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Odor Discrimination and Signal Transduction Mechanisms in Olfactory and Vomeronasal Systems

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by

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GENERAL INTRODUCTION

There are many types of internal receptors in our bodies for detecting chemical substances such as hormones, neurotransmitters or blood glucose. These internal receptors perceive only a limited number of substances such specified substances. On the other hand, olfactory receptors sensitively detect various chemical substances present in the external environment, where there are many types of substances.

During the past decade, a great deal of work in biochemistry, electrophysiology and molecular biology has enhanced our understanding of the mechanism of signal transduction in olfactory systems. As a results of these works, several concepts have been proposed for the mechanism by which olfactory neurons transduce a chemical stimulus into an electrical response (Fig. I-1). The most generally accepted one is that an olfactory response is induced by binding of an odorant to specific receptor proteins, followed by a G-protein-mediated increase in the internal cAMP and IP3 concentration to open the second messenger-activated cation channels on plasma membranes [1-12]. Recently Buck and Axel cloned the genes of an extremely large multigene family that encodes proteins with seven transmembrane domains and is expressed in the olfactory epithelium [13]. This report attracted a wide attention, but it is not as yet verified whether or not these proteins are true receptor proteins at present. Raming *et al.* reported the identification of another members of the gene family encoding putative odorant receptors and demonstrated that

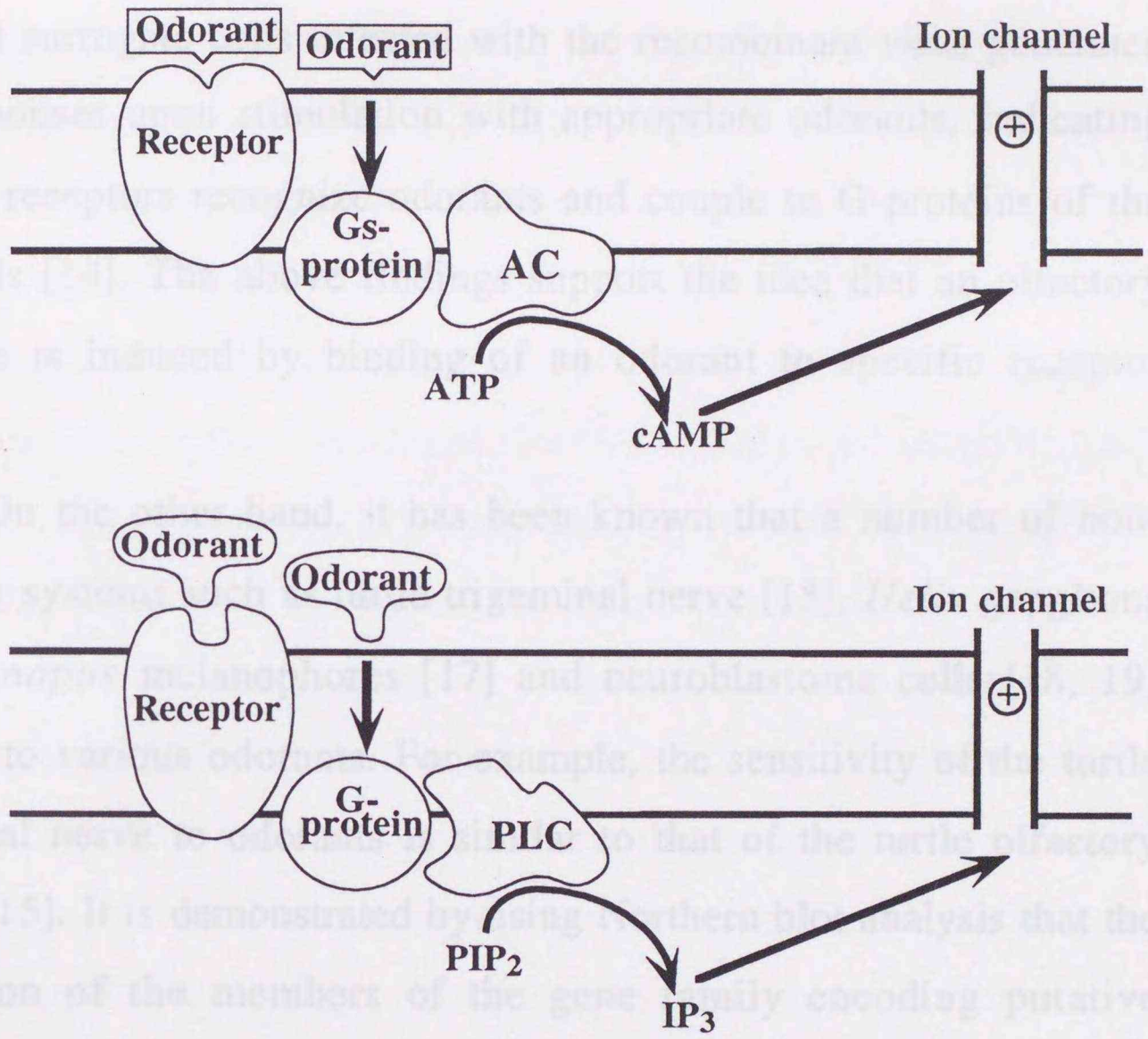

Figure I-1. A schematic drawing representing the several signal transduction pathways in olfactory neurons. See text for details. AC: adenylate cyclase. ATP: adenosine triphosphate. cAMP: cyclic adenosine monophosphate. PLC: phospholipase C. PIP_2: phosphatidyl inositol bisphosphate. IP_3: inositol trisphosphate.

they were transcribed in olfactory receptor neurons [14]. They infected a cell line derived from *Spodoptera frugiperda* (Sf9) with baculovirus harbouring the receptor-encoding complementary DNA. These non-neuronal surrogate cells infected with the recombinant virus generated IP_3 responses upon stimulation with appropriate odorants, indicating that the receptors recognize odorants and couple to G-proteins of the host cells [14]. The above findings support the idea that an olfactory response is induced by binding of an odorant to specific receptor proteins.

On the other hand, it has been known that a number of non-olfactory systems such as turtle trigeminal nerve [15], *Helix* ganglions [16], *Xenopus* melanophores [17] and neuroblastoma cells [18, 19] respond to various odorants. For example, the sensitivity of the turtle trigeminal nerve to odorants is similar to that of the turtle olfactory neuron [15]. It is demonstrated by using Northern blot analysis that the expression of the members of the gene family encoding putative olfactory receptors is restricted to the olfactory epithelium, and that within the olfactory epithelium it may be either predominantly or exclusively expressed by olfactory sensory neurons [13]. Thus these observations suggest that these specific receptor proteins are not required for odor reception in these non-olfactory systems and there is an olfactory transduction pathway which is not mediated by the receptor proteins. For instance, it is reported that the bitter tastant, quinine, directly activates transducin and Gi/Go-proteins [20]. Bitter tastants are generally hydrophobic as similar to odorants. There is a

possibility that some odorants penetrate the lipid layer of the olfactory receptor membranes and directly stimulate G-proteins.

Recently, Kashiwayanagi *et al.* reported that odorants, which did not increase IP_3 but increased cAMP in olfactory cilia, elicited large responses after application of high concentrations of forskolin, IBMX or cpt-cAMP, suggesting that there exists a pathway independent of a second messenger in olfactory transduction [21].

Putative olfactory receptor proteins are surrounded by membrane lipids, and G-proteins are located inside of the chemosensory membrane. Supposing that an odorant directly interacts with certain G-protein, the odorant has to traverse the lipid layers of the chemosensory membrane before contact with the G-protein. Odorants are generally hydrophobic and likely to interact directly with the lipid layers of the membrane. Thus it has been pointed out that interaction of odorants with lipid layers of olfactory receptor membranes may also play an important role in generation of olfactory responses even if olfactory transduction is mediated by either pathway including receptor proteins, or direct activation of G-proteins, or alternative pathways.

In the first Chapter of this study, we examine to what extent the olfactory system discriminates various odorants. It is well known that the olfactory system is exquisitely sensitive chemodetector recognizing odorants and discriminating among thousands of distinct odors. Although a great number of studies on odor discrimination have been carried out, there are few quantitative studies on the ability of olfactory

system to discriminate odorous compounds. In order to explore the mechanism of odor discrimination, it is necessary to examine to what extent the olfactory system discriminates various odorants. We measure the turtle olfactory bulbar responses to six pairs of highly pure optical isomers and compare the differences in odor intensity obtained between the optical isomers [22, 23]. The results indicate that there is no difference in odor intensity of optical isomers for all odorants examined. We also compare odor qualities of *d*- and *l*-isomers using a cross-adaptation method for quantitative analysis on odor quality [22, 23]. The results indicate that turtle olfactory system discriminates the odor quality difference between *l*- and *d*-isomers and that extent of discrimination of optical isomers varies among species of odorants. Recently, Hanada *et al.* reported that odor quality of *d*- and *l*-isomer is not discriminated at 40 °C [24], while it is well discriminated at 20 °C. This result suggests that odor is not recognized by binding of a ligand to a specific protein as seen in an interaction between enzyme and substrate or between a transmitter and its receptor. It is more likely that lipid layers of the olfactory receptor membrane are involved in the reception of odorant molecules.

In order to examine the role of lipids in the *in vivo* olfactory system, we treat the turtle olfactory epithelium with various lipids and examine its effects on the olfactory responses to various odorants [25-30] (Chapter II). The results indicate that the olfactory responses to various odorants are greatly affected by the treatment of olfactory

epithelium with various lipids, confirming that lipids play an important role in the odor reception in the olfactory system.

The vomeronasal organ is a chemoreceptive structure situated at the base of the nasal septum of most terrestrial vertebrates and is anatomically and functionally distinct from the main olfactory system. In most terrestrial vertebrates, the vomeronasal system plays important roles in the perception of chemical stimuli related to feeding, social and reproductive behavior [31, 32]. These stimuli can induce hormonal changes, affect the success of pregnancy, alter the course of puberty, modulate female cyclicity and ovulation, elicit courtship and attraction, and modulate reproductive behavior and aggression [32]. Our understanding of the functional properties of vomeronasal system in vertebrates has been mainly deepened by behavioral, biochemical and molecular biological studies. In contrast, few studies on the electrophysiological properties of vomeronasal receptor neurons in vomeronasal systems have been carried out. Chapter III describes the electrophysiological properties of the turtle vomeronasal receptor neuron with whole-cell patch clamp [33].

Up to date, no study has demonstrated the signal transduction mechanism of vomeronasal receptor neurons. In order to ascertain the existence of signal transduction pathways mediated by putative second messengers, which are considered to be involved in the main olfactory transduction, in the vomeronasal system, cAMP, cGMP (Chapter IV-2) and IP_3 (Chapter IV-3) were injected into turtle vomeronasal receptor neurons under whole-cell patch clamp. The results described in this

Chapter show that each intracellular application of cAMP, cGMP and IP_3 from the patch pipette to turtle vomeronasal receptor neurons elicits the membrane current under the condition of the whole-cell patch clamp, suggesting the existence of second messenger-mediated transduction pathways in the turtle vomeronasal system [33, 34].

Turtle vomeronasal organ senses general odorants as well as chemical stimuli of a social nature [35]. For instance, Hatanaka *et al.* and Shoji *et al.* recorded turtle accessory olfactory bulb wave induced by odor stimuli and showed that the vomeronasal organ responds to many kinds of odorants [37, 38].We examine to what extent cAMP-mediated pathway found in this study contributes to the generation of the vomeronasal response to general odorants in *in vivo* system by measuring the turtle accessory olfactory bulbar response (Chapter V) [36]. The results described in this Chapter indicate that a general odorant such as citralva, which does not increase IP_3 but increase cAMP in olfactory cilia of the bullfrogs and the rats, elicits a large response after application of high concentrations of forskolin. This suggests that the cAMP-mediated pathway does not greatly contribute to the generation of the vomeronasal response to a general odorant.

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LIST OF ABBREVIATIONS

PC; phosphatidylcholine from fresh egg yolk

PS; phosphatidylserine from bovine brain

PA; phosphatidic acid from egg yolk

CL; cardiolipin from bovine heart

PART 1

CHAPTER I
DIFFERENCE IN ODOR INTENSITY AND QUALITY BETWEEN *l*-AND *d*-OPTICAL ISOMERS

I-1 Difference in Odor Intensity Between *l*- and *d*-Optical Isomers

INTRODUCTION

Structure-activity relationships in odor reception have attracted a wide interest and been studied psychometrically by many investigators [1,2]. Among the relationships, whether there are differences in odor quality and intensity between optical isomers has been discussed as one of the central topics.

Laiterg *et al.* reported that threshold concentration of *l*-carvone is about 50 times lower than that of *d*-carvone [3]. Recently, Polak *et al.* reported that there are 78% of subjects whose threshold concentration of *l*-carvone was 3 to 14 times lower than that of *d*-carvone and 22% of subjects whose threshold concentration of *d*-carvone was 2 to 17 times lower than that of *l*-carvone [4]. They also reported that subjects' relative sensitivities for a-ionone optical isomers were found to diverge widely, some subjects being much more sensitive to *d*-α-ionone than to

l-α-ionone and vice versa [4]. The threshold of *d*-nootkatone was reported to be different from that of *l*-nootkatone [5], whereas there was a paper reporting no difference in odor intensity between them [6]. Thus the conclusions on the differences on odor intensity based on evaluation by human sense are ambiguous [1]. In addition, the lack of availability of pure optical isomers has hampered deduction of clear conclusions on odors of optical isomers.

In the present study [7,8], odor intensities of optical isomers are compared using highly pure odorants. Odor intensity is evaluated by measuring the summated olfactory bulbar responses in the turtle. The results clearly indicate that odor intensities of optical isomers are practically identical with each other for all odorants examined.

MATERIALS AND METHODS

Recording of olfactory bulbar response

Turtles (*Geoclemys reevesii*) weighing 160-240 g were used in the present study. Olfactory bulbar responses were recorded essentially as described by Kashiwayanagi *et al.* [9]. In brief, turtles were weakly anesthetized with the necessary and minimum amount of urethan to lessen pain in the operation of the animal, immobilized by an intramuscular injection of *d*-tubocurarine chloride (about 450 μg/100 g body wt im), and locally anesthetized with lidocaine at the wounded and head-fixation points. The olfactory bulb was exposed using a dental

drill, and the dura mater on the olfactory bulb was removed carefully. To eliminate the possible effect of the accessory olfactory bulb activities [10], the nerve from the vomeronasal organ was cut off before entry to the accessory bulb. The stimulant-induced brain waves (bulbar responses) were recorded by attaching a pair of silver bipolar electrodes to the medial part of the anterior bulb. The responses were amplified and then integrated by electric integrator (time constant 0.3 s).

Chemical stimulation

The irrigating and stimulating solutions were applied to the olfactory epithelium through a stainless steel tube. Before application of the stimulating solution, the olfactory epithelium was irrigated with turtle Ringer solution for about 10 min. Stimulating solution, which was prepared by dissolving odorants in the turtle Ringer solution, was applied to the epithelium at a flow rate of 27 ml/min. After each application of the stimulating solution on the epithelium, the epithelium was rinsed with the Ringer solution. About 10 min were interposed between successive stimulations. Composition of the turtle Ringer solution was (in mM) 116 NaCl, 4 KCl, 2 $CaCl_2$, and 0.5 Na_2HPO_4-NaH_2PO_4 (pH 7.4). All the experiments were carried out at 20 ± 3 °C.

Chemicals

Highly pure optical isomers of carvone [2-methy*l*-5-(1-methylethenyl)-2-cyclohexene-1-one], *β*-citronellol (3,7-dimethyl-6-

octen-1-ol), menthol [(1α,2ß,5α)-5-methyl-2-(1-methylethyl)cyclohexanol], hydroxycitronellal (7-hydroxy-3,7-dimethyloctanal), citronellal (3,7-dimethyl-6-octenal), and limonene [1-methyl-4-(1-methylethenyl)cyclohexene] were kindly supplied from Takasago International (Tokyo, Japan). The purity of the optical isomers evaluated by gas chromatography was 99% for *d*-carvone, 98% for *l*-carvone, >99% for *d*-*ß*-citronellol, >99% for *l*-*ß*-citronellol, 99% for *d*-menthol, >99% for *l*-menthol, 99% for *d*-hydroxycitronellal, 99% for *l*-hydroxycitronellal, 98% for *d*-citronellal, 99% for *l*-citronellal, 97% for *d*-limonene, and 96% for *l*-limonene. All chemicals used are of best grade available. Concentrations of stock solutions used were 10^{-2} M for hydroxycitronellal, 10^{-3} M for carvone, *ß*-citronellol, menthol and citronellal, and 10^{-4} M for limonene. These stock solutions were stored at +4 °C

RESULTS

Figure 1-1 shows the summated olfactory bulbar responses to *l*- and *d*-carvone of varying concentrations. The responses show a peak at onset of stimulation and decline rapidly during stimulation. The peak height of the summated bulbar response is taken as the magnitude of the response. The magnitudes of the responses to both *l*- and *d*-carvone are increased with an increase in their concentrations.

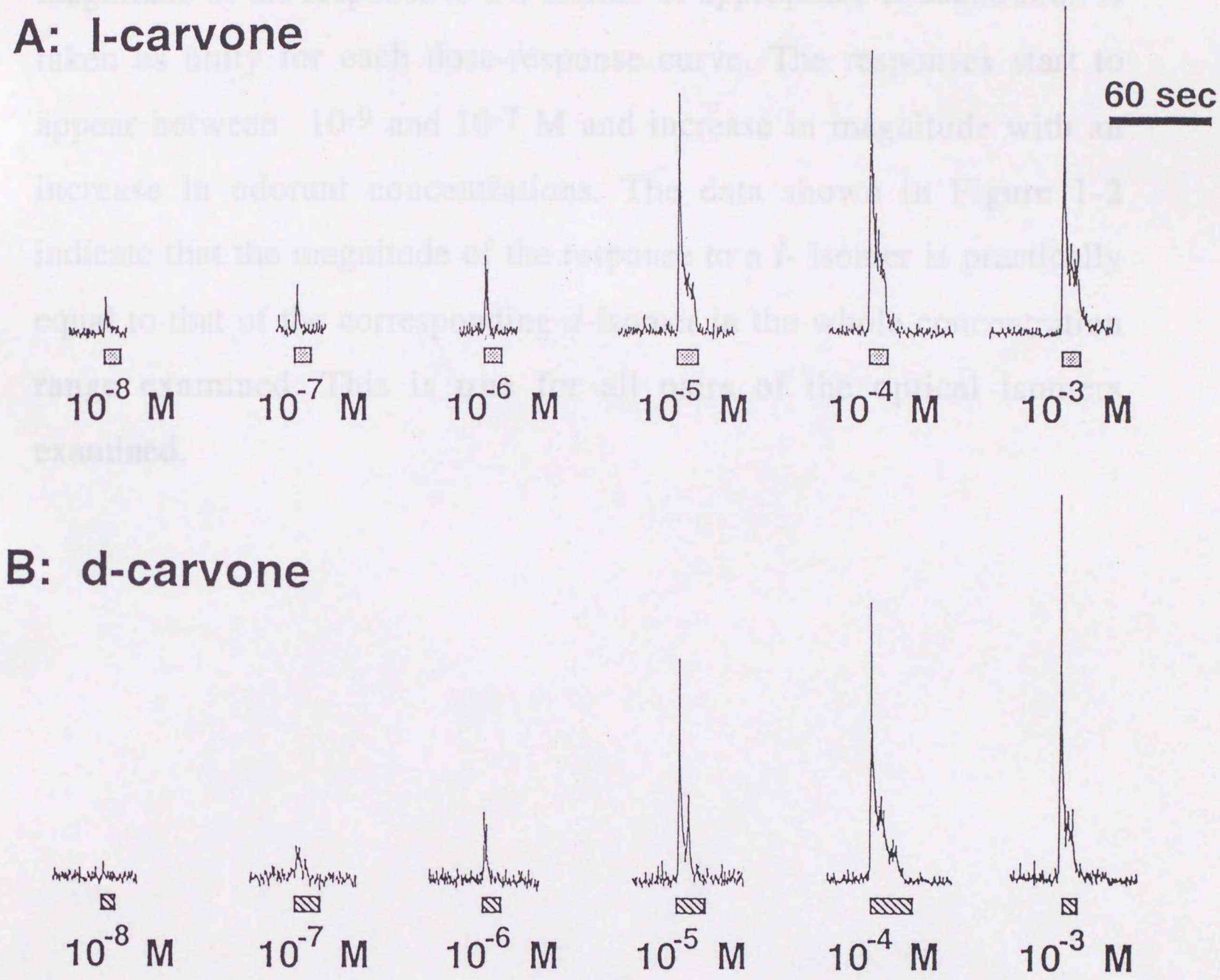


Figure 1-1. Turtle olfactory bulbar responses to *l*-carvone (**A**) and *d*-carvone (**B**) of varying concentrations. Bars at the bottom of each record represent duration of application of stimulants.

Figure 1-2 plots relative magnitudes of the responses to *l*- and *d*-optical isomers as a function of odorant concentration. Here the magnitude of the response to a *d*-isomer of appropriate concentration is taken as unity for each dose-response curve. The responses start to appear between 10^{-9} and 10^{-7} M and increase in magnitude with an increase in odorant concentrations. The data shown in Figure 1-2 indicate that the magnitude of the response to a *l*- isomer is practically equal to that of the corresponding *d*-isomer in the whole concentration range examined. This is true for all pairs of the optical isomers examined.

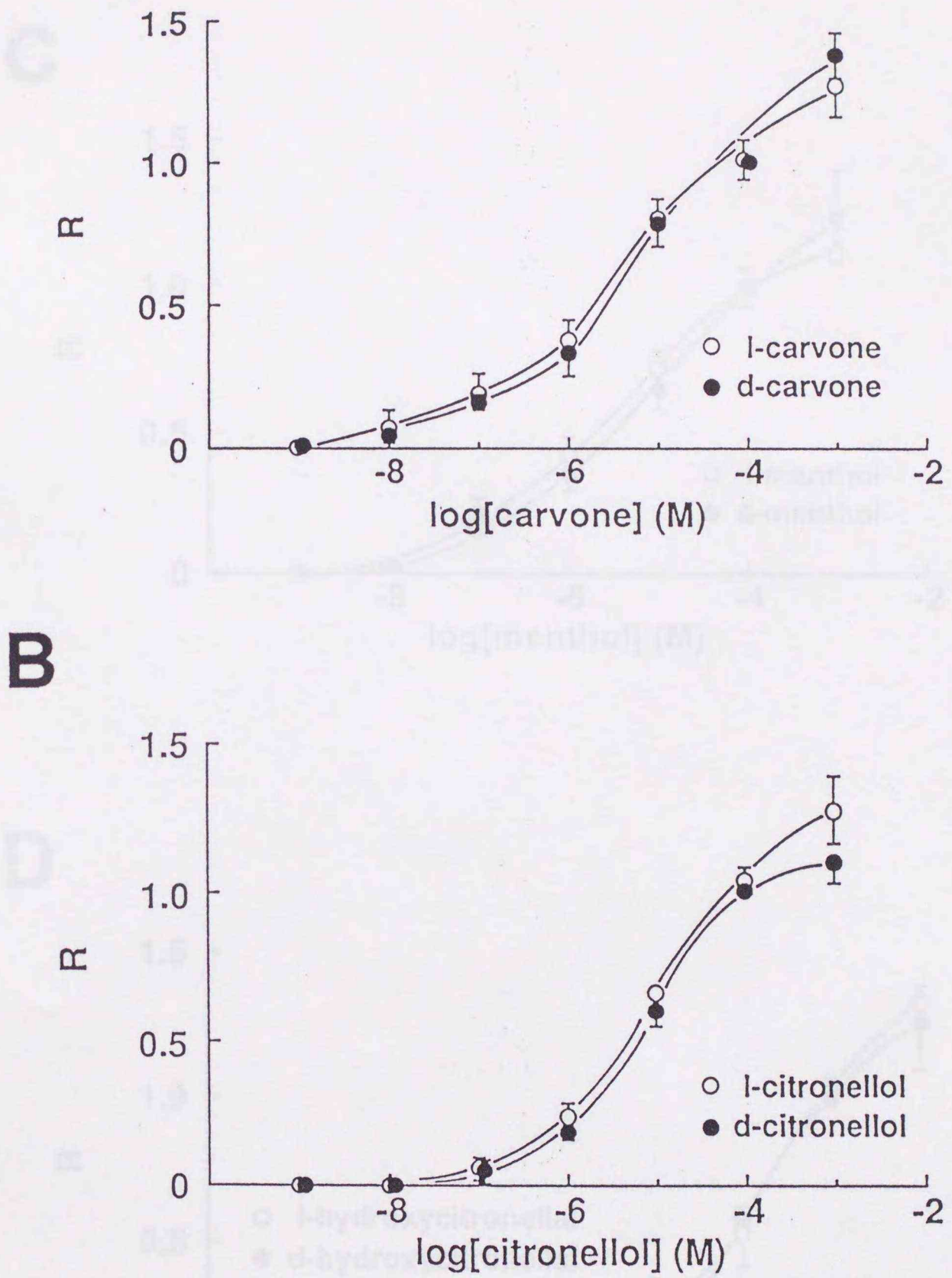
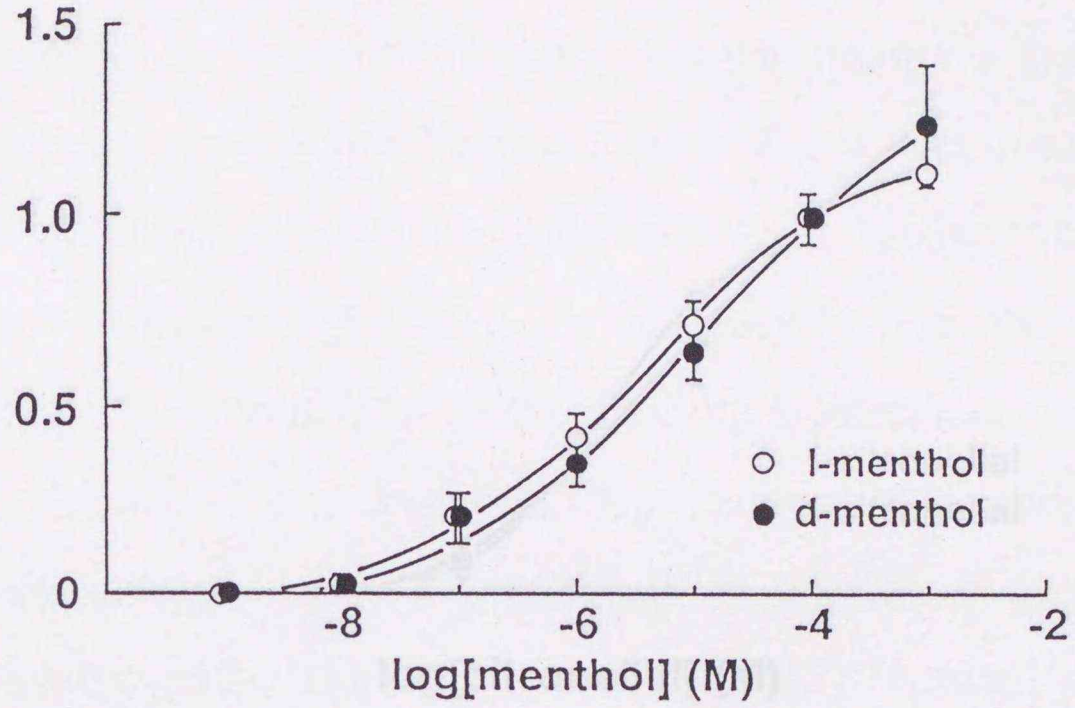


Figure 1-2. Relative magnitude of turtle bulbar responses to *d*-optical isomers (●) and corresponding *l*-optical isomers (○) as a function of odorant concentration. Magnitude of response is calculated relative to response to 10^{-4} M *d*-carvone (**A**), 10^{-4} M *ß-d*-citronellol (**B**), 10^{-4} M *d*-menthol (**C**), 10^{-4} M *d*-hydroxycitronellal (**D**), 10^{-4} M *d*-citronellal (**E**), or 5×10^{-5} *d*-limonene (**F**), respectively. Each point is mean ± S.E.M. of data obtained from at least 3 preparations.

C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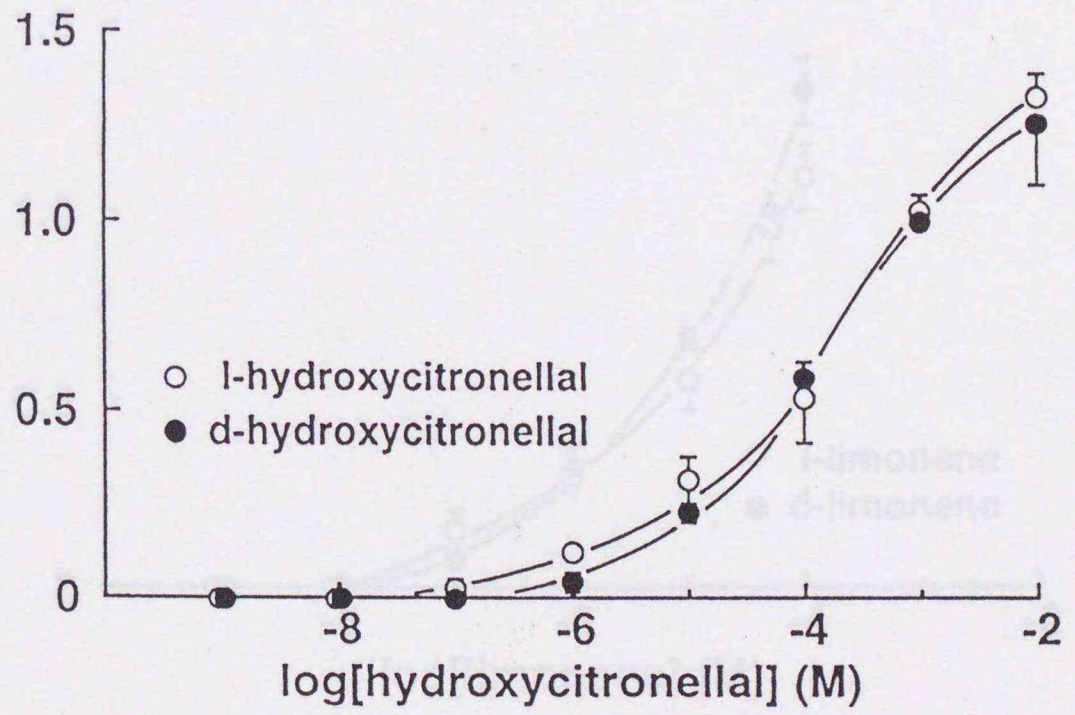
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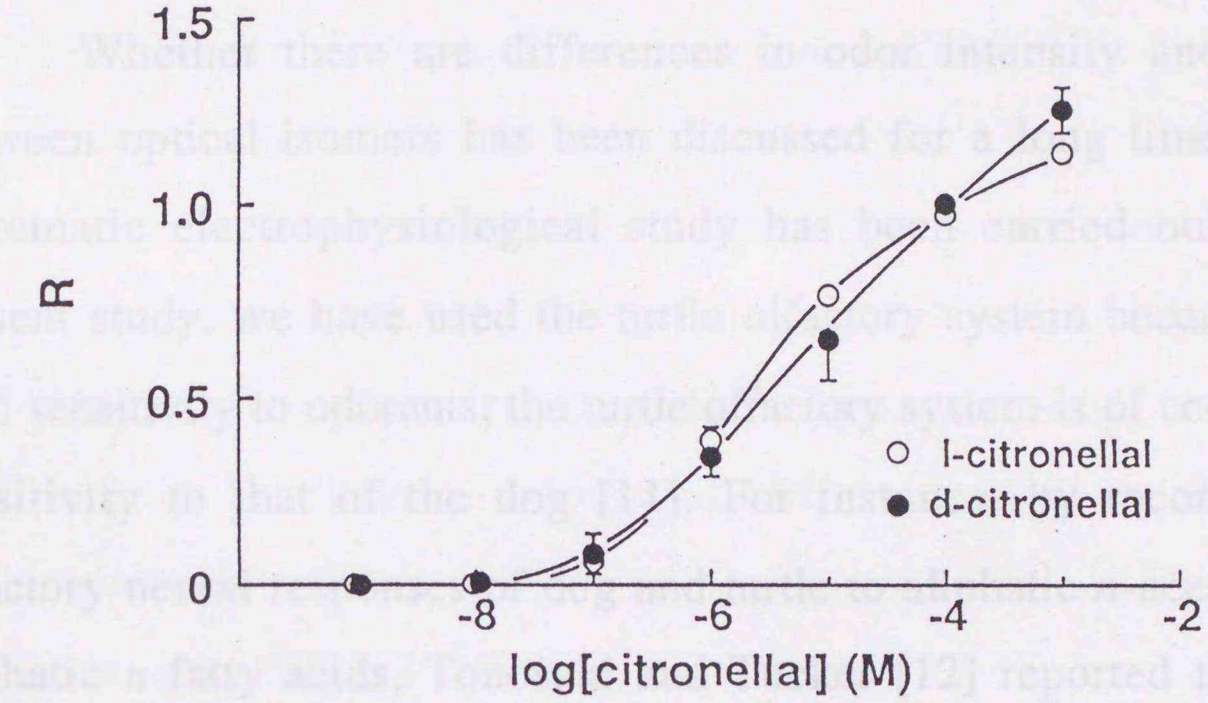
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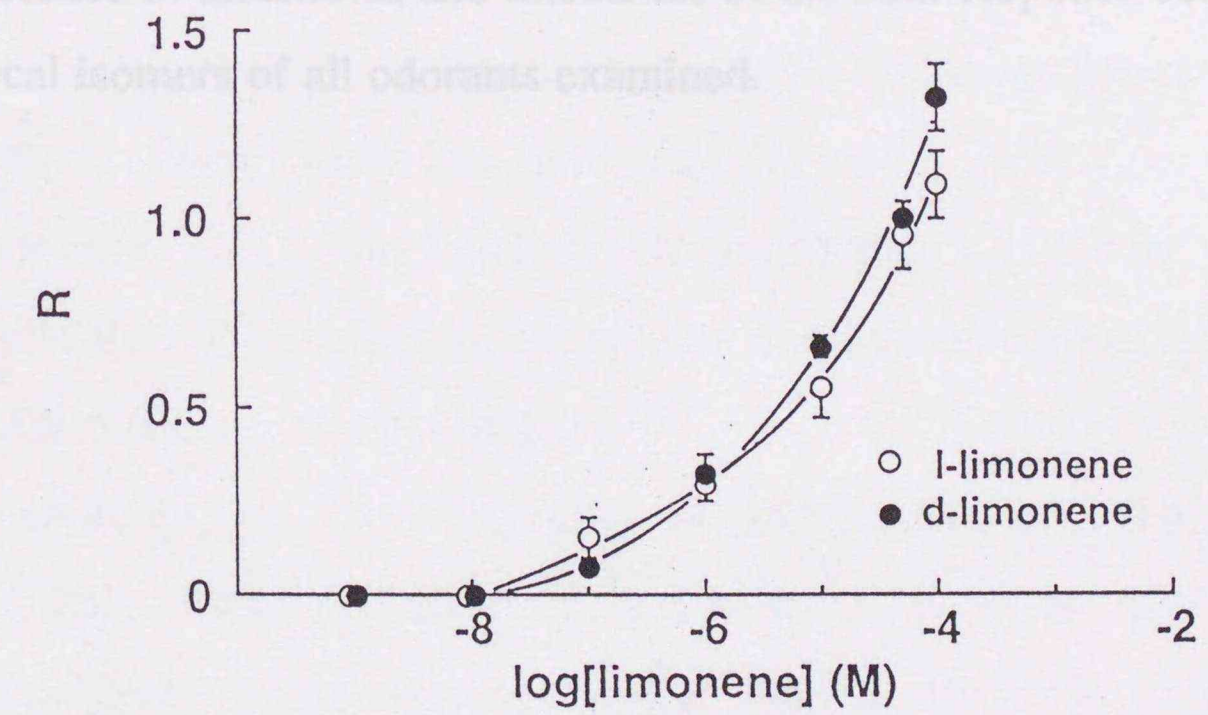
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DISCUSSION

Whether there are differences in odor intensity and quality between optical isomers has been discussed for a long time, but no systematic electrophysiological study has been carried out. In the present study, we have used the turtle olfactory system because of its high sensitivity to odorants; the turtle olfactory system is of comparable sensitivity to that of the dog [11]. For instance, by recording the olfactory neural responses of dog and turtle to aliphatic *n*-acetates and aliphatic *n*-fatty acids, Tonosaki and Tucker [12] reported that there was no practical difference between the thresholds of the dog and turtle olfactory responses to the odorants.

The present results clearly demonstrate that there is no difference in thresholds and intensities of the odor response between the optical isomers of all odorants examined.

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I-2. Difference in Odor Quality Between *l*- and *d*-Optical Isomers

INTRODUCTION

Chapter I-1 dealt with difference in odor intensity between optical isomers. In this section, difference in odor quality between optical isomers is focused on. It was reported that odor quality of *d*-carvone is distinctly different from that of *l*-carvone [3,4]. The odor quality of *d*-nootkatone was reported to be different from that of *l*-nootkatone [5]. The differences in odor qualities of optical isomers were also reported with limonene [6], *ß*-citronellol [7], and hydroxycitronellal [8]. However, the conclusions on the differences in odor quality based on evaluation by human sense are ambiguous [1,2].

The initial event of odor reception is adsorption of odorants on the receptor sites of olfactory cells. Odorants having different odor qualities must be adsorbed in different receptor sites. To analyze differences in the odor qualities of various odorants, a cross-adaptation method is useful. Usually cross-adaptation experiments have been carried out with one pair of chemical stimuli of fixed concentrations in order to observe whether a response to the stimulus applied secondarily appears. Thus the usual cross-adaptation method gives only qualitative data. In the present study, odor quality of optical isomers is compared

using highly pure odorants. To evaluate the difference in odor quality, we apply the quantitative cross-adaptation method [9,10] to the turtle olfactory system. The results indicate that the degree of cross adaptation greatly varies with species of odorants [11,12].

MATERIALS AND METHODS

Recording of olfactory bulbar response

Olfactory bulbar response was recorded essentially as described in Chapter I-1.

Chemical stimulation

The procedure of chemical stimulation was essentially similar to that described in Chapter I-1.

Chemicals

The sources for the reagents used except for cineole and the purity of the optical isomers evaluated by gas chromatography were described in Chapter I-1. Cineole (eucalyptol; 1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane) was purchased from Nakarai Chemicals (Kyoto, Japan). The concentration of stock solution used were 10^{-3} M for cineole. The concentrations of stock solutions of other odorants used were described in Chapter I-1. The stock solutions were stored at the same temperature as described in Chapter I-1.

RESULTS

Before the cross-adaptation experiment between optical isomers, the experiment between odorants having distinctly different odors was carried out as a control experiment. Figure 1-3A illustrates typical records for the cross-adaptation experiment between *l*-limonene having a lemonlike odor and cineole having a camphorlike odor. *l*-Limonene of varying concentrations is applied first, and then cineole of a fixed concentration (10^{-5} M) is applied after the response to *l*-limonene declines to a spontaneous level. As seen from the records, the magnitude of the response to cineole applied secondarily is decreased only slightly with an increase in concentration of *l*-limonene applied first.

Figure 1-3B plots the magnitude of the response to 10^{-5} M cineole (R_2) applied secondarily as a function of that to primarily applied *l*-limonene of varying concentrations (R_1). The reason why R_1 (magnitude of the response) is used instead of odorant concentration is as follows. In general, two different odorants of the same concentrations induce different magnitude of the response, and the magnitude of the response is a more valuable index than odorant concentration in the cross-adaptation experiment. The magnitudes of the responses (R_1 and R_2) are represented as relative magnitudes of the responses where the response to 10^{-5} M cineole applied alone is taken as

unity. In Figure 1-3B, R_1 = 1.0 means that the magnitude of the response to *l*-limonene is equal to that to cineole when each odorant is applied alone. Here the value of R_2 at R_1 = 1.0 is defined as the heterogeneity value. Hence, the heterogeneity value is some value between 0 and 1 where 1.0 implies that the receptor sites for two odorants are completely independent of each other, suggesting that the turtle olfactory system completely discriminates the quality of these two odorants. The heterogeneity value in the cross-adaptation between *l*-limonene and cineole is evaluated as 0.93, suggesting that the receptor site for cineole is almost independent of that for *l*-limonene.

Figure 1-4A illustrates typical records of the cross-adaptation experiment between *l*- and *d*-limonene. The response to 5 x 10^{-5} M *d*-limonene is decreased with an increase in concentration of *l*-limonene applied first. Figure 1-4B delineates the relationship between the magnitudes of R_1 to *l*-limonene and R_2 to *d*-limonene. As shown in Fig. 1-4, the magnitude of the response to *d*-limonene is greatly decreased with an increase in that to *l*-limonene applied first. The heterogeneity value is evaluated as 0.26.

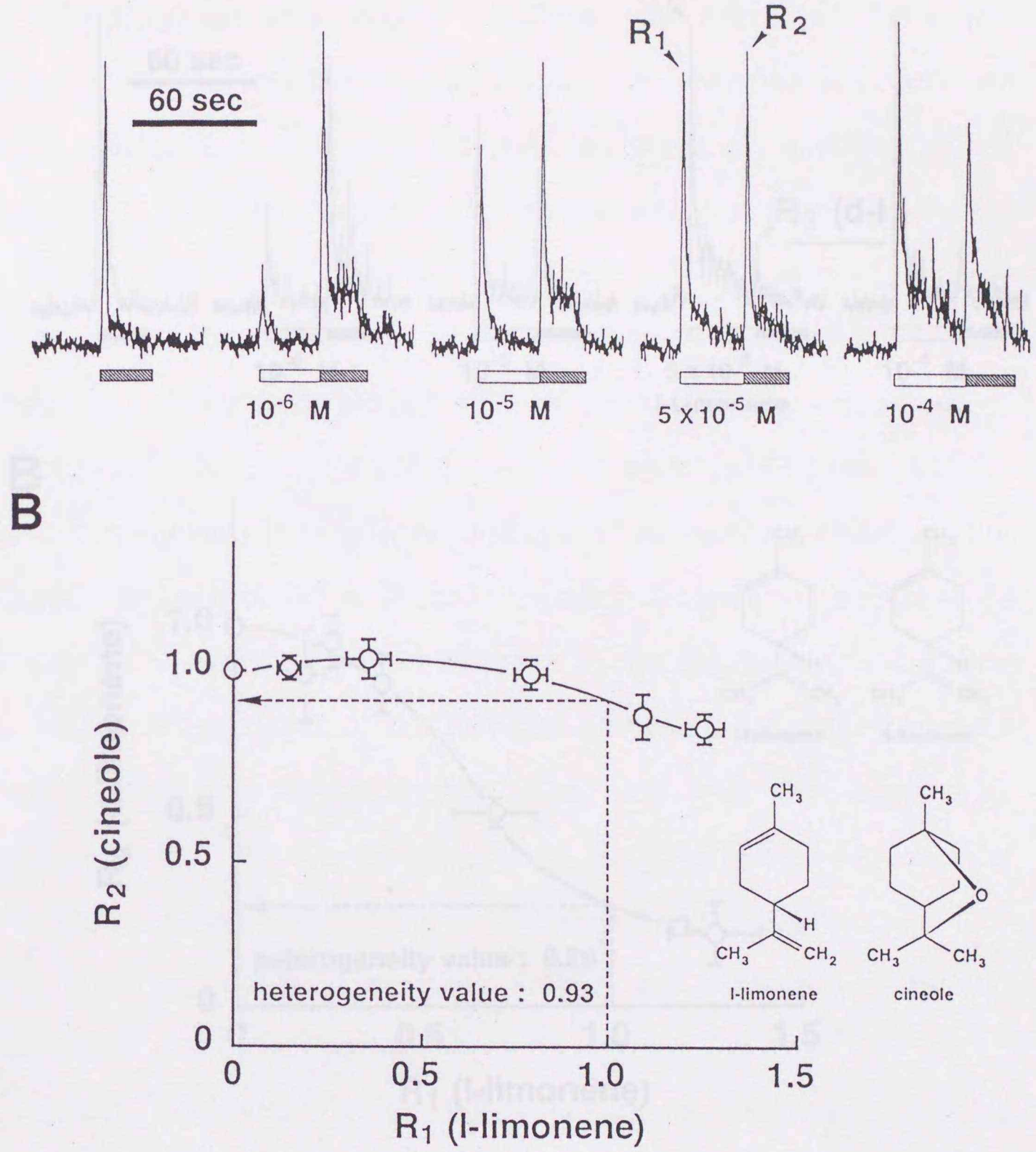


Figure 1-3. **A:** Typical records of response to 10^{-5} M cineole applied secondarily after application of odorants (open bars, *l*-limonene of varying concentrations; hatched bars, 10^{-5} M cineole). **B:** Relative magnitude of response to 10^{-5} M cineole applied secondarily as a function of magnitude of response to *l*-limonene applied first. Magnitudes of response (R_1 and R_2) are calculated relative to response to 10^{-5} M cineole. Each point is mean $\pm$ S.E.M. of data obtained from 4 preparations.

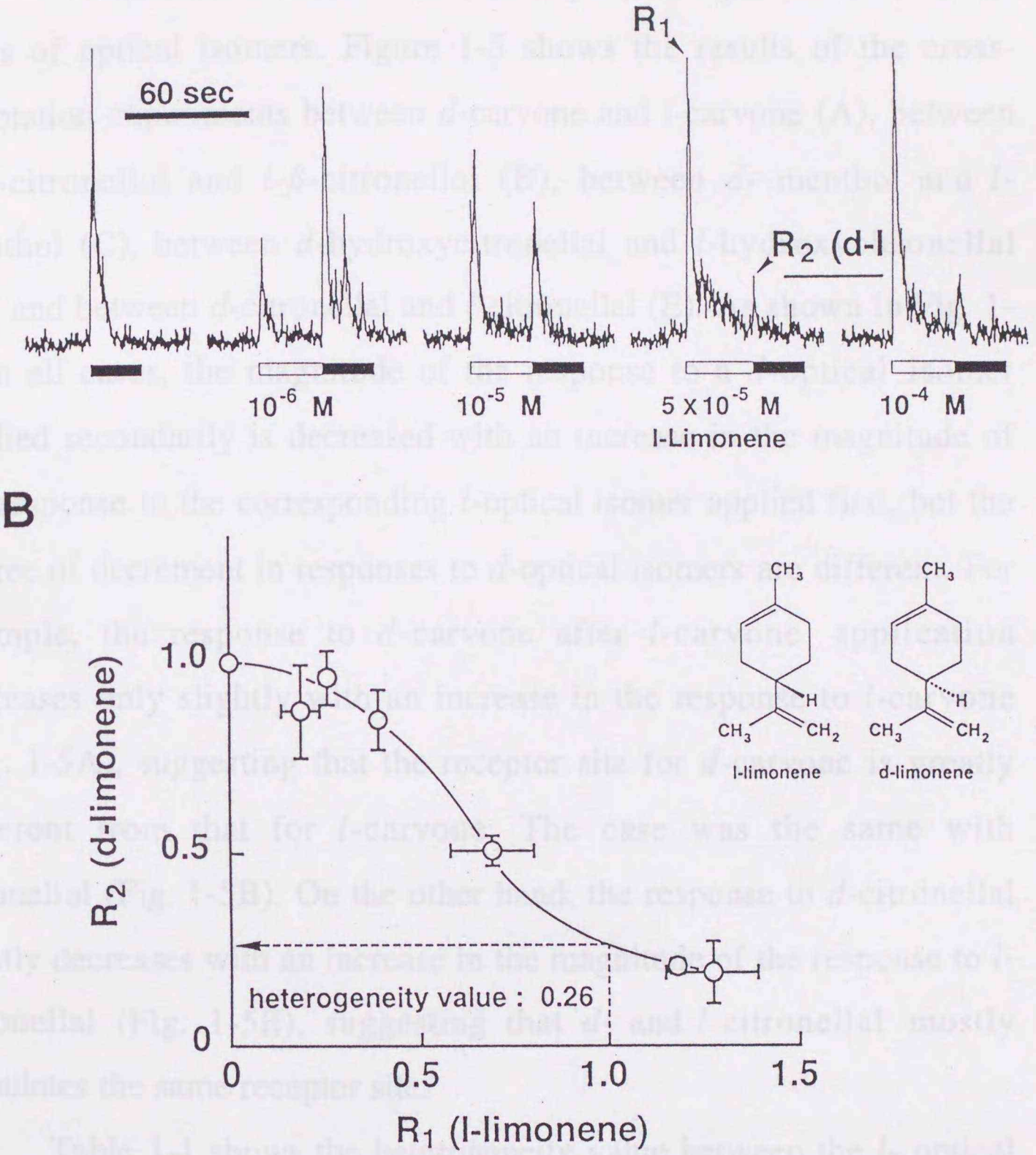


Figure 1-4. **A:** Typical records of response to 5×10^{-5} M *d*-limonene applied secondarily after application of *l*-limonene of varying concentrations applied first. Bars at the bottom of records represent duration of application of odorants (open bars, *l*-limonene of varying concentrations; closed bars, 5×10^{-5} M *d*-limonene). **B:** Relative magnitude of response to 5×10^{-5} M *d*-limonene applied secondarily as a function of magnitude of response to *l*-limonene applied first. Magnitudes of response (R_1 and R_2) are calculated relative to response to 5×10^{-5} M *d*-limonene. Each point is mean ± S.E.M. of data obtained from 3 preparations.

We also carried out the cross-adaptation experiments on other pairs of optical isomers. Figure 1-5 shows the results of the cross-adaptation experiments between *d*-carvone and *l*-carvone (A), between *d*-*ß*-citronellol and *l*-*ß*-citronellol (B), between *d*- menthol and *l*-menthol (C), between *d*-hydroxycitronellal and *l*-hydroxycitronellal (D), and between *d*-citronellal and *l*-citronellal (E). As shown in Fig. 1-5, in all cases, the magnitude of the response to a *d*-optical isomer applied secondarily is decreased with an increase in the magnitude of the response to the corresponding *l*-optical isomer applied first, but the degree of decrement in responses to *d*-optical isomers are different. For example, the response to *d*-carvone after *l*-carvone application decreases only slightly with an increase in the response to *l*-carvone (Fig. 1-5A), suggesting that the receptor site for *d*-carvone is greatly different from that for *l*-carvone. The case was the same with citronellol (Fig. 1-5B). On the other hand, the response to *d*-citronellal greatly decreases with an increase in the magnitude of the response to *l*-citronellal (Fig. 1-5E), suggesting that *d*- and *l*-citronellal mostly stimulates the same receptor site.

Table 1-1 shows the heterogeneity value between the *l*- optical isomer and the corresponding *d*-isomer. The values are distributed from 0.78 for carvone to 0.26 for limonene. Thus the degree of discrimination for optical isomers greatly varies with species of odorants.

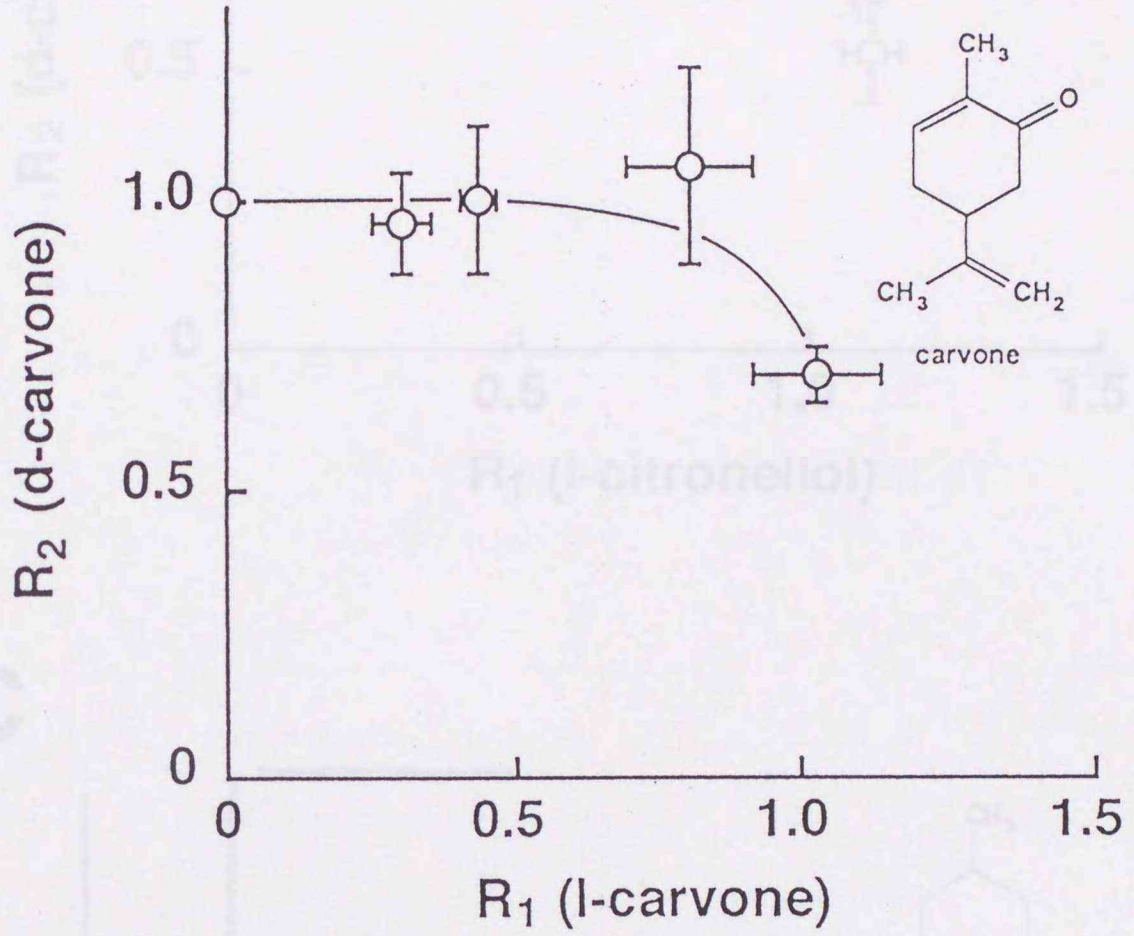
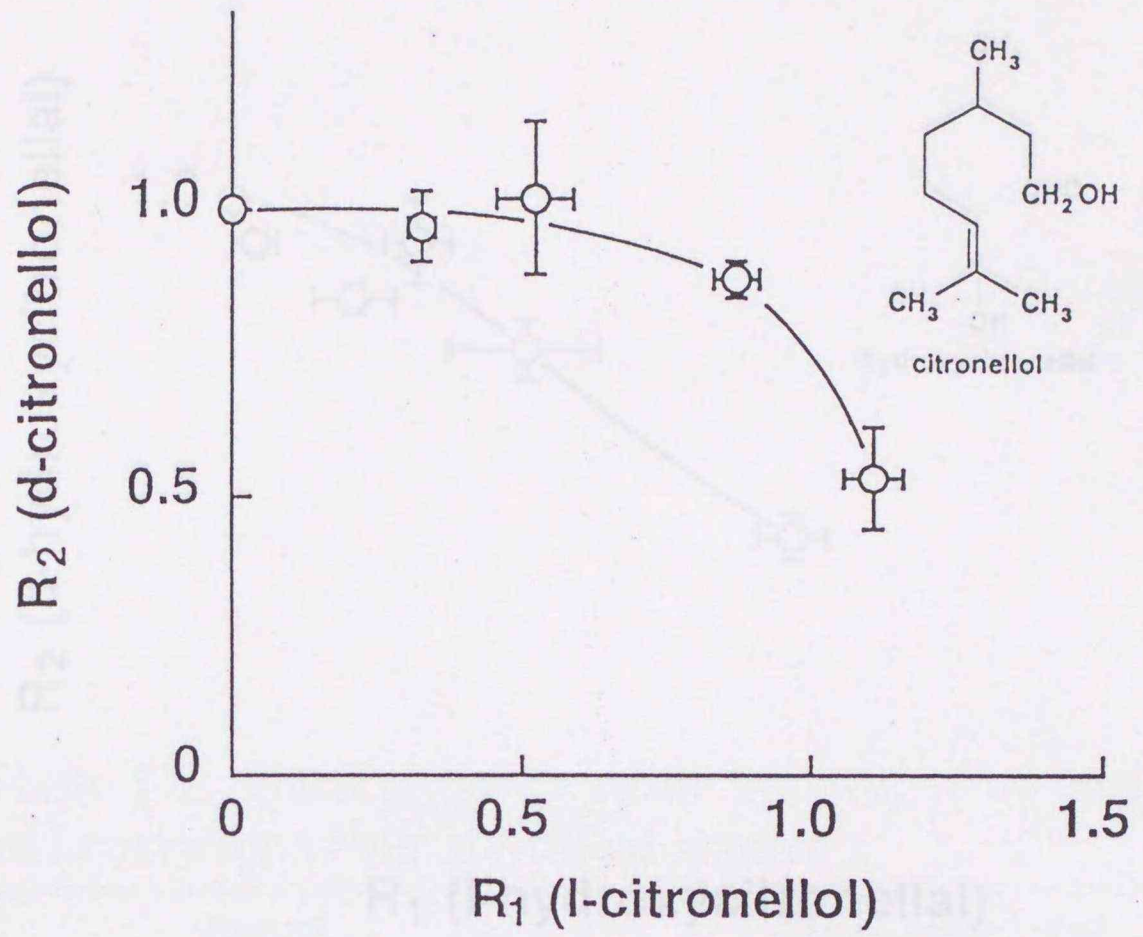
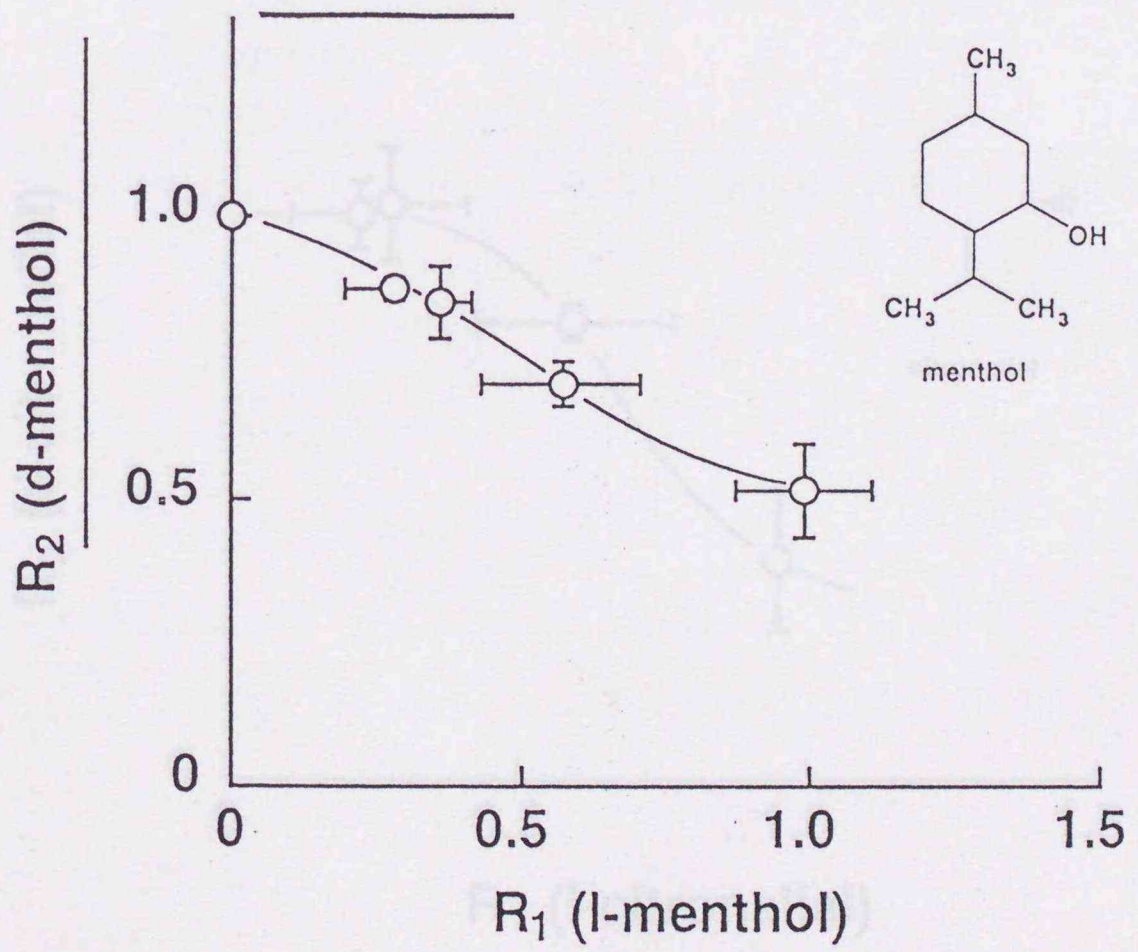


Figure 1-5. **A:** Relative magnitude of olfactory responses to *d*-optical isomers applied secondarily as a function of magnitudes of responses to corresponding *l*-optical isomers applied first. Magnitudes of response (R_1 and R_2) are calculated relative to response to 10^{-4} M *d*-carvone (**A**), 10^{-4} M *ß-d*-citronellol (**B**), 10^{-4} M *d*-menthol (**C**), 10^{-3} M *d*-hydroxycitronellal (**D**), or 10^{-4} M *d*-citronellal (**E**) applied alone, respectively. Each point is mean ± S.E.M. of data obtained from 3 preparations.

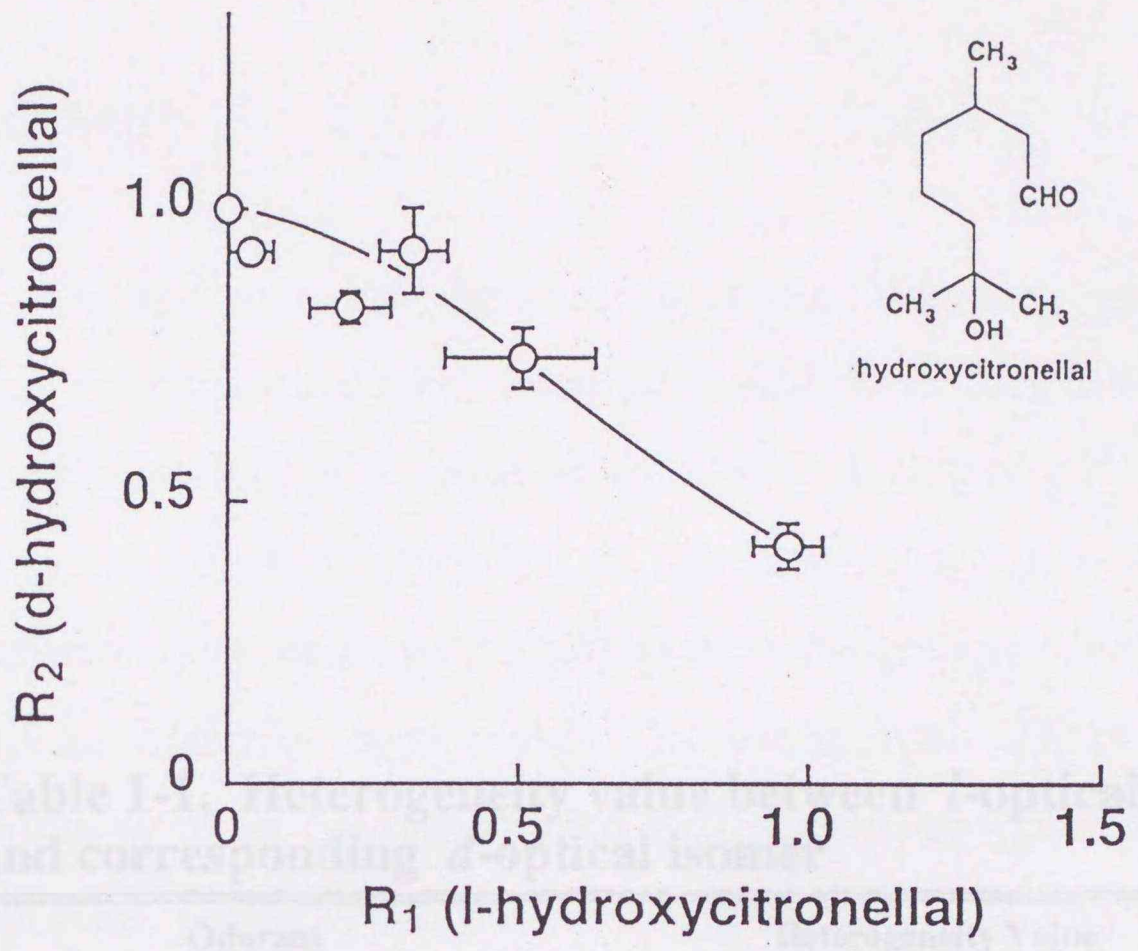
B


1.0
0.5
0
0
0.5
1.0
1.5
R2 (d-citronellol)
R1 (l-citronellol)
CH3
CH2OH
CH3
CH3
citronellol

C


1.0
0.5
0
0
0.5
1.0
1.5
R2 (d-menthol)
R1 (l-menthol)
CH3
OH
CH3
CH3
menthol

D


1.0
0.5
0
0
0.5
1.0
1.5
R2 (d-hydroxycitronellal)
R1 (l-hydroxycitronellal)
CH3
CHO
CH3
CH3
OH
hydroxycitronellal

E

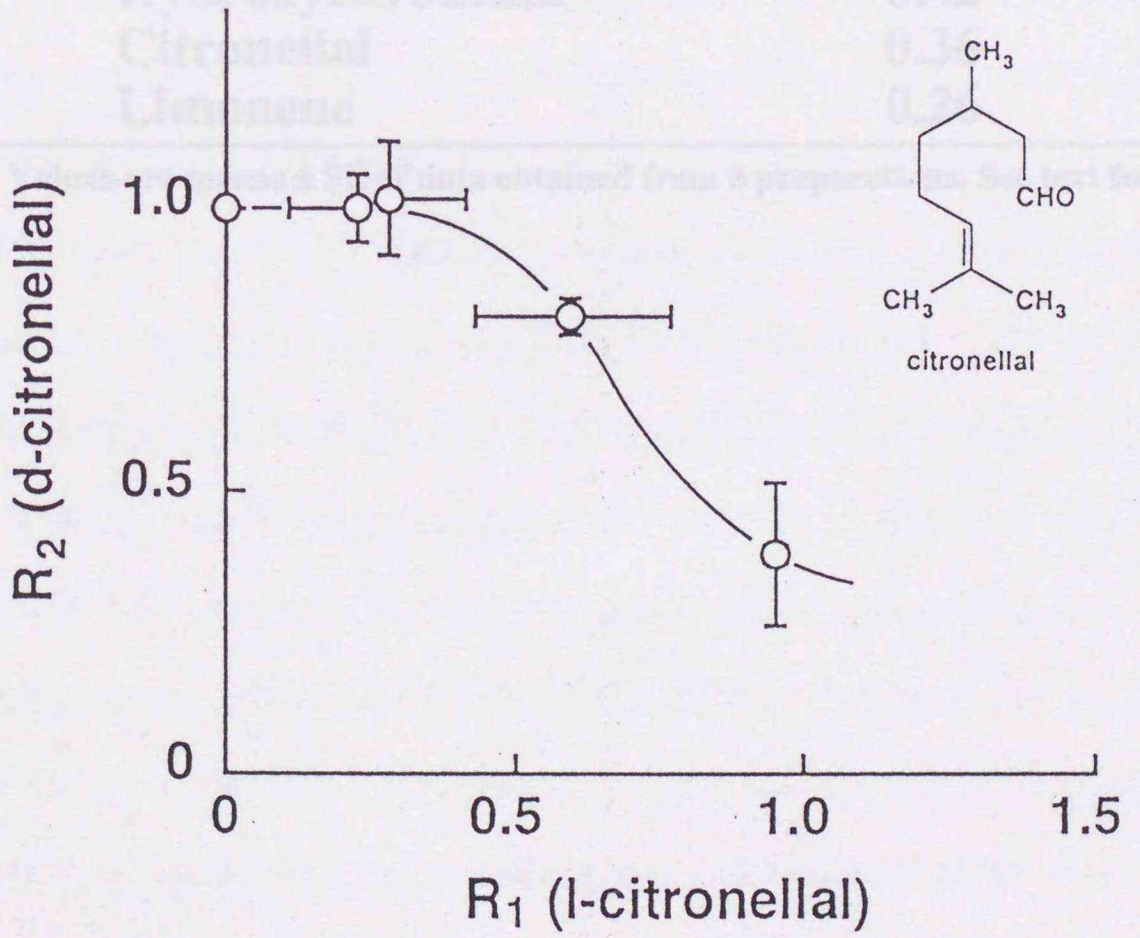

1.0
0.5
0
0
0.5
1.0
1.5
R2 (d-citronellal)
R1 (l-citronellal)
CH3
CHO
CH3
CH3
citronellal

Table 1-1. Heterogeneity value between *l*-optical isomer and corresponding *d*-optical isomer

Odorant	Heterogeneity Value
Carvone	0.78
β-Citronellol	0.75
Menthol	0.52
Hydroxycitronellal	0.42
Citronellal	0.36
Limonene	0.26

Values are means ± SE of data obtained from 3 preparations. See text for details.

DISCUSSION

Whether there are differences in odor intensity and quality between optical isomers has been discussed for a long time, but no systematic electrophysiological study has been carried out especially with regard to odor quality. In the previous cross-adaptation experiments using the frog olfactory system, the response to an odorant applied secondarily appreciably decreased with an increase in concentration of an odorant applied first even when odor qualities of two odorants used are quite different [10]. This is probably due to nonspecific inhibition of the response to odorant applied secondarily by odorant applied first because the frog olfactory system is not so sensitive to odorants and relatively high concentrations of odorants are used for the experiments. In the present study, we have used the turtle olfactory system because the turtle olfactory system is much more sensitive than the frog olfactory system. As seen in the combination of *l*-limonene and cineole whose odors are quite different from each other, the response to cineole applied secondarily dose not practically decrease with an increase in concentration of *l*-limonene applied first. Thus the turtle olfactory system has an advantage over the frog system on the basis that nonspecific inhibition in the cross adaptation is very small. In the present study, we have measured the olfactory bulbar response to odorants. In general, information on odor discrimination originated from the receptor cells is considered to be much more emphasized in

the central nerve system. Hence, when one pair of odorants is discriminated by the olfactory bulb, the odorants must be discriminated first by the receptor cells, and information on the discrimination may be emphasized in the bulb. Thus the olfactory bulbar response is a good index especially when odor discrimination is examined.

Many investigators have compared odor quality and intensity of optical isomers and reported that there are differences in thresholds as well as odor quality between optical isomers [3-5]. The results shown in Chapter I-1 clearly demonstrated that there is no difference in thresholds and intensities of the odor response between the optical isomers of all odorants examined. On the other hand, the results of the cross-adaptation experiments shown in this section indicate that there are differences in odor qualities between optical isomers. Carvone is known to be one of the typical odorants whose optical isomers have different odors [3,4]. This is consistent with the present results. The magnitude of the difference in odor quality between optical isomers greatly varies with species of odorants. In contrast to carvone, the differences in the odor quality are rather small in citronellal and limonene. The present study has offered first data in quantitative difference in odor quality between optical isomers.

The data in the present study were obtained at 20 $\pm$ 3 °C. Recently, Hanada *et al.* [13] found that the ability of turtle olfactory system to discriminate odor quality of odorants having similar structure such as *d*-carvone and *l*-carvone, *trans*-3-hexenol and *cis*-3-hexenol, geraniol and nerol was greatly reduced by increasing temperature up to

about 40 °C, while the ability to discriminate the odorants having different structure such as *l*-limonene and cineole, anisole and cineole was not greatly affected by increasing temperature. As shown in the present study, the difference in odor quality between *d*-carvone and *l*-carvone at about 20 °C was highest among six pairs of optical isomers. Hence it seems that the turtle olfactory system does not discriminate odor quality between a *d*-optical isomer and a corresponding *l*-optical isomer at 40 °C in general.

There are a number of possible explanations for the results described above. There is a possibility that the temperature change induces a conformational change of a specific receptor protein for an odorants, which leads to a change in the specificity of the receptor site of the protein to the odorant. In general, the specificity of a protein is not, however, unchanged by a small temperature change from 20 to 40 °C. For example, the receptor protein for *l*-amino acid does not accept *d*-amino acid even when temperature increases up to 40 °C. Hence these phenomena are not simply explained in terms of a conformational change of receptor protein. There is another possible explanation for the abolishment of odor-discriminating ability by the temperature increase. In this case, it is supposed that odorants are adsorbed on hydrophobic pockets composed of lipids and proteins in the receptor membranes. It has been pointed out that olfactory thresholds are closely related to partition coefficients of odorants between the organic solvent and water [14], and interaction of odorants with lipid layers mimics *in vitro* odor reception [15, 16]. Hanada *et al.* [13] reported that the

membrane fluidity of cells isolated from turtle olfactory epithelia and liposomes made of lipids extracted form the epithelia changed in a similar temperature range as for the decrease of the odor-discriminating ability, suggesting that an increase in membrane fluidity is correlated with the abolishment of the odor-discriminating ability. These studies support the above idea. According to this assumption, the mechanism for the phenomena is as follows. At room and lower temperatures, the lipid structure is rather rigid and different odorants are adsorbed on different pockets. At a higher temperature (40 ^{o}C), the fluidity of the lipid layers increases in magnitude and then the pockets for the odorants become flexible. At this temperature, the receptor pocket for a *d*-optical isomer accepts a corresponding *l*-optical isomer and hence the receptor pocket for a *d*-optical isomer is desensitized by application of a corresponding *l*-optical isomer. In any case, the fact that the turtle olfactory system does not discriminate optical isomers at 40 ^{o}C supports an idea that the lipids in the olfactory receptor membrane play an important role in odor reception.

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CHAPTER II

EFFECTS OF TREATMENT OF THE TURTLE OLFACTORY EPITHELIUM WITH VARIOUS LIPIDS ON OLFACTORY RESPONSES

INTRODUCTION

It is generally considered that an olfactory response is induced by binding of an odorant to specific receptor proteins in olfactory receptor membranes. Buck and Axel cloned the genes of an extremely large multigene family that encodes seven transmembrane domain proteins whose expression is restricted to the olfactory epithelium [1]. These proteins encoded by the genes are the most probable candidates for the receptor proteins.

On the other hand, as described in Chapter I-2, odor quality of *d*- and *l*-isomer is not discriminated at least at 40 °C [2], while it is well discriminated at 20 °C. This result suggests that lipid layers of the olfactory receptor membrane are also involved in the reception of odorant molecules.

It has been known that a number of non-olfactory systems such as *Tetrahymena* [3], fly [4], and frog [5] taste cells, turtle trigeminal nerves [6], *Helix* ganglions [7] and neuroblastoma cells [8, 9] respond to various odorants. There is a close relation between minimum concentrations of odorants to induce the responses in these systems and

those to induce a membrane potential change in liposomes [10-12]. These results suggest that there exists a non-receptor mediated pathway in odor reception.

In addition, Enomoto *et al.* [12] reported that the sensitivity of liposomes to odorants varied with the species of odorant. They also reported that addition of phosphatidylserine (PS) to phosphatidylcholine (PC) liposomes greatly enhances membrane potential changes, which were monitored with a fluorescence dye, in response to odorants, especially fatty acids such as *n*-valeric acid, isovaleric acid and *n*-butyric acid [13]. In order to examine whether PS enhances the response in *in vivo* olfactory system, the turtle olfactory epithelium is treated with lipids, including PS, cardiolipin (CL) and phosphatidic acid (PA), and its effects on the olfactory responses to various odorants are examined [14, 15]. The results obtained indicate that the effects of the lipid-treatment on the olfactory responses vary with species of lipids used for treatment. Especially the PS-treatment greatly enhances the responses to fatty acids [14-19], which corresponds to the results obtained with PS-containing liposomes system. On the basis of these results obtained, possible mechanisms of odor reception and discrimination are discussed.

MATERIALS AND METHODS

Recording of olfactory bulbar response

Olfactory bulbar response was recorded essentially as described in Chapter I.

In general, the magnitude of the turtle olfactory bulbar responses to an odorant gradually decreases during the experiments. The response to *n*-amyl acetate was practically unchanged before and after the lipid treatment. Hence the response to *n*-amyl acetate was often measured during the experiment as a control response and the magnitude of the olfactory responses to various odorants was calculated relative to a response to 10^{-4} M *n*-amyl acetate. The data obtained were statistically analyzed by the Student's *t*-test.

Chemical stimulation

The chemical stimulation was carried out as described in Chapter I. Because the relative magnitude of the responses to odorants such as fatty acids, lilial and lyral were relatively smaller than those to the others used in the present study, there is a possibility that responses to these odorants are obscured by background noise. To obtain the sufficient signal-to- noise resolution, we used the odorants of 10^{-4} M in the experiments descried in Chapter II except for that described in Fig. 2-3. At this concentration, turtle olfactory system discriminated the odorants used in this chapter and the magnitude of the response to any odorant used did not reach a plateau level.

Treatment of the olfactory epithelium with lipids

The lipid suspension was prepared as follows. Chloroform solution of lipid in a round-bottom flask was evaporated to dryness using a rotary vacuum evaporator. After chloroform was evaporated completely, glass beads were added to the flask and the dried lipid film was dispersed with turtle Ringer solution of an appropriate volume by shaking with a Vortex mixer at room temperature. The final concentration of lipid suspension was 20 mg/ml for PS-, CL- or PA-suspension. Before treatment of olfactory epithelium with lipid suspension, the epithelium was irrigated with the Ringer solution for about 10 min. Lipid suspension in the Ringer solution was applied to the olfactory epithelium through a stainless steel tube in the same way as that for application of irrigating solution. To save the lipid suspension, the lipid suspension dripped down from the turtle internal nostril was collected into a chamber and again applied to the epithelium using a reflux pump (AC-2110, ATTO Co., Ltd., Tokyo, Japan). The epithelium was incubated with the lipid suspension for 1 hour and washed out with the Ringer solution for 15 min. Then the olfactory bulbar responses to odorants were measured.

Measurement of [^{14}C]PS incorporated into olfactory epithelium

Figure 3-1 shows a schematic diagram illustrating procedure of the measurement of the amount of the [^{14}C]PS incorporated into olfactory epithelium. Cold PS (3.0 mg) and 0.03 mg of L-phosphatidyl-L-[3-^{14}C]serine ([^{14}C]PS, 1.96 GBq/mmol, Amersham, Japan) were

mixed and dissolved in chloroform solution in a flask was evaporated to dryness and 1.5 ml of Ringer solution was added to the flask. The PS suspension containing [^{14}C]PS was prepared by a similar method to that described above. The turtle internal nostril was covered with the surgical bond, the nasal cavity was filled with about 0.25 ml of the PS-suspension containing [^{14}C]PS. After incubation for 1 hour, the turtle internal nostril was opened again and the epithelium was washed out thoroughly with Ringer solution for 15 min. The turtle head was cut off along a median plane and the olfactory epithelium treated with PS-suspension containing [^{14}C]PS was carefully excised from the bone supporting the epithelium. The excised epithelium was collected in a scintillation vial and dissolved in the scintillation solution containing Triton X-100 and toluene (1:1, v/v). The radioactivity of the solution was then measured with a liquid scintillation counter and ratio of radioactivity incorporated into the epithelium to that of the PS suspension used was calculated. The experiments were carried out with six preparations and the mean ratio was obtained.

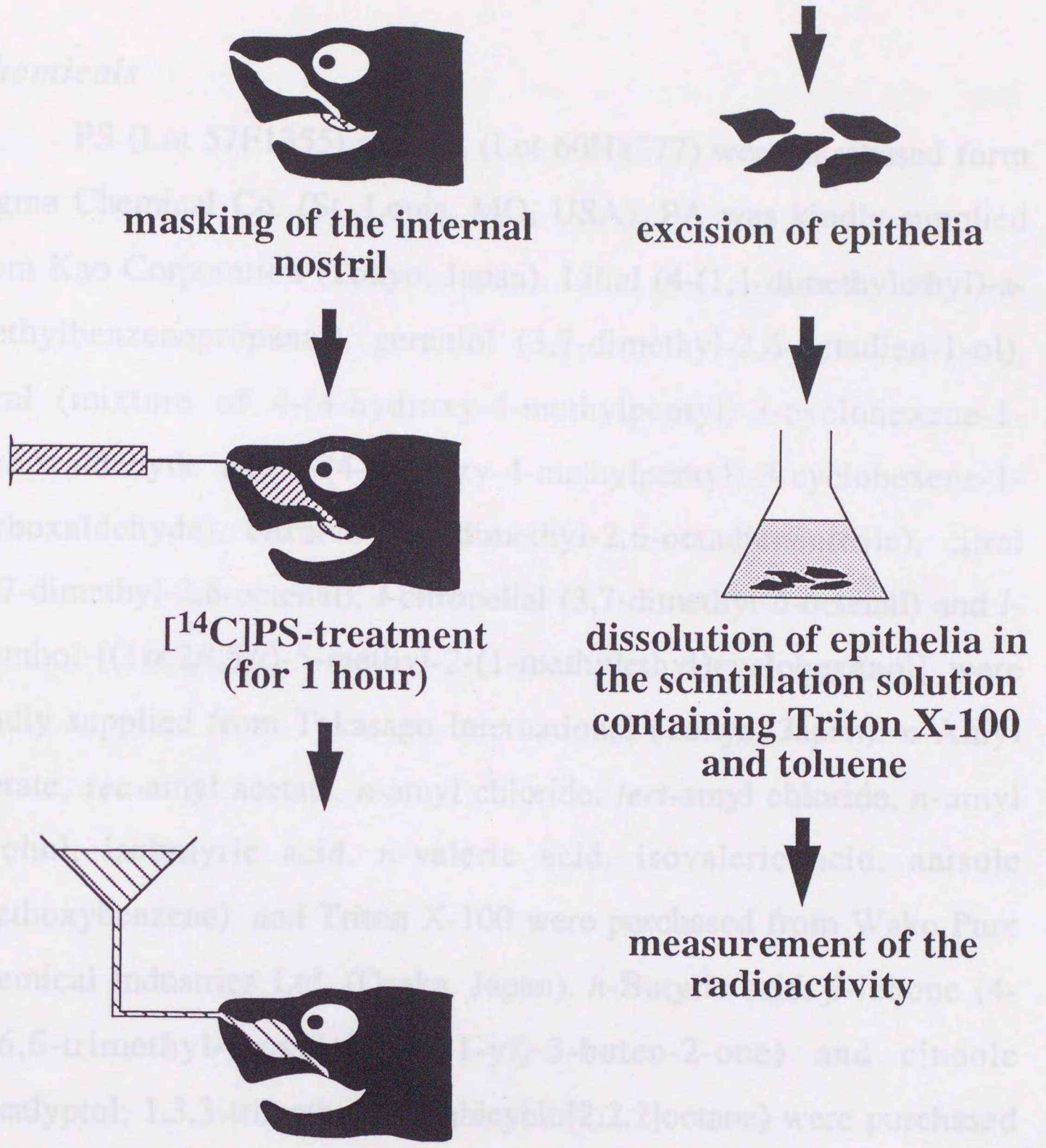

Figure 2-1. A diagram illustrating procedure of the measurement of the amount of [^{14}C]PS incorporated into olfactory epithelium. See text for details.

Chemicals

PS (Lot 57F1355) and CL (Lot 60H8377) were purchased form Sigma Chemical Co. (St. Louis, MO, USA). PA was kindly supplied from Kao Corporation (Tokyo, Japan). Lilial (4-(1,1-dimethylethyl)-a-methylbenzenepropanal), geraniol (3,7-dimethyl-2,6-octadien-1-ol), lyral (mixture of 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene-1-carboxaldehyde and 3-(4-hydroxy-4-methylpentyl)-3-cyclohexene-1-carboxaldehyde), citralva (3,7-dimethyl-2,6-octadienenitrile), citral (3,7-dimethyl-2,6-octenal), *l*-citronellal (3,7-dimethyl-6-octenal) and *l*-menthol [(1α,2β,5α)-5-methyl-2-(1-methylethyl)cyclohexanol] were kindly supplied from Takasago International (Tokyo, Japan). *n*-Amyl acetate, *sec*-amyl acetate, *n*-amyl chloride, *tert*-amyl chloride, *n*-amyl alcohol, isobutyric acid, *n*-valeric acid, isovaleric acid, anisole (methoxybenzene), and Triton X-100 were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). *n*-Butyric acid, β-ionone (4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one) and cineole (eucalyptol; 1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane) were purchased from Nakarai Chemicals (Kyoto, Japan). All chemicals used are of best grade available. Concentrations of stock solutions used were 10^{-4} M for all odorants examined.

RESULTS

Effects of the treatment of the olfactory epithelium with various lipids on the turtle olfactory bulbar responses

The turtle olfactory epithelium was treated with PS-suspension and its effects on the olfactory bulbar responses were examined. Figure 2-2 shows typical records of the turtle olfactory bulbar responses before and after PS-treatment. The response to *n*-amyl acetate was practically not affected. In contrast, the response to *n*-valeric acid before the PS-treatment was much smaller than that to *n*-amyl acetate, but the response to *n*-valeric acid after the PS-treatment became remarkably large. The responses to other fatty acids were also greatly increased by the PS-treatment as will be described later.

Figure 2-3 plots relative magnitudes of the responses to *n*-valeric acid before and after PS-treatment as a function of its concentration. Here the magnitude of the response to 10^{-3} M *n*-valeric acid before PS-treatment is taken as unity. The data shown in Fig. 2-2 indicate that the PS-treatment lowers the threshold of the response to n-valeric acid by a factor about 50 and enhances the magnitude of the responses over the concentration range examined. The enhanced responses to fatty acids by the treatment returned to the original level about 10 hour after the PS-treatment (data not shown).

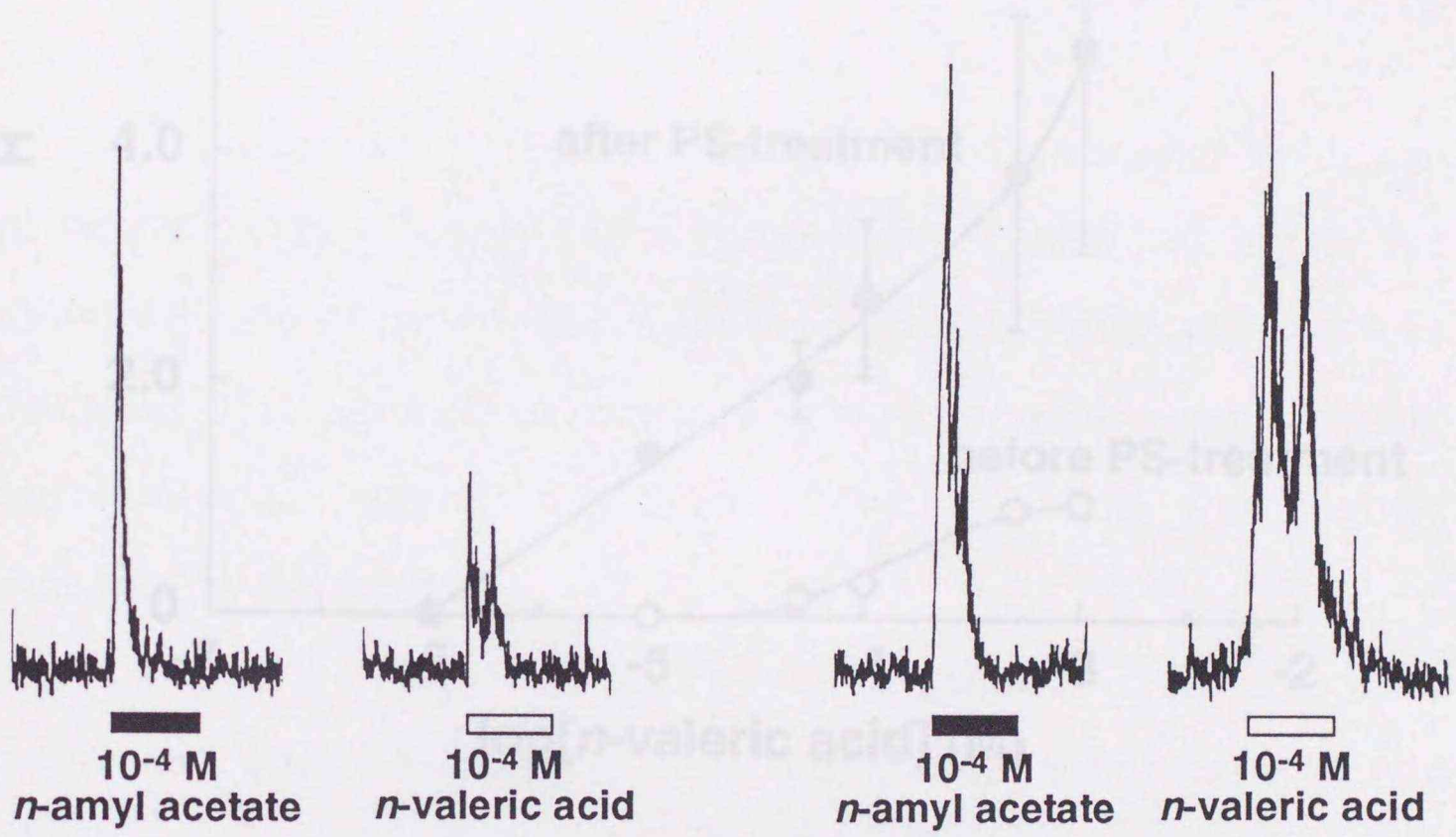

Figure 2-2. Typical records of the turtle olfactoy bulber responses before and after PS-treatment to *n*-amyl acetate and *n*-valeric acid. Bars under the records represent duration of stimurants (clased bars, 10^{-4} M *n*-amyl acetate; open bars, 10^{-4} M *n*-valeric acid).

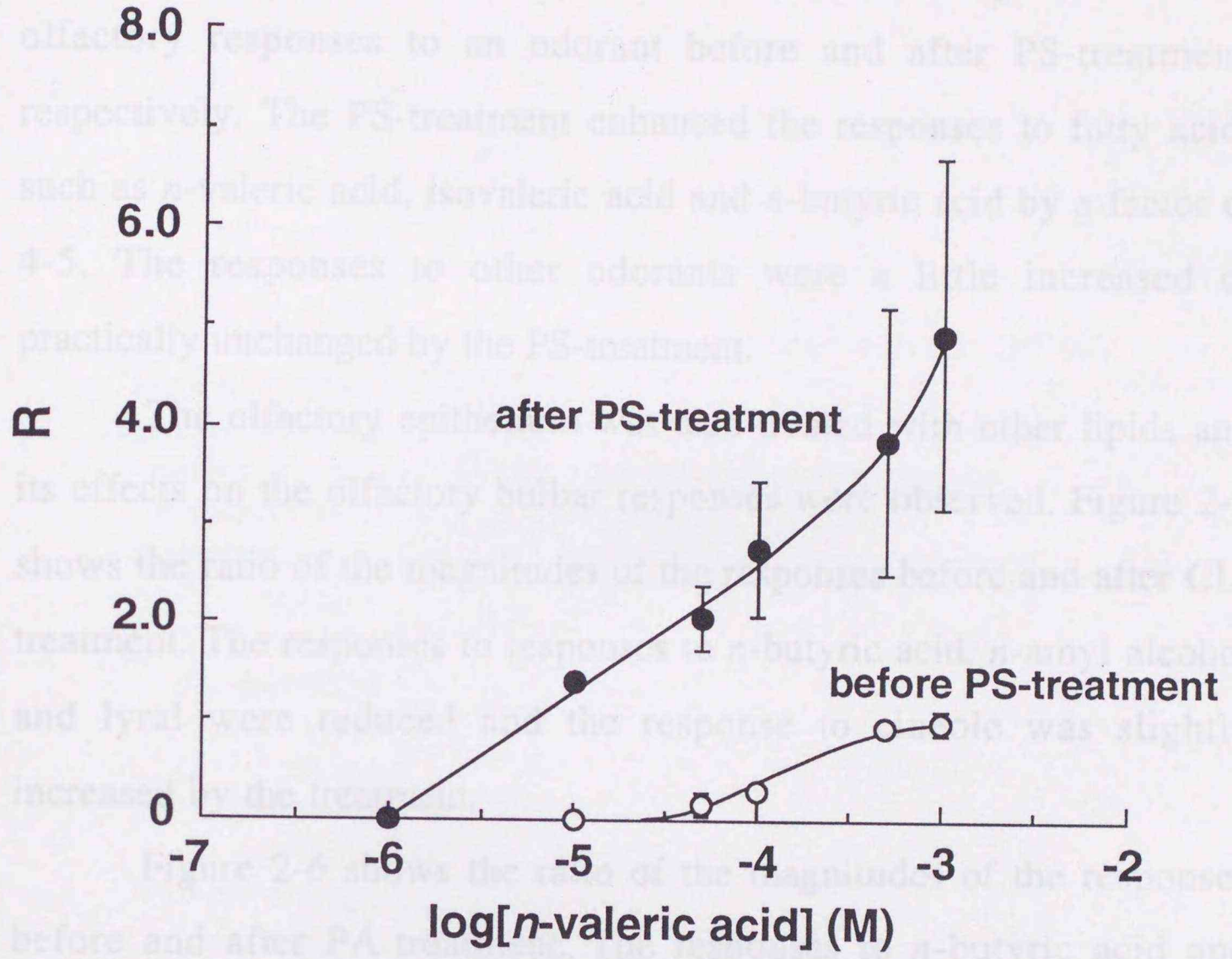

Figure 2-3. Relative magnitudes of turtle bulbar responses to *n*-valeric acid before and after PS-treatment as a function of odorant concentration. Magnitude of response is calculated relative to the response to 10^{-3} M *n*-valeric acid before PS-treatment. Each point is mean $\pm$ S.E.M. of data obtained from three preparations.

Figure 2-4 shows the effects of PS-treatment on the responses to various odorants. Here R_o and R represent the magnitude of the olfactory responses to an odorant before and after PS-treatment, respectively. The PS-treatment enhanced the responses to fatty acids such as *n*-valeric acid, isovaleric acid and *n*-butyric acid by a factor of 4-5. The responses to other odorants were a little increased or practically unchanged by the PS-treatment.

The olfactory epithelium was also treated with other lipids and its effects on the olfactory bulbar responses were observed. Figure 2-5 shows the ratio of the magnitudes of the responses before and after CL-treatment. The responses to responses to *n*-butyric acid, *n*-amyl alcohol and lyral were reduced and the response to cineole was slightly increased by the treatment.

Figure 2-6 shows the ratio of the magnitudes of the responses before and after PA-treatment. The responses to *n*-butyric acid and isovaleric acid were decreased by the treatment, while the responses to *n*-amyl acetate, *sec*-amyl acetate and anisole were not practically affected.

The results shown in Figs. 2-4, 2-5 and 2-6 indicate that the effect on the olfactory responses greatly varies with species of lipids used for the treatment of the olfactory epithelium.

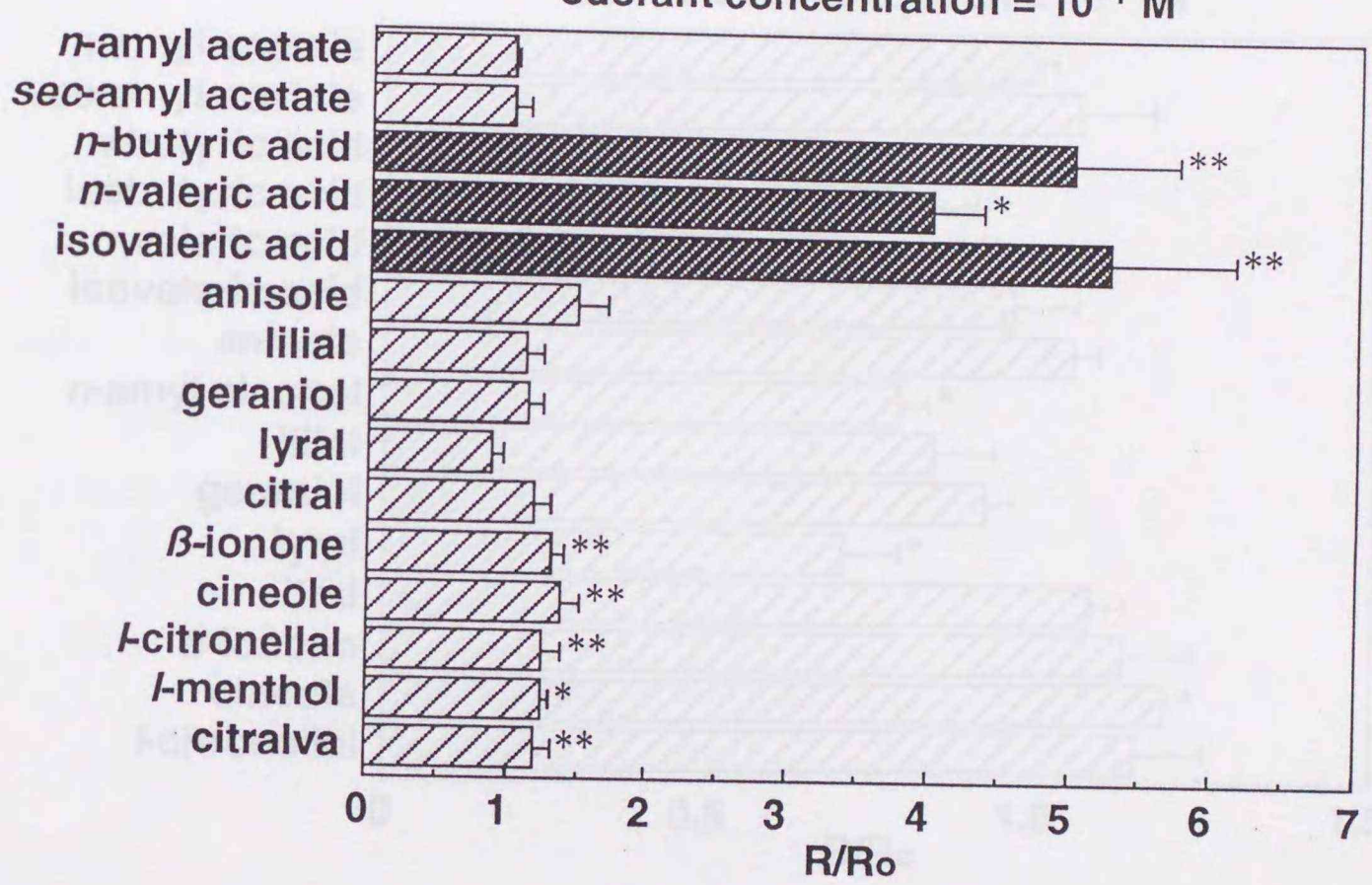

Figure 2-4. Ratio (R/Ro) of the olfactory bulbar responses to various odorants (10^{-4} M) before and after PS-treatment. Ro and R represent the magnitude of the olfactory bulbar response to an odorant before and after PS-treatment, respectively. The responses to *n*-valeric acid was increased by the treatment in a level of $P < 0.001$ (*). The responses to *n*-butyric acid and isovaleric acid were increased by the treatment in a level of $P < 0.05$ (**). The responses to *l*-menthol was slightly increased by the treatment in a level of $P < 0.001$ (*).The responses to *ß*-ionone, cineole, *l*-citronellal and citralva are slightly increased in a level of $P < 0.05$ (**). Each point is mean $\pm$ S.E.M. obtained from at least five preparations.

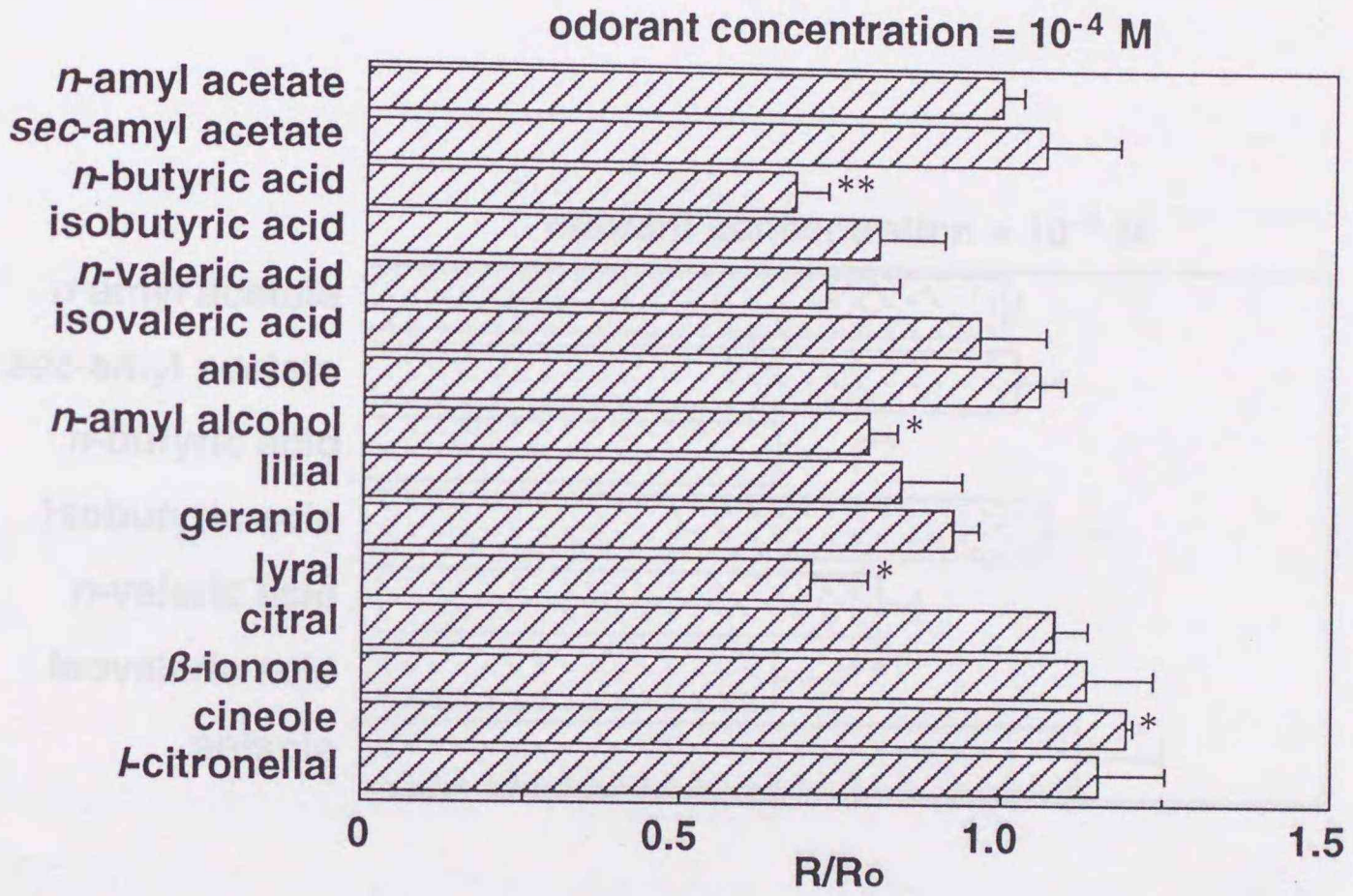

Figure 2-5. Ratio (R/Ro) of the olfactory bulbar responses to various odorants (10^{-4} M) before and after CL-treatment. Ro and R represent the magnitude of the olfactory bulbar response to an odorant before and after CL-treatment, respectively. The responses to n-amyl alcohol and lyral were decreased by the treatment in a level of $P < 0.001$ (*). The response to *n*-butyric acid was decreased by the treatment in a level of $P < 0.05$ (**). The response to cineole was slightly increased in a level of $P < 0.001$ (**). Each point is mean $\pm$ S.E.M. obtained from at least six preparations.

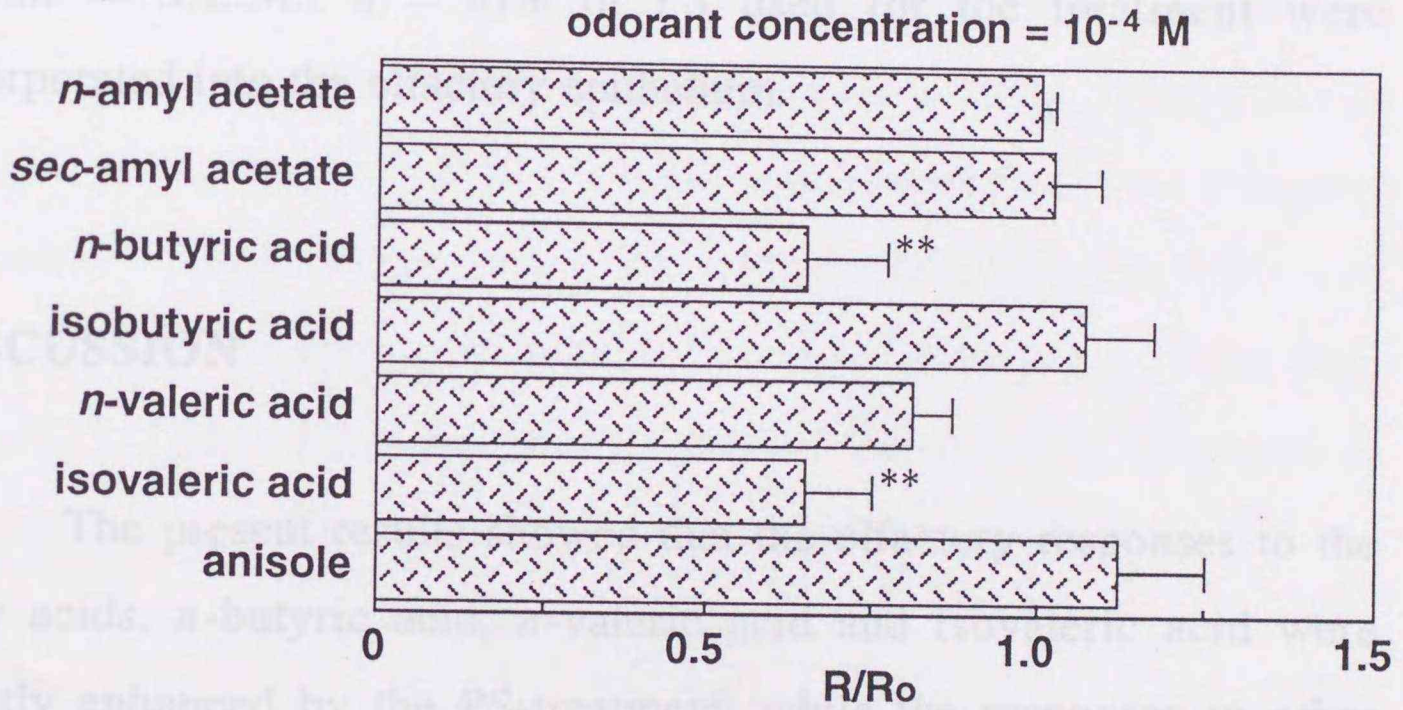

Figure 2-6. Ratio (R/Ro) of the olfactory bulbar responses to various odorants (10^{-4} M) before and after PA-treatment. Ro and R represent the magnitude of the olfactory bulbar response to an odorant before and after PA-treatment, respectively. The responses to *n*-butyric acid and isovaleric acid were decreased by the treatment in a level of $P < 0.05$ (**). Each point is mean ± S.E.M. obtained from six preparations.

Incorporation of PS into olfactory epithelium

As mentioned above, the PS-treatment greatly enhanced the olfactory responses to the fatty acids. In order to examine whether PS used for the treatment is incorporated into the olfactory epithelium, the epithelium was incubated with PS-suspension containing [^{14}C]PS for 1 hour and washed out thoroughly with the Ringer solution for 15 min (see Fig. 3-1). Among 0.51 mg PS used, 1.4 ± 0.2 (mean ± S.E.M., n = 6) μg PS were incorporated into one nostril. That is , 0.28 ± 0.04 (mean ± S.E.M., n = 6)% of PS used for the treatment were incorporated into the olfactory epithelium.

DISCUSSION

The present results showed that the olfactory responses to the fatty acids, *n*-butyric acid, *n*-valeric acid and isovaleric acid were greatly enhanced by the PS-treatment, while the responses to other odorants examined were a little enhanced or unaffected by the treatment. The experiment using [^{14}C]PS suggests that PS was incorporated into the olfactory epithelium. It is uncertain in what part of the epithelium PS was incorporated, but there is a possibility that PS was incorporated into the olfactory receptor membranes [20, 21]. It is well known that interaction of PC, one of the phospholipids, with erythrocytes eventually results in hemolysis [22, 23]. Tanaka *et al.* [21] reported that hemolysis occurred through incorporation of

dilauroylglyceroPC to human erythrocytes, supporting the above possibility in regard to demonstrating that phospholipids interact with animal cells by being incorporated into the plasma membranes. Hence a simple explanation for the present results is as follows. PS is incorporated into the olfactory receptor membranes and modifies the receptor site for the fatty acids so that affinity of the receptor membranes to the fatty acids is increased, which leads to enhancement of the olfactory response.

There is another possibility that PS is incorporated into the olfactory receptor membranes and increases the affinity of the receptor proteins to the fatty acids, which leads to enhancement of the olfactory response. It is, however, noted that addition of PS to PC liposomes greatly enhanced membrane potential changes in response to the fatty aids [13]. The fact that the enhancement of the olfactory responses to the fatty acids by PS-treatment closely resemble those observed with the liposomes supports the former mechanism.

As described in general introduction, Naim *et al.* [22] reported that quinine, the hydrophobic bitter tastant, directly activated transducin and Gi/Go-proteins [24], suggesting a possibility that some odorants penetrate the lipid layer of the olfactory receptor membranes and directly stimulate G-proteins. Therefore, it is possible to explain the mechanism of enhanced responses to fatty acids by PS-treatment as follows. PS incorporated into the lipid bilayer of the olfactory receptor membranes and modifies the receptor membranes. As a result, affinity and permeability to fatty acids to lipid layers of receptor membranes

are increased, which facilitates the direct activation of G-protein by fatty acids so that the olfactory responses to them were enhanced.

In general, phospholipids are amphiphilic substances [23]. It is, therefore, conceivable that certain proteins in the olfactory receptor membranes are solubilized by detergent effect of PS, which leads to enhancement of the olfactory response. However, the enhancement of the responses to the fatty acids was not observed by the treatment with CL or PA which has a similar detergent effect to that of PS. Therefore, the above possibility may be excluded.

It is known that PS activates adenylate cyclase [26] or protein kinase C [27]. Hence there is another possibility that PS is incorporated into the olfactory receptor membranes and activates the second messenger systems, which leads to enhancement of the olfactory responses. The olfactory response to isovaleric acid, which dose not induce an elevation of the cAMP level but induces an elevation of the IP_3 level [28], was greatly increased by the PS-treatment. The olfactory responses to geraniol, β-ionone and citronellal, which increase cAMP [29], were not much enhanced by the PS-treatment. It is the same case of the olfactory responses to lilial and lyral, which do not increase cAMP but increase IP_3 as well as isovaleric acid [28]. Hence it is unlikely that the PS-treatment enhanced the responses to the odorants via the cAMP [26, 29] or IP_3 second messenger cascade [27, 28].

The present results suggest an importance of lipid layers in odor reception. This notion was also demonstrated as follows. As mentioned previously in Chapter I-2, Hanada *et al.* [2] reported that an increase of

temperature of the turtle olfactory epithelium up to 40 °C has little effect on the magnitude of odor intensity, but abolishes the ability of the olfactory receptor to discriminate odorants having similar odors such as *d*-carvone and *l*-carvone, *trans*-3-hexenol and *cis*-3-hexenol and *n*-amyl acetate and isoamyl acetate. The decrease of odor-discriminating ability was closely related to an increase of the membrane fluidity of lipid layers of the cells isolated from turtle olfactory epithelium.

The present results support the following hypothesis for odor reception. It is assumed that odorants are adsorbed on hydrophobic pocket composed of lipids and proteins in olfactory receptor membranes. The composition of the lipids and proteins of each olfactory cell membrane is assumed to vary from cell to cell. Variation in combinations of lipids and proteins provides many different adsorption sites for odorants. The qualities of odors are recognized by firing pattern among various olfactory axons and the quality of odors is recognized in the brain which summarizes these firing one. As shown in general introduction (Fig. I-1), numerous theories regarding the molecular basis of odorant recognition have been proposed. The present results together with the above observations strongly suggest that lipid layers as well as the receptor proteins play an important role in odor reception.

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PART 2

CHAPTER III

ELECTROPHYSIOLOGICAL PROPERTIES OF TURTLE VOMERONASAL RECEPTOR NEURONS

INTRODUCTION

The nasal cavities of vertebrates are innervated by several nerves that may subserve different aspects of chemical sensation and perception (Fig. 3-1). A number of behavioral, biochemical, and molecular biological studies demonstrate that the vomeronasal system plays important roles in the perception of chemical stimuli related to animal behavior in most terrestrial vertebrates [1, 2]. Interaction between chemoattractants and vomeronasal receptor neurons results in depolarizing receptor potentials which activate voltage-gated channels and generate bursts of spikes. The voltage-gated currents underlying the excitatory response form an essential part of the vomeronasal transduction process. In spite of the exponential increase of interest in the structure and biological functions of the vomeronasal system, electrophysiological properties of vomeronasal receptor neurons have been little studied. Determining their electrical properties provides a basis for further analysis of molecular transduction mechanism in this system.

The electrophysiological properties of the vomeronasal receptor neurons were examined only with the frog. Trotier *et al.* reported that the resting membrane potential of the frog vomeronasal neurons was near -60 mV and overshooting repetitive action potentials were elicited by an injection of depolarizing current pulses in the range of 2 to 10 pA, which were measured using patch-clamp technique [3]. They also reported that an transient fast inward current and an outward K^+ current were activated in these neurons. No electrophysiological property of vomeronasal receptor neurons in other species of vertebrates has been described. The vomeronasal organ is well developed in reptiles [4] In the present study, the electrophysiological features of vomeronasal receptor neurons in the turtle were studied using the whole-cell patch clamp technique.

MATERIALS AND METHODS

Slice preparation of vomeronasal epithelium

Turtles, *Geoclemys reevesii*, weighing 140-240 g, were obtained from commercial suppliers and maintained at 22 °C. Turtles were cooled to 0 °C and decapitated. The nasal cavities were opened, and the vomeronasal neuroepithelia were dissected out carefully. The epithelia were cut into slices of about 120 μm thickness with a vibrating slicer in normal Ringer solution at 0 °C and stored at 4 °C. One slice of the epithelium was fixed on the glass at the bottom of a recording chamber.

The preparation was viewed under upright microscope (model OPTIPHOT, Nikon, Tokyo, Japan) using a x 40 water immersion lens.

Electron microscopy

The vomeronasal neuroepithelia and its slice preparation of 400 μm thickness were prepared as described above. After quick rinse with Ringer solution, the specimens were placed in immediately in 5% glutaraldehyde/ 4% formaldehyde, i.e. Karnovsky's fixative [5] in 100 mM sodium cacodylate buffer (pH 7.4). The fixation was carried out at room temperature for at least one night. The tissues were post-fixed in 1% osmium tetroxide aqueous solution for 2 hours and dehydrated in a graded series of ethanol. The samples, after dehydration, were critical-point dried from CO_2 and sputter-coated with gold (in model HCP-2, Hitachi Koki Co., Ltd., Ibaraki, Japan and model IB-3, Eiko engineering, Ibaraki, Japan, respectively) and examined in a Hitachi S-430 scanning electron microscope (Hitachi, Ltd., Tokyo, Japan) at 20 kV.

Data recording and analysis

Patch pipettes with resistances of 5-10 MΩ were made from borosilicate glass capillaries using a two-stage electrode puller (model PP853, Narishige Co., Tokyo, Japan) and then fire-polished. Membrane currents were recorded in the whole-cell configuration (holding potential, -70 mV). Data were continuously recorded using an EPC-7 patch clamp amplifier (List, Darmstadt, Germany) and stored on a

video cassette recorder via a digital audio processor. Data were filtered at 10 KHz and digitized at 10 KHz. Analysis was carried out on a personal computer using pCLAMP software (Axon Instruments, Foster City, CA, USA). All averages were given as mean ± S.E.M.

Solutions

Normal Ringer solution consisted of (in mM): 116 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 15 glucose, 5 Na-pyruvate, 10 HEPES-NaOH (pH 7.4). Patch pipettes were filled with a normal internal solution (in mM): 115 KCl, 2 $MgCl_2$, 10 HEPES-KOH (pH 7.6).

Chemicals

All chemicals used were of best grade available.

RESULTS

Cell morphology

A transverse section of the nasal cavity showed that the olfactory mucosa occupied the dorsal region and the vomeronasal one was located in the ventral region (Fig. 3-1). Three layers of supporting cells, receptor cells and basal cells could be distinguished in transverse section of the vomeronasal mucosa [6]. We examined electrical properties of neurons located in a receptor cell layer in the slice, having bipolar or ovoid shape (Fig. 3-2). It is reported that vomeronasal receptor neurons

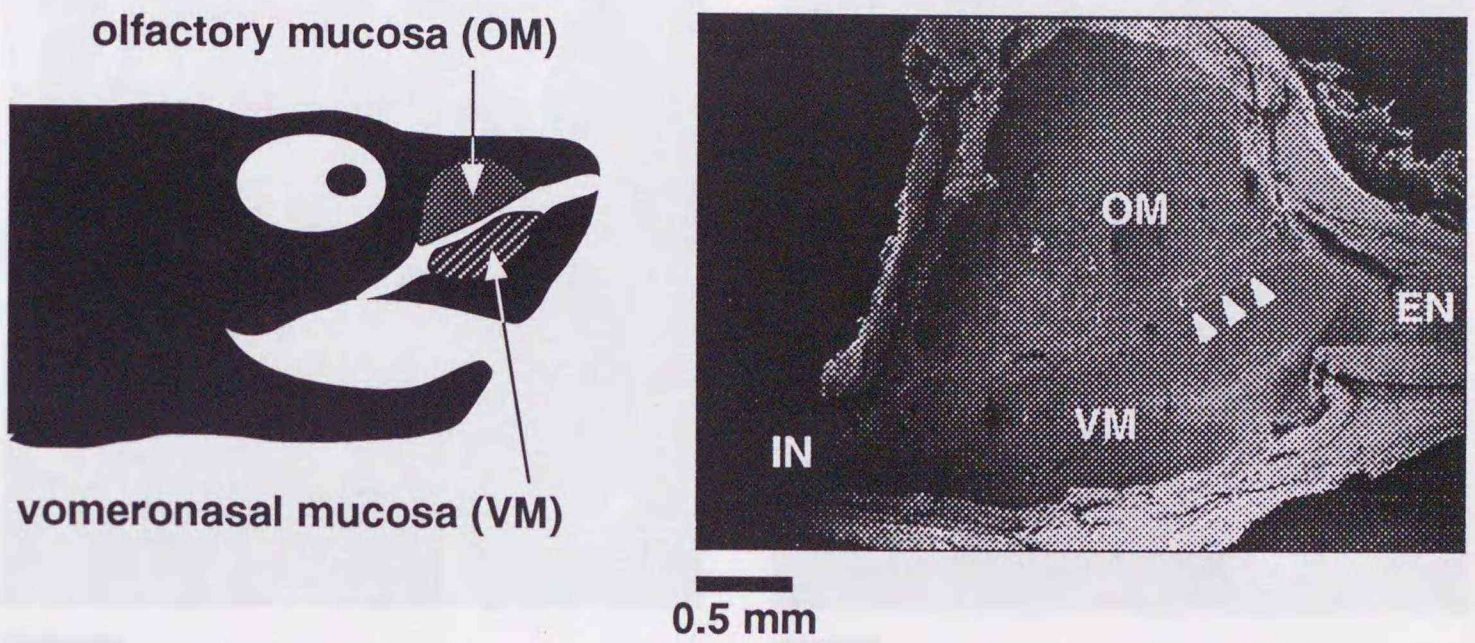

Fig. 3-1. The nasal cavity of the stink turtle. **A:** Schematic drawing of the sagittal section of a turtle nasal cavity corresponding to scanning micrograph. **B:** Low-magnification scanning micrograph of a sagittal section of turtle nasal cavity. ×37. There is a ridge-like structure indicated by arrow heads in the middle portion of the cavity. This ridge-like structure entirely separates the vomeronasal mucosa from the olcactory mucosa. That is, the olfactory mucosa occupied the dorsalregion and the vomeronasal mucosa was located in the ventral region. These two types of mucosa could be easily distinguished from their surface structure. EN: external nares. IN: internal nares. OM: olfactory mucosa. VM: vomeronasal mucosa.

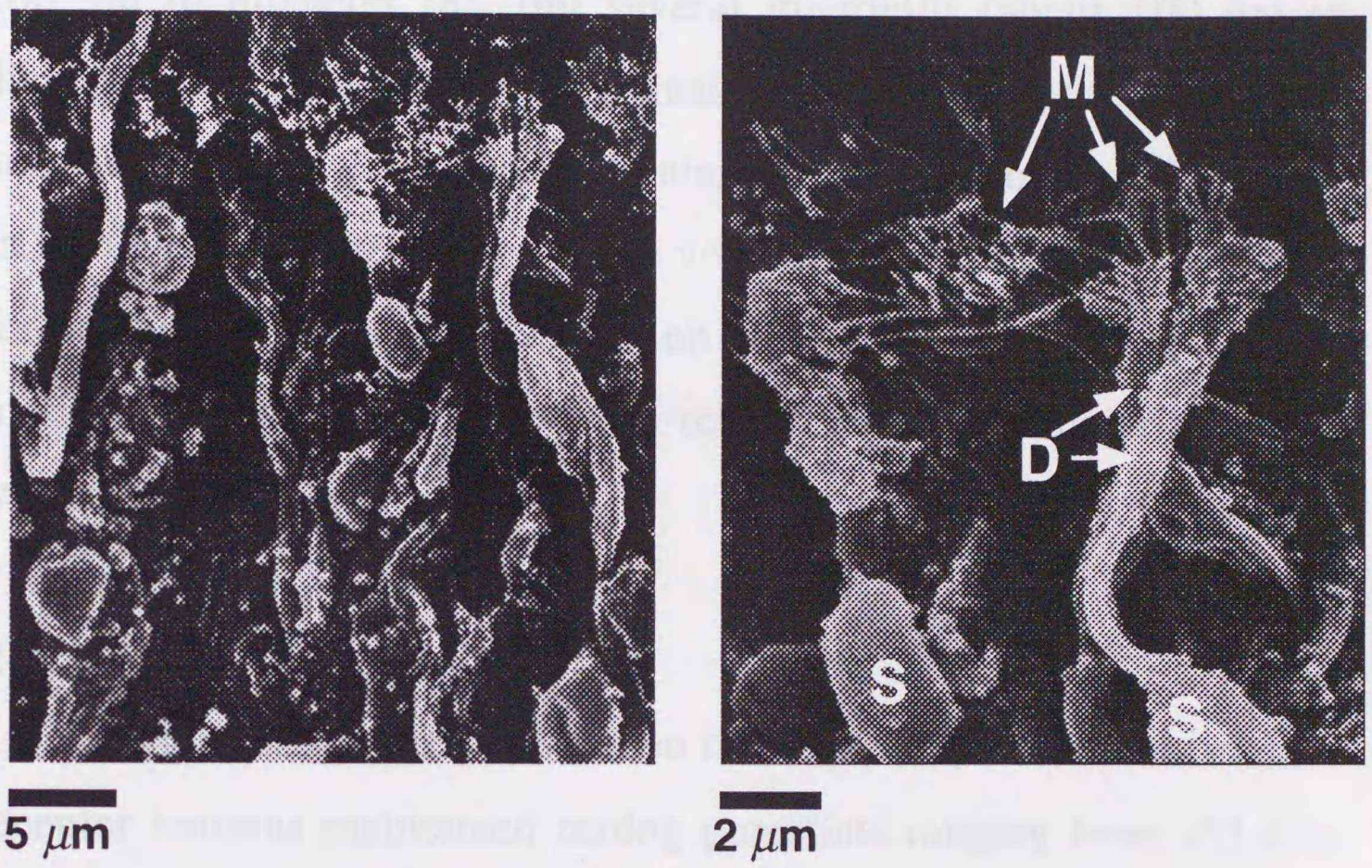

Figure 3-2. Lateral aspect of vomeronasal epithelium (**A**) and detailed view of microvilli at terminals of dendrites (**B**). It could be seen that receptor neuron dendrite extended from round soma to the mucosal surface through the supporting cell layer, and its terminal was little swelled. The higher-magnification scanning micrograph (B) taken from the same field as that in A clealy demonstrates that the terminals of the receptor neuron dendrites possess a number of microvilli. The magnification of the micrograph are ×2000 for A and ×8000 for B, respectively. D: receptor neuron dendrite. M: microvilli of the receptor neuron. S: soma of the receptor neuron.

are bipolar ones and their dendrites lack cilia and possess microvilli [7, 8]. The microvilli could not be identified with the optics used for viewing the electrophysiological experiments because they were only 100 nm in diameter [8]. But several microvilli (about 100 nm in diameter) extending from the terminal ends of the dendrite of receptor neurons could be viewed with scanning electron microscope (Fig. 3-2). In whole-cell voltage-clamp [9], the vomeronasal receptor neurons were further identified by the activation of a transient inward current followed by an outward current in response to step depolarization as will be shown below.

Resting potential

With normal internal solution in the pipette, turtle vomeronasal receptor neurons maintained resting potentials ranging from -33.0 to -71.5 mV (-48.1 ± 1.3 mV, n = 14). The input resistance was measured from the responses to injected currents of 1 sec ranging from -20 to +20 pA in 20 mV increments applied at the holding potential of -70 mV, and ranged from 0.7 to 2.8 GΩ (1.7 ± 0.1 GΩ, n = 14).

Voltage responses to injected current

In current-clamp recordings, step depolarization elicited by 7 pA stimulus current from a holding potential of about -70 mV produced an action potential (Fig. 3-3A) which had a relatively smooth rising phase. Twenty five of 30 (80%) neurons fired one to several action potentials in response to current steps of less than 30 pA form a

conditioning potential of about -70 mV. The threshold for action potential generation in vomeronasal receptor neurons was commonly between -45 and -55 mV. A variety of spiking patterns were seen, ranging from neurons that fired only a single action potential for any suprathreshold stimulus (data not shown) to neurons that generated brief trains of action potentials (Fig. 3-3B, for instance). In the example shown in Fig. 3-3B, the action potentials were generated repetitively in response to a depolarizing current pulse of 23 pA.

Several neurons required current injection of only 3 pA to depolarize to spike threshold (data not shown), suggesting that a vomeronasal receptor neuron in turtle has high sensitivity to injected currents similar to the frog vomeronasal receptor neurons [3]. Because the membrane potentials varied from neuron to neuron, the membrane potentials of neurons examined were held at -70 mV to measure the voltage response to injected current under the same experimental condition. Thus, spike threshold was increased and spike amplitude was decreased when the membrane was equal to the resting potential (data not shown).

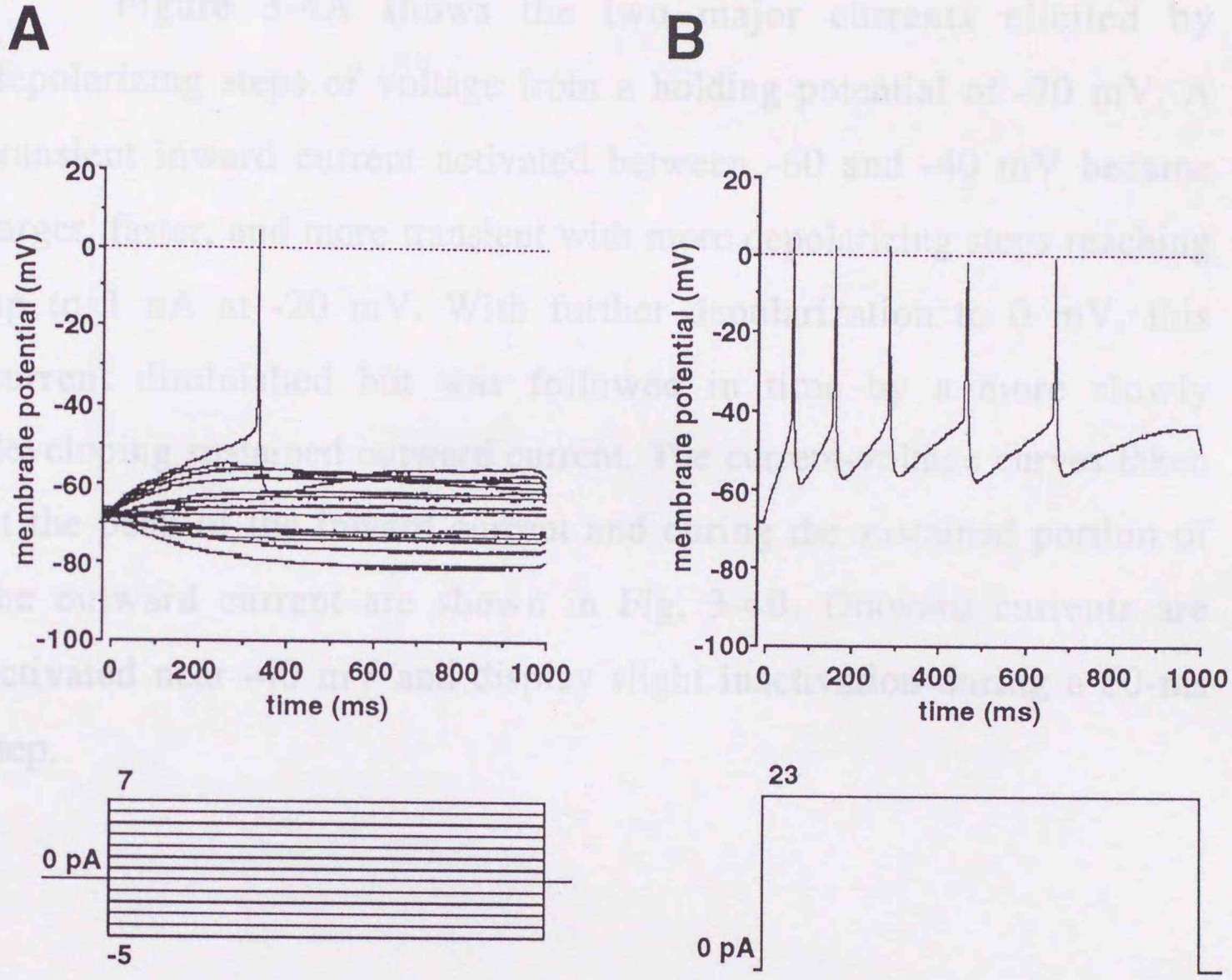

Figure 3-3. Electrical responses of a vomeronasal receptor neuron. **A:** Voltage response to current steps between -5 and 7 pA (in 1 pA increments). Resting level of this neuron was -70 mV and threshold was near -55 mV. **B:** Voltage response of the same neuron to a current step of 23 pA. In every case where the action potentials were generated repetitively, the interval between the few spikes increased with each subsequent spike (n=3). Each bottom traces shows the corresponding current pulse.

Whole-cell current

Figure 3-4A shows the two major currents elicited by depolarizing steps of voltage from a holding potential of -70 mV. A transient inward current activated between -60 and -40 mV became larger, faster, and more transient with more depolarizing steps reaching up to 1 nA at -20 mV. With further depolarization to 0 mV, this current diminished but was followed in time by a more slowly developing sustained outward current. The current-voltage curves taken at the peak of the inward current and during the sustained portion of the outward current are shown in Fig. 3-4B. Outward currents are activated near -40 mV and display slight inactivation during a 60-ms step.

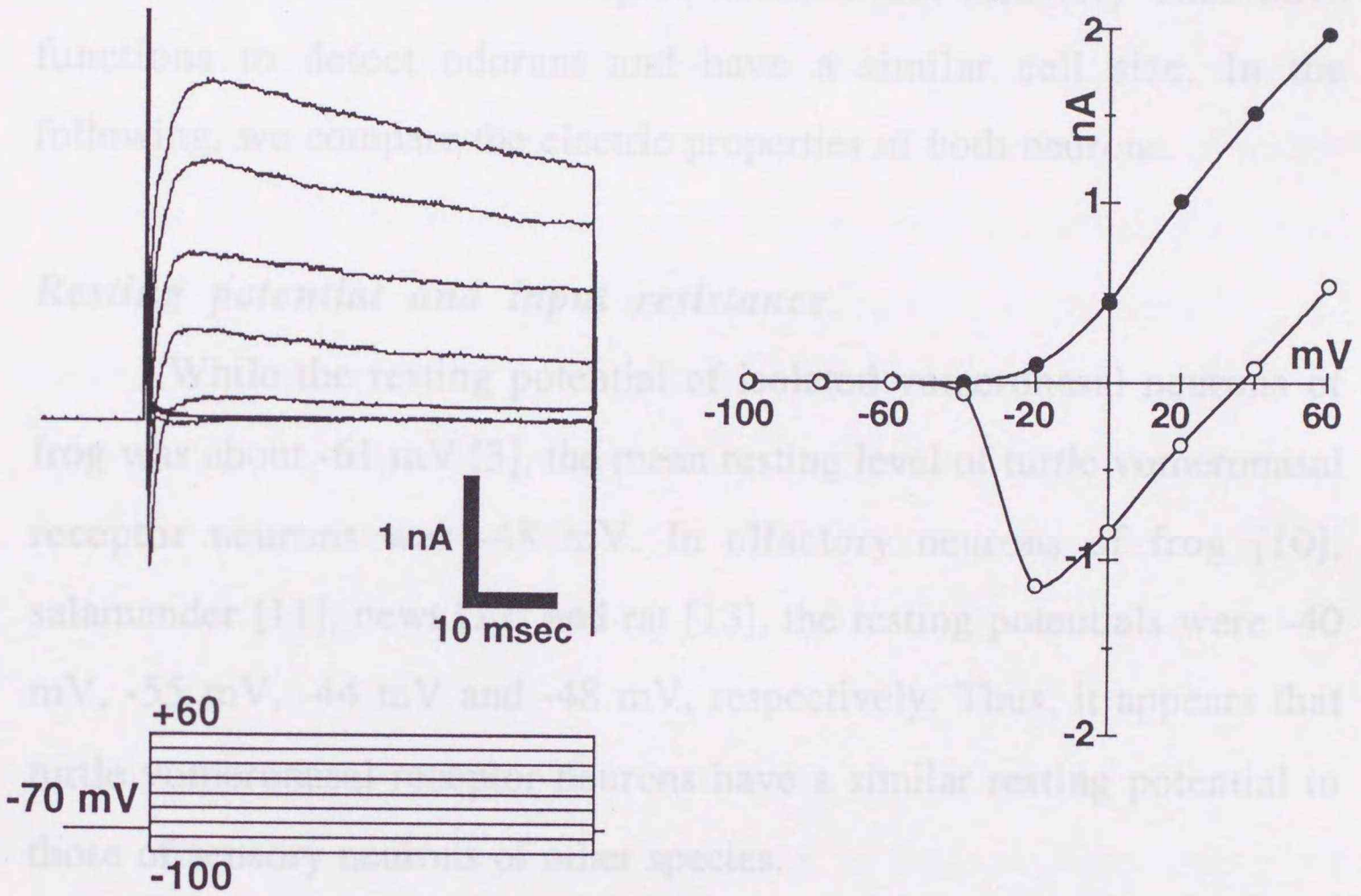

Figure 3-4. Typical whole-cell currents in an vomeronasal receptor neuron in a slice preparation. **A:** Responses to voltage steps. Step levels are shown in the bottom traces. Transient inward and delayed out ward currents were elicited in response to 60 ms voltage steps between -100 and 60 mV in 20 mV increments from a holding potential of -70 mV. **B:** Current-voltage relationships of peak inward currents (◯) and 60 ms after the onset of the voltage step during the sustained plateau of the outward current (●) measured from the records in A. The pipette contained normal internal solution and the bath contained Ringer solution.

DISCUSSION

Both vomeronasal receptor neurons and olfactory ones have functions to detect odorant and have a similar cell size. In the following, we compare the electric properties of both neurons.

Resting potential and input resistance

While the resting potential of isolated vomeronasal neurons of frog was about -61 mV [3], the mean resting level of turtle vomeronasal receptor neurons was -48 mV. In olfactory neurons of frog [10], salamander [11], newt [12] and rat [13], the resting potentials were -40 mV, -55 mV, -44 mV and -48 mV, respectively. Thus, it appears that turtle vomeronasal receptor neurons have a similar resting potential to those of sensory neurons of other species.

Input resistance of the receptor neurons which were observed in this study was near 1.7 GΩ. This value is slightly lower than that of olfactory neurons which was reported to be near 2-10 GΩ, but is not greatly different.

Voltage activated properties

Present results showed that the transient inward currents activated between -60 to -40 mV reached a peak at about -20 mV and inactivated rapidly (Fig. 3-4). These properties are similar to those measured in isolated rat olfactory neurons [13], rat olfactory neurons in

culture [14], salamander olfactory neurons [11] and frog vomeronasal receptor neurons [3], but differ from the properties of acutely isolated rat olfactory neurons where the sodium currents were activated at more negative potentials [14]. Outward currents were activated between -50 and -40 mV and displayed only slight inactivation during at 60-ms step. These properties are similar to those measured in isolated rat olfactory neurons [12-16]. A detailed analysis of these voltage activated currents was not carried out in the present study, but the transient inward currents and the outward currents were tentatively identified as sodium and potassium currents, respectively since the properties of there currents were similar to those of sodium and potassium currents observed in many neurons [17].

Voltage response and spike activity

Because of the high input resistance of these neurons, only 3 pA of injected current was required to reach spike threshold. This is similar to the case of rat olfactory neurons [18] and frog vomeronasal neurons [3]. The olfactory system is known to be an exquisitely sensitive chemodetector recognizing odorants at concentrations as low as the level of a few nM. It is desirable for sensory neurons to generate spikes by minimum membrane depolarization. That is, the smaller membrane depolarization of sensory neurons generates action potentials, the more sensitively the neurons can detect odorants.

As the results described above, turtle vomeronasal receptor neurons appear to be similar to olfactory neurons and frog vomeronasal receptor neurons in regard to their passive electrical characteristics being electrotonically compact and possessing a low spike threshold. They are also homogeneous in regard to their spike responses and the gated currents underlying those responses.

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CHAPTER IV
SIGNAL TRANSDUCTION MECHANISM IN VOMERONASAL RECEPTOR NEURONS

INTRODUCTION

In vertebrate olfactory transduction, it is generally considered that olfactory responses are triggered via the G-protein-linked cAMP pathway [1-6] or IP_3-dependent pathway [7-12]. Hence, there is the possibility that the second messenger-mediated pathway is involved in the signal transducing system in the vomeronasal system.

Recently, *in situ* hybridization experiment showed that some sensory neurons in the mouse vomeronasal organ expressed olfactory G-protein coupled receptor mRNA [13]. This observation also suggests that the vomeronasal system shares a similar transduction pathway to that in the olfactory system. In addition, Luo *et al.* reported that G-proteins (Gs, Gi and Go) were tentatively identified in the vomeronasal tissue membrane preparations of garter snakes using immunoreactivity and ADP-ribosylation techniques [14]. These G-proteins were suggested to be coupled with the receptor for ES20 [14] which was the chemoattractant for the snakes [15].

In spite of these interesting studies, no electrophysiological study on the signal transduction mechanism of vomeronasal receptor neurons has been carried out. The most suitable candidates for second messengers of vomeronasal transduction are considered to be cAMP and IP_3. In Section IV-1 and IV-2, we describe the responses induced

by intracellular injection and cAMP or IP_3 into the turtle vomeronasal receptor neurons, respectively.

IV-1 The Responses Induced by Intracellular Injection of cyclic Nucleotides into Turtle Vomeronasal Receptor Neurons

INTRODUCTION

Luo *et al.* [14] reported that both GTPγS and forskolin increased the cAMP level in vomeronasal receptor neurons of garter snakes. These observations suggest that there exists cAMP-dependent pathway in signal transduction in the vomeronasal receptor neurons.

In spite of this interesting observation, any study has demonstrated the existence of cAMP-activated membrane conductance in the vomeronasal system. In the present study, we injected cAMP into the turtle vomeronasal receptor neurons under whole-cell patch clamp and found that intracellular application of cAMP elicits the membrane current.

In the olfactory neurons, intracellular injection of cGMP also elicits inward current [16]. The studies using patches of ciliary membrane have clearly shown that both cGMP and cAMP directly activate a ciliary membrane conductance [3, 6, 17, 18]. In the present study [19], we found that intracellular application of cGMP from the patch pipette to turtle vomeronasal receptor neurons also elicits the membrane current under the whole-cell patch clamp.

MATERIALS AND METHODS

Preparations

The slice preparations were obtained as described in Chapter III.

Data recording and analysis

Membrane currents were recorded in the cell attached or whole-cell configurations of the patch clamp as described in Chapter III. In the whole-cell recordings, holding potential was -70 mV. Data were analyzed as described Chapter III.

Solutions

The compositions of normal Ringer solution and normal internal solution were the same as described in Chapter III. For the stimulation with adenosine 3' : 5'-cyclic monophosphate (cAMP) and guanosine 3' : 5'-cyclic monophosphate (cGMP), each cyclic nucleotide was dissolved in the internal solution to give the desired final concentrations. The stock solutions of cAMP and cGMP at appropriate concentrations derived into 1.8 ml aliquots were stored at -80 °C and thawed just prior to use. Stocked forskolin solution was prepared by dissolving it in ethanol at 10 mM and appropriate volume was added to Ringer solution to give desired concentrations. These forskolin solutions were prepared daily. The final concentration of ethanol never exceeded 0.5%. This

concentration of ethanol alone had no measurable effect on the electrical property of the neurons.

Gravity was used to deliver a constant stream of Ringer solution from the stimulating tube. Three electrically actuated valves were used to switch adapting Ringer solution and stimulating solutions. The stimulating tube with a lumen 160-200 μm in diameter was placed under visual control within about 500 μm of the neuron. The concentrations of stimuli were reported as the pipette concentration; no attempt was made to correct for dilution.

Chemicals

cAMP, forskolin and cGMP were purchased from Boehringer Mannheim GmbH (Mannheim, FRG), Wako Pure Chemical Industries Ltd. (Osaka, Japan) and Yamasa Shoyu Co. Ltd. (Choshi, Japan), respectively. All chemicals used were of best grade available.

RESULTS

As described in Chapter III in details, we examined electrical properties of neurons located in a receptor cell layer in the slice, having bipolar or ovoid shape. The vomeronasal receptor neurons were further identified by the activation of a transient inward current followed by an outward current in response to depolarizing voltage steps from a holding potential of -70 mV (Fig. 3-4).

Transient inward current induced by cAMP

In order to examine whether cAMP-mediated pathway exists in the vomeronasal receptor neurons, the response to forskolin, a direct activator of adenylate cyclase, was recorded from an vomeronasal receptor neuron of the turtle using the cell attached configuration (Fig. 4-1). Before application of the 10 μM forskolin, the vomeronasal receptor neurons generate spikes spontaneously. Bath application of 10 μM forskolin caused a remarkable increase in the spike rate, suggesting the possibility that there exists adenylate cyclase and cAMP-dependent ion channels in the vomeronasal receptor neurons.

To explore the existence of cAMP-mediated pathway in vomeronasal receptor neurons directly, cAMP was introduced into a proximal part of the dendrite or a part of cell soma by whole-cell dialysis. Figure 4-2 shows the currents induced by intracellular injection of cAMP of varying concentrations into vomeronasal receptor neurons.

When the pipette was filled with an cAMP-free inner solution, the neurons held a steady baseline over the test interval of about 3-10 min after membrane rupture (left trace). On the other hand, introducing cAMP into the neurons evoked prolonged, inward currents within a few seconds after membrane rupture. The magnitudes of the responses to cAMP introduced intracellularly were increased with an increase in their concentrations.

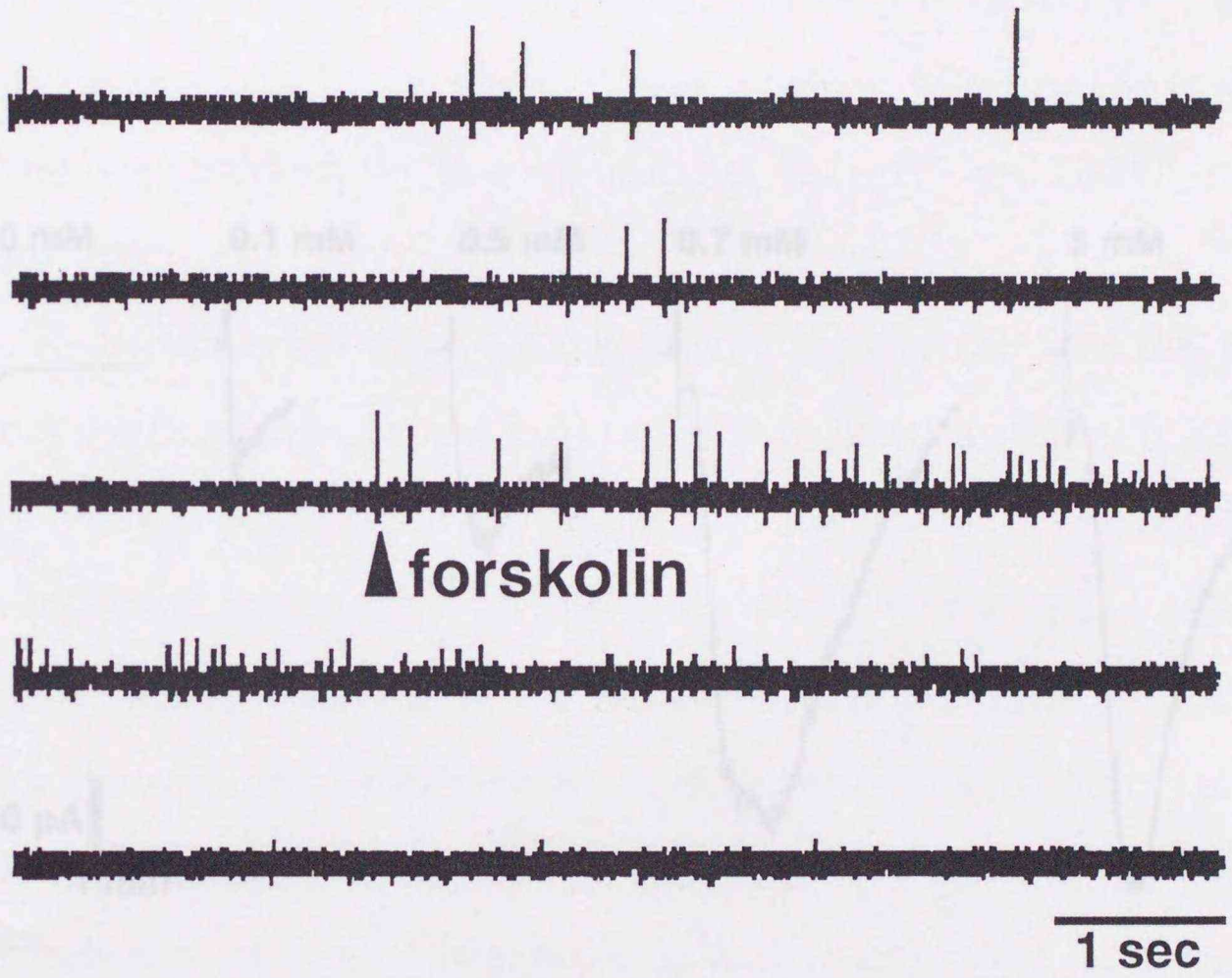

Figure 4-1. Cell-attached extracellular recordings of a single turtle vomeronasal receptor neuron *in situ*. An arrow-head represents time of 10 μM forskolin onset.

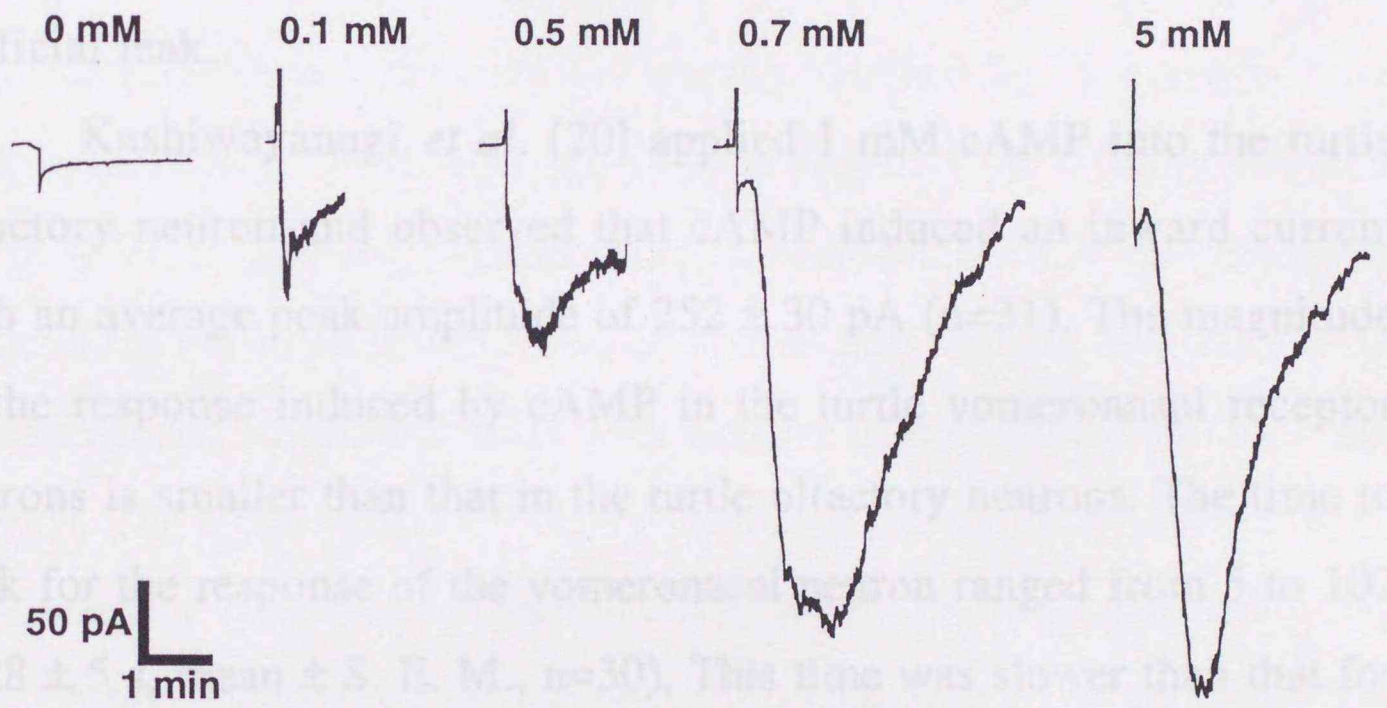

Figure 4-2. Response induced by intracellular application of cAMP from the patch pipette to a vomeronasal receptor neuron bathed in normal Ringer solution. The concentrations of cAMP contained in the pipette are shown at the top of each trace. Holding potential, -70 mV.

In the present study, 30 neurons were successfully stimulated by 1 mM cAMP. Twenty one neurons (70%) displayed an increase in inward current with adaptation of current after the peak response. The amplitude of the inward current induced by cAMP varied from 0 to 756 pA (176 ± 34 pA, mean ± S. E. M., n=30). In some neurons, the cAMP-induced current was not adapted. The data obtained from these neurons were excluded because it was unclear whether the current observed represented an inward current induced by cAMP or an artificial leak.

Kashiwayanagi *et al.* [20] applied 1 mM cAMP into the turtle olfactory neuron and observed that cAMP induced an inward current with an average peak amplitude of 252 ± 30 pA (n=31). The magnitude of the response induced by cAMP in the turtle vomeronasal receptor neurons is smaller than that in the turtle olfactory neurons. The time to peak for the response of the vomeronasal neuron ranged from 5 to 102 s (28 ± 5 s, mean ± S. E. M., n=30). This time was slower than that for cAMP-induced response of the newt olfactory neurons (near 4 s) [16], but similar to that of turtle olfactory neurons (26 ± 4 s; mean ± S.E.M, n=22) [20].

Figure 4-3 plots the magnitudes of the responses induced by intracellular application of cAMP as a function of cAMP concentration. Although the present method of nucleotide application allowed only a single dose to each neuron, cumulative results from a group of neurons indicated the dose-dependence of the response magnitude. The currents increase in a cAMP concentration-dependent manner, which reach the

maximum level at 1 mM cAMP. It should be noted that 0.1 mM cAMP can elicit the inward currents. The sensitivity of the turtle vomeronasal receptor neurons to cAMP applied intracellularly is similar to that of isolated olfactory neurons of the newt [16].

The current-voltage relationships were examined by applying a voltage ramp either from -70 to +50 mV (480 mV/s) or from -100 to +60 mV (43.7 mV/s) to voltage-clamped vomeronasal neurons before, during and after the response induced by cAMP (Fig. 4-4). There is no significant difference between I-V curves obtained by applying voltage ramp either from -70 to +50 mV (480 mV/s) or from -100 to +60 mV (43.7 mV/s). The I-V relationship measured before the response induced by intracellular injection of cAMP into neurons was similar to that measured in control cells with normal internal solution (data not shown). The slope of the I-V curve measured during the cAMP-induced response was steeper than that measured before the response, indicating that cAMP increases the membrane conductance. It returned not completely but reversibly to the basal level after the cAMP-induced response had been adapted. The reversal potential was estimated to be -14.8 ± 2.6 mV (n=12), which was more negative than the potentials observed in isolated newt olfactory neurons [6] and the patch membrane excised from the cilia of the frog [3, 18] and rat [18].

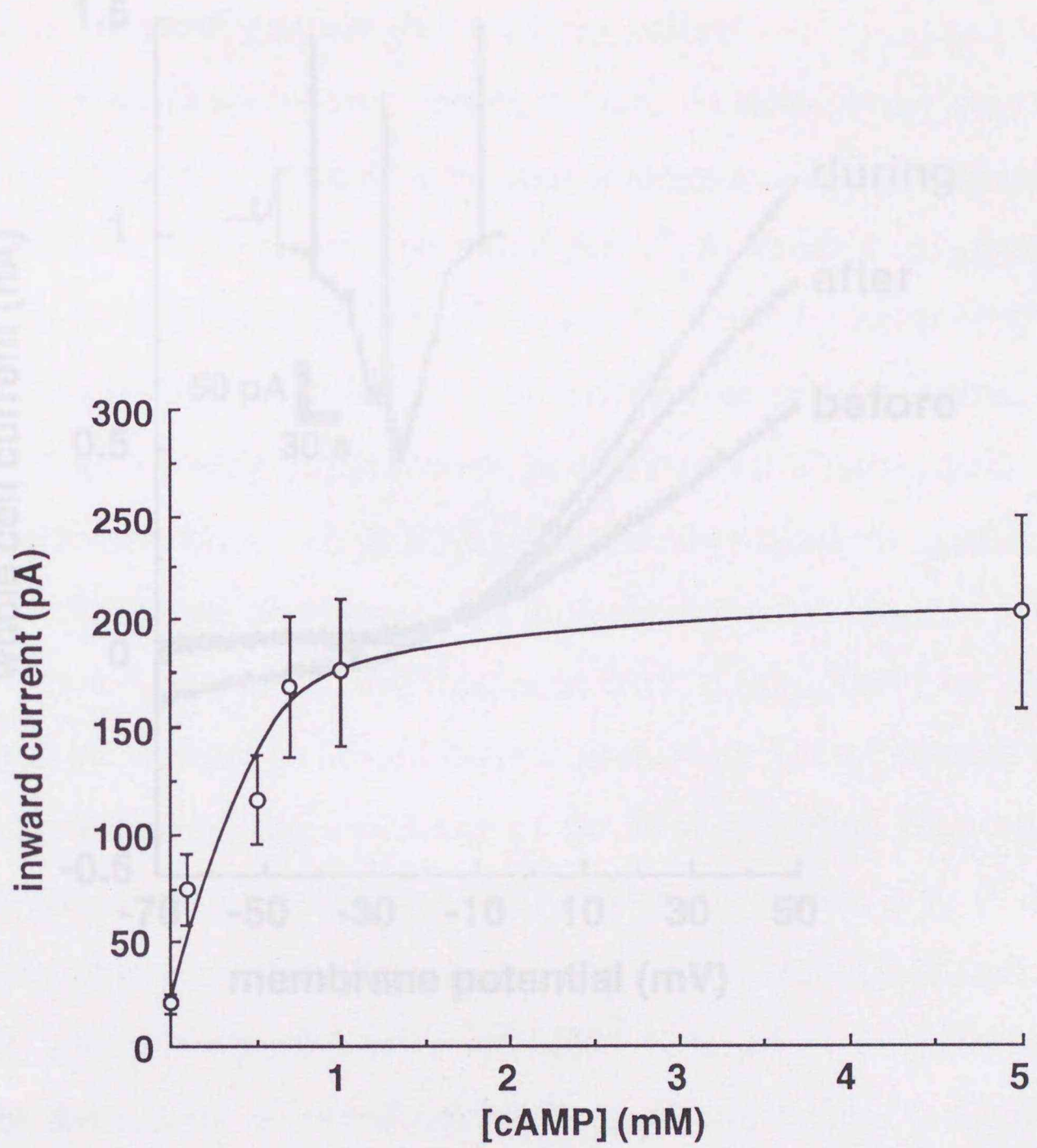

Figure 4-3. Dose-dependence of the response induced by intracellular injection of cAMP into turtle vomeronasal receptor neurons. Each point is mean ± S.E.M. of data obtained from at least 23 neurons.

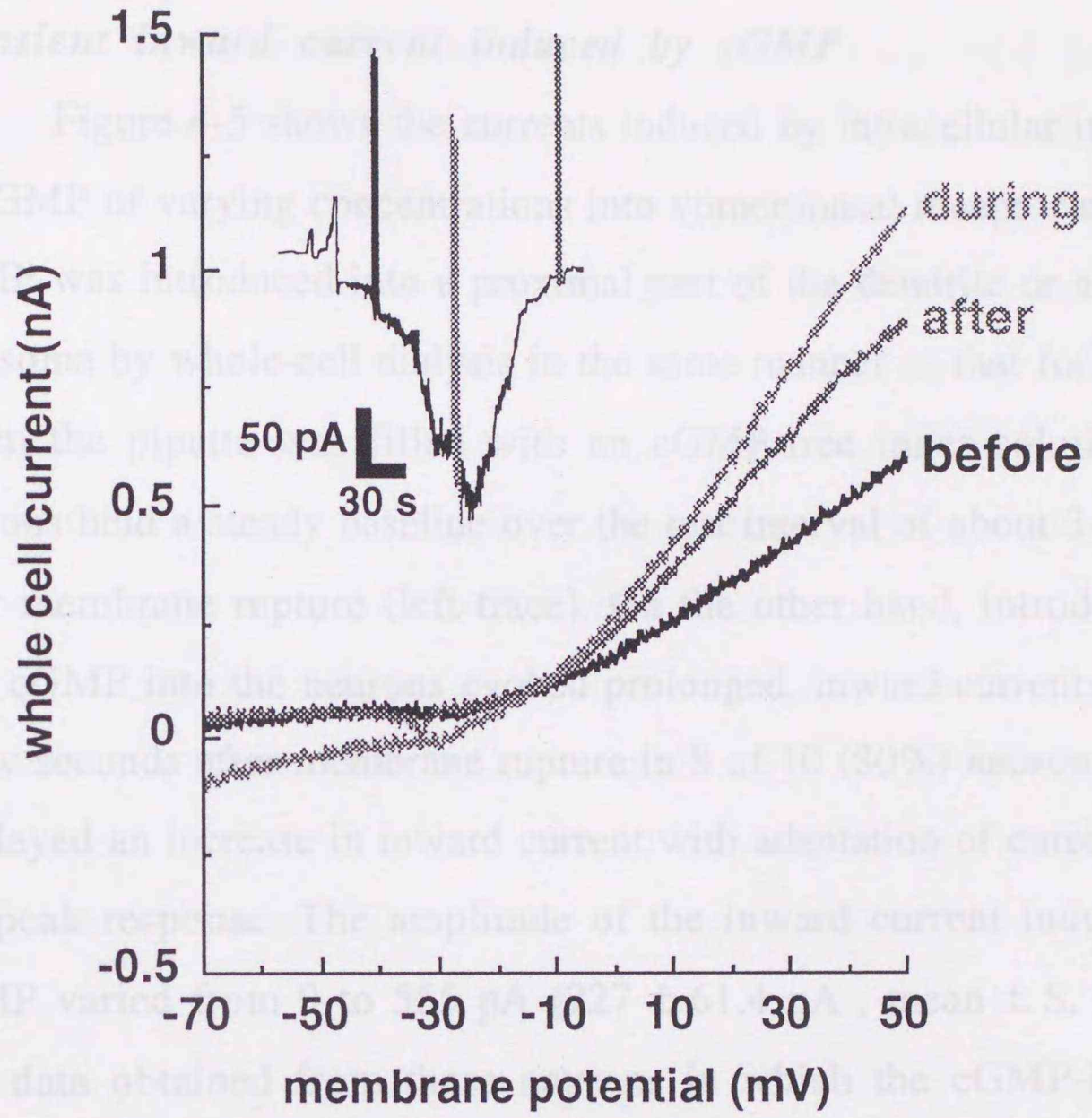

Figure 4-4. Whole-cell current-voltage relationships for the current evoked by intracellular application of 0.5 mM cAMP. The current was measured by applying a voltage ramp (480 mV/s) from -70 to +50 mV before, during and after the response induced by 0.5 mM cAMP. These traces were obtained from the same cell. The inset shows the record of the cAMP-induced response of this cell under the whole-cell voltage clamp at -70 mV. The current transients were produced by voltage ramps (480 mV/s) from -70 to +50 mV. The reversal potential of the current induced by intracellular application of 0.5 mM cAMP to this neuron was estimated to be -13.8 mV.

Transient inward current induced by cGMP

Figure 4-5 shows the currents induced by intracellular injection of cGMP of varying concentrations into vomeronasal receptor neurons. cGMP was introduced into a proximal part of the dendrite or a part of cell soma by whole-cell dialysis in the same manner as that for cAMP. When the pipette was filled with an cGMP-free inner solution, the neurons held a steady baseline over the test interval of about 3-10 min after membrane rupture (left trace). On the other hand, introducing 1 mM cGMP into the neurons evoked prolonged, inward currents within a few seconds after membrane rupture in 8 of 10 (80%) neurons which displayed an increase in inward current with adaptation of current after the peak response. The amplitude of the inward current induced by cGMP varied from 0 to 555 pA (227 ± 61.4 pA , mean ± S. E. M.). The data obtained from these neurons in which the cGMP-induced current was not adapted were excluded because of indistinctness whether the current observed represents an inward current induced by cGMP or an artificial leak. The time to peak for the response of the vomeronasal neuron varied from 4 to 55 s (28 ± 7, mean ± S. E. M., n=8). This time is similar to that for cAMP-induced response of the turtle vomeronasal receptor neuron.

Figure 4-6 shows the voltage dependence of the cGMP-induced currents examined by applying a voltage ramp from -100 to +60 mA (43.7 mV/s) to voltage-clamped vomeronasal neurons during and after the response induced by 1 mM cGMP. Because of difficulty of the

measurement, I-V relationships before the response induced by cGMP failed to be obtained. The I-V relationship measured after the introduction of cGMP into neurons was similar to that measured in control cells with normal internal solution (data not shown). The slope of the I-V curve measured during the cGMP-induced response is steeper than that measured after the response, indicating that cGMP increases the membrane conductance. The reversal potential was estimated to be -11.5 ± 8.7 mV (n=6), which was more negative than that observed in the patch membrane excised from the cilia of the frog [3, 18] and rat [18]. The reversal potential was similar to that observed in the response to intracellular application of cAMP to turtle vomeronasal receptor neurons as shown in Fig. 4-4, suggesting that the two nucleotides act at the same site.

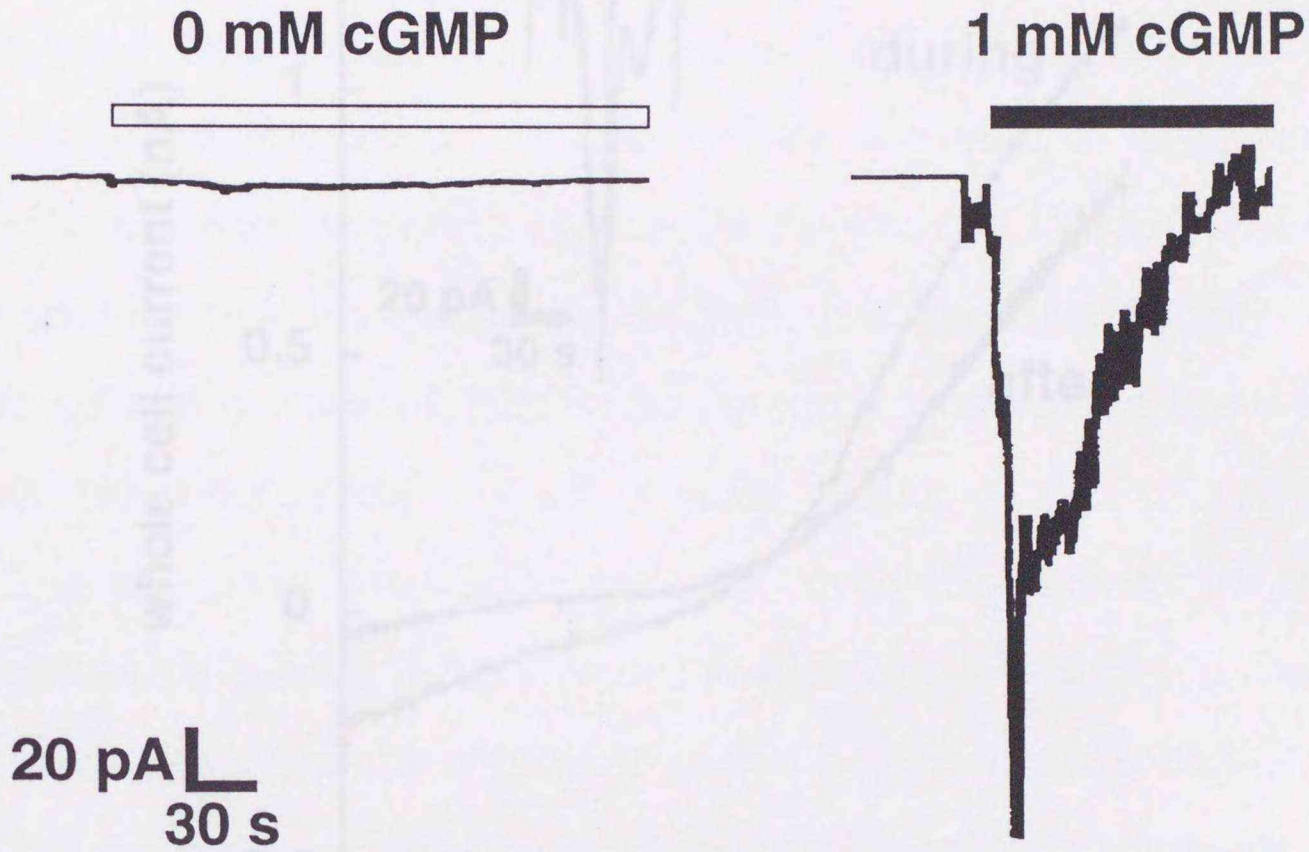

Figure 4-5. **A:** Response induced by intracellular application of 0 mM cGMP (normal internal solution) from the patch pipette to a vomeronasal receptor neuron bathed in normal Ringer solution (control). **B:** Response induced by intracellular application of 1 mM cGMP from the patch pipette to a vomeronasal receptor neuron bathed in normal Ringer solution. Open and closed bars above traces indicate period of intracellular dialysis of 0 and 1 mM cGMP in normal internal solution, respectively. Holding potential, -70 mV.

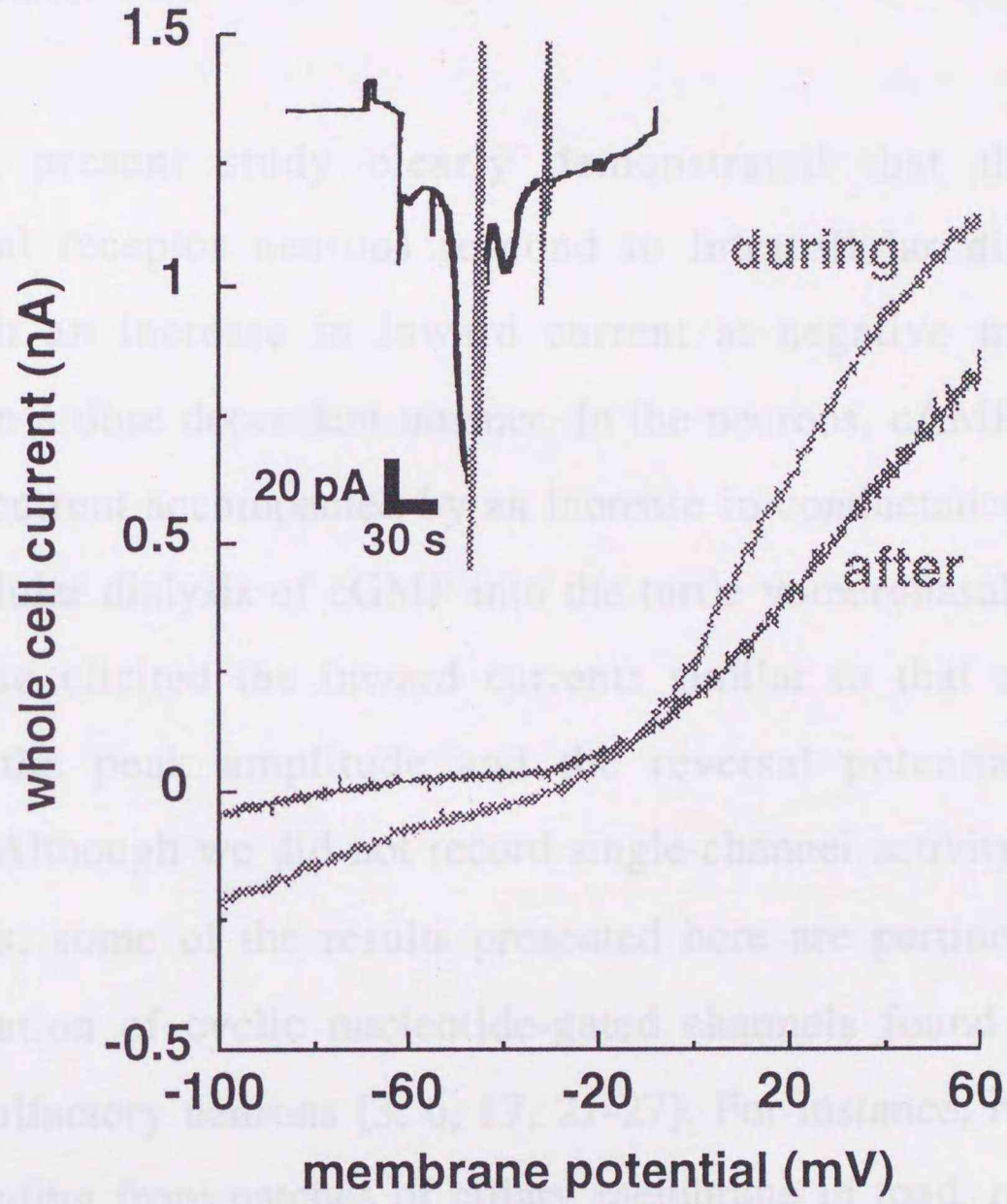

Figure 4-6. Whole-cell current-voltage relationships for the current evoked by intracellular application of 1 mM cGMP. The current was measured by applying a voltage ramp (43.7 mV/s) from -100 to +60 mV before, during and after the response induced by 1 mM cGMP. These traces were obtained from the same cell. The inset shows the record of the cGMP-induced response of this cell under the whole-cell voltage clamp at -70 mV. The current transients were produced by applying voltage ramps (43.7 mV/s) from -100 to +60 mV. The reversal potential of the current induced by intracellular application of 1 mM cGMP to this neuron was estimated to be -13.2 mV.

DISCUSSION

The present study clearly demonstrated that the turtle vomeronasal receptor neurons respond to intracellular dialysis of cAMP with an increase in inward current at negative membrane potentials in a dose dependent manner. In the neurons, cAMP induced an inward current accompanied by an increase in conductance (Fig. 4-4). Intracellular dialysis of cGMP into the turtle vomeronasal receptor neurons also elicited the inward currents similar to that of cAMP regarding the peak amplitude and the reversal potential of the responses. Although we did not record single-channel activity in these experiments, some of the results presented here are pertinent to the characterization of cyclic nucleotide-gated channels found in many vertebrate olfactory neurons [3, 6, 17, 21-27]. For instance, Nakamura *et al.*, recording from patches of ciliary membrane in toad, reported a cationic current that was directly activated by both cAMP and cGMP [3]. Therefore, it is likely that the cyclic nucleotide-gated channels in turtle vomeronasal receptor neurons are directly activated by both cAMP and cGMP

In vertebrate photo transduction, cGMP acts as a second messenger, directly activating the light-dependent membrane conductance [28, 29]. In the turtle vomeronasal receptor neuron, intracellular application of 1 mM cGMP elicited a rather large response

than that of cAMP at the same concentration. These results suggest that cGMP, rather than cAMP, is the second messenger for the signal transduction pathway. On the other hand, the turtle vomeronasal neurons responded to forskolin (Fig. 4-1). In addition, it was reported that forskolin increased the cAMP level in vomeronasal receptor neuron of garter snake. These observations indicate that vomeronasal receptor neurons have adenylate cyclase activities enough to elicit sufficient responses. As will be shown in Chapter V, forskolin elicits a large vomeronasal response of turtle. In turtle vomeronasal system, therefore, it is more likely that cAMP acts as a second messenger for signal transduction pathway.

In the frog, injection of cAMP into vomeronasal receptor neurons failed to elicit a membrane current [30]. This disagreement may be due to the difference in the preparations; enzymatically isolated olfactory neurons and neurons in slice preparation. In enzymatically isolated olfactory neurons, transient inward currents elicited by intracellular application of cAMP are, however, commonly observed [16, 20, 31]. Hence, it is not likely that enzymatic dissociation of the neurons inflicts unintentional damage on channel activities. It is more likely that this disagreement may due to the difference in species of animals used.

In the turtle vomeronasal organ, neither a chemoattractant nor a substance which activates the cAMP cascade has been identified. Therefore, biological roles of cAMP channels found in the present study are unknown. In general, the vomeronasal organ has been

reported to play important roles in feeding, social and reproductive behaviors [32, 33]. Hence cAMP channels in the turtle vomeronasal neurons seem to be involved in the transduction pathway for the chemicals related to the above behaviors. On the other hand, turtle vomeronasal system sensitively responds to various general odorant [34, 35]. Thus, there remains a possibility that the cyclic nucleotide-gated channels contribute to the transduction pathway for general odorants. This issue will be discussed in Chapter V.

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IV-2 The Responses Induced by Intracellular Injection of Inositol 1, 4, 5-Trisphosphate into Turtle Vomeronasal Receptor Neurons

INTRODUCTION

In the garter snakes, the binding of ES20, which was the chemoattractant for the snakes [1], to its receptors resulted in an increase in the basal level of IP_3 [2]. This observation supports the idea that IP_3 acts as a second messenger in signal transduction in the vomeronasal receptor neurons. In the present section [3], we show that intracellular application of IP_3 from the patch pipette to turtle vomeronasal receptor neurons elicits the membrane current under the whole-cell patch clamp.

MATERIALS AND METHODS

Preparations

The slice preparations were obtained as described in Chapter III.

Data recording and analysis

Membrane currents were recorded in the whole-cell configurations of the patch clamp as described in Chapter III (holding potential, -70 mV). Data were analyzed as described in Chapter III.

Solutions

The compositions of normal Ringer solution and normal internal solution were the same as described in Chapter III. For the stimulation with IP_3 from the patch pipette, IP_3 was dissolved in the internal solution to provide the appropriate final concentration. The stock solution of 0.1 mM IP_3 derived into 0.5 ml aliquots was stored at -80 ^oC in 0.5 ml aliquots and thawed just prior to use.

Chemicals

IP_3 and ruthenium red were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All chemicals used are of best grade available.

RESULTS

IP_3 was introduced into a proximal part of the dendrite or a part of cell soma by whole-cell dialysis. When the pipette was filled with an IP_3-free inner solution, the neurons held a steady baseline over the test interval of about 3-10 min after membrane rupture (Fig. 4-7A). On the other hand, introducing 0.1 mM IP_3 into the neurons evoked prolonged, inward currents within a few seconds after membrane rupture (Fig, 4-7B). In the present study, 98 neurons were successfully stimulated by 0.1 mM IP_3. Fifty two neurons (53%) displayed an

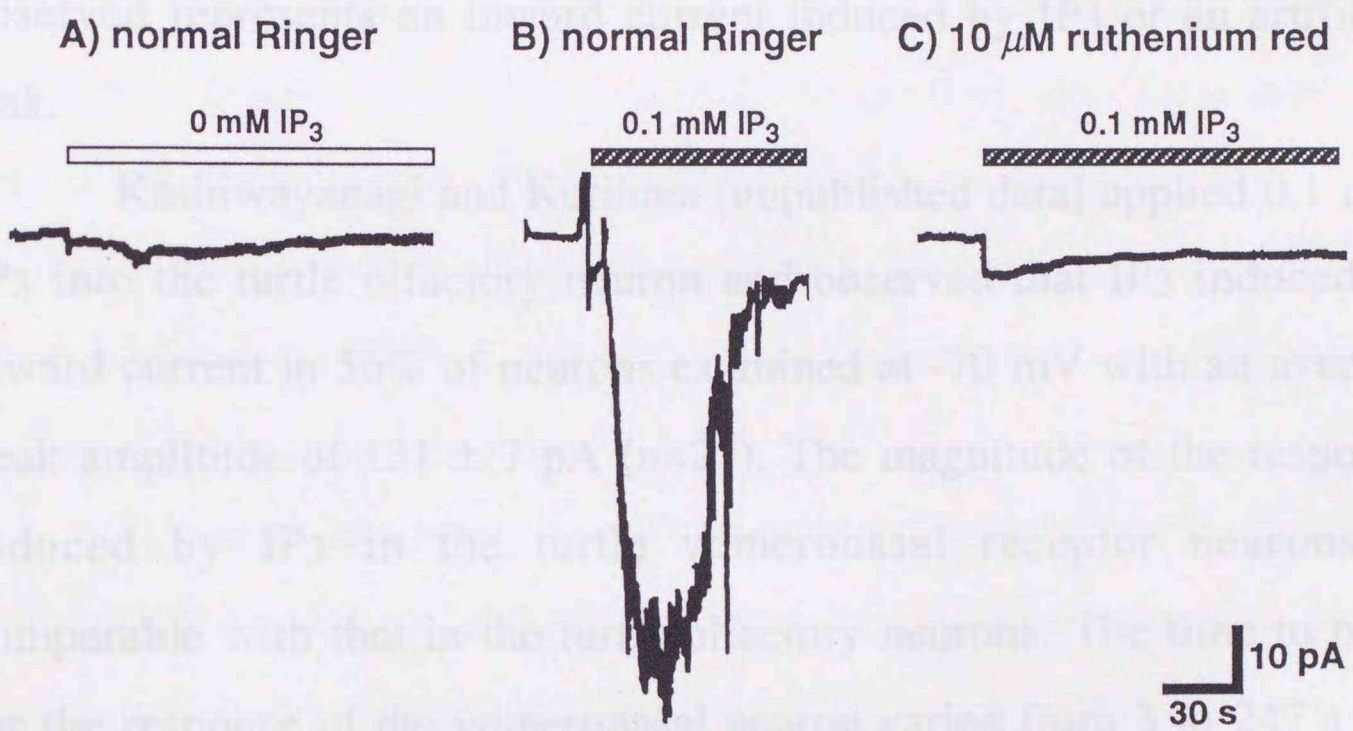

Figure 4-7. **A:** Response induced by intracellular application of 0 mM IP3 (normal internal solution) from the patch pipette to a vomeronasal receptor neuron bathed in normal Ringer solution (control). **B:** Response induced by intracellular application of 0.1 mM IP3 from the patch pipette into a vomeronasal receptor neuron bathed in normal Ringer solution. **C:** Response induced by intracellular application of 0.1 mM IP3 from the patch pipette to a vomeronasal receptor neuron bathed in Ringer solution containing 10 μM ruthenium red. Open and hatched bars above traces indicate period of intracellular dialysis of 0 and 0.1 mM IP3 in normal internal solution, respectively. Holding potential, -70 mV.

increase in inward current with adaptation of current after the peak response. The amplitude of the inward current induced by IP_3 varied from 0 to 736 pA (90 ± 11 pA , mean ± S. E. M.). In some neurons, the IP_3-induced current was not adapted. The data obtained from these neurons were excluded because it was unclear whether the current observed represents an inward current induced by IP_3 or an artificial leak.

Kashiwayanagi and Kurihara [unpublished data] applied 0.1 mM IP_3 into the turtle olfactory neuron and observed that IP_3 induced an inward current in 56% of neurons examined at -70 mV with an average peak amplitude of 131 ± 7 pA (n=27). The magnitude of the response induced by IP_3 in the turtle vomeronasal receptor neurons is comparable with that in the turtle olfactory neurons. The time to peak for the response of the vomeronasal neuron varied from 3 to 247 s (32 ± 5, mean ± S. E. M., n=52). This time is much faster than that for IP_3-induced response of the rat olfactory neuron (30 to 1200 s) [4].

In the olfactory neurons of the catfish [5] and lobster [6], the IP_3-induced response was inhibited by 10 μM ruthenium red. We examined the effect of ruthenium red on IP_3-induced response in the turtle vomeronasal neurons (Fig. 4-7C). Bathing the neurons in 10 μM ruthenium red solution greatly reduced IP_3-evoked inward currents. The peak amplitude of the inward current was reduced to 18.0 ± 4.6 pA (n=5) from 89.9 ± 10.9 pA (n=52).

The voltage dependence of the IP_3-induced currents was examined by applying a voltage ramp from -100 to +60 mA (43.7

mV/s) to voltage-clamped vomeronasal neurons before, during and after the response induced by 0.1 mM IP_3 (Fig. 4-8A). The I-V relationship measured before the introduction of IP_3 into neurons was similar to that measured in control cells with normal internal solution (data not shown). At negative membrane potentials, the slope of the I-V curve measured during the IP_3-induced response was steeper than that measured before the response, indicating that IP_3 increases the membrane conductance. It returned reversibly to the basal level after the IP_3-induced response had been adapted. The reversal potential was estimated to be -32.3 $\pm$ 1.5 mV (n=6), which was similar to that observed in rat olfactory neurons [4].

Figure 4-8B shows the IP_3-sensitive component obtained by subtracting the current measured before the response from that during the response. The IP_3-sensitive component was strongly outward rectifying at the membrane potential ranging from -30 to 0 mA, followed by large (4 of 5 cells) and small (1 of 5 cells) reduction in the current amplitude at positive potentials. A similar negative slope of the IP_3-induced current in the I-V curve was observed in the rat olfactory neurons [4]. Ca^{2+}-activated K^+ channel seems to contribute to the negative slope as Okada *et al.* [4] suggested.

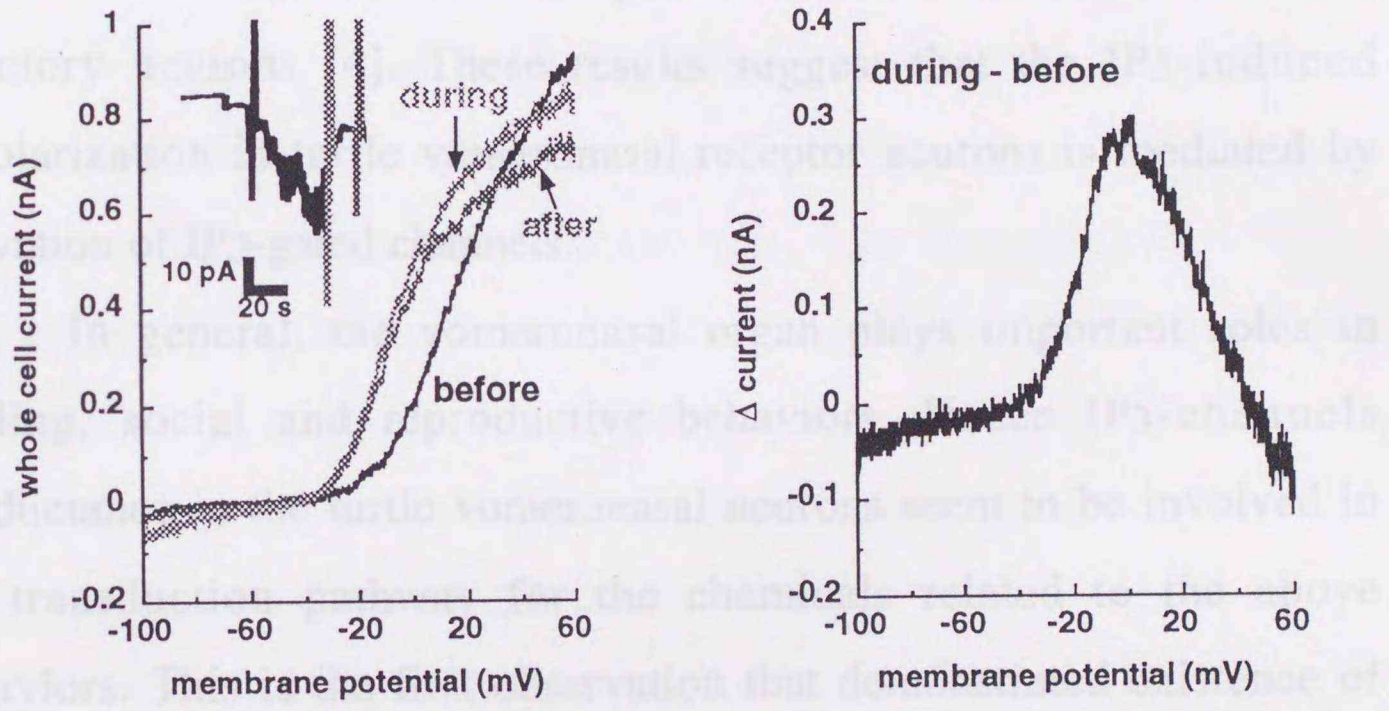

Figure 4-8. Whole-cell current-voltage relationships for the current evoked by intracellular application of 0.1 mM IP3. The current was measured by a voltage ramp (43.7 mV/s) from -100 to +60 mV before, during and after the response induced by 0.1 mM IP3 (**A**). These traces were obtained from the same cell. The inset shows the record of the IP3-induced response of this cell under the whole-cell voltage clamp at -70 mV. The current transients were produced by voltage ramps (43.7 mV/s) from -100 to +60 mV. The reversal potential of the current induced by intracellular application of 0.1 mM IP3 to this neuron was estimated to be -39.5 mV. IP3-sensitive component of this current was obtained by subtracting the current measured before the IP3-induced response from that measured during the response (**B**).

DISCUSSION

The present study demonstrated that turtle vomeronasal receptor neurons responded to intracellular dialysis of IP_3 with an increase in inward current at negative membrane potentials. This response was induced by IP_3 of mM concentration and inhibited by bath application of ruthenium red. The reversal potential was similar to that in rat olfactory neurons [4]. These results suggest that the IP_3-induced depolarization in turtle vomeronasal receptor neurons is mediated by activation of IP_3-gated channels.

In general, the vomeronasal organ plays important roles in feeding, social and reproductive behaviors. Hence IP_3-channels conductance in the turtle vomeronasal neurons seem to be involved in the transduction pathway for the chemicals related to the above behaviors. This is the first observation that demonstrated existence of IP_3-activated conductance in the vomeronasal receptor membranes.

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CHAPTER V

cAMP-DEPENDENT TRANSDUCTION PATHWAY DOES NOT GREATLY CONTRIBUTE TO THE TRANSDUCTION FOR GENERAL ODORANTS IN TURTLE VOMERONASAL NEURONS

INTRODUCTION

The results described in chapter IV demonstrated the existence of cyclic nucleotide or IP_3-mediated pathway in the vomeronasal receptor neurons. However, the biological role of these putative second messenger-mediated pathways in vomeronasal transduction is unknown, since neither a chemoattractant nor a substance which activates the putative second messenger cascade has yet been identified in the turtle vomeronasal organ.

On the other hand, the vomeronasal organ responds to general odorants as well as chemical stimuli of a social nature [1, 2]. It is reported that the turtle vomeronasal organ sensitively responds to many kinds of odorants [3, 4]. Recently, it was reported that the vomeronasal organ of garter snakes responded to chemical stimuli including general odorants as well as proteins in prey washes, indicating that the vomeronasal system is sensitive to a variety of odorants [5]. Hence there is the possibility that the putative second messenger-activated

conductance may be involved in transduction mechanism for general odorants as well as natural stimuli.

Data shown in Chapter IV indicated that the population of the turtle vomeronasal receptor neurons having cyclic nucleotide-mediated pathway is larger than that of neurons having IP3-mediated pathway. Thus, present study aims to examine to what extend the cyclic nucleotide-mediated pathway contributes to *in vivo* turtle vomeronasal transduction for general odorants [6]. The results obtained show that the turtle vomeronasal response to citralva appears even after complete desensitization of cAMP-mediated pathway by applying sufficient amounts of forskolin, suggesting that the cAMP-mediated pathway does not greatly contribute to the generation of the vomeronasal response to a general odorant.

MATERIALS AND METHODS

Recordings of main and accessory olfactory bulbar responses

Accessory olfactory bulbar response (vomeronasal response) was essentially recorded similarly to the recording of main olfactory bulbar response described in Chapter I. Turtles (*Geoclemys reevesii*) weighing 140-240 g were used in the present study. Turtles were weakly anesthetized with the necessary and minimum amount of urethane to lessen pain in the operation of the animal, immobilized by an intramuscular injection of d-tubocurarine chloride (450 μg/100 g

body weight), and locally anesthetized with lidocaine at the wounded and head-fixation points. The accessory olfactory bulb was exposed using a dental drill, and the dura mater on the accessory olfactory bulb was removed carefully. To eliminate the possible effect of the main olfactory bulb activities (3), the nerve from the main olfactory organ was cut off before entry to the main olfactory bulb. The stimulant-induced brain waves (bulbar responses) were recorded by attaching a pair of silver bipolar electrodes to the medial part of the anterior bulb. The responses were amplified and then integrated by electric integrator (time constant, 0.3 sec).

Activity of the main olfactory bulbar responses was recorded as described in Chapter I.

Chemical stimulation

The irrigating and stimulating solutions were applied to the vomeronasal epithelium instead of olfactory epithelium through a stainless steel tube. The other procedure of chemical stimulation was essentially similar to that described in Chapter I. The chemical stimulation of olfactory epithelium was carried out as described in Chapter I

Chemicals

Lilial (4-(1,1-dimethylethyl)-α-methylbenzenepropanal), geraniol (3,7-dimethyl-2,6-octadien-1-ol), lyral (mixture of 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene-1-carboxaldehyde and 3-(4-

hydroxy-4-methylpentyl)-3-cyclohexene-1-carboxaldehyde), citralva (3,7-dimethyl-2,6-octadienenitrile), hedione (3-oxo-2-pentylcyclopentaneacetic acid methyl ester), eugenol (2-methoxy-4-(2-propenyl)phenol), *l*-carvone (2-methyl-5-(1-methylethenyl)-2-cyclohexene-1-one), *d*-carvone, *d*-limonene (1-methyl-4-(1-methylethenyl)cyclohexene), *l*-citronellal (3,7-dimethyl-6-octenal), *d*-citronellal were kindly supplied from Takasago International (Tokyo, Japan). *n*-Amyl acetate, triethylamine, ethylvanillin and forskolin were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Cineole (eucalyptol; 1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane) was purchased from Nakarai Chemicals (Kyoto, Japan). All chemicals used were of best grade available.

RESULTS

Figure 5-1A shows the summated accessory olfactory bulbar responses to 5 x 10^{-5} M forskolin and 10^{-4} M citralva dissolved in Ringer solution. As seen from the figure, the responses show a peak at onset of stimulation and decline rapidly during stimulation. We take the peak height of the summated bulbar response as the magnitude of the response. The response to forskolin is much larger than that to citralva.

Figure 5-2 shows the relative magnitudes of the vomeronasal responses (A) and main olfactory responses (B) to various stimuli. Here the magnitudes of the main and vomeronasal responses to 10^{-4} M

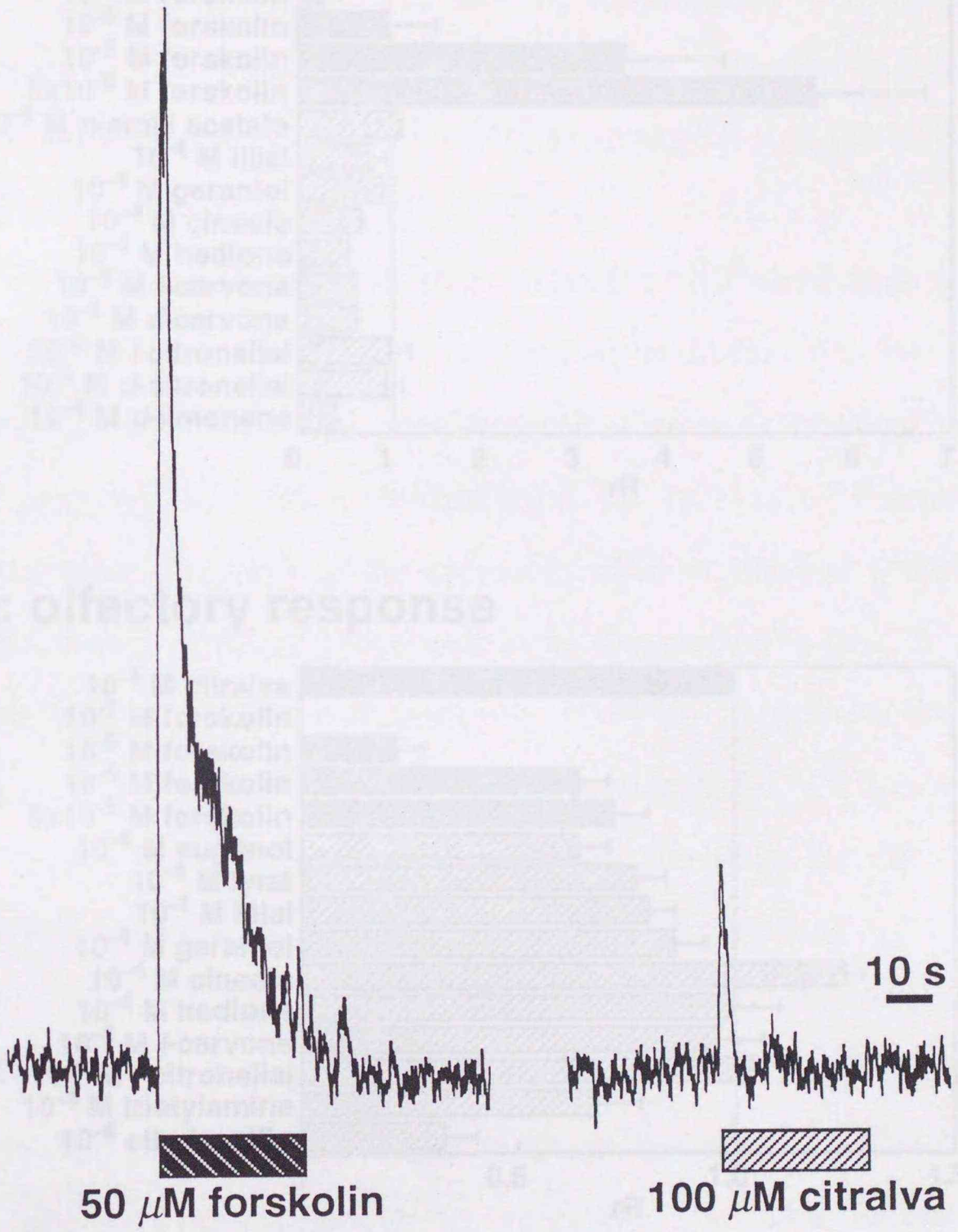
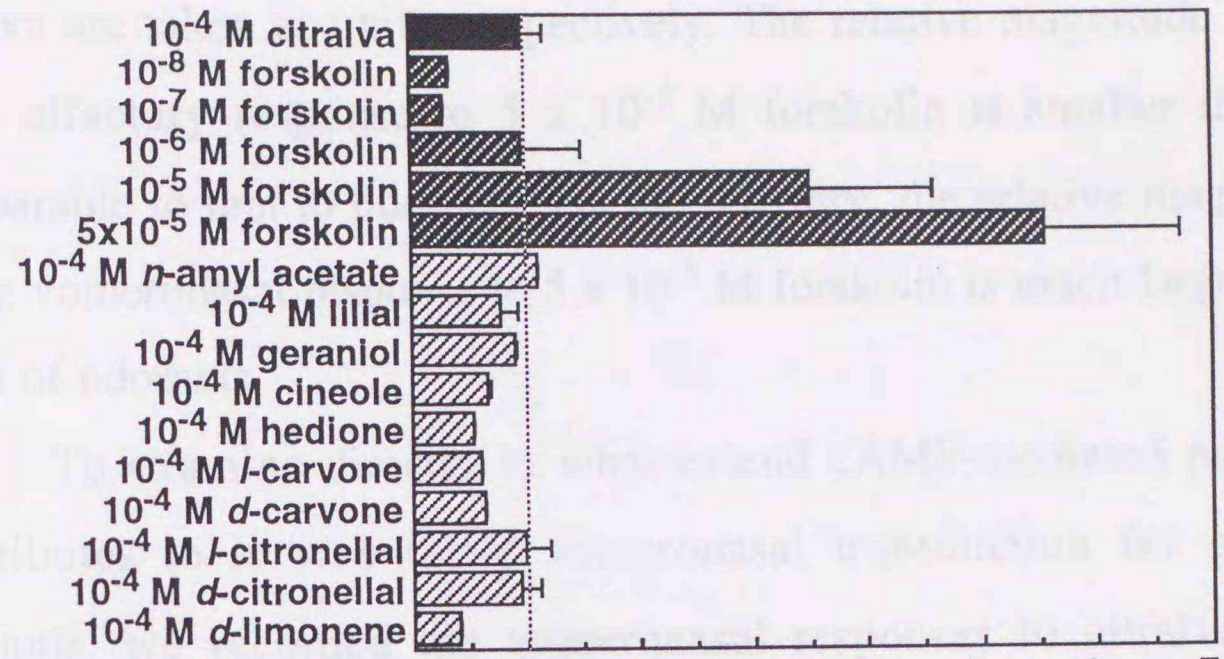


Figure 5-1. Typical records of the accessory olfactory bulbar responses to 50 μM forskolin and 100 μM citralva. Hatched bars under the records represent duration of stimulation.

A: vomeronasal response



B: olfactory response

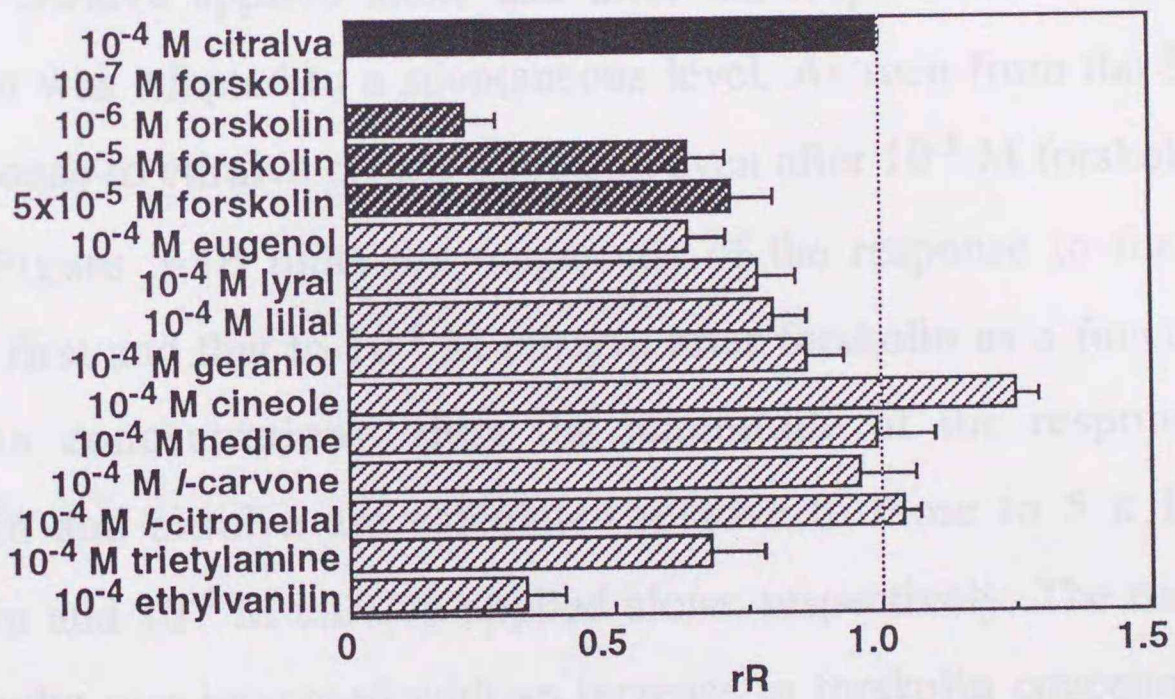


Figure 5-2. Relative magnitude of the accessory olfactory bulbar responses **(A)** and main olfactory bulbar responses to various stimuli **(B)**. Magnitude of accessory (A) and main (B) olfactory bulbar responses are calculated relative to the response to 10^{-4} M citralva, respectively. Each point is mean $\pm$ S.E.M.

citralva are taken as unity, respectively. The relative magnitude of the main olfactory response to 5 x 10^{-5} M forskolin is smaller than or comparable to that to odorants. On the contrary, the relative magnitude of the vomeronasal response to 5 x 10^{-5} M forskolin is much larger than those of odorants.

To examine directly to what extend cAMP-mediated pathway contributes to *in vivo* turtle vomeronasal transduction for general odorants, we recorded the vomeronasal responses to citralva after cAMP pathway had been desensitized by forskolin. Figure 5-3A illustrates typical records of the accessory olfactory bulbar responses to 10^{-4} M citralva applied alone and after the response to 5 x 10^{-5} M forskolin was adapted to a spontaneous level. As seen from the figure, the response to citralva clearly appeared even after 10^{-5} M forskolin.

Figure 5-3B plots the magnitude of the response to forskolin applied first and that to 10^{-4} M citralva after forskolin as a function of forskolin concentrations. Here the magnitude of the responses to forskolin and citralva are calculated relative to those to 5 x 10^{-5} M forskolin and 10^{-4} M citralva applied alone, respectively. The response to forskolin was increased with an increase in forskolin concentrations and reached a plateau level at 10-20 μM. The minimum concentration (threshold) of forskolin which elicits the vomeronasal response was 10^{-8} M. The response to a fixed concentration (10^{-4} M) of citralva applied after forskolin decreased with an increase in forskolin concentration up to 1-10 μM and plateaued. The fact that the response to citralva was partially suppressed by forskolin suggests that cAMP-mediated pathway

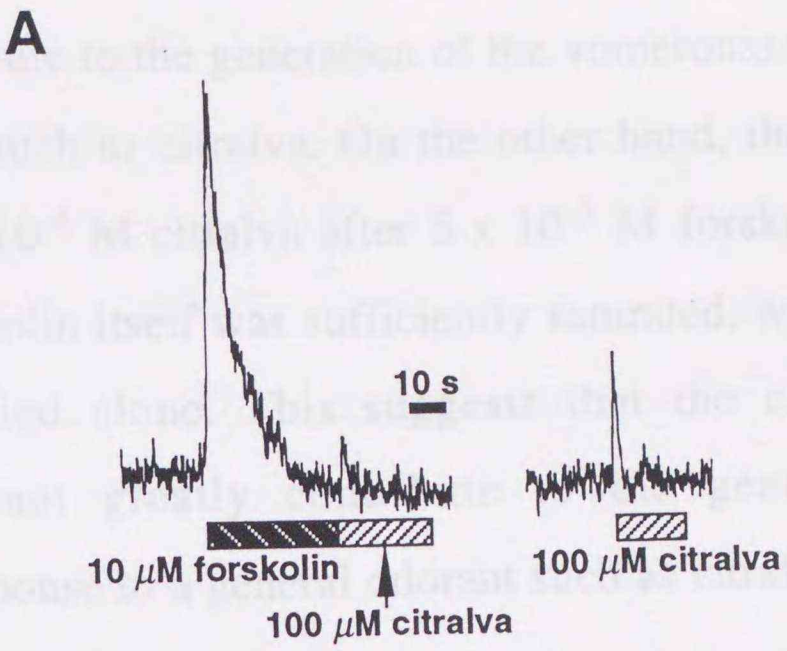


Figure 5-3. Effects of varying concentrations of forskolin on the response to citralva. **A:** typical records of response to 100 μM citralva applied alone (left trace) and 100 μM citralva applied secondarily after adaptation to 50 μM forskolin (right trace). Hatched bars, duration of application of stimuli. **B:** Relative magnitude of the accessory olfactory bulbar responses to forskolin of varying concentration applied alone (○) and citralva of a fixed concentration (100 μM) applied secondarily (●) as a function of forskolin concentration. Magnitudes of the responses to forskolin and citralva were measured from a basal level. Magnitudes of the responses to 50 μM forskolin and 100 μM citralva applied alone are taken as unity for the magnitude of the responses to forskolin applied alone (○) and that to citralva after adaptation to forskolin (●), respectively. Mean magnitude of the response to 50 μM forskolin was 4.9 times as large as that to 100 μM citralva applied alone.

somewhat contribute to the generation of the vomeronasal response to a general odorant such as citralva. On the other hand, the magnitude of the response to 10^{-4} M citralva after 5 x 10^{-5} M forskolin, where the response to forskolin itself was sufficiently saturated, was 65 % of that to citralva applied alone. This suggests that the cAMP-mediated pathway does not greatly contribute to the generation of the vomeronasal response to a general odorant such as citralva.

DISCUSSION

In the present study, we measured main and accessory olfactory bulbar responses to various stimuli. The whole-cell recordings offer direct information on olfactory transduction in a single neuron, but have some disadvantages. For example, function of isolated single neuron to respond to odorants is declined by repeated application of drugs such as forskolin. On the other hand, the accessory olfactory bulbar responses can be measured stably and reproducibly for a long time (e.g., 3 days). In addition, the recordings of the accessory olfactory bulbar responses allow us to perform repeated application of forskolin to the vomeronasal epithelium under *in vivo* conditions without any irreversible effect. Consequently, recordings from accessory olfactory bulb offer quantitative and reproducible data.

Present results obtained from measurements of the accessory olfactory bulbar responses indicated that forskolin elicits conspicuously

large responses in the turtle vomeronasal system. This is consistent with the results that forskolin elicits a large current in the turtle vomeronasal neurons as described in Chapter IV (Fig. 4-1). The present results also showed that forskolin reduces the response to citralva applied after adaptation to forskolin. This suggests the possibility that cAMP-mediated pathway partly contributes to vomeronasal transduction pathway for general odorants. Another possible reason for the suppression by forskolin is that the response to citralva applied secondarily is partly suppressed by nonspecific effects of forskolin, because forskolin has various actions besides activation of adenylate cyclase [7]. Thus, we cannot conclude that all of the forskolin-suppressive components in the responses to cAMP-producing odorants are cAMP-dependent components.

Present results also indicated that the accessory olfactory bulbar response to forskolin was much larger than those to general odorants. This does not support the idea that the vomeronasal response to a general odorant is mediated dominantly by the cAMP-dependent pathway. In addition, the response to citralva remained more than 60% of that applied alone even after application of high concentrations of forskolin, suggesting that the cAMP-independent pathway greatly contributes to the generation of the vomeronasal response to general odorants such as citralva.

The cAMP-insensitive component of the response to citralva may be generated via the pathways such as IP_3-dependent pathway, cGMP-dependent pathway, direct activation of G protein, or the other pathway

(see Fig. I-1). Further study will be needed to explore the transduction mechanism for general odorants and chemoattractants in the vomeronasal organ.

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SUMMARY

[I] Difference in odor intensity and quality between *l*- and *d*-optical isomers

(1) The turtle olfactory bulbar responses to six pairs of highly pure optical isomers were measured and the difference in odor intensity between the optical isomers was compared. With all odorants examined, there was no difference in odor intensity between optical isomers in the whole concentration ranges examined.

(2) We developed the quantitative cross-adaptation method in which the concentration of one odorant was varied and that of another remained constant. The difference in odor quality between optical isomers was evaluated by the quantitative cross-adaptation method. The degree of cross-adaptation between optical isomers were greatly different among species of odorants. The rank order of the magnitude of the difference in odor quality between optical isomers was carvone > β-citronellol > menthol> hydroxycitronellal > citronellal > limonene.

[II] Effects of treatment of the turtle olfactory epithelium with various lipids on olfactory responses

The turtle olfactory epithelium was treated with suspensions of various lipids and their effects on the olfactory responses were examined by measuring the olfactory bulbar responses.

(1) The phosphatidylserine (PS)-treatment greatly lowered the threshold for *n*-valeric acid and enhanced its responses at all concentrations examined. The responses to isovaleric acid and *n*-butyric acid were also greatly enhanced by the PS-treatment. The responses to twelve odorants examined were a little enhanced or practically unchanged by the PS-treatment. The enhanced responses to the fatty acids returned to the original level about 10 h after the treatment.

(2) The experiment using [^{14}C]PS indicated that PS was incorporated into the olfactory epithelium.

(3) In contrast to PS-treatment, the treatment of the epithelium with cardiolipin or phosphatidic acid unchanged or suppressed the olfactory responses to odorants including the fatty acids.

[III] Electrophysiological properties of turtle vomeronasal receptor neurons

The turtle vomeronasal receptor neurons in the slice preparation were studied with the whole-cell configuration of the patch clamp technique.

(1) Typical resting potentials were near -48 mV, and typical input resistance was 1-2 GΩ.

(2) The response to a current step consisted of either a single spike regardless of stimulus strength, or a train of spikes. Only 3 pA of injected current was required to depolarize the neuron to spike

threshold near -55 mV, suggesting that a turtle vomeronasal receptor neuron has high sensitivity to injected currents.

(3) Voltage-clamped vomeronasal receptor neurons displayed transient inward currents followed by sustained outward currents in response to depolarizing voltage steps. Because of their similar properties to Na^+-channels and K^+-channels found in many neurons, the transient inward currents were tentatively identified as Na^+-currents and the outward currents as K^+-currents, respectively.

(4) These results suggest that electrophysiological properties of turtle vomeronasal receptor neurons are similar to those of olfactory neurons.

[IV] Signal transduction mechanism in vomeronasal receptor neurons

cAMP, cGMP or IP_3 were injected into turtle vomeronasal receptor neurons in the slice preparation under the whole-cell patch clamp, and the evoked current was measured.

(1) In cell-attached recording, 10 μM forskolin added to the bath caused a transient increase of spike rate for 4 s, suggesting that there exists cAMP-dependent pathway in the vomeronasal receptor neurons.

(2) Intracellular application of cAMP evoked a prolonged, inward current in a dose-dependent manner. Intracellular application of 0.1 mM cAMP was enough to elicit an inward current in these

neurons. The magnitude of the response to cAMP reached a plateau level at 1 mM with an average peak amplitude of 176 ± 34 pA (n=30). The reversal potential of the response induced by cAMP was estimated to be -14.8 ± 2.6 mV (n=12).

(3) Intracellular application of 1 mM cGMP also evoked a prolonged, inward current with an average peak amplitude of 227 ± 61 pA. The reversal potential of the response induced by cGMP was estimated to be -11.5 ± 8.7 mV (n=6).

(4) The currents elicited by either intracellular cAMP or cGMP reversed near -14 mV and had similar current-voltage relationships, suggesting that the two nucleotides act at the same site.

(5) Application of 0.1 mM IP_3 also evoked a prolonged, inward current with an average peak amplitude of 89.9 ± 10.9 pA. The reversal potential of this response was estimated to be -32.3 ± 1.5 mV (n=6).

(6) The present results demonstrated the existence of second messenger-activated conductance in the vomeronasal receptor membranes.

[V] cAMP-dependent transduction pathway does not greatly contribute to the transduction for general odorants in turtle vomeronasal neurons

In order to examine to what extent cAMP-mediated pathway contributes to the generation of the vomeronasal response to

general odorants in *in vivo* system, the responses of the vomeronasal organ to various stimuli in the turtle were measured by recording the accessory olfactory bulbar responses.

(1) The vomeronasal response to forskolin was much larger than that to a general odorant.

(2) The cAMP-mediated pathway seems to be fully activated or desensitized when a sufficient amount of forskolin is applied to the olfactory neurons from external solutions. Large vomeronasal response to citralva appeared even after the response to forskolin itself was sufficiently saturated, suggesting that the cAMP-independent pathway greatly contributes to the *in vivo* vomeronasal transduction for general odorants such as citralva.

CONCLUDING REMARKS

The present study showed that there was no difference in odor intensity between optical isomers with all odorants examined. There was certain difference in odor quality between optical isomers. Especially, the odor quality was greatly different between *d*- and *l*-carvone. However, the difference in odor quality between *d*- and *l*-carvone disappeared at 40 °C. These results suggested that the receptor mechanism of the olfactory system is different from those of hormones and neurotransmitters. In addition, we showed that the PS-treatment of the olfactory epithelium greatly enhanced the responses to the fatty acids. All these results suggest that lipids as well as proteins play an important role in the olfactory reception.

The present study on signal transduction mechanisms in turtle vomeronasal system indicated that each intracellular application of cAMP, cGMP and IP_3 to the turtle vomeronasal receptor neurons elicits the membrane current. These results clearly demonstrated that the membranes of the turtle vomeronasal neurons carry cyclic nucleotide and/or IP_3-activated conductance. The present results also showed that the cyclic nucleotide-mediated pathway does not greatly contribute to vomeronasal transduction for general odorants. The present results raised the possibility that these second messenger-mediated pathways are involved in the transduction for the chemoattractant in the vomeronasal organ.

inches 1 2 3 4 5 6 7 8

cm 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Kodak Color Control Patches

Blue Cyan Green Yellow Red Magenta White 3/Color Black

Kodak Gray Scale

C Y M

A 1 2 3 4 5 6 **M** 8 9 10 11 12 13 14 15 **B** 17 18 19