

Dual Ca^{2+} modulation of glycinergic synaptic currents in rodent hypoglossal motoneurones

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Glycinergic synapses are implicated in the coordination of reflex responses, sensory signal processing and pain sensation. Their activity is pre- and postsynaptically regulated, although mechanisms are poorly understood. Using patch-clamp recording and Ca^{2+} imaging in hypoglossal motoneurones from rat and mouse brainstem slices, we address here the role of cytoplasmic Ca^{2+} (Ca_i) in glycinergic synapse modulation. Ca^{2+} influx through voltage-gated or NMDA receptor channels caused powerful transient inhibition of glycinergic IPSCs. This effect was accompanied by an increase in both the failure rate and paired-pulse ratio, as well as a decrease in the frequency of mIPSCs, suggesting a presynaptic mechanism of depression. Inhibition was reduced by the cannabinoid receptor antagonist SR141716A and occluded by the agonist WIN55,212-2, indicating involvement of endocannabinoid retrograde signalling. Conversely, in the presence of SR141716A, glycinergic IPSCs were potentiated postsynaptically by glutamate or NMDA, displaying a Ca^{2+} -dependent increase in amplitude and decay prolongation. Both presynaptic inhibition and postsynaptic potentiation were completely prevented by strong Ca_i buffering (20 mM BAPTA). Our findings demonstrate two independent mechanisms by which Ca^{2+} modulates glycinergic synaptic transmission: (i) presynaptic inhibition of glycine release and (ii) postsynaptic potentiation of GlyR-mediated responses. This dual Ca^{2+} -induced regulation might be important for feedback control of neurotransmission in a variety of glycinergic networks in mammalian nervous systems.

(Resubmitted 18 July 2005; accepted after revision 24 August 2005; first published online 25 August 2005)

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Glycinergic synapses in mature central nervous systems of vertebrates are implicated in the coordination of reflex responses, in the processing of sensory signals and in pain sensation. These synapses provide inhibitory neurotransmission by postsynaptic activation of Cl^- -selective glycine receptor (GlyR) channels (Hamill *et al.* 1983; Bormann *et al.* 1987). The functioning of GlyRs is regulated by several pathways, including cytoplasmic Ca^{2+} (Ca_i) (Ragozzino & Eusebi, 1993; Xu *et al.* 1999; Fucile *et al.* 2000), phosphorylation (Gu & Huang, 1998) and G-proteins (Yevenes *et al.* 2003). Particularly intriguing is the phenomenon of Ca^{2+} -induced fast potentiation of GlyRs (Fucile *et al.* 2000). This rapid transient increase in glycine-induced currents, resulting from Ca^{2+} elevation, is independent of both phosphorylation and G-proteins and, presumably, involves an unknown Ca^{2+} -binding protein. The phenomenon of Ca^{2+} -induced potentiation has been

well documented for extrasynaptic GlyRs (Fucile *et al.* 2000; Zhu *et al.* 2002; Diana & Bregestovski, 2005). Whether synaptically activated GlyRs are regulated by Ca_i during synaptic transmission is not known.

To answer this question, we analysed the effects of Ca_i on glycinergic inhibitory postsynaptic currents (IPSCs) in hypoglossal motoneurones (HMs) from rat and mouse brainstem acute slices. HMs are characterized by rhythmic activity and they are involved in a variety of motor functions, including breathing, chewing, sucking, swallowing and phonation (Peever & Duffin, 2001). HMs receive synaptic information from various parts of the brain due to dendritic arborization, and exhibit two remarkable features. The first is a low endogenous Ca^{2+} -buffering capacity, which determines both the selective vulnerability of HMs to Ca^{2+} -related excitotoxicity and the rapid dynamics of Ca_i (Lips & Keller, 1998; Palecek *et al.* 1999). The second is powerful glycinergic synaptic inputs,

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which present the major inhibitory drive in this brainstem nucleus (Umekiya & Berger, 1995; Singer *et al.* 1998; Donato & Nistri, 2000; Singer & Berger, 2000). HMs thus provide a convenient model for examination of the action of intracellular Ca^{2+} on glycinergic synapses.

We report here that Ca_i elevation in HMs modulates glycinergic synaptic transmission by two independent mechanisms of opposite sign: (i) a decrease in glycinergic IPSCs due to the reduction in presynaptic glycine release induced, predominantly, by retrograde action of endogenous cannabinoids; (ii) potentiation of postsynaptic GlyRs. Under normal physiological conditions in HMs, the postsynaptic effect is masked by powerful presynaptic inhibition.

Methods

Slice preparation

Experiments were carried out on brainstem slices from postnatal (P5–P9) Sprague-Dawley or Wistar rats and C57BL/6J or Swiss mice. Animals were anaesthetized with ether and killed by decapitation in agreement with the European Directive 86/609/EEC requirements. The brainstem was rapidly removed and placed in an oxygenated ice-cold saline buffer. Transverse 250- μm -thick brainstem slices were cut using a DSK (Dosaka, Japan) or HM 650V (Microm, Germany) vibrating microslicer in ice-cold oxygenated solution containing (mM): NaCl, 125; KCl, 3.5; CaCl_2 , 2; MgCl_2 , 1.3; NaH_2PO_4 , 1.25; NaHCO_3 , 26; and glucose, 10; equilibrated at pH 7.3 with 95% O_2 and 5% CO_2 . Prior to recording, slices were incubated at room temperature (22–25°C) for at least one hour to allow recovery. Experiments were performed in the same solution at room temperature.

Electrophysiological recordings

For patch-clamp recordings brainstem acute slices were visualized through a $\times 40$ water-immersion objective using an upright microscope (Axioskop, Zeiss, Germany). HMs were identified by their location in the hypoglossal nucleus (n.XII), their large somata (25–40 μm) and their dendritic arborization.

During measurements, slices were superfused with oxygenated saline (1.0–1.5 ml min⁻¹). Whole-cell borosilicate glass pipettes (Hilgenberg or Clark Capillaries) with a tip resistance of 2–5 M Ω were filled with a KCl-based intracellular solution containing (mM) KCl, 135; MgCl_2 , 1; Hepes, 10; Mg-ATP, 4; Na-GTP, 0.3; equilibrated at pH 7.3 with KOH. Different BAPTA concentrations (0.1–20 mM) were used as indicated in the text. To obtain about 50 nM free Ca_i in the internal solution, corresponding concentrations of CaCl_2 (0.03–6 mM) were added (calculating using developed by Dr F. Mendez (Gottingen) the 'Patcher's Power Tools'

subroutine of the 'Igor' program). In some experiments an alternative CsCl-based intracellular solution was used, containing (mM) CsCl, 140; MgCl_2 , 2; Hepes, 10; MgATP, 2; Na_2GTP , 0.4; CaCl_2 , 0.11; and EGTA, 0.5 (pH 7.3).

Membrane currents were recorded at 3–10 kHz with an Axopatch 200A (Axon Instruments, USA) or EPC-9 (HEKA Elektronik, Germany) amplifiers. Stored data were analysed using pClamp software (Axon Instruments) or the PulseFit program (HEKA Elektronik). The series resistances, ranging between 5 and 15 M Ω as estimated from slow transient cancellation, were compensated by 30–80% depending on the cell. The holding potential (V_h) was –70 mV. IPSCs were elicited by stimulating (2–100 V, 200–300 μs) at 0.5–1 Hz with glass bipolar electrodes. The stimulus intensity was adjusted to obtain about 20–50% failures. Stimulating electrodes were obtained by pulling theta-glass tubes to a final tip diameter of 1–2 μm and filling with external solution. Pairs of stimuli (at 50 ms intervals) were applied.

To isolate strychnine-sensitive glycinergic synaptic currents all experiments were performed in the presence of blockers of glutamate (CNQX, 10 μM ; AP-5, 40 μM) and GABA (bicuculline, 20 μM) receptors unless otherwise mentioned. Control experiments ($n=5$) showed that, at the concentrations used, bicuculline did not affect the amplitude of glycinergic IPSCs (see supplementary material). To record glycinergic miniature IPSCs (mIPSCs) TTX (1 μM) was added to the external solution in addition to the antagonists of glutamate and GABA receptors. Cannabinoid (CB) receptor agonist WIN55,212-2 (2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate) and antagonist SR141716A (*N*-piperidini-5-(4-chlorophenyl)-4-methyl-3-pyrazole-carboxamide) were bath-applied at concentrations of 3–5 μM . These concentrations are far above affinity constant (K_i) for CB1 receptors (Howlett *et al.* 2002) and similar concentrations have been used in a number of studies on brain slices (Hajos *et al.* 2001; Jennings *et al.* 2001; Diana *et al.* 2002; Marsicano *et al.* 2002; Yoshida *et al.* 2002). Adding WIN55,212-2 or SR141716A did not change leakage currents or input resistance of recorded HMs.

To increase the cytosolic Ca^{2+} , the recorded neurones were repetitively depolarized to 0 mV (1 s pulse duration). Each single depolarization was preceded by a 100 ms step to –100 mV to allow complete activation of voltage-dependent Ca^{2+} channels, and was followed by electrical stimulation to produce an IPSC after a delay of 100–300 ms. This protocol was repeated 20–40 times (30 times in most of experiments) and properties of averaged glycinergic IPSCs were compared in control and following depolarizing series.

For external application, agonists were applied via a puff pipette close to the cell soma. For glycine application, pipettes contained either low (30 μM) or high (0.5–5 mM)

concentrations of the agonist. Glutamate and NMDA were applied at a concentration of 200 μM . Glycine and NMDA were dissolved in the standard external solution (Ca²⁺ 2 mM; Mg²⁺ 1.3 mM). In experiments with application of glutamate, to maximize the influx of Ca²⁺ the puff pipette contained Mg²⁺-free extracellular solution with elevated Ca²⁺ (10 mM). In some experiments, at NMDA application cells were concomitantly depolarized (−20 mV) to give relief from Mg²⁺ block (Nowak *et al.* 1984). Effects of NMDA and glutamate were studied with AP-5 omitted from the external solution.

Antagonists and agonists were bath-applied via a gravity-driven perfusion system (unless otherwise specified). AP-5, bicuculline and WIN55,212-2 were from Tocris (UK), SR141716A was the gift from Sanofi–Aventis and TTX was from Latoxan (France). All the other chemicals were from Sigma (USA).

The results presented here have been obtained on 70 HMs from rat and 79 HMs from mouse brain slices. As data obtained on the two preparations were similar they have been pooled (unless otherwise specified).

Statistical analysis was done in Origin (OriginLab Corporation, USA) and SigmaStat (Systat Software Inc., USA) programs using Student's paired *t* test or Wilcoxon's signed-rank test for non-parametric data. The Kolmogorov–Smirnov statistical test was used to assess differences in distribution of glycinergic mIPSC intervals. Results are given as means $\pm$ S.E.M.

Calcium imaging

Monitoring of Ca_i was conducted using a customized digital imaging microscope allowing simultaneous monitoring of whole-cell currents and fluorescence signals. Fluo-5F (50–100 μM) or, in some experiments, Fura-2 (50–200 μM) (both from Molecular Probes, Netherlands) were included in the internal solution and allowed to diffuse into the neurone for at least 15 min before the beginning of the records. The single (480-nm) or dual (350/380-nm) wavelength excitation was achieved using a 1 nm bandwidth polychromatic light selector equipped with a 100 W xenon lamp (Polychrome II, TILL Electronics, Germany). Fluorescence was visualized using an upright microscope (Axioskop, Zeiss, Germany) and a 12-bit charge-coupled device (CCD) camera equipped with an image intensifier (PentaMax, Princeton Instruments, USA). Fluorescence signals were acquired with variable sampling rate (from 0.2 to 10 Hz), using MetaFluor software (Universal Imaging Co, USA). Because of high Ca_i transients in HMs, fluorescence signals recorded with Fura-2 showed a tendency to saturation. To avoid this problem, a lower sensitivity Ca²⁺ dye, Fluo-5F, was used in the experiments described here. To ensure that Ca²⁺ signals were recorded

within the dynamic range of Fluo-5F, the membrane permeability of HMs was artificially increased by a series of electrical discharges at the end of some experiments. Under these conditions the estimated maximal change in fluorescence (ΔF) was 2–5-fold higher than that induced by depolarization-induced Ca_i transients.

Results

Ca²⁺-dependent modulation of glycinergic synaptic currents in brainstem motoneurones

Properties of glycinergic IPSCs were studied in visually identified HMs. IPSCs were evoked with a theta-tube glass pipette placed either close (100–150 μm) to the recorded neurone or on the surface of small neurones (<10 μm) in the reticular formation, ventral to and immediately outside the hypoglossal nucleus, allowing extracellular monosynaptic stimulation (Umekiya & Berger, 1995). In the presence of antagonists of GABA and glutamate ionotropic receptors, stable IPSCs (range in different cells 50–800 pA, *n* = 149) were recorded at −70 mV, and their activity was completely abolished by strychnine (3 μM ; Fig. 1A). IPSC deactivation kinetics was best fitted by a single exponential. The decay time constant varied in different experimental conditions from 6 to 20 ms, consistently with previously described kinetic properties (Takahashi *et al.* 1992; Singer *et al.* 1998; Singer & Berger, 1999; Donato & Nistri, 2000).

To study Ca²⁺-dependent modulation of glycinergic currents with whole-cell recordings, we used relatively low concentrations of Ca²⁺ buffers in pipette solutions (0.1 mM BAPTA or 0.5 mM EGTA). An increase in Ca_i was induced by a series of somatic depolarizations of the recorded HMs (see details in Methods).

When the recording pipette contained 0.1 mM BAPTA and 0.1 mM Fluo-5F, depolarization caused a rapid Ca_i rise, whose amplitude was proportional to the number of depolarizing pulses (Fig. 1B). In these conditions, after 10–30 depolarizing pulses, the quasi-maximum values for Ca_i-induced relative fluorescence amplitudes (Fig. 1C) were reached. For this reason a depolarization protocol employing 30 pulses (otherwise mentioned) was chosen in the subsequent investigations.

Depolarization-induced Ca_i rise in HMs caused a significant decrease in glycinergic IPSC amplitude. IPSC depression was followed by a recovery of the amplitude within 3–5 min (Fig. 2A and B). As illustrated in Fig. 2C and D, the inhibition become more prominent with elevation of a number of depolarisations, and reached the quasi-maximum values after 20–30 pulses. These results were observed in rat and mouse HMs. In the rat HMs, the depolarization-induced inhibition was recorded with different Ca²⁺ buffers in the pipette solution. With BAPTA 0.1 mM, the mean depression was $49 \pm 3\%$ (*n* = 10, *P* < 0.001, *t* test; values are given

throughout as mean $\pm$ s.e.m.); with BAPTA 0.5 mM or 1 mM and with EGTA 0.5 mM depression was, respectively, $44\% \pm 4\%$ ($n = 5$, $P = 0.002$, t test; Fig. 2B), $38 \pm 3\%$ ($n = 10$, $P < 0.001$, t test) and $41 \pm 3\%$ ($n = 12$, $P < 0.001$, t test). A similar degree of inhibition was observed in the mouse HMs; at recordings with BAPTA 0.1 mM in the pipette the mean depression was $51 \pm 2\%$ ($n = 30$, $P < 0.001$, t test). These results demonstrate that even at

relatively strong Ca_i buffering (BAPTA 1 mM), a series of depolarizations caused a substantial inhibition of glycinergic IPSCs.

In addition to amplitude decrease, a weak but consistent reversible prolongation of the IPSC decay time constant was observed following depolarization-induced elevation of Ca_i (Fig. 3A and B). As summarized in Fig. 3B, at 0.1 mM BAPTA in the recording pipette, the mean decay time of glycinergic IPSCs before depolarization was 9.2 ± 0.7 ms; after depolarization it was 10% longer (10.1 ± 0.8 ms, $n = 10$, $P = 0.01$, Wilcoxon test) and 5 min later the decay kinetics had recovered to the control value (9.1 ± 0.8 ms).

With very strong Ca_i buffering (BAPTA 20 mM internal solution), depolarization failed to modulate the amplitude and kinetics of glycinergic IPSCs ($n = 7$, Fig. 3C and D). These results indicate that: (i) postsynaptic depolarization of HMs causes depression of IPSC amplitude and weak prolongation of its decay time; and (ii) both phenomena are Ca^{2+} dependent.

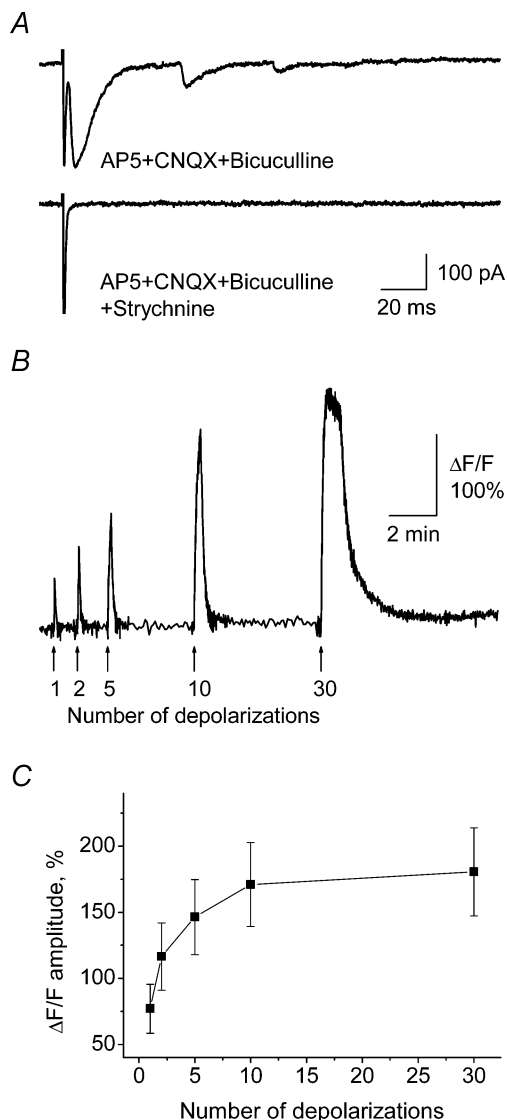


Figure 1. Glycinergic synaptic currents and depolarization-induced Ca_i dynamics in the hypoglossal motoneurons (HMs)

A, top trace, glycinergic IPSCs recorded in a voltage-clamped HM from an acute brainstem slice in the presence of bicuculline (20 μM), AP-5 (40 μM) and CNQX (10 μM). The trace was selected to also show spontaneous IPSCs. These currents were abolished by 3 μM strychnine (bottom trace). B, Ca_i fluorescence transients induced by repetitive depolarizations in HM dialysed with 0.1 mM BAPTA and 100 μM Fluo-5F. C, dependence of the amplitude of Ca_i fluorescence on the number of cell depolarizations; average data ($\pm$ s.e.m.) from seven cells are represented.

Ca^{2+} -induced potentiation of glycine-induced currents

Ca^{2+} -dependent depression of glycinergic IPSCs could be presynaptic in origin, due to inhibition of neurotransmitter release. Alternatively, it could be induced postsynaptically if the rise in Ca_i caused downregulation of GlyRs expressed in HMs, i.e. these receptors exhibit properties different from those described in spinal neurones and in HEK 293 cells expressing GlyRs (Fucile *et al.* 2000).

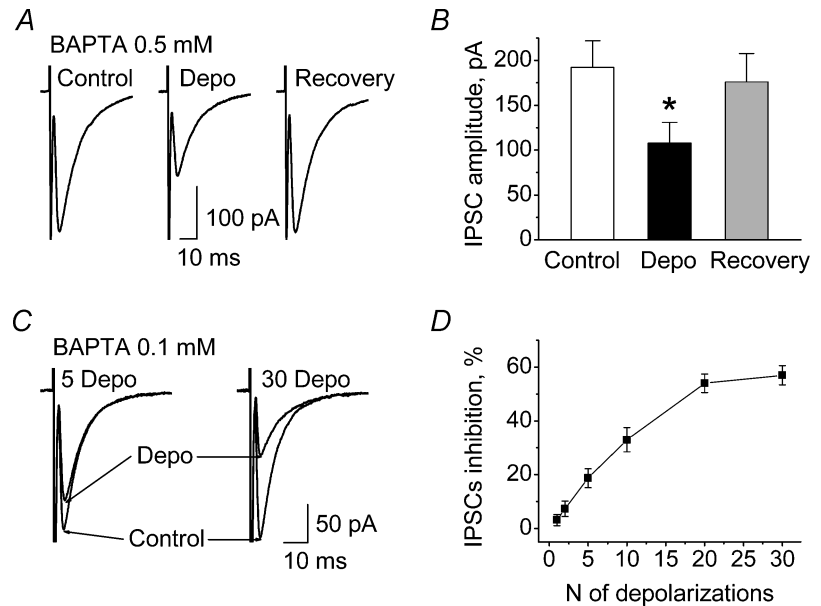
To address this point, we analysed the whole-cell currents evoked by pressure application of exogenous glycine from a puff pipette positioned close to the recorded HMs. Depending on the agonist concentrations and duration of application pulses, at a holding potential (V_h) of -70 mV, glycine induced inward currents (I_{Gly}) in the range 50–1000 pA.

When the Ca_i was increased by repeated depolarization, systematic modulation of I_{Gly} was not observed (not shown). A previous study (Fucile *et al.* 2000) demonstrated that, in spinal cord neurones, conditioning pulses of glutamate induce the potentiation of responses to exogenously applied glycine. As HMs express both NMDA and Ca^{2+} -permeable AMPA receptors (O'Brien *et al.* 1997; Essin *et al.* 2002), we used glutamate receptor ionic channels as a tool to elevate Ca_i during whole-cell recordings.

When a second puff pipette, containing glutamate (200 μM), was positioned close to the motoneurone's soma, a conditioning application of glutamate caused a large and reversible increase in the peak amplitude of I_{Gly} (Fig. 4A and B). No changes in the decay time of glycine-evoked currents were observed. This is in agreement with the fact that at puff application

Figure 2. Ca²⁺-dependent inhibition of glycinergic synaptic currents in HMs

A, effect of depolarization on glycinergic IPSCs recorded in HM with 0.5 mM BAPTA. Average traces, representing 30 individual evoked IPSCs, before (Control), in the first minute after (Depo) and 5 min after (Recovery) the series of depolarizations. **B**, average inhibition of glycinergic IPSCs induced by repeated depolarization (mean ($\pm$ S.E.M.) from five experiments). *significant difference ($P = 0.002$). **C**, average traces of glycinergic IPSCs before (Control) and after (Depo) of 5 (left traces) or 30 (right traces) repetitive depolarizations of HM. Note stronger inhibition with elevation of depolarizing pulses. **D**, dependence of degree of glycinergic IPSCs' inhibition on the number of cell depolarizations. Data ($\pm$ S.E.M.) from seven neurones.



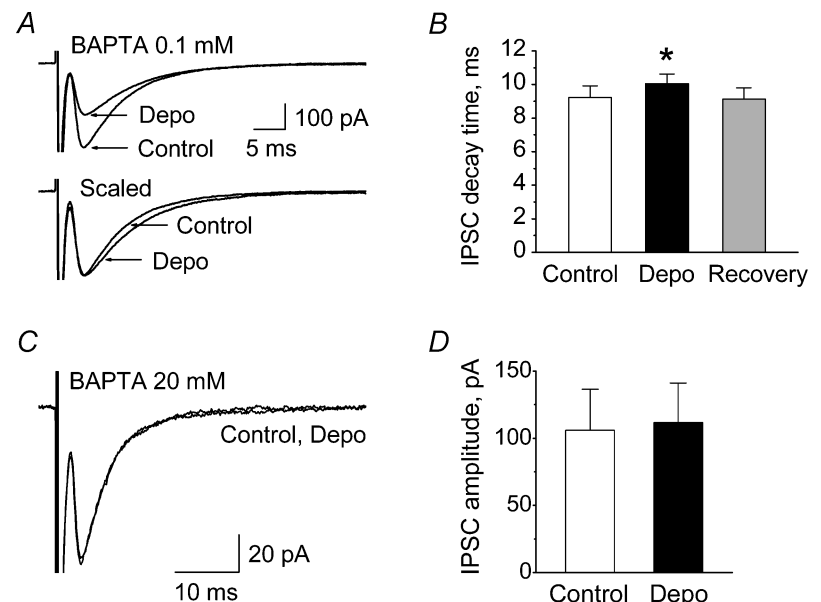
the decay time of I_{Gly} is determined by the relatively slow rate of glycine washout from the vicinity of recorded HM. On average, when recording with 0.1 mM BAPTA in the intracellular solution, after glutamate application I_{Gly} increased to $154 \pm 8\%$ ($n = 6$, $P = 0.001$, t test) and recovered to the control value ($110 \pm 3\%$) within 2–3 min (Fig. 4A–C). The extent of potentiation increased with prolongation of the conditioning glutamate pulse (Fig. 4B). Similar augmentation of I_{Gly} was observed with a conditioning application of NMDA ($200 \mu\text{M}$) dissolved in standard extracellular solution. After NMDA pulses (5–10 s), the I_{Gly} amplitude reversibly increased to $127 \pm 3\%$ ($n = 3$, $P = 0.013$, t test; Fig. 4D).

When the same protocol was performed in cells recorded in the presence of high- Ca_i buffer, no effect on the glycine-induced currents was observed. In five cells recorded with BAPTA 20 mM intracellular solution, amplitudes of I_{Gly} after NMDA application were similar to control ($102 \pm 1\%$, $n = 5$; Fig. 4D).

These results indicate that glutamate- or NMDA-dependent elevation of glycine-evoked currents is a Ca²⁺-dependent phenomenon. They also demonstrate that glycine receptors expressed in HMs are potentiated by Ca_i , similarly to GlyRs previously described (Fucile *et al.* 2000), raising the question of the mechanism of Ca²⁺-dependent depression of glycinergic IPSCs shown in Fig. 2.

Figure 3. Ca²⁺-dependent prolongation of glycinergic synaptic currents in HMs

A, depolarization-induced prolongation of glycinergic IPSCs. Top traces, average traces of IPSCs before (Control) and after (Depo) the series of depolarizations. Bottom traces, same traces as the top, scaled to control amplitude, showing the increase in the decay time of IPSCs after depolarization. **B**, summary of depolarization-induced decay time prolongation of glycinergic IPSCs (mean ($\pm$ S.E.M.) from 10 experiments). *Significant difference ($P = 0.01$). **C** and **D**, strong Ca²⁺ buffering suppressed depolarization-induced modulation of glycinergic IPSCs. **C**, superimposed traces representing IPSCs recorded in BAPTA 20 mM solution before (Control) and after (Depo) series of depolarizations. **D**, average of IPSC amplitudes in BAPTA 20 mM internal solution before (Control) and after (Depo) depolarization. Data ($\pm$ S.E.M.) from seven neurones.



Presynaptic nature of Ca^{2+} -dependent depression at glycinergic synapses

Depression of glycinergic IPSCs in HMs resembles the phenomenon called depolarization-induced suppression of inhibition (DSI), described originally for GABAergic synapses in cerebellar Purkinje cells (Llano *et al.* 1991). DSI is initiated postsynaptically due to a rise in Ca_i and expressed presynaptically as a decrease in neurotransmitter release. This phenomenon is accompanied by an increase in the failure rate (Vincent *et al.* 1992; Diana & Marty, 2003) and also an elevation in the paired-pulse ratio (PPR) (Yoshida *et al.* 2002; Diana & Marty, 2003).

To clarify whether the Ca^{2+} -dependent depression of glycinergic IPSCs was of presynaptic origin, we analysed changes in these parameters before and after Ca_i elevation in HMs. To measure the PPR, two consecutive pulses separated by 50 ms were delivered to the stimulating pipette. In the majority of neurones, we observed an increase in IPSC amplitude evoked by the second stimulus (I_2) with respect to the first one (I_1) (Fig. 5A and B). In whole-cell recordings with 0.5 mM EGTA in the intra-

cellular solution, the average value of the PPR, expressed as the ratio I_2/I_1 , was 1.13 ± 0.05 ($n = 10$, range 0.91–1.34; Fig. 5C). When a series of depolarizing steps was delivered to the recorded neurone, the PPR significantly increased. In the first minute after depolarization-induced Ca^{2+} influx, PPR increased to 1.36 ± 0.08 ($n = 10$, $P = 0.002$, Wilcoxon test) and recovered to the control value within 5 min (1.11 ± 0.05 ; Fig. 5C). The failure rate of glycinergic IPSCs also increased after the series of depolarizing pulses. On average, the failure rate increased from $34 \pm 4\%$ to $62 \pm 6\%$ ($n = 10$, $P = 0.002$, Wilcoxon test; Fig. 5D).

As another way of clarifying the origin of depolarization-induced depression, we analysed properties of spontaneous IPSCs (sIPSCs) as well as miniature IPSCs (mIPSCs) recorded in the presence of TTX ($1 \mu\text{M}$). In control conditions, the average values of amplitude and frequency of glycinergic mIPSCs in HMs were: $50 \pm 5 \text{ pA}$ and $0.83 \pm 0.36 \text{ s}^{-1}$ ($n = 7$), correspondingly. Series of depolarizations caused a reduction in frequency of glycinergic mIPSCs (Fig. 6A), as further illustrated by the cumulative interevent interval distribution ($n = 7$, $P < 0.001$, Kolmogorov–Smirnov

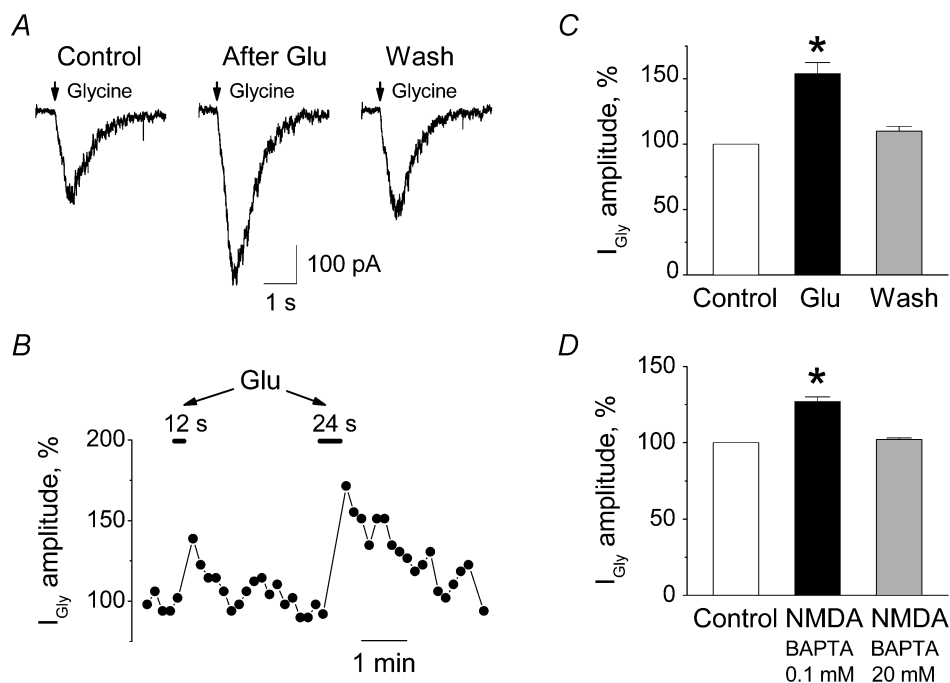


Figure 4. Ca^{2+} -dependent potentiation of glycine-evoked currents in HMs

A, glycine-evoked currents (I_{Gly}) recorded in HM with 0.1 mM BAPTA in intracellular solution are potentiated by a conditioning pulse of glutamate. Traces of whole-cell currents induced by pressure application of glycine (2 mM, 20 ms, arrows) recorded before, immediately after and 2 min after a glutamate pulse (200 μM , 24 s). B, time course of successive I_{Gly} peaks in an experiment when two conditional pulses of glutamate, respectively, of 12 and 24 s (indicated by bars), were applied to HM. Note reversible potentiation of I_{Gly} amplitude by glutamate. C, glutamate-induced potentiation of I_{Gly} from six neurones obtained in BAPTA 0.1 mM. Columns represent the amplitudes of I_{Gly} normalized with respect to control. *Significant difference ($P = 0.008$). D, average potentiation of I_{Gly} by NMDA (200 μM , 5 s) obtained in BAPTA 0.1 mM (three cells) and in BAPTA 20 mM (five cells) internal solutions. Current amplitudes are normalized to control amplitude before NMDA application. Note that in BAPTA 20 mM solution there is no potentiation of I_{Gly} . Data are mean $\pm$ S.E.M. *significant difference ($P = 0.013$).

Figure 5. Depolarization-induced inhibition alters paired-pulse facilitation of glycinergic IPSCs

A, superimposed traces showing glycinergic IPSCs evoked by a paired stimulation (50 ms interval) before (Control) and after (Depo) the series of depolarizations. *B*, same traces as in *A*, scaled to control amplitude, showing the increase in the paired-pulse facilitation after depolarization. *C*, paired pulse ratio (I_2/I_1) observed in 10 neurones before (Control), in the first minute after (Depo) and 5 min after (Recovery) the series of depolarizations. The mean values of PPR ($\pm$ S.E.M.) are represented by open circles. *D*, failure rate of glycinergic IPSCs estimated in the same conditions as in *C* for 10 cells. The mean values of failure rate are represented by open circles.

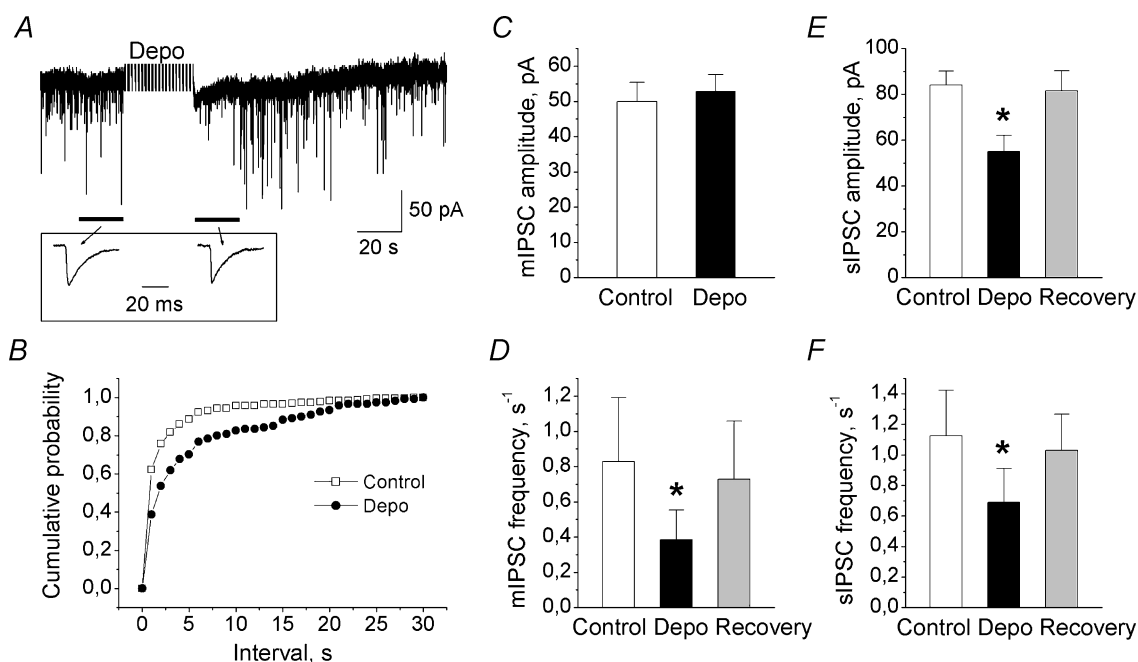
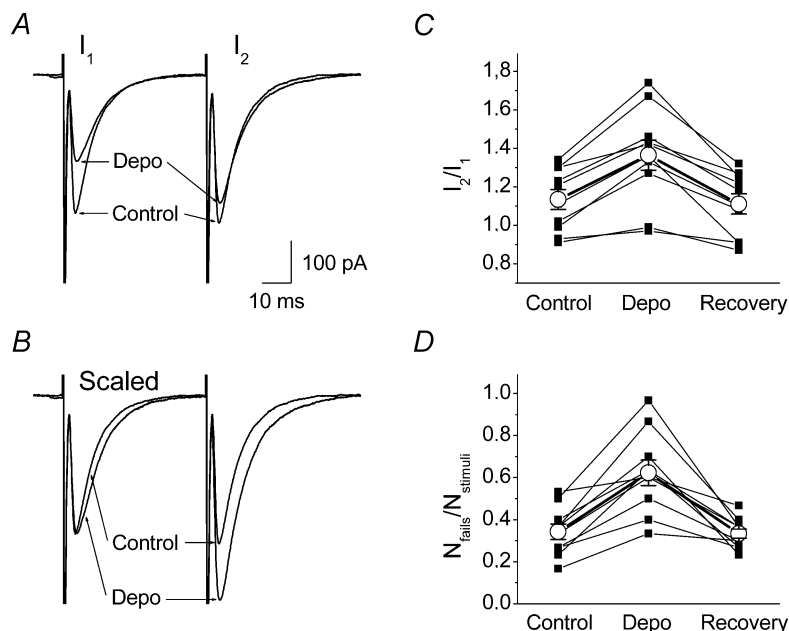


Figure 6. Effect of depolarization on glycinergic mIPSCs and sIPSCs

A, glycinergic mIPSCs recorded in the presence of TTX (1 μ M) before and after the series of 20 depolarizations. Note the clear reduction in frequency of mIPSCs following depolarization. Inset shows average traces of mIPSCs recorded during 20 s intervals before and immediately after the series of depolarizations. *B*, cumulative interevent interval histograms of glycinergic mIPSCs, showing the increase in the time intervals between mIPSCs after a series of 20 depolarizations. Data from seven motoneurons. *C* and *D*, average amplitude and frequency values of glycinergic mIPSCs from seven cells before (Control), in the first minute after (Depo) and 3–5 min after (Recovery) the series of depolarizations. Note the significant decrease only in the frequency of mIPSCs. *Significant difference ($P = 0.016$). *E* and *F*, average amplitude and frequency values of glycinergic sIPSCs before (Control), in the first minute after (Depo) and 3–5 min after (Recovery) the series of depolarizations. Note the significant decrease in both the amplitude and frequency of sIPSCs. Data ($\pm$ S.E.M.) from seven motoneurons. *Significant difference ($P = 0.007$, E ; $P = 0.027$, F).

test; Fig. 6B). On average, depolarization resulted in a decrease in the frequency of mIPSCs by $53 \pm 2\%$ ($n=7$, $P=0.016$, Wilcoxon test) without significant changes in their amplitude (Fig. 6C and D). Recorded in the absence of TTX, glycinergic sIPSCs exhibited depolarization-induced decreases in both the amplitude and frequency, by $35 \pm 10\%$ ($n=7$, $P=0.007$, t test; Fig. 6E) and $39 \pm 4\%$ ($n=7$, $p=0.027$, Wilcoxon test; Fig. 6F), correspondingly.

These results strongly suggest that Ca^{2+} -dependent depression of glycinergic IPSCs is of presynaptic origin and resembles DSI. For this reason we have called this phenomenon 'glycinergic DSI'.

Involvement of endocannabinoids in Ca^{2+} -dependent depression of glycinergic IPSCs

Several recent studies have demonstrated that the retrograde signalling for DSI is mediated by endo-

cannabinoids, whose synthesis is stimulated by the depolarization-induced rise in Ca_i (Maejima *et al.* 2001; Ohno-Shosaku *et al.* 2001; Wilson & Nicoll, 2001). To clarify whether such a mechanism could also be responsible for Ca^{2+} -dependent glycinergic DSI, we analysed the effects of the cannabinoid (CB) receptor agonist WIN55,212-2 and the CB receptor antagonist SR141716A on IPSCs.

The CB receptor agonist WIN55,212-2 ($3 \mu\text{M}$) caused decrease in IPSCs and partially or completely occluded glycinergic DSI (Fig. 7A). In 13 recorded cells, in low- Ca^{2+} -buffering conditions (0.5 mM EGTA or 0.1 mM BAPTA), IPSC suppression by WIN55,212-2 was $35 \pm 3\%$ ($P < 0.001$, t test; not shown). In these cells, control DSI achieved $46 \pm 3\%$ (Fig. 7B) while, in the presence of WIN55,212-2, the DSI value was, on average, only $12 \pm 5\%$ ($n=13$, $P < 0.001$, t test; Fig. 7B). In two cells WIN55,212-2 was not effective while in the other 11

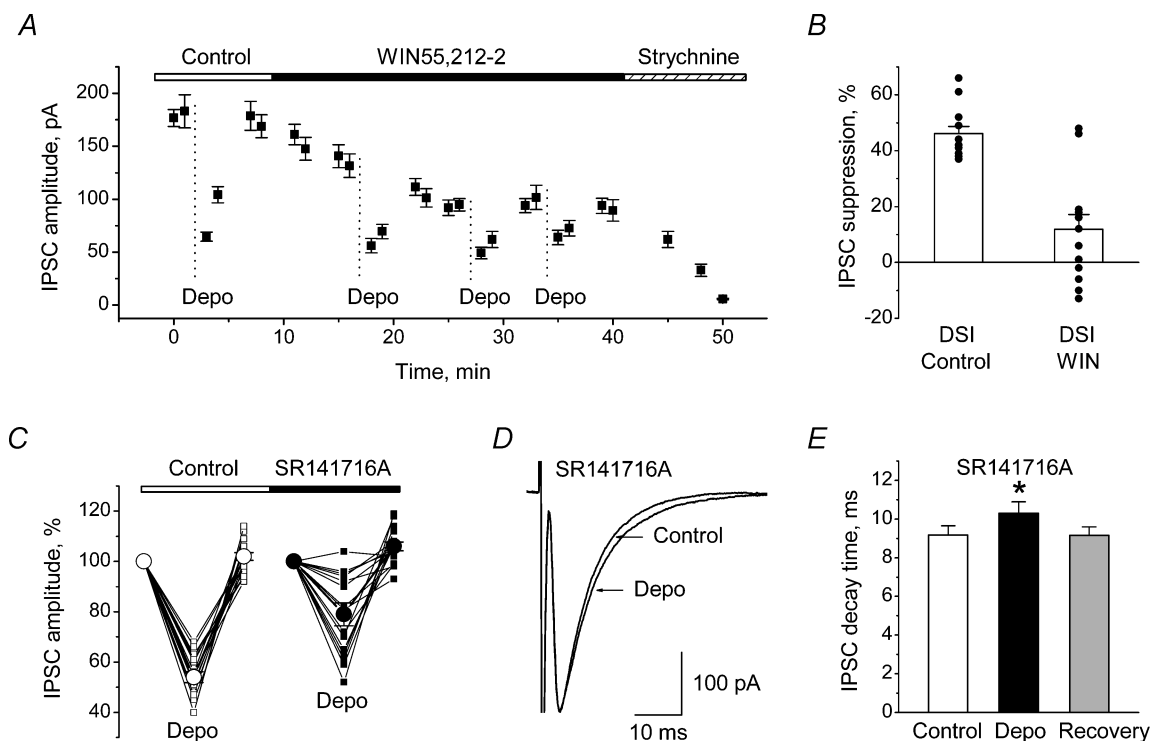


Figure 7. Effect of cannabinoid receptor agonist and antagonist on depolarization-induced inhibition of glycinergic IPSCs

A and B, effect of the cannabinoid receptor agonist WIN55,212-2 on glycinergic IPSCs. A, time course of IPSC amplitudes in control conditions, during application of WIN55,212-2 ($3 \mu\text{M}$) and after addition of strychnine ($1 \mu\text{M}$) to the external solution. Four depolarization series (Depo) were applied during the course of experiment. Note that WIN55,212-2 caused a decrease in IPSC amplitude and partially occluded DSI; strychnine completely antagonized IPSCs, confirming the glycinergic nature of the currents. B, average values of DSI in control and in the presence of $3 \mu\text{M}$ WIN55,212-2. Data from 13 neurones. C, effect of cannabinoid receptor antagonist SR141716A on glycinergic DSI; the data from 16 neurones for depolarization-induced inhibition of IPSCs in control ($\square$) and in the presence of 3–5 μM SR141716A ($\blacksquare$). The average values are shown by $\circ$ (Control) and $\bullet$ (SR141716A). Note that the effect of SR141716A varied markedly in the different cells. D, superimposed traces of glycinergic IPSCs recorded in the presence of SR141716A (3–5 μM) before (Control) and after (Depo) depolarization; each trace is the average of 30 single IPSCs. Note decay time prolongation of glycinergic IPSCs after depolarization. E, averaged data from 16 neurones for decay time of IPSCs before (Control), in the first min after (Depo) and 5 min after (Recovery) depolarization. Data are mean $\pm$ S.E.M. *Significant difference ($P < 0.001$).

cells the DSI values varied from -13 to $+19\%$ (Fig. 7B). Administration of WIN55,212-2 caused augmentation of the mean PPR value from 1.20 ± 0.10 to 1.45 ± 0.13 ($n = 13$, $P < 0.001$, Wilcoxon test; not shown), suggesting the presynaptic origin of glycinergic IPSC suppression by this CB receptor agonist.

In 16 HMs recorded in low Ca²⁺-buffering conditions (0.5 mM EGTA or 0.1 mM BAPTA), we compared glycinergic DSI before and after SR141716A. In control, the DSI value varied from 32% to 60% (mean $46 \pm 2\%$; Fig. 7C). SR141716A ($3\text{--}5\ \mu\text{M}$) antagonized glycinergic DSI, however, with varying efficacy. In six cells the DSI value was nearly completely eliminated (to 0–10%) without changes in the control amplitude of IPSCs; in four cells DSI decreased to 18–30%, and in the remaining six neurones it stayed in the range 35–48% (Fig. 7C). On average, in the presence of SR141716A the DSI value was $21 \pm 5\%$ ($n = 16$, $P < 0.001$, t test; Fig. 7C). In addition, SR141716A prevented the effect of WIN55,212-2 on the IPSC amplitude ($n = 10$; not shown).

These results indicate that, in the hypoglossal nucleus, the release of glycine is modulated presynaptically through the Ca²⁺-induced cannabinoid machinery (Wilson & Nicoll, 2002; Freund *et al.* 2003; Diana & Marty, 2004), while the extent of this modulation varied for different HMs.

Similarly to the control conditions (Fig. 3A and B), in the presence of SR141716A ($3\text{--}5\ \mu\text{M}$), glycinergic IPSCs exhibited depolarization-induced prolongation of decay kinetics (Fig. 7D and E). The mean decay time of IPSCs before depolarization was 9.2 ± 0.5 ms; after depolarization it was 11% longer (10.3 ± 0.6 ms; $n = 16$, $P < 0.001$, Wilcoxon test) and 5 min later it had recovered to the control value (9.1 ± 0.4 ms; Fig. 7E).

This weak but significant reversible increase of glycinergic IPSC decay time might reflect prolongation of postsynaptic glycine receptor channel openings during Ca_i elevation (Fucile *et al.* 2000). To check this hypothesis, we analysed glycinergic IPSCs when presynaptic modulation was suppressed by the CB receptor antagonist SR141716A and the Ca_i rise was transiently induced by activation of NMDA receptor channels.

Potentiation by NMDA of glycinergic IPSCs in the presence of a CB receptor antagonist

In the absence of a CB receptor antagonist, when the intracellular solution contained 0.1 mM BAPTA, application of NMDA (5–10 s pulses, $V_h = -70$ mV) resulted in a strong suppression of glycinergic IPSCs, similar to that caused by depolarization (Fig. 8A and B). On average, in six neurones analysed, the amplitude of glycinergic IPSCs after the NMDA pulse decreased to $47 \pm 8\%$ ($P = 0.001$, t test; Fig. 8B).

Moreover, pulses of NMDA caused a pronounced prolongation of glycinergic IPSC decay time constants (by $29 \pm 4\%$; $n = 6$, $p = 0.031$, Wilcoxon test; Fig. 8C), suggesting that, in addition to inhibition of presynaptic glycine release, postsynaptic GlyRs are profoundly modulated by this route of Ca_i elevation. This assumption was confirmed in the experiments when the presynaptic modulatory action of Ca²⁺ was diminished by a CB receptor antagonist.

In the presence of SR141716A ($3\ \mu\text{M}$), following NMDA pulses, synaptic currents exhibited both significant increases in peak amplitudes and prolongation of decay time constants (Fig. 8D–F). Both parameters reverted almost completely to control values within 1–2 min. On average, in the first minute following NMDA application, the amplitude of IPSCs increased to $123 \pm 6\%$ of control ($n = 6$, $p = 0.012$, t test; Fig. 8E). In these experiments, to give relief from Mg²⁺ block, cells were concomitantly depolarized to -20 mV during NMDA applications. This facilitation of Ca²⁺ influx resulted in a particularly powerful prolongation of the IPSC decay time constant (by $80 \pm 17\%$; $n = 6$, $P = 0.006$, Wilcoxon test; Fig. 8F). After the NMDA pulse, the IPSC amplitude and decay kinetics recovered to, respectively, $103 \pm 5\%$ and $119 \pm 13\%$ of control ($n = 6$; Fig. 8E and F).

When the same experiments were repeated with the BAPTA 20 mM internal solution, the NMDA-induced potentiation was absent: both IPSC amplitude ($103 \pm 6\%$, $n = 6$) and decay time constant ($105 \pm 4\%$, $n = 6$) measured following NMDA application were equal to control values (Fig. 8G, H and J).

Our observations demonstrate that GlyRs at glycinergic synapses can be strongly modulated by Ca²⁺ influx through glutamate receptor channels. This postsynaptic modulation could cause an increase in glycinergic transmission. However, in the HMs this phenomenon is masked by a powerful retrograde inhibition mediated partially by the endocannabinoid system.

Discussion

We have shown two novel roles for intracellular Ca²⁺ in modulation of glycinergic synaptic transmission in HMs: (i) presynaptic depression of neurotransmitter release; and (ii) postsynaptic potentiation of GlyR channels (Fig. 9). An inhibition of neurotransmitter release was induced by postsynaptic Ca²⁺ influx through voltage-gated Ca²⁺ channels or glutamate receptor channels, and it was observed even at relatively strong Ca²⁺ buffering (1 mM BAPTA in the recording pipette). This phenomenon is of presynaptic nature and the mechanism resembles endocannabinoid-stimulated inhibition of neurotransmitter release from presynaptic terminals (Ohno-Shosaku *et al.* 2001; Wilson & Nicoll, 2001; Diana & Marty, 2003). Potentiation of postsynaptic

GlyRs was observed preferably at activation of glutamate receptors, and it was only detectable when endocannabinoid-mediated depression was suppressed. This effect results in an increase in amplitude and prolongation of decay time of glycinergic IPSCs, and resembles the mechanism of a rapid Ca^{2+} -induced potentiation (Fucile *et al.* 2000). In hypoglossal brainstem motoneurons, the first phenomenon is highly predominant.

Depolarization-induced inhibition

Several arguments indicate that the glycinergic DSI has a presynaptic origin and is largely mediated by the endo-

cannabinoid system. First, we find that depolarization: (i) increases PPR and (ii) increases the failure rate of stimulation-induced IPSCs. Second, depolarization caused a decrease in the frequency of mIPSCs without changes in their amplitude. Third, SR141716A, a selective antagonist of the brain CB receptors, decreased glycinergic DSI without changes in IPSC amplitude. Finally, the CB receptor agonist WIN55,212-2 decreased the amplitude of IPSCs and occluded glycinergic DSI. The mechanism of Ca^{2+} -induced feedback regulation of inhibitory and excitatory synaptic transmission via retrograde endocannabinoid signalling is widely used in the mammalian nervous system (Jennings *et al.* 2001; Wilson & Nicoll, 2001; Yoshida *et al.* 2002; Azad *et al.* 2003).

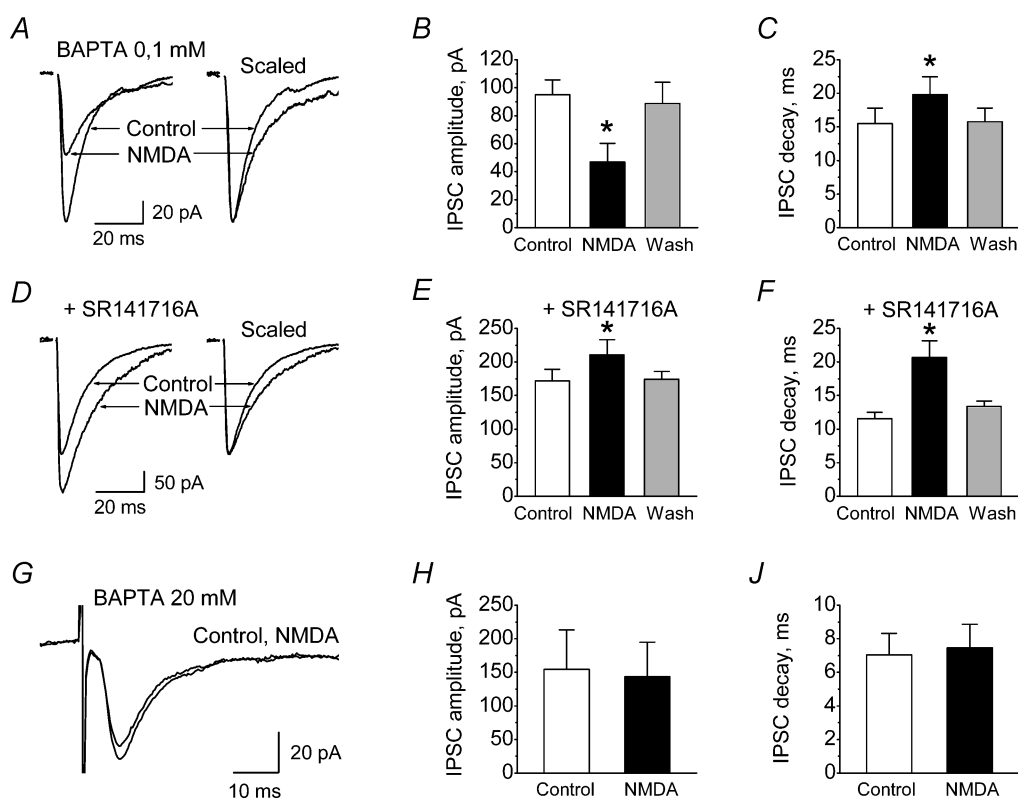


Figure 8. NMDA potentiates and prolongs glycinergic IPSCs in the presence of SR141716A

A, B and C, NMDA-induced suppression of glycinergic IPSCs in physiological conditions. A, superimposed traces before and after NMDA application (left) and normalized to the amplitude of control (right). Recording with BAPTA 0.1 mM intracellular solution; NMDA was applied focally from a puff pipette (200 μM , 5–10 s, $V_h = -70$ mV). Each trace is the average of 10–20 single IPSCs. B, mean IPSC amplitude and C, decay time constant before (Control), in the first minute after (NMDA) and 5 min after NMDA application (Wash). Data ($\pm$ S.E.M.) from six neurones. *Significant difference ($P = 0.001$, B; $P = 0.031$, C). Note pronounced increase in mean decay time after NMDA application. D, E and F, NMDA-induced potentiation of glycinergic IPSCs in the presence of the cannabinoid receptor antagonist SR141716A (3 μM). D, superimposed traces before and after NMDA application. Each trace is the average of 5–15 single IPSCs. E and F, similar presentation as in B and C. Mean data from six cells. Recording with 0.1 mM BAPTA. Note that NMDA caused an increase in IPSC amplitude and a prolongation of decay time, correspondingly by 23% and 80%. In this series of experiments, during the pulse of NMDA application the neurones were depolarized to -20 mV, which abolished Mg^{2+} block and, thus, elevated Ca^{2+} influx through NMDA receptor channels. *Significant difference ($P = 0.012$, E; $P = 0.006$, F). G, H and I, strong Ca^{2+} buffering in postsynaptic neurone abolishes NMDA-induced modulation of glycinergic IPSCs. Superimposed traces G, mean amplitudes H and decay times I of IPSCs in control and after NMDA application. For H and I, data are from six neurones. Recording with BAPTA 20 mM intracellular solution and with SR141716A (3 μM) in the extracellular solution.

Endocannabinoids can modulate the function of ionic channels independently on CB receptors. It has been shown that the acid-sensitive potassium (K⁺) channel TASK-1 is directly blocked by sub-micromolar concentrations of anandamide (Maingret *et al.* 2001). Moreover, anandamide, as well as WIN55,212-2, can also inhibit the activity of glycine receptors in heterologous systems (Lozovaya *et al.* 2005). While for HMs, a direct modulation of ionic channels by endocannabinoids is not excluded, several arguments suggest that this mechanism plays a minor role in glycinergic DSI. First, presynaptic inhibition of K⁺ channels should lead to membrane depolarization and, consequently, stimulation of

neurotransmitter release. In our experiments we observed the opposite effect. Second, postsynaptic inhibition by a CB receptor agonist of K⁺ TASK-1 channels should result in changes of both the input resistance and level of background current. However, WIN55,212-2 caused inhibition of glycinergic IPSCs without affecting the membrane properties of HMs. Third, SR141716A does not prevent the effects of anandamide and WIN55,212-2 on TASK-1 channels (Maingret *et al.* 2001), while in HMs this CB receptor antagonist effectively suppressed the action of WIN55,212-2. Finally, postsynaptic inhibition of ionic channels should be observed without changes in failure rate and PPR. In our experiments, these parameters were clearly modified by WIN55,212-2.

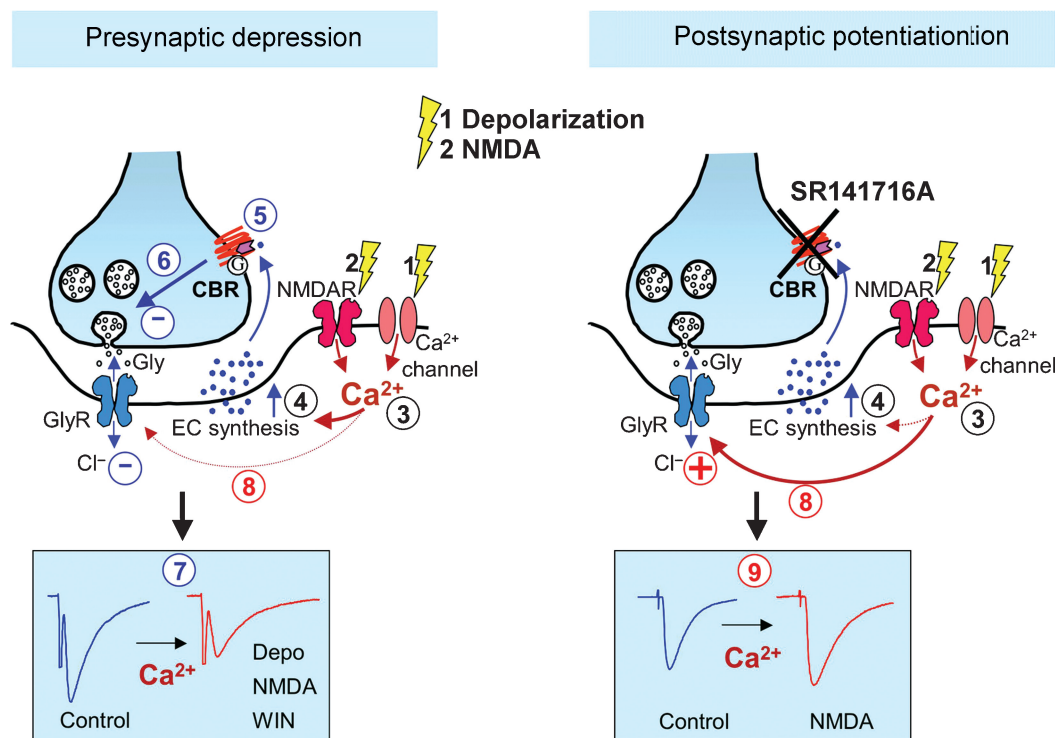


Figure 9. Scheme summarizing the mechanisms of glycinergic synaptic transmission modulation by intracellular Ca²⁺ in HMs

Presynaptic depression. Activation of postsynaptic voltage-gated Ca²⁺ channels (1) or NMDA receptor (NMDAR) channels (2), increase in Ca_i (3) and synthesis of endocannabinoids (EC) (4). Diffusion of endocannabinoids to presynaptic terminals leads to activation of CB receptors (CBR) (5), which causes the reduction in Ca_i transients in the presynaptic terminals and, consequently, a decrease (–) in release probability of glycine-containing vesicles (6); this results in a decrease in glycinergic IPSC amplitude (7). WIN55,212-2 (WIN), a CB receptor agonist, causes similar inhibition of IPSCs. In addition the increase in Ca_i modulates GlyRs (8), causing slight IPSC prolongation. **Postsynaptic potentiation.** In the presence of a CB receptor antagonist (SR141716A), the presynaptic mechanism of IPSC depression (steps 5–7) is strongly reduced. On the other hand, the increase in Ca_i generated by depolarization (1) and, much more effectively, by activation of NMDA receptor channels (2), potentiates postsynaptic GlyRs (8), increasing (+) the open-channel probability (Fucile *et al.* 2000). This results in the increase in amplitude and prolongation of decay time of glycinergic IPSCs (9). In brainstem HMs, under normal physiological conditions, IPSC presynaptic depression is highly predominant. Potentiation of postsynaptic GlyRs is only detectable when endocannabinoid-mediated depression is suppressed.

The fact that SR141716A did not completely suppress glycinergic DSI might indicate the possibility of an additional mechanism of Ca^{2+} -induced feedback regulation. Firstly, glycinergic neurotransmission in HMs could be regulated via a putative, as yet unidentified, cannabinoid receptor. Indeed, besides the cloned G-protein-coupled CB1 and CB2, it has been suggested that other cannabinoid-sensitive receptors (CBX) are present in the brain (Di Marzo *et al.* 2000; Breivogel *et al.* 2001; Hajos *et al.* 2001; Hajos & Freund, 2002). Alternatively, endocannabinoid-independent retrograde inhibition via activation of GABA_B or metabotropic glutamate receptors, similar to that described for glutamatergic EPSCs (Zilberter *et al.* 1999) or GABA_A ergic IPSCs (Zilberter, 2000) in rat neocortex neurones, could be involved. The GABA_B modulation pathway is particularly attractive as corelease of glycine and GABA from 'mixed' presynaptic terminals is well documented (Jonas *et al.* 1998; O'Brien & Berger, 1999). Moreover, in the auditory brainstem nucleus (Lim *et al.* 2000) and in HMs (O'Brien *et al.* 2004), GABA mediates presynaptic inhibition of glycinergic synapses via GABA_B receptors.

Ca^{2+} -induced potentiation

Our observations demonstrate that in glycinergic synapses, postsynaptic GlyRs are potentiated by Ca_i and this modulation strongly depends on the source of Ca^{2+} rise. Influx of Ca^{2+} through NMDA receptor channels was much more effective for potentiation of GlyRs than that through voltage-gated Ca^{2+} channels.

The organization of synaptic inputs and specificity of the cytosolic buffering system in HMs might account for these remarkable differences in Ca_i action. From light- and electron-microscopic observations, the vast majority (88–98%) of axonal projections on HMs forms axodendritic synapses (Zhang *et al.* 2001; Zhang *et al.* 2003). As levels of Ca^{2+} are regulated in a highly local environment, this would suggest that Ca^{2+} signals initiated in the soma and backpropagating to dendritically localized glycinergic synapses would be rapidly attenuated. Consequently, a depolarization-induced Ca_i rise in the vicinity of glycinergic synapses might be much weaker than those recorded in the soma. This is particularly critical for HMs, which exhibit a low concentration of endogenous Ca^{2+} buffers (Ca^{2+} binding ratio about 40) in the cytosol (Lips & Keller, 1998), which accounts for rapid relaxation times of Ca_i after depolarization. Analysis of Ca_i transients in HMs demonstrated that peak amplitudes of voltage-induced Ca_i rise are more than two-fold higher in the soma than in dendrites monitored 80 μm from the soma. Moreover, the decay time constant of Ca_i in dendrites is faster (Ladewig *et al.* 2003).

On the other hand, a high efficacy of Ca^{2+} entry through NMDA receptor channels might suggest that glutamatergic and glycinergic synapses are in close proximity on dendrites of HMs. It has been demonstrated on young brainstem slices that NMDA and non-NMDA receptors are colocalized on glutamatergic synapses in HMs; furthermore, these neurones exhibit a markedly higher proportion of NMDA receptor-mediated mEPSCs than do non-NMDA ones (O'Brien *et al.* 1997). On the basis of indirect evidence, the colocalization of NMDA and glycine receptors has been proposed in neurones of spinal cord (Fern *et al.* 1996) and brainstem hypoglossal nucleus (Berger *et al.* 1998). Thus, activation of dendritically situated NMDA receptors would produce a high local rise in Ca_i , and consequent potentiation of neighbouring GlyRs in glycinergic synapses.

The action of NMDA might be presynaptic in origin, as in some brain areas NMDA receptors are found on both excitatory and inhibitory terminals (Casado *et al.* 2000; Duguid & Smart, 2004). However, several lines of evidence indicate that the potentiation of IPSCs observed in our study is of postsynaptic origin and results from the increase in GlyR activity. First, NMDA similarly potentiated glycinergic IPSCs and ionic currents induced by exogenous application of glycine. Second, the increase in IPSC amplitude is associated with prolongation of decay time, as is expected for an increase in the apparent affinity of GlyR for the agonist (Fucile *et al.* 1999, 2000; Li & Pearce, 2000). Finally, the effect of NMDA was absent in the strong buffering of postsynaptic Ca^{2+} .

The most relevant feature of Ca^{2+} -induced postsynaptic potentiation is the reversible increase in duration of glycinergic IPSC decay time. We observed this, with different extents of prolongation, after a depolarization-induced Ca_i rise and after application of NMDA. An early study on HMs demonstrated that GlyR deactivation is the main determinant of glycinergic IPSC decay (Singer & Berger, 1999). As deactivation kinetics correlate with the apparent affinity of GlyR (Fucile *et al.* 2000; Li & Pearce, 2000), IPSC prolongation is consistent with a Ca^{2+} -induced decrease in the EC_{50} for glycine.

Prolongation of the IPSC decay during glycinergic DSI could be of presynaptic origin. This might result from the lower probability of liberation during glycinergic DSI and, consequently, more scattered latencies of individual vesicular release events in comparison with the control. Some presynaptic effects on IPSC decay time constant are not entirely excluded, while several observations do not support this suggestion. First, the CB receptor antagonist SR141716A, which decreased DSI, should also suppress changes in IPSC's decay time. Our results indicate that modulation of glycinergic currents' kinetics was similar in the two conditions (Fig. 3A and 7D). Second, following NMDA application, similar prolongation of IPSCs was observed in control conditions when the amplitude of

IPSCs was strongly depressed (Fig. 8A–C), as well as in the presence of SR141716A when the amplitude of IPSCs was augmented (Fig. 8D–F). For a presynaptic mechanism, the kinetics should follow the directions of amplitude variations. Finally, NMDA and depolarization caused similar suppression of amplitudes while NMDA-induced Ca_i rise resulted in much more pronounced prolongation of IPSC decay time. In the case of changes in the latencies of individual vesicular release, the effect should be similar in the two conditions.

The increase in glycinergic IPSC peak amplitude, although limited, is somewhat surprising because glycine-activated currents are not much affected by Ca²⁺-dependent potentiation at saturated concentrations of glycine (Fucile *et al.* 2000). Augmentation of IPSCs could indicate that GlyRs at glycinergic synapses of HMs are not saturated by transmitter release; this has been shown at glycinergic synapses in the zebrafish hindbrain (Rigo *et al.* 2003).

The double modulation of glycinergic synaptic transmission described here may be used for selective modification of neuronal activity under different physiological conditions. In particular, at early stages of postnatal development in some brain regions, such as hippocampus and neocortex, glycinergic (Ito & Cherubini, 1991) and GABAergic (Dammerman *et al.* 2000) responses are excitatory. Moreover, GlyRs may be activated in a non-synaptic fashion (Flint *et al.* 1998), suggesting that, before the establishment of mature chloride equilibrium, Ca²⁺-dependent potentiation of glycinergic currents potentially represents a positive feedback in the regulation of excitation, and may have a role in synaptic stabilization (Kirsch & Betz, 1998). This mechanism, however, would not be applicable for HMs as, immediately after birth, these motoneurons exhibit inhibition by synaptic glycinergic and GABAergic inputs (Marchetti *et al.* 2002).

Ca²⁺-induced potentiation might be critical at close colocalization of GlyRs with voltage-gated or receptor-operated Ca²⁺-permeable ion channels. This might be the case for the giant synapse of the calyx of Held, which contains presynaptic GlyRs; glycine spillover has been observed to activate these presynaptic receptors, regulating glutamate release (Turecek & Trussell, 2001). We suggest that, in a similar manner, the process of Ca²⁺-dependent potentiation of GlyRs might be relevant in the physiological regulation of synaptic activity.

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Acknowledgements

The authors wish to thank Drs P. Ascher, M. Diana, F. Eusebi, A. Milne and Y. Zilberter for helpful discussions and critical reading of the manuscript. We thank Sanofi–Aventis for the gift of SR141716A. This work was carried out as part of a CNR-INSERM cooperation project and D. R. was supported by FIRB grant. We thank NATO, IBRO and Fondation pour la Recherche Médicale (FRM) for financial support of M. M.

Supplemental material

The online version of this paper can be accessed at:
DOI: 10.1113/jphysiol.2005.094862
<http://jp.physoc.org/cgi/content/full/jphysiol.2005.094862/DC1>
and contains supplemental material consisting of a table entitled: Amplitudes and kinetics of glycinergic IPSCs in hypoglossal motoneurons at different concentrations of bicuculline
This material can also be found as part of the full-text HTML version available from <http://www.blackwell-synergy.com>