Excitatory Effects of GABA on Procerebrum Neurons in a Slug

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ABSTRACT

Classical neurotransmitters, such as glutamate and gamma-aminobutyric acid (GABA), often have different actions on invertebrate neurons from those reported for vertebrate neurons. In the terrestrial mollusk *Limax*, glutamate was found to function as an inhibitory transmitter in the procerebrum (PC), but it has not yet been clarified how GABA acts in the PC. We thus examined what effects GABA exerts on PC neurons in the present study. For this purpose, we first applied GABA to isolated PC preparations and recorded postsynaptic currents and potentials in PC neurons. The GABA application reduced the amplitude of inhibitory postsynaptic currents and depolarization-induced outward currents recorded in nonbursting neurons and increased the number of spontaneous spikes of nonbursting neurons. However, direct GABA-induced currents were not observed in either bursting or nonbursting neurons. These results suggest a potential direct effect of GABA on outward currents resulting in enhanced excitability of PC neurons. Next, we measured the change in $[\text{Ca}^{2+}]_i$ in cultured PC neurons by application of GABA. The GABA application increased spontaneous $\text{Ca}^{2+}$ events in cultured neurons. These $\text{Ca}^{2+}$ events were ascribable to the influx of extracellular $\text{Ca}^{2+}$. We then confirmed the presence of GABA and GABA receptors in the PC. The GABA-like immunoreactivity was observed in the neuropil layers of the PC, and the mRNAs for both $\text{GABA}_A$ and $\text{GABA}_B$ receptors were expressed in the PC. In particular, $\text{GABA}_B$ receptor mRNA, rather than $\text{GABA}_A$, was found to be more abundantly expressed in the PC. These results suggest that GABA functions as an excitatory modulator for PC neurons via mainly $\text{GABA}_B$ receptors.

Key words: $\text{Ca}^{2+}$ influx; excitatory modulator; GABA receptors; *Limax*
INTRODUCTION

Glutamate is a common neurotransmitter in mollusks and induces a wide variety of responses in molluskan neurons. A prominent characteristic of the molluskan glutamate receptors is the prevalence of inhibitory receptors (Cleland 1996; Rozsa 1984). Glutamate-gated Cl⁻ conductance has been reported in many invertebrate neurons (Bolshakov et al. 1991; Cleland and Selverston 1995; Jones et al. 1987; King and Carpenter 1989; Marder and Paupardin-Tritsch 1978; Sawada et al. 1984) but it is uncommon in vertebrate neurons. The receptors mediating these responses are categorized as inhibitory glutamate receptors and are thought to be ionotropic receptors (Cleland 1996). On the other hand, excitatory actions of gamma-aminobutyric acid (GABA) have been found in invertebrate central nervous systems (CNSs), such as in the respiratory neural network of the pond snail *Lymnaea stagnalis* (Cheung et al. 2006), and in the feeding system of the terrestrial snail *Helix lucorum* (Bravarenko et al. 2001), and excitatory GABAergic synaptic responses emerged after pairing of exogenous GABA with postsynaptic depolarization in the photoreceptor of the marine snail *Hermissenda crassicornis* (Alkon et al. 1992).

In the terrestrial mollusk *Limax*, which has been used as a model of elucidating the cellular and molecular mechanisms underlying higher olfactory functions (Gelperin 1975; Gelperin 1999; Matsuo et al. 2010; Watanabe et al. 2008), glutamate was found to function as an inhibitory transmitter in the olfactory center, the procerebrum (PC) (Matsuo et al. 2009; Watanabe et al. 1999, 2003). The PC possesses two types of neurons, which are classified by physiological features: bursting neurons and nonbursting neurons (Kleinfeld et al. 1994; Watanabe et al. 1998; Fig. 1A). Oscillatory activity of the local field potential (LFP) in the PC is produced by a synchronous inhibition by bursting neurons, which constitute ca. 10% of the
total PC neurons (Kleinfeld et al. 1994). K⁺ conductance is activated periodically in the
spontaneous synaptic currents in nonbursting neurons (Watanabe et al. 1999). These facts
support the hypothesis that glutamate is an inhibitory transmitter underlying the synchronized
membrane potential oscillation of PC neurons (Kleinfeld et al. 1994).

However, GABAergic actions in the PC have not yet been clarified. Previous studies showed
that there are GABA-like immunoreactive (GABA-LIR) neurons in the Limax CNS (Cooke
and Gelperin 1988), but there has been no detailed physiological analysis of such neurons in
the PC. In the tentacle ganglion, the primary olfactory system of Limax, the distribution of
GABA-LIR neurons has been clarified (Ito et al. 2003). Furthermore, a modulatory role for
GABA on the spontaneous oscillatory activity of the tentacle ganglion has been suggested (Ito
et al. 2001, 2004). Based on the finding that glutamate causes inhibition in the molluskan CNS,
we hypothesized that GABA exerts excitatory actions on PC neurons in Limax similarly to
other mollusks (Ito et al. 1994), and that those GABAergic actions may be involved in the
oscillatory olfactory network of the PC.

To test our hypothesis that GABA causes an excitatory response in the PC neurons, the effects
of GABA on PC neurons were tested using a combination of different techniques including
electrophysiological and Ca²⁺ imaging experiments. We first applied GABA to isolated PC
preparations and recorded the postsynaptic currents and potentials in PC neurons using
perforated patch-clamp recordings. Next, to examine whether GABA acts as an excitatory
transmitter (or modulator) in the PC, we applied GABA to cultured PC neurons and measured
their [Ca²⁺]. We then confirmed the presence of GABA and GABA receptors in the PC by
showing the distribution of GABA-LIR in the CNS, including the PC, and by showing the
expression of mRNAs for both GABA_A and GABA_B receptors in the PC. We found that the
PC not only receives GABAAergic innervation but also contains GABAAergic neuronal cell bodies, and spontaneous neuronal activities of PC neurons are modulated by the application of GABA, although GABA-induced currents were not observed. These results suggest that GABA functions as an "excitatory modulator" for the spontaneous activity of PC neurons via Ca\(^{2+}\) influx after metabotropic GABA receptor activation.

**MATERIALS AND METHODS**

**Animals**

All experiments were performed using adult (3 - 4 months after hatching) slugs, *Limax valentianus*. For CNS isolation, slugs were anesthetized by a body-cavity injection of Mg\(^{2+}\) buffer solution containing the following (in mM): 60.0 MgCl\(_2\), 5.0 glucose and 5.0 HEPES, pH 7.0. The CNSs were isolated in ice-cold high-Mg\(^{2+}\) saline containing the following (in mM): 35.0 NaCl, 2.0 KCl, 28.0 MgCl\(_2\), 4.9 CaCl\(_2\), 5.0 glucose and 5.0 HEPES, pH 7.0. The isolated ganglia were transferred to a dish filled with a *Limax* saline solution containing the following (in mM): 70.0 NaCl, 2.0 KCl, 4.9 CaCl\(_2\), 4.7 MgCl\(_2\), 5.0 glucose and 5.0 HEPES, pH 7.0.

**Perforated Patch-Clamp Recordings**

Perforated patch-clamp recordings and whole-cell patch clamp recordings were made using previously published experimental protocols (Watanabe et al. 1999, 2003). The cerebral ganglion was isolated, and the sheath on the surface of the PC was mechanically removed with fine forceps. The posterior surface of the PC was accessible with a patch pipette (the recording sites were around the area represented by arrows in Fig. 4C). The patch-clamp
recordings were performed under a differential interference contrast (DIC) microscope (BX51WI, Olympus, Tokyo, Japan) with a 40x water-immersion objective. The pipette solution contained the following (in mM): 70.0 potassium gluconate, 5.0 MgCl₂, 5.0 HEPES (pH 7.6) and 100 - 250 µg/ml nystatin (Wako Chemicals, Osaka, Japan). In whole-cell patch recordings, EGTA 1 mM was contained with the pipette solution (without nystatin). The resistance of the pipette was 8 - 12 MΩ. A patch-clamp amplifier (Axopatch 200B, Molecular Devices, Sunnyvale, CA) was used, and the signals were recorded on a computer via an A/D converter (Digidata 1322A, Molecular Devices). In the voltage-clamp recording, the membrane potential was held at -60 mV, and the amplitude of the outward current was measured by a depolarizing step to 0 mV. The input resistance was measured by protocol (average of five consecutive steps to -80 mV, 10 ms duration). Drugs were either puff-applied locally (focal application) or bath-applied to the whole chamber (bath application). Focal application was performed by a glass micropipette placed beside the soma (1 - 2 µm tip diameter, 10 - 30 kPa, 50 - 200 ms).

LFP Recordings

LFP recordings were made using previously published experimental protocols (Kobayashi et al. 2008, 2010). LFP was recorded from the posterior surface of the PC of the isolated CNS using a glass electrode filled with the Limax saline. The signals were differentially amplified and band-pass filtered at 0.5 - 30 Hz (DAM 80, World Precision Instruments, Sarasota, FL), and were recorded on a computer via an A/D converter (Digidata 1322A, Molecular Devices). All the experiments were done at room temperature.

Cell Culture

For Ca²⁺ imaging and GABA immunocytochemistry, the PCs of the isolated CNS were
digested with 1% protease (type IX, Sigma-Aldrich, St. Louis, MO) at 34°C for 1 h. Fifty μl of the cell suspension was placed on a poly-lysine-coated glass-bottomed dish (35 mm in diameter) at the final density of two PCs per dish. Two hours later, 2 ml of the Limax saline was added to the dish, and then the cells were further cultured at 20°C for 1 - 7 d.

**Ca²⁺ Imaging**

Ca²⁺ imaging was made using previously published experimental protocols (Watanabe et al. 2003). The cells were loaded with a Ca²⁺ indicator, Oregon Green 488 BAPTA-1/AM (Invitrogen, Carlsbad, CA), which had been dissolved in the Limax saline at a final concentration of 40 μM, at room temperature for 20 min. Image acquisition was performed with an imaging system (AQUACOSMOS, Hamamatsu Photonics, Hamamatsu, Japan) equipped with an electron multiplying CCD camera (C9100, Hamamatsu Photonics) and set on a fixed stage microscope (BX51WI, Olympus). A filter set (excitation 488 nm, emission 527 nm) was used for epifluorescent illumination. Image sets were acquired every 1 s during the session of 105 s, during the last 70 s of which the drug solution was perfused.

**GABA Immunohistochemistry**

GABA immunohistochemistry was performed using previously published experimental protocols (Matsuo et al. 2009). The isolated CNSs were fixed in 4% paraformaldehyde in phosphate-buffer (PB, pH 7.6) at 4°C for 6 - 9 h. The CNSs were cryoprotected in 20% sucrose in phosphate-buffered saline (PBS) at 4°C overnight and sectioned at 14 µm using a cryostat. Following the incubation in PBS containing 0.1% Triton X-100 for 10 min, the sections were pre-incubated in dilution buffer (DB) containing 2.5% normal goat serum, 2.5% bovine serum alubumin and 0.1% TritonX-100 in PBS at room temperature for 1 h and then incubated in rabbit anti-GABA antibody (1:500 in DB; Sigma-Aldrich) at room temperature
for 1 h. After incubation in the primary antiserum, the sections were washed three times in PBS, followed by incubation in Alexa Fluor 488-conjugated secondary antibody solution (1:500 in DB; Invitrogen) at room temperature for 1 h. After three times washing in PBS, the sections were immersed in 4, 6-diamidino-2-phenylindole (DAPI) solution (0.1 μg/ml DAPI in PBS) for 10 min and mounted in VectaShield with DAPI (Vector Laboratory, Burlingame, CA). The specimens were photographed with a fluorescence microscope (BX-51, Olympus) using an NIBA filter (excitation 470-490 nm, emission 515-550 nm) or a U-NUA filter (excitation 360-370 nm, emission 420-460 nm).

For GABA immunocytochemical analysis of cultured PC cells, the cells were fixed in 4% paraformaldehyde in PB (pH 7.6) at room temperature for 30 min, incubated in PBS containing 0.1% Triton X-100, and blocked in DB at room temperature for 1 h. The cells were then incubated in rabbit anti-GABA antibody (1:500 in DB; Sigma-Aldrich) at room temperature for 1 h. After three times washing in PBS, the cells were incubated in 1:500 Alexa Fluor 488-conjugated secondary antibody solution (in DB; Invitrogen) at room temperature for 1 h. Following two times washing in PBS, the sections were incubated in 0.1 μg/ml DAPI in PBS for 5 min, and then washed in PBS again. The specimens were photographed with an inverted fluorescence microscope (IX-51, Olympus) using an NIBA filter or a U-NUA filter. For pre-absorption test, the primary antibody (1:500) was pre-incubated with 10 mM GABA in DB overnight at 4°C, and then it was applied to the sections or cells.

Molecular Cloning and Expression Analysis of GABA<sub>A</sub> and GABA<sub>B</sub> Receptors by RT-PCR

The cDNAs were derived from the total RNA extracted from the Limax CNSs. They were synthesized as described previously (Fukunaga et al. 2006). To isolate GABA receptor
cDNAs, we first performed degenerate RT-PCR. The PCR primers for the *Limax* GABA<sub>A</sub> receptor (limGABA<sub>A</sub>-R) were designed (Primer A1, A2, A3, see below) on the basis of the nucleotide sequences of GABA<sub>A</sub> receptor beta subunit cDNAs from *Lymnaea* (GenBank accession number: X58638), *Sepia* (AY005810), *Drosophila* (NM_206746) and *Caenorhabditis elegans* (CAB07719). Briefly, degenerate hemi-nested RT-PCR was performed using Primer A1 and Primer A3 following the first degenerate RT-PCR using Primer A1 and Primer A2. The PCR primers for the *Limax* type 1 GABA<sub>B</sub> receptor (limGABA<sub>B</sub>-R) were designed (Primer B1, B2, see below) on the basis of the nucleotide sequences of type 1 GABA<sub>B</sub> receptor cDNAs from *Aplysia* (EB321140), *Apis* (XM_392294), *Drosophila* (AAF53431) and *Danio* (XM_689405). Based on the nucleotide sequences of the partial cDNA fragments, 5’- and 3’-RACE (Takara, Ohtsu, Japan) were performed using Primer A4-A7 (for limGABA<sub>A</sub>-R) and Primer B3-B5 for limGABA<sub>B</sub>-R according to the manufacturer’s instructions. The nucleotide sequences of limGABA<sub>A</sub>-R and limGABA<sub>B</sub>-R were registered in GenBank (accession numbers: AB473944 and AB477969, respectively). The nucleotide sequences of the PCR primers for limGABA<sub>A</sub>-R (Primer A1-A7) and limGABA<sub>B</sub>-R (Primer B1-B5) were as follows:

**Primer A1**

5’-ATGGA(T/C)TA(T/C)AC(C/G)AT(C/A)AC(C/A)ATGTA(T/C)CT(G/T)AA(T/C)CA(G/A)TA(T/C)TGG-3’

**Primer A2**

5’-GC(A/T/G)GC(A/G)AA(C/G)AC(A/G)AA(A/G/C)AC(A/G)AA(A/G)CACAT(C/G)ACCAG-3’

**Primer A3**

5’-GC(T/C)TC(A/G)TG(A/G)TT(A/G)ATCCA(A/G)AA(A/C)GAGACCCA(T/G/C)GA(C/G)AGCAT-3’
Primer A4
5’-CCAGTGGGTAGTTGTGAAGGTCCATCATGCAGGC-3’

Primer A5
5’-CCGTTGCCATAGAGACGCACCATCTTGTTC-3’

Primer A6
5’-CGACCAAGCTTTGCGGGGCTCCTCTCTAGAG-3’

Primer A7
5’-CGAGGTTCATCCTAGCCAGCTTTGACAGC-3’

Primer B1
5’-GACTCCCG(G/C)TTTGTGGGCATG-3’

Primer B2
5’-(A/G)AT(A/T)ATGGC(G/C)AGTGAGAC(A/G)AA(A/G)GC(A/G)AA-3’

Primer B3
5’-CCCAGCACACACTTACCCACTTGTCTGTCAGG-3’

Primer B4
5’-GAGAC(A/G)AAAGGCAAATGTGGCATCTTCCTGGTTGCC-3’

Primer B5
5’-GGGAGATCGGTGCAGTGATCACACAAAACCAC-3’

For the expression analysis, the cDNAs from three CNS regions, i.e., the PC, the cerebral ganglia without the PC, and the subesophageal ganglia, were prepared as described previously (Matsuo and Ito 2009). The PCR primers A5, A7, and B3, B5 were used to amplify limGABA_A-R and limGABA_B-R cDNAs, respectively. The PCR products were dissolved by electrophoresis in a 1% (w/v) agarose gel, and visualized using ethidium bromide under a UV illuminator.
Quantitative Real-Time PCR

The method for RNA analysis was modified from the previous report (Sadamoto et al. 2010). RNA was extracted using an RNAqueous-Micro kit (Applied Biosystems/Ambion, Foster City, CA) and treated with DNase I (Ambion). Ninety ng of total RNA was used for 20 μl of the reverse transcription reaction with M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA) and random primers, according to the manufacturer's instructions. Quantitative real-time PCR was performed with EXPRESS SYBR® GreenER™ qPCR SuperMix (Invitrogen) and analyzed with the 7900HT Fast Real-Time PCR System (Applied Biosystems). The primer sequences were as follows:

limGABA<sub>R</sub> forward 5’-CATGACTTTGACCGGAGCGTTTG-3’,
limGABA<sub>R</sub> reverse 5’-GCCATAAACCAGTGAGCCGTTG-3’,
limGABA<sub>B</sub> forward 5’-CACAGCCTGGCAAGTTTTAGATCC-3’,
limGABA<sub>B</sub> reverse 5’-CTGTGGCAGGAGTTCAATGTCC-3’.

A calibration curve was obtained using serial dilutions of plasmid DNA carrying either a cloned limGABA<sub>A</sub>-R cDNA or a limGABA<sub>B</sub>-R cDNA.

Statistical Analysis

The data were statistically analyzed by paired t-test and two-way ANOVA (or Friedman test) with the post hoc Scheffé test with a significance level of $P < 0.05$. The data are listed as the mean ± S.E. throughout.

RESULTS
We first examined the effects of a focal application of GABA on spontaneous excitatory postsynaptic currents (EPSCs, Fig. 1B, top) and excitatory postsynaptic potentials (EPSPs, Fig. 1C) of bursting neurons and on spontaneous inhibitory postsynaptic currents (IPSCs, Fig. 1B, bottom) and inhibitory postsynaptic potential (IPSPs, Fig. 1D) of nonbursting neurons by using voltage-clamp or current-clamp recordings. In bursting neurons, small effects were observed on spontaneous EPSCs or EPSPs by the application of 1 mM GABA (Fig. 1B, C). On the other hand, in nonbursting neurons, the application of 1 mM GABA decreased the amplitude of spontaneous IPSCs and IPSPs, but it did not change the frequency of such spontaneous synaptic events (Fig. 1B, D).

To confirm these GABAergic effects, we bath applied 1 mM GABA while the PC neuron was current-clamped. We observed an increase in the both the number of spikes and EPSPs in bursting neurons (Fig. 1E) that was thought to be caused by increments of excitatory inputs from nonbursting neurons. We next recorded the spontaneous activity of nonbursting neurons and found that bath application of 1 mM GABA resulted in an increase in the number of spikes in nonbursting neurons (Fig. 1F). In voltage-clamp recordings, the amplitude of spontaneous IPSCs in nonbursting neurons was significantly decreased 1 min after GABA application in a dose-dependent manner (10 μM, 85.5 ± 8.8%, n = 14, P < 0.05 vs. control, paired t-test; 100 μM, 74.9 ± 4.0%, n = 13, P < 0.01; 1 mM 74.2 ± 3.9%, n = 11, P < 0.01; Fig. 1G). In bursting neurons, the amplitude of the spontaneous EPSC was slightly decreased by GABA at the higher concentration (1 mM, 82.3 ± 5.8%, n = 11, P < 0.05 vs. control, paired t-test; Fig. 1G). In current-clamp, the number of spontaneously generated spikes in nonbursting neurons was significantly increased 1 min after 1 mM GABA application (Control, 6.3 ± 5.2 spikes/min; GABA, 93.2 ± 37.8 spikes/min; Wash, 11.0 ± 8.4 spikes/min;
n = 8 each, P < 0.01, Friedman test + post hoc Scheffé test, Fig. 1H). The resting membrane potentials were not changed (Control, 100%; GABA 1 mM, 99.1 ± 1.3%; Wash, 99.4 ± 2.2%; n = 8 each, P > 0.01, two-way ANOVA + post hoc Scheffé test). Direct GABA-induced currents were not observed in either bursting or nonbursting neurons.

However, in whole cell recordings in nonbursting neurons, the depolarization-induced outward current of these neurons was significantly decreased by GABA application (Control 83.5 ± 8.8 pA; 1 mM 76.9 ± 8.2 pA; Wash 6.7 ± 9.5 pA; P < 0.01, two-way ANOVA + post hoc Scheffé test; n = 12 each, Fig. 1 I and J, top). Application of vehicle alone had no effect on the depolarization-induced outward current of nonbursting neurons (Control 84.6 ± 3.6 pA; Vehicle 85.4 ± 3.1 pA; Wash 83.9 ± 3.6 pA; P > 0.01, two-way ANOVA + post hoc Scheffé test; n = 9 each). Furthermore, GABA application increased input resistance in nonbursting neurons (Control 100%; 1 mM 118.6 ± 4.8%; Wash 124.5 ± 10.5%; P < 0.01, two-way ANOVA + post hoc Scheffé test; n = 6 each). Application of vehicle alone had no effect on the input resistance in nonbursting neurons (Control 100%; Vehicle 99.2 ± 3.4%; Wash 100.1 ± 4.1%; P > 0.01, two-way ANOVA + post hoc Scheffé test; n = 9 each). GABA did not alter the depolarization-induced outward currents of bursting neurons (Control 77.0 ± 11.1 pA; 1 mM 76.9 ± 12.0 pA; Wash 76.3 ± 12.1 pA; P > 0.01, two-way ANOVA + post hoc Scheffé test; n = 11 each, Fig. 1I and J, bottom). All measurements of the membrane properties in PC neurons were performed during the intervals between the periodic synaptic inputs to avoid any effects of synaptic activity.

These effects suggest that GABA functions as an "excitatory modulator" in the PC. The presumable action sites of GABA in the PC are summarized in Fig. 6 (see Discussion section). On the other hand, the presumable action sites of GABA may not be confined to the synaptic...
terminal, because GABAergic action at perisomatic sites is well-known in other species (Mann and Paulsen 2007). This possibility is examined in the following experiments.

**Increase of Ca\(^{2+}\) Transients in Cultured PC Neurons by GABA Application**

To determine if GABA act as a neuromodulator on perisomatic sites, we tested the effects of GABA on [Ca\(^{2+}\)]\(_i\) of cultured PC neurons. Although there seem to be no synaptic connections between cultured PC neurons (1 - 7 d after culture), we observed spontaneous Ca\(^{2+}\) transients in a subpopulation of cultured PC neurons in the normal saline as reported previously (Rhines et al. 1993). Bath application of GABA at a low concentration (10 \(\mu\)M; Fig. 2A) to such a subpopulation of PC neurons increased the number of spontaneous Ca\(^{2+}\) transients (Control, 1.4 ± 0.1 times/min; 10 \(\mu\)M GABA application, 2.6 ± 0.3 times/min; \(P < 0.01\); \(n = 132\) cells each; Fig. 2B). However, in the other cultured PC neurons that did not exhibit spontaneous Ca\(^{2+}\) transients, bath application of GABA at the low concentration (10 \(\mu\)M) had no effects; but there was an effect at the high concentration (1 mM). The high concentration of GABA increased the basal level of intracellular Ca\(^{2+}\) concentration and also evoked Ca\(^{2+}\) transients (Fig. 2C).

Ca\(^{2+}\) transients in cultured PC neurons induced by 10 \(\mu\)M GABA were abolished in the presence of a Ca\(^{2+}\) chelator (1 mM EGTA) or a cocktail blockers for voltage-sensitive Ca\(^{2+}\) channels (Co\(^{2+}\), Ni\(^{2+}\) and Cd\(^{2+}\), 1 mM each) (Fig. 2D). The spontaneous Ca\(^{2+}\) oscillations also disappeared in the presence of these drugs (data not shown). That is, the Ca\(^{2+}\) transients in PC neurons were caused by influx of Ca\(^{2+}\) from the extracellular space. In addition, we found that a bath application of a GABA\(_A\) or a GABA\(_B\) receptor antagonist, bicuculline or phaclofen (200 \(\mu\)M each), inhibited the GABA-induced Ca\(^{2+}\) transients in some cultured PC neurons (Fig. 2E, F). However, there were both bicuculline-insensitive and the phaclofen-insensitive
cultured PC neurons. In some bicuculline-insensitive PC neurons (Fig. 2G), the GABA-induced Ca\textsuperscript{2+} transients in the presence of bicuculline were abolished by a phaclofen application, whereas the application of bicuculline did not abolish the GABA-induced Ca\textsuperscript{2+} transients in the phaclofen-insensitive PC neurons (Fig. 2H). A low concentration of GABA (10 μM) was bath-applied in these experiments (Fig. 2D-H). GABA responsive cells were made up 35% (97 of 376 cells) of the total number of cultured PC neurons, and bicuculline sensitive cells were 31% (20 of 64 cells) and phaclofen sensitive cells were 48% (44 of 92 cells) of total number of GABA responsive cells. These results showed that the GABA-induced Ca\textsuperscript{2+} transients in PC neurons may be mediated by a receptor that is related to GABA\textsubscript{A} receptors, GABA\textsubscript{B} receptors or both in most GABA responsive neurons.

**GABA-like Immunoreactivity in the CNS**

To confirm that GABA is present in the PC, we stained the cryostat sections of the Limax CNS with the anti-GABA antibody. Previously, Cooke and Gelperin (1988) found GABA-LIR neurons and fibers in the Limax CNS using the whole-mount preparations, but they did not pay attention to such GABA-LIR signals in the PC.

The PC consists of the three layers: the cell mass layer (CM, cell body region, the lateral and the dorsal side of the PC), the terminal mass layer (TM, neuropil region, the middle layer of the PC) and the internal mass layer (IM, neuropil region, the ventral side of the PC). In the sagittal sections, we found two types of GABA-LIR fibers in the neuropil regions of the PC (Fig. 3). One was the thick GABA-LIR bundle fibers innervating the TM, and the other one was a small number of nerve fibers innervating the IM (Fig. 3A, B). The thick bundle in the TM may have originated from cluster neurons in the ventral side of the cerebral ganglion (Cooke and Gelperin, 1988), and its terminal in the TM had fine varicose processes (Fig. 3C,
The fibers in the IM also possessed varicose processes in the terminal (Fig. 3C, arrowhead). These GABA-LIR fibers were observed only in the most medial region, i.e. the basal region of the PC (Fig. 3D).

The CM contains the cell bodies of a large number of nonbursting neurons, whose diameters are generally small (5 - 8 μm) (Ratté and Chase 1997; Watanabe et al. 1998). These nonbursting neurons projecting to the neuropil layers are thought to contain the input fibers from the tentacle nerves and the output fibers to the cerebral ganglia. On the other hand, a small number of bursting neurons (> ca. 10 μm diameter) are situated within and project into the cell body layer (Watanabe et al. 1998). We found GABA-LIR fibers in the TM and IM (Fig. 4A, B) and weakly stained GABA-LIR cell bodies in the CM (Fig. 4C, D).

Pre-absorption experiments showed substantially weaker GABA-LIR signals in the cerebral ganglion with the PC (Fig. 4E-H). In cultured PC neurons, a few GABA-LIR cells were also found (2 - 5% in the PC, Fig. 4I-K), whereas GABA-LIR cells were not found in cultured PC neurons in pre-absorption experiments (Fig. 4L-N). We found a large GABA-LIR cell with two neurites (Fig. 4O-Q). This cell has not been previously identified.

We found other GABA-LIR cells and fibers in the Limax CNS. The staining results were consistent with the previous report on Limax maximus (Cooke and Gelperin 1988). In the cerebral ganglia, two large GABA-LIR cells and two clusters of small GABA-LIR cells were observed on the ventral surface, and one large GABA-LIR cell and two clusters of small GABA-LIR cells were observed on the dorsolateral region, which is adjacent to the basal region of the PC (data not shown). Many GABA-LIR fibers were observed in all the interganglionic connectives and commissures, whereas most of the peripheral nerves or the connective tissue sheath surrounding the ganglia did not contain any immunostaining signals.
In each pedal ganglion, one immunostained cluster was located on the anterior ventral surface, and the other was located more posteriorly on the lateral surface of the ganglion. No staining was found in the cell bodies of the pleural, parietal, or visceral ganglia. The circular projection of the immunostained neuropils was found inside each of the cerebral ganglia in the coronal sections.

Expression of $\text{GABA}_A$ Receptor Beta Subunit and Type 1 $\text{GABA}_B$ Receptor in the PC

Finally, we confirmed the existence of GABA receptors in the PC. We isolated the lim$\text{GABA}_A$-R beta subunit and lim$\text{GABA}_B$-R type 1 subunit by a degenerate RT-PCR. We found that the deduced amino acid sequences of lim$\text{GABA}_A$-R and lim$\text{GABA}_B$-R are relatively conserved during evolution (see the GenBank accession numbers: AB473944 and AB477969). RT-PCR with cDNAs derived from the PC, the cerebral ganglia (without the PC) and the subesophageal ganglia (including parietal, visceral, pedal and pleural ganglia) revealed that the lim$\text{GABA}_A$-R beta subunit was expressed strongly in the subesophageal ganglia but weakly in the PC, whereas lim$\text{GABA}_B$-R expression in the PC was comparable to that in the subesophageal ganglia but low in the cerebral ganglia (Fig. 5A).

To compare the expression levels of the lim$\text{GABA}_A$-R beta subunit and type 1 lim$\text{GABA}_B$-R quantitatively, we used real-time PCR to determine the copy numbers of the gene transcripts in the PC. The expression level of the $\text{GABA}_B$ receptor was 20-fold higher than that of the $\text{GABA}_A$ receptor (cDNA copy number/1 ng total RNA; lim$\text{GABA}_A$-R beta, $214.3 \pm 40.9$; lim$\text{GABA}_B$-R type 1, $4715.4 \pm 1053.0$; $P < 0.05$; $n = 5$ animals each; Fig. 5B). The ratio of cDNA copy numbers ($\text{GABA}_A$ receptor/$\text{GABA}_B$ receptor = 0.047) was not largely different among the 5 slugs used (S.E. = 0.003). Note that a lower value of standard error indicates a more accurate estimation of the cDNA copy numbers.
DISCUSSION

In the present study, we found GABAergic modulation on the PC neurons, and three effective sites of GABA (see GABA 1-3 in Fig. 6) were suggested: (1) synaptic depression on the inhibitory inputs to nonbursting neurons; (2) enhancement of perisomatic membrane excitability in the PC neurons; (3) synaptic modulation on the input and output neuropil layer of the PC. Although these three effective sites may play distinct modulatory roles in the PC networks, the present results suggest that GABAergic effects result in the increased excitability or spike activity in nonbursting neurons.

The results from single-cell patch-clamp recordings of PC neurons as shown in Fig. 1 indicate that GABA acts primarily on nonbursting neurons. The inhibitory synaptic inputs from bursting neurons to nonbursting neurons and depolarization-induced outward currents of nonbursting neurons were depressed by GABAergic modulation. The depression in these synapses and outward currents may have resulted in an increase in the spike frequency of nonbursting neurons, causing an enhancement of the excitatory inputs (additional EPSPs and/or spikes) onto bursting neurons (Fig. 1E, F). Because bursting neurons project those neurites within the CM (Watanabe et al. 1998), the inhibitory synaptic connection from bursting neurons to nonbursting neurons is limited to the CM. This indicates that one of the presumable sites of GABAergic modulation in the PC neuron networks is the synapses from bursting neurons to nonbursting neurons in the CM (see GABA 1 in Fig. 6).

In a previous study by Rhines et al. (1993), loose-patch whole-cell recordings of cultured PC
neurons revealed that isolated and individual neurons can express spontaneous spike activity. This result, together with those of the present study, leads us to speculate that some of neurons without synaptic connection responded to GABA (Fig. 2A). In addition, we showed that extracellular Ca\(^{2+}\) plays an essential role in the generation of spontaneous Ca\(^{2+}\) activity in cultured PC neurons (Fig. 2D). Spontaneous fluctuations in Ca\(^{2+}\) activity is thought to reflect a spontaneous spiking activity in cultured PC neurons. In the present study, GABA increased the level of spontaneous Ca\(^{2+}\) activity in cultured PC neurons (Fig. 2A-C), whereas GABA application did not evoke any currents in the voltage-clamp recordings from single PC neurons (Fig. 1C, D). These results suggest that the GABA-induced increase in Ca\(^{2+}\) activity of cultured PC neurons corresponds to a GABA-induced spike increase in nonbursting neurons, and that GABA may change the membrane excitability or spike threshold of nonbursting neurons without any synaptic currents (Ito et al. 1994; Muzzio et al. 2001; Sakakibara et al. 2006). These results, in turn, suggest that the GABAergic modulation occurs at the level of the somata of PC neurons. Moreover, the results from Figure 1 show that GABA influences mainly nonbursting neurons. Taken together, these findings suggest that the other presumable sites of the GABAergic modulation are around the cell bodies of nonbursting neurons in the CM (see GABA 2 in Fig. 6).

A small number of weakly staining GABA-LIR cell bodies were observed in the cell body layer of the PC—i.e., the CM—and GABA-LIR neurons with their neurites were observed in cultured PC neurons (Fig. 4). These GABA-LIR neurons in the CM may be involved in the intrinsic GABAergic modulation in the neuronal activity of the PC. Because the inhibitory synaptic connections from bursting neurons to nonbursting neurons are limited to the CM (Watanabe et al. 1998), it is thought that a modulatory site of the spontaneous synaptic activity is localized in the area where the cell bodies of nonbursting neurons reside around the
cell bodies of nonbursting neurons. This also suggests that the sites of GABAergic modulation are in the CM (see GABA 1, 2 in Fig. 6).

The roles played by neuropil layers, TM and IM, in the PC have been suggested to be as follows: TM receives most of the inputs from the olfactory nerves (Kawahara et al. 1997), and IM predominantly possesses the output synapses of the PC (Ratte and Chase 2000). In GABA immunostaining, the extrinsic GABA-LIR fibers from the cerebral ganglion were found in the TM and IM of the PC (Figs. 3 and 4). In the electrophysiological analysis, suppressive effects of GABA on the spontaneous PSCs were larger in nonbursting neurons innervating TM and IM than in bursting neurons of the CM (Fig. 1). Taken together, these results lead us to propose that GABA-LIR fibers in the TM and IM may contribute modulatory effects to the input and output networks of the PC (see GABA 3 in Fig. 6). These two effective sites (that is, in the IM or TM; and the CM) may play distinct modulatory roles in the PC networks, and if these presumable sites are really effective, GABAergic effects may be reflected in the modulation on the input synapses of nonbursting neurons.

We found that a GABA\textsubscript{A} and a GABA\textsubscript{B} receptor blocker (bicuculline and phaclofen) had antagonizing effects on this GABA-induced increase in Ca\textsuperscript{2+} transients in cultured PC neurons (Fig. 2E-G). Furthermore, we succeeded in cloning a GABA\textsubscript{A} beta subunit and type 1 GABA\textsubscript{B} receptor that had highly similar amino acid sequences to the mammalian receptors (Casalotti et al. 1986; Jensen et al. 2002; Jones et al. 1998). By use of RT-PCR and real-time PCR, we found that the expression level of the GABA\textsubscript{B} receptor was higher than that of the GABA\textsubscript{A} receptor in the PC (Fig. 5). The application of GABA receptor antagonists on cultured PC neurons suggested that synaptic modulation via GABA\textsubscript{B} receptors was predominant in the neuronal activity of the PC (Fig. 2G, H). Activation of GABA\textsubscript{B} receptors modulates K\textsuperscript{+}
channel conductance via a G-protein activation in another mollusk, *Hermissenda* (Ito et al. 1994; Muzzio et al. 2001; Sakakibara et al. 2006). Thus, in *Limax*, K\(^+\) conductance alteration by GABA may underlie the change in the membrane excitability of PC neurons without eliciting any GABA-induced currents on the PC neurons. In nonbursting neurons, the spontaneous IPSCs and depolarization-induced outward currents are thought to be the outward K\(^+\) currents (Watanabe et al. 1999). Considering these results together, the GABA-induced decrements in K\(^+\) conductance and the increased input resistance may contribute to the enhanced membrane excitability and spike activity. As shown in Figure 1I, J, GABA-induced decrements in K\(^+\) conductance and the increased input resistance in nonbursting neurons were still observed 5 min after washout with saline. This irreversible effect of GABA may be caused by a long-lasting change in the K\(^+\) conductance, like that observed in the neuronal plasticity of type B photoreceptors via metabotropic GABA\(_B\) receptors in the classical conditioning of *Hermissenda* (Ito et al. 1994; Muzzio et al. 2001).

Furthermore, if GABA\(_A\) activation increases the Cl\(^-\) conductance as in the case of mammalian neurons, it may have excitatory effects on the membrane potential of PC neurons, because the equilibrium potential of Cl\(^-\) is more positive than the resting membrane potential in *Limax* PC neurons (Watanabe et al. 1999, 2003). The depolarizing effects of GABA and those of glutamate are caused by the higher Cl\(^-\) equilibrium potential in PC neurons, and these features in the *Limax* PC seem to be similar to those in the immature mammalian brain (Ben-Ari 2001; Ben-Ari et al. 1989; Owens et al. 1996). In any case, the enhancement of excitability and the increase in spikes in nonbursting neurons will result in the enhancement of the firing probability in bursting neurons, a pacemaker neuron in the oscillatory activity of the PC (Kleinfeld et al. 1994; Watanabe et al., 1998).
The PC was demonstrated to be the higher olfactory center in *Limax* (Gelperin 1999; Kasai et al. 2006; Matsuo et al. 2010). The GABAergic modulation found in the present study may be involved in the fine tuning of olfactory information processing through the modulatory action on nonbursting neurons, which are the major constituent of the PC.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).
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FIGURE LEGENDS

FIG. 1. GABAergic effects on spontaneous postsynaptic currents and potentials in PC neurons. A: Schematic diagram illustrating the PC network. Open circles and closed circles indicate excitatory synaptic connections and inhibitory synaptic connections, respectively. Output, output fibers from the PC; input, input fibers to the PC; B, bursting neurons; NB, nonbursting neurons; CM, cell mass layer; TM, terminal mass layer; IM, internal mass layer. B: Representative voltage-clamp recordings and effects of focal application of GABA (1 mM, arrowheads) on spontaneous synaptic currents in nonbursting neurons (indicate as NB) and bursting neurons (indicate as B). The holding potentials in voltage clamp recordings were -80 mV (bursting neuron) and -20 mV (nonbursting neuron). The two traces were not recorded simultaneously. C, D: Effects of focal application of GABA on spontaneous synaptic potentials (bottom) in bursting neurons (C) or nonbursting neurons (D). The resting membrane potentials in current clamp recordings were -50 mV (C) and -60 mV (D). The dotted lines indicate the peak level of the PSPs in both neurons (C, D). E, F: Current clamp recordings in bursting neurons (E) and nonbursting neurons (F). Ei: Spontaneous firings and EPSPs of bursting neurons (indicated as B) were recorded with the local field potentials (indicated as LFP). Each of the three traces is a series of simultaneous recordings in the currents of bursting neurons and the LFP. Eii: GABA (1 mM) bath-application for 1 min produced additional EPSPs (arrowheads) and firings (arrows). Eiii: The effects by GABA were abolished by wash out (about 5 min). Fi: Data for nonbursting neurons recorded in the same way as for bursting neurons. The spontaneous IPSPs and a firing (arrow) of nonbursting neurons were recorded with the LFPs. Fii: GABA bath-application (1 mM) for 1 min produced additional firings (arrows) but not for IPSPs. Fiii: Effects by GABA were abolished by wash out (about 5 min); that is, the firings disappeared. G: Summarized data of
GABAergic effects on PSC amplitudes of PC neurons. The IPSC amplitudes of nonbursting (NB) neurons were significantly decreased by GABA bath-application in a dose dependent manner (* $P < 0.05$, **$P < 0.01$ vs. control, 10 μM GABA, $n = 14$; 100 μM, $n = 13$; 1 mM, $n = 11$). The EPSC amplitudes of bursting (B) neurons were significantly decreased by 1 mM GABA application ($n = 11$). H: Changes in the spike number of nonbursting neurons after 1 min GABA application. The spike number of nonbursting neurons was significantly increased by GABA application (1 mM, **$P < 0.01$, $n = 8$). I: The depolarization-induced outward currents of nonbursting neuron and bursting neuron. The outward currents were observed when PC neurons were depolarized from holding potential (-60 mV) to 0 mV. Spontaneous IPSCs in nonbursting neurons are truncated (top traces). The dotted lines indicate the peak level of the outward currents in the control recordings. J: Changes in the depolarization-induced outward currents (arrow) of nonbursting neurons after 1 min GABA application. Although the outward currents of bursting neurons were not significantly changed by GABA application (1 mM, $n = 11$, bottom), the outward currents of nonbursting neurons were significantly decreased by GABA application (1 mM, **$P < 0.01$; $n = 11$, top).

FIG. 2. Changes in [Ca$^{2+}$] in cultured PC neurons by application of GABA. A: GABA application (10 μM) increased the number of Ca$^{2+}$ transients in spontaneously oscillating PC neurons. Each colored trace represents the recording from a single cultured PC neuron. B: Summarized data of GABAergic effects on the spontaneous Ca$^{2+}$ activity of cultured PC neurons. GABA (10 μM) increased the level of spontaneous activity for one minute (**$P < 0.01$; paired $t$-test; $n = 132$ cells each). C: When spontaneous Ca$^{2+}$ transients were not observed, GABA application (1 mM) increased the basal [Ca$^{2+}$], and induced Ca$^{2+}$ transients in PC neurons. D: Effects of Ca$^{2+}$-free saline on GABA-evoked Ca$^{2+}$ transients in PC neurons. These Ca$^{2+}$ transients in PC neurons were abolished in the Ca$^{2+}$-free saline that included...
EGTA (1 mM) or divalent cations (Co$^{2+}$, Ni$^{2+}$ and Cd$^{2+}$, 1 mM each). *E-H*: Effects of GABA$_A$ and GABA$_B$ receptor antagonists on GABA-evoked Ca$^{2+}$ transients in PC neurons. In some cultured PC neurons, the Ca$^{2+}$ transients disappeared 10 min after the application of 200 μM bicuculline (*E*) or phaclofen (*F*). *G*: Bicuculline-insensitive PC neurons. GABA-evoked Ca$^{2+}$ transients in the presence of bicuculline (200 μM, 10 min) were abolished after the application of phaclofen (200 μM, 10 min). *H*: Phaclofen-insensitive PC neurons. GABA-evoked Ca$^{2+}$ transients in the presence of phaclofen (200 μM, 10 min) were not abolished, but those frequencies and amplitudes were slightly decreased after the application of bicuculline (200 μM, 10 min).

**FIG. 3.** GABAergic innervation into the terminal mass (TM) layer and the internal mass (IM) layer in the PC. *A*: Immunohistochemical staining of the section of the PC and the cerebral ganglion by anti-GABA antibody. *B*: Nuclear staining with DAPI. The cell mass (CM) layer is observed in the dorsal side. *C*: A magnified view of GABA immunohistochemistry of the PC (the dotted box areas in Fig. *A* and *B*). An arrow and an arrowhead indicate the GABAergic innervations of the TM and IM layers, respectively. *D*: A schematic drawing of the *Limax* CNS. A blue dotted line indicates the sectioning plane in *A-C*. A, anterior; P, posterior; L, left; R, right. Scales: 200 μm.

**FIG. 4.** Immunohistochemistry and immunocytochemistry of GABA. *A*: Immunohistochemistry of GABA in the coronal section of the PC. An arrowhead indicates the GABAergic innervation into the IM layer in the PC. *C*: A magnified view of the CM layer in the PC. An arrow indicates the GABAergic somata of the CM layer in the PC (the arrowed areas also indicate the patch clamp recording sites in Fig. 1). *E, G*: The immunostaining images of the sections stained by the pre-absorbed GABA antibody. *B, D, F, H*: Nuclear
staining with DAPI. V, ventral; D, dorsal; L, lateral; M, medial. Scales: 200 μm. I, O:

Immunocytochemical staining of GABA in the dissociated culture of PC neurons. Arrows indicate the positively stained neurons. L: An immunostaining image of the cells stained by the pre-absorbed GABA antibody. J, M, P: Nuclear staining with DAPI. K, N, Q: The corresponding DIC images. Scales: 50 μm.

FIG. 5. Expression of limGABA<sub>A</sub>-R beta and limGABA<sub>B</sub>-R1 in the Limax CNS. A:

Expression of limGABA<sub>A</sub>-R beta and limGABA<sub>B</sub>-R1 mRNAs was examined in the PC, the cerebral ganglia (without PC) and the subesophageal ganglia (SEG). 18S rRNA served as an internal control for the amounts of template cDNAs. B: Real-time PCR analysis was performed to assess quantitatively the level of Limax GABA<sub>A</sub> and GABA<sub>B</sub> receptor gene expression in the PC. There was a 20-fold greater level of GABA<sub>B</sub> receptor gene expression than GABA<sub>A</sub> receptor expression (*P < 0.05; paired t-test; n = 5).

FIG. 6. Presumable sites of GABAergic modulation (GABA 1-3) in the PC network. Open circles with “-” and “+” indicate inhibitory and excitatory synaptic modulation by GABA, respectively.
**Figure Legend**

**A** Diagram showing the cell mass layer (CM) and neuropil layer (TM and IM).

**B** Illustration of bursting neuron (NB) and nonbursting neuron (iB).

**C** Bursting neuron recording showing the effects of GABA 1 mM.

**D** Nonbursting neuron recording showing the effects of GABA 1 mM.

**Ei** (Control)

**Bi** (Control)

**Li** (Control)

**Eii** (GABA)

**Bii** (GABA)

**Li** (GABA)

**Eiii** (Washed)

**Biii** (Washed)

**Li** (Washed)

**G** Graph showing amplitude changes in PSCs (%) for NB and B neurons.

**H** Bar graph showing number of spikes/min for NB and B neurons.

**I** Graph showing the effects of GABA and Wash on NB and B neurons.

**J** Bar graph showing depolarization-induced outward currents (nA) for NB and B neurons.
A

PC  CG  SEG

GABA_\text{A}R \beta
GABA_\text{A}R1
18S rRNA

B

Copy number / 1 ng total RNA

GABA_\text{A}R \beta  GABA_{\text{A}R1}

*
Cell mass layer (CM)
Neuropil layer (TM and IM)

GABA 1
GABA 2
GABA 3

Neuroplil ganglion

GABA 1
GABA 2
GABA 3

output
input