Distribution and Function of GABAB Receptors in Spider Peripheral Mechanosensilla


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Efferent fibers are immunoreactive to glial cells surrounding the neurons, and with each other. Most of these synapse with peripheral parts of the mechanosensory neurons, with the cuticle and dendrites attached to it. Numerous efferent fibers innervate the peripheral parts of arachnid mechanosensilla (Fabian-Fine et al. 1999a,b). We performed double-labeling experiments using a monoclonal antibody against synapsin and indicated synaptic vesicles on presynaptic locations to the GABAB receptors. Intracellular recordings from sensory neurons innervating the slit sensilla of the spider legs revealed that application of GABA receptor agonists attenuated voltage-activated Ca2+ current and enhanced voltage-activated outward K+ current, providing 2 possible mechanisms for controlling the neurons’ excitability. These findings support the hypothesis that GABAB 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 ionotopic receptors, GABA can modulate many signal transduction pathways by activating metabotropic G protein coupled GABA B 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; Staubli et al. 1999). Presynaptic GABAB receptors are believed to decrease Ca2+ influx and thus suppress neurotransmitter release, whereas postsynaptic receptors often decrease neuronal excitability by activating K+ currents (Bowery et al. 2002; Kaupmann et al. 1998). GABAB 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 GABAB receptor proteins, GABABR1 and GABABR2, have been cloned from mammalian nervous systems and both exist in several alternatively spliced forms (Billinton et al. 2001; Jones et al. 1998; Lopez-Bendito et al. 2002). The same two GABAB receptor subtypes and an additional GABABR3 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 GABABR1 and -R2 subunits, but the role of R3 protein is unknown (Filippov et al. 2000; Marjeta-Mitrovic et al. 2001; Mezler et al. 2001). Using Western blot analysis we tested specific antibodies against the two essential GABAB receptor subtypes in spider brain and peripheral nerve tissues and we located both receptor proteins on the mechanosensilla using immunocytochemistry. To learn how the GABAB 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).

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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 (*Ca. pinnipus 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. Lymphml 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 Lymphml 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% BSA, 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 over-night with 4°C. The primary antibodies were guinea pig anti-GABAaR1 (Chemicon International, Temecula, CA, AB1531, 1:2,500 dilution), guinea pig anti-GABAaR2 (Chemicon, AB5394, 1:500 dilution), and rabbit anti-GABAaR2 (Chemicon, AB5848, 1:500 dilution). The blot was then washed for 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 hypodermis" 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 penetrated 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 same primary antibodies as in the Western blot experiments were used in the following concentrations: guinea pig anti-GABAaR1 (1:2,500), guinea pig anti-GABAaR2 (1:1,000), and rabbit anti-GABAaR2 (1:5,000 to 1:500). Monoclonal mouse anti-synapsin (SYNORF1, Dr. E. Buchner Universität Würzburg, Germany; Klagg 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-GABAaR1 and anti-GABAaR2 (Jackson Laboratories, 106-165-006), goat anti-rabbit AlexaFluor 488 10 μg/ml (Molecular Probes, Eugene OR, A-11034) for the rabbit anti-GABAaR2, 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-L-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 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 experiment recordings. 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 with 25 mM tetraethylammonium chloride (TEA) and 25 mM 4-aminopyridine (4-AP). In most experiments Ba²⁺ was used as the current carrier by replacing the extracellular Ca²⁺ with the same concentration (8 mM) of Ba²⁺. In addition, the electrode-filling solution in these experiments was 3 M CsCl. For K⁺ current recordings 1 µM TTX was used to block the Na⁺ currents and 100 µM Ni²⁺ to block the Ca²⁺ currents. The Ni²⁺ application also caused inhibition of any intrinsic synaptic effects.

GABAᵦ 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 µl 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-GABAᵦ receptor antibodies on spider brain and peripheral nervous tissues. The peripheral tissue was obtained from the hypodermis in the leg femur and spider brain and peripheral nervous tissues. The peripheral 130-kDa molecular weight of the GABAᵦ R1A splice variant.

To learn whether GABAᵦ receptor labeling was postsynaptic to synaptic vesicles on the effector fibers we performed double-labeling experiments separately with both the guinea pig anti-GABAᵦ 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-GABAᵦ 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-GABAᵦ 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-GABAᵦ 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-GABAᵦ R2 antibody produced no labeling in the spider tissue at concentrations from 1:5,000 to 1:500. To our knowledge this antibody was previously used only for immunoblotting.

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Immunocytochemistry

Whole-mount preparations of pieces of hypodermis from spider patella were used for immunostaining with the same anti-GABAᵦ 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-GABAᵦ 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-GABAᵦ 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-GABAᵦ 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-GABAᵦ 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-GABAᵦ R2 antibody produced no labeling in the spider tissue at concentrations from 1:5,000 to 1:500. To our knowledge this antibody was previously used only for immunoblotting.

To learn whether GABAᵦ receptor labeling was postsynaptic to synaptic vesicles on the effector fibers we performed double-labeling experiments separately with both the guinea pig anti-GABAᵦ receptor antibodies plus a monoclonal antibody against Drosophila synapsin (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 antisynapsin 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
anti-GABA\(_B\)R2–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\(_B\)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\(_B\)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\(_B\)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\(_B\)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\(_B\)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\(_B\)R1 or anti-GABA\(_B\)R2 antibodies. In addition, those anti-synapsin–labeled fine fibers that were separated from the surrounding tissue did not show anti-GABA\(_B\)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\(_B\)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\(_B\)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\(_B\) receptor agonists**

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

We performed current-clamp experiments on preparations
where the Na$^+$ and K$^+$ currents were blocked and elicited 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 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

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**Fig. 3.** Fluorescent micrographs of anti-GABA$_B$R1 and anti-synapsin double staining of spider VS-3 sensilla. Anti-GABA$_B$R1 immunoreactivity (A and D) indicated by CY-3 fluorescent signal (red) and anti-synapsin immunoreactivity (B and E) indicated by Alexa 488 fluorescent signal (green). C and F: superimposed images. VS-3 organ in A–C has five sensory neurons: 3 partially overlap each other and are labeled so1; so2 and so3 are single cells. Orientation of neurons so1 and so3 is oblique and so3 is oriented face on. A: very strong immunoreactivity for anti-GABA$_B$R1 in the somata, axons, and parts of dendrites of VS-3 neurons. B: anti-synapsin immunoreactive punctae in fine fibers covering sensory axons (arrows), somata, and proximal parts of dendrites. Sensory neurons themselves are not stained with anti-synapsin. C: immunoreactive punctae are localized close to each other in many structures but there were also areas without anti-synapsin labeling with significant anti-GABA$_B$R1 labeling, especially in axonal parts. D–F: main leg nerve, which contains sensory axons, efferent fibers, and glial cells. D: anti-GABA$_B$R1 labeling was strong in whole leg nerve and its branches. E: strong anti-synapsin labeling in thin fibers (arrow) and in some unidentifed cells expanding out from nerve. F: anti-synapsin labeling overlaps with some but not nearly all of anti-GABA$_B$R1 labeling in main nerve. ax, axon; so, soma; de, dendrite. Scale bars: 20 $\mu$m in all figures.
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 GABA\(_B\) 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 GABA\(_B\) 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 Ca\(^{2+}\), we performed 5 similar experiments in which the I\(_{Ca}\) was blocked by 100 \(\mu\)M Ni\(^{2+}\). 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-GABA\(_B\) receptor immunoreactivity in the spider brain and mechanosensilla**

Functional metabotropic GABA\(_B\) receptors are formed from heterodimers of 2 proteins, GABA\(_B\)R1 and GABA\(_B\)R2 (Bowery et al. 2002; Jones et al. 1998; Kaufmann 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-GABA\(_B\)R1 antibody was developed against a common sequence in 2 GABA\(_B\) receptor isomers and produced a 130-kDa protein band corresponding to the GABA\(_B\)R1A isomer that is also prevalent in the rat neostriatum (Yung et al. 1999) and dorsal root ganglia (Towers et al. 2000). The other isomer, GABA\(_B\)R1B, has a molecular weight of 100 kDa (Kaufmann et al. 1998; Margeta-Mitrovic et al. 1999; Sloviter et al. 1999) and was not found in spider tissue. Both of the anti-GABA\(_B\)R2 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-GABA\(_B\)R2 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-GABA\(_B\)R1 immunoreactivity was more widespread than anti-GABA\(_B\)R2 labeling.
GABA<sub>B</sub> receptors in peripheral mechanoensilla

**FIG. 5.** GABA<sub>B</sub> receptor agonists baclofen and SKF97541 reversibly reduced LVA-ICa in VS-3 neurons. A: example of currents elicited from −100 mV holding potential to −30 mV test potential under control conditions, 5 min 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<sub>B</sub>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<sub>B</sub>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<sub>B</sub>R1 staining did not coincide with the antisynapsin staining, suggesting that this receptor subtype in the spider peripheral tissue is expressed extrasynaptically as well as synapticly. Most confocal sections through the spider sensory neurons showed immunoreactivity with both anti-GABA<sub>B</sub> receptor 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<sub>B</sub>R2 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 antisynapsin–stained effenter neurons. Clustering of GABA<sub>B</sub>R2 on the distal parts of the cell bodies, where there was also a large amount of anti-GABA<sub>B</sub>R1 staining, suggests that these receptors may play a critical role in modulating the sensory input close to the site of detection.

**FIG. 6.** GABA<sub>B</sub> receptor agonist effect on Ca<sup>2+</sup> spikes. In normal spider saline this neuron elicited a 46-mV transient action potential with duration of 7.5 ms in response to 750-pA current pulse from holding potential of −70 mV. When K<sup>+</sup>- and Na<sup>+</sup>-channel blockers (25 mM TEA, 25 mM 4-AP, and 1 μM TTX) were added to bath solution neuron fired Ca<sup>2+</sup> 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<sup>2+</sup> spike duration and amplitude was seen. In this experiment spike amplitude was 90 mV and duration 27 ms 1 min after SKF97541 application. Ca<sup>2+</sup> 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.

**GABA<sub>B</sub> receptor effector mechanisms in the VS-3 neurons**

The GABA<sub>B</sub> receptor agonists baclofen and SKF97541 both reduced the amplitude of LVA-ICa in the spider VS-3 neurons. In the mammalian nervous system the GABA<sub>B</sub> receptor–mediated effects on Ca<sup>2+</sup> 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<sub>B</sub> receptors elicit low-threshold Ca<sup>2+</sup> potentials, which give rise to high-frequency bursts of action potentials (Crutnell and Leresche 1991) and 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-ICa 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<sup>+</sup> currents are blocked, and produce spikes when the Na<sup>+</sup> channels are also inhibited (Sekizawa et al. 2000). Clearly, the GABA<sub>B</sub>-mediated reduction of LVA-ICa would attenuate Ca<sup>2+</sup> influx and, given that this effect is long-lasting, it may have a significant effect on intracellular processes that are mediated by Ca<sup>2+</sup>.

The GABA<sub>B</sub> receptor agonist SKF97541 induced a transient, small increase in the amplitude of the outwardly rectifying I<sub>K</sub> in the VS-3 neurons when the intrinsic synaptic...
activity was blocked by Ni²⁺. 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.

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

<table>
<thead>
<tr>
<th></th>
<th>R_m (MΩ)</th>
<th>E_m (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 (10)</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>
</tbody>
</table>

P (control/SKF97541) = 0.4001

P (control/wash) = 0.5628

Values are means ± SD with the number of experiments in parenthesis. R_m, membrane resistance; E_m, 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.

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found no evidence of presynaptic GABA_B receptors on the efferent neurons surrounding the sensory neurons that innervate the slits, tactile hairs, or spines, indicating that GABA_B 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 mechanosensilla is significantly lower during daytime than at night (Seyfarth 1980). It is possible that the GABA_B 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_B receptor immunolabeling or the GABA_B properties (Seyfarth and French 1994). We found no differences in the GABA_B receptor immunolabeling or the GABA_B properties (Seyfarth and French 1994).

The clearest long-term effect of GABA_B 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_K or decreased I_Ca. Because this effect was partially removed when Ca^{2+} currents were blocked with Ni^{2+}, it may have been produced by mechanisms initiated by reduced I_Ca. We have not seen similar effects in response to the GABA_B 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_B receptor activation would provide clarification to this question.

In conclusion, our findings suggest that functional GABA_B 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_B 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_B receptor activation on the most distal parts of the sensory afferents. This would probably change the neuron’s ability to detect different stimulus frequencies and amplitudes, and could cause a slowed, sustained inhibition when the neurons are subjected to a repeated stimulus or when a change is required by behavioral circumstances.

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