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$GABA_A$ RECEPTORS MEDIATE MOTONEURON TONIC INHIBITION IN THE TURTLE SPINAL CORD

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Abstract—$GABA_A$ receptors mediating tonic inhibitory currents are present in neurons from hippocampus, cerebellum, sensory cortex and thalamus. These receptors located at peri- and extra-synaptic sites are constituted mainly by $\alpha_{4/6}$ and α_5 subunits which confer them high affinity for GABA and low desensitization. Immunohistochemical and *in vitro* hybridization studies have shown the expression of these subunits, while functional studies have reported the presence of GABAergic tonic currents in spinal dorsal horn neurons. However, the presence of this inhibitory current has not been documented in motoneurons. In addition, we previously reported that the monosynaptic reflex is facilitated by furosemide, an antagonist of the $\alpha_{4/6}$ $GABA_A$ receptors, without affecting the dorsal root potential, which suggests the presence of a GABAergic tonic inhibitory current in motoneurons. The aim of this work was to investigate the presence of high affinity $GABA_A$ receptors in motoneurons. By intracellular recordings made with sharp electrodes and the whole-cell patch clamp recording technique we show here that the membrane input resistance and the monosynaptic excitatory postsynaptic potential (EPSPs) are significantly increased by bicuculline. Likewise, the depression of the EPSPs and the input membrane resistance normally induced by muscimol was partially reverted by 20 μM bicuculline and abolished when the concentration of the antagonist was raised to 100 μM. Last, bicuculline at low concentration did not affect the holding current as occur with the high concentration that block the tonic inhibitory GABAergic current. Together these results suggest that the excitability in motoneurons may be tonically inhibited by high affinity $GABA_A$ receptors.

Key words: GABA, excitability, extrasynaptic GABA receptors, motor control, channels.

$GABA_A$ receptors mediate phasic and tonic inhibition in neurons from hippocampus, cerebellum, thalamus and sensitive cortex (Farrant and Nusser, 2005; Walker and Semyanov, 2008). The phasic inhibition is produced by synaptic $GABA_A$ receptors and tonic inhibition by $GABA_A$ receptors located at peri- and extra-synaptic regions showing high affinity to GABA. Subunit composition contributes to determine the biophysical and pharmacological properties of $GABA_A$ receptors, as well as their cellular distribution (Farrant and Nusser, 2005; Walker and Semyanov, 2008). Activation of high affinity $GABA_A$ extra-synaptic receptors produces a tonic current that results in higher charge transfer than that flowing through the phasic synaptic receptors, conferring them an important role in controlling neuronal excitability (Chadderton et al., 2004; Farrant and Nusser, 2005; Walker and Semyanov, 2008). Extra-synaptic receptors are composed mainly of $\alpha_{4/6}$ and α_5 subunits in combination with γ_2, δ or ε subunits (Semyanov et al., 2003; Farrant and Nusser, 2005; Walker and Semyanov, 2008; Glykys et al., 2008). In the spinal cord, immunohistochemical and *in situ* hybridization evidence support the presence of distinct $GABA_A$ receptors comprising α_2, α_3, α_5, and γ_2 subunits through all laminas (Persohn et al., 1991; Wisden et al., 1991; Ma et al., 1993; Alvarez et al., 1996; Bohlhalter et al., 1994, 1996). Particularly, α_2, α_5, β_3 and γ_2 subunits have been detected in motoneurons (Alvarez et al., 1996; Persohn et al., 1991; Bohlhalter et al., 1994, 1996). Among these subunits, it has been documented that α_5 conform the extra-synaptic $GABA_A$ receptors in hippocampal pyramidal neurons (Glykys et al., 2008). Likewise, a GABAergic tonic current in neurons from laminas I–III and in some interneurons from the ventral horn has been recently reported (Ataka and Gu, 2006; Takahashi et al., 2006; Grasshoff et al., 2008; Takazawa and MacDermott, 2010). In ventral horn interneurons it has been suggested that this tonic current may play a role in anesthetic action given that the current was evoked by application of thiopental.

Interestingly, immunohistochemical studies also have demonstrated the presence of $GABA_A$ receptors located on the soma and dendrites of motoneurons, with some not matching pre-synaptic terminals (Todd et al., 1996; Persohn et al., 1991; Wisden et al., 1991; Alvarez et al., 1996; Bohlhalter et al., 1994, 1996). However, it is not known what role these $GABA_A$ receptors may be playing in motoneuron activity (Rekling et al., 2000). Electrophysiological evidence has shown that muscimol activation of $GABA_A$ receptors depresses the monosynaptic reflex and the monosynaptic motoneuron's evoked spinal potentials (ESPs), decreasing in parallel the membrane resistance and time constant of the motoneurons suggesting a postsynaptic action of the drug (Peng and Frank, 1989; Delgado-Lezama et al., 2004). Likewise, muscimol application also suppresses motoneuron plateau potentials (Alaburda et al., 2005). On the contrary, bicuculline facilitates the monosynaptic excitatory postsynaptic potential (EPSP)

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Abbreviations: DLF, dorsolateral funiculus; DRP, dorsal root potential; EPSP, excitatory postsynaptic potential; IPSP, inhibitory postsynaptic potential.

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evoked in motoneurons by a stimulus in the dorsolateral funiculus. The terminals of these fibers do not express $GABA_A$ receptors (Delgado-Lezama et al., 2004).

Recently, we showed that high affinity $GABA_A$ receptors may control motoneuron excitability because in the presence of an antagonist of the $\alpha_{4/6}$ subunit (furosemide) at a concentration that does not affect the NaCl transporter, the monosynaptic reflex evoked by dorsal root stimulation was facilitated without affecting the simultaneous induced dorsal root potential (DRP) (Bautista et al., 2010). Therefore, in this work we decided to investigate whether the high affinity $GABA_A$ receptors regulate excitability and mediate a tonic inhibitory current in motoneurons. To assess these issues, motoneurons were recorded intracellularly with sharp electrodes and whole-cell blind patch clamp in a slice preparation of the adult turtle lumbar spinal cord.

EXPERIMENTAL PROCEDURES

Preparation

Forty adult turtles (*Trachemys scripta spp.*, 15–20 cm carapace length) were anaesthetized with pentobarbitone (100 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO, USA). The plastron was opened and the blood removed by intraventricular perfusion with Ringer solution (~10 °C) of the following composition (in mM): 120 NaCl, 5 KCl, 15 $NaHCO_3$, 3 $CaCl_2$, 2 $MgCl_2$ and 20 glucose saturated with 2% CO_2 and 98% O_2 to attain pH 7.6. The lumbar spinal enlargement was isolated by a laminectomy and cut transversally to get slices of 2–3 mm thick. For the experiments the slices were placed in a recording chamber and superperfused with Ringer solution (20–22 °C). At the end of the dissection the animals were killed by decapitation.

All experimental procedures were carried out with the approval of the Cinvestav-IPN Experimental Ethics Committee and in accordance with the current Mexican Norm for Care and Use of Animals for Scientific Purposes. The animals were provided by the National Mexican Turtle Centre located in Mazunte, Oaxaca (Mexico) with the authorization (DGVS-03821/0907) by the Federal Mexican Government Ministry of Environment and Natural Resources (Secretaría de Medio Ambiente y Recursos Naturales, Semarnat).

Electrophysiology

Motoneurons were recorded intracellularly with a sharp electrode (40–50 ΩM) filled with potassium acetate (0.8 M) and KCl (0.2 M). Cells were classified as motoneurons if the input resistance was lower than 80 MΩ, the action potential presented a fast and slow posthyperpolarization and the firing pattern had adaptation (Hounsgaard et al., 1988; Delgado-Lezama et al., 2004). Monosynaptic motoneuron's EPSP were evoked by stimulating the dorsolateral funiculus (DLF; 3×T) with a bipolar electrode. The DLF terminals were selected because they do not have $GABA_A$ receptors (Delgado-Lezama et al., 2004). In some motoneurons a mix of excitatory (EPSP) and inhibitory (IPSP) postsynaptic potentials were elicited by DLF stimulation. Therefore, in all the experiments IPSPs were completely blocked by adding strychnine (2 μM) to the bath solution. The EPSP were considered monosynaptic if their latency measured from the beginning of the stimulus artifact was less than 3 ms (Yamashita, 1986) and followed high frequency stimulation.

In order to determine with accuracy the time at which the drug reached its maximal effect, an on-line analysis of the EPSP amplitude and membrane resistance was carried out as follows. The voltage responses to an intracellular current pulse (0.1–0.3 nA; 300 ms) and the EPSPs were recorded every 3 min. Using the Labview software (National Instruments) the average EPSPs amplitude and the voltage response of seven to nine sweeps measured on-line were plotted vs. time. Recordings were acquired at 5 kHz with a low-noise digitizer (National Instruments) and monitored in a computer using the Labview software. Recordings were stored in the hard disk of a personal computer for off-line analysis.

In other series of experiments, the presence of the GABAergic tonic current was determined in motoneurons using the blind patch technique in the whole-cell configuration. The electrodes were made from thick-walled borosilicate glass capillaries using a Sutter programmable horizontal micropipette puller. The patch pipettes with resistance of 5–10 MΩ were filled with the following solution (in mM): 122 K-gluconate; 5 Na_2-ATP; 2.5 $MgCl_2$; 0.0003 $CaCl_2$; 5.6 Mg-gluconate; 5 K-Hepes; 5 Hepes. Motoneurons were clamped at 0 mV using the MultiClamp-700B amplifier (Molecular Devices) and the maximal acceptable series resistance compensation was 15%. The membrane potential wad held at 0 mV to increase the driving force of the chloride current. Recorded signals were filtered (DC-2KHz) using the 4-pole bessel and butterworth low-pass filters of the amplifier, digitized at 20 kHz and stored in a hard disk for off-line analysis.

Drugs

$GABA_A$ receptors were activated with muscimol (5 μM) or GABA (1 mM) and blocked with bicuculline (20–100 μM) applied to the bath solution. Since, inhibitory propiospinal interneurons synapsing motoneurons send their axons through the DLF, strychnine (2 μM) was always added to the bath solution to evoke only EPSPs in the recorded motoneurons. All of the drugs used in this study were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Analysis

The activation and blockade of $GABA_A$ receptors in motoneurons recorded intracellularly were quantified by measuring the changes in input resistance and EPSP amplitude. The mean current recorded in voltage clamp experiments was calculated by generating all-point histograms of the current values recorded in control Ringer and in presence of the $GABA_A$ receptor blocker. A Gaussian distribution was fitted to the histograms. The change in the holding current was determined as the difference between the means of the Gaussians fitted to the histograms. The statistical differences between means were determined by Student t-, Mann–Whitney and Kolmogorov–Smirnov tests. Means were considered statistically different when $P<0.05$. Values are presented as the mean±SEM.

RESULTS

Motoneuron monosynaptic EPSPs evoked by DLF stimulation

Cells recorded with sharp electrodes from the spinal ventral horn were classified as motoneurons if they presented adaptation of the action potential firing elicited with a depolarizing current pulse ($n=13$). In some neurons ($n=5$) it was possible to evoke antidromic action potentials by ventral root stimulation. The average input resistance in these cells was 21±8 MΩ ($n=13$) and the membrane time constant was 31±4 ms, which is in agreement with the values reported previously for motoneurons (Hounsgaard et al., 1988; Delgado-Lezama et al., 2004). The EPSPs evoked by DLF stimulation at 3×T had the following basic proper-