications, respectively ($P < 0.05$, $n = 6$, Fig. 7*B*). On the other hand, the facilitatory effect of muscimol also slowly recovered after washout of bumetanide (258.1 ± 84.7 for 10 min and 444.8 ± 135.9 % for 20 min, respectively, Fig. 7*A* and *B*). However, these slow effects of bumetanide were probably not due to the direct blockade of presynaptic GABA$_A$ receptors, since 10 μM bumetanide itself did not affect GABAergic sIPSC amplitude or frequency (data not shown).

Since NKCC carries Na^+, K^+, and $2Cl^-$ simultaneously and electroneutrally, many studies regarding the function of NKCC have been performed by removing such ions from

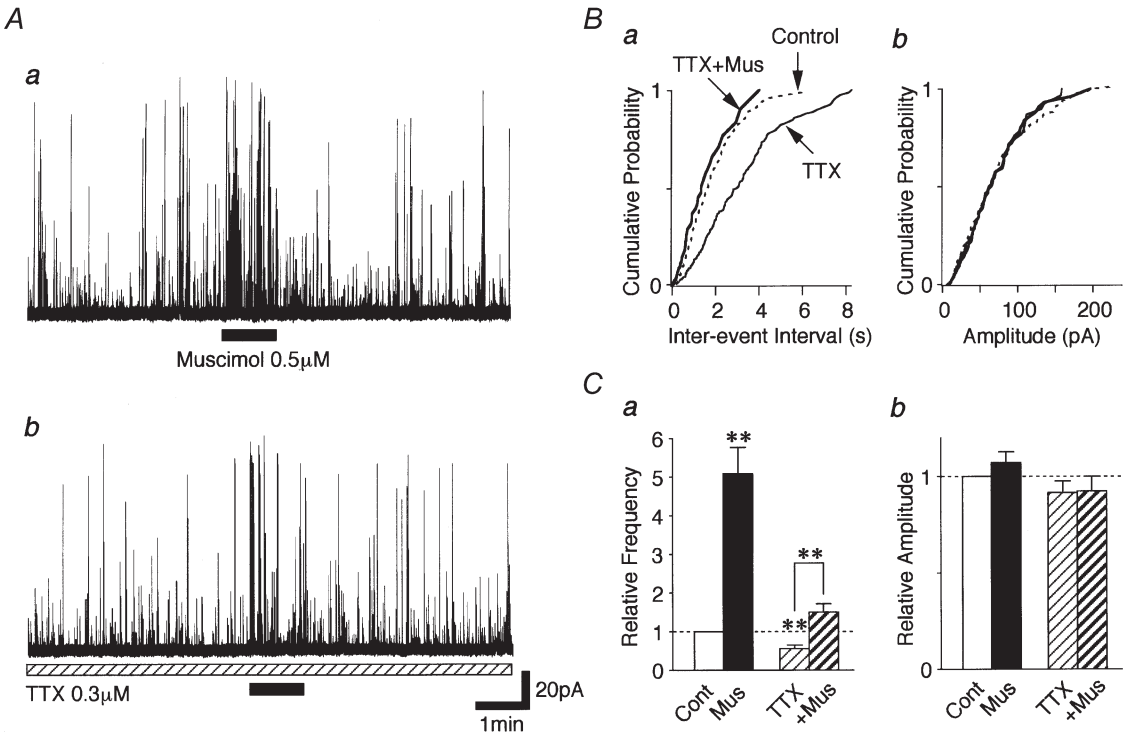

Figure 6. Muscimol-induced sIPSC facilitation in the presence of TTX

A, typical traces of glycinergic sIPSCs observed before, during and after adding 0.5 μM muscimol to a standard solution (*a*), and in the presence of 0.3 μM TTX (*b*). Both traces were obtained from the same neuron. *B*, cumulative probability plots for the inter-event interval (*a*; $P < 0.01$) and amplitude (*b*; $P = 0.41$) of sIPSCs shown in *A*; 458 events for the control, 346 events for TTX and 61 events for TTX plus muscimol were plotted. *C*, each bar is the mean of 8 neurons and normalized to the control. ** $P < 0.01$.

the external solution (Alvarez-Leefmans, 1990; Rohrbough & Spitzer, 1996; Altamirano *et al.* 1999). To confirm the involvement of NKCC in the maintenance of higher $[Cl^-]_i$, we investigated whether or not the action of muscimol on sIPSC frequency is maintained in a Na^+-free external solution. Since muscimol could directly activate VDCCs (Fig. 6), all experiments were performed in the presence of 0.3 μM TTX to exclude the recruitment of Na^+ channels. In the presence of TTX, muscimol facilitated sIPSC frequency to 259.1 ± 29.5 % of the TTX condition (Fig. 7*C*). As predicted, the action of muscimol on sIPSC frequency was also gradually attenuated in the Na^+-free external solution (Fig. 7*C* and *D*), in which the facilitation ratios were reduced from 221.6 ± 24.8 % for the first application to 146.8 ± 30.8 and 121.7 ± 15.8 % for the second and third applications, respectively ($P < 0.05$, $n = 5$, Fig. 7*D*). On the other hand, the action of muscimol quickly recovered after washout of the Na^+-free external solution (198.6 ± 21.6 for 10 min and 229.7 ± 31.4 % for 20 min, respectively, Fig. 7*C* and *D*). On the other hand, the replacement of standard external solution with Na^+-free external solution gradually increased the sIPSC frequency to 179.4 ± 46.4 % of the control ($P < 0.05$, $n = 5$, Fig. 7C). This increase in sIPSC frequency might be due to a Na^+–Ca^{2+} exchanger operating in a reverse mode (Fontana *et al.* 1995).

Effect of $GABA_A$ receptor-mediated presynaptic depolarization on glycinergic eIPSCs evoked by focal electrical stimulation of a single bouton

We tested whether $GABA_A$ receptor-mediated presynaptic depolarization induces presynaptic inhibition of electrically evoked glycinergic transmission. When focal stimulating pulses were applied to a single glycinergic nerve terminal (bouton) visualized with FM 1–43 (Fig. 8*Aa*), eIPSCs resulting from a single synapse were observed and reversibly blocked by adding 1 μM strychnine ($n = 6$,

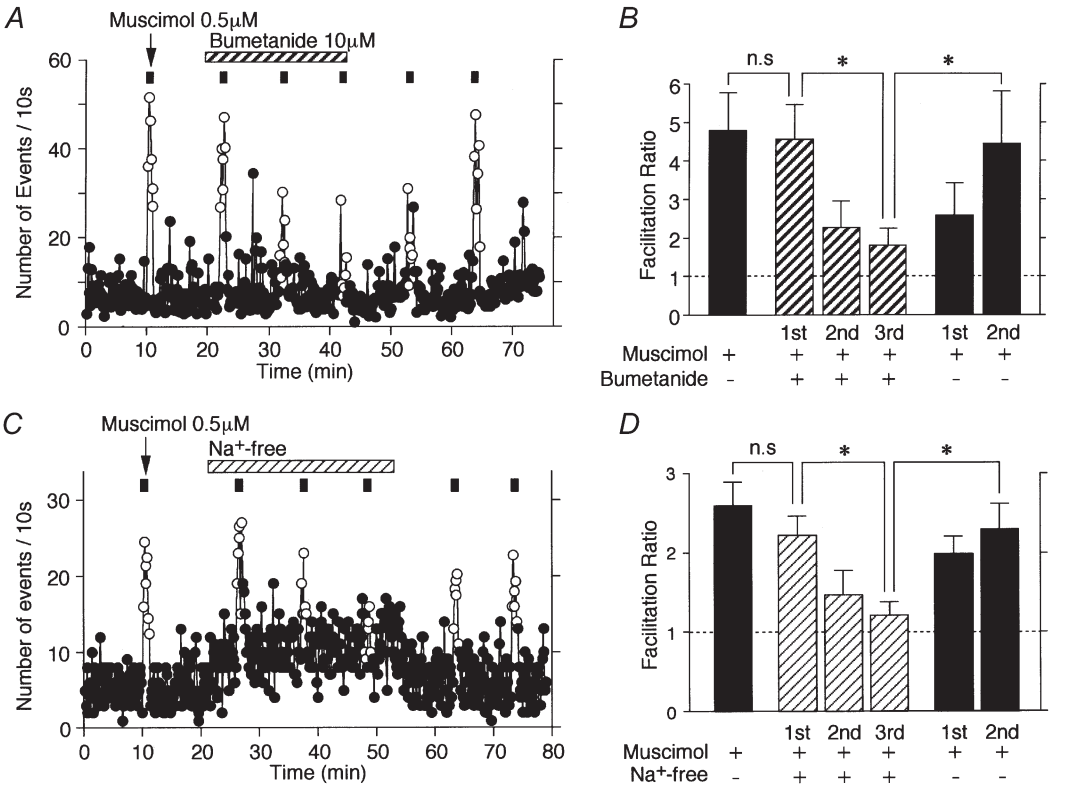

Figure 7. Effects of NKCC blocker and Na^+-free external solution on muscimol-induced sIPSC facilitation

A, typical time course of the event frequency of sIPSCs before, during and after application of 0.5 μM muscimol (○, presence of muscimol; ●, absence of muscimol) in the standard external solution with or without 10 μM bumetanide. The number of events for every 10 s time interval is plotted. *B*, facilitation ratios obtained by application of 0.5 μM muscimol with or without bumetanide. Each bar is the mean of 6 neurons and normalized to the control. The dotted line indicates the respective control. * $P < 0.05$. *C*, typical time course of the event frequency of sIPSCs before, during and after the application of 0.5 μM muscimol (○, presence of muscimol; ●, absence of muscimol) in Na^+-free external solution. The number of events for every 10 s time interval is plotted. *D*, facilitation ratios obtained by the application of 0.5 μM muscimol in the standard external solution or in Na^+-free external solution. Each bar is the mean of 5 neurons and normalized to the control. The dotted line indicates the respective control. * $P < 0.05$. Note that the muscimol-induced facilitation of sIPSC frequency gradually decreased in the presence of bumetanide or in Na^+-free external solution.

Fig. 8*Ab*), thus indicating that the eIPSCs are glycinergic but not GABAergic. Under such experimental conditions, muscimol (5 μM) significantly reduced eIPSC amplitude to 30.1 ± 13.5 % of the control in a concentration-dependent manner ($n = 6$, $P < 0.01$, Fig. 8*B* and *Ca*). In addition, muscimol (5 μM) greatly increased the failure rate of the evoked glycinergic transmission (33.8 ± 8.6 % for the control and 81.6 ± 8.7 % for 5 μM muscimol, $n = 6$, $P < 0.01$, Fig. 8*B* and *Cb*). These results clearly indicate that the activation of presynaptic GABA$_A$ receptors induces presynaptic inhibition of the evoked glycinergic transmission. In the same recorded neuron, on the other hand, muscimol also facilitated sIPSC frequency without affecting the distribution of sIPSC amplitude (Fig. 8*D*).

DISCUSSION

The present study has demonstrated that the activation of presynaptic GABA$_A$ receptors increases spontaneous glycine release but inhibits electrically stimulated glycine release, presumably by depolarizing presynaptic nerve terminals. In addition, this GABA$_A$ receptor-mediated depolarization is due to a higher $[Cl^-]_i$ within the glycinergic nerve terminals that is regulated by the inwardly directed Cl^- transporter NKCC.

Activation of GABA$_A$ receptors depolarizes the glycinergic presynaptic nerve terminals

In mature CNS neurons, GABA$_A$ receptor activation generally hyperpolarizes the neuronal membrane by increasing an inwardly directed Cl^- conductance based on a lower $[Cl^-]_i$. In immature neurons, however, GABA depolarizes and excites the neuronal membrane (Obrietan & van den Pol, 1995; Chen *et al.* 1996). In addition, GABA$_A$ receptor-mediated depolarization is observed in adult dorsal root ganglia and sympathetic neurons as well as hippocampal neurons (Ballanyi & Grafe, 1985; Misgeld, *et al.* 1986; Alvarez-Leefmans, 1990). In developing neurons, either synaptically released or exogenously applied GABA induces membrane depolarization, which results in an increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Leinekugel *et al.* 1995; Obrietan & van den Pol, 1995). Alternatively, GABA-induced depolarization could elicit an increase of $[Ca^{2+}]_i$ by the activation of Ca^{2+} channels via the recruitment of Na^+ channels (Hales *et al.* 1994; Owens *et al.* 1996). These neurons accumulate Cl^- at a higher

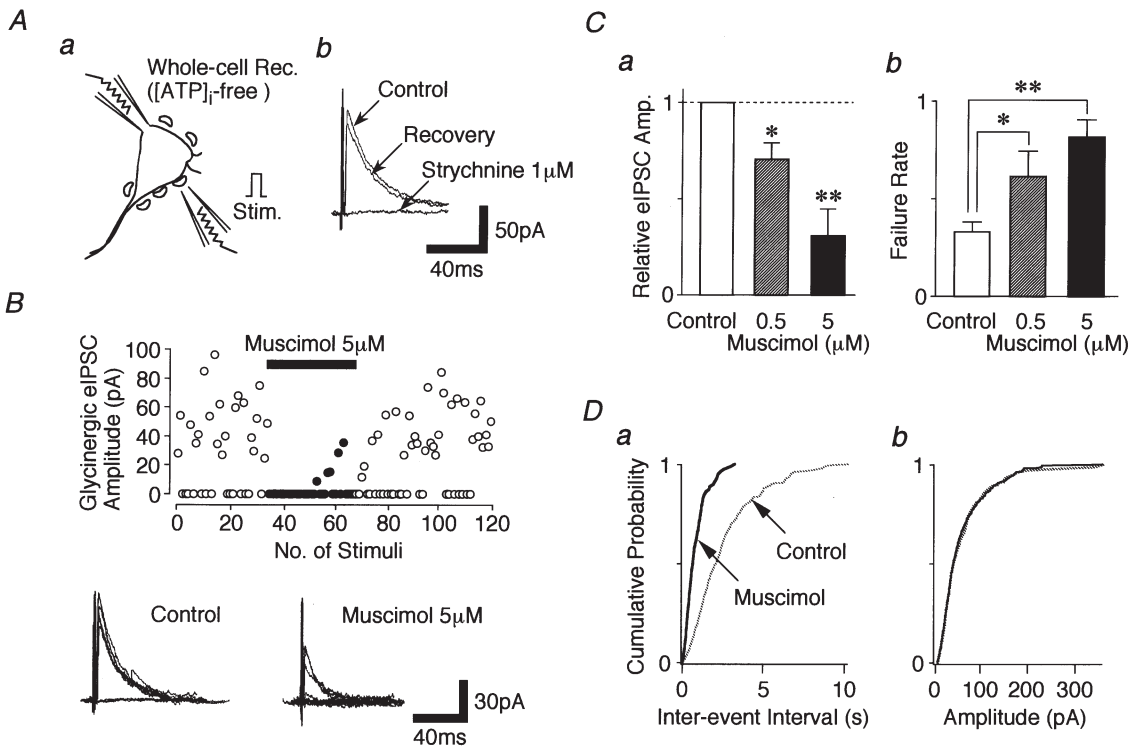

Figure 8. Muscimol inhibits evoked glycinergic transmission

Aa, schematic illustration of the focal stimulation. *b*, effect of 1 μM strychnine on eIPSCs obtained from a single synapse. *B*, scatter plot of the glycinergic eIPSC amplitude before, during and after the application of 5 μM muscimol (○, absence of muscimol; ●, presence of muscimol). Insets, typical traces of glycinergic eIPSCs in the absence (left) or presence (right) of 5 μM muscimol. Ten traces were superimposed, respectively. *C*, pooled data for the eIPSC amplitude (*a*) and its failure rate (*b*). Each bar is the mean of 6 neurons. Cont: control; * $P < 0.05$, ** $P < 0.01$. *D*, cumulative probability plots for inter-event interval (*a*; $P < 0.01$) and amplitude (*b*; $P = 0.74$) of sIPSCs recorded from *B*; 332 events for the control and 165 events for muscimol were plotted.

concentration than that predicted for passive distribution, and GABA equilibrium potential (E_{GABA}) is also more positive than the resting membrane potential (V_m). As a result, the depolarizing action of GABA is believed to result from activation of $GABA_A$ receptors, with a consequent efflux of Cl^-, which transiently drives V_m toward E_{GABA}. In the present study, the action of muscimol on glycinergic sIPSC frequency may be closely dependent on the Ca^{2+} influx passing through VDCCs, since exposure to either Cd^{2+} or Ca^{2+}-free external solution completely occluded the muscimol-induced sIPSC frequency facilitation. On the other hand, muscimol increased sIPSC frequency even in the presence of TTX, although its facilitation ratio was relatively small. In addition, muscimol still increased the sIPSC frequency even in a Na^+-free external solution. These results suggest that muscimol-induced depolarization is large enough to surpass the threshold for activation of VDCCs, and that the consequent Ca^{2+} influx through VDCCs are responsible for muscimol-induced sIPSC frequency facilitation.

Intraterminal $[Cl^-]_i$ is maintained higher by NKCC

Which mechanisms are involved in the accumulation of $[Cl^-]_i$ in the glycinergic nerve terminals? In immature neurons, the $[Cl^-]_i$ remains higher than predicted for passive distribution, and this results from the presence of inwardly directed Cl^- transporters, especially the NKCC (Alvarez-Leefmans, 1990; Kaila, 1994; Plotkin *et al.* 1997; Clayton *et al.* 1998; Kakazu *et al.* 1999; Russell, 2000; Jang *et al.* 2001). In the experiments examining the involvement of the NKCC, muscimol-induced facilitation of sIPSC frequency was gradually reduced either in the presence of bumetanide or in a Na^+-free external solution. These results suggest that repeated applications of muscimol gradually reduce the transmembrane Cl^- gradient after blockade of the NKCC. However, such slow effects were not mediated by direct $GABA_A$ receptor blockade because 10 μM bumetanide had no influence on $GABA_A$ receptor-mediated currents (data not shown). Accordingly, a bumetanide-sensitive NKCC might play an important role in the accumulation of a higher $[Cl^-]_i$ within the glycinergic nerve terminals projecting to SDCN neurons. This conclusion is consistent with our previous report that the NKCC contributes to the higher $[Cl^-]_i$ and to $GABA_A$ receptor-mediated presynaptic depolarization in the glutamatergic presynaptic nerve terminals (Jang *et al.* 2001).

On the other hand, we found that muscimol still increased glycinergic sIPSC frequency even in postnatal 30-day-old SDCN neurons. It would be interesting to know whether NKCC regulates the $[Cl^-]_i$ within these glycinergic presynaptic nerve terminals in mature neurons, because the increased expression of the K–Cl cotransporter, which is a major Cl^- extrusion mechanism, contributes to the conversion of the GABA-induced postsynaptic response from depolarization to hyperpolarization during postnatal development (Kakazu *et al.* 1999; Vu *et al.* 2000).

Physiological implications

The present results indicate that $GABA_A$ receptor-mediated presynaptic depolarization has differential effects on spontaneous *versus* evoked glycine release, that is, presynaptic facilitation of spontaneous glycinergic transmission and presynaptic inhibition of action potential-driven glycinergic transmission. One possible explanation for the reduction of eIPSC amplitude by muscimol is the depletion of the releasable pool due to a large increase in sIPSC frequency. However, this might not be the case because sIPSCs were continuously increased during muscimol application while eIPSCs were inhibited immediately after muscimol application. Alternatively, this presynaptic inhibitory action on evoked glycine release may result from the inactivation of Na^+ or Ca^{2+} channels based on membrane depolarization and/or shunt of the presynaptic membrane (see also Segev, 1990; Graham & Redman, 1994; Cattaert & El Manira, 1999). The present results suggest that the facilitation of spontaneous glycine release by presynaptic $GABA_A$ receptor activation leads to tonic inhibition of postsynaptic neurons, while the inhibition of evoked glycine release leads to disinhibition of postsynaptic neurons. Such differential effects on glycinergic transmission suggest that $GABA_A$ receptors on the glycinergic presynaptic nerve terminals might be involved in more complex regulation of inhibitory inputs and neuronal excitability.

In the spinal cord, GABAergic boutons onto the primary afferent terminals have been well described either morphologically (Todd *et al.* 1995; Maxwell *et al.* 1997) or electrophysiologically (Cattaert & El Manira, 1999). Although the present study has clearly demonstrated that exogenous muscimol depolarizes the glycinergic nerve terminals, it does not reveal the origin of the GABA that activates presynaptic $GABA_A$ receptors. One possibility is that GABAergic axo-axonic synapses are responsible for the activation of $GABA_A$ receptors on the glycinergic presynaptic nerve terminals. However, little is known about whether or not GABAergic axo-axonic synapses exist on the inhibitory presynaptic nerve terminals. Alternatively, synaptically released GABA might act on neighbouring glycinergic terminals via diffusion (spill-over). In the spinal cord, GABA is often colocalized with glycine (Todd & Spike, 1993) and the corelease of GABA and glycine has been demonstrated (Jonas *et al.* 1998; Chery & de Koninck, 1999). Chery & de Koninck (1999) suggested that coreleased GABA acts on extrasynaptic $GABA_A$ receptors by spill-over. Further study is necessary to resolve the origin of GABA for the presynaptic $GABA_A$ receptor activation.

The SDCN is known to be closely involved in nociceptive transmission (Honda, 1985; Ding *et al.* 1994; Lu *et al.* 1995;

Vizzard *et al.* 1995). The SDCN receives abundant afferent inputs from both visceral and somatic organs. The convergence of visceral and somatic inputs onto SDCN neurons has been reported both electrophysiologically (Honda, 1985) and anatomically (Lu *et al.* 1995). The SDCN also integrates the regulatory influences from the brainstem descending pathways (Jones & Light, 1990). Accordingly, $GABA_A$ receptor-mediated presynaptic modulation of glycinergic transmission may play an important role in regulation of the sensory process within the spinal cord.

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