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Properties of Wave Propagation in the Oscillatory Neural Network in *Limax marginatus*

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ABSTRACT—The olfactory center (procerebral lobe; PC lobe) of the terrestrial slug, *Limax marginatus*, shows oscillatory behavior with a frequency of about 1 Hz and an activity wave that propagates from the apical to distal regions of the PC surface. To study the dynamic properties of this oscillatory network, serotonin, glutamate and acetylcholine were applied to the PC lobe. Serotonin and acetylcholine increased the frequency of PC oscillation and decreased the velocity of wave propagation. The effect of serotonin on the frequency was long-lasting and there was a delay before it caused a decrease in the wave propagation velocity. In contrast, the effect of acetylcholine on the frequency was phasic, and no delay was observed. Glutamate first decreased, then increased, the frequency. However, specific changes in the wave propagation velocity were not observed. From these experimental results, it is suggested that the oscillatory neural network of PC lobe has a potential to represent odor information as a series of spatially and temporally distributed ensembles of coherent firing neurons.

INTRODUCTION

Researchers studying information processing in the nervous system have paid considerable attention to oscillatory activity (Hopfield, 1988; Sompolinski *et al.*, 1990; Tank, 1990). It has been suggested that coherent oscillation may coordinate complex responses to stimuli received by the mammalian visual system (Eckhorn *et al.*, 1988; Gray *et al.*, 1989; Engel *et al.*, 1990). In addition, oscillatory activity has been reported to occur in the olfactory bulb (Adrian, 1942; Freeman, 1978) and, at various frequencies, in several brain regions, including the thalamus (Striade and Llinas, 1988), hippocampus (Fox *et al.*, 1986), and cortex (Llinas *et al.*, 1991; Silva *et al.*, 1991). Oscillatory activity has also been reported in invertebrate nervous systems, for example in the olfactory center of a terrestrial slug (Gelperin and Tank, 1990) and in the mushroom bodies of the locust (Laurent and Naraghi, 1994) and honeybee (Stopfer *et al.*, 1997).

In this paper we describe a study on the dynamics of oscillatory activity in the olfactory organ, procerebral (PC) lobe, of the slug *Limax marginatus*. The PC lobe of *Limax* is a valuable model for studying the role of oscillatory activity in olfactory information processing in the nervous system. *Limax* species rely predominantly on olfaction to recognize their environment and appear to be able to learn from olfactory stimuli (Sahley *et al.*, 1981, 1990; Yamada *et al.*, 1992; Suzuki *et al.*, 1994). The anatomy of the PC lobe of *Limax* is well understood. The lobe contains a large number of neurons, about 10^5, the cell bodies of which are clustered on the surface and send neurites into the cerebral ganglion. Afferent fibers from olfactory receptors on both the inferior and superior tentacles terminate and interconnect extensively with PC interneurons in the olfactory lobe (Chase and Tolloczko, 1993; Gelperin *et al.*, 1993).

The local field potential of the PC lobe oscillates at about 1 Hz (Gelperin and Tank, 1990) and the wave of electrical activity travels from the apical to basal surface of the PC lobe (Kleinfeld *et al.*, 1994; Kawahara *et al.*, 1997). As fragments of the PC lobe also show oscillatory activity, the lobe is considered to be a network of a large number of oscillators (Kleinfeld *et al.*, 1994). The frequency is modulated by learned odors. This is shown by the capacity of aversively and appetitively conditioned odors to decrease and increase the frequency, respectively, and by memory-related changes in neuronal activities in the PC lobe (Kimura *et al.*, 1998a, b, c). In addition, it is reported that some neurotransmitters such as serotonin, dopamine and glutamate alter the frequency (Gelperin *et al.*, 1993). However the wave propagation observed on PC lobe surface has rarely been studied, though it

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is an important property of oscillatory networks.

Our present study was on the dynamic properties of the oscillatory network of the PC lobe with special focus on the wave propagation. To study the behavior of the network, serotonin, glutamate and acetylcholine were applied to PC lobe and the local field potential was observed with two electrodes placed on the different regions on PC surface.

MATERIALS AND METHODS

Experimental animal

The terrestrial mollusc, *Limax marginatus*, was maintained in laboratory culture on frog chow (Oriental Yeast Co. Ltd., Tokyo, Japan) with a 14-hr/10-hr light-dark cycle at 19°C. For the following experiments, animals 4–-6 months old were used (1.5–2.0g).

Electrophysiology

Each slug was anesthetized by injecting 200 µl of anesthetizing solution [50 mM $MgCl_2$ in 10 mM HEPES (pH 7.4)] into its body cavity. The cerebral ganglion was gently dissected from the body and placed in an experimental chamber filled with the anesthetizing solution. Then the anesthetizing solution was replaced by perfusing it with normal slug saline (flow rate, 1.5 ml/min). The saline consisted of (mM) 70.0 NaCl, 2.0 KCl, 4.9 $CaCl_2$, 4.6 $MgCl_2$, 5.0 glucose and 10 HEPES (pH 7.4).

The LFP of the procerebral (PC) lobe was recorded through two suction pipette electrodes of 50–80 µm tip diameter filled with saline. The electrodes were placed at two sites on the PC surface, one adjacent to the cell bodies of apical PC interneurons, and the other adjacent to basal PC interneurons (Fig. 1). Signals from the electrodes were filtered with a 0.08 Hz low-cut and 30 Hz high-cut passive filters and recorded by a PCM processing recorder (PC-108, Sony, Tokyo, Japan).

The experiments were carried out on the PC lobe with and without the other part of the cerebral ganglion.

Application of drugs

Serotonin (5-HT), glutamate (Glu) and acetylcholine (ACh), at a concentration of 10^{-6}–10^{-4} M in saline, were used in the experiments. Drugs were applied, with the dosage controlled by a solenoid valve, for 30 sec to the PC lobe with or without the other part of the cerebral ganglion. Each preparation was used to test a single transmitter. In

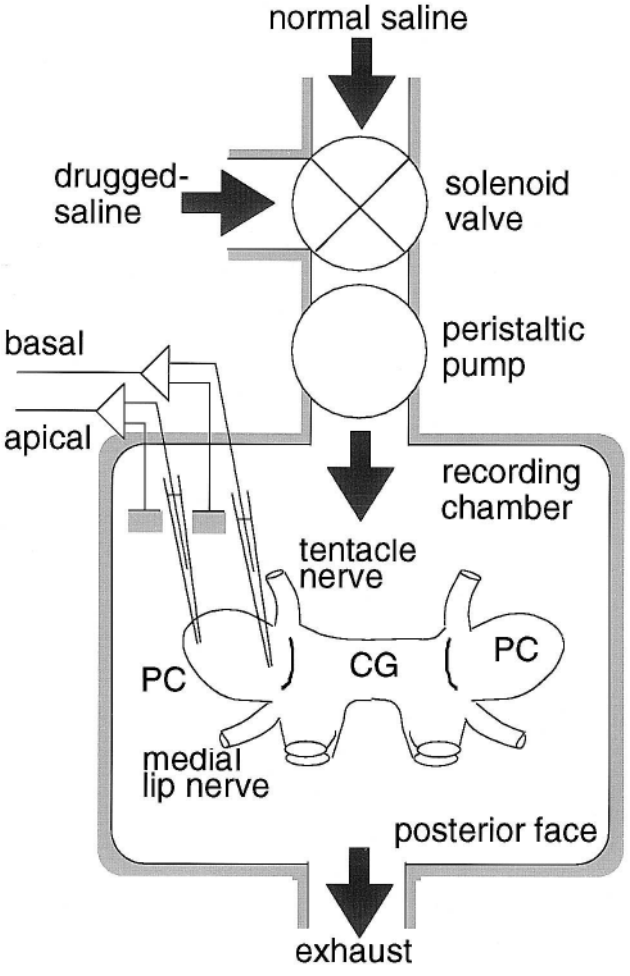

Fig. 1. Schematic drawing of the experimental setup used for local field potential (LFP) recording and drug application. The isolated cerebral ganglia (CG) were placed on the recording chamber over which fresh normal saline was continuously perfused. LFPs were simultaneously recorded from apical and basal regions of the posterior face of the procerebral (PC) lobe using two surface electrodes. For drug applications, a continuous flow of normal saline was replaced by one containing drugs using a peristaltic pump and solenoid valve.

most cases, 4 runs were made with each preparation, with a recovery time of 15 min between runs. The experimental chamber had a volume of 200 μl and the perfusion flow rate was 50 μl/s. The time for the solution to be completely exchanged was checked to be 15–20 sec.

Data analysis

To study the properties of the oscillatory neural network of the PC lobe, we focused on two variables. One was the instantaneous frequency and the other the velocity of wave propagation of neural activity on the PC surface. The instantaneous frequency was measured as a reciprocal of peak-to-peak time in each oscillatory cycle and wave velocity as the difference in peak time between the apical and basal LFP oscillations (ΔT, Fig. 2B–E). Generally, LFP signal had multiple peak (see Figs. 2E, 4B or 6C) and, in some case (ex. Fig. 2B), two or more clearly independent peaks were observed. In any cases, the position of a peak was defined as the point that the

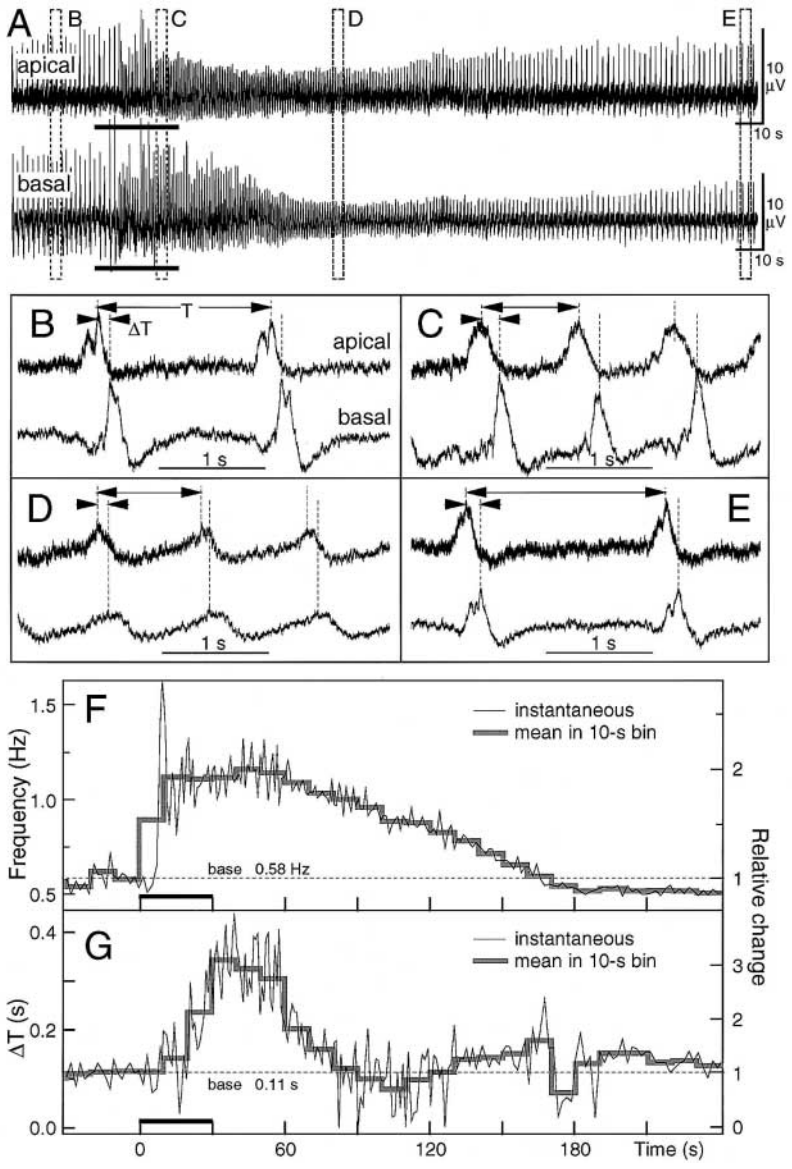

Fig. 2. A typical example of modulation of LFP oscillation by serotonin (5-HT). **A**: Oscillatory LFPs simultaneously recorded from apical and basal regions of the PC lobe. A 30-sec application of 10^{-4} M 5-HT (horizontal bars under traces) elicited an increase in oscillation frequency. **B**–**E**: Temporally expanded displays of 4 islets in panel A. In the LFP oscillations, delays (ΔT) in peak time at the basal recording from that at the apical recording are evident, indicating wave propagation from the apical to basal region. The instantaneous frequency defined as the reciprocal (1/T) of the interval between two adjacent peaks in a LFP record (T) is also increased by 5-HT. **F**: (thin lines) The instantaneous frequency change of LFP oscillation elicited by 10^{-4} M 5-HT. The data were taken from the apical record shown in panel A. (thick lines) The instantaneous frequency was averaged for the cycles over every 10-sec period. The left ordinate shows real values and the right ordinate the relative frequency. Relative frequency was obtained by dividing each frequency by the average calculated from the instantaneous frequencies of 100 cycles of spontaneous oscillations just before drug application (in this preparation, 0.58 Hz). **G**: (thin lines) The change of ΔT elicited by 10^{-4} M 5-HT. (thick lines) The ΔT averaged for the cycles in every 10 sec. The left ordinate shows real values and the right ordinate shows the relative ΔT which was obtained by dividing each ΔT by the average calculated from the ΔTs of 100 cycles of spontaneous oscillations just before the drug application (in this preparation, 0.11 sec).

largest voltage value was obtained in each oscillatory cycle.

The recorded data were transferred to a personal computer (Macintosh 8100/80AV, Apple Computer, Inc., Upertino, USA) via a 12 bit A–D converter with a 10 ms sampling speed (MacADIOS-8ain, GW Instruments, Somerville, USA). The position of each peak, instantaneous frequency and the difference in peak time were calculated by a commercial wave analysis program (IgorPro, WaveMetrics Inc., Lake Oswego, USA).

To compare the individual LFP oscillation frequencies, the instantaneous frequencies of 100 cycles before the application of a transmitter were averaged as basic frequency (F0) and every instantaneous frequency (F) was normalized as F/F0 (relative frequency). The relative frequencies were then averaged for the cycles that occurred during each 10-sec period to show trends in frequency change. The same calculation was done for the ΔT because the position of two electrodes was different for each sample. That is, the ΔT for 100 cycles before the application of a transmitter were averaged and every ΔT was normalized (relative ΔT). Then, the relative ΔT was averaged for the cycles in every 10 sec. As the effect of each drug was similar between the isolated PC lobe and the PC lobe with the other part of the cerebral ganglion, data from both preparations were combined.

RESULTS

Serotonin (5-HT)

Fig. 2A shows a typical result of 5-HT application to PC lobe with a cerebral ganglion. In this preparation, the PC lobe showed a spontaneous oscillatory activity of 0.58±0.06 Hz (mean±SD). As shown in Fig. 2B, the difference in peak time between two LFP oscillations recorded from the apical and basal regions (ΔT) was observed as 0.11±0.08 sec. When 5-HT (10^{-4} M) was applied to the PC lobe (solid lines in Fig. 2A), the frequency of oscillation increased and the amplitude decreased. In addition, ΔT increased, which means that the velocity of wave propagation decreased. Figure 2F and 2G shows relative frequency and ΔT, respectively, obtained from the results depicted in Fig. 2A. The values calculated from each oscillatory cycle (thin lines) and those averaged for the cycles within each 10-sec period (thick lines) are shown. In this preparation, frequency increased immediately after 10^{-4}M 5-HT was applied, reached its peak after about 20 sec, and returned to its spontaneous level more than 150 sec after the 5-HT had been first applied. On the other hand, ΔT increased after a delay, reached its peak 30 sec later, and returned to its spontaneous level about 90 sec after 5-HT had been first applied.

Fig. 3 summarizes the results obtained after applying 10^{-6}, 10^{-5} and 10^{-4} M 5-HT to 20 PC lobes. The data from the isolated PC lobes and those from PC lobes with the other parts of the cerebral ganglion have been combined. As the 5-HT concentration became higher, the increase in frequency and ΔT became more pronounced. For example, with 10^{-4} M 5-HT, the increase in frequency above spontaneous levels became 1.79±0.28 times (40–50 sec) and for ΔT became

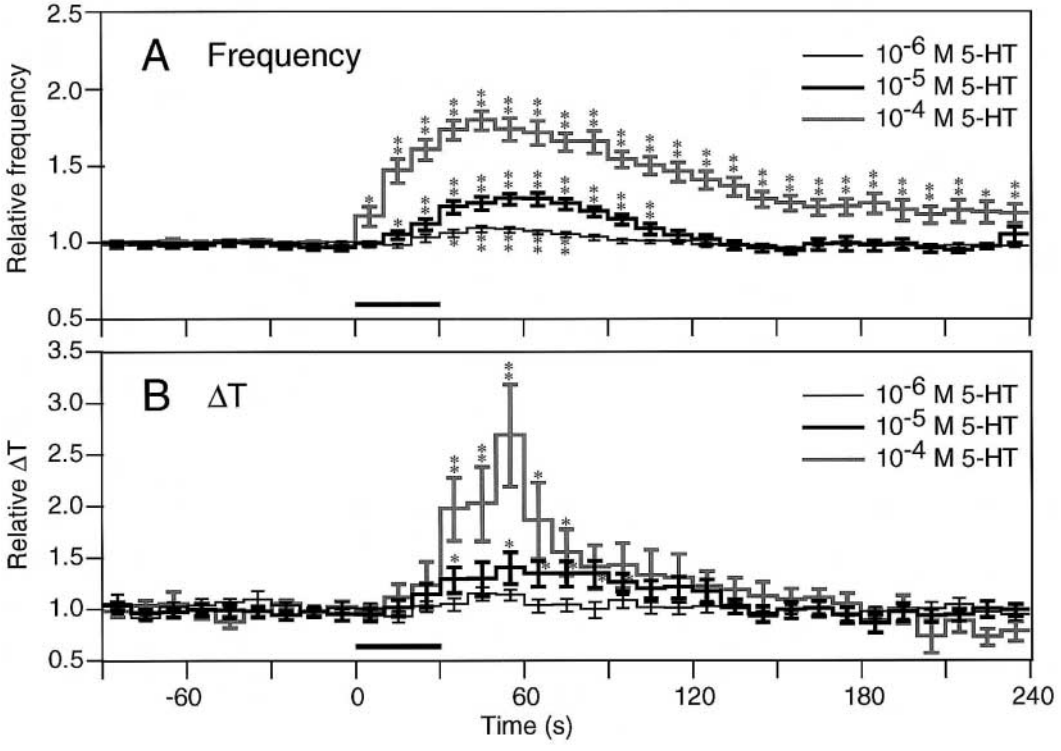

Fig. 3. Effects of 5-HT on the frequency and the ΔT of PC lobe oscillations. **A**: Frequency changes elicited by 10^{-6} M (thin line), 10^{-5} M (middle line), or 10^{-4} M (thick line) 5-HT. **B**: Changes of ΔT elicited by the corresponding concentrations of 5-HT. Each point indicates an average with a standard error of pooled data from 20 trials on 20 preparations; data of each trial are normalized by basic frequency or ΔT, and averaged for each 10-sec bin (e.g. Fig. 2E, thick line). Double and single asterisks show the results of stochastic analysis with two-tailed *t* test as $p<0.01$ and $p<0.05$, respectively.

1.86±1.66 times (50–60 sec). The temporal features of these changes were different between the frequency and ΔT. The frequency increased as soon as 5-HT had been applied and, for 10^{-4} M 5-HT, lasted more than 240 sec. On the other hand, there was a delay (about 30 sec) before ΔT increased and it returned to the spontaneous level within 120 sec after initial application of 5-HT.

Glutamate (Glu)

Fig. 4A shows a typical result after application of Glu to PC lobe with cerebral ganglion. Glu did not cause a dramatic change in the oscillation amplitude. As shown in Fig. 4F and 4G, the application of 10^{-4} M Glu first decreased the frequency from 0.84 to 0.38 Hz, and then increased it to 1.38 Hz. For ΔT, it decreased from 0.15 to 0.08 sec, and then increased to 0.25 sec.

Such a biphasic change in frequency was clearly observed in data obtained from 19 PC lobes and summarized in Fig. 5. Although we could not observe any change in frequency caused by 10^{-6} M Glu, statistically significant biphasic changes were revealed when the concentration was increased to 10^{-5} or 10^{-4} M Glu. For example, with 10^{-4} M Glu, the frequency first became 0.60±0.15 times smaller (10–20 sec) and then increased above the spontaneous level by 1.80±0.38 times

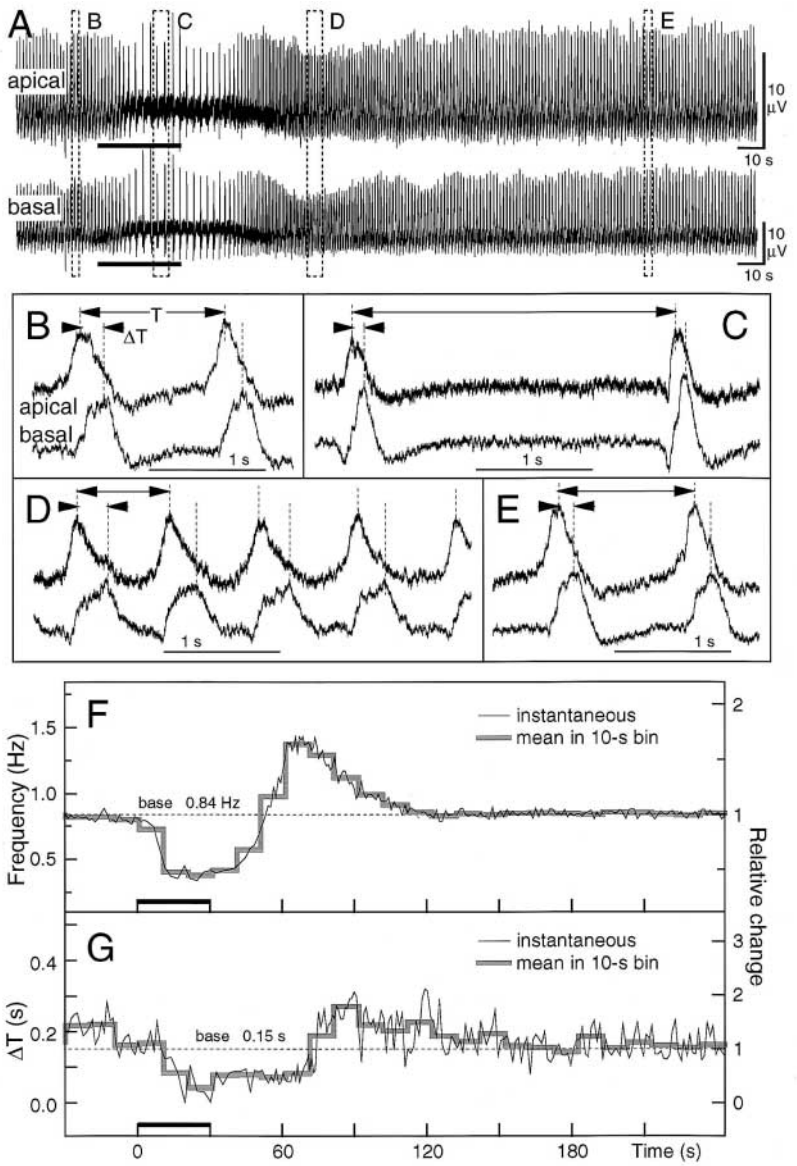

Fig. 4. Typical example of modulation of LFP oscillation by 10^{-4} M L-glutamate (Glu). **A**: Oscillatory LFPs simultaneously recorded from apical and basal regions of the PC lobe. **B**–**E**: Temporally expanded displays of 3 islets in panel A. **F**: (thin lines) The instantaneous frequency change of LFP oscillation elicited by 10^{-4} M Glu. The data were taken from the apical record shown in panel A. (thick lines) The instantaneous frequency averaged for the cycles in every 10 sec. **G**: (thin lines) The change of ΔT elicited by 10^{-4} M Glu. (thick lines) The ΔT averaged for the cycles in every 10 sec. In this preparation, the basic frequency and ΔT were 0.84 Hz and 0.15 sec, respectively. The other experimental setups or data analysis were the same as for Fig. 2.

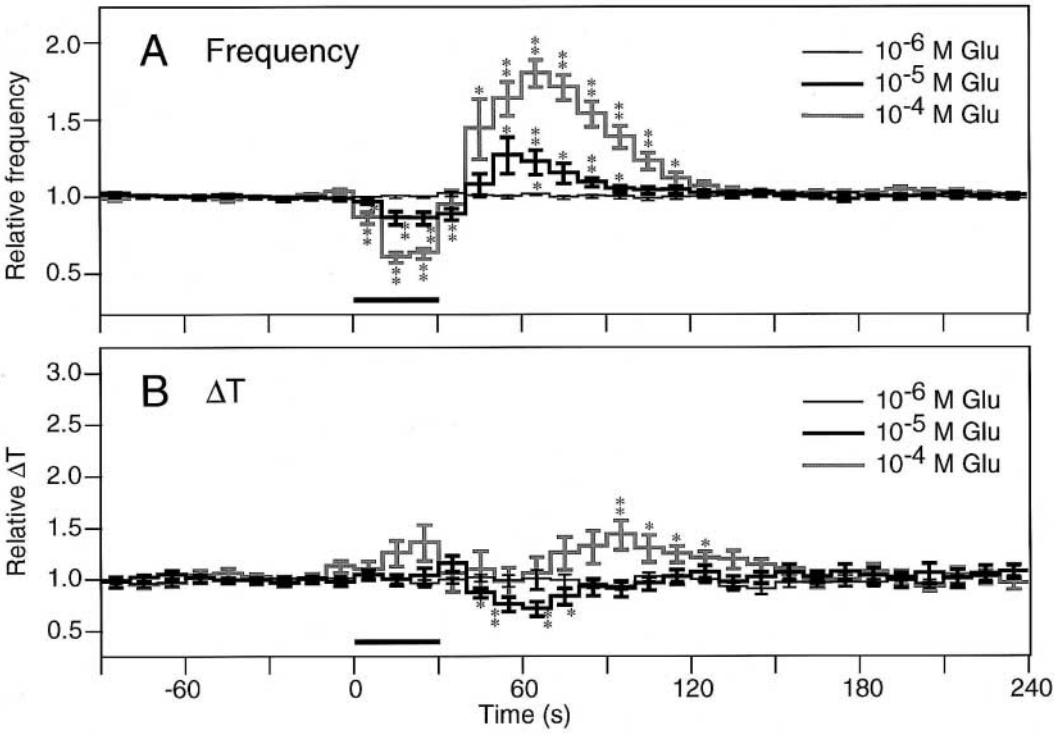

Fig. 5. Effects of Glu on the frequency and the ΔT of PC lobe oscillations. **A**: Frequency changes elicited by 10^{-6} M (thin line), 10^{-5} M (middle line), or 10^{-4} M (thick line) Glu. **B**: Changes in ΔT elicited by the corresponding concentrations of Glu. Each point indicates an average with a standard error of pooled data from 19 trials on 19 preparations. Double and single asterisks show the results of stochastic analysis using two-tailed t test as $p<0.01$ and $p<0.05$, respectively.

(60–70 sec). In contrast, there was no consistency in change in ΔT. For example, 10^{-5} M Glu caused the decrease of ΔT about 60 sec after the onset of the drug application, but 10^{-4} M Glu caused an increase about 90 sec after drug application.

Acetylcholine (ACh)

Bath application of ACh (10^{-4} M) caused an increase in frequency of the oscillating LFP, a decrease in amplitude, and an increase in ΔT (Fig. 6A). In this preparation, 10^{-4} M ACh increased the frequency from 0.69 to 1.39 Hz and ΔT from 0.13 to 0.44 sec (Fig. 6E and 6F).

The experimental results from 21 PC lobes are summarized in Fig. 7. Small increases in frequency and ΔT were caused by 10^{-5} M ACh, but no change was caused by 10^{-6} M ACh. With 10^{-4} M ACh, the frequency and ΔT increased 2.22±0.77 times (20–30 sec) and 1.99±0.56 times (30–40 sec), respectively. Although the changes in frequency and ΔT caused by ACh were similar to those caused by 5-HT, the temporal features were different. For ACh, the increase in the frequency and the ΔT occurred simultaneously within a few seconds after exposure to the drug, and returned to their spontaneous levels about 30 sec after the drug had been removed (cf. Figs. 7 and 3).

DISCUSSION

Frequency in LFP oscillation

5-HT caused a monophasic increase in LFP frequency (Fig. 3A). This feature is different from that reported by Gelperin *et al.* (1993), where 5-HT produced a biphasic response at 10^{-5} M, first increasing the frequency and then decreasing it, whereas the slow recovery observed after 5-HT removal in Fig. 3A is consistent with their finding. On the other hand, Glu caused a biphasic response in this study, but a monophasic decrease in the study by Gelperin *et al.* (1993). We consistently found that the increase in LFP frequency after the start of washing was always caused by 10^{-6}–10^{-4} M Glu. Thus, this rebound-like increase in the frequency is a general response of PC lobe preparation to Glu in the present experimental condition. In Gelperin *et al.* (1993), drugs were applied for about 300 sec with solution exchange time of 40–60 sec, which differed from ours (30 sec and 15–20 sec, respectively). Thus, the differences in response to 5-HT or Glu might be due to the difference in the drug application periods. For example, it is possible that a long exposure to drugs activates (5-HT) or inactivates (Glu) the secondary responses of neurons, though we have no direct evidence.

The effect of ACh on the LFP oscillation has not been studied. There is no direct evidence that ACh functions as a neurotransmitter in the PC lobe. However, it has been reported for *Limax* species that elevated dietary choline enhances blood

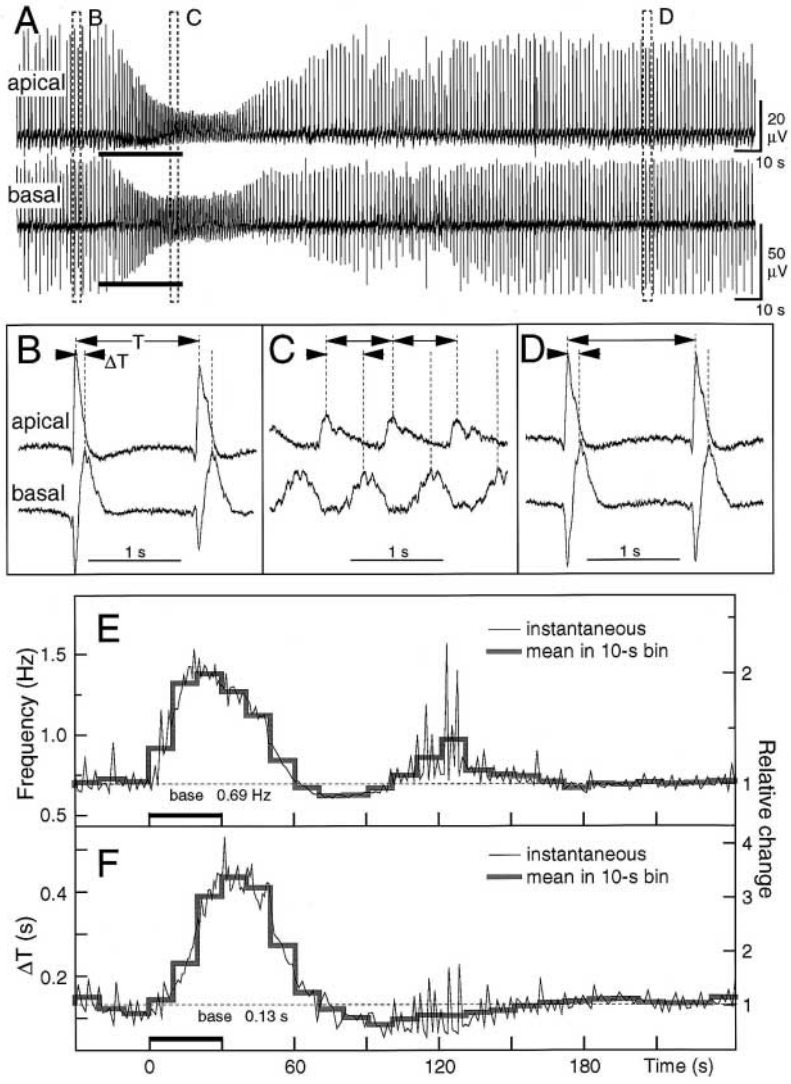

Fig. 6. Typical example of modulation of LFP oscillation by 10^{-4} M acetylcholine (ACh). **A**: Oscillatory LFPs simultaneously recorded from apical and basal regions of the PC lobe. **B**–**D**: Temporally expanded displays of 4 islets in panel A. **E**: (thin lines) The instantaneous frequency change of LFP oscillation elicited by 10^{-4} M ACh. The data were taken from the apical record shown in panel A. (thick lines) The instantaneous frequency averaged for the cycles in every 10 sec. **F**: (thin lines) The change in ΔT elicited by 10^{-4} M ACh. (thick lines) The ΔT averaged for the cycles in every 10 sec. In this preparation, the basic frequency and ΔT were 0.69 Hz and 0.13 sec, respectively. The other experimental setups or data analysis were the same as in Fig. 2.

choline levels and cholinergic transmission (Barry and Gelperin, 1982a, b), and that cholinergic synapses are involved in memory retention (Sahley *et al.*, 1986). Our results showing that ACh increased LFP frequency, when combined with the findings outlined above, suggest the involvement of ACh in odor information processing in *Limax*.

Amplitude and waveform

The LFP signals obtained here are a spatial summation of neuronal activities around an electrode. Thus, LFP amplitude is influenced not only by the amplitude of each neuronal activity (or activity of each oscillatory element), but the relation among the activities. In other words, when each element shows a phase-locked oscillation, the amplitude of LFP becomes large, but the amplitude becomes small when they show less phase-locked oscillation. So, there are two possibilities for changes in its amplitude. One is a change in the amplitude of each neuron (or oscillatory element) and the other is a change in coherency among the oscillatory elements. These possibilities are distinguishable by waveforms. The amplitude changes without changing waveform in the former case, but it changes with changing waveform in the latter case. Changes in the amplitude observed during or after the application of 5-HT and ACh were the latter case because the peak amplitude decreased with increasing the duration of positive voltage change (Figs. 2D and 6C). Thus, it is indicated that 5-HT and ACh both changed the coherency among the oscillatory elements.

LFP signals sometimes showed a doublet-shaped waveform as described in Materials and Methods section (Fig. 2B).

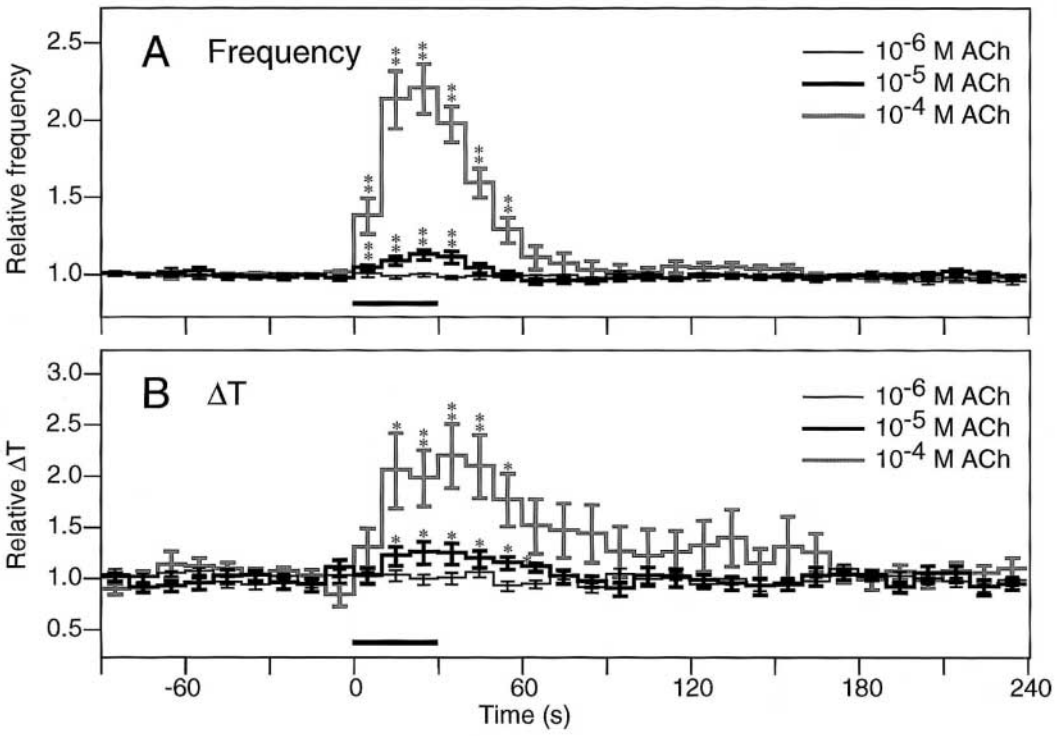

Fig. 7. Effects of ACh on the frequency and the ΔT of PC lobe oscillations. **A**: Frequency changes elicited by 10^{-6} M (thin line), 10^{-5} M (middle line), or 10^{-4} M (thick line) ACh. **B**: Changes of ΔT elicited by the corresponding concentrations of ACh. Each point indicates an average with a standard error of pooled data from 21 trials on 21 preparations. Double and single asterisks show the results of stochastic analysis with two-tailed *t* test as $p<0.01$ and $p<0.05$, respectively.

In such cases, two groups of coherent oscillatory elements would be formed around an electrode and there is possibility that two adjoining waves propagate in parallel. To elucidate the details of the propagation, we need to observe whole PC lobe with an optical recording method.

Wave propagation

Kleinfeld *et al.* (1994) reported the existence of a propagating wave on the PC surface. In addition, it is also reported that the collapse of the wave was caused by applying unlearned odor (Delany *et al.*, 1994) or learned odor (Gervais *et al.*, 1996) stimuli to the receptors on the superior tentacle. However changes in the velocity of wave propagation have not been reported.

The existence of a wave suggests a spatial gradient of excitability along the PC lobe (Kleinfeld *et al.*, 1994). Ermentrout *et al.* (1998) assigned the gradient of excitability to the gradient of population of bursting cells with coupling strength decreasing along the apical-basal axis. Their model also explained the collapse of the wave. However, their calculation did not take into account the decrease in velocity of the wave propagation. Based on our calculation (Yamada *et al.*, 1997), this can be explained effectively by a decrease in coupling strength among the bursting neurons (or oscillatory elements). In our calculation, the PC lobe was represented as a one-dimensional chain of Van der Pol oscillators with a gradient of intrinsic frequencies of each oscillator and with ohmic (bi-directional) interaction between neighboring oscillators. The velocity of wave propagation depends on the strength of interaction, that is, a stronger interaction results in a faster wave propagation (Kuramoto, 1984). Thus, the decrease in the velocity of wave propagation caused by 5-HT or ACh (Figs. 3 and 7) suggests a decrease in the strength of the interaction between oscillatory elements. This mechanism agrees well with the suggestion from the change in amplitudes described above, because it is reasonable that, when the strength of the interaction between oscillatory elements decrease, coherency between the activities of elements also decreased.

Gelperin *et al.* (1989) reported that 5-HT produces a clear elevation in intracellular calcium level and Glu suppresses the increase in intracellular calcium concentration induced by high potassium (10 mM) depolarization. In addition, it is known that elevation of intracellular calcium results in a reduction in intercellular coupling via gap junctions (Rose and Loewenstein, 1975). Though there has been no direct evidence that gap junctional (ohmic) interaction exists in the PC, it is possible that the decrease in wave propagation velocity by 5-HT or ACh is caused by a decrease in the strength of interaction between oscillatory elements. Furthermore, the decrease might be brought about by an increase in intracellular calcium levels of each of the PC interneurons. Consistent with this hypothesis is the finding that Glu did not change the velocity and that this is because Glu only decreases the elevated intracellular calcium level.

Oscillation and olfactory information processing

In this study, we showed that 5-HT, Glu and ACh could change not only the frequency or the amplitude of PC oscillation, but also the velocity of wave propagation. This suggests that oscillatory network of PC lobe has a potential to represent the olfactory information upon frequency, amplitude and wave propagation, in other words, spatio-temporal pattern of activities. As reported by Gervais *et al*. (1996) and Kimura *et al*. (1998c), changes in the frequency of PC oscillation are related to the olfactory information processing in *Limax* species. Recently, Ito *et al*. (1999) showed that electrical stimulation of the digit of superior tentacle increased the frequency of PC oscillation, while that of the middle or basal regions of inferior tentacle decreased it. The superior and inferior digits appear to be very similar in morphology and similar projection was observed to PC lobe from neurons in both digits (Kimura *et al*., 1998a). As stimulation to different oscillatory elements result in different states in an oscillatory network, it is possible that neurons in the superior and inferior tentacle digits stimulated different oscillatory elements in PC lobe, or simply, different cocktails of neurotransmitters are secreted from these neurons. Wave propagation is thought to be also involved in the olfactory information processing. According to our previous experiments (Kimura *et al*., 1994), aversively learned carrot and cucumber odors not only decreased the frequency of PC oscillation but also decreased the spontaneous fluctuation in the phase-lag of two procerebral regions. There has been no experimental evidence in *Limax* species that the amplitude of oscillation plays some role in the olfactory information processing. However, in a rabbit, it is well known that spatial patterns of neural activity encode odor information (Freeman, 1978).

Laurent *et al*. (1996) suggested in the locust olfactory system that odor information is represented as a series of spatially and temporally distributed ensembles of coherent firing neurons. Oscillatory network of PC lobe also has a potential to represent odors in the similar way.

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