Contributions of Voltage- and Ca\(^{2+}\)-Activated Conductances to GABA-Induced Depolarization in Spider Mechanosensory Neurons

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INTRODUCTION

\(\gamma\)-Aminobutyric acid (GABA)–mediated presynaptic inhibition involving an increase in membrane conductance (shunting) and depolarization [primary afferent depolarization (PAD)] was first discovered in vertebrate spinal cord (Eccles et al. 1961; Frank and Fuortes 1957). It has since been found throughout vertebrate and invertebrate nervous systems, and extensively reviewed (e.g., Miller 1998; Nusbaum et al. 1999; Rudomin and Schmidt 1999; Torkkeli and Panek 2002; Watson et al. 1992). Different types of receptors are involved in the presynaptic inhibitory effects of GABA, but activation of ionotropic GABA\(_A\) receptors that are Cl\(^-\)-selective ion channels comprises a widely distributed mechanism that mediates presynaptic inhibition in both vertebrate and invertebrate nervous systems (Zhang and Jackson 1993). In invertebrates, GABAergic presynaptic inhibition has been extensively studied in crustacean (Cattaert et al. 1992, 1994; El Manira and Clarac 1991) and locust (Burrows and Laurent 1993; Leitch and Laurent 1993) mechanosensory pathways and in the neural circuits of crustacean stomatogastric ganglia (Marder and Pauardin-Tritsch 1978; Swensen et al. 2000).

There are large differences in the amounts of membrane depolarization associated with GABA-mediated presynaptic inhibition in different neurons, from a few millivolts in locust proprioceptive afferents and crayfish stretch receptor neurons (Burrows and Laurent 1993; Kaila et al. 1992) to tens of millivolts in the crab lateral pyloric neurons, crayfish chordotonal organ, and sensory afferents of the spider slit sensilla (Cattaert et al. 1992; Panek et al. 2002; Swensen et al. 2000). These differences are believed to rise from the differences in the intracellular [Cl\(^-\)] in these neurons (Alvarez-Leefmans et al. 1998). However, the actual mechanism of GABA-induced depolarization and resulting inhibition is not fully understood. It is believed to inactivate voltage-gated Na channels (Alvarez-Leefmans et al. 1998), but it may also cause accumulation of K\(^+\) in the extracellular space (Kriz et al. 1974) or inactivation of voltage-gated Ca channels (Graham and Redman 1994; Wamsley et al. 1995). Cellular mechanisms of shunting and depolarization in centrally located afferent terminals have been difficult to unravel. However, GABA has similar effects on the peripherally located parts of arachnid and crustacean mechanosensory neurons making these models more experimentally accessible (Cattaert and El Manira 1999; Panek et al. 2002).

Spider, Cupiennius salei, mechanosensory neurons receive extensive efferent innervation in the periphery (Fabian-Fine et al. 2002). The efferent fibers contain GABA, glutamate, and acetylcholine, which all inhibit the responses of sensory neurons to step mechanical or electrical stimuli (Fabian-Fine et al. 2002; Panek and Torkkeli 2005; Panek et al. 2002; Widmer et al. 2006). Octopamine is also present in some of the efferent fibers and octopamine application has an excitatory effect on neuronal activity (Widmer et al. 2005). Intracellular recordings from neurons of VS-3 lyriform slit sensilla in the spider patella (Panek et al. 2002) and a simulated model based on these recordings (French et al. 2006) showed that GABAergic inhibition can be achieved by shunting or inactivation of Na channels and that these two factors can combine to cause stronger inhibition. The simulation demonstrated that depolarization caused by GABA partially inactivated and slowed the Na\(^+\) current. Surprisingly, it also demonstrated that the remaining Na\(^+\) current could contribute significantly to the GABA-induced depolarization (French et al. 2006). Here, we tested this possibility experimentally by blocking voltage-gated Na channels in spider VS-3 neurons prior to GABA application. We also tested the effects of blockers of voltage-gated K and Ca channels to learn whether...
currents through these channels contribute to the GABA-induced depolarization.

Rapidly activating and inactivating voltage-gated Na channels are responsible for the depolarization phase of the action potential in spider VS-3 neurons (Torkelii et al. 2001). This Na⁺ current and action potentials can be completely blocked by tetrodotoxin (TTX). The repolarization phase is produced by a K⁺ outward current with transient and noninactivating components (Sekizawa et al. 1999). Both components are sensitive to blockers of voltage-gated K channels, tetraethylammonium chloride (TEA) and 4-aminopyridine (4-AP). However, ≤50% of the noninactivating component was not blocked even with a high concentration of combination of these drugs or other K-channel blockers (Sekizawa et al. 1999). The only Ca²⁺ current that has been described in the VS-3 neurons is the transient low-voltage-activated (LVA) current, that can be completely blocked with Ni²⁺ or Cd²⁺ (Sekizawa et al. 2000). This current activated at about −50 mV and inactivated rapidly within 50–150 ms depending on the test voltage. This current produced large action potentials when all other voltage-activated Na⁺ and K⁺ currents were blocked. Blockers of Ca²⁺-activated K⁺ current \( I_{\text{K(Ca)}} \), such as charybdotoxin, apamin, and iberiotoxin, did not have any effect on the outward currents or firing behavior of these neurons (Sekizawa et al. 2000). However, Ni²⁺ and Cd²⁺ inhibit the outward current that remains after voltage-gated K⁺ currents are blocked by TEA and 4-AP, suggesting that an outward current component sensitive to Ca²⁺ is present in these neurons. In the present study, we found that blockade of Ca channels resulted in increased depolarization when GABA was applied. This finding led us to perform a more thorough investigation of the role of Ca²⁺ in the VS-3 neuron response to GABA, using voltage-clamp and ratiometric Ca²⁺ dye-imaging experiments.

**METHODS**

**Experimental animals and preparations**

A laboratory colony of Central American wandering spiders (*Cuipennius salei*, Keys) was maintained at room temperature (22 ± 2°C). Autotomized legs from adult spiders of both sexes were used for all experiments. Experiments followed protocols approved by the Dalhousie University Committee on Laboratory Animals (I4–28). For Ca²⁺ imaging experiments a “cuticular preparation” was used; a small piece of the patellar cuticle with its hypodermis membrane containing the VS-3 slit sensillum was dissected and placed on a Plexiglas holder where the slits could be stimulated mechanically (Fig. 1) (Juusola et al. 1994). For current- and voltage-clamp experiments a “hypodermis preparation” was used; the hypodermis membrane was detached from the cuticle with the VS-3 neurons attached and placed on a poly-L-lysine (1 mg/ml) coated coverslip in a recording chamber as described previously (Sekizawa et al. 1999). Preparations were continuously superfused 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)]. All chemicals were purchased from Sigma (Oakville, ON, Canada) if not otherwise indicated.

**Current- and voltage-clamp experiments**

Intracellular recordings were performed using the “hypodermis preparation.” The neurons were observed under bright-field optics (Axioskop 2FS, Carl Zeiss, Oberkochen, Germany). The somata were impaled by high-frequency oscillation (“buzzing”) followed by a 15-min stabilizing period before recordings. Electrodes were filled with 3 M KCl and they had resistances of 40–80 MΩ in solution. In Ca²⁺-current and Ca²⁺-spike experiments the electrode-filling solution was 3 M CsCl. Discontinuous single-electrode current- or voltage-clamp methods with an SEC-10 L amplifier (npi electronic, Tamm, Germany) were used as described before (Sekizawa et al. 1999). Switching frequencies of 20–25 kHz and a duty cycle of 1/4 (current passing/voltage recording) were used in all experiments.

All experiments were controlled by an IBM-compatible computer using custom-written software and a data acquisition board (NI6035E; National Instruments, Austin, TX). Voltage stimuli were provided by the computer via a 12-bit D/A converter. The membrane potential recording was low-pass filtered at 33.3 kHz and the current signal at 3.3 kHz by the voltage-clamp amplifier. Current and voltage were sampled by 16-bit A/D converters.

Ion channel blockers and transmitters, aliquoted and stored frozen at high concentrations, were diluted shortly before each experiment. The following blockers of voltage-gated ion channels were used: Na channels were blocked with 1 µM TTX, Ca-channels with 200 µM Ni²⁺, or 50 µM to 1 mM mibefradil dihydrochloride. K channels were blocked with a mixture of 25 mM TEA and 25 mM 4-AP, whereas the NaCl concentration was reduced from 223 to 173 mM to adjust osmolarity. In some of the Ca²⁺-current and Ca²⁺-spike experiments Ba²⁺ was used as the current carrier by replacing the extracellular Ca²⁺ with the same concentration (8 mM) of Ba²⁺.

GABA was applied using an iontophoretic drug ejection system (MVCS, npi electronic) to allow rapid local agonist application. Double-barrel borosilicate electrodes with 5- to 15-MΩ resistance
were used to allow stray capacitance compensation. The ejecting barrel was filled with 1 M GABA (pH 4.7) and the compensating barrel with 165 mM NaCl. The pipette tip was placed close to the neuron where GABA was ejected using 0.3- to 1-μA positive current pulses for 5–10 s. Leakage of GABA from the electrode was prevented by a retaining negative current until ejection.

Calcium imaging experiments

For the Ca\(^{2+}\) imaging experiments the “cuticular preparation” was used (Fig. 1). Ratiometric Ca\(^{2+}\) measurements were performed using Fura-Red, tetrapotassium salt (10 mM in 2 M KCl; Invitrogen, Burlington, ON, Canada) as described before (Höger et al. 2007). Cells were loaded iontophoretically through the glass microelectrodes and visualized with epifluorescence optics (XF2009 dichroic beam splitter and XF3012 emission filter; Omega Optical, Brattleboro, VT). Excitation light came from two light-emitting diodes (LEDs; V Star LXHL-LRC5 Royal Blue and LXHL-LE5C Cyan, Luxeon, Future Electronics, Pointe-Clair, QC, Canada) with peak emissions at 455 and 505 nm. Light from the two LEDs was projected onto the preparation by a custom-built branched fiber-optic light guide with randomized fibers, coupled to the LEDs by fiber-optic light injectors (Fraen, Reading, MA). The LEDs were driven (10-ms alternating stimulation periods at 1-s intervals) by a custom-built, computer-controlled current-regulated power supply. Fluorescence emission of the dye (640- to 690-nm band) was detected by an avalanche photodiode driven by a regulated power supply with thermal feedback (CS460 module, Hamamatsu Photonics Systems, Hamamatsu, Japan). Ratios were then calculated from the Fura-Red fluorescence due to cyan illumination divided by that due to royal blue illumination.

In these experiments, muscimol (100 μM) or GABA (1 mM) was transiently added to the gravity-fed superfusion solution while dye emission ratio was measured. In most experiments the cells were also impaled with intracellular electrodes and stimulated with low-frequency mechanical stimuli (0.2–0.4 Hz) using a piezoelectric stimulator (P-841.10 translator and a PZT controller; Physik Instrumente, Auburn, MA) during Ca\(^{2+}\) measurements (Fig. 1). In some experiments, 10 μM thapsigargin (LC Laboratories, Woburn, MA), diluted in dimethylsulfoxide (DMSO), was used to deplete intracellular Ca\(^{2+}\) stores.

Peak Ca\(^{2+}\) concentrations in response to muscimol or GABA were expressed as the change in fluorescence ratio/ratio before agonist application (ΔF/F). Ca\(^{2+}\) concentration was estimated by

\[
[K_{a}^{Ca^{2+}}] = K_{d} \frac{S_{2}(R - R_{min})}{S_{2}(R_{max} - R)}
\]

where \(K_{d}\) is the dissociation constant of the dye, \(R\) is the experimental fluorescence ratio, \(R_{max}\) is the ratio under saturated Ca\(^{2+}\) conditions, and \(R_{min}\) is the ratio under Ca\(^{2+}\)-free conditions (Grynkwicz et al. 1985; Lohr 2003). \(S_{2}\) and \(S_{2}^{*}\) are the fluorescence values for the Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free dye at 505 nm. Calibration was performed at the end of each experiment by applying 50 μM Ca\(^{2+}\) ionophore (Ionomycin, LC Laboratories) prepared from a frozen stock solution in DMSO. Normal spider saline with 8 mM [Ca\(^{2+}\)] was used to saturate the dye with Ca\(^{2+}\). In Ca\(^{2+}\)-free solution both Ca\(^{2+}\) and Mg\(^{2+}\) were removed from the spider saline.

Statistical analysis

Statistical differences were compared by a paired two-sample t-test when the data were normally distributed. In one data series (−50 mV nickel, control), the Wilcoxon signed-rank test was used for analysis.

RESULTS

Blockade of voltage-gated Na\(^{+}\) current during GABA-induced depolarization

GABA-induced depolarization was recorded from VS-3 neurons that were current-clamped to three different initial membrane potentials. Similar recordings were made under control conditions and after voltage-gated Na channels were blocked by 1 μM TTX (Fig. 2). TTX was previously shown to completely block the Na\(^{+}\) current in these neurons (Torkkeli et al. 2001). Examples of responses to GABA application at −50, −70, and −90 mV are shown in Fig. 2A and the mean peak depolarizations from seven different experiments with a linear fit drawn through the data in Fig. 2B. Depolarization amplitudes were statistically significantly reduced at membrane potentials of −70 mV (from 16.9 to 12.6 mV) and −90 mV (from 31.4 to 24.3 mV) in the TTX-treated neurons \(P = 0.04\). In most experiments the GABA response also lasted longer in TTX-treated neurons than under control conditions, resulting in a depolarization that did not completely recover during the recording period. The more negative the initial membrane potential, the larger the decrease in depolarization after TTX treatment. The reversal potential was −40 mV for both control and TTX-treated neurons.

![FIG. 2. Tetrodotoxin (TTX) reduced \(\gamma\)-aminobutyric acid (GABA)-induced depolarization. A: recordings at 3 different initial membrane potentials show depolarizations in response to iontophoretic application of GABA under control conditions (black) and after voltage-gated Na channels were blocked with TTX (gray). TTX reduced the depolarization when GABA was applied at initial membrane potentials of −70 and −90 mV. The depolarization also lasted longer than our recording period after TTX application than under control conditions. Time of GABA application is indicated by a line below each trace. B: the mean ± SE amplitudes of peak depolarizations in response to GABA application in 7 different experiments at 3 initial membrane potentials before (black circles) and after (gray squares) TTX was added to the superfusion solution. Depolarization was significantly reduced at −70- and −90-mV initial potentials when TTX was in the bath. Linear fits drawn through the data show that reduction was smaller near the reversal potential and both fitted lines reversed at −40 mV.](http://jn.physiology.org/)
Blockade of voltage-gated K channels during GABA-induced depolarization

Responses to GABA application were recorded under control conditions and after 25 mM TEA and 25 mM 4-AP were added to the superfusion solution to block the voltage-gated K channels. This drug combination does not completely block the outward currents, but sustained outward current remains at test potentials positive to −40 mV (see following text). Figure 3A shows voltage recordings from one cell at the three initial membrane potentials tested (−50, −70, and −90 mV). Figure 3B shows the mean peak depolarizations from all experiments with linear fits drawn through the data. There were no statistically significant differences in the depolarization amplitudes. However, the response lasted longer after K channels were blocked and the membrane did not return to resting level during the recording period (Fig. 3A). Sometimes the neuron fired action potentials at the beginning of the depolarization. Fitted lines in Fig. 3B are almost identical, with the reversal potentials at −39 and −37 mV under control conditions and after K channels were blocked, respectively.

Blockade of voltage-gated Ca channels during GABA-induced depolarization

Responses to GABA application were recorded under control conditions and after 200 µM Ni²⁺ was added to the superfusion solution to block the LVA Ca channels. This treatment was previously shown to block the LVA Ca²⁺ current in these neurons (Sekizawa et al. 2000). After the neurons were superfused by Ni²⁺ solution for a minimum of 20 min, the depolarization amplitude increased (Fig. 4A). This increase was statistically significant when results from six different experiments and three initial membrane potentials were compared (Fig. 4B) (P = 0.001 at −50 mV; P = 0.005 at −70 mV; and P = 0.003 at −90 mV). Linear fitted lines to the data showed a large change in the reversal potential from −37 mV under control conditions to −21 mV after Ni²⁺ treatment.

These results could not have been directly caused by elimination of Ca²⁺ current, since removal of an inward component of the GABA current would produce a smaller depolarization. Instead, these results suggested that Ca²⁺-activated K⁺ current [I_{K(Ca)}] could be involved. Depolarization following GABA application may activate I_{K(Ca)} under control conditions leading to membrane repolarization. Blockade of Ca²⁺ current would then cause a larger and longer-lasting depolarization and shift the reversal potential to a less negative value, as seen here. These findings led us to investigate whether I_{K(Ca)} is present in these neurons and whether there is an increase in intracellular [Ca²⁺] in response to GABA.

Effects of mibebradil on low-voltage–activated Ca²⁺ current and residual K⁺ current

When VS-3 neuron inward currents were recorded under voltage-clamp with TTX, TEA, and 4-AP in the superfusion solution, a small, slowly deactivating outward current was also present in all experiments. TTX completely blocks the voltage-activated Na⁺ current in these neurons (Torkkeli et al. 2001). Therefore the remaining inward current in the test potentials used here (−50 to 0 mV) was most likely the LVA Ca²⁺ current previously described in these neurons (Sekizawa et al. 2000). The sustained outward current cannot be blocked by K-channel blockers or conventional inhibitors of I_{K(Ca)} (Sekizawa et al. 1999). However, this current was partially inhibited by Ni²⁺, which blocks LVA Ca channels (Sekizawa et al. 2000), suggesting that it may be I_{K(Ca)} that is not sensitive to the conventional blockers of this currents. To further test this possibility, we investigated whether mibebradil, a potent blocker of LVA Ca channels (Bezprozvanny and Tsien 1995; Catterall et al. 2005), would have an effect on the sustained outward current along with the inward current. Figure 5A shows the transient Ca²⁺ current and a sustained outward current elicited by voltage steps to two different test potentials (−30 and −40 mV) from a holding potential of −100 mV. Application of 100 µM mibebradil to the superfusion solution blocked both currents (Fig. 5B). Similar results were obtained in six experiments where 100 µM mibebradil and in six experiments where 1 mM mibebradil was used. Complete inhibition of both currents occurred within 5–15 min after mibebradil application and was only partially reversible, even after a long rinsing period (>30 min). In three experiments, we...
Effects of mibefradil on Ca$^{2+}$ and Na$^{+}$ spikes

When spider VS-3 neurons were superfused by saline supplemented with TTX, which completely blocks the voltage-activated Na$^{+}$ current in these neurons (Torkkeli et al. 2001), and TEA and 4-AP that partially block the outward currents (Sekizawa et al. 1999), they fired large Ca$^{2+}$ spikes on step stimulation (Fig. 6A). When 100–500 μM mibefradil was added to the superfusion solution, similar stimuli did not produce Ca$^{2+}$ spikes, but a depolarization that lasted until the end of the step stimulus (Fig. 6A). Similar results were obtained in ten experiments with 100 μM mibefradil and ten experiments with 500 μM mibefradil. The effect was reversible in only three of these experiments. Interestingly, mibefradil also had an inhibitory effect on Na$^{+}$ spikes. When VS-3 neurons were initially superfused in normal spider saline without any blockers, current stimuli induced typical fast spikes (Fig. 6B). These spikes can be completely blocked with TTX (Torkkeli et al. 1999), indicating that they are driven by the voltage-activated Na$^{+}$ current. In six experiments where 100 μM mibefradil was added to the superfusion solution the Na$^{+}$ spikes were inhibited. In five of these experiments the inhibitory effect was reversible. Although mibefradil is usually considered as a specific blocker of Ca channels, there are previous reports of its inhibitory effects on Na channels (Eller et al. 2000).

GABA and muscimol induced Ca$^{2+}$ signals in VS-3 neurons

Application of 1 mM GABA to superfusion solution increased intracellular [Ca$^{2+}$], as indicated by the increased fluorescence ratio (Fig. 7A). In most experiments, intracellular recordings were performed simultaneously and neurons were stimulated with low-frequency mechanical step stimuli to produce action potentials (Fig. 7B). GABA application caused depolarization from a resting potential of −63.5 ± 3.4 mV to a peak amplitude of −42.8 ± 6.9 mV (mean ± SD, n = 13), and the neurons stopped firing action potentials. The fluorescence ratio increased in response to GABA application and reached a peak at the same time as the depolarization, but persisted longer than the depolarization or action potential inhibition (Fig. 7A).

In addition to rapidly activating GABA$_A$ receptors, VS-3 neurons also have metabotropic GABA$_B$ receptors that have been shown to mediate longer-term effects (Panek et al. 2003). To verify that the effect on fluorescence ratio was induced by activation of GABA$_A$ receptors, we performed similar experiments using muscimol, a specific agonist of these receptors (Fig. 8). Application of 100 μM muscimol increased the fluorescence ratio (Fig. 8A), depolarized the neurons from the resting potential of −65.5 ± 2.7 mV to a peak amplitude of −48.2 ± 6.1 mV (mean ± SD, n = 11), and inhibited the action potential response to mechanical step stimuli (Fig. 8B). The increase in fluorescence ratio was again longer lasting than the depolarization or inhibition.

Ten similar experiments were performed with 100 μM muscimol as an agonist and 14 experiments with GABA as an agonist (Fig. 9). The average peak change in fluorescence ratio was 0.22 with muscimol and 0.20 with GABA. There were no statistically significant differences between these values (P = 0.76). In 7 experiments, initial recordings were followed by depletion of intracellular Ca$^{2+}$ stores by incubation of the neurons in 10 μM thapsigargin for 50–60 min. The effects of muscimol (2 experiments) or GABA (5 experiments) on fluorescence ratio were then recorded again. The average change in fluorescence ratio in the thapsigargin-treated neurons was 0.18 (Fig. 9). This was not statistically significantly different from the fluorescence ratio measured before thapsigargin treatment (P = 0.69).

VS-3 neuron intracellular [Ca$^{2+}$] was previously shown to increase in response to action potential firing when the neurons were stimulated by repeated mechanical steps (Höger et al. 2007). Here, we used 15-Hz mechanical stimulation in 18 experiments to produce action potentials and observed a mean rise of 0.27 in the fluorescence ratio (Fig. 9). This value was not statistically significantly different from the fluorescence change observed when GABA or muscimol was applied. To compare data from the current experiments to the data in a
previous report (Höger et al. 2007), the actual Ca\(^{2+}\) concentration was estimated using Eq. 1. Resting [Ca\(^{2+}\)] in the present experiments was 144.9 ± 139 nM (mean ± SD, n = 22), somewhat higher than reported previously (67.3 nM; Höger et al. 2007). After GABA or muscimol treatment [Ca\(^{2+}\)] increased to 399.8 ± 325.2 nM (mean ± SD, n = 7), close to the previously reported saturated concentration of 400 nM in these cells (Höger et al. 2007).

To test whether the change in fluorescence ratio could be explained by membrane depolarization, we performed a linear regression analysis of 23 experiments using depolarization level as the independent variable and \(R/R\) as the dependent variable (Fig. 10). The regression analysis gave \(R^2 = 0.6\), indicating that depolarization could largely explain the increase in fluorescence ratio.

**DISCUSSION**

Activation of GABA\(_A\) receptors leads to opening of anion-selective ion channels. In mammalian and invertebrate mechanosensory neurons as well as neonatal brain preparations

**FIG. 5.** Mibefradil blocked Ca\(^{2+}\) and residual K\(^+\) currents. A: transient inward Ca\(^{2+}\) currents and small residual outward currents elicited by stimuli from −100 mV to 2 different voltages (−30 and −40 mV). Neurons were superfused by spider saline supplemented with TTX, TEA, and 4-AP. B: application of 100 μM mibefradil to the superfusion solution abolished both the inward and outward currents.

**FIG. 6.** Mibefradil inhibited Ca\(^{2+}\) and Na\(^+\) spikes. A: a large Ca\(^{2+}\) spike was recorded in response to 1.5-nA current pulse when Na\(^+\) currents were blocked by TTX and K\(^+\) currents by TEA and 4-AP (Control). After mibefradil was added to the superfusion solution, a similar stimulus failed to produce any Ca\(^{2+}\) spikes (Mibefradil). After a 20-min wash in spider saline without mibefradil this cell fired a similar Ca\(^{2+}\) spike as under control conditions (Wash). B: Na\(^+\) action potential elicited by a 1.5-nA current pulse was recorded when the neuron was superfused by normal spider saline (Control). Mibefradil application caused failure to produce action potentials with similar stimuli (Mibefradil). In this neuron the effect was reversible after 30-min wash in normal saline (Wash).
GABA current has a reversal potential that is less negative than the resting potential (Alvarez-Leefmans 1998; Marty and Llano 2005; Rudomin and Schmidt 1999; Torkkeli and Panek 2002). As a result, GABA-receptor activation in these cells leads to membrane depolarization. This depolarization inhibits mechanosensory neurons but has an excitatory effect in neurons of neonatal brain preparations (Marty and Llano 2005). It is not well understood why there is such a different effect in different cells. Inactivation of Na channels and increased membrane conductance (shunting) contribute to the inhibitory effect in at least some of the mechanosensory neurons (Cattaert and El Manira 1999; French et al. 2006), but other ionic mechanisms have also been suggested (Graham and Redman 1994; Kriz et al. 1974; Walmsley et al. 1995). In the present study, we demonstrated that a slow voltage-activated Na current contributes to the depolarization resulting from activation of GABA\textsubscript{A} receptors in spider VS-3 mechanosensory neurons. We also showed that intracellular Ca\textsuperscript{2+} concentration increased during depolarization, leading to efflux of K\textsuperscript{+} via Ca\textsuperscript{2+}-activated K channels (K\textsubscript{Ca} channels) and causing membrane repolarization aided by K\textsuperscript{+} efflux via voltage-gated K channels. A proposed model of these effects is presented in Fig. 11. Results from a previous investigation into the effects of GABA\textsubscript{B}-receptor activation on voltage-gated channels in VS-3 neurons (Panek et al. 2003) are also included in this model.

**Voltage-activated Na\textsuperscript{+} current contributes to GABA-induced depolarization**

When voltage-gated Na channels were blocked with TTX, GABA application to VS-3 neurons produced smaller depolarization than under control conditions. This confirms that the depolarization triggered by GABA and primarily caused by Cl\textsuperscript{−} efflux (step 1 in Fig. 11) is partially produced by slow Na\textsuperscript{+} influx (step 2 in Fig. 11). Previously, simulated VS-3 neurons were used to investigate how GABA treatment could affect Na\textsuperscript{+} current (French et al. 2006). When a step-current stimulus was applied to the simulated cells during GABA-induced depolarization, the resulting Na\textsuperscript{+} current was smaller and both activation and inactivation were slower than under control conditions. This model also suggested that Na\textsuperscript{+} current could contribute ≤5 mV depolarization in GABA-treated neurons. Our experimental results confirm this hypothesis by showing mean reductions of 4.2 mV and 6.7 mV in peak depolarization after TTX treatment from control depolarized membrane potential values of −53 and −59 mV, respectively. The present results and the simulated model both show that Na\textsuperscript{+} current is only
increased intracellular [Ca\textsuperscript{2+}] in response to GABA\textsubscript{A}-receptor activation was also demonstrated in those cells.

Charybdotoxin, apamin, andiberiotoxin, blockers of K\textsubscript{Ca} channels (Hille 2001), failed to inhibit the outward currents in the VS-3 neurons (Sekizawa et al. 1999; unpublished observations). However, when voltage-gated K channels were blocked by TEA and 4-AP, a portion of the outward current remained (Sekizawa et al. 1999), suggesting that an additional component of K\textsuperscript{+} current, resistant to these drugs, was present. Here, we eliminated this component with mibefradil, a blocker of voltage-gated Ca channels (Bezprozvanny and Tsien 1995; Catterall et al. 2005). Previously, Ni\textsuperscript{2+}, another Ca-channel blocker (Tsien et al. 1991) was shown to have similar, but somewhat less potent, effects on both the inward and residual outward currents in VS-3 neurons (Sekizawa et al. 2000). The residual outward current was not present when extracellular Ca\textsuperscript{2+} was replaced with the same concentration of Ba\textsuperscript{2+}, providing further proof that it was induced by Ca\textsuperscript{2+} influx. Taken together, these results strongly suggest that the residual outward current in the VS-3 neurons is \(I_{K(Ca)}\). The results here suggest that K\textsubscript{Ca} channels are activated in response to Ca\textsuperscript{2+} influx induced by GABA\textsubscript{A}-receptor activation (steps 3 and 4 in Fig. 11). There is also evidence that TEA blocks some types of K\textsubscript{Ca} channels (Hille 2001). Therefore it is possible that the total \(I_{K(Ca)}\) in VS-3 neurons is larger than recorded here after TEA was used to eliminate voltage-activated K\textsuperscript{+} current.

The only Ca\textsuperscript{2+} current that has been identified in VS-3 neurons is the LVA Ca\textsuperscript{2+} current that was estimated to activate at \(-50\) mV (Panek et al. 2003; Sekizawa et al. 2000). Both mibefradil and Ni\textsuperscript{2+} block LVA Ca channels, but they are not completely specific and can block other voltage-gated Ca channels (Cattaert et al. 2005; Tsien et al. 1991). Mibefradil was also previously shown to block voltage-gated Na channels (Eller et al. 2000), which probably explains our finding of elimination of Na\textsuperscript{+} action potentials by this drug. There are no completely specific blockers of LVA channels, making it difficult to separate them from other types of Ca channels. However, if GABA-induced depolarization in VS-3 neurons activates Ca channels, the most likely candidates would be the LVA channels, whose activation range is closest to the depolarization that occurred in response to GABA application (Panek et al. 2003; Sekizawa et al. 2000).

**Role of Ca\textsuperscript{2+} in the GABA response**

Our finding of increased depolarization in response to GABA after Ca channels were blocked was surprising. If the inward Ca\textsuperscript{2+} current itself contributed to depolarization, blockade would be expected to produce the opposite effect and hyperpolarize the cell. Therefore inhibition of a repolarizing process that normally occurs in response to Ca\textsuperscript{2+} influx probably caused the increased depolarization. At least two types of currents can be activated by increases in intracellular [Ca\textsuperscript{2+}]: Ca\textsuperscript{2+}-activated K\textsuperscript{+} current \(I_{K(Ca)}\) (Stockler 2004; Vergara et al. 1998) and Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} current \(I_{Cl(Ca)}\) (Hartzell et al. 2005). It is unlikely that inhibition of \(I_{Cl(Ca)}\) would increase GABA-induced depolarization since \(I_{Cl(Ca)}\) normally activates at more positive potentials and its activation would have a depolarizing effect (Hartzell et al. 2005). Inhibition of this current would be expected to cause the opposite effect to that observed here. On the other hand, \(I_{K(Ca)}\) is a repolarizing current (Stockler 2004) and its inhibition could lead to an increased GABA response. Previous single-channel recordings from mouse cerebellar interneurons showed that \(I_{K(Ca)}\) activated in response to muscimol application (Chavas et al. 2004).

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\Delta R/R = \frac{R_{new} - R_{old}}{R_{old}}
\]

**FIG. 9.** Fluorescence ratio (\(\Delta R/R\)) under different conditions: Application of 100 \(\mu\text{M}\) muscimol or 1 mM GABA produced similar increases in fluorescence ratio. These increases were not significantly different from the fluorescence ratios measured from neurons that were treated with 10 \(\mu\text{M}\) thapsigargin to deplete intracellular Ca\textsuperscript{2+} stores before GABA or muscimol application. High-frequency (15 Hz) mechanical stimulation produced a slightly larger change in fluorescence ratio than GABA or muscimol application, but this difference was not statistically significant.

**FIG. 10.** Relationship between depolarization and fluorescence ratio. Linear regression indicated that the change in fluorescence ratio could largely be explained by the amplitude of depolarization induced by GABA or muscimol application. \(R^2 = 0.60\) (\(n = 23\)).
A clear increase in intracellular \([\text{Ca}^{2+}]\) was observed in the \(\text{Ca}^{2+}\) imaging experiments in response to GABA or muscimol application. This increase had a similar time course with both agonists, suggesting that it was caused by activation of GABA\(_A\) receptors (step 2 in Fig. 11). Previously, activation of metabotropic GABA\(_B\) receptors was shown to reduce the activity of K\(_C\) channels as shown in Fig. 11. Decrease of intracellular \([\text{Ca}^{2+}]\) could remove a current source at a specific equilibrium potential. This increase had a similar time course with both GABA\(_A\) receptors. Decrease of intracellular \([\text{Ca}^{2+}]\) increase initiated by activation of GABA\(_A\) receptors, whereas data for metabotropic GABA\(_B\)-receptor effects on voltage-gated ion channels is from a previous study (Panek et al. 2003). GABA binding to GABA\(_A\) receptors induces Cl\(^-\) efflux and depolarization (step 1). The depolarization is further increased by a slow Na\(^+\) influx via voltage-gated Na channels (step 2). During the depolarization low-voltage–activated (LVA) Ca channels open allowing Ca\(^{2+}\) influx (step 3). Increase in intracellular \([\text{Ca}^{2+}]\) leads to opening of K\(_C\) channels, K\(^+\) efflux, and hyperpolarization (step 4). K\(^+\) also exits via voltage-gated K channels, hyperpolarizing the neuron to resting level. GABA\(_B\) receptors act by activating G-proteins leading to an increase in intracellular cAMP concentration. This cascade is followed by larger outward current via voltage-gated K channels and reduced inward current via LVA Ca channels (Panek et al. 2003). The latter effect would also reduce the activity of K\(_C\) channels.

**Membrane properties**

Selective blockade of a specific ionic current not only can increase the membrane resistance and time constant, but also can remove a current source at a specific equilibrium potential. Separating these effects is difficult in current-clamped cells. However, the blocking experiments each produced different results that were specific, rather than due to any general increase in membrane resistance. Blockade with TTX reduced the GABA-induced depolarization, probably by removing the depolarizing Na\(^+\) current, whereas an increase in membrane resistance alone would be expected to increase the effect of Cl\(^-\) current. Blockade of Ca\(^{2+}\) current increased the depolarization, probably by removing a hyperpolarizing K\(^+\) current, as well as by increasing membrane resistance. However, blockade of K\(^+\) currents that probably also increased membrane resistance did not have any effect on the amplitude of GABA-mediated depolarization.

**Mechanisms of intracellular \([\text{Ca}^{2+}]\) increase**

Increases in intracellular \([\text{Ca}^{2+}]\) in response to activation of GABA\(_A\) receptors have been reported in several preparations. In some, the GABA current reversal potentials are less negative than the resting potentials (Obrietan and van den Pol 1995; Panek et al. 2002; Reichling et al. 1994); in others they are closer to the resting membrane potential (Connors et al. 1987). Because of this, there is some controversy about the mechanism of \([\text{Ca}^{2+}]\) increase. In cells that have a depolarizing response to GABA, the increase is believed to result from depolarization above the level of Ca-channel activation (Marty and Llano 2005). For instance, GABA depolarized cultured rat dorsal horn neurons and produced a \([\text{Ca}^{2+}]\) increase that could be prevented by removing Ca\(^{2+}\) from the extracellular solution (Reichling et al. 1994). In rat embryonic hippocampal neurons GABA induced a \([\text{Ca}^{2+}]\) increase that could be removed with Ca-channel blockers (Obrietan and van den Pol 1995).

In cells where the GABA current reversal potential is close to the resting membrane potential this mechanism seems less likely (Marty and Llano 2005). In hippocampal slice preparations the intracellular \([\text{Cl}^-]\) increased when GABA\(_A\) receptors were activated and this was shown to lead to a less negative \text{Cl}^--\text{equilibrium potential followed by an increase in intracellular \text{Ca}^{2+}} via voltage-gated channels (Woodin et al. 2003). Another theory is that the \text{Cl}^- accumulation could lead to osmotic tension and an osmotically or mechanically induced \([\text{Ca}^{2+}]\) increase (Chavas et al. 2004).
There is also evidence that the increase is at least partially caused by Ca\(^{2+}\) release from internal stores (Chavas et al. 2004; Segal 1993). Since the [Ca\(^{2+}\)] increase in VS-3 neurons was not affected by thapsigargin, it is more likely that Ca\(^{2+}\) entered from the extracellular solution. The osmotic stress theory is also unlikely to explain Ca\(^{2+}\) influx in VS-3 neurons, since most of the depolarization is probably caused by Cl\(^{-}\) efflux rather than influx, as suggested for rat cerebellar neurons (Chavas et al. 2004). The GABA-induced depolarization recorded in the Ca\(^{2+}\) imaging experiments here would be adequate to activate LVA Ca channels in these neurons (Panek et al. 2003; Sekizawa et al. 2000). However, other mechanisms could also contribute to the [Ca\(^{2+}\)] increase.

**Ionic mechanism of presynaptic inhibition**

GABA-mediated depolarization and inhibition in spider VS-3 neurons is not exactly presynaptic, since it occurs in the peripherally located axosomatic region of these neurons rather than in the axon terminals (Gingl et al. 2004). However, the depolarization, shunting, and inhibition are very similar (Panek et al. 2002). Although the increase in Cl\(^{-}\) conductance in response to GABA\(_A\) receptor activation is generally accepted as the primary mechanism behind the primary afferent depolarization, there is still controversy about the ionic mechanism that leads to inhibition. Previous results in the VS-3 neurons indicated that inactivation of voltage-gated Na channels during GABA\_mediated depolarization and membrane “shunting” can both cause inhibition (French et al. 2006; Panek et al. 2001), agreeing with results in many other neurons (e.g., Alvarez-Leefmans et al. 1998; Cattaert and El Manira 1999). Kritz et al. (1974) suggested that in the spinal cord accumulation of K\(^{+}\) in the extracellular space could also produce depolarization leading to inhibition. Since K\(^{+}\) accumulation on the extracellular side would be prevented when K channels are blocked, our results indicate that this is not the case in the VS-3 neurons since there was no change in the amplitude of GABA-mediated depolarization when K channels were blocked. Previous modeling studies have also suggested that inactivation of voltage-gated Ca channels in response to GABA\(_A\) receptor activation may be important in presynaptic inhibition since this would reduce transmitter release (Graham and Redman 1994; Walmsley et al. 1995). Our results show that intracellular [Ca\(^{2+}\)] is actually increased by GABA-mediated depolarization in the VS-3 neurons. Similar to the spider VS-3 neurons, GABA\(_A\) and GABA\(_B\) receptors are both present in the primary afferent terminals of mammalian spinal cord (Curtis and Lacey 1994; Page and Blackshaw 1999). Although GABA\(_B\) receptor activation leads to reduction in Ca\(^{2+}\) influx in the VS-3 neurons (Panek et al. 2003) and spinal neurons (Dolphin 1995), it is a slower effect and may be designed to turn off the reaction initiated by GABA\(_A\) receptor activation, rather than be a primary cause of inhibition.

It would be interesting to learn whether [Ca\(^{2+}\)] increase occurs in other neurons with similar GABA response and to understand the major consequences of this increase. Whatever the mechanism of [Ca\(^{2+}\)] increase in VS-3, or other neurons, it has at least one significant consequence: activation of K\(_{Ca}\) channels. This is particularly interesting in a mechanosensory neuron, since interactions between currents through voltage-gated Ca channels and K\(_{Ca}\) channels are important in the frequency tuning of mechanosensory hair cells in some vertebrate inner ears (Fettiplace and Fuchs 1999). It is possible that GABA, or other transmitters, released by the efferent networks surrounding VS-3 neurons, would have more complex effects on the ways these neurons detect and process information from their environment than previously recognized.

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