Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 261–264, January 1988
Neurobiology

Olfaction by melanophores: What does it mean?

(olfactory receptors/adenylate cyclase)

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Communicated by Aaron B. Lerner, August 27, 1987

ABSTRACT **Hypotheses on general olfaction can be divided into two broad groups: those that predict the existence of olfactory-specific olfactory receptor proteins and those that do not. Recently, much attention has been paid to the discovery of an odorant-stimulated adenylate cyclase in purified olfactory cilia. This finding has, for the most part, been accepted as evidence that the former hypotheses are correct. Here we report that frog melanophores, which are nonolfactory in nature, disperse their melanosomes in response to the same types and concentrations of odorants used in the investigations of olfactory cilia and that pigment dispersion is accompanied by rises in intracellular cAMP levels. The effects show that the existence of a cAMP-based second messenger system in olfactory cilia is not in itself proof of the existence of olfactory-specific olfactory receptor proteins. Also they explain the basis of Ottoson's pioneering work of 30 years ago on the electrical responses of frog olfactory epithelium to stimulation with alcohols. The results suggest that there could be two mechanisms that are important for the detection of odorants: one based on specific receptors, the other nonspecific, but both working through activation of cAMP.**

Many cellular processes are mediated by cAMP and recently olfaction has been added to this list (1–3). The sense of smell in vertebrates is a property of the olfactory neuroepithelium. While this epithelium contains several types of cells that are important for olfaction, the bipolar receptor neurons are ultimately responsible for odorant detection. These neurons are physically striking for the tuft of cilia sprouting from the bulbous expansion that marks the end of each dendrite (4). The cilia, which in some animals exceed 100 μM in length, form an interlacing meshwork intercalated into mucus covering the epithelium. Because of their physical location the olfactory cilia have long been thought to be the primary detectors of odorants. In support of this premise cilia have now been shown to contain high levels of the GTP binding protein G_s, a cAMP-gated conductance, and an adenylate cyclase that can be activated by incubation of the cilia with odorants applied at concentrations in the range of 100 to 250 μM (1–3, 5). However, the molecular nature of olfactory receptors themselves has remained obscure.

Many proteins have been suggested as candidates for being olfactory receptors (6–9). That no specific receptor proteins may be required for general olfaction has also been suggested (10–12). We decided to determine whether odorants could activate adenylate cyclase in cells that were neither neuronal nor olfactory in nature. Melanophores were chosen because activation of adenylate cyclase results in a rapid and easily observable change in phenotype.

Melanophores, which are found in many vertebrates, contain organelles called melanosomes that are filled with the dark brown pigment melanin. The melanosomes can be moved either centripetally or centrifugally within the cells in response to specific stimuli. For example, melatonin causes frog melanophores to aggregate their melanosomes while melanocyte-stimulating hormone, which activates adenylate cyclase, brings about their dispersion (13). Melanophores from most species disperse their melanosomes in response to any agent that leads to increases in cAMP (14).

Here we report that application of odorants to frog melanophores causes activation of adenylate cyclase and dispersion of melanosomes. The possible relevance of these observations to olfaction in human beings is discussed.

MATERIALS AND METHODS

Melanophore-Based Odorant Assay. Squares (2 × 2 mm) were etched into 9-mm tissue culture dishes with a steel stylus. Cells from a culture of pure *Xenopus* melanophores established by methods that were similar to one previously reported (15) were plated onto culture dishes so that 50–100 cells attached within any given square. After incubation overnight in 1 ml of L-15 medium supplemented with 20% fetal bovine serum, dishes were used for odorant assays. Melatonin was added to a concentration of 0.5 nM and 30 min was allowed to elapse, during which time the majority of cells aggregated their melanosomes. A photographic record was then made of all the cells in a square. Next an odorant was added in ethanol (except as noted in *Results*) to a specific final concentration. After incubation with the odorant for 15 min another photograph was taken of the same square. In this way a record was obtained for an identifiable set of cells before and after exposure to a known concentration of an odorant. The distribution of melanosomes for every cell in the square was examined before and after treatment with odorant, and the percentage of cells that dispersed their melanosomes either partially or completely in response to the odorant was determined.

Determination of cAMP Levels in Melanophores Following Exposure to Odorants. Melanophores were plated in 9-mm tissue culture dishes and allowed to grow to confluency. For experiments, the volume of medium was adjusted to 1 ml. As above, melatonin was added to a final concentration of 0.5 nM. After 30-min odorants, melanocyte-stimulating hormone or forskolin was added. Five minutes later the medium was removed and 1 ml of ice-cold 5% trichloroacetic acid was added to each dish. The dishes were placed at 4°C for 30 min. The acid solution was then collected from each dish and the remaining cells were washed with another 1 ml of 5% trichloroacetic acid. The washes were combined with the original acid extracts, and the combined extracts were extracted with three 5-vol portions of ether and then lyophilized. The samples were then dissolved in 0.5 ml of sodium acetate, pH 6.2. All experiments were performed in triplicate. Measurements of cAMP were done in duplicate on each sample according to Steiner *et al.* (16) with a Rianen cAMP RIA kit from New England Nuclear. Protein was measured by the method of Bradford (17). cAMP levels are expressed as pmol/mg of protein.

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RESULTS

Melanophores Disperse Their Melanosomes in Response to Melanocyte-Stimulating Hormone and Citralva. As shown in Fig. 1*A*, in cells treated with 0.5 nM melatonin for 30 min, the melanosomes have aggregated, forming what look like single black discs. Subsequent exposure of these cells to 10 nM melanocyte-stimulating hormone for 15 min causes dispersal of the melanosomes (Fig. 1*B*). Citralva (100 μM; ref. 2) and citral (250 μM; ref. 1) have been used as standards in studies concerning the odorant dependent activation of adenylate cyclase in isolated olfactory cilia. Fig. 1*C*, like Fig. 1*A*, shows cells that have been incubated with 0.5 nM melatonin for 30 min. Fig. 1*D* shows the same cells 15 min after treatment with 100 μM citralva. Clearly the cells have responded to odorant, as to melanocyte-stimulating hormone, by dispersal of their melanosomes.

Melanophores Disperse Their Melanosomes in Response to Many Odorants. The effects of four odorants—β-ionone, citralva, octanol, and ethyl vanillin—on melanophores are summarized in Fig. 2*A*, in which percentage of responding cells is plotted against concentration of applied odorants. The odorants differ in their effectiveness at stimulating melanophores, with β-ionone the most potent and ethyl vanillin the least.

To be consistent from one experiment to another, the odorants were diluted in ethanol so that the addition of 1 μl of a solution containing odorant in ethanol gave the desired final concentration. Exceptions were experiments in which the odorants were tested at concentrations that required greater than 1 μl of pure chemical, in which case the appropriate volume of chemical was added. One microliter of ethanol, which corresponds to 17 mM, had no effect on the melanophores (see Fig. 4).

Odorants Activate Adenylate Cyclase in Melanophores. To determine whether or not the odorant-induced dispersal of melanosomes is associated with an increase in cAMP concentration, as it is with melanocyte-stimulating hormone stimulation, 9-mm tissue culture dishes containing confluent cultures of melanophores were exposed to the odorants and the accumulation of cAMP in cells was measured by RIA 5 min later. The relationship between concentration of odorant and resulting cAMP level is shown in Fig. 2*B*.

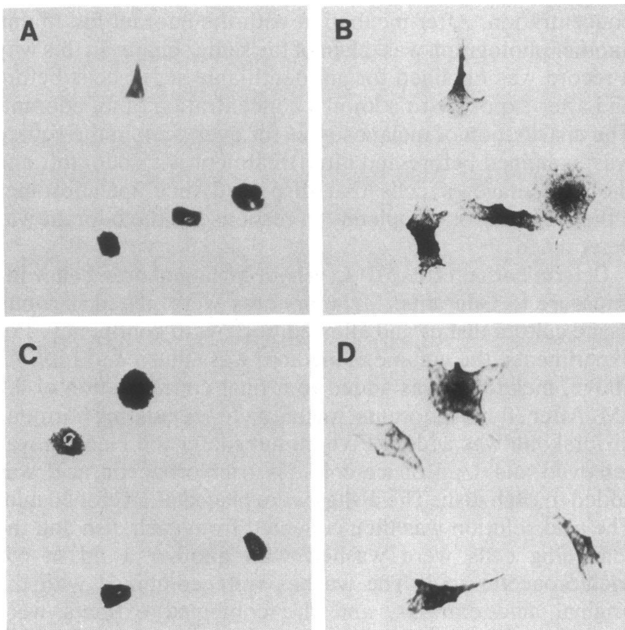

FIG. 1. Dispersal of melanosomes in melanophores exposed to melanocyte-stimulating hormone or citralva. (*A*) Four melanophores 30 min after addition of 0.5 nM melatonin. (*B*) The same melanophores 15 min after addition of 10 nM melanocyte-stimulating hormone. (*C*) A second set of four melanophores 30 min after addition of 0.5 nM melatonin. (*D*) The same melanophores shown in *C* 15 min after addition of 100 μM citralva. Bright-field micrographs are shown.

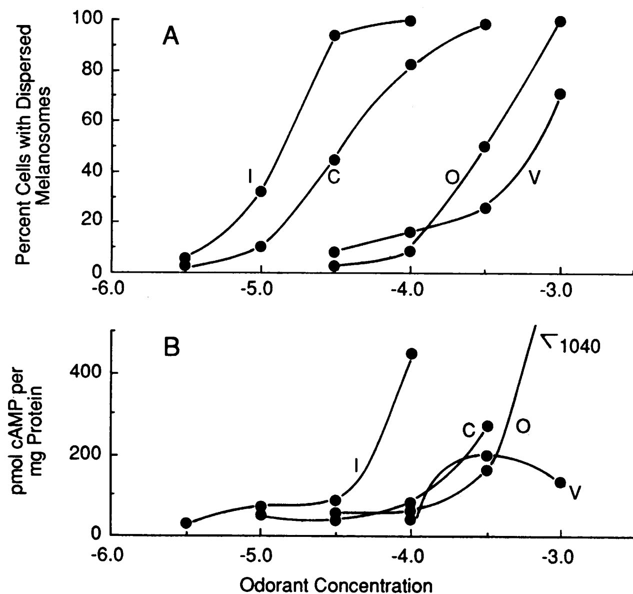

FIG. 2. Comparison between the ability of melanophores to disperse their melanosomes after exposure to odorants and the amount of cAMP generated by cells in response to odorant stimulation. (*A*) Dose–response curves: percentage of melanophores that disperse melanosomes versus the concentration of odorants to which they were exposed for 15 min: I, β-ionone; C, citralva; O, octanol; V, ethyl vanillin. The units for the abscissa are logarithm of the molar concentration. (*B*) Dose–response curves: amount of cAMP generated per mg of protein during a 15-min exposure to the same concentrations of the four odorants used in *A*.

The Response of Melanophores to Alcohols Depends on the Length of the Hydrocarbon Chains. The percentages of melanophores that responded to butanol, pentanol, hexanol, heptanol, and octanol at 1 mM and to methanol, ethanol, propanol, butanol, pentanol, and hexanol at 10 mM are shown in Fig. 3. For either concentration, the longer the hydrocarbon chain, the more effective an alcohol was at inducing melanosome dispersion. The responses of melanophores to several different concentrations of ethanol, butanol, hexanol, octanol, and decanol are shown in Fig. 4. The longer the hydrocarbon chain, the lower the concentration required to effect melanosome dispersal.

Comparison Between the Sensitivity to Alcohols of the Human Olfactory System and Frog Melanophores. The logarithms of the concentrations of the alcohols predicted from Fig. 4 to cause melanosome dispersal in 40% of melanophores are plotted against the logarithms of the concentrations that represent their olfactory thresholds in human beings. The 40% response level is taken as a threshold response because, as shown in Fig. 4, this is about the lowest response that is typically seen in the assay. The olfactory threshold for humans means the smallest concentration of an odorant dissolved in air that can be smelled. Because the alcohols were applied to the melanophores in an aqueous solution whereas the human olfactory threshold values are for alcohols dissolved in air, the latter were corrected by taking into account the air/water partition coefficients of the alcohols. Human threshold values and the partition coefficients were taken from published data (19).

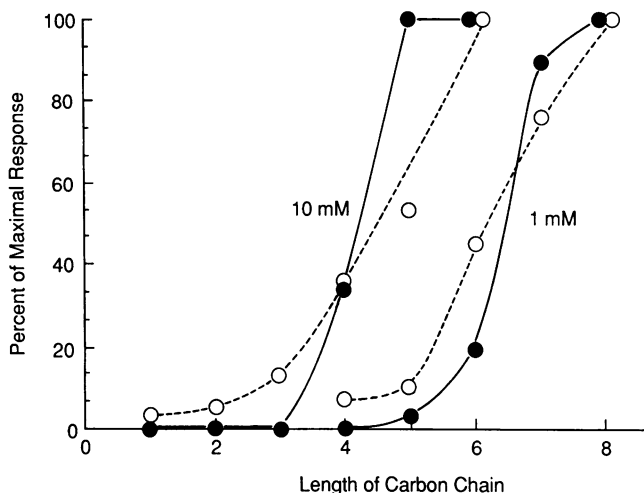

FIG. 3. Comparison between the ability of frog melanophores and the olfactory epithelium to respond to alcohols. ●, Results from experiments in which the percentage of melanophores that disperse melanosomes in response to a 15-min exposure to 1 or 10 mM concentrations of straight-chain alcohols is plotted against length of the hydrocarbon chain of the alcohol. ○, Results from experiments in which percentage of a maximal electrical response of frog olfactory epithelium to straight-chain alcohols is plotted against length of the hydrocarbon chain of the alcohol; data are from ref. 18.

DISCUSSION

Hypotheses about olfaction of general odorants fall into two groups: those that invoke the existence of special olfactory receptor proteins, and those that do not. The ability of fish to smell amino acids [and studies probing their olfactory cilia with radiolabeled amino acids that reveal binding sites for specific amino acids (20, 21)] and the ability of insects to detect very specific chemicals—their pheromones—strongly support a specific receptor model. It has also become attractive to think of olfaction involving common odorants as being based on a specific set of olfactory receptor proteins that might be related to each other as immunoglobulins are (1). On the other hand, several reports have suggested that specific olfactory receptor proteins are not required. For example, Persaud and Dodd (10) have built a model system with three semiconductor transducers that, while differing from each other only slightly in their ability to detect different odorants, could as a whole discriminate among many chemicals. And Kashiwayanagi and Kurihara (12) have suggested that the lipid makeup of olfactory receptor cells might be the key to their ability to detect odorants. They found that cells from a nonolfactory neuroblastoma cell line would depolarize to different degrees when exposed to specific odorants at concentrations similar to those used here and that addition of various lipids to the cells altered their responses to the selected odorants in different ways.

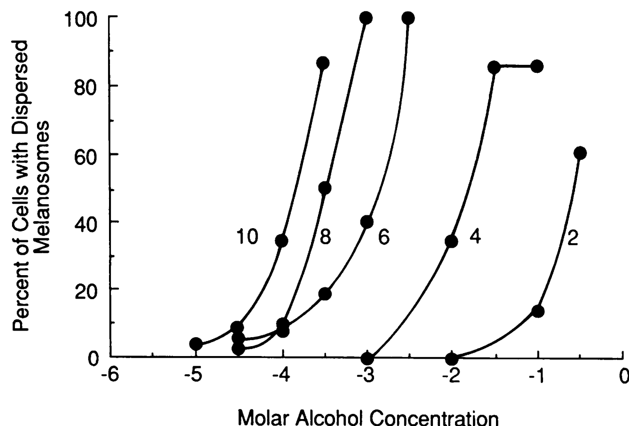

FIG. 4. Sensitivity of melanophores to alcohols of different hydrocarbon chain lengths. The percentage of cells with dispersed melanosomes after incubation with different concentrations of ethanol (curve 2), butanol (curve 4), hexanol (curve 6), octanol (curve 8), or decanol (curve 10) for 15 min is shown. The units for the abscissa are the logarithm of the molar concentration of the alcohol.

Recently, a cAMP-based second messenger system for general olfaction has become well established, indicating that the mechanism of olfactory transduction is analogous to that for visual and hormonal systems (3) and that olfactory-specific olfactory receptor proteins for general olfaction almost certainly exist. Two recent reports concerning the activation of adenylate cyclase in olfactory cilia by odorants have been thought to support this concept (1, 2). However, we have found that melanophores respond to odorants at concentrations that are identical to those required to activate adenylate cyclase in olfactory cilia. For example, 100 μM citralva is an effective stimulant of both melanophores and olfactory cilia whereas 100 μM ethyl vanillin and ethanol are not (2). Although odorants vary in their effectiveness to induce pigment granule dispersion, the order of potency for the four odorants shown in Fig. 2—β-ionone > citralva > octanol > ethyl vanillin—is also the order of their effectiveness in promoting the production of cAMP. Further, odorant activation of adenylate cyclase is not limited to olfactory cilia and melanophores. For example, it is known that lipophilic molecules such as ethanol and butanol, which have distinct odors, can increase the activity of adenylate cyclase in mouse striatal membranes (22). These two alcohols have also been shown to exert their effects through the GTP binding protein G_s. However, it has been reported that the odorants have no effect on the adenylate cyclase in membranes from heart, liver, or brain (1, 2). This was taken as evidence that the cyclase system in olfactory cilia was being activated in an odorant-specific manner. We believe that the adenylate cyclase from any type or tissue can be activated by odorants, although the effects may be difficult to detect in some systems. We speculate that the reason olfactory cilia and melanophores are more sensitive to odorants than membranes for any liver, brain, or heart is either that they have especially abundant amounts of G_s and adenylate cyclase or that membrane preparations from tissues are inadequate controls for structurally intact olfactory cilia in adenylate cyclase assays. Thus, odorant activation of adenylate cyclase as it is currently measured in olfactory cilia, melanophores, and probably any other type of cell shares a common mechanism that does not require olfactory-specific proteins.

Does this shared mechanism have any relevance for olfaction *in vivo*? In an attempt to find out, the response of melanophores to a series of alcohols whose odorant properties had already been studied in frogs was examined. Thirty years ago Ottoson (18) measured the electrical response generated by frog olfactory epithelium to two series of straight-chain alcohols. The first set, which included alcohols having two to six carbon atoms, was studied at 10 mM, while the second set, which included alcohols having four to eight carbon atoms, was studied at 1 mM. The responses of melanophores are compared with Ottoman's results in Fig. 3. The sets of curves are similar and suggest to us that the mechanism by which alcohols activate adenylate cyclase in isolated olfactory cilia, melanophores, and other cells is identical to that by which they initiate electrical responses from frog olfactory epithelium. The alcohol-induced activation of electrical responses from frog olfactory epithelium appears to occur by induction of adenylate cyclase and does not need olfactory-specific proteins.

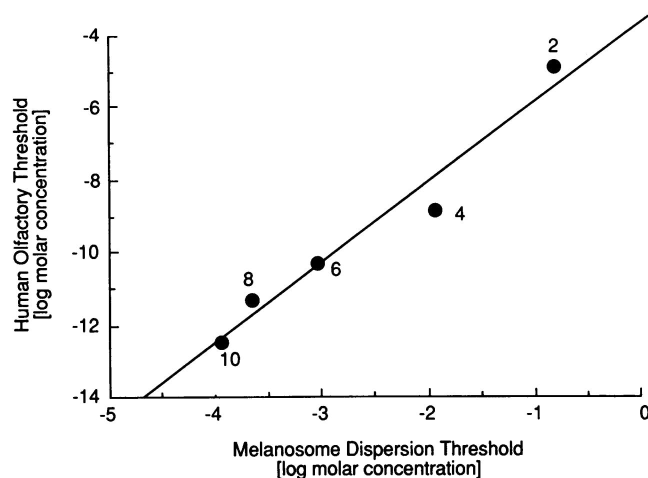

FIG. 5. Comparison between the olfactory threshold of humans and the sensitivity of frog melanophores to straight-chain alcohols (point 2, ethanol; point 4, butanol; point 6, hexanol; point 8, octanol; point 10, decanol). Human olfactory threshold data are from Laffort (19) and are corrected for the air/water partition coefficients of the alcohols. Melanophore threshold data are from Fig. 4 and represent concentrations of alcohols that cause dispersal of melanosomes in 40% of cells.

Still, it is difficult to relate these observations to the way in which human beings smell odorants and so the ability of melanophores to detect alcohols was compared with the ability of humans to smell them. The limits of detection—the olfactory threshold—of many alcohols by human beings have been determined by psychophysical studies (10), and Fig. 4 shows dose–response curves of melanophores to a series of straight-chain alcohols—ethanol, butanol, heptanol, octanol, and decanol—having even numbers of carbon atoms. Because the curves are quite steep, the first detectable response to a given alcohol was often registered by ≈40% of the melanophores, so this concentration of alcohol was taken to be the sensitivity threshold. The melanophore and human olfactory thresholds are compared for these alcohols in Fig. 5. Because the studies in humans were carried out with alcohols dissolved in air whereas those with melanophores were done using aqueous solutions, the human thresholds were modified to take into account the air/water partition coefficients of the alcohols (10). As the chain length of alcohols increases both frog melanophores and the human sense of smell become increasingly sensitive and there appears to be a distinct relationship between melanophore and human olfactory sensitivity to alcohols of different chain length. However, the results also show that the human nose is more sensitive than melanophores to alcohols by several orders of magnitude, with the slope of the line in Fig. 5 being 2.2 instead of 1. These points indicate that there are differences in the apparati used by the human nose and a frog melanophore to detect alcohols. Although the simplest explanation for the difference in sensitivities is that there are olfactory-specific receptor proteins for alcohols, in the absence of actual identification of such receptors, two factors must be ruled out. First, olfactory cilia are surrounded by mucus that contains an odorant-binding protein (23). This protein is thought to carry hydrophobic organic molecules from the air to the cilia and as such may act to increase the sensitivity of olfactory cilia to stimulatory agents. Second, the amount of alcohol required to activate sufficient adenylate cyclase in cilia to generate action potentials in enough receptor neurons to lead to a signal that is detectable for a few seconds by a human brain may be much lower than the amount needed to cause melanosome dispersal, which can require 15 min of sustained exposure. If these points are found to account for the difference in sensitivity to alcohols between the human nose and frog melanophores, it would indicate that the ability to smell alcohols does not require receptor proteins that are specific to olfactory neurons. So, while olfactory-specific odorant receptor proteins almost certainly will eventually be shown to account for part of general olfaction, it is possible that some aspects of odorant reception are mediated by a nonspecific mechanism whose signal is transduced by the cAMP-based second messenger system.

We thank Moo Ming Poo, Charles Stevens, Randall Reed, and Geoffrey Gold for helpful advice. This work was supported by the Helen Hay Whitney Foundation, the McKnight Foundation, National Science Foundation Grant CHE-8509626, and the Howard Hughes Medical Institute.

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