SYNAPTIC STRENGTH BETWEEN MOTONEURONS AND TERMINALS OF THE DORSO-LATERAL FUNICULUS IS REGULATED BY GABA RECEPTORS IN THE TURTLE SPINAL CORD.

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SYNAPTIC STRENGTH BETWEEN MOTONEURONS AND TERMINALS OF THE DORSO-LATERAL FUNICULUS IS REGULATED BY GABA RECEPTORS IN THE TURTLE SPINAL CORD.


The role of GABA\textsubscript{A} and GABA\textsubscript{B} receptors in modulation of excitatory synaptic transmission between motoneurons and terminals from dorso-lateral funiculus (DLF) was studied in \textit{in-vitro} spinal cord slices of adult turtles. Muscimol –a GABA\textsubscript{A} receptor agonist- depressed the monosynaptic excitatory postsynaptic potential (EPSP) induced by stimulation of the DLF, and shortened its duration. The input resistance and the membrane time constant also were strongly reduced. The input membrane resistance, the amplitude and the halfwidth of the EPSP were reduced at the same rate in the presence of muscimol. Bicuculline –a GABA\textsubscript{A} receptor antagonist- increased the EPSPs amplitude and the input membrane resistance. The EPSP amplitude ratio elicited by a paired pulse protocol did not change significantly. Our results suggest that a) muscimol acts mainly by activation of postsynaptic GABA\textsubscript{A} receptors located on the motoneuron, and b) the synaptic strength on motoneurons may be modulated by tonic activation of postsynaptic GABA\textsubscript{A} receptors. Baclofen –a GABA\textsubscript{B} receptor agonist- also depressed DLF-motoneuron synaptic transmission. However, it did not affect the falling phase of the EPSPs or the motoneuron membrane time constant but induced a small decrement in input resistance. In the presence of baclofen, the amplitude ratio produced by a paired pulse protocol increased significantly. This suggests that baclofen decreased the synaptic strength by inhibition of
neurotransmitter release from the DLF terminals via activation of presynaptic GABA\textsubscript{B} receptors.
INTRODUCTION

Gamma-amino-butyric acid (GABA) is the most important inhibitory neurotransmitter in the central and peripheral nervous system (Princivalle et al. 2001; Sivilotti and Nistri 1991). GABA receptors are of three types: GABA\(_A\), GABA\(_B\), and GABA\(_C\). With exception of GABA\(_C\) receptors, which are mainly located in the retina, these receptors are found in the entire nervous system at the postsynaptic and as well as the presynaptic level (Princivalle et al. 2001; Towers et al. 2000; Sivilotti and Nistri 1991). In the spinal cord, presynaptic inhibition of afferent fibers occurs via activation of both ionotropic GABA\(_A\) and metabotropic GABA\(_B\) receptors (Rudomin and Schmidt 1999). Immunocytochemical studies have shown the existence of GABA\(_A\) and GABA\(_B\) receptors on motoneurons and on terminals synapsing on them (Magul et al. 1987; Alvarez et al. 1996; Bohlhalter 1996; Sivilotti and Nistri 1991; Towers et al. 2000; Yang K et al. 2001). In neonatal spinal cord, one action potential elicited in one interneuron evoked a unitary compound IPSCs on motoneurons, mediated by activation of GABA\(_A\) and glycine receptors. In the same preparation, a subset of miniature synaptic currents was mediated by activation of GABA\(_A\) and glycine receptors (Jonas et al. 1998). Few electrophysiological studies deal with the role of GABA\(_A\) and GABA\(_B\) receptors on motoneurons in modulation of the synaptic strength. It is known, that axons synapsing rat spinal cord motoneurons are modulated by activation of presynaptic GABA\(_B\) receptors (Jonas et al. 1998). In bullfrogs, monosynaptic EPSPs evoked on motoneurons by stimulation of a single muscle Ia afferent in the presence of muscimol were depressed by activation of presynaptic and postsynaptic GABA\(_A\) receptors (Peng and Frank 1989b). In the same preparation, activation of presynaptic GABA\(_B\) receptors by baclofen inhibited the monosynaptic EPSPs (Peng and Frank 1989a). The same action of baclofen has been shown in synapses between Ia afferents and
motoneurons (Curtis and Lacey 1998; Jiménez et al. 1991; Edwards et al. 1989; Peshori et al. 1998; Lev-Tov et al. 1988; Lacey 1996; Stuart and Redman 1992). This evidence points out that in the ventral spinal cord activation of GABA_A and GABA_B receptors could regulate synaptic strength at the presynaptic as well as at the postsynaptic level. In contrast to the Ia-motoneuron synapse, there are few studies on GABAergic modulation of synaptic strength between descending or propriospinal fibers and motoneurons. It is known that the vestibular and the rubrospinal terminals are not modulated by presynaptic GABA_A receptors (Curtis et al. 1984a, b; Rudomin et al. 1991). Nevertheless, baclofen depressed monosynaptic motoneuron EPSPs, evoked by stimulation of the ventromedial funiculus, which contains vestibulo-spinal and reticulo-spinal fibers, by activation of presynaptic GABA_B receptors (Jiménez et al. 1991). No information is available regarding modulation of the DLF-motoneuron synapses by GABA receptors. DLF contains rubrospinal and propriospinal fibers and evoke mainly EPSPs in flexor motoneurons and IPSPs and/or EPSPs in extensor motoneurons in cats (Kostyuk et al. 1971). In the present study, we have investigated the modulation of synaptic efficacy between terminals of the dorso-lateral funiculus and motoneurons. We found that this synapse was modulated by activation of postsynaptic muscimol-sensitive receptors and presynaptic baclofen-sensitive receptors.
METHODS

Preparation

Adult turtles 15-20 cm long (Kinosternon) were anesthetized with intraperitoneal injection of sodium pentobarbital (100 mg/Kg) and perfused with a cold Ringer solution containing (mM): 120 NaCl, 5 KCl, 15 NaHCO₃, 2 MgCl₂, 3 CaCl₂ and 20 glucose, saturated with 98% O₂ and 2% CO₂ to obtain a pH of 7.5. A laminectomy was made to isolate the lumbar enlargement. Turtles were subsequently killed by decapitation. Transverse slices of 2-3 mm were cut from the lumbar enlargement. Slices were placed in a recording chamber bathed with Ringer solution at room temperature (20-22°C). All experiments were carried out with the approval of the Cinvestav Experimental Ethics Committee and in accordance with the current Mexican Norm for Care and Use of Animal for Scientific Purposes.

Stimulation of DLF and recording of motoneurons

Intracellular recordings of motoneurons were performed in bridge-mode (Axoclamp-2B, Axon Inc) with a sharp electrodes filled with 1 M potassium acetate (50-60 MΩ). A neuron was classified as a motoneuron according to the criteria described by Hounsgaard et al. (1988): the input resistance, the time constant, the configuration of action potential, and the firing pattern during a depolarizing current pulse were determined for each neuron recorded. Only motoneurons with resting membrane potential more negative than -65 mV and action potential of more than 80 mV were studied. When voltage excursions were larger than 10 mV, a sag in voltage response during hyperpolarizing current pulses appeared followed by an overshoot at the end of the pulse (Fig. 1C). This is a Cs⁺ sensitive time-dependent inward rectification (Hounsgaard et al. 1988). This rectification did not affect the results because the control and the drug action were recorded with the same
electrode. The input resistance was obtained as the slope of the line fit to the IV plot built with voltage responses smaller than 10 mV (Fig. 1D).

Recordings were digitized by Digidata 1322A (Axon Instruments Inc.), visualized in a computer with the Axoscope program (Axon Instruments Inc.) and stored in the hard disk for off-line analysis. All the average parameter values are given with the standard error. In order to stimulate the dorsolateral funiculus (DLF; Fig. 1A) a pair of wire stimulus electrodes was placed on the dorso-lateral portion of the spinal cord slice and rectangular pulses (500 $\mu$s duration, 0.3-1 Hz) were applied. The threshold (T; 5-10 $\mu$A) of the DLF stimulation was determined as the stimulus intensity at which a mono-postsynaptic potential started to appear in the motoneuron. The maximal stimulus strength was 2T. In some motoneurons a mix of excitatory (EPSP) and inhibitory (IPSP) postsynaptic potentials were elicited by DLF stimulation. Elicited IPSPs were completely blocked by adding strychnine (10 $\mu$M) to the bath solution. The EPSPs were considered monosynaptic if their latency (measured from the beginning of the stimulus artifact) were less than 3 ms (Yamashita 1986).

To determine if the muscimol and the baclofen action was at presynaptic and/or postsynaptic sites, the following variables were measured before and after the drug was added to the bath solution: the motoneuron input resistance, the maximal amplitude, the halfwidth (duration at half peak amplitude), and the time constant of the falling phase of the EPSP. The time constant was determined from the slope of the falling phase of a semilogarithm plot of the EPSP. A paired pulse facilitation protocol was applied as an additional test to assess the presynaptic action of muscimol and baclofen.
**Histology**

At the end of the electrophysiological study, neurons were filled with biocytin (2% in wt) by action of depolarizing current pulses (500 ms, 0.5 nA at 1 Hz for 30 min). After biocytin injection, slices were kept in Ringer solution for 30 min and then immersed in 4% paraformaldehyde and 0.1% glutaraldehyde for 36 h. Finally, they were transferred to a buffer with sucrose (30%). For histochemistry, serial transverse sections (60 µm thick) were cut in a cryostate (Leica, CM 1850) and rinsed in PBS. Slices were first incubated in a hydrogen peroxide solution (1%) in PBS for 30 min to inactivate endogenous peroxides and then incubated in an avidin-peroxidase complex (Vector ABC kit) diluted in PBS and 0.3% Triton X-100 for 2 h. After rinsing in PBS, slices were reacted in diaminonobenzidine (10mg/25ml 10mM PBS) and hydrogen peroxide (0.01%) for 10 min and contrasted with nickel sulfate (30%). The tissue was air-dried and mounted with Permount onto glass slides. The images were acquired and digitized by Metamorph Imaging System and the neuron was reconstructed with a computer program (Photoshop; Fig. 1A).
Drugs used

Since this study was focused on GABAergic modulation of excitatory DLF terminal-motoneuron synapse, 17 neurons were recorded in Ringer added with strychnine (10 μM; Sigma) and 8 without the glycine receptor blocker. Muscimol (1-10 μM; Sigma) was used to activate GABA$_A$ receptors whereas picrotoxin (20 μM; Tocris) and bicuculline (20 μM; Tocris) were added to block GABA$_A$ receptors. Baclofen (10 μM; Tocris) was used to activate GABA$_B$ receptors. Substance list for histology: biocytin (2 % wt, Sigma), paraformaldehyde (4 %, Sigma), glutaraldehyde (0.1 %, Ted Pella Inc.), avidin-peroxidase complex (ABC kit pk-4000 Vector labs), Triton X-100 (0.3 %, Sigma), diaminonobenzidine (10mg/25ml 10mM PBS, Sigma), hydrogen peroxide (0.01%, Baker), nickel sulfate (30%, Sigma).
RESULTS

**Motoneuron classification**

To determine if the recorded cell was a motoneuron, we searched for distinctive motoneuron properties such as the configuration of the action potential and the subthreshold responses (Hounsgaard et al. 1988). The input resistance and the time constant of the ventral horn neurons recorded were $28.3 \pm 3.9 \, \text{M} \Omega$ (n = 17, mean ± se; 18-55 MΩ, Hounsgaard et al. 1988) and $36.2 \pm 4.1$ ms (n= 17; 12-61 ms, Hounsgaard et al. 1988) respectively. Action potentials had a rise time of $0.52 \pm 0.02$ ms (n = 20; 0.4-0.5 ms Hounsgaard et al. 1988)) and a duration of $0.97 \pm 0.03$ ms (n = 20; 0.9-1.2 ms, Hounsgaard et al. 1988) as measured 10 mV above threshold. The spike amplitude was $93.5 \pm 8.4$ mV (n = 20; 95-100.5 mV, Hounsgaard et al. 1988). All cells included in this study had the typical firing pattern of motoneurons (Hounsgaard et al. 1988). Furthermore, in some of these cells (n=3) bistability (Hounsgaard and Kiehn 1985) was induced when 5-HT (10 μM) was added to the bath solution (not shown). The morphology of a typical motoneuron filled with biocytin is illustrated in a transverse section of the spinal cord in Fig 1A. The dimension of the soma and the distribution of the dendritic tree coincided with that reported by others (Hounsgaard et al. 1988; Ruigrok et al. 1985).

*Time course of muscimol effect on the motoneuron membrane resistance and on EPSPs*

The time course of muscimol effect on the membrane resistance, the amplitude and the halfwidth of the EPSPs was evaluated recording in the same trace the voltage response and the EPSP elicited by DLF stimulation (2T) every 3 s, in Ringer solution and in the presence of muscimol (5 μM). In Fig 1B, the average of five consecutive traces were shown in each row, the first from the top to the bottom correspond to the control responses. Fifteen
seconds after muscimol application, the voltage response, the amplitude and the halfwidth of the EPSP started to decrease (Fig. 1B, second row), and reach the maximal decrement 120 s latter. This was better illustrated when the input resistance, the amplitude and the halfwidth of the EPSP, measured from the average traces were normalized and plotted against the time (Fig. 1C). The three quantities were reduced almost at the same rate and they reached the maximal reduction at the same time. Similar results were obtained from four cells recorded, suggesting that the input resistance may be the most important factor accounting for the EPSP depression. To know, whether muscimol affected the motoneuron membrane potential, this was continuously recorded monitoring the input resistance by means of the voltage response to a current pulse of -0.3 nA (300 ms) every 2 s (Fig. 1E). After some seconds of recording the control membrane potential muscimol was added (left vertical arrow in Fig. 1E), approximately 27 s later the input resistance started to decrease (right vertical arrow in Fig. 1E) reaching a stable value after additional 32 s. The membrane potential was not affected. In this motoneuron, the input resistance was reduced from 14.7 MΩ in control medium to 4.4 MΩ in muscimol (Fig. 1D). Similar results were obtained from 11 cells with an average reduction in input resistance of 54.7 ± 5.4 % (mean ± se).

Effect of muscimol on monosynaptic postsynaptic potentials (EPSP)

To evaluate whether the reduction in the ESPS amplitude and its halfwidth by muscimol was due to activation of postsynaptic GABA<sub>A</sub> receptors the EPSP time course, the membrane input resistance, and the time constant of the motoneurons were determined in Ringer solution added with strychnine (10 µM) and in the presence of muscimol (5 µM). Stimulation of the DLF (0.3 Hz, 1.1-1.6T), evoked monosynaptic excitatory EPSPs in
motoneurons (Fig. 2A) with a latency of $2.49 \pm 0.15$ ms ($n = 11$) measured from the stimulus artifact and an amplitude of $2.16 \pm 1.16$ mV ($n = 11$) (Yamashita 1986). Muscimol (5-10 µM) consistently depressed the EPSP amplitude by $41.4 \pm 13.4$ % ($n = 11$; Fig. 2A) measured after the muscimol action on membrane resistance was stable, and shortened the halfwidth by $59.43 \pm 8.4$ % ($n = 11$). The EPSP rise time was not affected but the time course of the falling phase was faster (14 out of 14 cells, Fig. 2A). This was more evident when the EPSPs were normalized (Fig. 2B, left). The time constant calculated from an EPSP semilogarithmic plot was consistently decreased by muscimol ($66.8 \pm 8.7$ %, $n = 11$). For the motoneuron shown in Fig. 2B (right), muscimol decreased the membrane time constant from $43.4$ ms in control medium to $15.25$ ms. After muscimol washout, the membrane time constant returned to $42.7$ ms. In cat spinal cord motoneurons most of the synaptic current is capacitive which makes the EPSP amplitude independent of the postsynaptic membrane resistance unless the latter is reduced in such a way that the membrane time constant is comparable to the EPSP time to peak (Finkel and Redman 1983). On average, the membrane time constant was reduced by muscimol from 7 times EPSP time to peak in control medium to 2.5 times. According to the theory (Finkel and Redman 1983) this strongly suggests that the decrement of the membrane resistance could account for the EPSP depression. In motoneurons where mono and polysynaptic EPSP were elicited by DLF stimulation, EPSP shortening could be due to a muscimol-induced block of polysynaptic EPSPs. However, in two neurons, polysynaptic EPSPs were blocked by stimulation of the DLF at a higher frequency (10 Hz) resulting in a reduction of the halfwidth by 12.5 % on average and the membrane time constant by 25%. These reductions were smaller than those produced by muscimol. In addition, baclofen clearly blocked
polysynaptic EPSPs in two neurons with a reduction of the halfwidth by about 20% but without any significant change in the membrane time constant. This means that blocking polysynaptic EPSPs could contribute to EPSP shortening but does not explain the large reduction in both the halfwidth and the membrane time constant in the presence of muscimol. The fact that the EPSP shortening and the reduction of the membrane time constant induced by muscimol were reverted by bicuculline (n = 3) and picrotoxin (n = 3), GABA<sub>A</sub> antagonists, suggests that receptors could mediate such depression (Fig 2C). In the neonatal spinal cord, strychnine applied at concentration higher than 1.5 µM partially blocked GABA<sub>A</sub> receptors on motoneurons (Jonas et al. 1998). To clarify this point EPSPs in Ringer solution and the presence of muscimol (5 µM) were recorded. When the muscimol action was stable strychnine (10 µM) added to the bath solution did not change the EPSPs (n = 3, data not showed), suggesting that the glycine receptor antagonist (10 µM) did not blocked GABA<sub>A</sub> receptors activated by muscimol. One possibility may be that GABA<sub>A</sub> receptors have higher affinity for strychnine on neonatal rat than on turtle motoneurons.

To assess if EPSP depression was also due to activation of muscimol-sensitive presynaptic receptors, paired pulse facilitation (PPF) protocol was applied. Facilitation of the second EPSP (A<sub>2</sub>) relative to the first EPSP (A<sub>1</sub>) is a consequence of a non-linear relationship between [Ca<sup>++</sup>]<sub>i</sub> and neurotransmitter release. It is believed that the probability of release after a presynaptic action potential is higher due to some residual calcium that remains at the sites of transmitter release (Kats and Miledi 1968, 1970; Zuker 1989). PPF could be induced either by reduction of [Ca<sup>++</sup>]<sub>o</sub> or by presynaptic inhibition affecting Ca<sup>++</sup> entry at the terminals (Katz and Miledi 1968, 1970; Chen and Regehr, 2003). In cat Ia-motoneuron synapses, presynaptic inhibition, mediated mainly by activation of GABA<sub>A</sub> receptors,
induced PPF (Stuart and Redman 1992) and depressed the EPSP by decreasing the number of quanta release without changing the quantal size (Clements et al. 1987). In Fig. 3A a pair of EPSPs elicited with a stimulus strength of 1.5T and a time interval of 100 ms in control medium (upper trace), in muscimol (middle trace), and after muscimol washout (bottom trace) are shown. To compare the time course and the relative amplitude between the first and the second EPSP, they were normalized by taking the amplitude of the first EPSP as 100% for each condition. As illustrated in Fig. 3B, the amplitude ratio in control medium was $A_2/A_1 = 1.08$. In the presence of muscimol, both EPSPs were depressed in approximately the same proportions with an amplitude ratio of 1.13. In this cell, muscimol reduced both the input resistance and the membrane time constant by 76 % and 72.5 %, respectively, which corroborates the strong action on motoneuron excitability exerted by activation of postsynaptic muscimol-sensitive receptors. The average amplitude ratio ($A_2/A_1$) in control condition was $1.13 \pm 0.05$ (n = 11) and with muscimol it was $1.13 \pm 0.04$ (n = 11). When a paired two population t-test was applied to both samples of data they were not significantly different with a P < 0.05, suggesting that muscimol did not activate muscimol-sensitive presynaptic receptors and supporting the possibility that postsynaptic muscimol-sensitive receptors could account for the EPSP depression.

**Tonic release of GABA**

To kwon, whether GABA$_A$ receptors were tonically activated, a GABA$_A$ receptor antagonist was added to the bath solution. Bicuculline (40 µM) increased the EPSPs amplitude by $22.3 \pm 2.3$ % (n = 4) and the membrane input resistance by $24.6 \pm 0.66$ % (n = 4, Fig 3C). If the DLF terminals were not regulated by presynaptic GABA$_A$ receptors then our result suggests that GABA$_A$ receptors on motoneurons may be tonically activated.
On rat spinal cord motoneurons, a subset of spontaneous miniature IPSPs has been reported to be mediated by activation of GABA\textsubscript{A} and glycine receptors (Jones et al. 1988). It remains to know whether unitary and spontaneous compound IPSCs are evoked on turtle motoneurons.

*Effect of baclofen on the DLF-motoneuron synapse*

The effect of baclofen (10 µM) on DLF terminal-motoneuron synapses was evaluated. The major action of baclofen was a systematic depression of the EPSPs in all cells (64.8 ± 5.5%; n = 7; Fig. 4A). In contrast, the halfwidth of the EPSP decreased by 0 to 27% (n = 6; Fig. 4B). In all cells baclofen produced a small reduction in the membrane resistance (7.7 ± 2 %; n = 6; Fig. 4C). The small decrement in the membrane resistance could account for a small decrement in the membrane time constant from 35.43 ± 4.54 ms (n = 6) in control medium to 30.80 ± 3.76 ms (n = 6) in the presence of baclofen. Therefore, according to Finkel and Redman (1983), the small decrement of the postsynaptic membrane resistance (7.7 ± 2 %) could not explain the large EPSP depression (64.8 ± 5.5%). One possibility is that baclofen affected the neurotransmitter release from the DLF terminals.

*Paired pulse protocol to evaluate baclofen action*

To evaluate whether baclofen inhibited neurotransmitter release from DLF terminals a paired pulse protocol was applied. Fig. 4D shows that baclofen increased the EPSP amplitude ratio (A2/A1) from 0.9 in control medium to 1.08. In all cells (n = 6), the change in amplitude ratio from 0.95 ± 0.17 in control medium to 1.075 ± 0.19 in baclofen was statistically different when a paired two population t-test (P<0.05) was applied. This result suggests that baclofen may inhibit neurotransmitter release from DLF terminals.
DISCUSSION

Presynaptic control of vestibulo-spinal, reticulo-spinal, and rubro spinal terminals synapsing on motoneurons is not mediated by activation of GABA_A receptors (Curtis et al. 1984a, b; Rudomin et al. 1991). It is thought that neurotransmitter release from vestibulo-spinal terminals could be inhibited by GABA_B receptors, as is the case for Ia afferents or interneuron terminals synapsing motoneurons (Edwards et al. 1989; Jiménez et al. 1991; Stuart and Redman 1992; Curtis and Lacey 1998; Peshori et al. 1998; Lev-Tov et al. 1998; Jonas et al. 1998). The present findings provide evidence that the strength of the DLF terminal-motoneuron synapse may be controlled by activation of presynaptic GABA_B receptors and postsynaptic GABA_A receptors.

GABA_A-induced postsynaptic inhibition

In all recorded motoneurons, muscimol produced a large depression in amplitude and a shortening of the EPSP falling phase, which were always accompanied by a decrement in the membrane resistance and a large reduction of the membrane time constant, making it comparable to the EPSP time to peak. Theoretically, this finding (Finkel and Redman, 1983) agrees with the hypothesis that muscimol depresses the EPSPs by a postsynaptic action. This conclusion is supported by the result that muscimol decreased the input membrane resistance, the amplitude and the halfwidth of the EPSPs at the same rate. Additionally, muscimol did not change the EPSP amplitude ratio obtained with the paired pulse protocol. This suggests that muscimol-sensitive receptors were not activated at the presynaptic level. Our results are consistent with two findings: a) vestibulo-spinal and reticulo-spinal terminals synapsing on motoneurons are not subjected to presynaptic inhibition of the type acting on Ia terminals (Rudomin et al. 1991), which is characterized by a terminal depolarization (PAD) and mainly mediated by activation of GABA_A receptors
(Rudomin and Schmidt 1999); b) rubro-spinal terminals do not have bicuculline-sensitive receptors as determined by measurement of their terminal threshold in the presence of GABA (Curtis and Malik 1984a). The fact that muscimol-induced shortening and depression of EPSPs, and the decrease in the membrane resistance were blocked by bicuculline or picrotoxin indicates the involvement of GABA\textsubscript{A} receptors. Therefore, although we cannot completely rule out a presynaptic effect of muscimol, it seems more likely that the synaptic efficacy of DLF terminals synapsing on motoneurons is controlled by activation of postsynaptic GABA\textsubscript{A} receptors.

**Presynaptic inhibition of neurotransmitter release by baclofen**

Our results show that baclofen - a GABA\textsubscript{B} receptor agonist - produced a large EPSP depression of approximately 68% in all motoneurons recorded without any significant postsynaptic change suggesting that such depression could be presynaptic in origin. This possibility is supported by the significant increment in EPSP amplitude ratio obtained with the paired pulse protocol. Similar result was obtained in Ia-motoneuron synapse (Stuart and Redman, 1991), and in the retinogeniculate synapse where activation of presynaptic GABA\textsubscript{B} receptors by baclofen depressed EPSC amplitude, turned paired pulse depression into paired pulse facilitation, and decreased calcium influx in the presynaptic terminals (Chen and Regehr, 2003). Presynaptic inhibition induced by baclofen has been predicted by PPF and has been confirmed by intracellular, whole cell, and double recordings (Shen and Johnson 1997; Mougnot et al. 1998; Takahashi et al. 1998). Therefore, it is reasonable to assume that GABA\textsubscript{B} receptors should have been activated on DLF terminals and thereby blocking neurotransmitter release. This interpretation is in line with other findings showing that baclofen presynaptically depresses synaptic transmission in the spinal cord (Lev-Tov 1988; Peng and Frank 1989a; Jimenéz et al. 1991; Rudomin et al. 1991; Stuart and Redman...
1992; Curtis and Lacey 1998; Russo et al. 1998; Jonas et al. 1998). As in descending fibers in the cat (Jiménez et al. 1991), baclofen never completely eliminated the postsynaptic response to low threshold stimulation of DLF terminals but it produced a strong depression and sometimes abolished the postsynaptic response to Ia afferent, dorsal root and spinal axon stimulation in cats (Jimenez et al. 1991), in turtles (Russo et al. 1998), and in rats (Jonas et al. 1998), respectively. The differential effect of baclofen could be due to a) not all DLF terminals having GABA<sub>B</sub> receptors, b) the density of GABA<sub>B</sub> receptors being less in DLF terminals than in intraspinal terminal afferents and c) the density of GABA<sub>B</sub> receptors is similar in both kind of fibers but with different affinity to baclofen (Jiménez et al. 1991). The presence of GABA<sub>B</sub> receptors in motoneurons, in dorsal horn neurons, and in primary afferents has been revealed by immunocytochemical studies (Towers et al. 2000; Yang K et al. 2001). The small baclofen-induced decrement in motoneuron membrane resistance may be explained by activation of a Ba<sup>2+</sup>-sensitive current. This current is activated by baclofen in motoneurons and interneurons in the spinal cord and leads to a decrease in membrane resistance and a down regulation of plateau potentials in both types of neurons (Svirskis and Hounsgaard 1998; Russo et al. 1998). A postsynaptic effect of baclofen could not be excluded. AMPA receptors, which mediate motoneuron EPSPs, need phosphorylation to be activated (Song and Huganir 2002). Baclofen acting through G protein-coupled pathways could affect phosphorylation of AMPA receptors and thereby reducing the EPSPs. In the calyx of Held giant synapse, that possibility was discarded because baclofen depressed the postsynaptic response only by reducing calcium influx at the presynaptic terminal (Takahashi et al 1998). In addition, AMPA receptors dephosphorylated during LTD did not change paired pulse facilitation (Lee et al. 1998) as occurs with DLF-motoneuron synapse in the presence of muscimol.
Functional implications

Presynaptic inhibition via axo-axonic GABAergic synapses controls neurotransmitter release from thick myelinated primary afferents by activation of GABA$_\mathrm{A}$ receptors (PAD; Rudomin and Schmidt, 1999). The prominent postsynaptic effect that activation of GABA$_\mathrm{A}$ receptors by muscimol or synaptic inputs showed, strongly suggests that GABA$_\mathrm{A}$ receptors also may play an important role in controlling Ia-motoneuron synaptic efficacy by shunting the postsynaptic element. Physiological activation of motoneuron GABA$_\mathrm{A}$ receptors has been shown. In rat spinal cord motoneurons unitary and miniature spontaneous IPSCs mediated by GABA$_\mathrm{A}$ and glycine receptors were evoked (Jonas et al. 1998). Activation of cat interneurons by Group I afferents produced a slow strychnine-resistant inhibitory potential on motoneurons (Rudomin et al. 1990), which was blocked by picrotoxin. Likewise, Kellerth and Szumski (1966) found strychnine-resistant and picrotoxin-sensitive postsynaptic inhibition elicited on motoneurons. Therefore, it could be speculated that motoneuron GABA$_\mathrm{A}$ receptors, like GABA$_\mathrm{B}$ receptor, may down regulate or prevent activation of plateau potentials when they are tonically activated (Svirskis and Hounsgaard 1998). In conclusion, according to our results GABA may regulate the DLF terminals-motoneuron synaptic strength by activation of presynaptic GABA$_\mathrm{B}$ and postsynaptic GABA$_\mathrm{A}$ receptors. The presynaptic GABA$_\mathrm{B}$ receptors inhibit neurotransmitter release and the postsynaptic GABA$_\mathrm{A}$ receptors decrease motoneuron excitability.
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FIGURE LEGENDS

Figure 1. Histology of a motoneuron and time course of muscimol effect.

A, Photomicrograph of the transverse section of the spinal cord showing a motoneuron stained with biocytyn in the ventral horn, which is delineated with a dashed line. The elliptic area indicates the area electrically stimulated (DLF). To know the time course of the muscimol effect on the membrane resistance, the amplitude and the halfwidth of the EPSP voltage response to an intracellular hyperpolarizing current (-0.3 nA) and the EPSPs elicited by the DLF stimulation, every 3 s were recorded. In B, from the top to the bottom the averages of five consecutive sweeps are shown. The column number represent the time elapsed after 5 consecutive sweeps. The fist trace is the control response (0 s). The next traces were the averages in the presence of muscimol (15 to 135 s; 5 µM). The input resistance calculated from the average voltage response traces, and values of the amplitude and the halfwidth of the EPSPs obtained from B were normalized and plotted against the time (C). Observe that the normalized quantities decreased at the same rate. When the input resistance reached the stable value both the amplitude and the halfwidth were also stable.

D, the membrane resistance calculated as the slope of the best linear fit to the IV plot was 14.7 MΩ in control medium (fill circles) and 4.4 MΩ (empty circles) in muscimol. Voltage responses plotted were lesser than 10 mV in amplitude. E, the membrane potential was continuously recorded and the input resistance was monitored by means of the voltage response (bottom sweep) to an intracellular current pulse of -0.3 nA (upper trace). Vertical arrows indicate times at which muscimol was added to the bath solution (left arrow) and at which input resistance started to decrease (right arrow). Insets show two amplified voltage
responses in control medium (left) and when muscimol effect was stable (right). B-C and D-E were from different neurons.

Figure 2. Muscimol depressed EPSP.

A, the motoneuron EPSP elicited by DLF stimulation at 1.5T: at the top, in control medium (strychnine 10 µM); at the middle, in the presence of muscimol (5 µM) recorded after muscimol action on input resistant was stationary; at the bottom, after muscimol washout. To show muscimol effect on time course EPSPs from A were normalized (B, left). The effect of muscimol on membrane time constant is evaluated by plotting the natural logarithm of the EPSP amplitude (B, right). Washout of muscimol recovers the EPSP time course (B, left) and the membrane time constant (B, right). Picrotoxin blocked muscimol effect, time course (C, left) and the membrane time constant (C, right) were recovered. All traces in this and in next figures were averages of 50 responses elicited at 0.3 Hz. A-B and C are from different neurons.

Figure 3. Evaluation of muscimol effect by the paired pulse protocol and bicuculline effect on EPSPs. To assess if EPSP depression by muscimol was due to presynaptic inhibition of neurotransmitter release a paired pulse protocol was applied. A, Upper trace, pair of control EPSPs elicited by DLF stimulation at 1.5T and an interstimulus interval of 100 ms, the amplitude ratio \( A_2/A_1 \) was 1.08. Muscimol depressed both EPSPs in approximately the same proportion with no significant change in amplitude ratio \( A_2/A_1 = 1.13 \); middle trace), which is better appreciated when EPSPs were normalized taking the maximal amplitude of the first EPSP as 100 % for each condition (B). After washout of muscimol the time course
of both EPSPs was almost recover (A bottom trace, B). C, bicuculline added to the Ringer solution increased the EPSPs recorded in Ringer solution without strychnine. A-B and C were from different neurons.

Figure 4. Baclofen effect on the DLF-motoneuron synaptic efficacy.

EPSP elicited by DLF stimulation at 1.5T (A, upper trace) was depressed by baclofen (10 μM) added to the bath solution (A, bottom trace). Normalized EPSPs in control medium and in baclofen had the same time course (B). Input resistance was reduced from Ri = 56 MΩ (C, black circle) in control medium to Ri = 54.4 MΩ (C, empty circle) in baclofen. To assess whether EPSP depression by baclofen was due to inhibition of neurotransmitter release from DLF terminals a paired pulse protocol was applied. The amplitude ratio A2/A1 changed from 0.9 in control medium to 1.08 in baclofen, which is better appreciated when EPSPs were normalized (D) taking the maximal amplitude of the first EPSP as 100 % for each condition. A-D were from the same neuron.
Figure 1
Figure 3
Figure 4