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Tonic inhibition in spinal ventral horn interneurons mediated by α_5 subunit-containing $GABA_A$ receptors

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ABSTRACT

$GABA_A$ receptors mediate synaptic and tonic inhibition in many neurons of the central nervous system. These receptors can be constructed from a range of different subunits deriving from seven identified families. Among these subunits, α_5 has been shown to mediate GABAergic tonic inhibitory currents in neurons from supraspinal nuclei. Likewise, immunohistochemical and *in situ* hybridization studies have shown the presence of the α_5 subunit in spinal cord neurons, though almost nothing is known about its function. In the present report, using slices of the adult turtle spinal cord as a model system we have recorded a tonic inhibitory current in ventral horn interneurons (VHIs) and determined the functional contribution of the α_5 subunit-containing $GABA_A$ receptors to this current. Patch clamp studies show that the GABAergic tonic inhibitory current in VHIs is not affected by the application of antagonists of the $\alpha_{4/6}$ subunit-containing $GABA_A$ receptors, but is sensitive to L-655708, an antagonist of the $GABA_A$ receptors containing α_5 subunits. Last, by using RT-PCR and immunohistochemistry we confirmed the expression of the α_5 subunit in the turtle spinal cord. Together, these results suggest that $GABA_A$ receptors containing the α_5 subunit mediate the tonic inhibitory currents observed in VHIs.

1. Introduction

Except for the monosynaptic reflex, all spinal reflexes involve the activation of interneurons between the afferent fibers and the motoneurons. Therefore, understanding the functional organization and the basic properties of spinal interneurons is crucial to understand how motor control is integrated and regulated in the spinal cord [1]. Nevertheless, only a few studies documenting the intrinsic properties of ventral horn interneurons (VHIs) have been performed in the adult spinal cord [2]. In particular, the function of $GABA_A$ receptors in VHIs has received scarce attention [2–4].

Although tonic inhibition has not been clearly documented in VHIs, seminal studies by Grasshoff and co-workers suggested that these cells may play a role in the anesthetic-induced immobility. By applying the barbiturate thiopental, these authors unmasked a tonic inhibitory current mediated by the activation of $GABA_A$ receptors in the mouse spinal cord [5]. Likewise, immunohistochemical and *in situ* hybridization studies have shown the expression of α_5 subunit-containing $GABA_A$ receptors in VHIs. Interestingly, these receptors are located at extrasynaptic regions [3] and are thought to be tonically activated by ambient GABA in CA_1/CA_3 pyramidal and dentate gyrus granule cells of the hippocampus [6].

Likewise, previous studies have shown the presence of tonic inhibition mediated by high affinity $GABA_A$ receptors activated by ambient GABA in the dorsal horn interneurons of the mouse spinal cord [7,8]. However, the molecular nature of the receptors involved is presently unknown. In this paper, with the aid of the blind patch technique we recorded a GABAergic tonic inhibitory current from VHIs using a slice preparation of the adult turtle spinal cord as a model. Our results show that the holding current (I_h) required to keep the cell at a fixed potential was not affected by application of antagonists of the $\alpha_{4/6}$ subunit-containing $GABA_A$ receptors. In sharp contrast, the use of bicuculline (100 μM) and L-655708, an antagonist of the α_5 subunit-containing $GABA_A$ receptors [6], effectively blocked the tonic inhibitory current recorded. Last, gene and protein expression analysis of α_5 subunit by RT-PCR and immunohistochemistry corroborated the results of the functional assay. Therefore, our results show that VHIs in the adult spinal cord express a tonic inhibitory current mediated by $GABA_A$ receptors containing the α_5 subunit.

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2. Materials and methods

2.1. Preparation

Adult turtles (*Trachemys scripta*) were anaesthetized with pentobarbitone (100 mg/kg, I.P.). 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 (pH 7.6). The lumbar spinal enlargement was isolated by a laminectomy and cut transversally to get 2–3 mm thick slices. For electrophysiology the slices were placed in a recording chamber and superfused with Ringer solution (20–22 °C). 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 Center (Mazunte, Mexico) with the authorization (DGVS-03821/0907) of the Ministry of Environment and Natural Resources (Semarnat).

2.2. Electrophysiology

The blind patch technique in the whole-cell configuration was used to determine the presence of the GABAergic tonic current in VHIs. Patch pipettes with a resistance of 8–10 MΩ were filled with a solution of the following composition (in mM): 122 K-gluconate; 5 Na_2-ATP; 2.5 $MgCl_2$; 0.003 $CaCl_2$; 5.6 Mg-gluconate; 5 K-Hepes; 5 Hepes. VHIs were clamped at 0 mV using the MultiClamp-700B amplifier (Molecular Devices) and the series resistance was compensated (<15%). Recorded signals were filtered at 2 kHz, digitized at 20 kHz and stored in the hard disk of a personal computer for off-line analysis.

2.3. Drugs

$GABA_A$ receptors were blocked with bicuculline (20–100 μM), gabazine (20–100 μM), furosemide (100–200 μM) or L-655708 (5 μM) applied to the bath solution. IPSCs were blocked with strychnine (2 μM). $GABA_A$ receptor antagonists were purchased from Sigma–Aldrich. All other chemicals were of reagent grade.

2.4. Data analysis

A 1 pA bin histograms of 1 min current recordings for each condition (1.2×10^6 points) were generated. In order to compare the current amplitude in each condition, a Gaussian distribution was fitted to the histograms. The change in I_h was calculated as the difference between the means of the fitted Gaussian distributions. Statistical differences between means were determined by *t*-Student, Mann–Whitney and Kolmogorov–Smirnov tests. Means were considered different when $p < 0.05$. Values are presented as mean ± SE.

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from the lumbar segment of the turtle spinal cord with Trizol reagent (Invitrogen) and dissolved in DEPC-treated water. The cDNA was synthesized from 5 μg total RNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. PCR primers were designed to amplify a conserved α_5 subunit mRNA region of several species. The forward primer sequence was 5′-TTTTTCCGACAAAGCTGGAA-3′ and the reverse primer sequence was 5′-CCCATCAGGTGGTACTGGTT-3′. PCR reaction was carried out in a total volume of 50 μl containing 5 μl of cDNA, 1× PCR buffer (20 mM Tris–HCl, 50 mM KCl, pH 8.4), 0.2 mM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 0.5 μM of each primer, and 2.5 U of Taq DNA polymerase (Invitrogen) on a PCR thermal cycler (Thermo Fisher Scientific). The PCR was performed as follows: an initial step at 94 °C for 5 min was followed by 30 cycles (45 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C), and finally by a 10 min extension step at 72 °C. After amplification, PCR products were analyzed on a 1.5% agarose gel. The specific product for the α_5 subunit of the turtle spinal cord $GABA_A$ receptor was purified by QIAquick PCR Purification Kit (Qiagen) and the purified DNA fragment was sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

2.6. Immunohistochemistry

Spinal cord segments (2–3 mm) were fixed in 4% paraformaldehyde in PBS for 24 h. After fixation, samples were cryoprotected by suspending them overnight in PBS containing 30% sucrose at 4 °C and then sliced using a cryotome (30 μm). Free-floating tissue sections were made permeable (0.3% Triton X-100 in PBS) for 10 min and blocked in PBS blocking solution 1 (1% gelatin and 10% FBS) for 30 min at room temperature. Overnight incubation with the primary antibody (1:50; Sigma–Aldrich) was performed in PBS blocking solution 2 (0.1% Triton X-100, 2% BSA and 5% FBS) at 4 °C, and subsequently incubated with the secondary antibody (1:100; Dylight 549-conjugated anti-rabbit IgG; Jackson ImmunoResearch Laboratories) in PBS blocking solution 2 for 60 min at room temperature. Sections were then placed on slides and coverslipped with Vectashield. Images were acquired with a Leica TCS SP2 confocal microscope (Leica). Images were obtained using the necessary filter set for Dylight 549 using the 20× and the 40× oilimmersion plan apochromat objectives (NA 0.5 and 0.8, respectively) with the pinhole set to one Airey unit.

3. Results

3.1. Spontaneous IPSCs in VHIs are sensitive to strychnine

Initial electrophysiological analysis indicated that all VHIs recorded presented spontaneous inhibitory postsynaptic currents (IPSCs). We therefore used antagonists of glycine and $GABA_A$ receptors (strychnine and bicuculline) to investigate what kind of receptor could be mediating this inhibitory synaptic activity. Fig. 1A illustrates representative traces recorded from one interneuron in control Ringer showing that the application of strychnine (2 μM) abolishes IPSCs without affecting I_h. Similar results we observed in all VHIs recorded ($n = 16$) suggesting that spontaneous synaptic inhibitory activity targeting interneurons is most likely mediated by activation of glycine synaptic receptors. This observation suggests also that synaptic activation of glycine receptors did not produce tonic inhibition in VHIs, as has been reported for supraspinal neurons [9]. It is worth mentioning, however, that in some dorsal horn interneurons from lamina I to III of adult mice, strychnine (1 μM) has been shown to block a tonic inhibitory current [10].

Considering that glycine and GABA are co-released in neonatal rat VHIs [11], we next decided to assess whether synaptic $GABA_A$ receptors were active in the VHIs of the turtle spinal cord. It is known that bicuculline (20 μM) blocks synaptic $GABA_A$ receptors in supraspinal nuclei neurons [3,4], and prevents presynaptic inhibition of primary afferents [12] which is mediated by synaptic activation of $GABA_A$ receptors. Fig. 1B shows representative IPSCs recorded in one interneuron in control Ringer and in presence of 20 μM bicuculline. As can be seen, the IPSCs

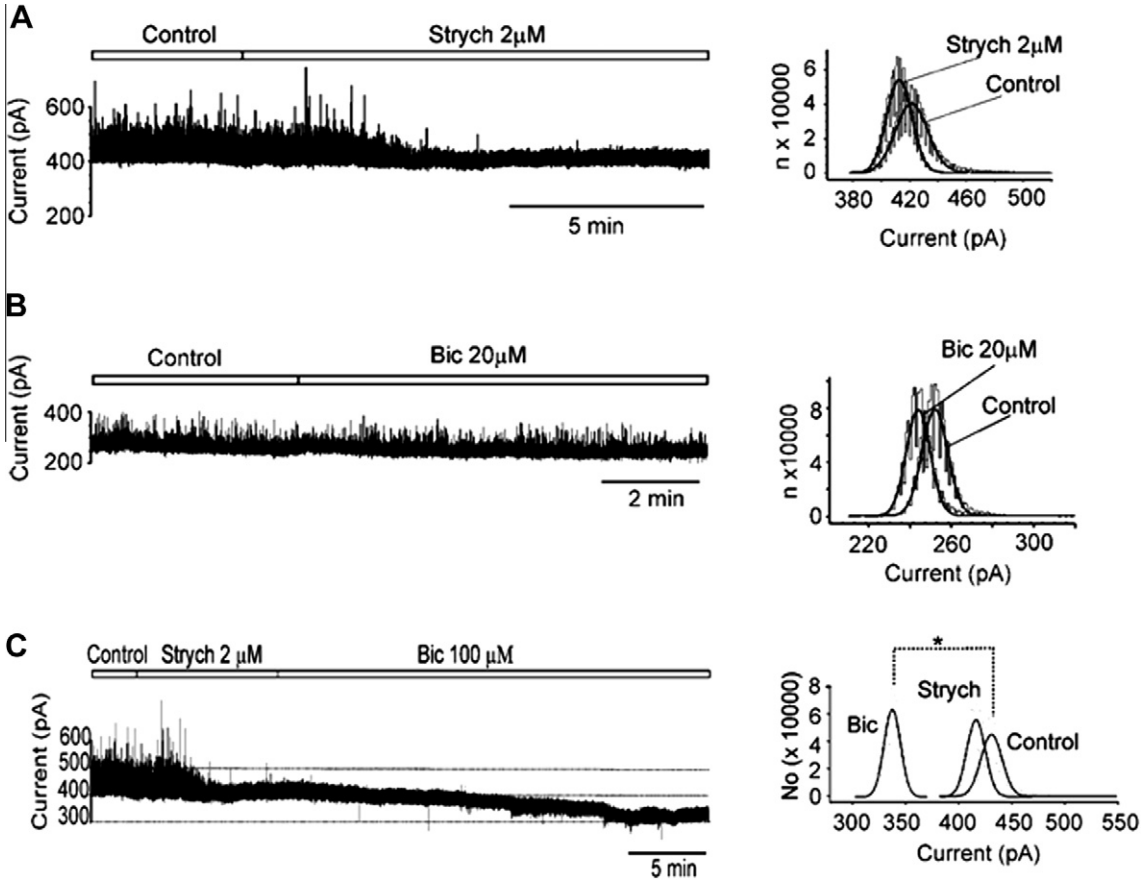

Fig. 1. Spontaneous synaptic activity and tonic inhibition in VHIs. Holding current (I_h) recorded in interneurons voltage-clamped at a holding potential (V_h) of 0 mV. (A) Example of spontaneous activity recorded in VHIs with an input resistance (R_{in}) of 340 MΩ (trace) in absence (control) and presence of 2 μM strychnine. (B) Example of spontaneous GABAergic IPSCs recorded in VHIs with a R_{in} of 300 MΩ (trace) in absence (control) an in presence of 20 μM bicuculline. Right panels show the I_h values in the control condition and after the addition of the drugs. (C) Example of spontaneous GABAergic IPSCs recorded in VHIs (V_h = 0 mV) in absence (control) and in presence of 2 μM strychnine followed by the addition of 100 μM bicuculline (trace). The right panel shows the comparison of I_h in the control condition and after the addition of the drugs. The asterisk indicates statistical significance ($p < 0.05$; Kolmogorov–Smirnov test).

and the I_h were not affected by the $GABA_A$ receptor antagonist suggesting the presence of a tonic inhibitory current insensitive to this concentration of the drug. In addition, these results suggest that the inhibitory interneurons synapsing VHIs may not co-release glycine and GABA, and that the fast inhibitory glycinergic synaptic activity may not be mediating tonic inhibition in this cells.

3.2. Inhibitory tonic currents in VHIs

It has been reported that bicuculline at a concentration of 100 μM effectively blocks the GABAergic tonic current recorded in mice VHIs in the presence of thiopental [5], as well as in supraspinal neurons [3,4]. Therefore, we next evaluated the actions of 100 μM bicuculline in our preparation to investigate whether an inhibitory current could be active tonically in VHIs. Fig. 1C summarizes the results of this analysis. As in Fig. 1A, the IPSCs were abolished by strychnine without affecting I_h. Interestingly, bicuculline (100 μM) produced a change in I_h of 72 ± 24 pA (n = 4). This change in I_h is similar to that recorded previously in neurons from supraspinal nuclei and considered to be mediated by high sensitivity $GABA_A$ receptors tonically active located at peri- and extra-synaptic regions [3,4]. Therefore, our finding suggests that VHIs express a tonic inhibitory current mediated by high sensitive $GABA_A$ receptors.

3.3. The tonic inhibitory current in VHIs is not sensitive to gabazine or furosemide

In a previous paper we showed that gabazine (SR-95531), a high affinity antagonist of $GABA_A$ receptors, at a concentration of 1 μM suppressed presynaptic inhibition mediated by synaptic $GABA_A$ receptors in the adult turtle spinal cord [12]. Though this concentration of gabazine also blocks IPSCs in hippocampal interneurons and CA_1 pyramidal cells without affecting I_h [3,9,13], gabazine (10 μM) has also been shown to effectively block the tonic GABAergic inhibitory current in cerebellar granule cells [14]. Therefore, we decide to test whether the tonic current recorded in VHIs was sensitive to this GABA receptor antagonist. Fig. 2A shows that gabazine at 20 and 100 μM did not affect the tonic GABAergic inhibitory current recorded in VHIs in the presence of strychnine. Likewise, it has been reported that furosemide, an antagonist of the $GABA_A$ receptor comprising the $\alpha_{4/6}$ subunits, blocks the tonic GABAergic inhibitory current in cerebellar granulle cells [15]. In addition, we have found that this antagonist (at 100 and 200 μM) facilitates the monosynptic reflex without affecting the dorsal root potential in the turtle spinal cord, suggesting that $\alpha_{4/6}$ subunit-containing $GABA_A$ receptors are present in motoneurons but not in primary afferents [12]. Therefore, we next decided to investigate whether $GABA_A$ receptors containing these subunits were mediating the tonic current observed in VHIs.

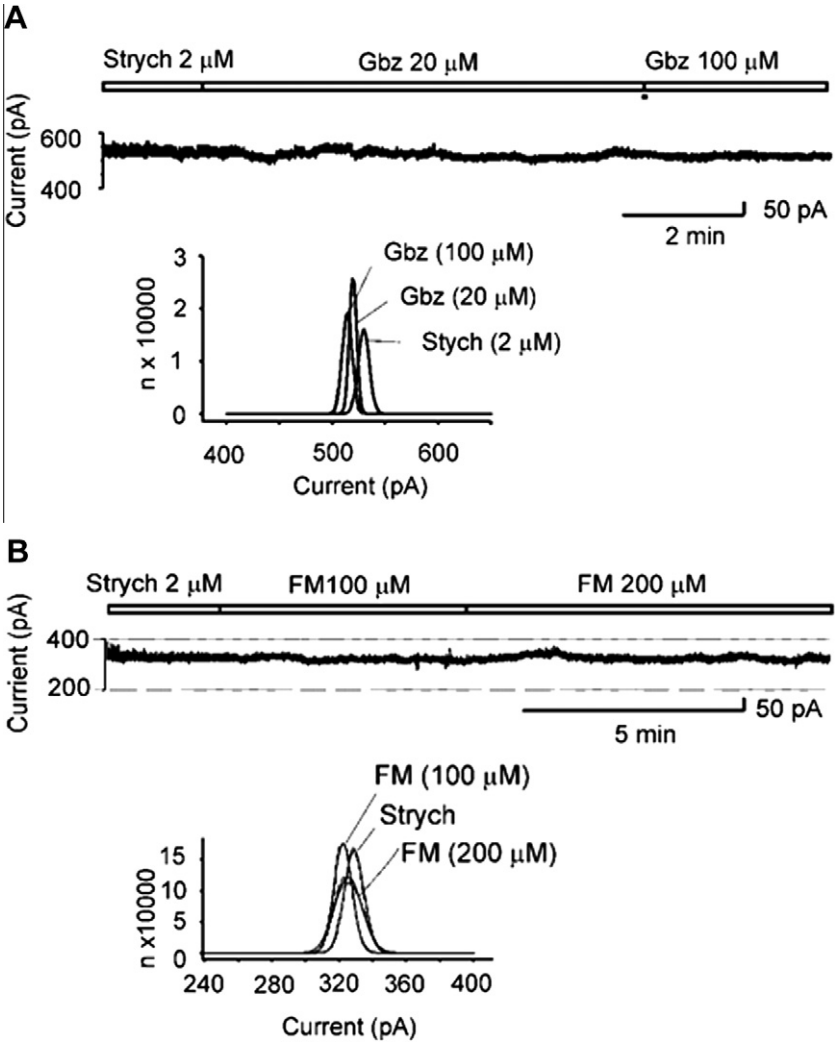

Fig. 2. High sensitivity $GABA_A$ receptors are not blocked by gabazine and furosemide. (A) Example of I_h recorded in VHIs (V_h = 0 mV) in presence of strychnine (2 μM) followed by increasing concentrations of gabazine (20 and 100 μM) (top trace). (B) Example of I_h recorded in VHIs (V_h = 0 mV) in presence of 2 μM strychnine and furosemide (100 and 200 μM). Histograms below traces show the I_h values in the control condition and after the addition of the drugs.

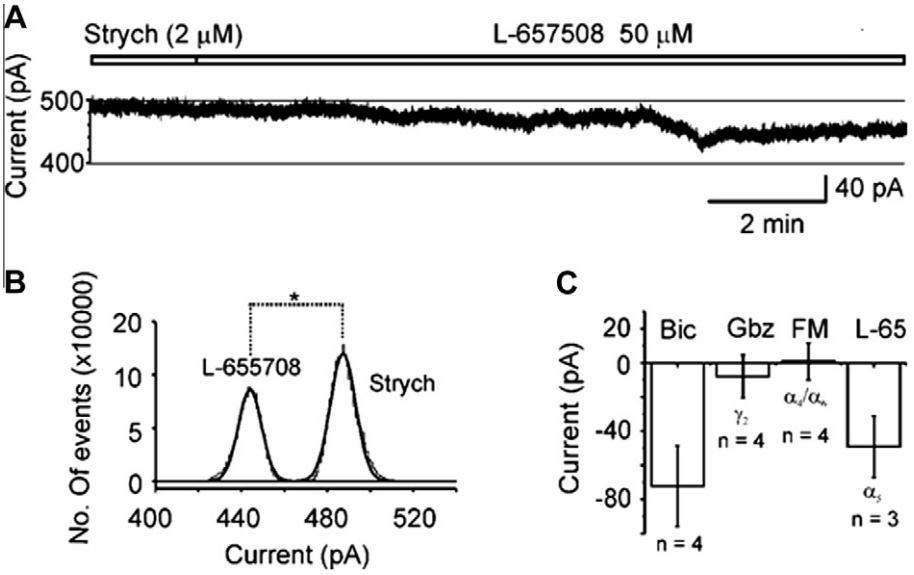

Fig. 3. $GABA_A$ receptors containing the α_5 subunit mediate tonic inhibition in VHIs. (A) Holding current (I_h) recorded from VHIs (V_h = 0 mV) in presence of strychnine plus L-657508. (B) Current distribution for each condition. The asterisk indicates statistical difference with $p < 0.05$; Kolmogorov–Smirnov test. (C) Bar plot summarizing the action of different $GABA_A$ receptor antagonists on I_h recorded in VHIs.

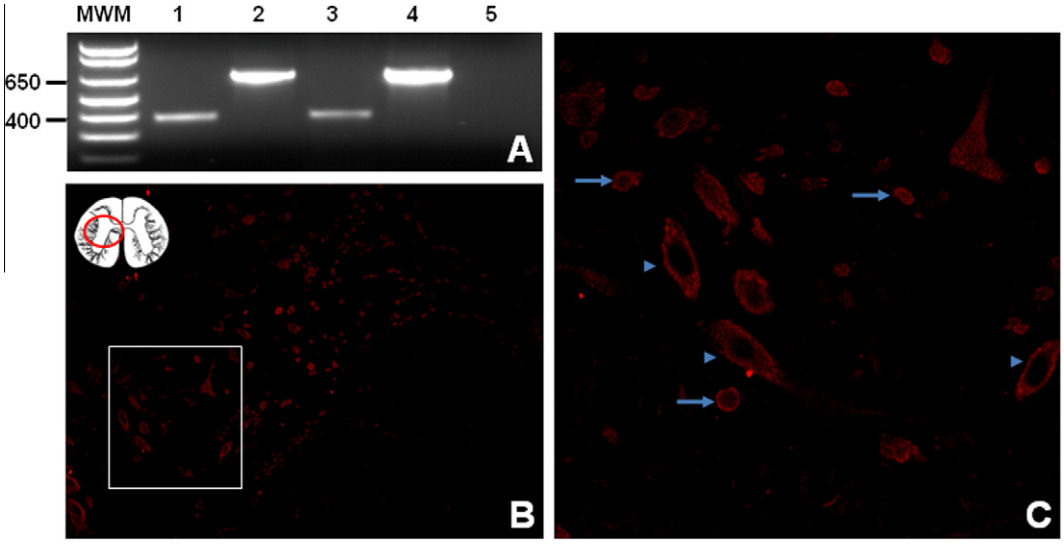

Fig. 4. Expression of the $GABA_A$ receptor α_5 subunit in the adult turtle spinal cord. (A) Detection of α_5 mRNA by RT-PCR. MWM, molecular weight markers; lane 1, turtle spinal cord α_5 subunit; lane 2, turtle β-actin; lane 3, mouse α_5 subunit; lane 4, mouse β-actin; lane 5 negative PCR control. (B) Immunohistochemical staining of the turtle spinal cord. An image of a transversal section of the lumbar region is shown. Immunoreactivity for $GABA_A$ receptor α_5 subunit in the outlined area is shown at a higher magnification in (C). Interneurons (arrows) at the medial surface of lamina VIII in the lumbar spinal cord could be identified in the section. Also visible are $GABA_A$ α_5 subunit positive motoneurons (arrow heads) in the lateral and medial columns.

Fig. 2B shows that I_h recorded from interneurons in the presence of strychnine was not affected by furosemide at concentrations of 100 and 200 μM ($n = 4$). These results indicate that the GABAergic tonic inhibitory current in VHIs sensitive to 100 μM bicuculline is not blocked by gabazine or furosemide, therefore suggesting that the high sensitivity $GABA_A$ receptors mediating this current may not contain $\alpha_{4/6}$ subunits.

3.4. The tonic inhibitory current in VHIs may be mediated by α_5 subunit-containing $GABA_A$ receptors

It has been previously reported that the GABAergic tonic inhibitory current in hippocampal pyramidal cells is partially blocked by L-655708, an antagonist of α_5 subunit-containing $GABA_A$ receptors [6,16]. Therefore, we decided to assess the action of this antagonist on I_h from VHIs. As described earlier, strychnine did not affect the holding current in VHIs (Figs. 1 and 2), however in presence of L-655708 (5 μM) a significant change ($p < 0.05$) in I_h of 49 ± 18 pA was observed (Fig. 3). This result strongly suggests that the tonic inhibitory current in VHIs may be mediated by α_5-subunit containing $GABA_A$ receptors.

3.5. Expression of α_5 subunit in the turtle spinal cord

The expression of the $GABA_A$ α_5-subunit receptor was confirmed by two independent lines of evidence. First, regions of the α_5 gene that were conserved among different species were used to design primers for RT-PCR amplification. Gene expression by RT-PCR using these oligonucleotides revealed a band of the expected size for the α_5 subunit product in the turtle spinal cord (Fig. 4A). The identity of the amplicon was confirmed by automated sequencing. Conventional multiple sequence alignment of the turtle spinal cord $GABA_A$ α_5-subunit receptor revealed >53% overall identity within different species (Supplemental Fig. 1). Likewise, a phylogenetic tree based on the DNA sequence was constructed using Molecular Evolutionary Genetics Analysis (MEGA) software. This tree has two major branches (Supplemental Fig. 2) and indicates that the $GABA_A$ α_5-subunit receptor from the turtle (*T. scripta*) clusters with that of zebrafish (*Danio rerio*), zebra finch (*Taenopygia guttata*) and chicken (*Gallus gallus*). The sequence of $GABA_A$ α_5-subunit receptor from *T. scripta* will be deposited in GenBank. The second line of experimental evidence supporting the expression of the $GABA_A$ α_5-subunit receptor in the turtle spinal cord was obtained using antibodies. Immunohistochemical staining was performed on transverse slices of the turtle lumbar spinal cord. Immunostaining was prominent in motoneurons but could also be found in VHIs (Fig. 4B and C), where signal was restricted to the cell bodies and proximal dendrites, sparing the nucleus. Interneurons immunopositive for the $GABA_A$ α_5-subunit receptor had similar morphology; they had small, round bodies with scant cytoplasm (Fig. 4C). These small interneurons were located along the midline in segments though they did not show an obvious organization in medial clusters.

4. Discussion

4.1. Spontaneous IPSCs in VHIs: comparison with previous studies

In our experiments, all recorded interneurons showed IPSCs that were abolished by strychnine and were not affected by 20 μM bicuculline. This result contrasts with patch clamp recordings from mouse ventral horn interneurons where spontaneous IPSCs are mediated by activation of glycinergic and GABAergic synaptic receptors [5]. Absence of GABAergic IPSCs in turtle spinal cord VHIs probably occurs because GABAergic interneurons contacting these cells may be silent as reported in cats, where motoneuron IPSPs mediated by the activation of $GABA_A$ receptors is observed only after antidromic activation of Clark neurons [17]. The lack of GABAergic IPSCs may also imply that turtle spinal cord inhibitory interneurons do not co-release glycine and GABA as may occur in neonate rats [11].

4.2. A GABAergic tonic inhibitory current is present in VHIs

The tonic inhibitory current in turtle spinal cord VHIs was blocked by bicuculline at 100 μM which is in agreement to what has been reported in some neurons from supraspinal nuclei, where this current is mediated by activation of high sensitivity $GABA_A$

receptors located at peri- and extra-synaptic regions [3,4]. Interestingly, though bicuculline 100 μM abolishes the IPSCs without affecting I_h in mouse VHIs, a tonic inward current with a slow onset is activated in presence of thiopental. This current is also blocked by 100 μM bicuculline, and it is not observed initially due to the low ambient GABA concentrations present during the recordings [5]. It is worth mentioning that in our preparation the ambient GABA concentration seems to be high enough to activate the tonic inhibitory current from the beginning of the recording, and this is because we used thick slices (segments of 2–3 mm) and recorded from cells that were located at >100 μm below the surface.

4.3. Pharmacological properties of the $GABA_A$ receptors mediating the tonic current in VHIs

The tonic inhibitory current in turtle spinal cord VHIs was not sensitive to gabazine at concentrations that block synaptic $GABA_A$ receptors [3] and furosemide, an antagonist of the $\alpha_{4/6}$ subunit-containing $GABA_A$ receptors [15], that at concentrations like the ones used in our studies do not block Cl^- transporters [18]. In sharp contrast, the tonic current observed in VHIs was effectively blocked by L-655708 suggesting that this current is mediated by α_5 subunit-containing $GABA_A$ receptors. In support of this, a series of molecular biology and immunohistochemical experiments unambiguously showed the expression of the α_5 subunit at the ventral horn of the turtle spinal cord. These results are in agreement with *in situ* hybridization, immunohistochemical and RT-PCR studies showing the presence of this subunit at peri- and extra-synaptic regions in the rat spinal ventral horn [19–22].

Taken as a whole, our results strongly suggest that $GABA_A$ receptors containing the α_5 subunit may be mediating a major fraction of the tonic inhibitory current observed in VHIs. Activation of these receptors by ambient GABA could have important functional implications in controlling neuron excitability and stabilizing neuronal networks involved in motor control.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.07.026.

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