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Odor-induced phosphorylation of olfactory cilia proteins

(second messenger/protein kinases/protein kinase inhibitors/protein phosphatase)

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ABSTRACT **Stimulation of isolated rat olfactory cilia in the presence of [γ-^{32}P]ATP leads to a significantly enhanced incorporation of [^{32}P]phosphate. Depending on the type of odorants applied, the induced phosphorylation is completely blocked by specific inhibitors of either protein kinase A or protein kinase C. Time-course experiments indicate that the odor-induced modification of ciliary proteins is transient; the intensity of labeling decayed over time (1–10 sec). Separation of ciliary proteins by SDS/polyacrylamide gel electrophoresis followed by autoradiography demonstrated that upon stimulation with lilial, a single polypeptide (50,000 Da) was phosphorylated; the size of the modified protein is in line with the hypothesis that odorant receptors are phosphorylated subsequent to activation by specific odors.**

The responses of olfactory systems and sensory cells to odor stimulation attenuate very rapidly (< 1 sec) and change from phasic to tonic during continuous stimulus application (1, 2). This immediate desensitization is mimicked by the rapid termination of odor-induced second messenger signals in olfactory cilia, which can be monitored in "stopped flow" experiments (3, 4). Recently, desensitization has also been observed in primary olfactory neuronal cultures (5). The transient responsiveness to adequate stimuli is a characteristic feature of sensory cells, such as olfactory neurons, that respond to repeated stimulation. In a previous study it was demonstrated that the odorant-induced second messenger responses are rapidly switched off by negative-feedback loop reactions involving specific kinases; in the presence of inhibitors for the protein kinases desensitization was prevented (6). Subsequent experiments have shown that odorant-activated second messenger signaling pathways are turned off by only those kinases that are stimulated by the second messengers generated in the active cascade: cAMP formation is affected by protein kinase A but not by protein kinase C; inositol trisphosphate (IP_3)/diacylglycerol (DAG) generation is affected only by protein kinase C. These observations have led to the concept that uncoupling of the transduction cascade is brought about by phosphorylating key elements within the transduction apparatus, thus implying that exposure to odorants may lead to a stimulus-dependent phosphorylation of specific proteins in olfactory receptor cells. The existence of characteristic phosphoproteins in preparations from the olfactory neuroepithelium has recently been reported (7).

In the present study we provide evidence that the incorporation of [^{32}P]phosphate into rat olfactory ciliary proteins is significantly enhanced upon stimulation with odorants in a dose-dependent manner. Autoradiographic identification of the phosphorylated polypeptides supports the hypothesis that odorant receptor proteins are modified by specific kinases.

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MATERIALS AND METHODS

Materials. Sprague–Dawley rats were obtained from the Zentralinstitut für Versuchstierzucht (Hanover, F.R.G.). Citralva (3,7-dimethyl-2,6-octadienenitrile), ethylvanillin (3-ethoxy-4-hydroxybenzaldehyde), eugenol [2-methoxy-4-(2-propenyl)phenol], hedione (3-oxo-2-pentylcyclopentane-acetic acid methyl ester), lilial [4-(1,1-dimethylethyl)-α-methylbenzene-1-propanol], and lyral [4-(4-hydroxy-4-methylpentyl)-3-cyclohexene] were kindly provided by W. Steiner (Baierbrunn, F.R.G.). [γ-^{32}P]ATP was supplied by DuPont/New England Nuclear. Protein kinase A inhibitor (Walsh inhibitor) was purchased from Sigma (catalog no. P 0393). Calphostin C was obtained from Calbiochem. Okadaic acid was purchased from BioTrend (Cologne, F.R.G.). The purity of all chemicals used in this study was >99%.

Methods. *Isolation of olfactory cilia.* Partially purified chemosensory cilia from rat olfactory epithelia were prepared according to the procedures described previously (8, 9). Rat olfactory epithelia were dissected and collected in Ringer solution (120 mM NaCl/5 mM KCl/1.6 mM K_2HPO_4/1.2 mM $MgSO_4$/25 mM $NaHCO_3$/7.5 mM glucose, pH 8.0). This and all subsequent operations were carried out at 4°C. Cilia were detached from the epithelia by the calcium shock procedure, replacing the medium by Ringer solution containing 10 mM $CaCl_2$. After agitation in an end-over-end shaker for 20 min, the deciliated epithelia were pelleted by centrifugation at 7000 × *g* for 15 min and the supernatant was collected. The pellet was resuspended in Ringer solution containing 10 mM $CaCl_2$ and then agitated and centrifuged. The combined supernatants containing the detached cilia were centrifuged at 27,000 × *g* for 15 min. The protein concentration was determined by the Bradford procedure, with bovine serum albumin as the standard.

Phosphorylation procedure. The phosphorylation experiments were performed by following a modification of the procedure described by Wilden and Kühn (10). Isolated cilia were resuspended in a small volume of buffer (10 mM Tris·HCl/3 mM $MgCl_2$/2 mM EDTA, pH 8.0) giving a final protein concentration of 1 mg/ml. The odorous compounds were dissolved in dimethyl sulfoxide, giving a stock solution of 100 mM. To obtain the appropriate odorant concentration, different volumes of the stock solution were added to the "stimulation buffer" (200 mM NaCl/10 mM EDTA/50 mM Mops/2.5 mM $MgCl_2$/1 mM dithiothreitol/0.05% sodium cholate/1 mM ATP/1 μM GTP and a free Ca^{2+} concentration of 20 nM, pH 7.4). Both solutions were thoroughly mixed in an ultrasonic water bath at 25°C and used immediately. The reaction was started by adding 20 μl of the cilia preparation to 80 μl of warm (25°C) stimulation buffer containing odorants at the appropriate concentrations and in addition 1 μCi (37 kBq) of [γ-^{32}P]ATP. After incubation for the appropriate time interval, usually 1 sec, the reaction was stopped by adding 300 μl of an ice-cold mixture of 25% trichloroacetic acid and 5 mM phosphoric acid. The samples were placed on

Abbreviations: IP_3, inositol trisphosphate; DAG, diacylglycerol.

ice for 30 min and then the precipitated proteins were thoroughly mixed. Aliquots (100 μl) of each sample were spotted onto Whatman 3 MM filter paper (4 × 4 cm) and immersed in a solution of 10% trichloroacetic acid and 5 mM phosphoric acid. After thorough washing, the filters were dried at room temperature for 2 hr. The radioactivity adsorbed to the filters was quantitated by scintillation counting.

Identification of phosphorylated polypeptides. The reaction was started by adding 5 μl (30 μg) of cilia protein to 29.5 μl of warm (25°C) stimulation buffer containing 1 μM lilial as odorous compound and 2 μCi of [γ-^{32}P]ATP. The reaction was stopped after 5 or 10 sec by adding 8 μl of electrophoresis buffer containing 3.5% SDS and 0.2% dithiothreitol. After the samples had been boiled for 3 min 20-μl aliquots were subjected to SDS/polyacrylamide gel electrophoresis according to the method of Laemmli (11), using homogeneous 12.5% slab gels. Gels were stained for 30 min in 0.2% Coomassie blue R/7% (vol/vol) acetic acid/50% (vol/vol) methanol and destained in 7% acetic acid/20% methanol overnight. After the gels had dried, labeled polypeptides were visualized by exposure to Fuji RX films at −80°C with intensifying screens for 14 days.

RESULTS

Incubation of isolated olfactory cilia with [γ-^{32}P]ATP resulted in an incorporation of [^{32}P]phosphate into ciliary protein; after an incubation time of 1 sec 102 ± 19 pmol of phosphate per mg of ciliary protein was transferred. A similar level of phosphorylation (125 pmol/mg of protein) was recently observed in preparations of synaptic membranes (12). The basal rate of phosphorylation in olfactory cilia was significantly enhanced upon application of odorants. Challenging olfactory cilia with 1 μM of the fruity odorant citralva, which activates the cAMP pathway (3), led to a significantly enhanced labeling (Fig. 1); after a period of only 1 sec an incorporation of 271 ± 20 pmol/mg of protein was determined. A similar level of phosphorylation (306 ± 21 pmol/mg protein) was found 1 sec after stimulation with the floral odorant lilial, which activates the IP_3/DAG cascade (13).

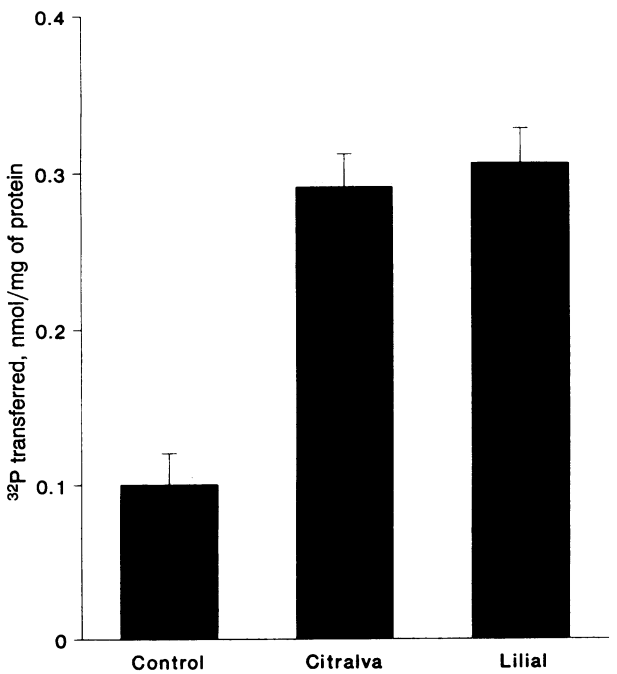

FIG. 1. Odorant-induced phosphorylation of olfactory ciliary proteins. Isolated olfactory cilia were incubated with [γ-^{32}P]ATP for 1 sec in the absence (control) or presence of odorants (citralva or lilial, 1 μM). The results are expressed as nmol of phosphate transferred per mg of ciliary protein. Data are the mean ± SD of four experiments.

To measure the sensitivity of the system, the relationship between stimulus intensity and amount of [^{32}P]phosphate incorporation into isolated olfactory cilia was determined. In Fig. 2 the dose–response curves for odorants inducing either cAMP or IP_3 are displayed. The data in Fig. 2*A* represent the concentration-dependent incorporation of [^{32}P]phosphate induced by an equimolar mixture of citralva, hedione, and eugenol; these odorants stimulate adenylate cyclase and rapidly induce a cAMP signal (3, 14). In Fig. 2*B* the concentration-dependent phosphorylation after application of a mixture of lyral, lilial, and ethylvanillin, odorants which all activate the IP_3 cascade (13), is demonstrated. In both cases the reaction showed an apparent saturation at an odorant concentration of about 1 μM; a half-maximal activation was observed at about 50 nM. These dose–response curves are reminiscent of the results obtained in recent experiments monitoring the odor-induced second messenger responses (3).

In a previous study we have shown that the termination of odorant-induced second messenger signals is mediated by specific kinases, which are activated by the messenger generated in the primary reaction cascade (6). To analyze if the odorant-induced phosphorylation of olfactory cilia protein is catalyzed by the kinases that are under the control of the generated second messenger, we employed specific kinase inhibitors. The data in Fig. 3*A* demonstrate that the basal level of [^{32}P]phosphate incorporation was hardly affected by the Walsh inhibitor, the specific blocker of protein kinase A (15). However, the incorporation of labeled phosphate induced by cAMP-generating odorants was almost completely prevented by the inhibition of kinase A, indicating that the odor-induced elevation of cAMP leads to an activation of the cAMP-dependent kinase A, which in turn catalyzes the phosphorylation of olfactory cilia protein.

To determine if an analogous mechanism is realized in the IP_3/DAG pathway, the effect of calphostin C, a highly specific inhibitor of protein kinase C (16, 17) was examined. Fig. 3*B* shows that a cocktail of odorants that generate IP_3 induced [^{32}P]phosphate incorporation about 4 times higher than under control conditions. In the presence of 1 μM calphostin C the odor-induced incorporation of phosphate was almost completely inhibited; no effect of the Walsh inhibitor was observed (data not shown). These results may be considered as an indication that the odor-induced phosphorylation of olfactory cilia protein is controlled by kinases which are activated by the second messenger generated in the primary reaction cascade.

To approach the question of whether the dual pathways of protein phosphorylation induced by the two odor classes and mediated by two different kinases are independent or interfere with and affect each other, the odor-induced incorporation of [^{32}P]phosphate was examined by stimulating with a cocktail of odorants that induces both cAMP and IP_3. To explore if the effects of the two classes of odorants are additive, both mixtures were applied at saturating concentration (each odorant at 1 μM). As is shown in Fig. 4, application of a mixture containing citralva, hedione, eugenol, lilial, lyral, and ethylvanillin leads to a significantly higher [^{32}P]phosphate incorporation. In the presence of a kinase A inhibitor, the odorant-induced phosphorylation rate was reduced to the level that was reached by stimulating solely with IP_3-inducing odorants (see Fig. 3*B*). A similar effect was observed when calphostin C was used to block protein kinase C; the remaining [^{32}P]phosphate incorporation corresponds to the level induced by cAMP-inducing odorants (Fig. 3*A*). The inactivation of both kinases completely blocked the odor-induced phosphorylation. These results

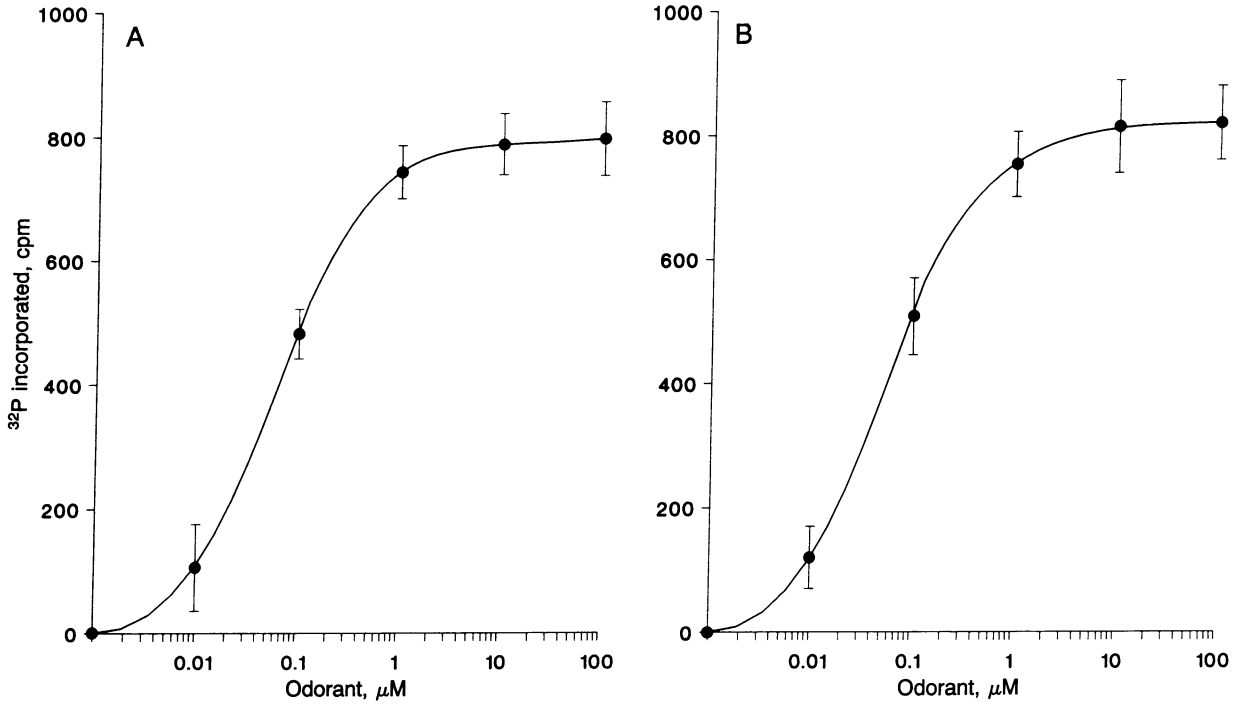

FIG. 2. Relationship between stimulus intensity and amount of phosphorylation. Cilia preparations were stimulated for 1 sec with different concentrations of odorous compounds. The scale gives the concentration of each individual odorant. Data are the mean ± SD of three or four experiments. Basal incorporation (250 cpm) has been subtracted. (*A*) Dose–response curves of a mixture of cAMP-generating odorants (citralva, hedione, and eugenol) inducing [^{32}P]phosphate incorporation into an olfactory cilia preparation. (*B*) Amount of cilia phosphorylation depends on the concentration of a mixture of IP_3-generating odorants (lilial, lyral, and ethylvanillin).

indicate that the effects of the two kinases are additive, suggesting that upon activation of the two second messenger cascades different substrates are phosphorylated and that the pathways are independent.

The concept that kinase-mediated modification of key elements uncouples the primary reaction cascade and thus terminates the second messenger signal (6) implies that the odor-induced phosphorylation of ciliary proteins must be a rapid and transient event. In fact, time course experiments between 1 and 30 sec indicate that the odor-induced incorporation of [^{32}P]phosphate does not increase over time, but in contrast, that the highest level of labeling is detected

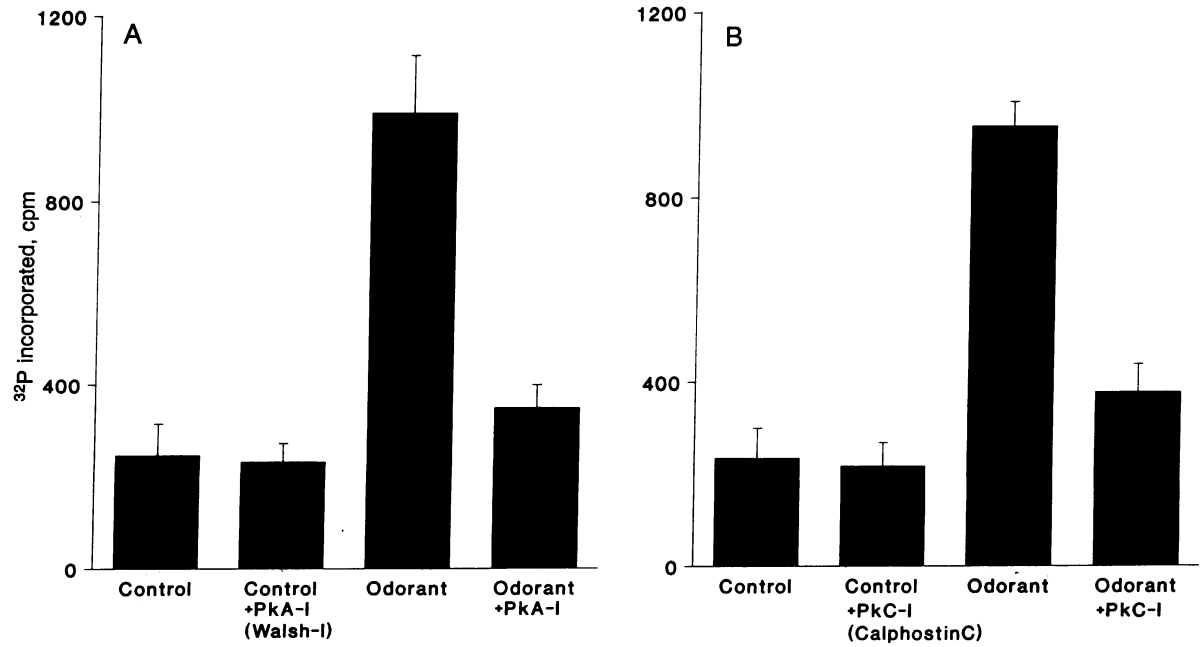

FIG. 3. Odorant-induced phosphorylation is prevented by specific kinase inhibitors. Incorporation of [^{32}P]phosphate under control conditions was affected neither by the protein kinase A inhibitor (PkA-I) nor by the protein kinase C inhibitor (PkC-I). Data are the mean ± SD of three or four experiments. (*A*) Incorporation of [^{32}P]phosphate into ciliary proteins within 1 sec in the absence (control) or presence of cAMP-generating odorants (citralva, hedione, and eugenol; 1 μM each). Protein kinase A was inhibited by Walsh inhibitor (3.8 μM). (*B*) Incorporation of [^{32}P]phosphate into olfactory cilia preparation in the presence of IP_3-generating odorants (lilial, lyral, and ethylvanillin; 1 μM each). Protein kinase C was inhibited by the specific blocker calphostin C (1 μM).

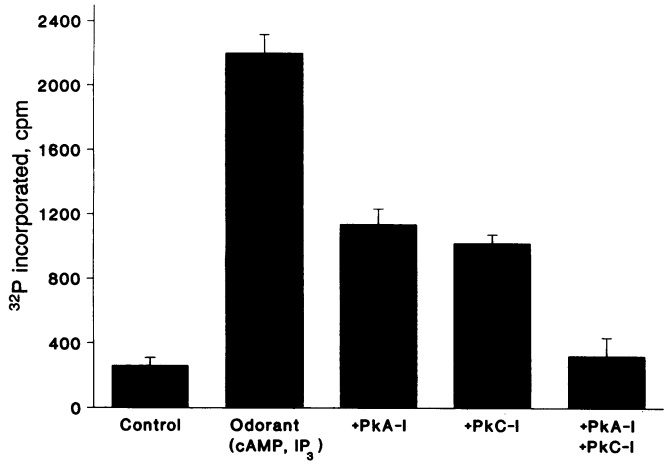

FIG. 4. Phosphorylation of olfactory ciliary proteins induced by applying both cAMP- and IP_3-generating odorants. Cilia preparations were incubated with [γ-^{32}P]ATP and a mixture of six odorants that produces both cAMP and IP_3 responses; the incorporation of [^{32}P]phosphate was determined after 1 sec. Note the significantly higher degree of labeling compared with Fig. 3. Application of either one of the protein kinase inhibitors (Walsh inhibitor or calphostin C) partially blocked the phosphorylation. In the presence of both kinase inhibitors the odorant-induced incorporation of [^{32}P]phosphate was completely prevented. Data are the mean ± SD of three to five experiments.

already after the shortest incubation period (1 sec); thereafter the degree of phosphorylation decayed to the basal level within 10 sec (Fig. 5). This observation suggests that the

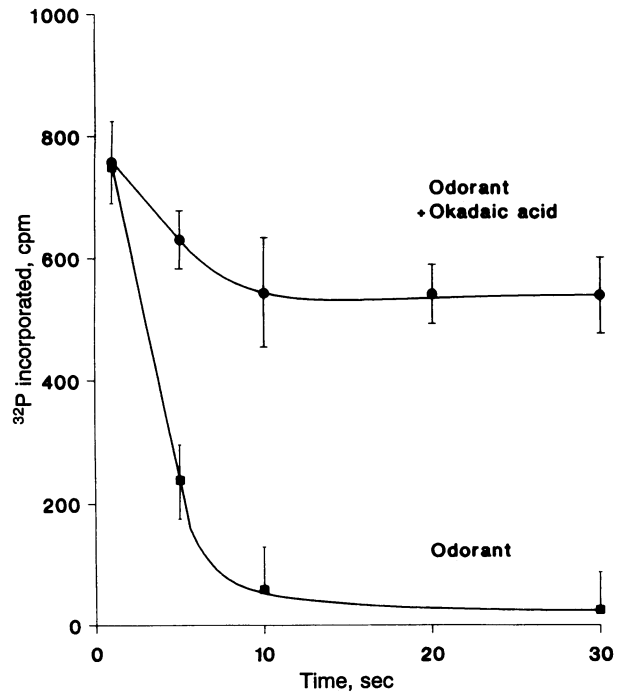

FIG. 5. Time course of odorant-induced phosphorylation of olfactory cilia. Olfactory cilia preparations were incubated for the indicated intervals with [γ-^{32}P]ATP and a mixture of citralva, hedione, and eugenol, 1 μM each; the amount of [^{32}P]phosphate incorporated was determined at various times between 1 and 30 sec and was corrected for basal incorporation (250 cpm). In the presence of the phosphatase inhibitor okadaic acid (1 μM) the decay of protein labeling was significantly reduced, indicating that okadaic acid-sensitive phosphatases are actively involved in the phosphorylation/dephosphorylation cycle. Data are the mean ± SD of three to five experiments.

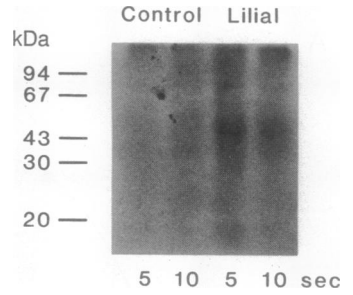

FIG. 6. Autoradiographs of olfactory cilia proteins separated by SDS/PAGE, illustrating odorant-induced phosphorylation. Isolated cilia were incubated for 5 or 10 sec with [γ-^{32}P]ATP in the absence (control) or presence of odorant (lilial, 1 μM). The samples were solubilized and fractionated by SDS/PAGE, and the gels were stained and dried. The incorporation of [^{32}P]phosphate into polypeptides was visualized by autoradiography. The first two lanes show an autoradiogram of a control sample exposed to the solvent used to administer the odorants; lanes 3 and 4 show the autoradiography of samples stimulated with lilial for 5 or 10 sec. Note the significant labeling of a polypeptide band at about 50 kDa.

modified proteins are very rapidly dephosphorylated. To verify that active phosphatases are involved, okadaic acid, a specific phosphatase inhibitor (18), was employed. The data in Fig. 5 indicate that in the presence of okadaic acid (1 μM) the level of phosphorylation remained stable over a long period of time. This observation indicates that the transient labeling is due to active, okadaic acid-sensitive phosphatases. Thus, a rapid cycle of phosphorylation and dephosphorylation may underly the desensitization and reactivation of the transduction pathways in olfactory cilia.

Toward an identification of the proteins which are phosphorylated upon stimulation of olfactory cilia with odorants, labeled ciliary proteins were separated by SDS/polyacrylamide gel electrophoresis and visualized by autoradiography. After a few seconds, labeled polypeptides could not be detected in unstimulated controls; however, upon stimulation with 1 μM lilial a single labeled polypeptide band in the range of about 50,000 Da was detectable after 5 sec (Fig. 6). The degree of labeling in this band was significantly reduced after 10 sec, a feature expected from the time-course experiments (Fig. 5). Considering the key elements of the phosphatidylinositol pathway, which is activated by lilial, the labeled polypeptide band is too small for a phospholipase C and too large to be a G protein; however, it corresponds to the predicted molecular mass of the putative odorant receptors (19). On the basis of size, the phosphorylated proteins may in fact represent odorant receptor proteins.

DISCUSSION

The rapid disappearance of odorant-induced signals in olfactory receptor cells is apparently due to an uncoupling of the olfactory reaction cascades mediated by specific kinases (6). In this study we have presented evidence indicating that upon odorant stimulation of olfactory cilia specific proteins undergo a rapid modification by covalent incorporation of phosphate groups. This reaction is catalyzed by either protein kinase A or protein kinase C, depending on the type of odorant applied. On the basis of analogy to other signal transduction cascades, such as the visual system (10) or the β-adrenergic system (20, 21), it has been suggested that uncoupling of the olfactory reaction cascade may be due to phosphorylation of the receptors for odors. A multigene family that is thought to encode odorant receptors has recently been discovered; the receptor proteins have several putative sites for phosphorylation and a molecular mass of about 50,000 Da (19). Interestingly, this corresponds to the

size of the ciliary polypeptides that are labeled upon odorant stimulation (Fig. 6). Experiments using receptor-specific antibodies to immunoprecipitate the phosphorylated proteins may solve the issue. If in fact odorant receptors are phosphorylated upon stimulation, this process is reminiscent of the homologous or agonist-specific desensitization described for the β-adrenergic receptor-coupled adenylate cyclase system (22). It will be of interest to evaluate if mechanisms of heterologous desensitization also exist in olfactory receptor cells.

Time-course experiments have indicated that the odorant-induced incorporation of phosphate groups into olfactory ciliary proteins is apparently very rapid and transient; the degree of labeling decreases over the time period (1–10 sec) investigated; for technical reasons, these experiments could not yet be performed in the apparently relevant subsecond time range. Nevertheless, the short incubation time (seconds) is probably one of the reasons why only a single polypeptide band was labeled when olfactory cilia were incubated with [γ-^{32}P]ATP and stimulated with odorants. In a previous study a variety of phosphoproteins in olfactory cilia that undergo phosphorylation in a cyclic nucleotide dependent manner have been identified (7). However, in this investigation the labeled polypeptides have been analyzed after an incorporation period of 10 min. Furthermore, modulation of protein phosphorylation by odorants has not been reported before, to our knowledge.

The cycle of phosphorylation and dephosphorylation of ciliary proteins upon odorant stimulation indicates that the modified proteins are reactivated by a fast removal of the covalently bound phosphate groups. These observations imply that not only specific kinases but probably also specific phosphatases are active in olfactory receptor cells. A coordinated interplay of phosphorylation and dephosphorylation governed by specific kinases and phosphatases is proposed to control the proportion of modified and thus inactivated ciliary proteins. A functional role of specific phosphatases has recently also been suggested for the regeneration of phosphorylated metarhodopsin (23). Studies exploring the types of phosphatases and the regulation of their activity in olfactory receptor neurons may contribute to understanding the molecular mechanisms underlying the phasic-tonic responses of olfactory systems to odor stimulation.

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1. Getchell, T. V. & Shepherd, G. M. (1978) *J. Physiol. (London)* **282,** 541–560.
2. Kurahashi, T. & Shibuya, T. (1990) *Brain Res.* **515,** 261–268.
3. Boekhoff, I., Tareilus, E., Strotmann, J. & Breer, H. (1990) *EMBO J.* **9,** 2453–2458.
4. Breer, H., Boekhoff, I. & Tareilus, E. (1990) *Nature (London)* **345,** 65–68.
5. Ronnett, G. V., Parfitt, D. J., Hester, L. D. & Snyder, S. H. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2366–2369.
6. Boekhoff, I. & Breer, H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 471–474.
7. Heldman, J. & Lancet, D. (1986) *J. Neurochem.* **47,** 1527–1533.
8. Anholt, R. R. H., Aebi, U. & Snyder, S. H. (1986) *J. Neurosci.* **6,** 1962–1969.
9. Chen, Z., Pace, U., Heldman, J., Shapira, A. & Lancet, D. (1986) *J. Neurosci.* **6,** 2146–2154.
10. Wilden, U. & Kühn, H. (1982) *Biochemistry* **21,** 3014–3022.
11. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
12. Kearney, K. & Gurd, J. W. (1986) *J. Neurochem.* **46,** 1683–1691.
13. Breer, H. & Boekhoff, I. (1991) *Chem. Senses* **16,** 19–29.
14. Sklar, P. B., Anholt, R. R. H. & Snyder, S. H. (1986) *J. Biol. Chem.* **261,** 15538–15543.
15. Walsh, D. A., Ashby, C. D., Gonzalez, C., Calkins, D., Fischer, H. & Krebs, E. G. (1971) *J. Biol. Chem.* **246,** 1977–1985.
16. Kobayashi, E., Nakano, H., Morimoto, M. & Tamaoki, T. (1989) *Biochem. Biophys. Res. Commun.* **159,** 548–553.
17. Tamaoki, T. & Nakano, H. (1990) *Biotechnology* **8,** 732–735.
18. Cohen, P., Holmes, C. F. B. & Tsukitani, Y. (1990) *Trends Biochem. Sci.* **15,** 98–102.
19. Buck, L. & Axel, R. (1991) *Cell* **65,** 175–187.
20. Sibley, D. R., Benovic, J. L., Caron, M. G. & Lefkowitz, J. (1987) *Cell* **48,** 913–922.
21. Hausdorff, W. P., Caron, M. G. & Lefkowitz, R. J. (1990) *FASEB J.* **4,** 2881–2889.
22. Sibley, D. R. & Lefkowitz, R. J. (1985) *Nature (London)* **317,** 124–129.
23. Fowles, C., Akhtar, M. & Cohen, P. (1989) *Biochemistry* **28,** 9385–9391.