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Phosphorylation and Inhibition of Olfactory Adenylyl Cyclase by CaM Kinase II in Neurons: a Mechanism for Attenuation of Olfactory Signals

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Summary

Acute desensitization of olfactory signaling is a critical property of the olfactory system that allows animals to detect and respond to odorants. Correspondingly, an important feature of odorant-stimulated cAMP increases is their transient nature, a phenomenon that may be attributable to the unique regulatory properties of the olfactory adenylyl cyclase (AC3). AC3 is stimulated by receptor activation and inhibited by Ca^{2+} through Ca^{2+}/calmodulin kinase II (CaMKII) phosphorylation at Ser-1076. Since odorant-stimulated cAMP increases are accompanied by elevated intracellular Ca^{2+}, CaMKII inhibition of AC3 may contribute to termination of olfactory signaling. To test this hypothesis, we generated a polyclonal antibody specific for AC3 phosphorylated at Ser-1076. A brief exposure of mouse olfactory cilia or primary olfactory neurons to odorants stimulated phosphorylation of AC3 at Ser-1076. This phosphorylation was blocked by inhibitors of CaMKII, which also ablated cAMP decreases associated with odorant-stimulated cAMP transients. These data define a novel mechanism for termination of olfactory signaling that may be important in olfactory responses.

Introduction

The olfactory system has an exquisite capacity to discriminate among an immense variety of odorants that are present at low concentrations in the environment. Olfactory cues are detected by sensory neurons at two locations in mammals: the olfactory epithelium (OE) in the nasal cavity and the neuroepithelium of the vomeronasal organ (VNO). Volatile chemicals released from a variety of sources, including food, are detected in the OE. The VNO appears to detect pheromones that are emitted by other animals and that convey information for social and reproductive behavior. Since the VNO neurons do not express several of the key signal transduction components expressed in the OE (Berghard et al., 1996) and express a family of G protein–coupled receptors that are specific to the VNO (Ryba and Tirindelli, 1997), VNO sensory transduction very likely differs from that in the OE.

Olfactory signal transduction in the OE is mediated by second messenger cascades (reviewed by Breer et al., 1994; Shepherd, 1994; Ache and Zhainazarov, 1995; Restrepo et al., 1996; Ronnett and Payne, 1995) and is initiated by the interaction of odorants with receptors encoded by a multigene family (Buck and Axel, 1991; Chess et al., 1992). These odorant receptors exhibit structural characteristics of the seven transmembrane G protein–coupled receptor superfamily. The application of rapid kinetics methodology has shown that odorants elicit a rapid elevation of either cAMP or IP_3 (Breer et al., 1990). The colocalization of a unique G protein, G_{olf} (Jones and Reed, 1989), the olfactory adenylyl cyclase (AC3) (Bakalyar and Reed, 1990), and cyclic nucleotide–gated (CNG) cation channels (Nakamura and Gold, 1987) in the olfactory cilia suggests an important role of cAMP in olfactory signal transduction. Indeed, a variety of odorants stimulate adenylyl cyclase activity in membrane preparations from OE (Pace et al., 1985; Sklar et al., 1986; Lowe et al., 1989). Odorant receptor activation increases intracellular cAMP through interactions with G_{olf} (Belluscio et al., 1998). This leads to the opening of CNG cation channels, membrane depolarization, and the generation of action potentials (Nakamura and Gold, 1987). It was recently discovered that excitatory responses to both cAMP and IP_3-producing odorants are undetectable in mice lacking functional olfactory CNG channels, further suggesting that cAMP may be a major second messenger mediating olfactory signaling (Brunet et al., 1996). Although the cAMP regulatory system may play a major role in olfactory signal transduction, there may also be contributions from other signaling systems. For example, one subset of olfactory sensory neurons in rodents expresses cGMP-stimulated phosphodiesterase (PDE2) and receptor guanylyl cyclase-D (Juilfs et al., 1997). Furthermore, olfactory imprinting in Coho salmon is apparently due to sensitization of olfactory guanylyl cyclase in olfactory cilia (Dittman et al., 1997). Nevertheless, the coupling of olfactory receptors to adenylyl cyclase is a major mechanism for olfactory signal transduction.

One of the characteristic features of odorant-induced second messenger signaling is the transient responsiveness to odorant. Rapid increases and subsequent decreases in cAMP or IP_3 have been observed both in olfactory cilia (Breer et al., 1990) and in primary olfactory neuron cultures (Ronnett et al., 1991). In vitro biochemical approaches and studies with transgenic mice have suggested several mechanisms for desensitization, including odorant receptor phosphorylation (Boekhoff et al., 1992; Peppel et al., 1997), activation of PDEs (Borisy et al., 1992), and ion channel regulation (Kramer and Siegelbaum, 1992; Kurahashi and Menini, 1997). Although increases in intracellular Ca^{2+} are thought to terminate olfactory signaling (Kurahashi and Shibuya, 1990; Jaworsky et al., 1995; Torre et al., 1995; Restrepo et al., 1996), mechanisms for inhibition of olfactory signaling by Ca^{2+} are not well defined. Ca^{2+} influx through the CNG channels (Kurahashi et al., 1990) may trigger adaptation of odorant responses by targeting multiple steps in the cAMP signaling cascade. For example, olfactory receptor neurons express a Ca^{2+}/calmodulin-(CaM) activated PDE (Borisy et al., 1992; Yan et al., 1996), and PDE inhibitors prolong odor-induced cAMP formation in olfactory cilia (Boekhoff and Breer, 1992).

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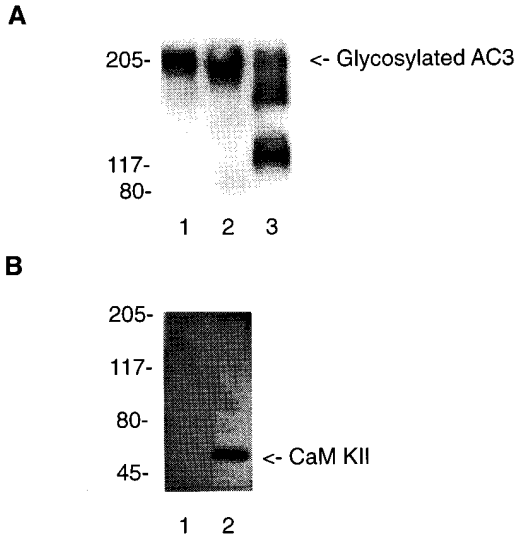

Figure 1. Western Analysis for AC3 and CaMKII in Mouse Olfactory Cilia

Olfactory cilia were submitted to Western analysis for AC3 (A) or CaMKII (B) as described in Experimental Procedures.

(A) Protein samples were not treated (lane 1), incubated with glycosidase buffer only at 37°C (lane 2), or incubated with the glycosidase PNGase F (37°C) before Western analysis (lane 3).

(B) The CaMKII antibody was pretreated with (lane 1) or without (lane 2) purified CaMKII protein (1 μg/ml) before Western analysis.

This suggests that a CaM-sensitive PDE may contribute to signal termination. Intracellular Ca^{2+} also decreases the sensitivity of the CNG channel to cAMP (Kramer and Siegelbaum, 1992), suggesting that Ca^{2+}-regulated channels may also participate in the adaptation process.

Odorant stimulation of cAMP increases in the OE is mediated by AC3 (Bakalyar and Reed, 1990), a Ca^{2+}-inhibited adenylyl cyclase (Wayman et al., 1995b). Inhibition of AC3 by intracellular Ca^{2+} is mediated by CaM kinase II (CaMKII) phosphorylation of AC3 at Ser-1076 (Wei et al., 1996); the mutation of Ser-1076 to alanine renders AC3 insensitive to Ca^{2+} inhibition. Since odorant stimulation of intracellular cAMP is accompanied by increased intracellular Ca^{2+}, CaMKII phosphorylation of AC3 may contribute to the cAMP transients in olfactory cilia. To test this hypothesis, we generated a polyclonal antibody specific for AC3 phosphorylated at Ser-1076 (Wei et al., 1996). Here, we report that AC3 and CaMKII are both expressed in the cilia of olfactory neurons, that the phosphorylation of AC3 at Ser-1076 is significantly enhanced upon stimulation with an odorant, and that this phosphorylation is mediated by CaMKII.

Results

Colocalization of AC3 and CaMKII in Olfactory Cilia

A prerequisite for CaMKII regulation of AC3 in olfactory signal transduction is the expression of these proteins together in olfactory cilia. Since the presence of CaMKII in OE had not been reported, Western analysis and immunohistochemistry were performed using affinity-purified anti-peptide antibodies directed against AC3 or CaMKII. On Western blots, the anti-AC3 antibody recognized a 200 kDa protein in olfactory cilia (Figure 1A, lanes 1 and 2), consistent with previous reports concerning the migration of AC3 on SDS gels (Bakalyar and Reed, 1990; Wei et al., 1996). Treatment of olfactory cilia with the glycohydrolase peptide N-glycosidase F (PNGase F) shifted the mobility of AC3 (lane 3) from an apparent molecular weight of 200 kDa to 129 kDa, because AC3 is a glycoprotein (Bakalyar and Reed, 1990; Wei et al., 1996). Anti-CaMKII antibody detected a 60 kDa protein in olfactory cilia (Figure 1B, lane 2), consistent with the M_r of CaMKII (Fukunaga et al., 1988).

An immunohistochemical analysis of OE detected AC3 primarily in the apical ciliary layer (Figure 2, red). There was little or no AC3 detectable in olfactory receptor neuron cell bodies. CaMKII immunoreactivity was also evident in the apical ciliary layer as well as neuron cell bodies (Figure 2, green). The merged image indicates that AC3 and CaMKII are coexpressed in olfactory cilia (Figure 2, yellow), the structures that contain olfactory receptors and are the primary source of olfactory signaling (Lowe and Gold, 1991). Therefore, CaMKII has the potential to regulate AC3 activity in olfactory neurons during olfactory signaling, since they are both expressed in the cilia of olfactory sensory neurons.

Phosphorylation of AC3 at Ser-1076 in Mouse Olfactory Cilia in Response to Odorant Stimulation

To determine whether odorants stimulate CaMKII phosphorylation of AC3 at Ser-1076 in olfactory cilia, we generated a polyclonal antibody against a Ser-phosphorylated peptide corresponding to amino acids 1066–1079 of AC3 (1066-NTVNVASRMES[PO_4]TGV-1079). This antibody, AC3P, was purified by peptide affinity chromatography. By using an enzyme-linked immunoabsorbent assay (ELISA), AC3P interacted with the phosphorylated peptide but not with the corresponding nonphosphorylated peptide. Citralva was used as an odorant, since it has been shown to potently stimulate the cAMP pathway in olfactory cilia and olfactory sensory neurons (Sklar et al., 1986; Boekhoff et al., 1990; Sato et al., 1991). In unstimulated cilia, there was generally some basal phosphorylation of AC3 at Ser-1076 that varied somewhat from one cilia preparation to another (Figure 3A). This may reflect a dynamic phosphorylation/dephosphorylation of AC3 in cilia. Treatment of olfactory cilia with citralva for 15 s induced a significant increase in phosphorylation of AC3 on Ser-1076 (Figure 3A). This band was eliminated by preabsorption of the AC3P antibody with pep-p, the phosphopeptide used to generate the antibody (Figure 3C), but not with the corresponding unphosphorylated peptide (pep, Figure 3D). The Western blot shown in Figure 3A was stripped and reprobed with an anti-AC3 antibody to ensure that equal amounts of protein were loaded on the gel (Figure 3B). The phosphorylated AC3 may have a slightly slower mobility than unphosphorylated AC3. However, this apparent difference may also be due to microheterogeneity of the AC3 protein. These data indicate that Ser-1076 of AC3 is rapidly phosphorylated after citralva treatment, consistent with the hypothesis that AC3 is phosphorylated in response to odorant stimulation.

The kinetics for citralva-induced phosphorylation of

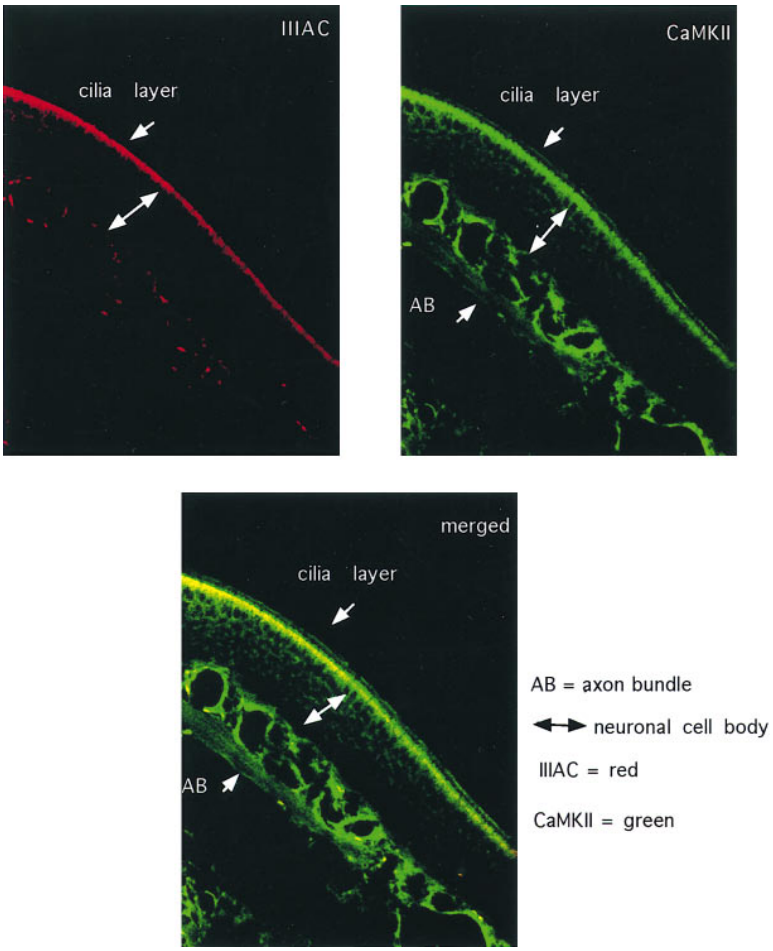

Figure 2. Confocal Microscope Double-Labeled Images of AC3 (red), CaMKII (Green), and Merged Signals (Yellow) Showing Colocalization of AC3 and CaMKII in Mouse Olfactory Cilia

Anti-AC3 antibody primarily labeled olfactory cilia. Anti-CaMKII antibodies labeled olfactory cilia and axon bundles.

AC3 at Ser-1076 were characterized in the presence and absence of okadaic acid, a general phosphatase inhibitor (Figure 4A). In the absence of phosphatase inhibitor, phosphorylation was maximal 10 s after addition of odorant and returned to baseline by 20 s. In the presence of okadaic acid, phosphorylation of AC3 was sustained and did not decrease after 20 s. This suggests that the enzyme is normally rapidly phosphorylated and dephosphorylated following exposure to odorant. We also compared phosphorylation of AC3 in response to citralva, isoamyl acetate (IA), and forskolin using forskolin as an index of the maximum level of phosphorylation (Figure 4B). IA is another model odorant used in olfactory studies (Lowe and Gold, 1991). Compared with

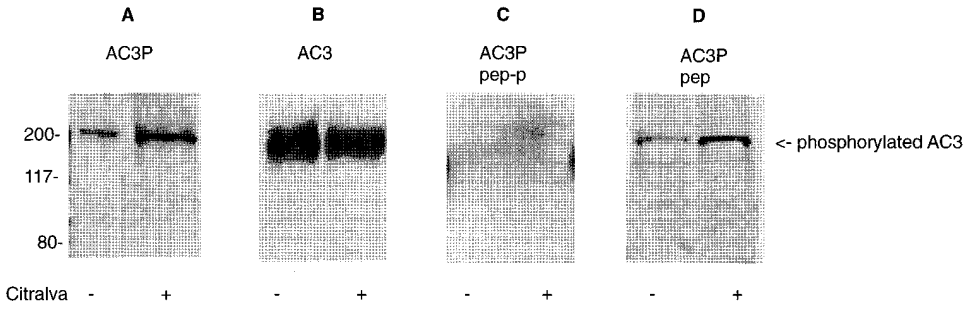

Figure 3. Citralva Stimulates the Rapid Phosphorylation of AC3 in Mouse Olfactory Cilia

Mouse cilia (20 μg) were treated with 10 μM citralva and subjected to Western analysis using an antibody against AC3 phosphorylated at Ser-1076 (AC3P) or an antibody for AC3 as described in Experimental Procedures. The blot was first probed with AC3P (A), stripped, and then reprobed with anti-AC3 antibody (B). Phosphorylation of AC3 at its CaMKII phosphorylation site increased within 15 s after the odorant was applied (A) without affecting the amount of AC3 (B). The AC3P-immunoreactive band was eliminated by preadsorption of AC3P with pep-p, against which AC3P was generated (C). The phosphorylated AC3 band was still detectable when AC3P was preadsorbed with the corresponding nonphosphorylated peptide (pep) (D).

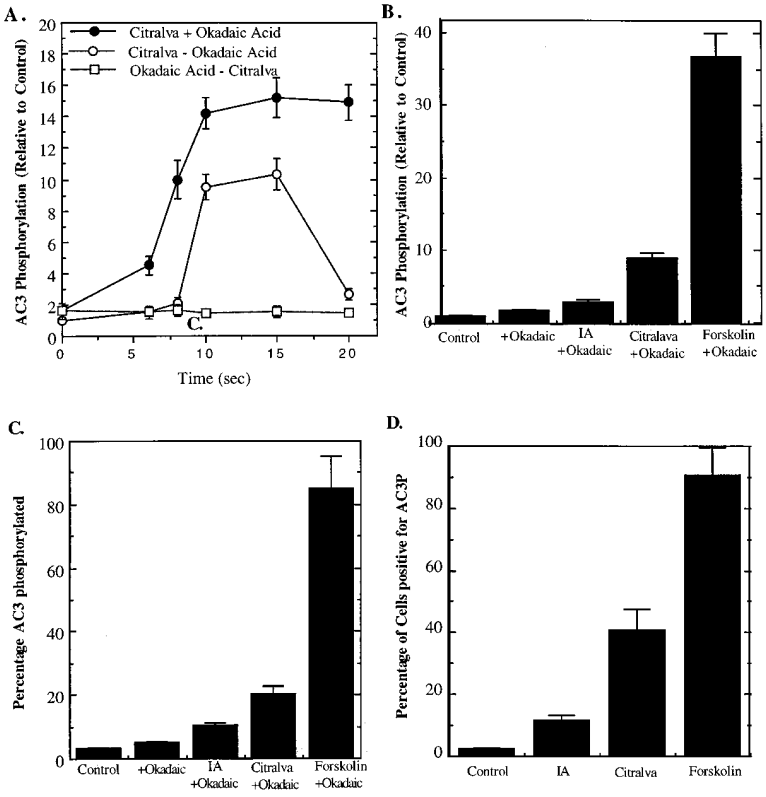

Figure 4. Kinetics for Citralva-Induced Phosphorylation of AC3

(A) Mouse cilia (20 μg) were treated with 10 μM citralva ± 1.0 μM okadaic acid or with 1.0 μM okadaic acid without citralva and subjected to Western analysis using an antibody against AC3 phosphorylated at Ser-1076 (AC3P) as described in Experimental Procedures. Western blots were scanned, and data are normalized relative to controls not treated with okadaic acid or citralva. Data are the average of three independent experiments. When present, okadaic acid was preincubated with cilia for 10 min prior to addition of odorant.

(B) Cilia samples were treated with 1.0 μM okadaic acid, 100 nM IA + 1.0 μM okadaic acid, 10 μM citralva + 1.0 μM okadaic acid, or 10 μM forskolin + 1.0 μM okadaic acid for 15 s. Samples were then analyzed for phosphorylation of AC3 as described in (A), and data are reported relative to untreated controls.

(C) Cilia samples were treated with 1.0 μM okadaic in the absence or presence of 10 μM IA, 10 μM citralva, or 10 μM forskolin and submitted to sequential immunoprecipitation using the AC3P and AC3 antibodies. These samples were submitted to Western analysis using the AC3 antibody, and the percentage of AC3 phosphorylated at Ser-1076 was estimated as decribed in Experimental Procedures.

(D) Primary cultured neurons were treated for 30 s with 100 nM IA, 10 μM citralva, or 10 μM forskolin and then fixed and stained for phosphorylated AC3 by using the AC3P antibody as described in Experimental Procedures. n = 200 neurons for each treatment.

untreated controls, okadaic acid caused a 60% ± 7% ($p < 0.008$) increase in basal phosphorylation of AC3. IA, citralva, and forskolin stimulated phosphorylation of AC3 2.0 ± 0.09–fold ($p < .003$), 5.6 ± 0.47–fold ($p < 0.001$), and 23 ± 3.1–fold ($p < 0.001$), relative to samples treated only with okadaic acid. Citralva stimulation of AC3 phosphorylation was ~24% of the maximum signal. A related experiment was carried out by sequential immunoprecipitation with the AC3P and AC3 antibodies followed by Western blotting with the AC3 antibody to provide a quantitative estimate of the percentage of AC3 phosphorylated (Figure 4C). In the absence of odorants, only 3% ± 0.6% of the AC3 was phosphorylated. With okadaic acid in the presence of IA, citralva, or forskolin, 10.2% ± 1.1% ($p < 0.001$), 20.3% ± 2.9% ($p < 0.001$), and 85.1% ± 10.4% ($p < 0.001$) of AC3 was phosphorylated, respectively.

To determine what fraction of olfactory neurons responds to odorants, cultured olfactory neurons were treated with citralva or IA for 30 s and immunostained for phosphorylated AC3. Only 2% ± 0.4% of the cells showed a detectable signal in the absence of odorant, whereas 11.0% ± 1.6% ($p < 0.001$), 40% ± 7.0% ($p < 0.001$), and 91% ± 9.2% ($p < 0.001$) of the cells were positive for phosphorylated AC3 when olfactory neurons were treated with IA, citralva, or forskolin, respectively (Figure 4D). The high percentage of cells activated by citralva is not unexpected; citralva is a complex mixture of odorants that undoubtedly stimulates multiple receptors in olfactory cilia. Representative fields of olfactory neurons stained for phosphorylated AC3 after treatment with IA or forskolin are reported in Figure 5.

Phosphorylation of AC3 at Ser-1076 Is Blocked by Inhibitors of CaMKII

To determine which protein kinase(s) is required for the phosphorylation of AC3, odorant-induced phosphorylation of AC3 was monitored in olfactory cilia (Figure 6A) and cultured olfactory neurons (Figure 6B) in the presence of various protein kinase inhibitors. Phosphorylation of AC3 was monitored by Western analysis using the AC3P antibody. In isolated olfactory cilia, treatment with 10 μM citralva for 15 s stimulated phosphorylation of AC3 (Figure 6A, lane 2), which was completely inhibited by pretreatment with the CaMKII inhibitory peptide (CaMKII-IP) (Figure 6A, lane 4). Preincubation with 10 μM protein kinase A inhibitory peptide (PKA-IP) (Figure 5A, lane 3) or 10 μM protein kinase C inhibitory peptide (PKC-IP) (Figure 6A, lane 5) did not affect odorant-induced phosphorylation of AC3. Similar results were obtained with mouse primary olfactory neuron cultures. Citralva stimulated phosphorylation of AC3 at Ser-1076 (Figure 6B, lane 2), which was blocked by KN-62, an inhibitor of CaM kinases (Figure 6B, lane 3). Preincubation of neurons with myristylated PKA- or PKC-IPs failed to inhibit the phosphorylation of AC3. Since the AC3P antibody was generated against the CaMKII phosphorylation sequence of AC3, and CaMKII inhibitors blocked this phosphorylation, we conclude that odorant-induced phosphorylation of AC3 is likely due to CaMKII.

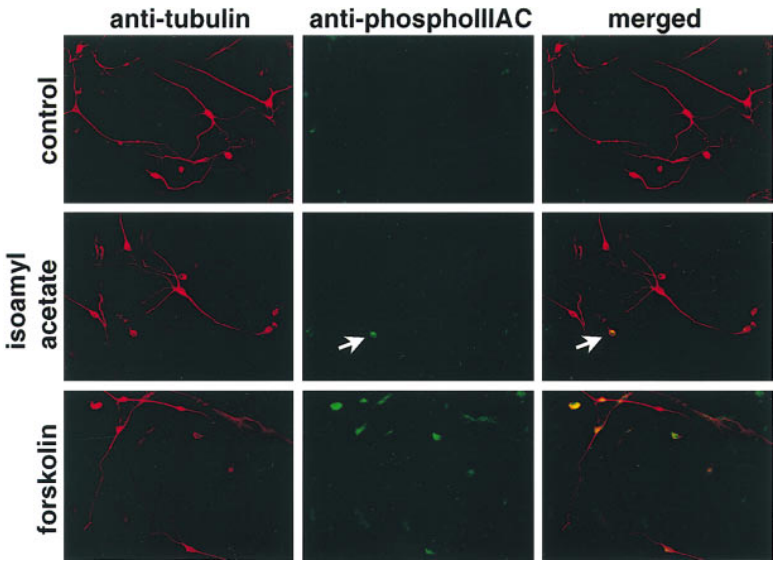

Figure 5. IA Treatment Promotes the Phosphorylation of AC3 in a Subset of Olfactory Neurons

Cultured olfactory neurons were treated with IA (100 nM) or forskolin (10 μM) for 2 min, fixed, and stained immunocytochemically for phosphorylated AC3 (phosphoIIIAC, green) and tubulin (red). The representative images show increased phosphorylated AC3 in only one IA-treated neuron out of seven neurons, while the majority of forskolin-treated neurons show increased AC3 phosphorylation.

Effects of CaMKII Inhibitors on cAMP Transients in Mouse Primary Olfactory Neurons

A key feature of odorant-induced cAMP increases in olfactory neurons or cilia isolated from olfactory neurons is their transient nature. Odorants generally cause at least two cAMP transients in olfactory cilia: one within milliseconds and another within 5–10 s after application of the odorant (Jaworsky et al., 1995). The decrease in cAMP signals associated with these transients may be due to stimulation of PDEs, Ca^{2+} inhibition of AC3, or a combination of both. Although it is not possible to measure the fast cAMP transient in neurons, since they cannot be submitted to stopped flow kinetics, the slower transient was measured manually. Treatment of mice olfactory neurons with citralva elicited a cAMP transient that was maximal 30 s after treatment (Figure 7). The decrease in cAMP associated with this transient was unaffected by pretreatment with isobutylmethylxanthine (IBMX), an inhibitor of PDEs (Figure 7A). However, the CaM kinase inhibitors KN-62 and AIP, a myristylated CaMKII-IP, both attenuated the cAMP decrease associated with this cAMP transient (Figures 7B and 7C).

Effect of CaMKII Inhibitor on cAMP in Mouse Olfactory Cilia

To determine the contribution of PDE and CaMKII for cAMP transients at the millisecond time scale, mouse olfactory cilia were preincubated with either 1 mM IBMX, a potent PDE inhibitor, or 10 μM CaMKII-IP prior to stopped flow experiments. Cilia were then mixed rapidly with a buffer containing 10 μM citralva for various periods of time, and cAMP was then measured. Citralva-stimulated cAMP increases were detectable within 20 ms after addition of the odorant (Figure 8). The cAMP response peaked at 50 ms and returned to basal levels within 200–300 ms. In the presence of IBMX, cAMP did not return to basal levels. This suggests that IBMX-sensitive PDEs are primarily responsible for the cAMP signal attenuation at the millisecond time scale (Figure 8A). The CaMKII inhibitor partially attenuated the cAMP decrease, suggesting that CaMKII inhibition of AC3 may contribute to this cAMP transient (Figure 8B).

The effects of PDE and CaMKII inhibitors on the second peak of cAMP, which reaches a maximum in seconds, were also examined. Samples containing cilia

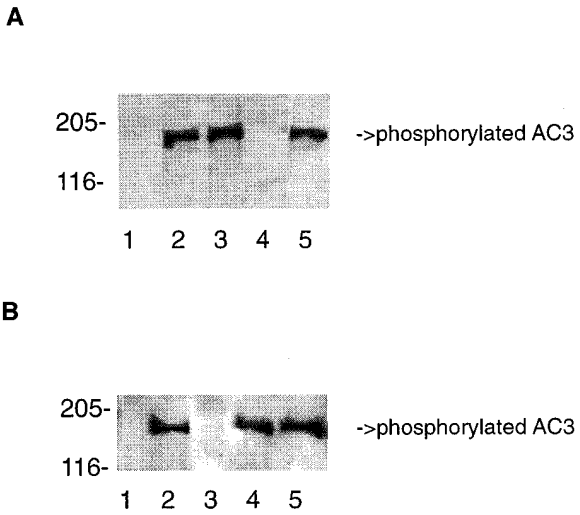

Figure 6. CaMKII Inhibitors Block Odorant-Stimulated Phosphorylation of AC3

(A) Mouse olfactory cilia were preexposed for 1 hr to various nonmyristylated protein kinase inhibitors and then treated with citralva for 15 s after incubation in phosphorylation buffer for 1 min. Western analysis was performed using the AC3P antibody as described in Experimental Procedures. Lane 1, untreated; lane 2, citralva; lane 3, citralva + PKA-IP; lane 4, citralva + CaMKII-IP; lane 5, citralva + PKC-IP. When present, citralva and kinase inhibitors were at 10 μM. (B) Primary cultures of mouse olfactory neurons were preincubated with protein kinase inhibitors for 1.5 hr and then treated with 100 nM citralva for 30 s. Western blots for AC3P were carried out as described in Experimental Procedures. Lane 1, untreated; lane 2, citralva; lane 3, citralva + KN-62; lane 4, citralva + myristylated PKA-IP; lane 5, citralva + myristylated PKC-IP. When present, citralva was at 100 nM and protein kinase inhibitors were at 20 μM.

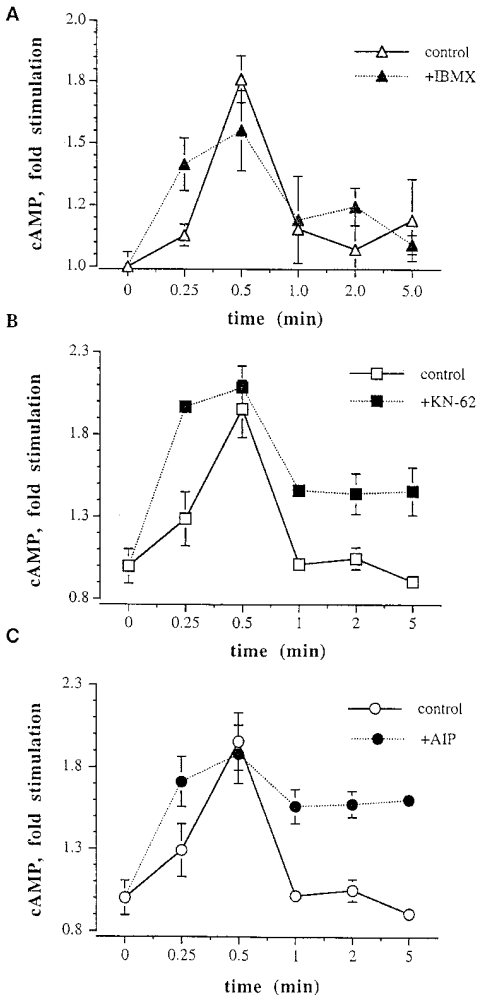

Figure 7. Effect of CaMKII and PDE Inhibitors on cAMP Transients in Cultured Olfactory Neurons

Mouse primary olfactory neurons were preincubated with 1 mM IBMX (A), 10 μM KN-62 (B), or 20 μM AIP (C) for 1.5 hr before treatment with 100 nM citralva (Cit). The reactions were quenched with 5% TCA after the indicated times. cAMP accumulations were measured as described in Experimental Procedures. The data are the mean ± SD of triplicate assays and are plotted as the increase in cAMP relative to controls without citralva treatment. Similar results were obtained in three independent experiments.

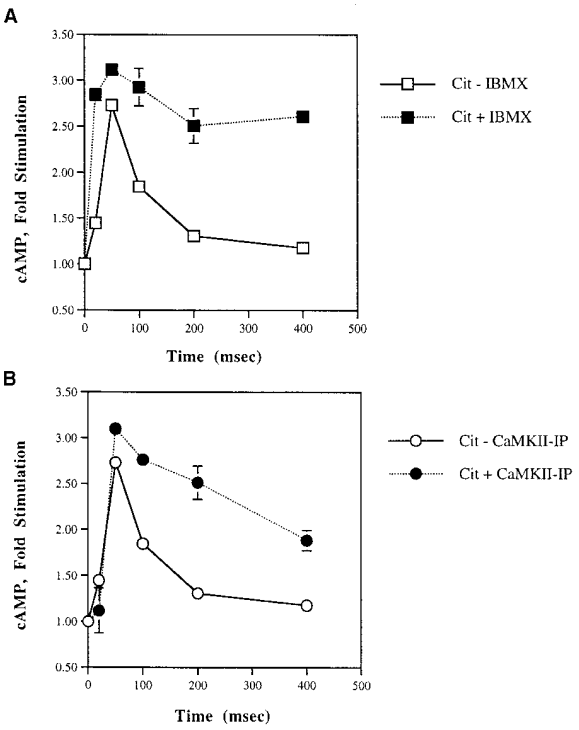

Figure 8. Effects of CaMKII and PDE Inhibitors on Fast cAMP Transients in Cilia

Mouse olfactory cilia were pretreated with 1 mM of IBMX for 30 min (A) or 10 μM of CaMKII-IP for 1 hr (B). Cilia were then incubated with reaction mix containing 10 μM citralva (Cit). cAMP concentrations were obtained at millisecond time points and performed using rapid stopped flow analysis. Data are presented as stimulation relative to basal and are averages of triplicates. Similar results were obtained in three independent experiments.

were mixed with 10 μM citralva and were quenched manually with trichloroacetic acid (TCA). In cilia, the second peak of odorant-stimulated cAMP accumulation occurred at ~8–10 s (Figure 9). IBMX had little or no effect on this cAMP transient (Figure 9A). However, the CaMKII-IP completely blocked the decrease in cAMP (Figure 9B). Our data suggest that IBMX-sensitive PDEs are important in modulating cAMP signals at the millisecond time scale, whereas CaMKII is critical for modulating the slower cAMP transient that occurs within seconds.

Discussion

For an animal to respond to olfactory cues in its environment, olfactory signal transduction must be rapid and reversible. Although olfactory signaling may be synchronized by the sniff frequency (Bressler and Freeman, 1980), an animal cannot sense an olfactory gradient unless it can turn off the olfactory signal and resample during movement through that gradient. Furthermore, smelling and sniffing induce activation of different parts of the human olfactory cortex (Sobel et al., 1998). Several mechanisms have been proposed for attenuation of olfactory signaling, including modulation of receptor function and ion channel activities as well as regulation of signal transduction machinery (Boekhoff et al., 1992; Kramer and Siegelbaum, 1992). Mechanisms for ablation of odorant-stimulated cAMP increases have not been completely defined, although cAMP PDEs clearly play an important role for cAMP transients generated within milliseconds (Boekhoff and Breer, 1992). The unique regulatory properties of AC3 suggested another mechanism for attenuation of cAMP signals that was investigated in this study.

When olfactory sensory neurons are treated with odorants, free Ca^{2+} increases from an average concentration of 20–50 nM to 150–250 nM. This Ca^{2+} transient is preferentially detected in the dendritic knob; however, Ca^{2+} also increases in the soma (Tareilus et al., 1995). Although it has been hypothesized that Ca^{2+} may attenuate olfactory signaling (Jaworsky et al., 1995; Torre et

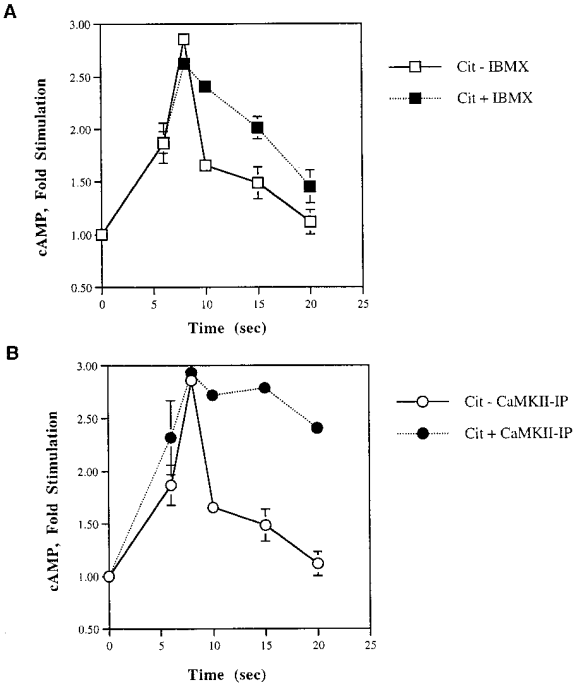

Figure 9. Effects of CaMKII and PDE Inhibitors on Slow cAMP Transients in Cilia

The procedure was the same as described in Figure 7 except that cAMP concentrations were measured manually. Data are presented as stimulation relative to basal and are averages of triplicate determinations.

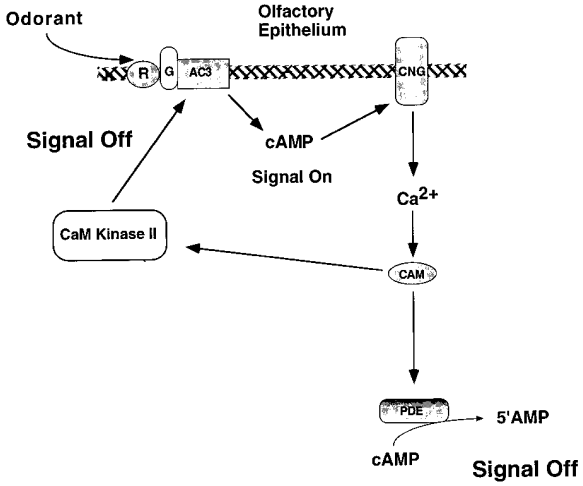

Figure 10. Model for Odorant Stimulation of cAMP Transients

Activation of odorant receptors increases cAMP and leads to stimulation of CNG. This depolarizes the membrane and increases intracellular Ca^{2+}. It is hypothesized that cAMP increases are attenuated by PDE and CaMKII inhibition of AC3.

al., 1995; Restrepo et al., 1996), the role of CaMKII in olfactory signal termination had not been previously considered. Interestingly, CaMKII is inactive at resting levels of Ca^{2+} (80 nM) and is activated by 250–500 nM free Ca^{2+} (Schulman et al., 1995). Since odorant-stimulated cAMP increases are accompanied by increases in intracellular Ca^{2+} (Restrepo et al., 1993), we hypothesized that Ca^{2+} inhibition of AC3 may play an important role in odorant-stimulated cAMP transients (Wayman et al., 1995b). The objectives of this study were to determine if AC3 and CaMKII are coexpressed in olfactory cilia and to ascertain if an odorant stimulates CaMKII phosphorylation of AC3. We also wanted to define the role of CaMKII in modulating cAMP transients in olfactory neurons and cilia.

Citralva Stimulates CaMKII Phosphorylation of AC3 in Cultured Olfactory Neurons and Isolated Cilia

Our data indicate that AC3 and CaMKII are coexpressed in mouse olfactory cilia. AC3 is almost exclusively localized to cilia, whereas CaMKII is found in cilia and neuron cell bodies. Using a polyclonal antibody that recognizes AC3 phosphorylated on Ser-1076, the CaM kinase phosphorylation site of AC3 (Wei et al., 1996), we discovered that AC3 is rapidly phosphorylated when citralva or IA is applied to cilia isolated from olfactory sensory neurons. This phosphorylation was blocked by specific inhibitors of CaMKII but not by PKA or PKC inhibitors. This leads us to believe that phosphorylation of AC3 in olfactory sensory neurons is mediated by Ca^{2+} activation of CaMKII. Our data also indicate that PDEs are primarily responsible for the cAMP decreases associated with millisecond transients, and that CaMKII attenuation of AC3 plays a major role for the slower cAMP transients. In isolated cilia, the second cAMP transient and AC3 phosphorylation occurred 8–10 s after odorant was added. Presumably, these kinetics reflect the time required for Ca^{2+} activation and for diffusion of CaMKII to the cilia membrane, where AC3 is localized.

There Are Two Mechanisms for Reversal of cAMP Increases in Olfactory Cilia

What is the mechanistic basis for odorant induction of cAMP transients, and why do they respond differentially to PDE and CaMKII inhibitors? The data in this study suggest a plausible hypothesis for the induction of multiple cAMP transients by odorants (Figure 10). Presumably, odorants increase cAMP by stimulating receptors coupled to AC3 through G_{olf} (Bakalyar and Reed, 1990; Belluscio et al., 1998) or G_s. Elevated cAMP opens CNG channels, causing membrane depolarization and Ca^{2+} influx. It is notable that the first peak of cAMP, maximal at 50 ms, is very sharp compared with the second slower peak, which reaches a maximum in seconds. PDEs clearly play a major role in relaxing cAMP signals generated within milliseconds, since IBMX blocks the cAMP decrease associated with these transients. CaM-stimulated PDEs, which are expressed in olfactory sensory neurons (Borisy et al., 1992; Juilfs et al., 1997; Yan et al., 1996), are likely candidates for this cAMP decrease, because they are rapidly activated by Ca^{2+} at the membrane surface. After the first cAMP transient, intracellular Ca^{2+} is lowered, and the activity of the CaM-sensitive PDE decreases. PDE activity may also be inhibited by the initial burst of cAMP, since PKA phosphorylation of a Ca^{2+}-stimulated PDE significantly lowers its Ca^{2+} sensitivity (Sharma and Wang, 1985). When the PDE(s)

are inactivated, this resets the system and allows the odorant to generate subsequent cAMP transients. Ca^{2+} increases associated with the second cAMP transient activate CaMKII, which inhibits AC3. Presumably, the kinetics for the development and decay of the second cAMP peak are much slower than the initial cAMP transient, because they depend upon kinase phosphorylation of the PDE followed by CaMKII phosphorylation of AC3. This mechanism would explain why the first cAMP transient is quite sensitive to PDE inhibitors, whereas the second cAMP transient is sensitive to CaMKII inhibitors and relatively insensitive to PDE inhibitors. Although this model is clearly speculative, it is consistent with the available data and may explain why olfactory sensory neurons have two independent mechanisms for attenuating odorant-stimulated cAMP increases.

Multiple Odorant-Induced cAMP Transients May Be Important for Olfactory Responses

What is the physiological significance of multiple cAMP transients caused by odorants? Although olfactory signaling may be synchronized by the sniff frequency (Bressler and Freeman, 1980), an animal cannot sense an olfactory gradient unless it can turn off the olfactory signal and resample during movement through an odorant gradient. We speculate that rodents may need multiple peaks of odorant-stimulated cAMP increases to detect odorant gradients. The initial rapid cAMP increase is crucial, since it presumably alerts the animal to the presence of a specific odorant in its environment. The animal may then respond by moving toward or away from the odorant source, assuming that it can compare a second odorant signal within the time scale that it takes to move through a gradient. In principle, a rodent can determine if it is moving up a positive odorant gradient by comparing the amplitude of the first cAMP transient with subsequent signals. It is also interesting that one can generate low frequency Ca^{2+} oscillations when AC3 is stimulated by hormones or forskolin (Wayman et al., 1995a). This suggests that multiple cAMP and Ca^{2+} transients may be generated in olfactory cilia by olfactory signals.

In summary, CaMKII phosphorylation of AC3 in olfactory receptor neurons is one of two mechanisms for attenuation of odorant-stimulated cAMP increases. This is the first evidence for CaMKII phosphorylation of AC3 in olfactory signal transduction, and it provides significant new insights concerning mechanisms for olfactory responses.

Experimental Procedures

Antibody Production

Anti-AC3P antibody was generated in rabbits immunized with a peptide corresponding to amino acids 1066–1079 of mouse AC3, phosphorylated on Ser-1076 (peptide AC3P, 1066-NTVNVASRMES [PO_4]TGV-1079). The AC3P antibody was purified with an affinity column prepared from the phosphorylated peptide. The AC3P antibody did not adsorb to an affinity column prepared from the nonphosphorylated peptide. It reacted with the phosphorylated peptide AC3P but not with nonphosphorylated peptide by enzyme-linked immunoabsorbent assay (ELISA) (Cocalico Biologicals). The specificity of this antibody in Westerns and immunostaining of olfactory neurons or OE was confirmed by elimination of the phosphorylated AC3 bands (Figure 3) following preabsorption with the phosphorylated peptide (0.1 μg/ml) but not with the unphosphorylated peptide (0.1 μg/ml).

Primary Cultured Olfactory Neurons

Primary cultures of mouse olfactory neurons were prepared as described (Ronnett et al., 1991). Four to five litters of 2-day-old mice (Swiss Webster) were sacrificed by decapitation, and olfactory tissue was dissected and immediately placed in Dulbecco's modified Eagle's medium containing 4.8 gm/1 HEPES buffer (DMEM-H). The turbinates were washed twice with DMEM-H, minced to obtain tissue fragments of ~1 mm in size, resuspended in DMEM-H, and centrifuged at 500 × g for 7 min. The preparation was then incubated with DMEM-H containing 1% (w/v) bovine serum albumin (BSA) (Sigma), 1 mg/ml hyaluronidase (Sigma), 50 μg/ml DNase (Sigma), 1 mg/ml collagenase (Worthington Biochemicals), and 5 mg/ml dispase (Boehringer-Mannheim Biochemicals) for 50 min at 37°C, with agitation. The cell suspension was passed through a 150 μm wire mesh and centrifuged at 500 × g for 5 min. The supernatant was aspirated, and the cell pellet was resuspended in plating medium composed of modified Eagle's medium containing D-valine (MDV, Gibco) with 10% (v/v) dialyzed fetal bovine serum (dFBS, Gibco), 5% (v/v) Nu serum (Collaborative Research), 10 μM cytosine arabinoside (Ara C, Sigma), and 25 ng/ml nerve growth factor (NGF, Gibco). After resuspension, cells were filtered through 50 and then 10 μm nylon mesh filters (Small Parts) to remove clumps of cells. Cells were plated at a density of 7×10^5 cells/cm^2 into tissue culture dishes coated overnight with MDV containing laminin at 25 μg/ml (Gibco). Cultures were placed under 5% CO_2 in a humidified 37°C incubator. After 3 hr, the medium was aspirated to remove dead cells, and MDV containing 15% dFBS, 50 μg/ml gentamicin, 100 μg/ml kanamycin, 25 ng/ml NGF, and 10 μM Ara C was added. Every day thereafter, cells were given the same fresh growth medium.

Olfactory Cilia Preparations and Pharmacological Treatments

Cilia were prepared from 25 g adult mice (C57BL/6 male) according to the procedure of Sklar et al. (1986). Mice were sacrificed by decapitation, and nasal turbinates were dissected, pooled, and washed in Kreb's Ringer's solution (120 mM NaCl, 5 mM KCl, 1.6 mM K_2HPO_4, 25 mM $NaHCO_3$, and 7.4 mM glucose [pH 8.0]) at 4°C. The tissue was centrifuged at 5,000 × g for 5 min. The pellet was resuspended in 100 mM Tris-HCl (pH 8.0). The bathing medium was supplemented with 10 mM $CaCl_2$ and agitated gently on an end-over-end shaker for 20 min at 4°C. The deciliated epithelium was removed by centrifugation for 5 min at 1,500 × g. The supernatant containing the detached cilia was centrifuged for 10 min at 12,000 × g, and the resulting pellet containing the isolated cilia was washed twice in 10 mM Tris-HCl, 3 mM $MgCl_2$, and 1 mM EDTA (pH 8.0). The final cilia pellet was resuspended in a small volume of 10 mM Tris-HCl, 3 mM $MgCl_2$, and 1 mM EDTA (pH 8.0). The concentrated cilia were aliquoted and stored at −70°C. Protein concentrations were determined according to the method of Bradford using BSA as a standard (Bradford, 1976).

To monitor phosphorylation of AC3 in cilia by protein kinases, phosphorylation buffer was used containing 50 mM HEPES (pH 7.5), 10 mM magnesium acetate, 0.4 mM ATP, and 0.5 mM $CaCl_2$. For treatment with nonmyristylated protein kinase inhibitors, mouse cilia were preincubated with 10 μM CaMKII-IP (amino acids 281–309, Calbiochem), 10 μM PKA-IP (amino acids 5–24, BIOMOL), or 10 μM PKC-IP (amino acids 19–36, BIOMOL) for 1 hr. After incubation in phosphorylation buffer for 1 min, cilia were treated with odorant for various amounts of time. Cilia were then solubilized by adding 5 × Laemmli buffer, and immunoblots were performed as described below.

Neuron Cell Extracts and Pharmacological Stimulation

For Western analysis, neurons were plated in 60 mm tissue culture dishes (Falcon) at a density of 7×10^5 cells per cm^2. Cells were used between days 5 and 7 in culture. Before odorant stimulation, cells were incubated in DMEM-H for 2 hr. For protein kinase inhibitor treatment, the cells were preincubated with myristylated, cell-permeable protein kinase IPs, 20 μM CaMKII-IP (AIP, amino acids 281–309, BIOMOL), 20 μM PKC-IP (amino acids 18–28, BIOMOL), or 20

μM PKA-IP (amino acids 14–22 of PKI, BIOMOL) for 1.5 hr. Immediately before use, citralva was diluted from a 100 mM stock in ethanol to the final concentration specified in each experiment. Citralva was added to cells and at intervals thereafter, and the medium was removed. After washing with PBS, cells were harvested in solubilization buffer (PBS, 1 mM DTT, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA and EGTA, 50 mM NaF, 1 mM Na_3VO_4, and 10 mM sodium pyrophosphate) with a protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg/ml aprotonin, 5 μg/ml leupeptin, and 10 μg/ml pepstatin). The lysates were passed several times through a 21 gauge needle to shear the DNA and then incubated on ice for 40 min. After incubation, cell lysates were microcentrifuged at 12,000 × g for 20 min at 4°C, and protein concentrations were determined by the method of Bradford using BSA as a standard (Bradford, 1976).

Western Analysis and Immunoprecipitation

For AC3 Westerns, olfactory cilia protein samples (20 μg) were solubilized in 1% SDS and boiled for 5 min. Samples were incubated with or without 5 μl PNGase F for 1 hr at 37°C in glycosidase incubation buffer made according to the instructions of the manufacturer (Boehringer-Mannhiem). After addition of Laemmli buffer, protein samples were subjected to SDS-PAGE (7.5%) and transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore). Immunoblots were probed with anti-AC3 antibody (1:100, Santa Cruz), incubated with goat anti-rabbit horseradish peroxidase (HRP) (1:5000, Gibco), and detected by enhanced chemiluminescence (ECL) (NEN). The CaMKII and phosphorylated AC3 Westerns were carried out by boiling of samples in Laemmli buffer for 10 min, which were then treated as described previously. The anti-AC3P and anti-CaMKII (ZYMED) antibodies were diluted 1:100. Westerns were scanned with a Hewlett Packard Scan Jet II, and relative band intensities were determined with Deskscan II and NIH image 1.58. Immunoprecipitation of AC3 in detergent-solubilized cilia was carried out with the AC3P and AC3 antibodies by the general method previously described (Wei et al., 1996) using antibodies diluted at 1:100 to maximize immunoprecipitations. The immunoprecipitates sequentially isolated with the AC3P and AC3 antibodies were run on Westerns, scanned, and used to estimate the percentage of AC3 phosphorylated in the presence of odorants. Westerns were scanned with a Hewlett Packard Scan Jet II, and relative band intensities were determined in the linear range with Deskscan II and NIH image 1.58. The bands detected by NEN ECL were in the linear range and below saturation.

Immunocytochemistry of OE and Cultured Neurons

Adult mice (C57BL/6 male) were anesthetized with sodium pentobarbital injected intraperitoneally and transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (pH 7.4). Nasal structures were dissected and fixed in 4% PFA for 2 hr. The fixation was followed by decalcification in 0.5 M EDTA (pH 7.4) overnight. Washed tissues were embedded in Tissue-TEK cryomolds (MILES). Cryostat sections, 20 μm thick, cut with a cryostat were mounted on microscope slides (VWR Scientific). The sections were preincubated in Histomouse-sp kit (ZYMED) for 1 hr. Next, they were treated with rabbit anti-AC3 antibody (1:5000, Santa Cruz) in blocking buffer (PBS with 1 mg/ml BSA, 0.1% Triton X-100, and 5% goat serum) for 1 hr at room temperature. After several PBS washes, the sections were incubated with Texas Red goat anti-rabbit antibody (1:500, Molecular Probe) for 1 hr. After washing with PBS four times, the sections were treated with mouse anti-CaMKII antibody (1:20, ZYMED) in blocking buffer overnight at 4°C. CaMKII was visualized with goat anti-mouse antibody conjugated to FITC (1:200, Molecular Probes). For immunocytochemistry, cells were plated on tissue culture coverslips previously treated with MDV containing 25 μg/ml laminin. After 5–7 days in culture, cells were first incubated in DMEM-H for 30 min. Immediately before use, odorant was diluted from a 100 mM stock and added to cells. After a 30 s treatment, medium was removed. The coverslips were rinsed three times in PBS (pH 7.4) and fixed in 4% (w/v) PFA for 15 min. After washing three times with PBS, coverslips were first blocked (blocking buffer: 5% BSA, 1% goat serum, and 0.1% Triton X-100 in PBS) for 1 hr at room temperature and then treated with primary AC3P antibody (1:100) overnight at 4°C. The next day, coverslips were washed with PBS and incubated with goat anti-rabbit antibody conjugated to FITC (1:500, Molecular Probe) for 1 hr at room temperature. Images were analyzed on a Bio-Rad MRC 600 confocal microscope with Comos 6.01 software.

Odorant Stimulation of Mouse Olfactory Cilia

A quench flow apparatus (Biologic) equipped with three syringes was used to monitor cAMP transients generated within the millisecond time scale. The first syringe contained cilia suspended in 10 mM Tris-HCl, 3 mM $MgCl_2$, and 10 mM EDTA. The second syringe contained 50 mM morpholinepropanesulfonic acid (MOPS), 2.5 mM $MgCl_2$, 1.0 mM dithiothreitol (DTT), 1.0 mM ATP, 1.0 mM GTP, and 0.05% cholic acid (pH 7.4), with or without odorant. The third syringe contained 13.5% TCA to quench the reaction. The reaction was performed by combining one part cilia to four parts reaction mix followed by four parts quench. Citralva was prepared in an ethanol stock; the final concentration of ethanol in the reaction mixture was $<$0.1%. Controls lacking citralva also contained 0.1% ethanol. In all cases, the free Ca^{2+} concentration was 10 μM. cAMP increases are expressed as changes relative to those samples lacking citralva. The absolute levels of cAMP in isolated mouse cilia varied somewhat from one preparation to another. When using enzyme inhibitors, 1 mM IBMX (Sigma), a PDE inhibitor, or 10 μM CaMKII-IP (Calbiochem), a specific CaMKII inhibitor, was preincubated with cilia for 1 hr. The protein concentration in the first syringe averaged 200 μg/ml of cilia buffer. cAMP assays were performed using 10 μg of protein. Quenched samples were collected on ice and spun 10 min in a microfuge at 4°C. The cAMP assay was performed on the supernatant by using a radioimmune assay system (NEN).

cAMP Accumulation Assay

Changes in intracellular cAMP were measured by determining the ratio of [^{3}H] cAMP to a total ATP, ADP, and AMP pool in [^{3}H]adenine-loaded cells as described by Wong et al. (1991). Although the absolute numbers for cAMP accumulation generally show some variation between experiments using different sets of cells (Dittman et al., 1994), relative changes in cAMP were consistent between experiments. Mouse primary olfactory neurons were plated in 24 well plates. After 5–7 days in culture, neurons were incubated in MDV containing [^{3}H]adenine (3.0 μCi/ml, ICN) for 16–20 hr. Thirty minutes before odorant treatment, neurons were fed with DMEM-H. Odorant stimulation was performed as described previously. After the designated times, the media were aspirated, the neurons were washed with 150 mM NaCl, and reactions were terminated by adding 1.0 ml of ice-cold 5% TCA containing 1 μM cAMP. Acid-soluble nucleotides were separated by ion exchange chromatography as described previously (Dittman et al., 1994). Reported data are the averages of triplicate determinations ± SD.

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