Cell Suspensions from Porcine Olfactory Mucosa

Changes in Membrane Potential and Membrane Fluidity in Response to Various Odorants

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ABSTRACT A suspension of olfactory epithelial cells was prepared from porcine olfactory mucosa and the physiological functions of the suspension were examined. (a) The membrane potential of the cell suspension, which was monitored by measuring the fluorescence changes of rhodamine 6G, was depolarized by an increase in the K+ concentration in the external medium. (b) Various odorants depolarized the cell suspension in a dose-dependent fashion. (c) The magnitude of depolarization by odorants was either unchanged or slightly increased by a reduction of the concentration of Na+, Ca2+, and Cl− in the external medium, which suggests that changes in the permeabilities of specific ions are not involved in depolarization by odorants. (d) The application of various odorants to the cell suspension induced changes in the membrane fluidity at different sites of the membrane that were monitored with various fluorescent dyes [8-anilino-1-naphthalene sulfonate, n-(9-anthroyloxy) stearic acids, 12-(9-anthroyloxy) oleic acid, and (1,6-diphenyl-1,3,5-hexatriene)], which suggests that the odorants having different odors are adsorbed on different sites in the membrane. On the basis of these results, a possible mechanism of odor discrimination is discussed.

INTRODUCTION

Olfactory systems have the characteristic of being able to respond sensitively to various chemicals and discriminate among the structures of numerous chemicals. Although we know that olfactory systems have this characteristic, the fundamental mechanisms of olfactory reception are still unknown. That is, we do not yet know how odorants induce receptor potentials and how olfactory cells discriminate among numerous odorants.

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To explore the mechanism of generation of receptor potentials, we examined, in a previous study (Yoshii and Kurihara, 1983), the effect of changed ionic environments on the olfactory responses in the carp, the rainbow trout, and the bullfrog. The elimination of Na⁺, K⁺, Ca²⁺, and Cl⁻ from a solution perfusing the olfactory epithelium did not significantly affect the olfactory responses, which suggested that changes in the permeability of such ions across the apical membranes of olfactory cells are not involved in the generation of receptor potentials. However, there is a possibility that ionic permeability changes occur at the basal part of the olfactory cell. This possibility cannot be tested with in vivo preparations since the perfusion of the olfactory epithelium with a salt solution does not change the salt composition of the intercellular fluid covering the basal part of the cells. The above possibility could be tested using an olfactory cell suspension capable of responding to odorants.

Another important theme in olfactory reception is how olfactory cells discriminate among numerous odorants. A number of theories on odor discrimination (Wright, 1964; Amoore, 1970; Davies, 1971; Hornung and Mozell, 1981) have been proposed but none of them has been verified. To explore the mechanism of odor discrimination, it is desirable to examine directly the interaction between odorants and olfactory cell membranes. This interaction can be examined using isolated olfactory cells.

Several investigators have attempted to isolate olfactory cells (Cancalon, 1978; Hirsch and Margolis, 1979, 1981; Noble et al., 1984; Anderson and Ache, 1985). They have obtained cell suspensions from the olfactory tissues of the rat, catfish, and lobster, but none of them have reported that the cells obtained are capable of responding to odorants. In the present study, we have succeeded in obtaining a cell suspension from porcine olfactory mucosa, which has the ability to respond to various odorants. The cell suspension is depolarized by various odorants in a dose-dependent manner; this depolarization is monitored with a fluorescent dye. The magnitude of depolarization by odorants is practically unchanged by the elimination of specific ions such as Na⁺, Ca²⁺, and Cl⁻ from the external solution. We also measured the changes in the membrane fluidity in response to various odorants using five different fluorescent dyes and we show that different species of odorants induce changes in the membrane fluidity at different regions of the membranes. We discuss a possible mechanism of odor discrimination based on the results obtained.

METH ODS

Preparation of Cell Suspension 1 from Porcine Olfactory Mucosa

Porcine olfactory mucosae were gently removed with a spatula within 90 min after the animal was killed. We sometimes encountered animals whose mucosae were very tender. Such mucosae were not used for the experiments. The mucosae were washed with Dulbecco's modified Eagle's medium and centrifuged at 200 g for 5 min. The pellets were dispersed in Ringer solution by gentle pipetting. Nondispersed tissue was eliminated by filtration through nylon cloth. The cell suspension obtained, which was referred to as cell suspension I, was kept on ice before use. Cell viability, as judged by the ability to exclude a 1% nigrosin solution, was ~50%.
Preparation of Cell Suspension II

Cell suspension I was fractionated further by a bovine serum albumin (BSA) density gradient essentially according to Hirsch and Margolis (1979). Cell suspension I was layered over separate 6-ml discontinuous gradients of BSA. BSA was dissolved in a normal solution (Ringer solution containing 14 mM glucose, 40 µg/ml DNAse, and 20 µg/ml hyaluronidase). DNAse and hyaluronidase were added to prevent aggregation of the cells. Each centrifuge tube contained, from bottom to top, 1 ml of 10% BSA, 4 ml of 4% BSA, 1 ml of 2% BSA, and 1.5 ml of cell suspension. The tubes were centrifuged at 3.6 g for 25 min. The fraction of 4% BSA, which contained the greatest number of olfactory cells, was diluted with normal solution and centrifuged at 200 g for 5 min. The cell suspension obtained will be referred to as cell suspension II. Cell viability, as determined with nigrosin, was ~50%.

Cell Labeling with DPH

Suspension I or suspension II was labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH) by a modification of the method described by Shinitzky and Inbar (1974). DPH was added to the cell suspension (5 × 10^5 cells/ml). After the mixture was stirred for 20 min at 30°C, the polarization was measured. The concentration of DPH was 2 µM.

Cell Labeling with n-(9-Anthroyloxy) Fatty Acids

The procedure of labeling with n-(9-anthroyloxy) stearic acids (7-AS and 12-AS) and 12-(9-anthroyloxy) oleic acid (12-AO) has been described in previous articles (Schachter and Shinitzky, 1977; Kashiwayanagi and Kurihara, 1985). The fluorescent fatty acids were added to cell suspension I (5 × 10^5 cells/ml). The mixture was stirred for 20 min at 30°C and then the polarization was measured. The concentrations of the probes were 0.63 µM for 7-AS and 12-AS and 1.25 µM for 12-AO.

Cell Labeling with ANS

Cells were labeled with 8-anilino-1-naphthalene sulfonate (ANS) by the method described previously (Kashiwayanagi and Kurihara, 1985). 50 µM ANS was added to the cell suspension (5 × 10^5 cells/ml). After the mixture was stirred for 10 min at 30°C, the polarization was measured.

Measurement of Fluorescence Polarization

The polarization of dye fluorescence in the membrane was measured in a fluorescence spectrophotometer (650-10S, Hitachi Ltd., Tokyo, Japan) as described in a previous article (Kashiwayanagi and Kurihara, 1985). All experiments were carried out at 30°C. The degree of polarization was calculated as P = (I₁ - I₂)/(I₁ + I₂). Here, I₁ and I₂ stand for the fluorescence intensity parallel to and that perpendicular to the plane of polarization of the excitation beam, respectively. The background intensity was subtracted from the measured fluorescence intensity.

Measurement of Membrane Potential

Changes in the membrane potential of the cell suspensions were monitored with a fluorescent dye, rhodamine 6G (Rh6G). The method was essentially the same as that described in a previous article (Kashiwayanagi and Kurihara, 1985). 1 µl of 0.01 mM Rh6G was added to a 2-ml cell suspension (3 × 10^6 cells/ml) in the cuvette. After the cell suspension containing Rh6G was stirred for 30 min at 30°C, the fluorescence intensity (F₀) was measured. A small volume of odorant solution dissolved in ethanol was then
added to the cuvette. Ethanol itself, at the concentration added (<0.2 M), did not affect the membrane potential of the cell suspension. After the cell suspension containing an odorant was stirred for 10 min at 30°C, the fluorescence intensity (F) was measured. When the effects of nonodorous chemicals such as glycerol, glucose, glycine, and Tris on the fluorescence intensity of the cell suspension were examined, the NaCl concentration in the Ringer solution was reduced to hold the osmolarity of the solutions constant.

The change in the fluorescence intensity, \( \Delta F \), was defined as \( \Delta F = \frac{F - F_0}{F_0} \). Here, \( F \) and \( F_0 \) stand for fluorescence intensities in the odorant and the odorant-free solution, respectively.

**Solutions**

Stock dye solutions were as follows: 2 mM DPH dissolved in tetrahydrofuran, 10 mM ANS dissolved in distilled water, 0.25 mM 12-AS, 0.25 mM 7-AS, and 2.5 mM 12-AO dissolved in ethanol. The composition of the Ringer solution was as follows: 155 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 0.8 mM MgCl\(_2\), and 5 mM HEPES. Solutions containing different compositions of K\(^+ \) were prepared by replacing Na\(^+ \) in the Ringer solution with K\(^+ \), thereby keeping the total concentrations of these ions constant. The pH of the Ringer solution was adjusted to 7.3.

**Chemicals**

The sources of chemicals used were as follows: DNAse I and hyaluronidase (type I-S) was from Sigma Chemical Co., St. Louis, MO; bovine serum albumin (fraction V) was from Wako Pure Chemical Industry, Osaka, Japan; Rh6G was from Chroma-Gesellschaft Schmid & Co., Stuttgart, Federal Republic of Germany; 3,3'-dipropylthiocarbocyanine iodide [diS-C\(_5(5)\)] was from Nihon Sensitive Dye Laboratories Co., Okayama, Japan; ANS and DPH were from Tokyo Chemical Industry Co., Tokyo, Japan; and 7-AS, 12-AS, and 12-AO were from Molecular Probes Inc., Junction City, OR. The odorants used were of the best grade available.

**RESULTS**

Fig. 1A shows a light micrograph of cell suspension I isolated from porcine olfactory mucosae. There are cells that have the characteristic structure of the olfactory cell. These cells are composed of cilia, an olfactory knob, a cell soma, and a neural axon (Fig. 1B). The cells with such morphology in Fig. 1A are indicated by the arrows. The average content of the cells with such morphology was at least ~10% of the living cells in cell suspension I. Cell suspension I was subjected to further fractionation by a BSA density gradient. The micrograph of cell suspension II thus obtained is shown in Fig. 2. The content of cells with the morphology of the olfactory cell in the suspension was ~30% of the living cells.

Changes in the membrane potential of the cell suspensions in response to various odorants were monitored with a voltage-sensitive fluorescent dye, Rh6G. The changes in the membrane potential of cell suspension II in response to

**Figure 1.** (opposite) Phase-contrast photomicrographs of cell suspension I (A) and typical olfactory cells (B) isolated from porcine olfactory mucosae. The cells indicated by the arrows (A) show the cells that have the characteristic structure of the olfactory cell. Calibration bars, 20 \( \mu \)m (A) and 10 \( \mu \)m (B).
various odorants were essentially similar to those of cell suspension I (data not shown) and hence cell suspension I was used in the following experiments. Fig. 3 shows changes in the fluorescence intensity of cell suspension I as a function of the $K^+$ concentration in the external solution ($[K]_o$). The fluorescence intensity increased with an increase in $[K]_o$, which indicates that the membrane potential of the cell suspension was depolarized by an increase in $[K]_o$.

**Figure 2.** Phase-contrast photomicrograph of cell suspension II isolated from porcine olfactory mucosae. Calibration bar, 20 μm.

Fig. 4 shows changes in the fluorescence intensity of the cell suspension in response to various odorants as a function of their concentrations. The figure shows that various odorants depolarize the cells in a dose-dependent manner. The membrane potential of the cell suspensions in response to odorants was also monitored with diS-C$_3$(5). The relationships between the $ΔF$ value of diS-C$_3$(5)
FIGURE 3. The magnitude of the changes in fluorescence intensity ($\Delta F$) of Rh6G added to cell suspension I as a function of [KCl]. An increase in the value of $\Delta F$ represents depolarization of the membrane potential. Each point is the mean value ± SD of data obtained from at least three preparations.

and the odorant concentrations are similar to those between the $\Delta F$ value of Rh6G and the odorant concentrations (data not shown).

Nonodorous chemicals such as glycerol (100 mM), cholesterol (26 μM), glucose (310 mM), glycine (310 mM), and Tris (140 mM) did not affect the membrane potential of the cell suspensions. The cells treated weakly with 0.01% Triton X-100 lost the ability to respond to various odorants, although the cell morphology was unchanged. In addition, the cells that were allowed to stand in Ringer solution containing only salts for, e.g., 8 h also lost the ability to respond to odorants. These results suggest that nonviable cells were not depolarized by odorants.

FIGURE 4. The magnitude of the change in fluorescence intensity ($\Delta F$) of Rh6G added to cell suspension I as a function of the concentration of odorants added. Each point is the mean value ± SD of data obtained from at least three preparations.
To explore the mechanism of depolarization by odorants, the effects of the elimination of specific ions on depolarization were examined. Fig. 5 shows the effects of the elimination of specific ions in Ringer solution on the fluorescence intensity changes induced by the application of the odorants; the upper and lower panels show the relative magnitude of the fluorescence intensity change induced by 0.02 mM muscone and 2 mM menthone, respectively, where the ΔF value in normal Ringer solution is taken as unity. A reduction of the concentrations of Na⁺ and Cl⁻ had practically no effect on the magnitude of the fluorescence intensity changes, which indicates that the depolarization induced by muscone and menthone is not affected by the reduction of the concentrations of these ions. A reduction in the Ca²⁺ concentration leads to a small increase in the magnitude of the response to muscone and essentially does not affect the response to menthone.

The effects of various odorants on the membrane fluidity of the cell suspension were examined. Fig. 6 plots relative changes in the degree of the polarization (P/P₀ value) monitored with DPH in response to various odorants as a function of their concentrations. P₀ and P indicate the degree of the polarization in odorant-free and odorant solutions, respectively. The application of muscone,
amyl acetate, cyclooctane, nonanol, and menthone decreased the \( P/P_0 \) values in a dose-dependent manner, which indicates that these odorants increase the membrane fluidity in the domain where DPH is localized. On the other hand, the application of \( \beta \)-ionone increased the \( P/P_0 \) value in a dose-dependent fashion, which indicates that the membrane fluidity in the domain where DPH is localized was decreased. The DPH polarization of the cell suspension was essentially unchanged when the cell suspension was depolarized by 100 mM KCl (data not shown). This suggests that changes in the membrane fluidity induced by odorants are not brought about by depolarization of the cells.

Fig. 7 compares minimum concentrations of odorants at which the cell suspension was depolarized with those at which the membrane fluidity monitored with
DPH was changed. The minimum concentrations of odorants at which depolarization and the fluidity change occur were determined by extrapolating the curves in Fig. 4 to the zero level and those in Fig. 6 to the control level. There exists a good correlation between both minimum concentrations, which suggests that the depolarization by each odorant occurs at a concentration similar to that at which the membrane fluidity monitored with DPH changes.

Fig. 8 shows changes in the membrane fluidity monitored with various fluorescent probes when different concentrations of muscone were applied to the cell suspension. The $P/P_0$ value of ANS polarization was either unchanged or decreased by the application of muscone. The $P/P_0$ values of 7-AS, 12-AS, 12-AO, and DPH decreased with an increase in the muscone concentration, which indicates that muscone increases the membrane fluidity in the domains monitored with various fluorescent probes.

Fig. 9 summarizes the changes in the membrane fluidity monitored with various probes in response to different species of odorants. The concentrations of odorants used are those at which the odorants change the fluorescence intensity by 10–20%. As the figure shows, the profiles of membrane fluidity in response to odorants with different odors differ from each other. For example, muscone, which has a musky odor, increases the membrane fluidity monitored with 7-AS, 12-AS, 12-AO, and DPH. Cyclooctane, which has a camphoraceous odor, increases only the membrane fluidity monitored with DPH. $\beta$-ionone, which has a floral odor, decreases the membrane fluidity monitored with 12-AS,
FIGURE 9. Effects of various odorants on changes in the polarization of various fluorescent dyes. The $P/P_0$ value in the ordinate is defined in the legend to Fig. 6. The concentrations of odorants were $2 \times 10^{-5}$ M muscone, $2 \times 10^{-3}$ M menthone, $1 \times 10^{-4}$ M cyclooctane, $2 \times 10^{-4}$ M $\beta$-ionone, $2 \times 10^{-4}$ M nonanol, and $5 \times 10^{-5}$ M amyl acetate. Each point in the figure is the mean value ± SD of data obtained from at least eight preparations.

12-AO, and DPH. These results suggest that odorants with different odors are adsorbed at different sites in the membrane.

DISCUSSION

A number of attempts to isolate olfactory cells have been made. In these studies, the olfactory cells of the rat (Hirsch and Margolis, 1979, 1981; Schubert et al., 1985), the catfish (Cancalon, 1978), and the lobster (Anderson and Ache, 1985) were dispersed with enzymes such as collagenase, hyaluronidase, pancreatin, trypsin, and DNase. Cells of the nasal ethmoturbinates from newborn rats (Nobel et al., 1984) were mechanically dispersed, but the yield of isolated cells was very low. On the other hand, porcine olfactory epithelial cells were easily dispersed simply by gentle pipetting of the mucosa. DNase and hyaluronidase were used for the preparation of cell suspension II, but these enzymes were used
only for preventing aggregations of the cells dispersed by pipetting. Thus, the porcine olfactory cell suspension was obtained rapidly under much milder conditions than those employed in previous studies. The use of such a rapid and mild method seems to have enabled the isolation of olfactory cells with the physiological capability of responding to various odorants.

Cells that exhibit morphology typical of the olfactory cell corresponded to \(~10\%\) of cell suspension I and \(~30\%\) of cell suspension II. During the preparation of the cell suspensions, rounding up of some cells occurred. Such rounded cells were not counted as olfactory cells because the cell types of rounded cells are not easily determined. Hence, the actual content of the olfactory cells in the cell suspensions seems to be higher than the content described above. The cell suspensions obtained \(~50\%\) of nonviable cells, as judged by the ability to exclude a nigrosin solution. The leaky cells treated with Triton X-100 and the cells that were allowed to stand at room temperature in Ringer solution containing only salts lost the ability to respond to odorants. Hence, the presence of such nonviable cells seems not to affect the depolarizing responses of the cell suspensions to various odorants. The physiological functions (depolarization by increasing \([K]_o\) or the odorant concentration and the membrane fluidity changes monitored with DPH in response to odorants) of cell suspension II were not greatly different from those of cell suspension I (data not shown), although cell suspension II contained a higher content of olfactory cells. This is probably because the physiological functions were partly lost during the longer preparation procedures of cell suspension II. It is also possible that cell suspension I includes many deformed (e.g., round) olfactory cells that respond to various odorants, and that the deformed cells are eliminated by the BSA density gradient used for the preparation of cell suspension II; the actual content of cells that respond to odorants may not be increased in cell suspension II.

Since the size of the porcine olfactory cell is too small for its membrane potential to be measured with a microelectrode, the membrane potential of the cell suspensions was monitored with a voltage-sensitive fluorescent dye, Rh6G. We have demonstrated previously (Kashiwayanagi and Kurihara, 1985) that changes in the fluorescence intensity of Rh6G correspond closely to membrane potential changes of the neuroblastoma cell (N-18 clone) induced by odorants: the relationships between the \(\Delta F\) value of the N-18 cell–dye suspension in response to odorants and their concentrations were closely related to those between the membrane potential changes measured with a microelectrode and their concentrations. In the present study, we also used another voltage-sensitive dye, diS-C3(5), to monitor the depolarization of cell suspension I by various odorants and obtained results similar to those obtained with the use of Rh6G. Thus, changes in the fluorescence intensity of Rh6G seem to correspond closely to the changes in the membrane potential of the cell suspensions. The fluorescence measurements make it possible to monitor easily the average value of the membrane potential changes in numerous cells. In the present study, we applied odorants to the cell suspension as it was being stirred gently. The fluorescence changes in response to odorants reached a steady level within 90 s. This time course seems to be due to the method of odorant application (bath application)
employed in the present study. The magnitude of the fluorescence changes stayed at a constant level for at least 15 min.

As shown in Fig. 3, an increase in \([K]\), led to depolarization of cell suspension I. The value of \(\Delta F\) induced by 100 mM KCl was much smaller than that induced by odorants (e.g., 5 mM menthone). The absolute value of depolarization by odorants is unknown but it does not seem to be as large as 28 mV (the maximum value of the olfactory receptor potential reported by Trotier and MacLeod, 1983). Hence, the magnitude of the depolarization per 10-fold change in \([K]\) seems to be much smaller than that predicted by the Nernst equation for an ideal K+ electrode. This suggests that the cells isolated from the olfactory mucosa have a rather low density of K+ channels open in the resting state. This notion is consistent with the recently proposed idea (Getchell, 1977; Masukawa et al., 1983, 1985; Trotier and MacLeod, 1983; Anderson and Ache, 1985) that olfactory cells have low resting potentials and an extremely high input resistance. The reduction of the concentrations of Na+, Ca2+, and Cl- in the external solution for cell suspension I did not affect the resting membrane potential of the cell suspension (data not shown), which suggests that the diffusion potential of these ions does not contribute significantly to the resting membrane potential of the cells. It has been noted that the K+ channels of the N-18 cell, which is depolarized by various odorants, are mostly closed at the resting state (Miyake and Kurihara, 1983a, b; Miyake et al., 1984).

In a previous study (Yoshii and Kurihara, 1983), we demonstrated that the elimination of Na+, Ca2+, and Cl- from the solution perfusing the olfactory epithelium did not affect the olfactory responses of the frog, the carp, and the rainbow trout, and suggested that permeability changes of specific ions at the receptive membrane are not involved in depolarization induced by odorants. In the present study, the ionic composition of the solution contiguous to the whole-cell membranes was changed. The magnitude of the depolarization of cell suspension I induced by odorants was practically unchanged or slightly increased by a reduction of the concentrations of Na+, Ca2+, and Cl-. These results suggest that permeability changes of specific ions at the basal membranes of the cells as well as the receptive membranes are not involved in the depolarization induced by odorants.

In a previous article (Kashiwayanagi and Kurihara, 1984), we showed that the mouse neuroblastoma cell (N-18) was depolarized by various odorants. The elimination of Na+, Ca2+, or Cl- from the external solution did not affect the depolarization induced by odorants, as observed in the present study with the porcine olfactory cell suspension. Various odorants induced changes in the membrane fluidity of the N-18 cell (Kashiwayanagi and Kurihara, 1985). The profiles of the membrane fluidity changes monitored with various fluorescent dyes were quite similar to those observed in the present study. These results suggest that the receptor mechanism of odorants in cell suspension I is similar to that in the N-18 cell. The N-18 cell is unrelated to olfactory cells and hence it is unlikely that specific receptor proteins for odorants are involved in odorant reception in the N-18 cell. The fact that the profiles of the membrane fluidity changes in cell suspension I are similar to those of the N-18 cell suggests that
specific receptor proteins unique to olfactory cells are not involved in odorant reception in the porcine olfactory system.

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