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PKA-Dependent Regulation of Synaptic Enhancement between a Buccal Motor Neuron and Its Regulatory Interneuron in *Lymnaea stagnalis*

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ABSTRACT—The cerebral giant cell (CGC) is known to play a crucial role in the regulation of feeding response in the pond snail, *Lymnaea stagnalis*. However, the mechanisms of signal transduction from the CGC to the following buccal motor neurons are not clear. In the present study, we examined whether cyclic AMP (cAMP)-dependent protein kinase (PKA) contributes to enhancement of a monosynaptic connection between the presynaptic CGC and the postsynaptic buccal motor neuron 1 (B1 cell). Injection of cAMP into the CGC or inhibition of phosphodiesterase by isobutylmethylxanthine in the CGC increased the amplitude of excitatory postsynaptic potential (EPSP) in the B1 cell, whereas no changes were detected in the electrical properties of the CGC. The synaptic enhancement in the B1 cell was completely blocked by inhibition of PKA in the CGC but did not require a *de novo* protein synthesis due to a PKA phosphorylation. The increase in the EPSP amplitude of B1 cell was associated with the increase in the amount of serotonin release from the CGC. These results hence provided the physiological evidence of the direct regulation of a synaptic enhancement by PKA in the CNS of *L. stagnalis*, indicating the completely different mechanism from that in the well-studied siphon- and gill-withdrawal reflex in *Aplysia*.

INTRODUCTION

The neural system for feeding response in the pond snail, *Lymnaea stagnalis*, has been studied by physiological, histological and ethological approaches (Yeoman *et al*., 1996; Kobayashi *et al*., 1998; Sadamoto *et al*., 1998; Staras *et al*., 1998; Nakamura *et al*., 1999a, b). One of interneurons, which plays a crucial role in the regulation of feeding response, is a cerebral giant cell (CGC). The CGC has a gating and enabling function in the central pattern generator (CPG) for feeding response (Yeoman *et al*., 1994). The depolarization of the CGC excites and/or inhibits the CPG interneurons, influencing the duration of radula protraction and that of rasping (Yeoman *et al*., 1996). Some synaptic connections from the CGC to the directly following buccal motor neurons are also involved in the formation of feeding behavior (Kemenes *et al*., 1986; McCrohan and Audesirk, 1987; Elphich *et al*., 1995). Further, the CGC receives appetitive and aversive taste signals from the lip and tentacles through their own nerves (Nakamura *et al*., 1999a, b).

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The regulation of feeding response is varied via some synaptic enhancement in the feeding circuitry by a conditioning. *L. stagnalis* can be readily conditioned to inhibit feeding response to a previously appetitive stimulus, when repeatedly and specifically paired with an aversive reinforcement (Kojima *et al*., 1996, 1997, 1998; Yamanaka *et al*., 1999). This is referred to as a 'conditioned taste aversion'. In this conditioning, inhibitory postsynaptic potential (IPSP) in a CPG interneuron by activation of the CGC was larger and lasted longer in the conditioned snails than in the control snails (Kojima *et al*., 1997). However, the mechanisms of signal transduction in this synaptic enhancement have not yet been determined. In general, the studies of the second-messenger system in *L. stagnalis* have not been advanced very far (Dreijer and Kits, 1995). Only the work by McCrohan and Gillette (1988) suggested that cyclic AMP (cAMP) is a candidate of the second messenger in the CGC by demonstrating the appearance of Na^+ current by the cAMP injection. The roles of cAMP have been extremely studied in synaptic enhancements in *Aplysia*. Intracellular cAMP in the sensory neuron plays an important role in the siphon- and gill-withdrawal reflex (Ocorr *et al*., 1985; Pollock *et al*., 1985; Schacher *et al*., 1988; Klein, 1993). In addition, the cAMP-dependent protein kinase (PKA) pathway and the cAMP-response element binding protein (CREB) in

the siphon- and gill-withdrawal reflex of *Aplysia* are known to act critically in the consolidation of short-term changes in the synaptic plasticity into long-term memory (Dash *et al.*, 1990; Kaang *et al.*, 1993; Martin *et al.*, 1997). Taken together, the effects of cAMP on the synaptic plasticity should be first of all examined in gastropod neurons, such as the CGC in *L. stagnalis*.

The buccal motor neuron 1 (B1 cell) is a salivary gland motor neuron which receives a monosynaptic excitatory input from the CGC (Benjamin *et al.*, 1979; McCrohan and Benjamin, 1980). The firing of the B1 cell definitely synchronizes with the radula-protraction phase of feeding cycle (Rose and Benjamin, 1979). The excitation of the B1 cell by activation of the CGC is thus considered to evoke secretion of saliva and to be important for harmonious behavior in feeding.

In the present study, we examined a synaptic enhancement by cAMP and PKA between the CGC and the B1 cell in the isolated central nervous system (CNS) of *L. stagnalis*. First, the changes in the excitatory postsynaptic potentials (EPSPs) in the B1 cell were examined as the synaptic enhancement between the CGC and the B1 cell, when cAMP was injected into the CGC. Second, to confirm that this synaptic enhancement was induced by the increase in intracellular cAMP concentration, a phosphodiesterase inhibitor was applied to the CGC. Third, whether PKA activated by cAMP is involved in the synaptic enhancement was studied by a competitive antagonist of cAMP. Fourth, the effect of *de novo* protein synthesis was examined on the synaptic enhancement by protein synthesis inhibitors. Finally, as taking into account that the CGC is serotonergic (Boer *et al.*, 1984; Tuersley and McCrohan, 1988; Yeoman *et al.*, 1996; Sadamoto *et al.*, 1998), the B1 cell was treated with serotonin antagonists to test the contribution of amount of serotonin release to the synaptic enhancement.

MATERIALS AND METHODS

CNS preparations

Specimens of *Lymnaea stagnalis*, originally supplied from Vrije Universiteit in Amsterdam, were kept in tap water and fed on lettuce on a 12:12 light-dark cycle at 20°C. The CNSs were isolated from adults with the shell length of 2–2.5 cm after anesthetization by immersion in 25% Listerine® for 5 min. The isolated CNS was immersed in *Lymnaea* saline and pinned in a Sylgard-lined dish for recording. *Lymnaea* saline contained: 24 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 4 mM $CaCl_2$, 0.3 mM D-glucose, 0.1 mM NaH_2PO_4, and 35.4 mM HEPES-NaOH (pH 7.9). Then the CNS was incubated with protease (1 mg/ml, type XXIV, Sigma, St. Louis, MO) for 10 min and washed out thoroughly with the saline. To stabilize the excitation of the CNS after dissection, the preparation was kept in the recording dish filled with the saline at 4°C for 2 hr before intracellular recording.

Intracellular recording

The B1 cell and the CGC were impaled with glass microelectrodes filled with 2 M potassium acetate, giving tip resistances of 20–60 MΩ. The EPSP evoked in the B1 cell was amplified with an intracellular recording amplifier (NEURO DATA, Model IR-283, New York, NY), monitored with an oscilloscope (TEKTRONIX, 2213, Wilsonville, OR), and recorded with a computer (Quark, IBM-compatible type, Sapporo, Japan). A train of 10 spikes in the CGC, which was produced by a current injection for 1 sec, evoked a large compound EPSP in the B1 cell as a superposition of 10 individual EPSPs. Only the B1 cells that showed responses of less than 15 mV amplitude as the initial compound EPSPs were used in the present study. The time course of the EPSP enhancement after the cAMP injection and that after the 5'AMP injection consist of the data obtained from six and three preparations, respectively. The combinations of the data recorded in the same preparation were as follows: 0 and 0.5 hr, 0 and 1 and 2 hr, 0 and 3 hr, 0 and 6 hr, and 0 and 24 hr. The data at 0 hr were recorded before drug applications. Therefore, the electrodes were changed with the electrodes for injection or removed until the next recording time.

Drug applications

Either cAMP (Sigma) or 5'AMP (Sigma), which was filled in a glass microelectrode (80–120 MΩ as a 200 mM solution dissolved in 20 mM Tris buffer (pH 7.5), was injected into the CGC by passing hyperpolarizing current pulses (50 msec on, 50 msec off) of 4 nA for 20 min. This injection period was decided by conferring with the work of *Aplysia* (Scholz and Byrne, 1988). Isobutylmethylxanthine (IBMX, Sigma), Rp-adenosine 3',5'-cyclic monophosphothioate (Rp-cAMPS, Sigma), anisomycin (Sigma), and cycloheximide (Sigma) were applied in the recording chamber. The concentrations and application periods of three drugs were specified in each experiment. IBMX is a cAMP phosphodiesterase inhibitor. Rp-cAMPS is a cAMP competitive antagonist for PKA. Anisomycin and cycloheximide are protein synthesis inhibitors.

When the isolated CNS except the buccal ganglia was incubated with these drugs, the buccal ganglia were completely partitioned off by a silicone grease wall. Thus, the B1 cell was not affected by these drugs. When the EPSP in the B1 cell was recorded, IBMX, but not Rp-cAMPS nor the protein synthesis inhibitors, was washed out to avoid the bursting of the CGC. Neither Rp-cAMPS nor the protein synthesis inhibitors elicited the bursting of the CGC. Ketanserin (RBI, Natick, MA) and 3-tropanyl-3,5-dichlorobenzoate (MDL 72222, RBI), which are an antagonist for the metabotropic serotonin (5-HT_1 and 5-HT_2) receptor and that for the ionotropic serotonin (5-HT_3) receptor, respectively, were applied onto the whole CNS.

Analysis of data

The changes in the amplitudes of EPSPs were normalized by the results at the start of recording (0 hr). The data were presented by mean±SEM. The Student's *t* test (two-tailed) was used to test the significance of the EPSP changes by injection or application of drugs. In dose-response of the EPSP amplitude to applied MDL 72222, the half maximum blockade was estimated by the curve fitting to the data points with Boltzmann equation, which was presented by $y = (A_1 - A_2)/[1 + \exp\{(x - x_0)/ x\}] + A_2$. y and x indicate the EPSP amplitude and the concentration of the drug, respectively. A_1, A_2, x_0, and x are the initial value of y, the final value of y, x value at a half decay of y, and the width of x value during a decay of y, respectively. The software for this curve fitting was 'Microcal Origin' (ver. 4.10, Microcal Software, Northanpton, MA).

RESULTS

Synaptic enhancement between a buccal motor neuron and its regulatory interneuron by cAMP

When cAMP was injected into the CGC, we observed the firing of the CGC. This firing, however, disappeared 30 min after the injection. We measured the changes in the EPSPs in the B1 cell by activation of the CGC after the cAMP injection. Three hours after the onset of cAMP injection, the CGC was depolarized by a constant current (1.0 nA, 500 msec) to fire

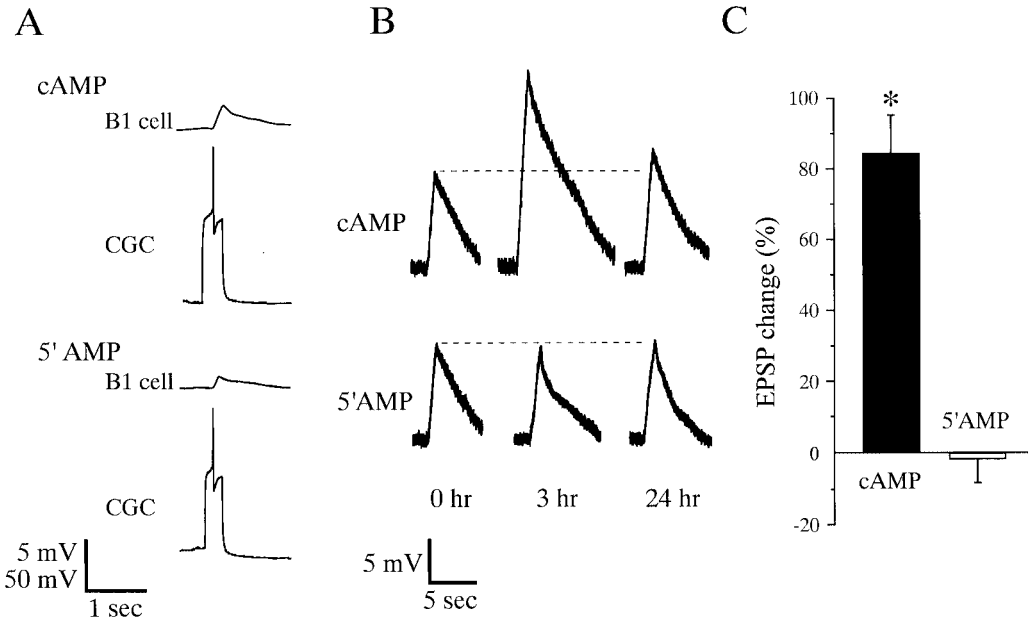


Fig. 1. Synaptic enhancement between a buccal motor neuron (B1 cell) and its regulatory interneuron (CGC) injected with cAMP. (**A**) A single EPSP in the B1 cell evoked by firing an action potential in the cAMP-injected CGC with a constant current depolarizing pulse was about twice larger than that by an activation of the 5'AMP-injected CGC. Both EPSPs were recorded 3 hr after the onset of injection and filtered with FFT. The injection period was 20 min. (**B**) A train of 10 spikes in the CGC evoked a large compound EPSP in the B1 cell, providing the clearer effects of cAMP. The effect of cAMP injection into the CGC was produced 3 hr later and extinguished in 24 hr. No significant changes were observed by the 5'AMP injection. (**C**) The changes in the amplitudes of the compound EPSPs in the B1 cells were summarized. The data were recorded 3 hr after the onset of injections of cAMP and 5'AMP into the CGC. * indicates $p < 0.001$.

an action potential, offering a single EPSP in the B1 cell (Fig. 1A). This single EPSP in the preparation of the cAMP-injected CGC was about twice larger than that in the preparation of the 5'AMP-injected CGC (Fig. 1A). Because the single EPSP in the B1 cell was very small (< 3 mV), we often failed to detect it depending on the preparation. We thus noticed a large compound EPSP in the B1 cell that was evoked by a train of 10 spikes in the CGC. The large compound EPSPs provided very clear effects of cAMP. The effect of cAMP injection was apparent 3 hr later and extinguished in 24 hr (Fig. 1B), while no significant changes were observed in the 5'AMP-injected preparations. The changes in the amplitudes of compound EPSPs in the B1 cells were summarized in Fig. 1C. The amplitudes of the compound EPSPs 3 hr after the onset of the cAMP injection (n = 8) were significantly greater ($p < 0.001$) than that in the preparations of the 5'AMP injection (n = 7).

To examine the time-dependency of the effect of cAMP, we measured the amplitudes of the compound EPSPs at seven intervals (0, 0.5, 1, 2, 3, 6, and 24 hr) after the onset of the cAMP injection or at four intervals (0, 0.5, 3, and 24 hr) after the onset of the 5'AMP injection into the CGC (Fig. 2, n = at least 7). Both EPSPs by the cAMP injection and the 5'AMP injection were slightly enhanced at 0.5 hr, but not presented any significant differences. The facilitatory effect of cAMP on the synaptic transmission between the CGC and the B1 cell was thus produced 3 hr later. The effect persisted for more than 6 hr ($p < 0.001$ vs. all the data for 5'AMP) and extinguished in 24 hr. Thus, the compound EPSP 3 hr after the

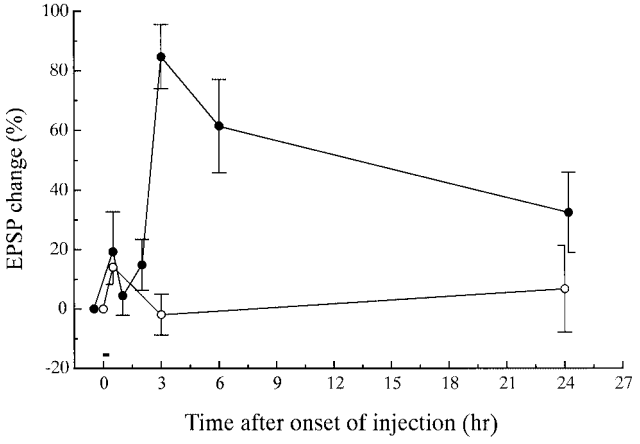


Fig. 2. Time course of the EPSP enhancement in the B1 cell induced by the cAMP injection into the CGC. The solid circles and the open circles show the data of the cAMP injection and the 5'AMP injection, respectively. The synaptic enhancement persisted for more than 6 hr after the injection of cAMP, and almost recovered to the initial amplitude in 24 hr. Bar, the injection period of 20 min.

cAMP or 5'AMP injection was monitored in the following experiments.

Changes in excitability of the CGC by cAMP

To test the effect of cAMP on the excitability of the CGC, we compared the input resistance, the resting potential, the duration of the current-evoked spike and the spontaneous

Table 1. Electrical properties of cAMP- and 5'AMP-injected CGCs 3 hr after the onset of injection

	cAMP (n = 8)	5'AMP (n = 7)
Input resistance (MΩ)	54 ±12	46 ± 7
Resting potential (mV)	–55 ± 3	–56 ± 2
Spontaneous spike duration* (msec)	12.1 ± 0.5	11.5 ± 0.7
Evoked spike duration* (msec)	13.0 ± 0.7	14.2 ± 0.9
Depth of afterhyperpolarization in the spontaneous spike (mV)	9.1 ± 0.7	9.0 ± 0.6
Duration of afterhyperpolarization in the spontaneous spike * (msec)	94.4 ± 12.0	90.6 ± 12.4

Values are mean±SEM.
* indicates the width at half maximum.

spike, and the depth and duration of the afterhyperpolarization in the spontaneous spike between the cAMP-injected CGCs and the 5'AMP-injected CGCs. We found no significant differences in these values between the cAMP- and the 5'AMP-injected CGCs (Table 1). The data were recorded 3 hr after the onset of injections.

Effect of IBMX on the EPSP in the B1 cell

By using IBMX, we proved that the increase in intracellular cAMP concentration in the CGC can enlarge the compound EPSP in the B1 cell. An incubation with IBMX caused the spontaneous firing of the CGC which presented the same pattern as observed by the cAMP injection. The effective concentration of IBMX was determined to be 300 μM by taking the dose-response to this firing of the CGC (data not shown). The incubation with 300 μM IBMX on the cerebral ganglion for 20 min that was the identical period as the cAMP-injection period did not mimic the enlargement of the compound EPSP in the B1 cell, even if the data were recorded 3 hr later (Fig. 3A). The 20-min incubation with IBMX, together with the cAMP injection, increased the amplitude of the compound EPSP 3 hr after the treatment. This increase was similar to that by the cAMP injection only (see Figs. 1B and 3A). The 3-hr incubation with IBMX did mimic the large compound EPSP 3 hr after the incubation (Fig. 3A). The results summarized in Fig. 3B show that the significantly larger EPSPs ($p < 0.001$) in the B1 cells were elicited by the long-term (3 hr) application of IBMX (n = 7) than by the short-term (20 min) application (n = 7).

Effect of PKA inhibitor on the EPSP in the B1 cell

We applied Rp-cAMPS onto the cerebral ganglion, in which the CGC was injected with cAMP, for 4 hr that included 1-hr pre-incubation period before the cAMP-injection. The effective concentration (500 μM) of Rp-cAMPS, which was reported in the work for *Aplysia* by Ghirardi *et al.* (1992), blocked the increase in the EPSP in the B1 cell, and further reduced it below the initial level (Fig. 4A). The results summarized in Fig. 4B show that the significantly smaller EPSPs ($p < 0.01$) in

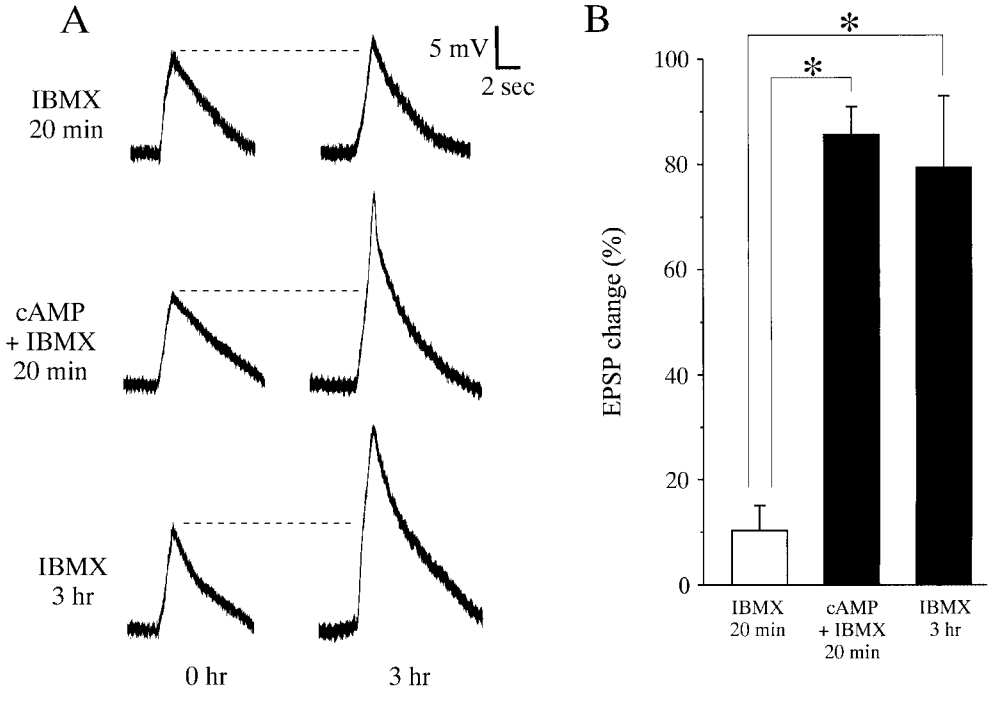


Fig. 3. Effect of IBMX on the EPSP enhancement in the B1 cell. (**A**) The IBMX application on the cerebral ganglia during the identical period (20 min) as that for the cAMP injection did not induce the EPSP enhancement in the B1 cell when recorded 3 hr after the onset of treatment. Both the IBMX application and the cAMP injection at the same time elicited the large enhancement. The long-term (3 hr) application of IBMX enhanced the EPSP in the B1 cell to the same level as the cAMP injection. (**B**) The changes in the amplitudes of the compound EPSPs in the B1 cells were summarized. The data were recorded 3 hr after the onset of IBMX application on the cerebral ganglia and/or that of cAMP injection into the CGC. * indicates $p < 0.001$.

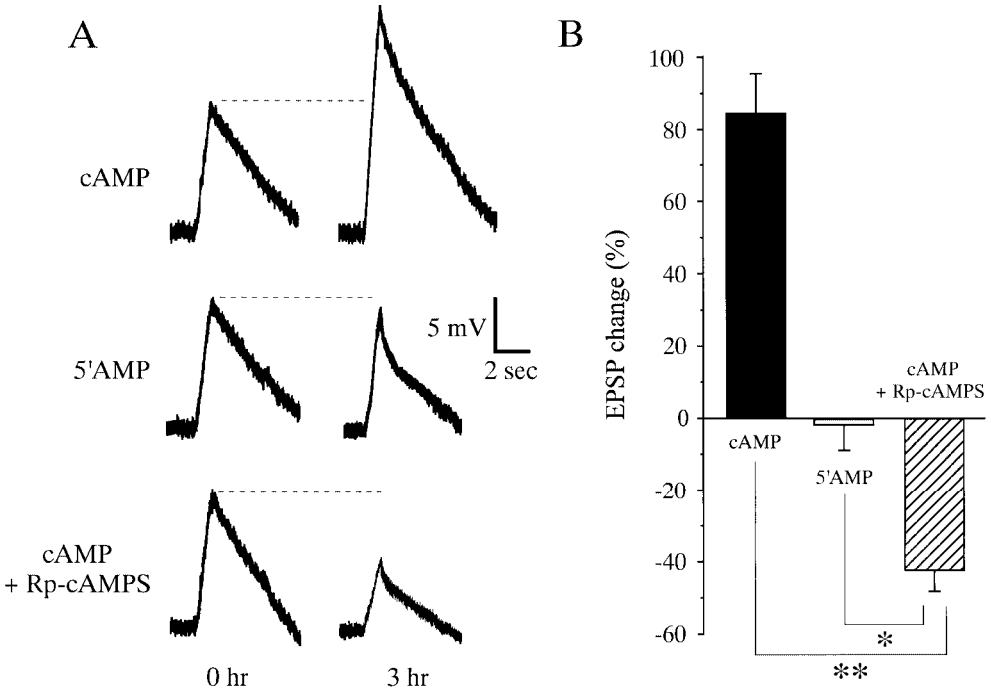


Fig. 4. Blockade of the increase in EPSP amplitude in the B1 cell by Rp-cAMPS application on the cerebral ganglia. (**A**) Even though cAMP was injected into the CGC, the long-term (4 hr) treatment of Rp-cAMPS, including the pre-incubation period of 1 hr, prevented the increase in EPSP in the B1 cell, and further suppressed it under the control level of 5'AMP injection. (**B**) The EPSP changes in the B1 cell were summarized. * and ** indicate $p < 0.01$ and $p < 0.001$, respectively.

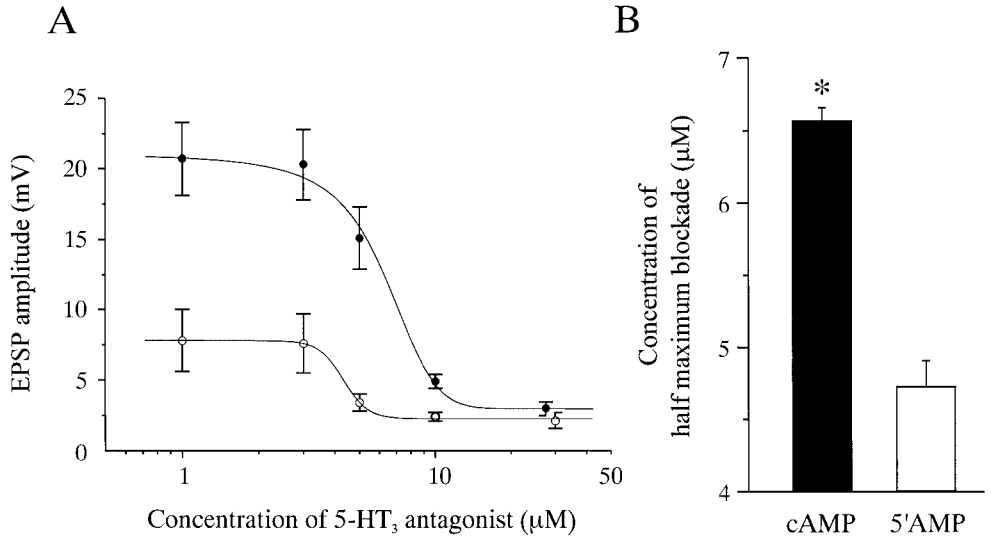


Fig. 5. Dose-response of the EPSP amplitude in the B1 cell to 5-HT_3 antagonist. (**A**) The amplitudes of the compound EPSPs in the B1 cell were recorded 3 hr after the onset of injection of cAMP (solid circles) or 5'AMP (open circles) into the CGC. During the last 30 min of this 3 hr, 5-HT_3 antagonist, MDL 72222, was applied on the whole CNS. (**B**) The concentrations of MDL 72222 for the half maximum blockade were summarized. The concentration for half maximum blockade for the EPSP evoked by activation of the cAMP-injected CGC was significantly higher (*$p < 0.001$) than that for the 5'AMP-injected CGC.

the B1 cells were elicited by the cAMP injection into the CGCs together with the application of Rp-cAMPS (n = 5) than by the 5'AMP injection into the CGCs without Rp-cAMPS (n = 7). The electrical properties of the CGC were not changed by the incubation of Rp-cAMPS (data not shown).

Effect of protein synthesis inhibitor on the EPSP in the B1 cell

We incubated the cerebral ganglion, in which the CGC was injected with cAMP, together with the protein synthesis inhibitor (anisomycin or cycloheximide) for 4 hr that included

1-hr pre-incubation period before the cAMP injection. By the cAMP injection, the EPSPs were enlarged with an increase of 85%±8% (n = 3) and 76% ± 4% (n = 3) even in the presence of 30 μM anisomycin and 100 μM cycloheximide, respectively. These increases were the same as that by the cAMP injection only (see Fig. 1B). The concentrations of the applied drugs were three times higher than those for the cultured cells of *Aplysia* (Montarolo *et al.*, 1986; Ghirardi *et al.*, 1995) for a good penetration through the sheath of ganglia.

Dose-response of the EPSP in the B1 cell to serotonin antagonist

Wolcourt-Ambakederemo and Winlow (1994a, b, 1995) identified two metabotropic subtypes (5-HT_1- and 5-HT_2-like receptors) and one ionotropic subtype (5-HT_3-like receptor) of serotonin receptors in the CGC and some motor neurons (B1, B2, and B4 cells) of *L. stagnalis*. To determine if the increase in the amplitude of the compound EPSP in the B1 cell by cAMP is due to the increase in the serotonin release from the CGC, we examined the dose-responses of the EPSP amplitudes to the antagonists, ketanserin and MDL 72222. If the amount of released serotonin increases, the dose-response curve must shift. These antagonists were applied onto the whole CNS for 30 min before the EPSP recording. The large compound EPSP in the B1 cell by cAMP was not suppressed by 100 μM ketanserin (data not shown). This concentration was considered to be effective from the results of Wolcourt-Ambakederemo and Winlow (1994b), if the metabotropic serotonin receptors acted in the CGC-B1 cell synapse. On the other hand, the EPSP was decreased by MDL 72222 in a dose-dependent manner, and the dose-response for the preparations in the synaptic enhancement was shifted (Fig. 5A). The EPSP amplitudes in the preparations injected by cAMP and the 5'AMP were saturated to decrease by the applications of about 10 μM and 5 μM of MDL 72222, respectively (Fig. 5A). Figure 5B shows the concentrations of MDL 72222 for the half maximum blockade for the EPSPs. The concentrations for half maximum blockade for the EPSPs evoked by activation of the cAMP-injected CGCs (n = 3) were significantly higher ($p < 0.001$) than for the 5'AMP-injected CGCs (n = 3).

DISCUSSION

We demonstrated that the synaptic enhancement as the increase in the amplitude of the compound EPSP in the buccal motor neuron 1 (B1 cell) by activation of its regulatory interneuron (cerebral giant cell, CGC) in which the intracellular cAMP concentration was elevated by the cAMP injection or the phosphodiesterase inhibition (Figs. 1 and 3). The characteristics of this synaptic enhancement were as follows. (1) The compound EPSP became the largest 3 hr after the onset of cAMP injection, and its enhancement persisted for more than 6 hr (Fig. 2). (2) This synaptic enhancement was not caused as a result of the prolongation of the action potential in the presynaptic neuron, the CGC. (3) The PKA inhibitor completely blocked the effect of cAMP on the compound EPSP and, further, significantly reduced the amplitude of the compound EPSP below the control level (Fig. 4). (4) The synaptic enhancement was independent from a *de novo* protein synthesis. (5) The dose-response of the compound EPSP in the B1 cell to the antagonist for the ionotropic serotonin receptor was shifted for the preparations in the synaptic enhancement (Fig. 5).

The synaptic enhancement by cAMP in the present study seemed to be similar to the case in *Aplysia*. However, there are two remarkable differences between these two preparations: (1) the prolongation of an action potential in a presynaptic cell and (2) a *de novo* protein synthesis. The short- and long-term facilitation in *Aplysia* caused by cAMP cascade required the prolongation of an action potential and a *de novo* protein synthesis, respectively (Siegelbaum *et al.*, 1982; Sweatt and Kandel, 1989; Hochner and Kandel, 1992; Schacher *et al.*, 1993; O'Leary *et al.*, 1995; Sugita *et al.*, 1997; Abel and Kandel, 1998). Even in the CNS of *Hermissenda* in which intracellular Ca^{2+} plays an important role in the signal transduction (Alkon *et al.*, 1992; Ito *et al.*, 1994), the long-lasting depolarization due to a reduction in K^+-channel conductance and the increase in specific mRNA were reported in the synaptic enhancement in a classical conditioning (Alkon *et al.*, 1985; Nelson and Alkon, 1990). To gain a deeper insight into the excitability of the presynaptic CGC, the duration of the current-evoked and the spontaneous spikes at 30% and 70% from their bottoms were also examined (see the methods in Klein (1993). We found no changes in the results after the cAMP injection (data not shown). Because the data were recorded in the somata of the CGCs, we cannot role out a possibility of changes in action potential in the synaptic terminal.

The 500 μM concentration of Rp-cAMPS is known to give the maximal level of inhibition of the enhancement of sensorimotor synapses in *Aplysia* (Ghirardi *et al.*, 1992). The enhanced EPSP amplitudes in these systems, however, were only reduced to the initial level, but not below it. Therefore, the results using Rp-cAMPS shown in Fig. 4 strongly suggest that the activation of PKA in the CGC is required to even evoke the usual EPSP in the B1 cell. In other words, a physiological substrate phosphorylated by PKA always works in the process of neurotransmitter release. One possible explanation for this phenomenon is a cAMP-dependent regulation of the synaptic vesicle fusion. Recently, some works for cAMP-dependent long-term potentiation in the rat hippocampus reported that the PKA phosphorylation of a synaptic vesicle protein such as rabphilin regulates the synaptic vesicle fusion in presynaptic terminal, causing an increase in neurotransmitter release (Castillo *et al.*, 1997; Lonart and Südhof, 1998). This synaptic plasticity did not need the prolongation of the presynaptic action potential or the *de novo* protein synthesis. We should examine the phosphorylation of rabphilin-like proteins in the CGC in a future work.

If the PKA activation is directly involved in the synaptic vesicle fusion, the period of 3 hr to express the increase in the

EPSP amplitude (Fig. 2) is considered to be the axonal diffusion time of cAMP from the cell body to the synaptic terminal in the CGC. When a fluorescent dye, lucifer yellow (MW = 457), was injected into the cell body of CGC, its axonal diffusion time to reach the synaptic terminal took 4 hr at 4°C. Because cAMP has the comparable molecular weight (MW = 351), the period of 3 hr may be reasonable to produce the maximal effect on the EPSP amplitude in the B1 cell.

We injected cAMP into the CGC by using a hyperpolarizing current, and applied the drugs for cAMP cascade onto only the ganglia including the CGC. That is, we never stimulated the postsynaptic B1 cell. These conditions strongly suggest that Fig. 5 shows the increase in the amount of serotonin release from the presynaptic CGC after the cAMP injection, but not the changes in the sensitivity or the density of serotonin receptors in the postsynaptic B1 cell. In addition, because the electrical properties, such as the input resistance etc, were not changed in the CGC after the cAMP injection, the appearance of new release sites of serotonin in the presynaptic terminal was not supposed. Only the antagonist for the ionotropic serotonin receptor could suppress the EPSP amplitude in the B1 cell. This result seems to reflect the difference in the physiological properties of the subtypes of serotonin receptor. Wolcourt-Ambakederemo and Winlow (1994b, 1995) reported that the antagonist for ionotropic receptor and that for metabotropic receptor reduced the depolarizing and the hyperpolarizing responses, respectively, in the CNS of *L. stagnalis* by the application of serotonin.

The increase in intracellular concentration of cAMP in the CGC has not yet been confirmed by taste stimulation. The CGC has metabotropic serotonin receptors which activate adenylyl cyclase (Wolcourt-Ambakederemo and Winlow, 1994a, b) and receives taste signals from the sensory neurons in the lip and tentacles (Kemenes *et al*., 1986; McCrohan and Audesirk, 1987; Nakamura *et al*., 1999a, b). Because the lip nerves include serotonergic fibers (Sadamoto *et al*., 1998), some of the sensory neurons for taste are considered to be serotonergic. Therefore, the increase in cAMP in the CGC possibly occurs during feeding.

In conclusion, the synaptic enhancement elicited by cAMP-PKA cascade was first observed in the synaptic connection between the buccal motor neuron and its regulatory interneuron in the feeding circuitry of *L. stagnalis*. The cellular mechanism of this synaptic enhancement included the increase in the amount of serotonin release from the presynaptic interneuron which was regulated by the PKA phosphorylation, but not required any changes in the electrical properties or a *de novo* protein synthesis in the presynaptic interneuron. Therefore, the mechanism of the synaptic enhancement in the present study was completely different from the well-studied mechanisms in *Aplysia* and *Hermissenda*. The present results provided the physiological evidence of the direct regulation of a synaptic enhancement by PKA.

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