Distribution and Function of GABA<sub>B</sub> Receptors in Spider Peripheral Mechanosensilla

Izabela Panek, Shannon Meisner, and Päivi H. Torkkeli

1Department of Physiology and Biophysics, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada; and 2Department of Biophysics, Institute of General and Molecular Biology, N. Copernicus University, 87-100 Torun, Poland

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Panek, Izabela, Shannon Meisner, and Päivi H. Torkkeli. Distribution and function of GABA<sub>B</sub> receptors in spider peripheral mechanosensilla. J Neurophysiol 90: 2571–2580, 2003. First published June 11, 2003; 10.1152/jn.00321.2003. The mechanosensilla in spider exoskeleton are innervated by bipolar neurons with their cell bodies close to the cuticle and dendrites attached to it. Numerous efferent fibers synapse with peripheral parts of the mechanosensory neurons, with glial cells surrounding the neurons, and with each other. Most of these efferent fibers are immunoreactive to γ-aminobutyric acid (GABA), and the sensory neurons respond to agonists of ionotropic GABA receptors with a rapid and complete inhibition. In contrast, little is known about metabotropic GABA<sub>B</sub> receptors that may mediate long-term effects. We investigated the distribution of GABA<sub>B</sub> receptors on spider leg mechanosensilla using specific antibodies against 2 proteins needed to form functional receptors and an antibody that labels the synaptic vesicles on presynaptic sites. Both anti-GABA<sub>B</sub> receptor antibodies labeled the distal parts of the sensory cell bodies and dendrites but anti-GABA<sub>B</sub>R1 immunoreactivity was also found in the axons and proximal parts of the cell bodies and some glial cells. The fine efferent fibers that branch on top of the sensory neurons did not show GABA<sub>B</sub> receptor immunoreactivity but were densely labeled with anti-synapsin and indicated synaptic vesicles on presynaptic locations to the GABA<sub>B</sub> receptors. Intracellular recordings from sensory neurons innervating the slit sensilla of the spider legs revealed that application of metabotropic GABA<sub>B</sub> receptors agonists attenuated voltage-activated Ca<sup>2+</sup> current, providing 2 possible mechanisms for controlling the neurons’ excitability. These findings support the hypothesis that GABA<sub>B</sub> receptors are present in the spider mechanosensilla where their activation may modulate information transmission.

INTRODUCTION

A dense network of fine efferent fibers innervates the peripheral parts of arachnid mechanosensilla (Fabian-Fine et al. 1999a,b, 2000; Foelix et al. 2002). In the tropical wandering spider (Cupiennius salei, Keys.) the efferent fibers form synapses with the axons, somata, and dendrites of the mechanosensory neurons. In addition, the efferent fibers synapse with each other and with glial cells surrounding the sensory neurons (Fabian-Fine et al. 1999b, 2000). The majority of efferent neurons are immunoreactive to an antibody against the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Fabian-Fine et al. 1999b) and the sensory neurons are inhibited by agonists of ionotropic GABA receptors (Panek et al. 2002). The mechanism of inhibition is similar to the presynaptic inhibition that modulates most known mechanoreceptor afferent terminals located in the CNS (e.g., Nusbaum et al. 1997; Rudomin and Smith 1999; Torkkeli and Panek 2002).

In addition to relatively fast inhibition by ionotropic receptors, GABA can modulate many signal transduction pathways by activating metabotropic G protein coupled GABA<sub>B</sub> receptors that play critical roles in long-term modulation of synaptic transmission (Bowery 1993). These receptors are common in mammalian brain (Bowery 1993; Balon et al. 2002) and spinal cord (Towers et al. 2000) in both pre- and postsynaptic locations, where they mediate mechanisms that play fundamental roles in long-term potentiation, muscle relaxation, and nociception (Bowery et al. 2002; Malcangio and Bowery 1996; Staubb et al. 1999). Presynaptic GABA<sub>B</sub> receptors are believed to decrease Ca<sup>2+</sup> influx and thus suppress neurotransmitter release, whereas postsynaptic receptors often decrease neuronal excitability by activating K<sup>+</sup> currents (Bowery et al. 2002; Kaupmann et al. 1998). GABA<sub>B</sub>-receptors also exist in arthropod CNS (e.g., Bai and Sattelle 1995; Mezler et al. 2001) but very little is known about their distribution, mechanisms of action, or the types of responses they mediate.

Two different GABA<sub>B</sub> receptor proteins, GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2, have been cloned from mammalian nervous systems and both exist in several alternatively spliced forms (Billington et al. 2001; Jones et al. 1998; Lopez-Bendito et al. 2002). The same two GABA<sub>B</sub> receptor subtypes and an additional GABA<sub>B</sub>R3 protein have also been isolated from Drosophila (Mezler et al. 2001). In both mammalian and Drosophila systems the functional receptor constitutes a heterodimer with the GABA<sub>B</sub>R1 and -R2 subunits, but the role of R3 protein is unknown (Filippov et al. 2000; Margeta-Mitrovic et al. 2001; Mezler et al. 2001). Using Western blot analysis we tested specific antibodies against the two essential GABA<sub>B</sub> receptor subtypes in spider brain and peripheral nerve tissues and we located both receptor proteins on the mechanosensilla using immunocytochemistry. To learn how the GABA<sub>B</sub> receptor immunoreactivity correlated with the synaptic sites and whether these receptors were present in the efferent fibers we performed double-labeling experiments using a monoclonal antibody against synapsin that has previously been shown to specifically label the synaptic vesicles on the fine efferent fibers on the spider sensilla (Fabian-Fine et al. 1999a,b). We
also tested whether specific GABAB receptor agonists modulate the voltage-activated currents and the excitability of identified peripheral mechanosensory neurons.

**METHODS**

**Experimental animals**

A laboratory colony of Central American wandering spiders (*C. piemontii salei*, Keys.) was kept at room temperature (22 ± 2°C). For immunocytochemical and electrophysiological experiments legs were autotomized, and for Western blot analysis the brains and peripheral tissue were removed from spiders that were killed by deep CO2 anesthesia following protocols approved by the Dalhousie University Committee on Laboratory Animals (00-2971). Adult spiders (older than 10 months) of both sexes were used for all experiments.

**Western blot analysis**

All chemicals were purchased from Sigma (Oakville, Ontario, Canada) unless otherwise indicated. The spider brain tissue was rapidly frozen with liquid nitrogen and ground with a precooled mortar and pestle, dissolved in phosphate-buffered saline (PBS) and mixed by vortex. Laemmli sample buffer solution (1:1) and 4% protease inhibitor cocktail solution (Roche, Laval, Quebec, Canada) were added and the homogenate was kept frozen until use. The spider leg peripheral nerves are embedded in a "hypodermis" membrane that can be isolated from the surrounding tissue. We collected 16 to 24 membranes from the proximal parts of the legs into a microtissue grinder tube (Fisher Scientific, Nepean, Ontario, Canada) on ice, added Laemmli sample buffer and the tissue was ground, followed by addition of 4% protease inhibitor cocktail. We ran 5- to 30-μl samples on a SDS-acrylamide gel (Bio-Rad Laboratories, Mississauga, Ontario, Canada), and blotted them to nitrocellulose membrane. Rainbow markers (BioRad 161-0305) were used as molecular weight markers.

The blot was incubated in blocking buffer (Tris buffer saline, 1% skim milk powder, and 0.6% Triton X-100 in PBS) for 1 h followed by incubation in the primary antibody in blocking buffer at 4°C. All immunocytochemical and electrophysiological experiments were performed in the following concentrations: guinea pig anti-GABA B R1 (Chemicon International, Temecula, CA, AB1531, 1:2,500 dilution), guinea pig anti-GABA B R2 (Chemicon, AB5394, 1:500 dilution), and rabbit anti-GABA B R2 (Chemicon, AB5848, 1:50 dilution). The blot was then washed 30 min in Tris buffer saline with 1% Tween 20, followed by incubation in peroxidase-conjugated anti-guinea pig (Jackson Immunoresearch Laboratories, West Grove, PA, 1:10,000 or 1:1,500 dilution) or anti-rabbit secondary antibody (Jackson Laboratories, 1:5,000 dilution). Immunoreactive protein bands were visualized using an ECL plus chemiluminescent kit (Amersham Biosciences, Montreal, Quebec, Canada) according to the manufacturer’s instructions.

**Immunocytochemistry**

A piece of cuticle from the anterior patella containing the lyriform VS-3 slit sense organ [nomenclature according to Barth and Libera (1976)] was dissected free from the surrounding tissue. The tissue was fixed with 4% paraformaldehyde (Fisher) in PBS for 20 min followed by several rinses in PBS. Tissue was then permeabilized with 1% Triton X-100 in PBS for 1 h followed by incubation in the blocking solution [5% normal goat serum, 1% bovine serum albumin Fraction V (Gibco, Burlington, Ontario, Canada), 3% skim milk powder, and 0.6% Triton X-100 in PBS] for 2 h. The primary antibodies were dissolved in fresh blocking solution and the tissue was incubated in this solution overnight at 4°C. The primary antibodies were dissolved in fresh blocking solution and the tissue was incubated in this solution overnight at 4°C. The same primary antibodies as in the Western blot experiments were used in the following concentrations: guinea pig anti-GABA B R1 (1:2,500), guinea pig anti-GABA B R2 (1:1,000), and rabbit anti-GABA B R2 (1:5,000 to 1:500). Monoclonal mouse anti-synapsin (SYNORF1, Dr. E. Buchner Universität Würzburg, Germany; Klages et al. 1996) was used in 1:100 dilution. Tissue was then washed 4 times in 0.6% Triton X-100 in PBS followed by overnight incubation in the secondary antibodies in blocking solution at 4°C. The secondary antibodies were goat anti-guinea pig CY-3 (1:1,000) for the guinea pig anti-GABA B R1 and anti-GABA B R2 (Jackson Laboratories, 106-165-006), goat anti-rabbit AlexaFluor 488 10 μg/ml (Molecular Probes, Eugene OR, A-11034) for the rabbit anti-GABA B R2, and goat anti-mouse Alexa Fluor 488 10 μg/ml (Molecular Probes, A-11029) for anti-synapsin. Tissue was then washed 4 times in 0.6% Triton X-100 in PBS followed by 5 washes in PBS. The leg hypodermis were then carefully removed from the cuticle with the sensilla attached and placed on a microscope slide and mounted in media containing Mowiol dissolved in 0.2 M Tris buffer enriched with 2.5% DABCO (1,4-diazabicyclo[2.2.2]octane) to reduce fading. For controls, one or both of the primary antibodies were omitted and/or the primary antisera were substituted with normal sera of the same species at the same dilution. For the monoclonal mouse antibody, mouse ascites fluid served as a control. For double-labeling experiments the 2 primary and 2 secondary antibodies were used simultaneously.

The preparations were examined under epifluorescence optics with an Axiovert 100 inverted microscope (Carl Zeiss, Oberkochen, Germany) and/or under a laser scanning confocal microscope (LSM 510, Carl Zeiss) with an argon-krypton laser for Alexa Fluor 488 (488 nm) and a helium-neon laser for CY-3 (543 nm). Digital images of the immunoreactive structures were captured and analyzed. The final images were enhanced in Adobe Photoshop 7.

**Electrophysiology**

Similar dissection of the anterior leg patella was performed as for the immunocytochemistry. The hypodermis membrane with the VS-3 slit sense organ was detached from the cuticle and attached to a coverslip coated with 1 mg/ml poly-γ-lysine (Sigma P5899) and then placed in a recording chamber as described previously in detail (Sekizawa et al. 1999). The preparation was superfused continuously with spider saline [in mM: 223 NaCl, 6.8 KCl, 8 CaCl2, 5.1 MgCl2, 5 sucrose, and 10 HEPES, pH 7.8 (Höger et al. 1997)] using a BPS-4 solution exchange system and PR10 pressure regulator (ALA Scientific, New York, NY). The drugs were injected either using the solution exchange system or manually with a syringe and a needle.

Intracellular recordings were performed from the somata of the VS-3 neurons using the discontinuous single-electrode current- and voltage-clamp methods (Finkel and Redman 1984) with a SEC-10 L amplifier (NI-P Electronic, Tamm, Germany). The conditions for successful single-electrode voltage- and current-clamp were described previously (Torkkeli and French 1994), and the same methods were used in VS-3 neurons to study voltage-activated currents (Sekizawa et al. 1999, 2000; Torkkeli et al. 2001). A horizontal puller (P-2000, Sutter Instrument, Novato, CA) was used to pull microelectrodes from borosilicate glass (1 mm OD and 0.5 mm ID). Electrodes were filled with 3 M KCl or CsCl (see following text) and their resistances were 40–80 MΩ in solution. Switching frequencies of 20–25 kHz and a duty cycle of 1/4 (current passing/voltage recording) were used in all experiments.

The neurons were located and observed under bright-field optics (Axioskop 2FS, Carl Zeiss). They were impaled by high-frequency oscillation (‘buzzing’) followed by a 15-min stabilizing period before recordings. All experiments were controlled by an IBM-compatible computer with custom-written software (courtesy of Dr. A. S. French, Dalhousie University, Halifax, NS, Canada). Current or voltage stimuli were provided by the computer by a 12-bit D/A converter. The membrane potential recording was low-pass filtered at 33.3 kHz and the current signal was filtered at 3.3 kHz by the voltage-clamp amplifier.

For Ca2+ current and Ca2+ spike recordings the voltage-activated Na+ currents were blocked with 1 μM tetrodotoxin (TTX) and K+ currents were blocked with 5 μM 4-AP.
currents with 25 mM tetraethylammonium chloride (TEA) and 25 mM 4-aminopyridine (4-AP). In most experiments Ba\(^{2+}\) was used as the current carrier by replacing the extracellular Ca\(^{2+}\) with the same concentration (8 mM) of Ba\(^{2+}\). In addition, the electrode-filling solution in these experiments was 3 M CsCl. For K\(^{+}\) current recordings 1 \(\mu\)M TTX was used to block the Na\(^+\) currents and 100 \(\mu\)M Ni\(^{2+}\) to block the Ca\(^{2+}\) currents. The Ni\(^{2+}\) application also caused inhibition of any intrinsic synaptic effects.

\(\text{GABA}_B\) receptor agonists were aliquoted and kept frozen until shortly before the experiment. The following pharmacological agents and solutions were used: SKF97541 hydrochloride (3-aminopropylmethylphosphonic acid hydrochloride; Sigma, A196) and 3-aminopropylphosphonic acid (3-APA) (Sigma, A7162) were initially dissolved in distilled water. (±)-Baclofen (Sigma, B5399) was initially dissolved in concentrated HCl. The total volume of the recording chamber was 500 \(\mu\)l and the concentrations of the pharmacological agents shown in the RESULTS section indicate the final concentrations in the chamber.

Statistical analysis was performed by comparing differences between means using two-sided unpaired \(t\)-test with Prophet 6.0 software (AbTech Corporation, Charlottesville, VA).

RESULTS

Western blot analysis

Western blot analysis was used to test the specificity of commercially available anti-\(\text{GABA}_B\) receptor antibodies on spider brain and peripheral nervous tissues. The peripheral tissue was obtained from the hypodermis in the leg femur and patella. The anti-\(\text{GABA}_B\)R1 antibody produced a discrete darkly stained band at about 130 kDa in both tissues (Fig. 1). This antibody is directed against 2 splice variants of the \(\text{GABA}_B\)R1 receptor, and our findings are consistent with the 130-kDa molecular weight of the \(\text{GABA}_B\)R1A splice variant. The guinea pig anti-\(\text{GABA}_B\)R2 antibody produced a clear band at 105 kDa in both homogenates, corresponding closely to the values given in literature (e.g., Bowery et al. 2002). This antibody required a relatively high protein concentration to produce specific staining and because we were not able to obtain large amounts of the peripheral tissue we also increased the secondary antibody concentration for the hypodermis homogenate. This resulted in the additional less discrete bands in the hypodermis homogenate, probably representing unspecific staining. The rabbit anti-\(\text{GABA}_B\)R2 antibody produced a clear band at 105 kDa in brain tissue, corresponding exactly to the manufacturer’s description. We did not test this antibody on peripheral tissue because of the negative results with immunocytochemistry (see following text).

Immunocytochemistry

Whole-mount preparations of pieces of hypodermis from spider patella were used for immunostaining with the same anti-\(\text{GABA}_B\) receptor antibodies that were used in the Western blot experiments. In this preparation the VS-3 slit sensilla, one of the lyriform slit sense organs that detect strains on the spider cuticle (Fig. 2A), and numerous other sensilla remain attached to a hypodermis membrane. The VS-3 neurons expressed strong immunoreactivity against the guinea pig anti-\(\text{GABA}_B\)R1 and -R2 antibodies but the effluent neurons were not labeled and therefore not visible in the fluorescent images (Fig. 2, B and C). The anti-\(\text{GABA}_B\)R1 immunoreactive punctae were not limited to the neurons, but were also present in tissues surrounding the neurons, including glial cells. The staining pattern of the anti-\(\text{GABA}_B\)R2 antibody was more specific: clusters of immunoreactive punctae were seen in the distal halves of all of the VS-3 neurons but the proximal parts were not stained. The glial cells surrounding the neurons were not immunoreactive to this antibody. Intense immunoreactivity against both anti-\(\text{GABA}_B\) receptor antibodies was also found in the small sensory neurons that innervate the trichobothrial (filiform) hairs, which are used for measuring air movements, and on larger groups of neurons that form the proprioceptive joint receptors. Because of the small size of most of these neurons the subcellular localization of staining in the cell bodies was difficult to determine, but it was also present on the axonal parts. Punctate staining was not seen in the control preparations (Fig. 2D), but fluorescing spots were often present in the peripheral regions of the cell bodies. The intensity of these spots was distinctly different from that produced by the fluorochromes that were used in this study and they were previously shown to be caused by lipofuscin granules that appear in the neurons of older spiders (Fabian and Seyfarth 1997). The rabbit anti-\(\text{GABA}_B\)R2 antibody produced no labeling in the spider tissue at concentrations of 1:5,000 to 1:500. To our knowledge this antibody was previously used only for immunoblotting.

To learn whether \(\text{GABA}_B\) receptor labeling was postsynaptic to synaptic vesicles on the effenter fibers we performed double-labeling experiments separately with both the guinea pig anti-\(\text{GABA}_B\) receptor antibodies plus a monoclonal antibody against \(\text{Drosophila}\) synaptic (Klagges et al. 1996). Previous work using fluorescence staining and epifluorescence optics as well as electron microscopic immunogold labeling has shown that the same synapsin antibody specifically labels synaptic vesicles in the effenter neurons forming contacts with mechanosensory neurons in the spider leg with no staining in the sensory neurons or the glial cells (Fabian-Fine et al. 1999a,b). The specificity of this antibody in the spider tissue was also demonstrated in Western blot analysis (Fabian-Fine et al. 1999a). Our results indicated a clear and specific anti-synapsin immunoreactivity in the fine fibers surrounding the axons, somata, and the dendrites of the VS-3 neurons (Figs. 3, B and E; 4, B and E). It labeled thin fibers in the main leg nerve (Fig. 3E) and immunoreactive punctae extended to the neurons that innervate tactile spines and hairs (Fig. 4H). Many of the

**FIG. 1.** Western blot analysis of \(\text{GABA}_B\) receptor subunit proteins. Guinea pig anti-\(\text{GABA}_B\)R1 antibody revealed distinct band of about 130 kDa in spider brain and leg hypodermis homogenates (left). Hypodermis tissue mainly consists of peripheral sensory nerves and glial cells. Guinea pig anti-\(\text{GABA}_B\)R2 (GP) antibody produced a discrete band at 105 kDa, but additional, less discrete bands were present in hypodermis homogenate (middle). Rabbit anti-\(\text{GABA}_B\)R2 antibody induced only one band at about 105 kDa in spider brain homogenate (right).
anti-GABA<sub>B</sub>R1–labeled parts in the sensory neurons were in close contact with the anti-synapsin–labeled elements. However, this antibody also labeled elements that were not in contact with the anti-synapsin immunoreactive sites (Fig. 3, C and F).

Anti-GABA<sub>B</sub>R2 labeling coincided with anti-synapsin labeling in the distal parts of the VS-3 neuron cell bodies and dendrites (Fig. 4, C and F), but there was very little anti-GABA<sub>B</sub>R2 staining on the axons or proximal parts of the VS-3 neurons that were covered with anti-synapsin–labeled fine efferent fibers (Fig. 4, B and E). Groups of 3 neurons innervating the tactile hairs and spines had staining patterns similar to those of the VS-3 neurons, with clusters of anti-GABA<sub>B</sub>R2 immunoreactivity on the distal parts of the cell bodies, and the largest cell body having more staining than the 2 smaller ones (Fig. 4G). Because the proximal parts of the sensory cell bodies were only faintly labeled with the anti-GABA<sub>B</sub>R2 antibody, and there was very little background staining on the sensory neurons, the shapes of the cell bodies are somewhat difficult to conceive from the projected confocal images. Therefore we increased artificially the brightness of Fig. 4H to allow a clearer view to the whole neuron. The shape of the large neuron and the pattern of anti-GABA<sub>B</sub>R2 immunolabeling in this image are similar to the VS-3 neurons and very thin branches of anti-synapsin labeled fibers extend to these cell bodies (Fig. 4, H and I).

Confocal 0.5-μm sections through the different sensilla revealed that the anti-synapsin–labeled efferent branches that extended onto the neurons innervating the slits or the tactile hairs and spines were not stained with anti-GABA<sub>B</sub>R1 or anti-GABA<sub>B</sub>R2 antibodies. In addition, those anti-synapsin–labeled fine fibers that were separated from the surrounding tissue did not show anti-GABA<sub>B</sub>R1-receptor immunoreactivity in the projected confocal images (e.g., Fig. 4F). Similarly, the sensory neurons did not express any anti-synapsin immunoreactivity. The extensive immunoreactivity with anti-GABA<sub>B</sub>R1 antibody in the main leg nerve (Fig. 3D) originates most probably from the sensory axons and the glial cells. Similar difference in the R1 and R2 subunit immunoreactivity has been demonstrated in the rodent nervous system (Ng and Yung 2001; Yung et al. 1999). The anti-GABA<sub>B</sub>R1 also produced some staining in the hypodermis membrane, but this staining was significantly sparser than that seen in the sensory neurons and may be unspecific staining.

**Modulation of voltage-activated currents by GABA<sub>B</sub> receptor agonists**

Spider VS-3 neurons produce a large Ca<sup>2+</sup> current at low voltages (−40 to −20 mV). This LVA-I<sub>Ca</sub> produces Ca<sup>2+</sup> spikes when the voltage-activated K<sup>+</sup> and Na<sup>+</sup> currents are blocked (Sekizawa et al. 2000). We tested the effects of the GABA<sub>B</sub> receptor agonists baclofen and SKF97541 on the LVA-I<sub>Ca</sub> and on the Ca<sup>2+</sup> spikes. The voltage-clamp recordings were performed using Ba<sup>2+</sup> as the current carrier instead of Ca<sup>2+</sup>. At 400 μM concentration both GABA<sub>B</sub> receptor agonists significantly reduced the Ba<sup>2+</sup> current (I<sub>Ba</sub>) and this reduction was reversible, although it often took a long time (10 min–1 h) before complete reversal (Fig. 5A). The I<sub>Ba</sub> reduction was largest at the peak of the Ca<sup>2+</sup> current at −40 to −30 mV (Fig. 5B).

We performed current-clamp experiments on preparations...
Ca\(^{2+}\) spikes with electrical stimulation to test whether they could be modulated by the GABA\(_B\) receptor agonist SKF97541. Figure 6 shows an example of this type of recording. TTX blocked the Na\(^+\) current completely but the neuron fired large action potentials in response to current stimulus when the K\(^+\) currents (I\(_K\)) were also blocked. In intracellular recordings with sharp microelectrodes complete inhibition of outward K\(^+\) currents is not possible, given that the intracellular K\(^+\) cannot be replaced. The I\(_K\) inhibition developed slowly and some outward I\(_K\) may still have been present when the neurons were depolarized strongly, probably contributing to the repolarization of the Ca\(^{2+}\) spike. The normal trend of the Ca\(^{2+}\) spike development was a rapid increase in spike amplitude and gradual increase in its duration, probably reflecting the effectiveness of K\(^+\) current block. When SKF97541 was added to the bath solution, a significant decrease in Ca\(^{2+}\) spike duration was recorded in 3 experiments and in 2 of these experiments the spike amplitude was also slightly reduced. These effects were transient and fully reversible even without wash in normal spider saline.

GABA\(_B\) receptors on postsynaptic membranes often activate an inwardly rectifying K\(^+\) conductance. To learn whether this occurs in the VS-3 neurons, we tested GABA\(_B\) receptor agonist effects on preparations where Ca\(^{2+}\) currents were blocked by 100 \(\mu\)M Ni\(^{2+}\), which also blocks any possible intrinsic synaptic effects. Neither baclofen nor SKF97541 (400–800 \(\mu\)M) produced inward currents at the voltage ranges expected (−140 to −60 mV) in 15 experiments. We also investigated whether SKF97541 modulates the voltage-activated outward K\(^+\) currents (I\(_K\)), previously investigated in the VS-3 neurons (Sekizawa et al. 1999). A small but distinct increase in the I\(_K\) amplitude was seen in all 3 experiments (Fig. 7). This increase occurred immediately after agonist application and reversed relatively quickly, in <15 min without wash with normal saline. The time scale of this effect was similar to the change in the Ca\(^{2+}\) spike duration (Fig. 6).

**GABA\(_B\) receptor mediated long-term effects on VS-3 neurons**

Long-term synaptic effects on VS-3 neurons can be investigated for several hours when intracellular sharp electrodes are used. We performed experiments using the following GABA\(_B\) receptor agonists: 200–800 \(\mu\)M SKF97541 (13 experiments), 400 \(\mu\)M baclofen (4 experiments), and 200–400 \(\mu\)M 3-APA (3 experiments) to see whether they modulated VS-3 neuron excitability and/or passive membrane properties. The results of the experiments with SKF97541 are summarized in Table 1. The membrane resistance or potential did not change in response to agonist
application. There were small but not statistically significant changes in the thresholds for firing action potentials or the actual numbers of action potentials that the cells fired in response to step-current stimuli. Experiments with baclofen and 3-APA produced similar results. These results suggest that the physiological effects of GABAB receptor activation in spider VS-3 neurons develop very slowly or may be more obvious when mechanical rather than electrical stimuli are applied.

A clear difference in the cell’s response to step-current stimuli was seen in 15 of 16 experiments. Figure 8A shows a neuron that fired a brief burst of action potentials and Fig. 8B is an example of a neuron that fired a maximum of 2 action potentials in response to a step stimulus. In both cases the stimulus induced depolarization was larger within 2–5 min after GABAB receptor agonist application and increased during washing periods of 1 to 2 h. This effect did not reverse in any of the experiments. In 5 experiments the action potentials became smaller and fused (Fig. 8A). The change in the stimulus-induced depolarization was not correlated with changes in passive membrane resistance (Table 1). To learn whether this effect was mediated by Ca2+, we performed 5 similar experiments in which the Ica was blocked by 100 μM Ni2+. In 3 of these experiments a significantly smaller increase in the stimulus-evoked depolarization developed more slowly than in the control experiments; in one experiment the depolarization was actually reduced and in one there was no change.

**DISCUSSION**

**Anti-GABAB receptor immunoreactivity in the spider brain and mechanosensilla**

Functional metabotropic GABAB receptors are formed from heterodimers of 2 proteins, GABABR1 and GABABR2 (Bowery et al. 2002; Jones et al. 1998; Kaupmann et al. 1998; Mezler et al. 2001). In this report we demonstrate that both of these receptor proteins are abundantly present in the spider central and peripheral nervous tissues. The commercially available guinea pig anti-GABABR1 antibody was developed against a common sequence in 2 GABABR receptor isomers and produced a 130-kDa protein band corresponding to the GABABR1A isomer that is also prevalent in the rat neostriatum (Yung et al. 1999) and dorsal root ganglia (Towers et al. 2000). The other isomer, GABABR1B, has a molecular weight of 100 kDa (Kaupmann et al. 1998; Margeta-Mitrovic et al. 1999; Sloviter et al. 1999) and was not found in spider tissue. Both of the anti-GABABR2 antibodies that we tested in the spider brain homogenate produced an expected 105-kDa band in the Western blot analysis and the guinea pig antibody also produced a similar band in the peripheral tissue. The rabbit anti-GABABR2 antibody, which was previously tested only in immunoblotting, produced no labeling by immunocytochemistry, but the guinea pig antibody revealed a very specific staining pattern in spider mechanosensilla.

In the spider leg hypodermis the anti-GABABR1 immunoreactivity was more widespread than anti-GABABR2 labeling.
as synaptically. Most confocal sections through the spider peripheral tissue is expressed extrasynaptically as well as synapsin staining, suggesting that this receptor subtype in the spider VS-3 neurons.


duced LVA-I Ca in VS-3 neurons. 

potentials under control conditions, after 400 μM baclofen application and 80 min after washing are shown. B: peak currents (±SE) from 7 similar recordings plotted against membrane potentials under control conditions, after 400 μM baclofen (3 experiments) or SKF97541 (4 experiments) application and after wash.

The former antibody stained all parts of the neurons and some of the glial cells, whereas the latter staining was restricted to the sensory neurons. Some of the anti-GABA_B R1 staining may represent nonfunctional precursor molecules. Similar findings have been made with the same antibodies in human and rodent CNS and this has led to speculation that additional receptor subtypes may exist that could also dimerize with GABA_B R1 to form functional receptors (Billinton et al. 2000; Ng and Yung 2001; Yung et al. 1999). However, the significant amount of the anti-GABA_B R1 staining did not coincide with the anti-synapsin staining, suggesting that this receptor subtype in the spider peripheral tissue is expressed extrasynaptically as well as synthaptically. Most confocal sections through the spider sensory neurons showed immunoreactivity with both anti-GABA_B receptors antibodies, suggesting that the receptor proteins are present intracellularly as well as in the plasma membrane.

Previous electron microscopic studies from the rat cerebellum have indicated that, although the majority of both receptor proteins are found in the plasma membrane, they are also present intracellularly (Ige et al. 2000), suggesting that nonfunctional subunits that have not yet been transported to their final locations were also stained. Because the anti-GABA_B receptor antibodies that produced immunostaining in spider tissue were both made in the same host, we could not perform double-labeling experiments to test how these proteins are co-localized. However, both proteins were found in the sensory neurons in locations that were postsynaptic to the anti-synapsin-stained efferent neurons. Clustering of GABA_B R2 on the distal parts of the cell bodies, where there was also a large amount of anti-GABA_B R1 staining, suggests that these receptors may play a critical role in modulating the sensory input close to the site of detection.

The GABA_B receptor agonists baclofen and SKF97541 both reduced the amplitude of LVA-I Ca in the spider VS-3 neurons. In the mammalian nervous system the GABA_B receptor-mediated effects on Ca^{2+} conductances usually involve presynaptic P/Q- and N-type channels (Bowery et al. 2002; Chen and van der Pol 1998). However, in thalamocortical neurons GABA_B receptors elicit low-threshold Ca^{2+} potentials, which give rise to high-frequency bursts of action potentials (Crunelli and Lerescu 1991) and a low-voltage-activated T-type current in a rat cultured dorsal root ganglion neurons is modulated by baclofen. In the latter case, low baclofen concentration enhanced the current but high concentration caused a reduction (Scott et al. 1990). The LVA-I Ca in spider VS-3 neurons does not produce high-frequency firing and its physiological function is unknown. A previous investigation concluded that this current does not play any part in the spike generation or frequency modulation. However, it can increase the action potential amplitude and duration when K^+ currents are blocked, and produce spikes when the Na^+ channels are also inhibited (Sekizawa et al. 2000). Clearly, the GABA_B-mediated reduction of LVA-I Ca would attenuate Ca^{2+} influx and, given that this effect is long-lasting, it may have a significant effect on intracellular processes that are mediated by Ca^{2+}.

The GABA_B receptor agonist SKF97541 induced a transient, small increase in the amplitude of the outwardly rectifying I_K in the VS-3 neurons when the intrinsic synaptic

GABA_B receptor effector mechanisms in the VS-3 neurons

The GABA_B receptor agonist SKF97541 both reduced the amplitude of LVA-I Ca in the spider VS-3 neurons. In the mammalian nervous system the GABA_B receptor-mediated effects on Ca^{2+} conductances usually involve presynaptic P/Q- and N-type channels (Bowery et al. 2002; Chen and van der Pol 1998). However, in thalamocortical neurons GABA_B receptors elicit low-threshold Ca^{2+} potentials, which give rise to high-frequency bursts of action potentials (Crunelli and Lerescu 1991) and a low-voltage-activated T-type current in a rat cultured dorsal root ganglion neurons is modulated by baclofen. In the latter case, low baclofen concentration enhanced the current but high concentration caused a reduction (Scott et al. 1990). The LVA-I Ca in spider VS-3 neurons does not produce high-frequency firing and its physiological function is unknown. A previous investigation concluded that this current does not play any part in the spike generation or frequency modulation. However, it can increase the action potential amplitude and duration when K^+ currents are blocked, and produce spikes when the Na^+ channels are also inhibited (Sekizawa et al. 2000). Clearly, the GABA_B-mediated reduction of LVA-I Ca would attenuate Ca^{2+} influx and, given that this effect is long-lasting, it may have a significant effect on intracellular processes that are mediated by Ca^{2+}.

The GABA_B receptor agonist SKF97541 induced a transient, small increase in the amplitude of the outwardly rectifying I_K in the VS-3 neurons when the intrinsic synaptic action potential with duration of 7.5 ms in response to 750-pA current pulse from holding potential of +70 mV. When K^+ and Na^+ channel blockers (25 mM TEA, 25 mM 4-AP, and 1 μM TTX) were added to bath solution neuron fired Ca^{2+} spikes in response to similar stimulus. By 15 min after blocker application spike amplitude was 99 mV and duration 42 ms. When 80 μM SKF97541 was added to bath solution, a transient reduction of Ca^{2+} spike duration and amplitude was seen. In this experiment spike amplitude was 90 mV and duration 27 ms 1 min after SKF97541 application. Ca^{2+} spike then gradually returned to its original shape. In this experiment spike amplitude 10 min after SKF97541 application was 100 mV and duration 42 ms.
activation was blocked by Ni\(^{2+}\). However, neither SKF97541 nor baclofen activated inward K\(^{+}\) currents, as has been found in postsynaptic locations of many mammalian and invertebrate preparations. In cockroach motor neurons (Bai and Sattelle 1995) and cloned Drosophila GABA\(_{B}\) receptors expressed in oocytes (Mezler et al. 2001) the only effects induced by GABA\(_{B}\) agonists were inwardly rectifying K\(^{+}\) currents. However, agonist effects on I\(_{Ca}\) or on voltage-activated outwardly rectifying I\(_{K}\) were not investigated in those reports. Whereas activation of an inwardly rectifying K\(^{+}\) current is the most commonly found modulatory effect of GABA\(_{B}\) receptors on postsynaptic sites, other types of K\(^{+}\) currents are also often involved (Bowery et al. 2002). For example, in Xenopus embryonic spinal cord baclofen caused a reversible enhancement of outward I\(_{K}\) as well as reduction of I\(_{Ca}\) (Wall and Dale 1994), closely corresponding to the findings here.

**FIG. 7.** SKF97541 effect on voltage-activated outward I\(_{K}\) in VS-3 neurons. A: currents elicited by depolarizing cell from holding potential of −70 to +50 mV are shown under control conditions, 5 min after application of 200 μM SKF97541 and 15 min after wash. B: steady-state current–voltage curves (±SE) from 3 similar recordings. Current values represent data at end of 200-ms pulse at potentials from −70 to 80 mV at 10-mV intervals. Increase in I\(_{K}\) amplitude after SKF97541 application was larger at higher depolarizing voltages. This increase was fully reversible.

**TABLE 1.** SKF97541 effects on VS-3 neuron membrane properties

<table>
<thead>
<tr>
<th></th>
<th>R(_{m}) (MO)</th>
<th>E(_{\text{rest}}) (mV)</th>
<th>Current Threshold (pA)</th>
<th>Number of Spikes</th>
<th>Voltage Threshold (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>151 ± 65 (13)</td>
<td>−67 ± 5 (12)</td>
<td>712 ± 224 (13)</td>
<td>2.2 ± 1.8 (13)</td>
<td>31.3 ± 7.1 (13)</td>
</tr>
<tr>
<td>SKF97541</td>
<td>176 ± 76 (13)</td>
<td>−66 ± 5 (12)</td>
<td>712 ± 267 (13)</td>
<td>1.8 ± 1.1 (13)</td>
<td>32.7 ± 8.4 (13)</td>
</tr>
<tr>
<td>Wash</td>
<td>169 ± 77 (11)</td>
<td>−66 ± 5 (10)</td>
<td>818 ± 252 (11)</td>
<td>1.7 ± 1.4 (11)</td>
<td>35.7 ± 7.2 (11)</td>
</tr>
<tr>
<td>P (control/SKFR97541)</td>
<td>0.4001</td>
<td>0.4414</td>
<td>0.999</td>
<td>0.5270</td>
<td>0.6546</td>
</tr>
<tr>
<td>P (control/wash)</td>
<td>0.5628</td>
<td>0.5493</td>
<td>0.2851</td>
<td>0.4730</td>
<td>0.146</td>
</tr>
</tbody>
</table>

Values are means ± SD with the number of experiments in parenthesis. R\(_{m}\), membrane resistance; E\(_{\text{rest}}\), resting potential; Current Threshold, current stimulus needed to elicit an action potential; Voltage Threshold, membrane potential increase needed to elicit an action potential. SKF97541 concentrations used for these experiments were from 200 to 800 μM.

**FIG. 8.** GABA\(_{B}\) receptor agonist effects on VS-3 neuron voltage response. A: neuron that fired a burst of action potentials in response to 1.25-nA current stimulus (continuous line) produced fused action potentials and larger depolarization in response to similar stimulus 5 min after application of 200 μM SKF97541 (dashed line). Evoked membrane depolarization increased with time even when preparation was washed with normal saline for 15 min (dotted line). B: neuron that fired 2 action potentials in response to 1.5-nA current pulse (continuous line). By 2 min after application of 800 μM SKF97541 a slightly larger depolarization was produced (dashed line) in response to similar stimulus with no change in action potential amplitude or duration. Evoked depolarization continued to increase when preparation was washed for 55 min with normal saline (dotted line).

**GABA\(_{B}\)** receptor–mediated modulation of sensory neuron excitability

GABA\(_{B}\) receptor–mediated modulation can provide fine control of a cell’s excitability by multiple mechanisms. Those mechanisms that regulate the voltage-activated conductances in VS-3 neurons can cause decreased excitability and reduction of neurotransmitter release: Reduction of I\(_{Ca}\) decreases Ca\(^{2+}\) entry during action potentials and increased outwardly rectifying I\(_{K}\) may shorten the action potential duration. In addition to modulation of K\(^{+}\) and Ca\(^{2+}\) channels, several other GABA\(_{B}\) receptor–mediated mechanisms may be involved, but were not studied here. For example, they can stimulate phospholipase A, inhibit adelylate cyclase, and regulate inositol phosphate hydrolysis (Misgeld et al. 1995). In the mammalian spinal cord and brain, presynaptic GABA\(_{B}\) receptor activation has also been shown to modulate glutamate, ACh, and dopamine release (Balon et al. 2002; Li et al. 2002; Towers et al. 2000). We
found no evidence of presynaptic GABA<sub>B</sub> receptors on the efferent neurons surrounding the sensory neurons that innervate the slits, tactile hairs, or spines, indicating that GABA<sub>B</sub> receptors do not serve as autoreceptors on the GABAergic efferents and that functional receptors are present only on the sensory neurons. Because the majority of these receptors were strategically located on the distal parts of the sensory neurons, their most obvious function is in regulating the transduction of sensory signals. The normal locomotory activity of Cupiennius has been shown to be restricted to the dark period of the day and the responsiveness of the mecanosensilla is significantly lower during daytime than at night (Seyfarth 1980). It is possible that the GABA<sub>B</sub> receptor-mediated inhibition is responsible for the lower responsiveness during daytime. The 2 sensory neurons that innervate each slit in the spider lyriform sensilla were previously shown to have different adaptation properties (Seyfarth and French 1994). We found no differences in the GABA<sub>B</sub> receptor immunolabeling or the GABA<sub>B</sub> agonist–induced effects in the different neuron types, suggesting that both types can be modulated by GABA<sub>B</sub>-mediated mechanisms.

The clearest long-term effect of GABA<sub>B</sub> receptor agonists that we detected in VS-3 neurons was the depolarization in response to a current stimulus. This could not be a direct consequence of either increased I<sub>K</sub> or decreased I<sub>Ca</sub>. Because this effect was partially removed when Ca<sup>2+</sup> currents were blocked with Ni<sup>2+</sup>, it may have been produced by mechanisms initiated by reduced I<sub>Ca</sub>. We have not seen similar effects in response to the GABA<sub>B</sub> receptor activation reported previously in other preparations. However, all previous intracellular work was done using whole cell patch-clamp where the neurons are not viable for such a long time as with sharp electrode recordings. The increased stimulus-induced depolarization was more clearly visible about 15 min after agonist application and continued to increase in recordings that lasted for up to 2 h. Physiologically, this effect would make it more difficult for the neurons to respond to high-frequency stimuli, given that the action potentials would not be able to repolarize and therefore new spikes would not be fired. Future research into the possible changes in the neuron’s frequency sensitivity in response to GABA<sub>B</sub> receptor activation would provide clarification to this question.

In conclusion, our findings suggest that functional GABA<sub>B</sub> receptors are abundantly present in spider brain and peripheral sensilla. Immunocytochemical localization of these receptors has not been described in any invertebrate tissue, although it is known that they exist in the CNS. In addition, although GABA<sub>B</sub> receptors have been shown to modulate mammalian mechanoreceptors and nociceptors, they have not been described in locations that are close to the sensory endings. Our findings suggest that mechanosensory input in spider peripheral sensilla may be finely tuned by GABA<sub>B</sub> receptor activation on the most distal parts of the sensory afferents. This would probably enable the neuron’s ability to detect different stimulus frequencies and amplitudes, and could cause a slow, sustained inhibition when the neurons are subjected to a repeated stimulus or when a change is required by behavioral circumstances.

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**DISCLOSURES**

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